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PHYSIOLOGICAL AND PHARMACOLOGICAL PERSPECTIVES OF MONOAMINE REGULATION IN THE FETOPLACENTAL UNIT

Doctoral Dissertation

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STATEMENT OF AUTHORSHIP

I hereby declare that I am the sole author of this thesis. I have not used any materials previously published or written by someone else except those listed in the bibliography and identified as references. I further declare that I have not submitted this work at any other institution to obtain a degree.

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ABSTRACT

During pregnancy, the placenta is a vital organ for fetal growth and development, and its proper formation and function are essential for a successful gestational course. Current research indicates that maternally derived factors, such as disease or pharmacotherapy, may alter the functions of its key structure, the trophoblast. This layer, consisting of terminally differentiated syncytiotrophoblasts and their progenitor cells, the cytotrophoblasts, plays a significant role in the regulation of primary monoamine homeostasis. Monoamines such as serotonin, norepinephrine, and dopamine are involved in fetal organ development, including that of the brain. Thus, maintaining proper monoamine homeostasis in the fetoplacental unit is critical for healthy fetal programming, with implications for later life.

To ensure this balance, the trophoblast is equipped with regulatory proteins like those found in the brain. Monoamine synthetic and degradation enzymes, along with monoamine transporters, regulate monoamine homeostasis in the fetoplacental unit. However, the functional characteristics of these systems in the trophoblast layer, as well as their activity in undifferentiated cytotrophoblast progenitor cells, remain incompletely understood. Additionally, the gestation-related regulation of monoamine pathway components in the placenta is not well characterized. Moreover, monoamine transporters are known to be susceptible to drugs commonly used during pregnancy, such as antidepressants and antidiabetics (metformin).

Therefore, in this work, we aimed to 1) investigate the regulation of trophoblast monoamine transporters and monoamine handling during the cyto-/syncytiotrophoblast transition, 2) evaluate the synthesis, transport, and metabolism of monoamines in the fetoplacental unit based on gestation age, and 3) examine the effects of pharmacotherapy commonly used in pregnancy (antidepressants and metformin) on monoamine transport in the placenta.

To achieve our aims, we employed a range of experimental approaches, including cell models of syncytium development (primary human trophoblasts, BeWo, and JEG-3 cells), fresh villous human term placenta isolated fragments, and human term placenta isolated placental membrane vesicles. Additionally, rat placenta-derived HRP-1 cells and rat term placenta perfusions were used as animal models. Gene and protein expression of key monoamine transporters were assessed via quantitative PCR and Western blotting, respectively. The effects of trophoblast differentiation and inhibitors on monoamine uptake were investigated through functional studies.

The outcomes of this research elucidate monoamine transport alterations in differentiating trophoblasts and describe the expression patterns of monoamine regulatory components in the fetoplacental unit during gestation. Furthermore, this study highlights the expressional and functional differences between placenta-derived cell models, which is crucial for selecting the optimal model for monoamine research. Lastly, our findings indicate that several drugs commonly used during pregnancy interact with monoamine transport systems, inhibit their function, and thereby alter monoamine homeostasis in the fetoplacental unit.

ABSTRAKT

Během těhotenství je placenta životně důležitým orgánem pro růst a vývoj plodu a její správný vývoj a funkce jsou nezbytné pro úspěšný průběh těhotenství. Současný výzkum naznačuje, že faktory odvozené od matky, jako je nemoc nebo farmakoterapie, mohou ovlivnit funkci její klíčové struktury, trofoblastu. Tato vrstva, skládající se z terminálně diferencovaných syncytiotrofoblastů a jejich progenitorových buněk, cytotrofoblastů, hraje významnou roli v regulaci primární homeostázy monoaminů. Monoaminy, jako je serotonin, noradrenalin a dopamin, se podílejí na vývoji orgánů plodu, včetně mozku. Udržení správné homeostázy monoaminů ve fetoplacentární jednotce je proto zásadní pro zdravé programování plodu, s potenciálními důsledky pro život.

K zajištění této rovnováhy je trofoblast vybaven regulačními proteiny podobnými těm, které se nacházejí v mozku. Enzymy pro syntézu a degradaci monoaminů spolu s monoaminovými transportéry regulují homeostázu monoaminů ve fetoplacentární jednotce. Funkční charakteristiky těchto systémů ve vrstvě trofoblastu a jejich aktivita v nediferencovaných cytotrofoblastových progenitorových buňkách však zůstávají neúplně pochopeny. Navíc není dobře charakterizována regulace složek monoaminové dráhy v placentě související se stupněm těhotenství. Monoaminové transportéry jsou také citlivé na léky běžně používané během těhotenství, jako jsou antidepresiva a antidiabetika (metformin).

Proto jsme se v této práci zaměřili na: 1) studium regulace monoaminových transportérů v trofoblastu a monoaminové rovnováhy během přechodu z cyto- na syncytiotrofoblasty, 2) hodnocení syntézy, metabolismu a transportu monoaminů ve fetoplacentární jednotce v závislosti na stupni gestace a 3) zkoumání účinků v těhotenství běžně používaných léčiv (antidepresiva a metformin) na transport monoaminů v placentě.

Kvůli přísným etickým omezením výzkumu placenty *in vivo* byly použity alternativní modely placenty. K dosažení našich cílů jsme použili řadu experimentálních přístupů, včetně buněčných modelů vývoje syncytií (primární lidské trofoblasty, BeWo a JEG-3 buňky), vilózní fragmenty a membránové vezikuly izolované z lidské placenty po porodu v termínu. Dále byly použity buňky HRP-1 odvozené z placenty potkana a perfúze placenty potkana před termínem, jako zvířecí modely. Exprese genů a proteinů klíčových monoaminových transportérů byla hodnocena pomocí kvantitativní PCR a Western blottingu. Účinky diferenciace trofoblastů a inhibitorů na vychytávání monoaminů byly zkoumány prostřednictvím funkčních studií.

Výsledky našeho výzkumu objasňují změny v transportu monoaminů v diferencujících se trofoblastech a popisují vzory exprese regulačních složek monoaminů ve fetoplacentární jednotce během těhotenství. Dále tato studie zdůrazňuje expresní a funkční rozdíly mezi modely buněk odvozených z placenty, což je klíčové pro výběr optimálního modelu pro výzkum monoaminů. Nakonec naše zjištění naznačují, že léky běžně používané během těhotenství interagují s monoaminovými transportními systémy, inhibují jejich funkci a tím mění homeostázu monoaminů ve fetoplacentární jednotce.

LIST OF ABBREVIATIONS

5-HIAA – 5-hydroxyindoleacetic acid

5-HT – serotonin (5-hydroxytryptamine)

ADHD – attention deficit hyperactivity disorder

ATP – adenosine triphosphate

BCRP – breast cancer resistance protein

BM – basal membrane

COMT – catechol-O-methyltransferase

CTB – cytotrophoblast

DA – dopamine

DAT – dopamine transporter

DBH – dopamine β-hydroxylase

DDC – dopa decarboxylase

DOHaD – Developmental Origins of Health and Disease

ELISA – enzyme-linked immunosorbent assay

GDM – gestational diabetes mellitus

hCG – human chorionic gonadotropin

MAO – monoamine oxidase

MDCKII – Madin-Darby canine kidney

MRP – multidrug resistance-associated protein

MVM – microvillous membrane

NE – norepinephrine

NET – norepinephrine transporter

OCT3 – organic cation transporter 3

P-gp – P-glycoprotein

PHT – primary human trophoblast

PNMT – phenylethanolamine N-methyltransferase

SERT – serotonin transporter

SLC – solute carrier

SNRIs – serotonin and norepinephrine reuptake inhibitors

SSRIs – selective serotonin reuptake inhibitors

STB – syncytiotrophoblast

TH – tyrosine hydroxylase

TPH – tryptophan hydroxylase

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1 INTRODUCTION

The placenta, a temporary fetal organ formed during pregnancy, serves as a crucial interface for immune [1] and xenobiotic [2] protection of the fetus. Additionally, it synthesizes key biogenic amines, essential for fetal and placental development [3, 4], particularly in the early stages of pregnancy. Monoamines like serotonin (5-HT), norepinephrine (NE), and dopamine (DA), pivotal neuromodulators, play significant roles in regulating fetal organ development [5]. NE and DA are also known as catecholamines, a specific subgroup within the broader class of monoamines. However, in this dissertation thesis, all three will be referred to only as monoamines. Their meaning is evident especially in the brain, where they influence neuronal cell proliferation and migration [6]. Research on monoamine synthesis, metabolism, and transport mechanisms within the fetoplacental unit has gained momentum due to its association with fetal programming. It is presumed that perturbations in monoamine signaling may lead to aberrant fetal brain development, potentially resulting in behavioral or psychiatric disorders later in life [4, 7-9].

To maintain monoamine homeostasis, the placenta is equipped with transport systems and a variety of enzymes, primarily located within the trophoblast layer [syncytiotrophoblast (STB) and cytotrophoblast (CTB)]. The STB layer is further polarized into two distinct membranes. The serotonin transporter (SERT) [10] and norepinephrine transporter (NET) [11] have been found expressed on the maternal-directed microvillous membrane (MVM), and the organic cation transporter 3 (OCT3) [12] on the fetal-directed basal membrane (BM). In addition, OCT3 was described earlier in the past as bidirectional [13]. Altogether, these transporters facilitate the movement of monoamines across the placental barrier.

Monoamine synthesis is mediated through a complex of enzymes (Fig. 1), including tyrosine hydroxylase (TH) and dopa decarboxylase (DDC), to yield DA; dopamine β-hydroxylase (DBH) is involved in NE synthesis, which eventually serves for epinephrine production through phenylethanolamine N-methyltransferase (PNMT). 5-HT is created from tryptophan via tryptophan hydroxylase (TPH) and 5-hydroxytryptophan decarboxylase (5-HTPD). Degradation of these monoamines is ensured by monoamine oxidase (MAO) or catechol-o-methyltransferase (COMT), both highly expressed in the placenta [12, 14] (Fig. 1).

While the existence of monoamine transport systems within the trophoblast is known, the specifics of monoamine passage across this layer remain insufficiently explored. The transport mechanisms for 5-HT have been previously studied by our team [12], however, NE and DA handling in the fetoplacental unit remains unclear. Similarly, the expression and functional characteristics of monoamine

transporters in the trophoblast according to its differentiation require further investigation. Monoamine pathway synthesis, metabolism, and transport regulation in the fetoplacental unit across gestation is similarly obscure. Moreover, possible interactions of certain inhibitors, like antidepressants [15] and antidiabetic drugs such as metformin [16-18], drugs commonly used during pregnancy, with monoamine transporters in the placental barrier remain not completely elucidated.

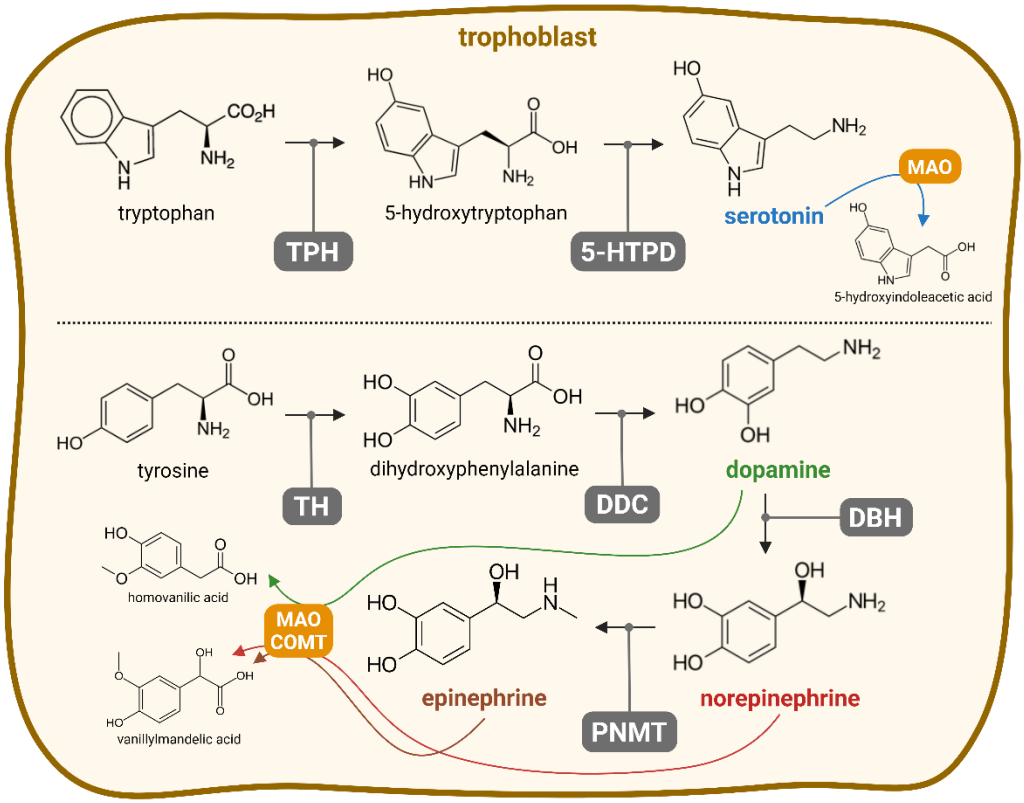

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Fig. 1. Schematic representation of key enzymes involved in monoamine synthesis and metabolism in the trophoblast.

The synthesis of monoamines in the trophoblast is facilitated by a complex cascade of enzymes that convert the precursors tryptophan into 5-HT, and tyrosine into NE, DA, and epinephrine. Subsequently, these monoamines are metabolized by MAO and COMT into their respective metabolites.

Abbreviations: 5-HTPD – 5-hydroxytryptophan decarboxylase, COMT – catechol-O-methyltransferase, DBH – dopamine β-hydroxylase, DDC – dopa decarboxylase, MAO – monoamine oxidase, PNMT – phenylethanolamine N-methyltransferase, TH – tyrosine hydroxylase, TPH – tryptophan hydroxylase

Ethical and technical constraints limit the direct investigation of monoamine homeostasis in the human placenta *in vivo*. However, various experimental approaches were designed, including cell lines derived

from human or rat placenta, such as primary trophoblasts [19, 20], choriocarcinoma-derived BeWo [21], JEG-3 [22] or HRP-1 [23] cells. Experiments using human or rat placental tissues also offer valuable insights into placental monoamine transport mechanisms and regulatory dynamics, such as fresh villous human term placenta isolated fragments [24], vesicles isolated from human term placenta [12, 25], or rat term placenta perfusions [26].

Our work provides comprehensive insights into placental monoamine handling during trophoblast differentiation. We highlight the significance of the CTB and OCT3 in this process. Additionally, our study elucidates the expression of monoamine-related enzymes and transporters, as well as the dynamic regulatory mechanisms governing monoamine handling throughout gestation. Understanding the differences between human and rat placental models is crucial for selecting appropriate *in vitro* models for placental monoamine research. Finally, our findings suggest that pharmacotherapy during pregnancy, such as antidepressants or metformin, may impact monoamine transporter functionality, potentially disrupting monoamine homeostasis in the fetoplacental unit.

2 THEORETICAL BACKGROUND

2.1 PLACENTA AND ITS SIGNIFICANCE IN PREGNANCY

Although transient in nature, the placenta plays indispensable roles in fetal development throughout pregnancy. Serving as a maternal-fetal barrier [27], it safeguards the fetus from potential harm while assuming the functions of the fetal digestive, excretory, respiratory, immune, and endocrine systems within the uterine environment [28]. Additionally, the placenta synthesizes vital substances crucial for fetal development [29]. Alterations in maternal or placental health can have enduring effects on fetal growth and development. This phenomenon, known as fetal programming, may predispose offspring to develop diseases later in life [30]. Therefore, precise placental functions are extremely important during the entire pregnancy.

2.1.1 PLACENTAL DEVELOPMENT

The placenta is a fetal organ sharing genetic makeup with the fetus and extraembryonic membranes [27]. Its development is complicated and involves many key steps. Following fertilization, a 16-cell morula forms from a zygote. During transit to the uterus, the morula absorbs fluid, forming a blastocyst [31, 32], composed of trophectoderm as its outer layer (placenta) and inner cell mass (fetus), creating subjacent extraembryonic mesoderm [33]. Extensive proliferation and differentiation of trophectoderm stem cells give rise to two primary trophoblast lineages: mononucleated CTBs and multinucleated STBs [28]. The STBs form a syncytial layer in direct contact with maternal blood, while CTBs further differentiate into villous and extravillous subtypes [34].

Villous CTBs remain within the placental villi, contributing to their growth. In contrast, extravillous CTBs migrate into the decidua and become invasive extravillous trophoblasts, facilitating the erosion of maternal tissues and remodeling of spiral arteries. Subsequently, cavities filled with maternal blood develop within the syncytial layer, forming the intervillous space [35]. The erosion of maternal tissue leads to the formation of primary villi, which transform into secondary and tertiary villi as extraembryonic mesoderm migrates into them, giving rise to placental vessels [32, 34, 35]. Invasive extravillous trophoblasts derived from the trophoblastic shell penetrate deeper into decidual tissue, replacing the endothelial cells in maternal spiral arteries to establish efficient maternal-fetal blood flow [34]. Blood supply is mediated by two umbilical arteries and one umbilical vein within the umbilical cord [28]. The placental membrane facilitates nutrient and gas exchange, ensuring no mixing of maternal and fetal blood [36].

Placentation involves crucial steps of vascular remodeling, including decidua- and trophoblast-associated stages [37]. Abnormal trophoblast invasion during early pregnancy may lead to placental insufficiency development [38], resulting in conditions such as pre-eclampsia, intrauterine growth restriction, preterm birth, or stillbirth, affecting 10-15% of pregnancies [39].

The fetoplacental unit comprises both fetal and maternal tissues, with the fetal component derived from the chorionic sac and the maternal component from the endometrium. Within this unit lies the intervillous space, the region between the chorionic blood vessels branching from the umbilical vessels and the maternal basal plate. Here, intricately branched villous structures housing the functional units of the placenta facilitate materno-fetal exchange. These villous structures are encased in a highly polarized interface, densely covered with microvilli on their apical surface [27]. The placental membrane, separating maternal and fetal blood within the intervillous space, comprises four layers: the endothelium of fetal capillaries, connective tissue of the villus, and two cellular layers – fetal-facing CTBs and maternal-facing STBs [28]. While it was traditionally believed that the CTB layer diminishes as gestation progresses until its disappearance [40], recent findings underscore its sustained metabolic activity and significance even in later stages of gestation [41, 42].

2.1.2 PROCESS OF SYNCYTIALIZATION

Trophoblast fusion, known as syncytial fusion from its nascent stage, is a pivotal tissue phenomenon crucial for the establishment and progression of pregnancy. This intricate process entails the activation of numerous intracellular pathways and the presence of fusogenic proteins. It manifests as the merging of plasma membranes of closely adjacent cells, culminating in the formation of a multinucleated structure termed a syncytium (STB). Fused cells intermix cytoplasmic contents including RNA, proteins, lipids, and organelles such as the nucleus, endoplasmic reticulum, and mitochondria. The genesis of the first STB occurs during implantation from trophectoderm cells, uniquely equipped to breach the uterine epithelium and facilitate embryo implantation [43].

Although the molecular underpinnings of this process remain incompletely elucidated, it is understood that STB produces human chorionic gonadotropin (hCG), which orchestrates syncytialization via autocrine and paracrine mechanisms [44]. hCG binds to LH/CG-R receptors, triggering intracellular cAMP production in trophoblast cells [45]. Subsequent activation of protein kinase A initiates a cascade leading to the phosphorylation and/or upregulation of specific genes (mediated by the CREB transcription factor, cAMP response element-binding protein, CBP/CREB-binding protein, and P300 [46]), encoding fusogenic proteins like syncytins, cadherin, and connexin [47-50]. This hCG-induced cAMP signaling pathway also amplifies hCG production and secretion in trophoblasts [51].

In vitro, the process of trophoblast fusion can be observed through isolated and purified CTBs aggregating and fusing to form a non-proliferative yet hormonally active multinucleated structure [43, 52]. Choriocarcinoma cells such as BeWo or JEG-3 cells undergo syncytium formation primarily through a cAMP-driven mechanism. Stimulation of BeWo cells with non-physiological agents like forskolin induces substantial alterations in gene and protein expression, ultimately culminating in fusion [53]. Notably, the BeWo cell's syncytialization process is known distinct in syncytium formation with differences seen in epigenetics and regulatory units when compared to physiological primary human trophoblast (PHT) cells [54, 55].

2.1.3 PLACENTAL FUNCTIONS

Throughout gestation, the placenta serves a wide range of functions, yet for the purpose of this study, its transport and endocrine functions are of primary importance. Placental transmission of essential components is crucial for fetal development, including gases, maternal antibodies, and various nutrients such as oxygen, carbohydrates, amino acids, fatty acids, and vitamins [28]. Furthermore, the placenta can produce pregnancy hormones, such as estrogens [28, 56, 57]. In primates, the placenta also typically generates hCG, which stimulates progesterone production by the corpus luteum [58]. In addition, in some animal species, the placenta produces hormones that stimulate maternal blood cell production and increase blood volume [59-62]. Notably, it secretes metabolic hormones like placental growth hormone and placental lactogens, impacting insulin production and inducing insulin resistance in maternal tissues, leading to elevated glucose concentrations in fetal tissues [63, 64]. Moreover, the placenta synthesizes various other substances, such as cytokines, chemokines, eicosanoids, and others [28]. Finally, the placenta can produce monoamines such as 5-HT, NE, and DA [4].

Additionally, the placenta fulfills a crucial immune protection function [65], by supplying the fetus with immunoglobulin G, preparing it for the postnatal period when the fetal immune system remains immature [66]. Simultaneously, the placenta eliminates potentially harmful waste substances from the fetal circulation and safeguards the fetus against pathogens [67]. To shield the fetus from xenobiotics, the placenta is equipped with efflux transporters, such as ATP-binding cassette transporters, expressed on the maternal side of the STB. Notable examples include p-glycoprotein (P-gp) [68] and breast cancer resistance protein (BCRP) [69], which return harmful substances to the maternal circulation. However, the passage of all noxious substances to the fetus cannot be prevented, including anti-Rh antibodies, live vaccines, or certain maternal medications [32].

2.2 FETAL PROGRAMMING

Epidemiological investigations have revealed that placental responses to specific stimuli and insults during critical stages of embryonic and fetal development enable fetal survival, however, at the cost of permanent developmental adaptations [70]. Consequently, these structural, physiological, and metabolic adjustments predispose offspring to various health conditions including mental, cardiovascular, metabolic, and endocrine disorders later in life [71]. This phenomenon, recognized as "fetal programming", "Developmental Origins of Health and Disease (DOHaD)", or the "Barker hypothesis", named after its originator, has been well-established for over two decades [72].

Although a comprehensive elucidation of the molecular mechanisms underlying fetal programming remains elusive, it is postulated that regulatory, metabolic, and endocrine pathways facilitating the communication between maternal and fetal compartments might be responsible [73]. For instance, perturbations such as altered expression of 5-HT receptors or perturbed levels of 5-HT in serotonergic neurons due to external factors like antidepressant usage can lead to deviations in fetal brain development, potentially resulting in anxiety disorders later in life [9]. Furthermore, investigations have demonstrated associations between prenatal conditions (e.g., maternal malnutrition, stress, infections, or exposure to xenobiotics) and the onset of central nervous system disorders, such as psychiatric conditions, autism spectrum disorders, depression, or attention-deficit/hyperactivity disorder (ADHD) in adulthood [74-77].

2.3 MONOAMINES IN THE PLACENTA

Monoamines represent vital endogenous compounds renowned for their multifaceted functions within the nervous system. Notably, 5-HT, NE, and DA serve as chemical messengers facilitating communication between neurons upon interaction with their respective receptors. However, recent insights have highlighted the pivotal involvement of monoamines in fetal development, sparking considerable research interest in placental monoamine handling.

2.3.1 SEROTONIN (5-HT)

Isolated and characterized in 1948 [78], 5-HT is a fundamental biogenic monoamine that functions as a hormone, neurotransmitter, and mitogen [79]. In addition, 5-HT plays a pivotal role in regulating vascular tone, blood pressure, and vasoconstriction within blood vessels [80, 81]. These mechanisms are particularly relevant in pregnancy, as elevated levels of 5-HT observed in pregnant women may contribute to gestational vascular pathologies such as preeclampsia, a condition marked by abnormal blood vessel function [82]. Furthermore, 5-HT is essential to key processes of embryonic development, including placentation, neurogenesis, and cardiac morphogenesis [83].

The synthesis of 5-HT begins with the hydroxylation of the amino acid tryptophan, a reaction catalyzed by a rate-limiting enzyme TPH [84]. The product, 5-hydroxy-L-tryptophan, is then decarboxylated by aromatic L-amino acid decarboxylase to yield 5-HT [84, 85]. Notably, the placenta itself contains the necessary enzymatic machinery for 5-HT synthesis, further highlighting its importance in early fetal development [86]. Once synthesized, 5-HT is transported by SERT and is primarily degraded by MAO-A to yield 5-hydroxyindoleacetic acid (5-HIAA) as its main metabolite [87, 88].

At the onset of gestation, the fetus relies on the placental transport of 5-HT due to its inability to produce it autonomously. However, as pregnancy progresses, the fetus gains the capacity for endogenous synthesis, diminishing its reliance on placental production [3]. Recent investigations underscore the involvement of 5-HT in fetal programming, frequently linked to the genesis of mental disorders in individuals across various life stages. This association is attributed to 5-HT's participation in critical developmental processes such as cell proliferation, migration, and neural circuit formation [9]. Experimental studies in mice have demonstrated that perturbations in prenatal 5-HT signaling can precipitate anxiety disorders in adulthood [89]. Disruptions in the expression of 5-HT receptors or alterations in 5-HT levels, for instance, due to maternal antidepressant use, can lead to aberrant neuronal pathway formation [3, 90]. Hence, it is evident that both genetic predispositions and environmental influences exert profound impacts on the intricate developmental trajectory of the fetal brain, potentially contributing to the onset of mental disorders during childhood or later in life.

2.3.2 NOREPINEPHRINE (NE)

NE, first identified in the 1940s by Swedish physiologist Ulf von Euler [91], serves a multifaceted role in the body as both a neurotransmitter and stress hormone. NE plays a key role in cell proliferation and differentiation, as well as neuronal growth and migration [92]. Furthermore, NE is a crucial factor for postnatal adaptations [93].

NE synthesis commences with the conversion of the aromatic amino acid tyrosine by the rate-limiting enzyme TH to dihydroxyphenylalanine (DOPA), which is subsequently decarboxylated by DDC to DA. The vesicular monoamine transporter facilitates the transport of DA into vesicles, where it undergoes conversion to NE by DBH. NE may be metabolized to epinephrine by PNMT [91]. NE is a substrate of NET [94] and undergoes metabolism via MAO-A through oxidation or COMT through O-methylation [91].

NE emerges as a pivotal factor in fetal organ development, detectable in brain tissue on the 13th day of gestation [95, 96]. It orchestrates the maturation of both target brain structures and noradrenergic neurons during critical fetal developmental periods. Disruptions in this process can precipitate enduring alterations in brain function, thereby influencing behavior, cognition, and mental health

across the lifespan. Notably, noradrenergic pathways attain full maturity only after 5-10 weeks of fetal development, underscoring the criticality of the period preceding their full maturation. Therefore, any aberrations in NE levels during this developmental window can detrimentally impact the establishment of neuronal connections [92]. Research indicates that prenatal circumstances, such as maternal malnutrition, can perturb the neuronal system's development by modulating noradrenergic signaling [97]. Additionally, NE assumes a critical role in heart development. A study involving pregnant mice revealed that constraining NE synthesis and supply from the mother resulted in fetal demise, likely attributed to the heart's incapacity to sustain cardiac rhythm and contractility [98]. These findings underscore the indispensability of proper NE levels for fetal cardiac function and survival.

2.3.3 DOPAMINE (DA)

DA stands as an essential neurotransmitter and a precursor for other monoamines such as NE and epinephrine [99]. It functions as a key regulator of cellular proliferation and differentiation, neuronal growth and migration, and cell cycle modulator [100-103]. In addition, DA also influences placental endocrine functions through the regulation of human placental lactogen and hCG production [104, 105].

The biosynthesis of DA and other monoamines is governed by the rate-limiting enzyme TH [106], which catalyzes the hydroxylation of tyrosine to DOPA [87], a precursor that DDC subsequently decarboxylates to yield DA [91]. DA is a substrate of DAT and is metabolized by the MAO-A or MAO-B and COMT [99].

Human and animal studies have illuminated a significant association between DA transmission and depression [107]. Depressed mothers often exhibit diminished DA levels, exerting a detrimental influence on DA concentrations in their newborns [108]. Behavioral investigations have corroborated these findings, revealing that reduced DA levels in children can adversely impact behavior and temperament [109, 110]. Moreover, disruptions in DA balance, such as those induced by cocaine use, can have harmful effects on neurogenesis [103], potentially manifesting in later-life conditions such as schizophrenia, autism spectrum disorder, or ADHD [99, 103]. Hence, it becomes apparent that a nexus exists between maternal depression, DA levels, and impairment of fetal development. This underscores DA's critical role in maternal mental health and offspring neurodevelopmental outcomes.

2.4 MONOAMINE TRANSPORT IN THE PLACENTA

To ensure the safe passage of essential substances for fetal development, the placenta employs a variety of transport mechanisms. These are crucial for the transport of nutrients, endogenous

substances, and other essential compounds to the developing fetus. However, for the purposes of this thesis, only the transport of monoamines is discussed in detail.

Within the trophoblast layer, transporters crucial for monoamine homeostasis during gestation are situated, akin to those found in the brain. Specifically, these transporters are localized within the STBs, featuring a maternal-facing MVM and a fetal-facing BM (Fig. 2) [111]. Current understanding of monoamine transporter expression in the placenta reveals the localization of high-affinity/low-capacity transporters such as SERT and NET primarily within the MVM [10, 11, 112], while information regarding the presence or absence of the dopamine transporter (DAT) remains limited (Fig. 2). Additionally, the low-affinity/high-capacity OCT3 transporter is situated in the BM, facilitating the uptake of 5-HT from the fetal circulation [12].

Transporter proteins embedded within the placental barrier facilitate transport for hydrophilic or charged molecules incapable of passive diffusion. Facilitated diffusion mediated by transporter proteins occurs bidirectionally and at a higher velocity [113]. A typical example of a transporter functioning via facilitated diffusion is a high-capacity/low-affinity transporter OCT3 [114-117]. On the other hand, active transport requires the expenditure of energy in the form of ATP. This transport mechanism is typical for high-affinity/low-capacity transporters SERT, NET, and DAT [118, 119].

2.4.1 TRANSPORTERS EXPRESSED ON MICROVILLOUS MEMBRANE

This chapter describes SERT, NET, and DAT transporters, characterized as neurotransmitter Na^+-symporters [120]. These secondary active proteins exploit the Na^+ gradient to facilitate the inward transport of monoamines from the extracellular milieu via a cotransport (symport) mechanism, counteracting the prevailing concentration gradient. Throughout this transport process, Na^+ and Cl^- ions are translocated in opposite directions, with the driving force stemming from the ion concentration gradient established by the Na^+/K^+-ATPase [121].

The physiological significance of these transporters is extensive, exerting influence over myriad biological processes encompassing synaptic transmission, metabolic regulation, and fluid homeostasis [119]. Moreover, their pivotal role within the brain is evident as they mediate the termination of nerve signaling via monoamine reuptake for subsequent reuse [122]. Consequently, they represent prime targets for pharmacological intervention, notably in the realm of antidepressant therapy, wherein their inhibition augments monoamine concentration within the synaptic cleft [123].

2.4.1.1 NOREPINEPHRINE TRANSPORTER (SLC6A2/NET)

The NET transporter exhibits a widespread expression pattern across various organs, including the brain [124], kidney [125], lung [126], and placenta [11, 127]. Its expression in the placenta remains

relatively stable throughout gestation, with a marginal increase noted during the second trimester followed by a subsequent decrease [128].

Within the binding site of NET, specific amino acid residues facilitate Na^+ ion binding, thereby modulating transporter functionality [129]. Normally mobile within the cytoplasm, NET undergoes immobilization upon Na^+ binding. Consequently, the Na^+ gradient established by the Na^+/K^+-ATPase governs the localization of NET within the cell membrane, with the ions themselves playing a pivotal role in the active transport of substrates [130]. Transport occurs via the utilization of one molecule each of Na^+ and Cl^- [131], resulting in NE transport across the membrane at a 1:1:1 $NE/Na^+/Cl^-$ stoichiometry and ensuing positive charge transfer [132]. Additionally, K^+ is transiently bound and subsequently released during transport [133], although some studies suggest transport remains unaffected even under conditions of complete K^+ deficiency [134].

Given NE's vasoconstrictive properties [135], NET-mediated uptake from maternal circulation assumes critical importance in preventing high resistance within chorionic veins, thereby ensuring adequate maternal blood flow through the placenta. Furthermore, placental NE uptake is indispensable for fetal development, particularly during early gestation when the fetus lacks the capacity for endogenous NE synthesis [136]. Additionally, 5-HT and DA are recognized as substrates of NET [94, 137, 138] which has been confirmed in the placenta (Fig. 2) [139].

Inhibition of NET function can be achieved with certain therapeutic drugs used in depression, ADHD, or anxiety management. Such inhibition leads to increased NE availability for binding to postsynaptic receptors, thereby modulating adrenergic neurotransmission. Notably, atomoxetine stands as a specific NET inhibitor [140]. Other inhibitory agents include antidepressants, albeit with less selectivity, as they may also inhibit SERT [141]. Importantly, inhibitor binding to NET is contingent upon the presence of Na^+ [142].

2.4.1.2 DOPAMINE TRANSPORTER (SLC6A3/DAT)

DAT expression is predominantly observed within dopaminergic innervation pathways of the brain, encompassing the mesostriatal, mesocorticolimbic, and nigrostriatal pathways [143]. Notably, DAT expression has also been detected in immune cells such as lymphocytes [144]. The presence of DAT in placental tissue remains uncertain, with current conjecture suggesting that the transport of DA in the placenta is mediated by SERT, NET, and OCT3 (Fig. 2) [138, 139, 145, 146].

Two distinct binding sites are discernible on DAT, one capable of binding DA while the other binds non-competitive and allosteric DAT inhibitors [147]. Analogous to NE transport, the transport of DA is contingent upon a single Cl^- ion, although two Na^+ ions participate in the process. Consequently, the

stoichiometry for DA transport is established at 1:2:1 for DA/Na^+/Cl^- [148]. Similarly to NET, K^+ is transiently bound and subsequently released during transport [133].

Any perturbation in DAT activity may precipitate behavioral disorders, including depression, bipolar disorder, or ADHD [149]. Furthermore, DAT represents a target not only for medications but also for addictive substances that modulate DAT function, such as cocaine, amphetamine, or methamphetamine [150, 151].

2.4.1.3 SEROTONIN TRANSPORTER (SLC6A4/SERT)

SERT, regarded as the most extensively characterized transporter so far, exhibits a broad expression profile involving various tissues, including the brain [152], intestine [153], rat adrenal chromaffin cells [154], mast cells, and platelets [155], lungs [156], skin cells [157] along with placental tissue [10, 112]. Notably, SERT gene expression is initially low in the placenta in the first trimester but escalates as pregnancy progresses [128].

The substrate-binding site, juxtaposed with an ion-binding site, is situated approximately midway through the membrane layer and is comprised of five transmembrane helices [158]. An additional allosteric binding site for small molecules is situated extracellularly on the transporter [159], where antidepressants, such as specific serotonin reuptake inhibitors (SSRIs, 5-HT reuptake blockers), can bind [160]. Transport entails the co-transport of 5-HT with one Na^+ and one Cl^-, facilitated by the energy derived from the gradient established by the Na^+/K^+-ATPase [161]. Consequently, the stoichiometry of this transport process is established at 1:1:1 for 5-HT/Na^+/Cl^-. The binding of 5-HT, Na^+, and Cl^- to SERT elicits a conformational alteration in the transporter, exposing the binding site on the opposite membrane surface and concomitantly translocating the substrate and ions. This reorientation necessitates an additional step involving the binding and outward transport of intracellular K^+ [121, 133].

During early pregnancy, the fetus heavily relies on 5-HT supplied via SERT, as endogenous 5-HT synthesis is not yet available, although this dependency diminishes with gestation age [3]. The absorption of 5-HT from maternal circulation assumes significance due to its vasoconstrictive effects [162]. Intriguingly, SERT serves not only as a dedicated 5-HT transporter but also mediates the transport of NE [163] and DA [164], as evidenced by studies utilizing knock-out technology in mice. Furthermore, a recent study of ours has also confirmed that SERT contributes to placental NE and DA uptake (Fig. 2) [139].

SERT manifests sensitivity to various inhibitors, including antidepressants from the SSRI class, which exert their effects by inhibiting 5-HT reuptake [165]. Other inhibitors of SERT function include tricyclic

antidepressants (e.g., imipramine) and substances that induce transport reversal (e.g., amphetamine derivatives MDMA or ecstasy) [166].

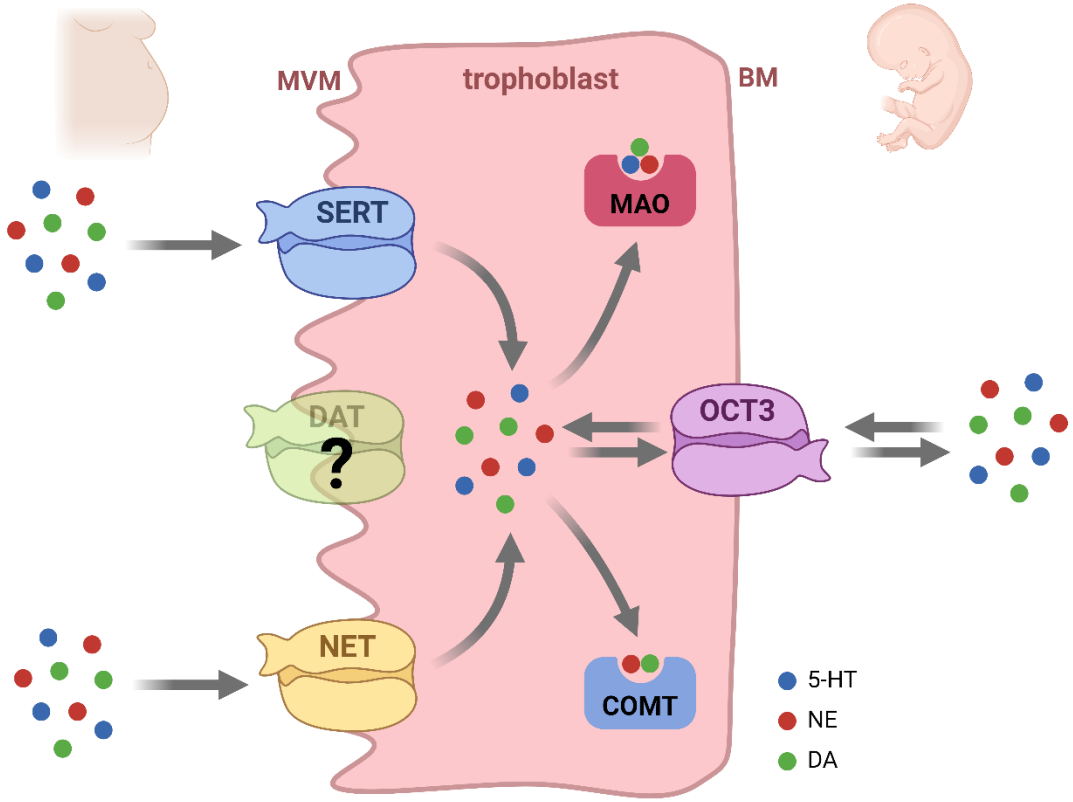

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Fig. 2. Summary of monoamine transport mechanisms in the human trophoblast.

Monoamines are transported into the trophoblast from the maternal circulation via SERT and NET. The presence of the DAT in the human placenta and its role in monoamine transport within the trophoblast remains controversial. Once within the trophoblast, monoamines are exported into the fetal circulation by OCT3. Due to the bidirectional nature of OCT3, monoamines can also be transported back from the fetal side into the trophoblast, where they can be metabolized by MAO or COMT.

Abbreviations: 5-HT – serotonin (5-hydroxytryptamine), BM – basal membrane, COMT – catechol-O-methyltransferase, DA – dopamine, DAT – dopamine transporter, MAO – monoamine oxidase, MVM – microvillous membrane, NE – norepinephrine, NET – norepinephrine transporter, OCT3 – organic cation transporter 3, SERT – serotonin transporter

2.4.2 TRANSPORTERS EXPRESSED ON BASAL MEMBRANE

2.4.2.1 ORGANIC CATION TRANSPORTER 3 (SLC22A3/OCT3)

OCT3 exhibits abundant expression in the human heart [167], with notable expression levels also detected in skeletal muscle, prostate, adrenal, and salivary glands [168]. Lower levels of expression have been documented in tissues such as the liver, colon, lung, small intestine, stomach, testis, bone marrow, adipose tissue, trachea, thyroid gland, uterus, and placenta. Minor expression has been observed in the kidney, urinary bladder, brain, and monocytes [116]. Its expression in the placenta remains relatively stable throughout gestation, with only a slight increase observed [169].

Operating via facilitated diffusion, the OCT3 transporter translocates substrates across the plasma membrane bidirectionally, with the driving force being the electrochemical gradient of the transported molecules [170]. However, its functionality remains unaltered by Na^+ [171].

OCT3 plays a crucial role in maintaining monoamine homeostasis, with *in vivo* studies demonstrating that OCT3-mediated transport influences extracellular monoamine concentrations [172-174]. Moreover, it is recognized that this transporter actively takes up 5-HT from the fetal bloodstream and transports it to the trophoblast, where it undergoes subsequent degradation. This protective mechanism serves to prevent excessive fetal concentrations of 5-HT [12]. In addition to biogenic amines such as 5-HT, NE, DA (Fig. 2), and histamine [116, 139, 146, 175], OCT3 is capable of transporting drugs such as metformin [168, 176]. Notably, OCT3 activity can be attenuated by the specific inhibitor corticosterone [173].

2.5 FACTORS AFFECTING MONOAMINE HOMEOSTASIS IN THE FETOPLACENTAL UNIT

During pregnancy, various pathological conditions or exposure to xenobiotics can disrupt monoamine homeostasis within the fetoplacental unit. Pharmacotherapeutic agents that can cross the placenta may enter the fetal circulation, potentially compromising fetal development either directly or by altering monoamine regulation in the placenta. Disruptions in monoamine balance within the fetoplacental unit can subsequently influence fetal programming, leading to long-term adverse effects in the offspring. Therefore, this dissertation explores the impact of commonly prescribed pharmacotherapies during pregnancy, including antidepressants and antidiabetic medications such as metformin, on monoamine transport in the placenta.

2.5.1 DEPRESSION IN PREGNANCY AND ITS TREATMENT

Depression during pregnancy has emerged as a prevalent concern in recent times, affecting approximately 20-40% of pregnant women [177]. According to the monoamine hypothesis, depression arises from dysregulated monoamine neurotransmission [178]. Antidepressants are widely acknowledged as highly efficacious in alleviating depressive symptoms, yet they are also prescribed for various other psychiatric conditions, including anxiety disorders. Despite warnings issued by the Food and Drug Administration, the prevalence of antidepressant use during pregnancy continues to rise, with at least 13% of women reported to have taken at least one antidepressant during this period [179]. Major depressive disorder during pregnancy poses substantial risks to fetal health, including increased infant morbidity and mortality rates, such as preterm birth, low birth weight, gestational diabetes mellitus (GDM), and hypertensive disorders [180, 181].

The most frequently used antidepressants during pregnancy belong to the class of SSRIs and selective noradrenaline reuptake inhibitors (SNRIs), both are considered the first-choice pharmacotherapy [182, 183]. These medications exert their therapeutic effects by inhibiting the reuptake of monoamines from the synaptic cleft, thereby elevating their extraneuronal concentrations [184]. Indeed, while the inhibition of monoamine reuptake occurs rapidly after initiating treatment with antidepressants, the therapeutic effects often manifest with a delay of 2 to 4 weeks. This delayed onset of action might be explained by the downregulation of presynaptic inhibitory receptors over time, followed by enhanced synthesis and release of monoamines into the synaptic cleft [185].

2.5.1.1 SELECTIVE SEROTONIN REUPTAKE INHIBITORS (SSRIs)

SSRI antidepressants are commonly regarded as first-line pharmacotherapy for depression treatment, owing to their high efficacy and favorable tolerability profile [186]. This class of medications comprises six main drugs with differing chemical structures but similar mechanisms of action: fluoxetine, citalopram, escitalopram, paroxetine, sertraline, and fluvoxamine [187]. Common side effects associated with SSRI treatment include anxiety, restlessness, insomnia, dry mouth, weight gain, nausea, diarrhea, sweating, headaches, dizziness, decreased libido, tremors, and ejaculation problems [188]. A potentially life-threatening side effect that warrants attention is 5-HT syndrome [189]. However, in comparison to other classes of antidepressants such as MAO inhibitors or tricyclic antidepressants, SSRIs exhibit much greater specificity for the SERT, thereby minimizing side effects associated with the blockade of antimuscarinic, antihistamine, or antiadrenergic receptors [190]. Among SSRIs, escitalopram demonstrates the highest specificity for the SERT receptor and is nearly twice as effective as its counterpart citalopram [191].

Metabolism of SSRI antidepressants occurs primarily through the cytochrome P450 system in the body. Fluvoxamine, fluoxetine, and sertraline inhibit specific cytochrome P450 enzymes, which can lead to drug-drug interactions. Moreover, fluoxetine, paroxetine, and fluvoxamine inhibit their metabolism, potentially posing a significant challenge in cases of concurrent liver or kidney disease, or in elderly individuals [192]. Notably, all SSRIs inhibit the CYP2D6 enzyme, with fluoxetine and paroxetine exhibiting the strongest inhibition [193].

2.5.1.2 SEROTONIN AND NOREPINEPHRINE REUPTAKE INHIBITORS (SNRIs)

SNRIs, or 5-HT and NE reuptake inhibitors, encompass antidepressants such as venlafaxine and duloxetine. Their mechanism of action entails inhibiting the reuptake of both 5-HT and NE from the synaptic cleft. These antidepressants are commonly prescribed for severe depression as well as anxiety disorders.

Venlafaxine exhibits a thirty times greater affinity for inhibiting 5-HT reuptake than NE [194]. Consequently, inhibition of 5-HT reuptake precedes that of NE reuptake, leading to the manifestation of side effects primarily associated with 5-HT reuptake inhibition, such as nausea, headache, sexual dysfunction, and fatigue, followed by side effects from NE reuptake inhibition, including dry mouth and night sweats [195]. As a result, SNRIs are often more effective in achieving treatment response and remission than SSRIs. In contrast, duloxetine displays approximately three times lower selectivity for inhibiting 5-HT reuptake. Relative to SSRIs, SNRIs have relatively short half-lives, approximately 4 hours for venlafaxine (longer half-life of 10 hours for the active metabolite) and 12 hours for duloxetine [196].

Metabolism primarily occurs in the liver via the cytochrome P450 system; venlafaxine is primarily metabolized by the 2D6 and 3A4 isoenzymes, while duloxetine is metabolized by the 2D6 and 1A2 isoenzymes. Additionally, venlafaxine undergoes metabolism to the active metabolite o-desmethyl venlafaxine, also known as desvenlafaxine [195].

2.5.1.3 EFFECT OF ANTIDEPRESSANTS ON PREGNANCY AND FETUS

It is reasonable to speculate that drugs affecting 5-HT handling, such as SSRIs or SNRIs, may impact the development of the embryo or fetus's brain, potentially leading to various neurobehavioral disorders. Given their ability to cross the placenta [197, 198] and blood-brain barrier [199], these medications can elevate brain 5-HT levels and modify 5-HT pathways, thus influencing behavior later in life. Therefore, it is not surprising that the use of antidepressants during pregnancy has been associated with the emergence of behavioral symptoms in offspring [200]. Various neurological and psychiatric disorders, including ADHD, autism spectrum disorder, and depression have been observed [201-204].

Moreover, even antidepressant use in the third trimester is not devoid of adverse effects on the fetus, as hyperglycemia, respiratory difficulties, muscle weakness, and general restlessness have been reported [205]. Additionally, symptoms such as vomiting, tremors, irritability, feeding difficulties, cyanosis, apnea, or spasms may also manifest [206-209]. Hence, these effects on the fetus could stem from direct or indirect actions of SSRIs or SNRIs, or they may represent withdrawal symptoms. In certain cases, the clinical presentation resembles that of 5-HT syndrome [182].

Furthermore, findings from numerous cohort and case-control studies suggest that antidepressant use during pregnancy may elevate the risk of cardiac and other serious congenital malformations in the fetus [210-215]. Cardiac malformations, including ventricular septal defects and atrial septal defects, were initially reported with the antidepressant paroxetine [216-218], while malformations have also been documented with fluoxetine [217, 219]. Additionally, antidepressant use has been associated with a higher incidence of spontaneous abortions and stillbirths [182, 220]. *In vitro* studies utilizing placental cell lines have demonstrated that SSRIs can induce placental dysfunction via their mechanism of action [221-223].

2.5.2 GESTATIONAL DIABETES MELLITUS AND ITS TREATMENT

GDM represents the most prevalent complication during pregnancy, affecting approximately 13.2% of pregnant women [224], defined as glucose intolerance diagnosed during pregnancy [225]. GDM is closely associated with adverse pregnancy outcomes, including increased birth weight, premature birth, and preeclampsia, and often necessitates cesarean section [226, 227]. Furthermore, the detrimental effects of GDM can extend to the fetus; maternal hyperglycemia induces fetal hyperinsulinemia, which may lead to various metabolic, cardiac, neurological, or hematological disorders [228, 229].

Traditionally, insulin is the preferred treatment for GDM and is considered the primary therapeutic option [230]. Insulin molecules generally do not traverse the placental barrier [231] due to their molecular size, rendering them safe for the fetus. Metformin, an oral antidiabetic medication, offers certain advantages over insulin, such as non-invasive administration and lack of weight gain effects [232]. However, unlike insulin, metformin has been shown to cross the placenta [233-235]. Despite numerous studies investigating its safety for fetal development [236-238], caution is advised when considering its use during pregnancy.

2.5.2.1 METFORMIN

Metformin, a derivative of biguanide and a prototype of galegine — an alkaloid sourced from *Galega officinalis* [239] — exerts its blood glucose-lowering effects by reducing hepatic glucose production

and intestinal glucose absorption [240]. Discovered in the 1920s, metformin's efficacy in lowering blood glucose levels was swiftly recognized [241]. Today, it is primarily prescribed for managing type 2 diabetes mellitus, demonstrating efficacy in obese patients, with additional cardioprotective benefits and weight-reducing effects [242]. Beyond its diabetic indications, metformin is utilized for preventing diabetes and treating polycystic ovary syndrome, where it improves menstrual cycle regularity and enhances fertility in women [243].

Following oral administration, approximately 70% of metformin is absorbed from the small intestine into the bloodstream, with the remaining 30% excreted in feces [244]. Metformin plasma concentrations typically range between 8-24 µmol/l post-absorption [245]. Upon absorption, metformin predominantly accumulates in the intestines, liver, kidneys, and bladder via OCTs [244, 246]. Notably, metformin undergoes minimal metabolism in the body and is excreted unchanged in urine via active tubular secretion [247].

The mechanism of metformin's action in reducing blood glucose levels is multifaceted and involves various pathways that enhance insulin utilization. Consequently, metformin belongs to the class of "insulin sensitizers". In the liver, metformin reduces gluconeogenesis and, to a lesser extent, glycogenolysis, thereby diminishing endogenous glucose production. Specifically, metformin activates adenosine monophosphate kinase by inhibiting mitochondrial function [248], subsequently downregulating key gluconeogenesis enzymes in the liver [249].

2.5.2.2 EFFECT OF METFORMIN ON PREGNANCY AND FETUS

The use of metformin during pregnancy remains a subject of debate, despite some studies suggesting it may offer advantages over insulin in terms of risk-benefit profiles [250]. However, evidence indicates that metformin use during pregnancy may lead to adverse outcomes compared to insulin, including reduced birth weight, accelerated postnatal growth, and various cardiac and metabolic abnormalities [251, 252]. Additionally, research suggests that metformin's negative effects on the fetus could potentially be mediated through epigenetic mechanisms, such as DNA methylation or altered activity of histone modification enzymes [253].

Moreover, metformin has been identified as both a substrate and an inhibitor of OCT3 [16, 17]. This interaction could impact fetal development, as demonstrated by a study in mice linking prenatal metformin exposure and OCT3 transporter activity to alterations in offspring social behavior [254]. Therefore, the use of metformin during pregnancy warrants caution, as evidence suggests it may affect fetal metabolism [255] and potentially alter fetal programming [256, 257].

3 AIMS OF THE DISSERTATION THESIS

The dissertation aims to investigate the monoamine homeostasis systems in the trophoblast, focusing on their expression, functionality, and regulation. Specifically, it seeks to achieve the following objectives:

a) Characterize the regulation of monoamine transporters in the trophoblast:
   - investigate the gene and protein expression of SERT, NET, DAT, and OCT3 in PHT and BeWo cells throughout trophoblast differentiation
   - describe the physiological functionality of these transporter systems in the trophoblast

b) Analyze the synthesis, metabolism, and transport of monoamines in the fetoplacental unit:
   - study the expression and differentiation changes (where possible) of monoamine pathway-related enzymes and transporters in trophoblast cell models:
     i. human – PHT, BeWo, JEG-3
     ii. rat – HRP-1
   - examine the regulation of monoamine synthesis, metabolism, and transport in the placenta across different gestational ages:
     i. human placenta – first trimester, term
     ii. rat placenta – GD 15, GD 18, GD 21

c) Determine the inhibitory potential of selected clinically used drugs on 5-HT homeostasis in the placenta:
   - antidepressants:
     i. SSRIs – citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline
     ii. SNRI – venlafaxine
   - antidiabetics (metformin)

4 METHODOLOGY

When investigating monoamine homeostasis in the placenta, conducting *in vivo* studies directly in humans is not feasible due to technical and ethical limitations. Therefore, a variety of alternative experimental approaches have been developed to investigate placental physiology and pharmacology. In this dissertation, the following methods were used (Fig. 3):

1. *In vitro* models: Culturing human or rat placental cells in a controlled laboratory environment allows to study the monoamine transport, metabolism, and regulation under controlled conditions. This approach enables to manipulate variables and assess their effects on monoamine homeostasis.

2. *Ex vivo* methods: Utilizing placental tissue obtained from human donors provides a more physiologically relevant setting compared to *in vitro* cell culture. *Ex vivo* experiments allow for the assessment of monoamine transporter function in placental tissue (fragments or membranes), maintaining the tissue's native architecture and cellular interactions as closely as possible.

3. *In situ* techniques: Employing methodology such as rat term placenta perfusions allows to study the monoamine handling in a setting that closely mimics *in vivo* conditions. By perfusing the placental tissue with a controlled medium, it is possible to assess the transport of monoamines across the placental barrier and evaluate the impact of various factors on monoamine homeostasis.

4.1 *IN VITRO* CELLULAR MODELS

Cellular *in vitro* models form a substantial part of this dissertation. PHT cells freshly isolated from healthy term placentas through trypsin digestion and Percoll gradient centrifugation serve as a physiological representation of the placenta. Additionally, human-derived BeWo choriocarcinoma cells, commonly used in placental trophoblast research, are employed. In differentiation studies, PHT-CTB cells are cultured for 72 hours, spontaneously differentiating into PHT-STB cells. Conversely, BeWo cells require induction with 20 µM forskolin to initiate differentiation, occurring over 48 hours. The successful differentiation of cells into STB was confirmed by assessing the release of hCG, a marker of trophoblast differentiation, via ELISA (Fig. 3).

Additionally, JEG-3 cells, another choriocarcinoma-derived cell line, and the rat trophoendodermal cell line HRP-1 were utilized in studies involving monoamines and gestational age. Finally, Madin-Darby canine kidney II (MDCKII) parental cells, along with MDCKII cells transfected with human efflux

transporters P-gp (MDCKII-P-gp), BCRP (MDCKII-BCRP), and MRPs (MDCKII-MRP2) were included in the antidepressant study.

Experimental procedures using cell cultures are very briefly described. *In vitro* monoamine uptake studies were performed with 96-well TPP culture plate setup where PHT or BeWo cells were seeded. Uptake of monoamines was performed by preincubation containing blank or inhibitor/drug of interest for 10 minutes, followed by incubation with ^{3}H-(monoamine), ascorbic acid, phenelzine, and entacapone (the latter for NE and DA only) with or without the inhibitor/drug of interest for 15 minutes at 37˚C. Uptake was stopped by ice-cold Dulbecco's Phosphate Buffered Saline at 4˚C. Finally, cells were lysed in 0.5 M KOH or 0.02% SDS respectively, accumulated levels of ^{3}H-(monoamine) were measured by scintillation counting, and protein content was measured by BCA-assay (Fig. 3). *In vitro* bidirectional transport of selected antidepressants studies was performed using MDCKII cells on Transwell® polycarbonate membrane inserts. ^{3}H-(antidepressant) has been added in OptiMEM into the donor compartment. Samples were consequently collected from the acceptor compartment and measured by scintillation counting. For detailed descriptions of the experimental methods, readers are referred to the individual publications.

However, it is worth noting that the use of BeWo and HRP-1 cell lines is not recommended for studies of monoamine homeostasis. This recommendation stems from notable differences observed at both the transcriptional and functional levels when compared to the human or rat placenta, respectively.

4.2 *EX VIVO* FRESH VILLOUS HUMAN TERM PLACENTA ISOLATED FRAGMENTS

The *ex vivo* methodology utilizing fresh villous fragments isolated from a human term placenta constitutes an approach with preserved tissue integrity, encompassing both CTB and STB cells. Human placental fragments were affixed to hooks and sequentially immersed in an equilibrium solution of Tyrode´s buffer and Dulbecco´s Modified Eagle Medium (DMEM) for 30 minutes at 37°C, followed by a pre-wash solution containing blank or the inhibitor/drug of interest for 15 minutes at 37°C. Incubation has been initiated by immersing the fragments into the solution of the ^{3}H-(monoamine), ascorbic acid, and phenelzine with or without the inhibitor/drug of interest for an additional 15 minutes at 37°C. The reaction was terminated by subjecting the tissue to wash in Tyrode's buffer twice, after which they were left overnight in distilled water to facilitate the release of radioactivity. To determine protein concentrations within the samples, the fragments were lysed in a 0.3 M NaOH solution for 6-8 hours at 37°C with continuous agitation (Fig. 3).

4.3 *EX VIVO* HUMAN TERM PLACENTA ISOLATED VESICLES

The isolation of membrane vesicles (BM and MVM) from the human term placenta entails a series of different centrifugation steps, magnesium ion (Mg^{2+}) precipitation, and sucrose gradient fractionation. This methodological approach proves invaluable for facilitating high-throughput screening aimed at elucidating transporter-mediated mechanisms operative across distinct placental membranes. A notable limitation of this method is the inherent challenge of achieving consistent and successful isolation of the membrane vesicles, which can be prone to damage, particularly upon repeated thawing and freezing cycles during storage.

Briefly, the uptake of ^{3}H-(monoamine) into MVM or BM vesicles was measured at room temperature with the use of a rapid vacuum filtration technique. MVM or BM vesicle suspension was preincubated with a solution of blank or the inhibitor/drug of interest for 10 minutes at room temperature followed by incubation with a solution of ^{3}H-(monoamine) and ascorbic acid with or without the inhibitor/drug of interest for 10 minutes at room temperature. Reactions were stopped by ice-cold STOP-solution and samples were filtered through a mixed cellulose ester filter under vacuum. Radioactivity captured on the filter has been measured by liquid scintillation counting. Uptake was compared to that of inhibitor-free controls (Fig. 3). For detailed descriptions of the experimental method, readers are referred to the specific publication (appendix No. 3).

4.4 *IN SITU* RAT TERM PLACENTA PERFUSIONS

Placental perfusion stands as a pivotal methodology for investigating the transport dynamics of monoamines across the placenta. While *ex vivo* perfusion of the human placenta is employed in certain regions, providing a remarkably faithful representation of *in vivo* conditions, this dissertation utilized *in situ* perfusion of the rat placenta. Through placental perfusion, researchers can meticulously track the extraction of substances across the placenta, discern their transit between maternal and fetal circulation, and observe metabolic processes.

In this dissertation, both unilateral and dual perfusion techniques were employed. The procedure involved cannulation of umbilical (and uterine, depending on the experimental setup) arteries and veins. A perfusion solution containing blank or the inhibitor/drug of interest was introduced for 10 mins at 37°C, followed by a solution of ^{3}H-(monoamine) and ascorbic acid with or without the inhibitor/drug of interest, and samples were systematically collected at 5-minute intervals over 40 minutes. Subsequently, the radioactivity content of these samples was quantified via scintillation counting, facilitating the elucidation of monoamine transport kinetics across the placenta (Fig. 3).

4.5 mRNA AND PROTEIN EXPRESSION DETERMINATION

Following the isolation of mRNA and protein from cellular or tissue samples, their respective expression levels were assessed utilizing PCR (qPCR, and ddPCR) and Western blot techniques.

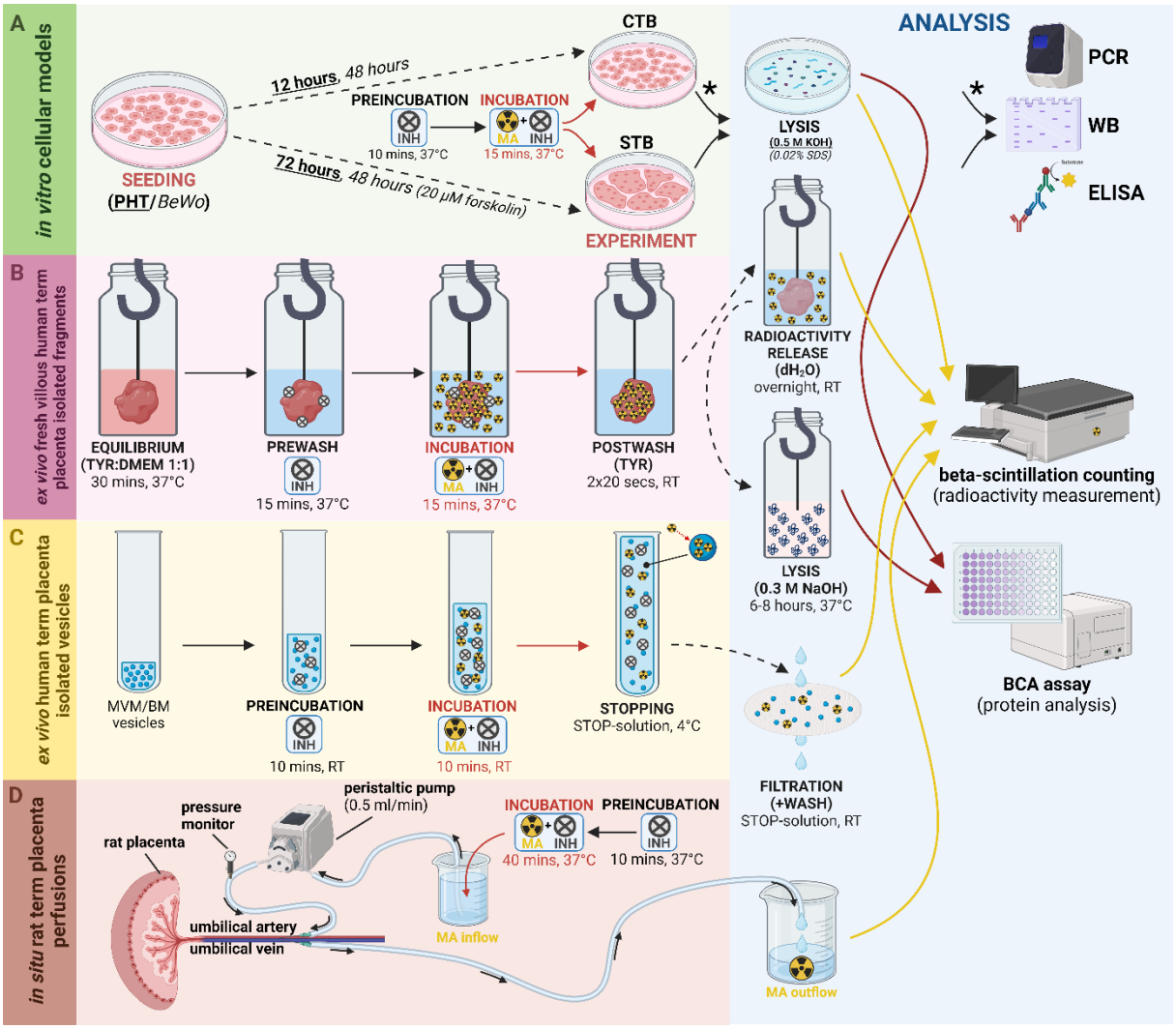

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Fig. 3. Summary scheme of selected methods used in the study.

(A) PHT and BeWo cells, cultured under specific conditions to differentiate into CTB or STB phenotypes, were employed as *in vitro* models. Experiments involved preincubation with inhibitors, followed by incubation with radiolabeled monoamines. After cell lysis, intracellular radioactivity was quantified using beta-scintillation counting, and protein content was determined via the BCA-assay. (B) Villous fragments were isolated from the human placenta, suspended on hooks, equilibrated, and prewashed with inhibitor solution before incubation with radiolabeled monoamines. Post-incubation, the fragments were washed, and the released radioactivity was measured by beta-scintillation counting.

The fragments were subsequently lysed, and protein content was measured using the BCA-assay. (C) After isolation, MVM and BM vesicles were preincubated with inhibitors and then incubated with radiolabeled monoamines. Uptake was halted by adding ice-cold stopping solution, and the vesicles were vacuum-filtered, adhering to the filter. Captured radioactivity was quantified using beta-scintillation counting. (D) For *in situ* rat term placenta perfusions, the placenta was cannulated from the fetal side, and an inhibitor solution, followed by a solution with radiolabeled monoamine, was infused. The effluent was collected, and the washed-out radioactivity was measured using beta-scintillation counting.

Abbreviations: BM – basal membrane, CTB – cytotrophoblast, DMEM – Dulbecco´s Modified Eagle Medium, INH – inhibitor, MA – monoamine, MVM – microvillous membrane, PHT – primary human trophoblast, RT – room temperature, STB – syncytiotrophoblast, TYR – Tyrode's buffer

5 COMMENTS ON INDIVIDUAL PUBLICATIONS AND CONTRIBUTION OF THE CANDIDATE

The dissertation comprises four publications featured in international journals with established impact factors. Within this body of work, the candidate assumes the role of first author in two instances, shares joint first authorship in one publication, and contributes as a co-author to another.

5.1 FUNCTIONAL REORGANIZATION OF MONOAMINE TRANSPORT SYSTEMS DURING VILLOUS TROPHOBLAST DIFFERENTIATION: EVIDENCE OF DISTINCT DIFFERENCES BETWEEN PRIMARY HUMAN TROPHOBLASTS AND BeWo CELLS

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Reproductive biology and endocrinology, 2022 Aug 4;20(1):112, **(IF = 5.211/Q1, AIS = 1.094/Q1)**

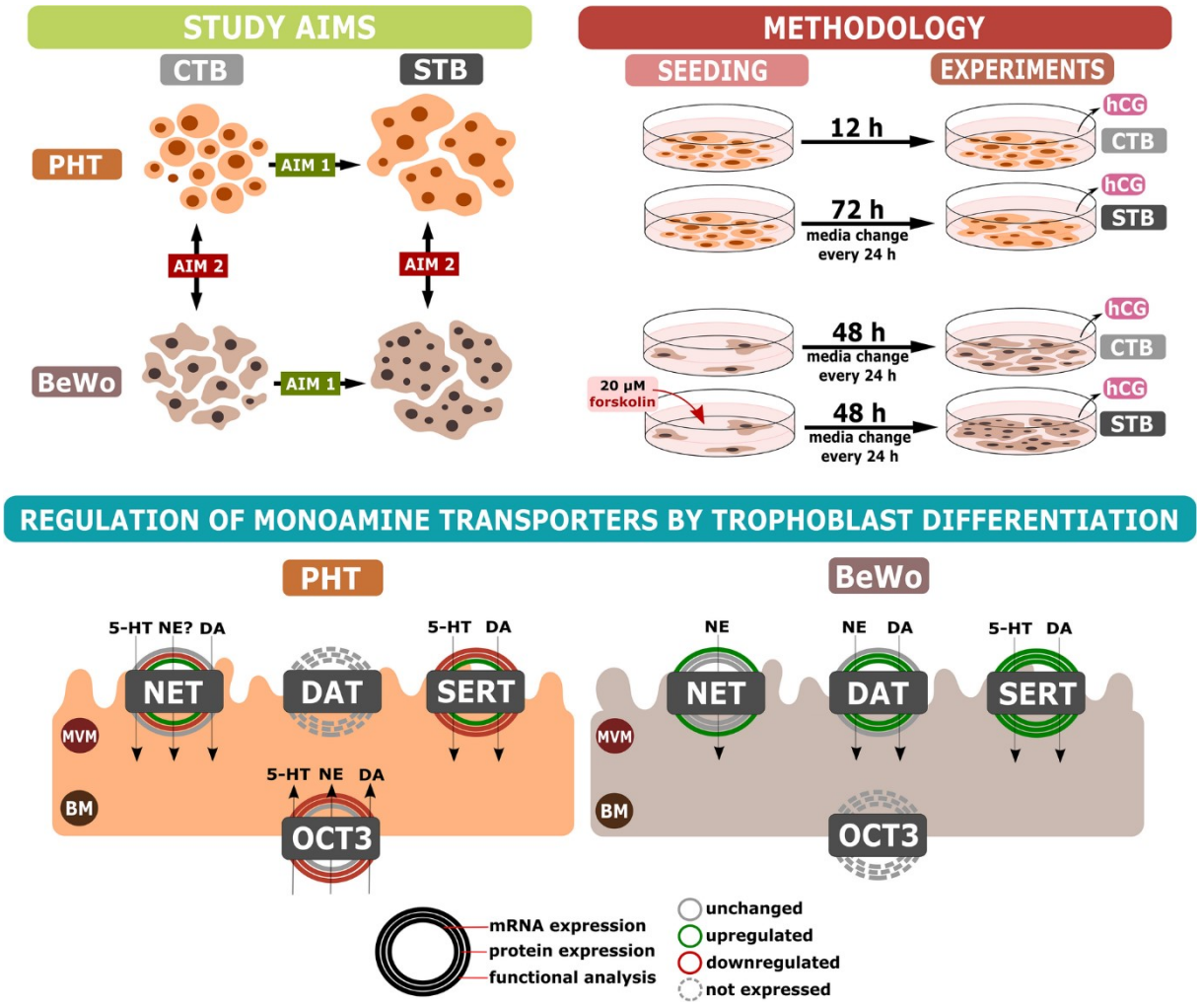


Fig. 4. Graphical abstract of the objectives, methodology, and results of the study.

Abbreviations: 5-HT – serotonin (5-hydroxytryptamine), BM – basal membrane, CTB – cytotrophoblast, DA – dopamine, DAT – dopamine transporter, hCG – human choriogonadotropin, MVM – microvillous membrane, NE – norepinephrine, NET – norepinephrine transporter, OCT3 – organic cation transporter 3, PHT – primary human trophoblasts, SERT – serotonin transporter, STB – syncytiotrophoblast

Biogenic amines (5-HT, NE, and DA) play indispensable roles in fostering optimal fetal growth and development. The transporters governing this regulation (SERT, NET, and OCT3) are localized within the placental trophoblast. Previous research has established that trophoblast differentiation is concomitant with modulation in the expression of genes encoding functionally related proteins. However, to date, no studies have comprehensively elucidated the expression and functional alterations of monoamine transport systems in the placenta throughout trophoblast differentiation, particularly in undifferentiated CTB.

Thus, our investigation utilized two complementary *in vitro* placental models (PHT and BeWo) in conjunction with molecular techniques, including qPCR and Western blot analysis, to delineate the gene and protein expression patterns of SERT, NET, and OCT3 during trophoblast phenotypic transitioning. Both expressional and functional studies involved syncytialization of the cells in culture. Following syncytialization, the cells were exposed to radiolabeled 5-HT, NE, and DA, and time-dependent uptake studies were conducted. The uptake of these monoamines was further subjected to inhibitory studies, wherein SSRIs and SNRIs were assessed for their inhibitory efficacy.

Our findings reveal significant alterations in the expression and functionality of these transporters throughout trophoblast differentiation (Fig. 4). Notably, we observed a decrease in OCT3 expression with differentiation, challenging previous assumptions regarding its role primarily in the STB and highlighting its substantial metabolic involvement in CTB. Moreover, disparities were evident not only between differentiated and undifferentiated states but also among the cell types themselves. Of particular interest was the absence of OCT3 in BeWo cells, contrasted with the presence of DAT. Such marked discrepancies also manifested in the functional characteristics of these cells, as evidenced by experiments with known OCT3 inhibitors.

Contribution of the candidate:

- isolation of PHT cells, cultivation of BeWo cells

- mRNA and protein isolation

- conducting experiments, more precisely:

    - qPCR, Western blot

    - functional analysis (*in vitro* cell experiments)

    - ELISA (hCG)

- data analysis, interpretation, and visualization of results

- manuscript preparation

The paper is available online at: https://pubmed.ncbi.nlm.nih.gov/35927731/

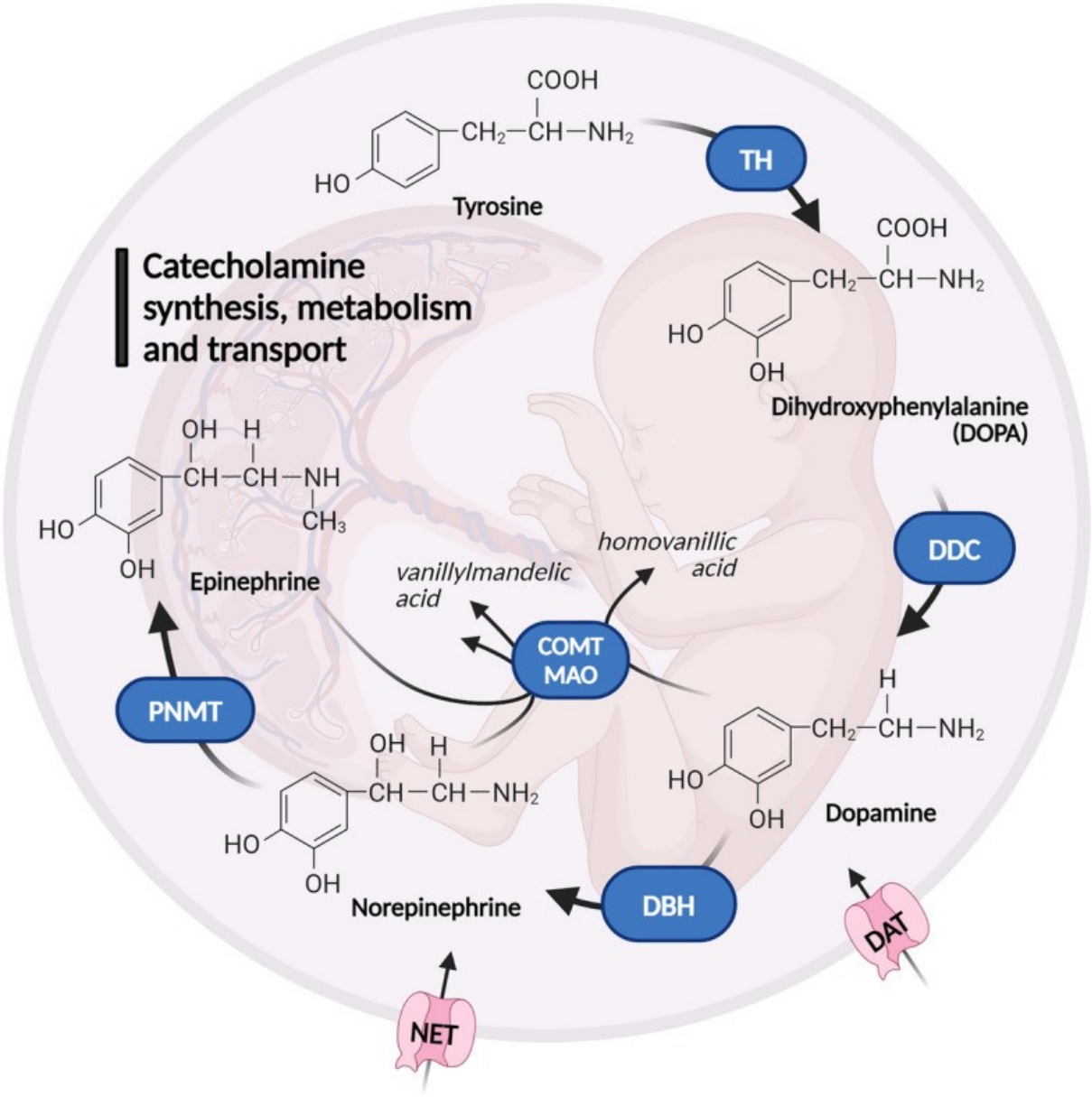
Publication in printed form is available as an appendix No. 1.

5.2 DEVELOPMENTAL EXPRESSION OF CATECHOLAMINE SYSTEM IN THE HUMAN PLACENTA AND RAT FETOPLACENTAL UNIT

Rona Karahoda[†], **Veronika Vachalova[†]**, Ramon Portillo, Filip Mahrla, Mireia Vinas Noguera, Cilia Abad, Frantisek Staud

[†] The authors contributed equally to this work.

Scientific Reports, 2024 Mar 23;14(1):6948, **(IF = 4.6/Q2, AIS = 1.129/Q2)**



Created in BioRender.com

Fig. 5. Summary depiction of key enzymes and transporters involved in monoamine synthesis, metabolism, and transport.

Abbreviations: COMT – catechol-O-methyltransferase, DAT – dopamine transporter, DBH – dopamine β-hydroxylase, DDC – dopa decarboxylase, MAO – monoamine oxidase, NET – norepinephrine transporter, PNMT – phenylethanolamine N-methyltransferase, TH – tyrosine hydroxylase

Monoamines serve as pivotal neurotransmitters and hormones, exerting physiological effects crucial for fetal development and programming. Key aspects of monoamine handling by the placenta encompass synthesis, degradation, and transport (Fig. 5). However, besides the well-documented synthesis of 5-HT, the placenta also emerges as a potential source and modulator of other monoamines, such as NE, DA, and epinephrine, within the fetoplacental unit. Notably, the intricate machinery responsible for placental monoamine handling remains largely undescribed.

Thus, our study aimed to comprehensively evaluate the synthesis, metabolism, and transport of monoamines within the fetoplacental unit. Initially, we delineated the cellular elements involved in their handling using various placental cell models (BeWo, JEG-3, HRP-1, and PHT). Notably, only PHT cells exhibited transcriptional profiles akin to human placental tissue, while unique expression of DAT was discerned solely in BeWo and JEG-3 cells, underscoring the limitations of alternative *in vitro* models. Similarly, the expression patterns observed in HRP-1 cells did not parallel those of rat placenta. Subsequently, we investigated the impact of gestational age on monoamine regulation in both human and rat models. Our findings elucidate dynamic changes in the expression of transporters and enzymes throughout gestation, suggesting their role in organogenesis and endocrine adaptations during the postnatal period. Finally, we explored the expression of pathway genes related to monoamine handling in fetal rat organs, revealing distinct developmental expression patterns. Noteworthy observations include the expression of DDC in the liver and kidneys of adult rats, as well as PNMT expression in juvenile rat lungs, aligning with previous research. Moreover, the heightened expression of these enzymes at term corroborates their significance in fetal organ tissue development.

Contribution of the candidate:

- isolation of PHT cells, cultivation of BeWo cells

- placenta and tissue sample collection

- mRNA and protein isolation

- conducting experiments (Western blot)

- contribution to interpretation and visualization of Western blot results

The paper is available online at: https://pubmed.ncbi.nlm.nih.gov/38521816/

Publication in printed form is available as an appendix No. 2.

5.3 EFFECT OF SELECTED ANTIDEPRESSANTS ON PLACENTAL HOMEOSTASIS OF SEROTONIN: MATERNAL AND FETAL PERSPECTIVES

Hana Horackova, Rona Karahoda, Lukas Cerveny, **Veronika Vachalova**, Ronja Ebner, Cilia Abad, Frantisek Staud

Pharmaceutics, 2021 Aug 20;13(8):1306, **(IF = 6.321/Q1, AIS = 0.903/Q2)**

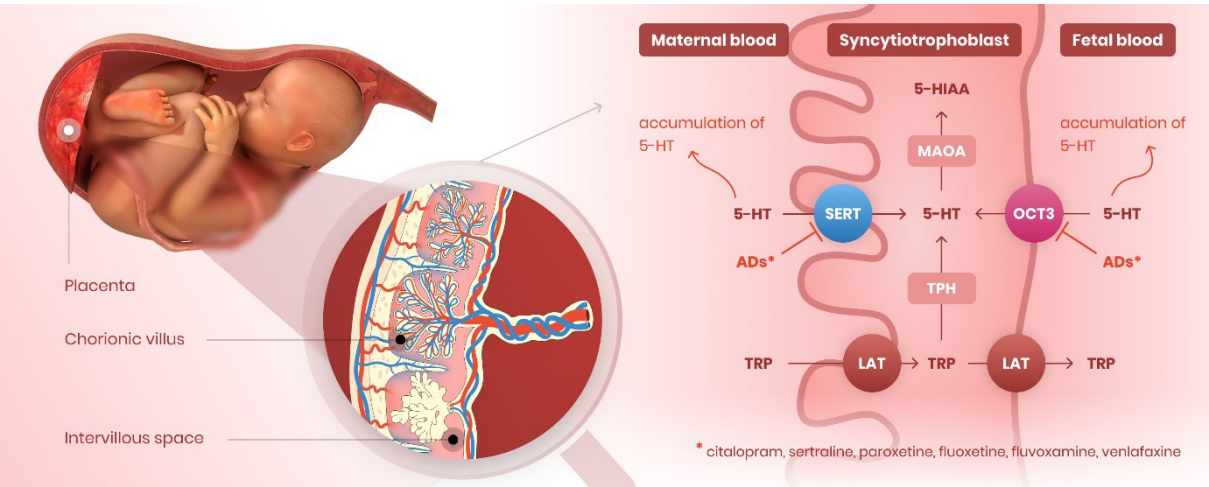


Fig. 6. Effect of antidepressants on 5-HT homeostasis in the placenta.

Abbreviations: 5-HIAA – 5-hydroxyindoleacetic acid, 5-HT – serotonin (5-hydroxytryptamine), ADs – antidepressants, LAT – L-type amino acid transporter, MAO-A – monoamine oxidase A, OCT3 – organic cation transporter 3, SERT – serotonin transporter, TPH – tryptophan hydroxylase, TRP – tryptophan

The use of antidepressants during pregnancy has become increasingly prevalent. A correlation has been identified between maternal use of SSRIs and SNRIs for the treatment of depression and the risk of perinatal complications as well as long-term health consequences. Previous studies have demonstrated that antidepressants can disrupt 5-HT homeostasis within the fetoplacental unit, yet the underlying molecular mechanisms remain inadequately characterized. SERT and OCT3 are the primary regulators of 5-HT transport in the placenta. While SERT is a long-time known SSRI and SNRI antidepressant target, OCT3 sensitivity to these inhibitors in the placental barrier has not been fully elucidated. Consequently, this study aimed to investigate the impact of clinically used antidepressants on SERT and OCT3-mediated 5-HT transport in the placenta.

Initially, we assessed the concentration-dependent effects of antidepressants on 5-HT uptake in MVM and BM placental membranes. The findings revealed that 5-HT uptake in the BM (mediated by OCT3) could not be fully inhibited, even at high antidepressant concentrations, likely due to the high-capacity nature of OCT3 and the increased fluidity of the BM membrane. These observations were further

validated in rat term placenta perfusion experiments, which also uncovered sex-dependent effects. Additionally, our results indicated that efflux transporters do not significantly impede the transfer of antidepressants from the mother to the fetus. Finally, the identified IC50 values are therapeutically attainable within the fetal circulation.

Therefore, this publication elucidates the interaction between antidepressants and SERT/OCT3 in the placenta (Fig. 6). The outcome of this interaction may result in the accumulation of 5-HT in both maternal and fetal circulations. Ultimately, this effect could lead to suboptimal 5-HT concentrations in the fetoplacental unit, potentially impacting placental blood circulation, together with fetal development and programming.

Contribution of the candidate:

- isolation of placental BM and MVM vesicles, purity assays

- conducting experiments (rat term placenta perfusions)

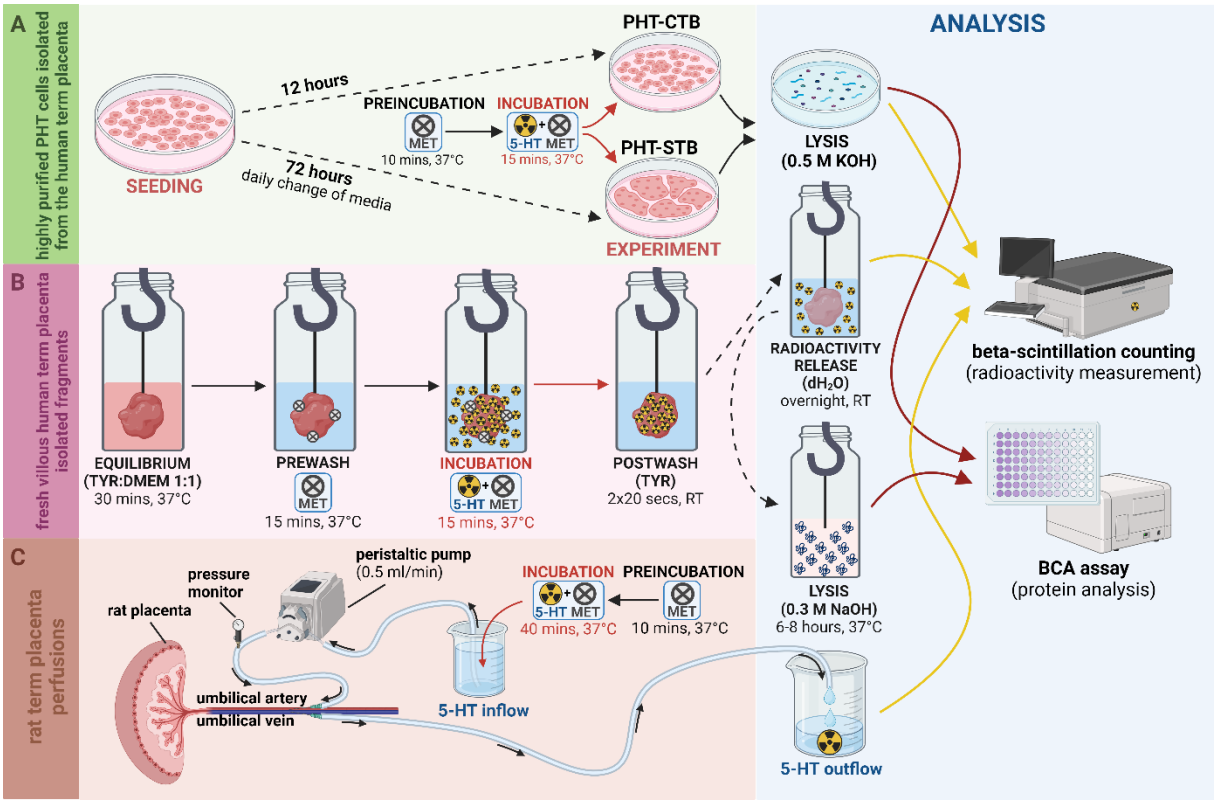
The paper is available online at: https://pubmed.ncbi.nlm.nih.gov/34452265/

Publication in printed form is available as an appendix No. 3.

5.4 METFORMIN INHIBITS OCT3-MEDIATED SEROTONIN TRANSPORT IN THE PLACENTA

Veronika Vachalova, Fiona Kumnova, Tetiana Synova, Kasin Yadunandam Anandam, Cilia Abad, Rona Karahoda, Frantisek Staud

Biomedicine & Pharmacotherapy, 2024 Oct:179:117399, **(IF = 6.9/Q1, AIS = 1.139/Q1)**



Created in BioRender.com

Fig. 7. Graphical representation of the methodology used in the study.

Abbreviations: 5-HT – serotonin (5-hydroxytryptamine), CTB – cytotrophoblast, DMEM – Dulbecco´s Modified Eagle Medium, MET – metformin, PHT – primary human trophoblast, RT – room temperature, STB – syncytiotrophoblast, TYR – Tyrode's buffer

During pregnancy, the placenta serves as a crucial source of primary monoamines, such as 5-HT. This monoamine is vital for the development of the fetal brain and other organs, necessitating stringent homeostasis throughout gestation. The mechanism by which the placenta maintains precise 5-HT homeostasis within the fetoplacental unit is mediated by bidirectional OCT3. However, maternal factors, including drug therapy, can disrupt OCT3 function and, consequently, 5-HT homeostasis. Metformin, an antidiabetic drug deemed safe for use during pregnancy, has previously demonstrated

inhibitory effects on OCT3 and adverse effects on the fetus with potential long-term health consequences.

Therefore, this study aimed to investigate the effects of metformin on 5-HT transport via OCT3. We employed several experimental approaches derived from human and rat models, including highly purified PHT cells isolated from human term placenta (cellular level), fresh villous human term placenta fragments (tissue level), and rat term placenta perfusions (organ level) (Fig. 7). Using these models, we monitored the uptake of 5-HT and its modulation by metformin at three different concentrations: therapeutic (10 µM), subtherapeutic (1 µM), and supratherapeutic (100 µM). The results indicate that metformin affects 5-HT transport in the trophoblast. Through experiments employing specific inhibitors and methods isolating only the BM, we inferred that this inhibition occurs via OCT3.

Our study provides evidence that metformin interacts with OCT3 in the trophoblast, thereby altering 5-HT homeostasis in the fetoplacental unit. This alteration poses a risk of health disorders associated with improper fetal programming due to disrupted 5-HT regulation.

Contribution of the candidate:

- isolation of PHT cells

- conducting experiments, more precisely:

    - functional analysis (*in vitro* cell experiments)

    - isolation of fragments, experiments with fragments

    - rat term placenta perfusions

- data analysis, interpretation, and visualization of results

- manuscript preparation

The paper is available online at: https://pubmed.ncbi.nlm.nih.gov/39243433/

Publication in printed form is available as an appendix No. 4

6 SUMMARY

During pregnancy, the placenta plays a crucial role in fetal development and programming. It mediates essential functions for the developing fetus, such as oxygen and nutrient supply, waste removal, hormonal regulation, immunological barrier formation, and protection from xenobiotics and other potentially harmful substances. Additionally, the placenta supplies the fetus with key factors necessary for its proper development, including biogenic monoamines such as 5-HT, NA, and DA [4, 86]. These monoamines are critical for fetal organ development such as the brain, heart, and lungs [98, 258, 259]. Therefore, to ensure accurate fetal development and programming, it is essential to maintain these monoamines at optimal levels in the fetoplacental unit. For this purpose, the placenta is equipped with a complex network of synthetic, transport, and metabolic systems. These mechanisms enable the placenta to deliver monoamines to the fetus and to maintain their levels within the optimal ranges necessary for healthy development. Therefore, disruption of these transport systems can result in maldevelopment, manifesting later in life as neuropsychiatric disorders, for instance [3].

Research of monoamine homeostasis in the human placenta is intensively pursued using various experimental models. These models include those derived directly from human placenta or animals, such as rats. Our studies employed several different approaches to examine this issue from various perspectives. *In vitro* cell experiments (using isolated human PHT cells and cell lines such as BeWo and JEG-3, or rat HRP-1) provided insights at the cellular level. Tissue-derived models, such as *ex vivo* studies with fresh villous human placenta fragments and membrane vesicles isolated from human term placentas, offered a closer approximation to physiological conditions. Finally, *in situ* rat term placenta perfusions were utilized as a whole-organism approach.

A key aspect of monoamine homeostasis research in the placenta is determining the presence and expression levels of monoamine transporters. SERT [10, 112], NET [11], and OCT3 [12] are known to be expressed in the trophoblast, but alterations in their expression during trophoblast differentiation had not been previously examined. Our study described changes in the gene and protein expression of these transporters in PHT (Vachalova et al., 2022, appendix No. 1), finding that SERT and NET mRNA expression increases with differentiation while protein expression decreases. OCT3 showed no significant change in mRNA expression but a reduction in protein expression. BeWo cells, a popular trophoblast model, exhibited different expression patterns from physiological PHT cells, likely due to their carcinogenic origin. BeWo cells showed increased SERT expression at both mRNA and protein levels, unchanged NET expression, absence of OCT3, and presence of DAT, most probably associated with tumor cell growth [100, 260]. Functional studies confirmed that reduced protein expression in

PHT cells after differentiation corresponded with decreased monoamine uptake in syncytialized form, whereas BeWo cells showed the opposite pattern. The presence of high-capacity OCT3 in PHT cells likely accounts for their higher monoamine uptake capacity. Finally, the study highlights the impact of antidepressants on 5-HT uptake, impeaching the suitability for BeWo monoamine uptake research. Thus, although these cells appear similar to the physiological PHT cells, there have been differences reported in syncytium formation, epigenetics, and regulators [54, 55].

Additionally, we examined monoamine homeostasis and identified distinct monoamine transcriptional profiles for monoamines in different cell models (Karahoda/Vachalova et al., 2024, appendix No. 2). Notably, PHT cells did not express TH or DAT, unlike choriocarcinoma-derived cells. HRP-1 exhibited similar differentiation from physiological rat cells. Understanding these differences is crucial for selecting appropriate cell models for monoamine homeostasis studies. Protein expression profiles in human and rat placentas, including PNMT, COMT, and NET, were found to be similar. Increased PNMT expression corresponds to heightened NE and DA synthesis and transplacental NE transfer [261]. These findings underscore the complexity of species-specific regulatory pathways and necessitate further exploration of placental physiology. Finally, we investigated the expression of monoamine-related enzymes and transporters in fetal organs, noting that monoamine levels increase significantly at birth to support postnatal adaptations. Precise regulation of monoamines during embryonic development is essential, with intrinsic cardiac monoamine biosynthesis and renal contributions to amniotic fluid dopamine levels playing critical roles in late gestation.

The use of drugs during pregnancy remains a controversial topic. Although there are documented cases linking some pharmacotherapy (like antidepressants or metformin) during pregnancy to altered fetal development [251, 252, 258, 262, 263], the underlying mechanisms of these effects have not been thoroughly elucidated. Therefore, adequate approaches to prevent this adverse influence cannot be implemented. Given the critical role of 5-HT in fetal organ development, we hypothesized that factors negatively impacting the regulatory elements of 5-HT homeostasis in the placenta could significantly affect its levels in the fetal brain and other organs. This disruption could result from an insufficient supply or inadequate removal of 5-HT from the fetal circulation, especially during the later stages of gestation when the fetus begins synthesizing 5-HT independently [3].

Indeed, our research about the effects of antidepressants on monoamine homeostasis in the placenta (Horackova et al., 2021, appendix No. 3) revealed that some drugs, like those of SSRIs and SNRIs, do not only inhibit SERT, a key monoamine transporter commonly targeted in their mechanism of action, but also OCT3, altogether potentially disrupting placental function and fetal monoamine balance. Concentration-dependent inhibition experiments on human placenta membranes revealed that

common therapeutic doses of antidepressants strongly inhibit 5-HT uptake from both maternal and fetal circulation, as indicated by calculated IC50 values. This inhibition was further validated in rat models, confirming the significant impact of antidepressants on fetal 5-HT uptake. Out of all, paroxetine was identified as the most potent OCT3 inhibitor, with male fetuses more susceptible to this inhibition, possibly explaining sex-dependent behavioral differences and increased risk of neurodevelopmental disorders [264, 265]. Therefore, our studies highlighted previously unknown mechanisms by which antidepressants may compromise fetoplacental 5-HT homeostasis.

In another study, we investigated the impact of metformin, an antidiabetic drug commonly used during pregnancy, on OCT3-mediated 5-HT transport (Vachalova et al., 2024, appendix No. 4). Metformin is known as a substrate and inhibitor of OCT3 [16-18, 266, 267] in both placenta and brain, therefore, we speculated that its use during gestation might alter the transporters' function displayed by lowered capacity of 5-HT uptake. Such intervention might alter 5-HT levels in the fetus manifested by inaccurate fetal development and programming. We have used multiple alternative experimental approaches that enable us to study the effect of metformin on 5-HT uptake at the cellular, tissue, and organ levels. All models demonstrated that metformin affects OCT3 function, showing inhibition like that observed with the specific OCT3 inhibitor decynium 22 [268]. These findings were confirmed through rat term placenta perfusions, where we perfused solely the fetal side of the placenta expressing OCT3. Therefore, our results indicate that metformin interacts with OCT3, which might disturb 5-HT homeostasis in the fetoplacental unit and cause irreversible alterations in fetal development and programming.

We are aware that in both studies, involving antidepressants and metformin, only the acute effects of the drugs on 5-HT transport in the fetoplacental unit can be observed. However, in the treatment of depression or GDM, these drugs are administered almost exclusively on a chronic basis. Therefore, further long-term studies are needed to monitor the chronic effects of these drugs on 5-HT homeostasis in the placenta. In the case of paroxetine, such a study has already been conducted (Horáčková, unpublished data). Paroxetine was administered to rats orally for the entire duration of pregnancy. The results of this study confirm the impact of paroxetine on the homeostasis of monoamines in the rat placenta and fetal brain, as well as its effect on uteroplacental and fetoplacental circulation, as determined by Doppler ultrasonography. A long-term metformin exposure study has been performed by Garbarino et al. (2019) on pregnant mice and results compared to OCT3 knock-out mice. Results detected behavioral changes in male pups supposedly caused by an alteration in 5-HT homeostasis either in the brain or placenta [254].

In conclusion, our work focused on monoamine homeostasis in the fetoplacental unit, determining the expression of key monoamine transporters in the trophoblast, and describing their changes during differentiation. We identified significant expressional and functional differences between BeWo cells and physiological PHT cells, suggesting that BeWo is unsuitable for studying monoamine homeostasis. We also described differences in enzyme and transporter expression profiles for monoamines in JEG-3 and HRP-1 cells. Furthermore, we detailed the expression of enzymes and transporters involved in monoamine synthesis, release, degradation, and transport in the human placenta and fetal organs, emphasizing the importance of these processes for proper fetal development and programming. Our studies on the effects of drugs such as antidepressants and metformin revealed their impact on 5-HT homeostasis in the fetoplacental unit.

7 LIST OF OTHER OUTPUTS OF THE CANDIDATE

7.1 OTHER ORIGINAL ARTICLES RELATED TO THE TOPIC OF THE DISSERTATION

- Hana Horackova, Rona Karahoda, **Veronika Vachalova**, Helena Turkova, Cilia Abad, Frantisek Staud: Functional characterization of dopamine and norepinephrine transport across the apical and basal plasma membranes of the human placental syncytiotrophoblast; *Scientific Reports,* 2022 Jul 8;12(1):11603, **(IF = 4.997/Q2, AIS = 1.208/Q2)**

- Hana Horackova, **Veronika Vachalova**, Cilia Abad, Rona Karahoda, Frantisek Staud: Perfused rat term placenta as a preclinical model to investigate placental dopamine and norepinephrine transport; *Clinical Science (London),* 2023 Jan 4;CS20220726, **(IF = 6.876/Q1, AIS = 1.458/Q1)**

- Frantisek Staud, Xin Pan, Rona Karahoda, Xiaojing Dong, Petr Kastner, Hana Horackova, **Veronika Vachalova**, Udo R. Markert, Cilia Abad: Characterization of a human placental clearance system to regulate serotonin levels in the fetoplacental unit; *Reproductive biology and endocrinology,* 2023 Aug 23;21(1):74, **(IF = 4.4/Q2Q1, AIS = 1.124/Q2Q1)**

7.2 SCIENTIFIC CONFERENCES WITH DATA PRESENTATIONS RELATED TO THE TOPIC OF THE DISSERTATION

7.2.1 ORAL PRESENTATIONS

- **Veronika Vachalova**, Rona Karahoda, Martina Ottaviani, Kasin Yadunandam Anandam, Cilia Abad, Christiane Albrecht, Frantisek Staud – Dynamic changes in monoamine transport upon trophoblast differentiation: Evidence for transcriptional and functional variability between primary human villous and BeWo trophoblasts; *12. Postgraduální a 10. Postdoktorandská vědecká konference Farmaceutické fakulty UK, Hradec Králové, 2022*

- **Veronika Vachalova**, Rona Karahoda, Martina Ottaviani, Kasin Yadunandam Anandam, Cilia Abad, Christiane Albrecht, Frantisek Staud – Monoamine transport in the trophoblast: Effect of trophoblast differentiation and drugs commonly used in pregnancy; *13. Postgraduální a 11. Postdoktorandská vědecká konference Farmaceutické fakulty UK, Hradec Králové, 2023*

- **Veronika Vachalova**, Rona Karahoda, Fiona Kumnova, Tetiana Synova, Martina Ottaviani, Kasin Yadunandam Anandam, Ramon Portillo, Filip Mahrla, Mireia Vinas-Noguera, Cilia Abad, Christiane Albrecht, Frantisek Staud – Monoamine homeostasis in the fetoplacental unit:

Effect of trophoblast differentiation, gestation age and pharmacotherapy; *14. Postgraduální a 12. Postdoktorandská vědecká konference Farmaceutické fakulty UK, Hradec Králové, 2024*

- **Veronika Vachalova**, Rona Karahoda, Fiona Kumnova, Tetiana Synova, Martina Ottaviani, Kasin Yadunandam Anandam, Ramon Portillo, Filip Mahrla, Mireia Vinas-Noguera, Cilia Abad, Christiane Albrecht, Frantisek Staud – Physiological and pharmacological perspectives of monoamine regulation in the fetoplacental unit; *17th EPPW, Rotterdam, Netherlands, 2024*

7.2.2 POSTER PRESENTATIONS

- **Veronika Vachalova**, Rona Karahoda, Martina Ottaviani, Kasin Yadunandam Anandam, Cilia Abad, Christiane Albrecht, Frantisek Staud – Functional reorganization of monoamine transport systems during villous trophoblast differentiation: Evidence of distinct differences between primary human trophoblasts and BeWo cells; *DOHaD world congress, Vancouver, 2022*

7.3 SCIENTIFIC EXPERIENCE ABROAD

- Six-month scientific internship at the Universitätsklinikum Jena, Jena, Germany (supervisors Prof. Dr. med. Udo Markert and Dr. rer. nat. med. habil. Diana Maria Morales Prieto); March 2023 - September 2023; specialization on human term placenta perfusions, placenta and blood-brain barrier on-a-chip, and trophoblast spheroid invasion

7.4 AWARDS ATTAINED DURING THE STUDIES

- 1st place at Angelini University Award (team competition) - Angelini Pharma Czech Republic; September 2020

7.5 GRANT PROJECTS

7.5.1 MAIN INVESTIGATOR

- The Charles University Grant Agency (GAUK 358821/C/2021), 2021–2023, main investigator; Title: Pharmacotherapy in pregnancy; effect on monoamine homeostasis in the placenta

7.5.2 TEAM MEMBER

- Program START/MED/069, 2021–2023, co-investigator; Title: Pregnancy disorders; effect on fetal development and programming

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9 LIST OF APPENDICES

A1. Functional reorganization of monoamine transport systems during villous trophoblast differentiation: evidence of distinct differences between primary human trophoblasts and BeWo cells

Veronika Vachalova, Rona Karahoda, Martina Ottaviani, Kasin Yadunandam Anandam, Cilia Abad, Christiane Albrecht, Frantisek Staud

Reproductive biology and endocrinology, 2022 Aug 4;20(1):112, **(IF = 5.211/Q1, AIS = 1.094/Q1)**

The paper is available online at: https://pubmed.ncbi.nlm.nih.gov/35927731/

A2. Developmental expression of catecholamine system in the human placenta and rat fetoplacental unit

Rona Karahoda[†], **Veronika Vachalova[†]**, Ramon Portillo, Filip Mahrla, Mireia Vinas Noguera, Cilia Abad, Frantisek Staud

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A3. Effect of selected antidepressants on placental homeostasis of serotonin: maternal and fetal perspectives

Hana Horackova, Rona Karahoda, Lukas Cerveny, **Veronika Vachalova**, Ronja Ebner, Cilia Abad, Frantisek Staud

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A4. Metformin inhibits OCT3-mediated serotonin transport in the placenta

Veronika Vachalova, Fiona Kumnova, Tetiana Synova, Kasin Yadunandam Anandam, Cilia Abad, Rona Karahoda, Frantisek Staud

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APPENDIX No. 1

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Veronika Vachalova, Rona Karahoda, Martina Ottaviani, Kasin Yadunandam Anandam, Cilia Abad, Christiane Albrecht, Frantisek Staud

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APPENDIX No. 3

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Pharmaceutics, 2021 Aug 20;13(8):1306, **(IF = 6.321/Q1, AIS = 0.903/Q2)**

APPENDIX No. 4

Metformin inhibits OCT3-mediated serotonin transport in the placenta

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