Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Effect of inflammation on placental metabolism of tryptophan

Diploma thesis

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Hradec Králové 2023   Anna Orbisová

STATEMENT OF AUTHORSHIP

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

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ABSTRACT

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Maternal inflammation throughout pregnancy has been firmly linked to the development of neuropsychiatric disorders in the offspring. Furthermore, intrauterine infections, whether viral or bacterial, are widely recognized as significant risk factors for conditions like autism and schizophrenia. Tryptophan metabolism has been suggested as a plausible pathway through which maternal inflammation during gestation can hinder fetal brain development and programming. This occurs as tryptophan is metabolized through the serotonin (5-HT) pathway, producing metabolites that exert direct effects on the development of the fetal brain. In this work, using an *ex vivo* model, we assess the impact of bacterial (LPS) and viral (poly I:C) placental infection on the 5-HT pathway. Human term placenta explants were treated with LPS or Poly I:C for different times (4 or 18 hours). Subsequently, the impact on gene and protein expressions of the key enzymes within the 5-HT pathway, along with their functional enzymatic activities, was assessed. Our results confirm that the expression and function of the main enzymes of the 5-HT pathway are affected by inflammation. Tryptophan hydroxylase (TPH), the first and rate-limiting enzyme for the 5-HT pathway, declined significantly at gene, protein as well as at functional levels in explants treated with LPS. Conversely, the gene expression of the 5-HT-metabolizing enzyme, monoamine oxidase A (MAO-A), was upregulated by inflammation. We conclude that placental inflammation impairs 5-HT homeostasis in the placenta and thus may affect the neurodevelopmental programming of the fetus.

ABSTRAKT

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Názov diplomovej práce: Vplyv zápalu na placentárny metabolizmus tryptofánu

Zápalová aktivita v tele ženy počas obdobia tehotenstva je veľmi známym rizikovým faktorom pre vznik neuropsychiatrických porúch u potomstva. Zatiaľ čo intrauterinná bakteriálna infekcia je známym rizikovým faktorom autizmu, systémová virálna infekcia u matky je dokazaným rizikovým faktorom pre vývoj autizmu a schizofrénie. Značná pozornosť sa venuje pochodom, kde zmena daná zápalovou aktivitou počas tehotenstva môže ovplyvniť vývoj a programovanie mozgu plodu. Mezi také pochody patrí aj serotonínová (5-HT) dráha metabolizmu tryptofánu. V tejto diplomovej práci hodnotíme vplyv bakteriálnej (LPS) a vírusovej (poly I:C) placentárnej infekcie na dráhu 5-HT pomocou *ex vivo* modelu. Vzorky ľudskej placenty boli vystavené LPS alebo Poly I:C počas 4 alebo 18 hodín; hodnotená bola expresia génov a proteínov, ako aj funkčná enzymatická aktivita hlavných enzýmov 5-HT dráhy. Z našich výsledkov vyplýva, že expresia a funkcia viacerých enzýmov dráhy 5-HT boli ovplyvnené zápalovou aktivitou. Expresia rozhodujúceho enzýmu zodpovedného za syntézu serotonínu z tryptofánu, tryptofán hydroxyláza (TPH), sa významne znížila v explantátoch vystavených bakteriálnej infekcii (LPS), zatiaľ čo expresia génu pre enzým degradujúci 5-HT, monoaminooxidázy A (MAO-A), bola zápalom zvýšená. Dospeli sme teda k záveru, že placentárny zápal narúša homeostázu 5-HT vo fetoplacentárnej jednotke, a tým môže ovplyvniť priebeh vývoja nervovej signalizácie.

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1. LIST OF ABBREVIATIONS

3-OH-KYN – 3-hydroxy-kynurenine

5-HT – Serotonin

5-OH-TRP – 5-hydro tryptophan

ABS – Absorbance

AOAA – Amino acetic acid

API – Aqua pro injection

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

CNS – Central nervous system

CTB – Cytotrophoblast

ddPCR – Droplet digital PCR

DOHaD – Developmental Origins of Health and Disease

DTT – Dithiothreitol

ELISA – Enzyme-linked Immunosorbent Assay

HIAA – 5-hydroxyindoleacetic acid

HPLC – High-performance liquid chromatography

IDO – Indolamine dioxygenase

IL-6 – interleukin 6

IL-8 – interleukin 8

IL-1β – interleukin 1 beta

KAT – Kynurenine amino transferase

KMO – Kynurenine monooxygenase

KYN – Kynurenine

KYNA – Kynurenine acid

LDH – Lactate dehydrogenase

LPS – Lipopolysaccharides

MAO – Monoamine oxidase

MIA – Maternal immune activation

MTT – Thiazolyl blue tetrazolium bromide

NAD – Nicotinamide adenine dinucleotide

OCT3 – Organic cation transporter 3

Poly I:C – Polyinosinic–polycytidylic acid sodium salt.

qPCR – Quantitative reverse transcriptase polymerase chain reaction

QUIN – Quinolinic acid

SSD – Schizophrenia spectrum disorders

STB – Syncytiotrophoblast

TDO – Tryptophan 2,3-dioxygenase

TMB – Tetramethylbenzidine

TNF – tumor necrosis factor

TPH – Tryptophan hydroxylase

TRP – Tryptophan

WB – Western blot

2. INTRODUCTION

While the placenta is the most crucial organ in the body during pregnancy, yet interestingly, it remains the least comprehended. Throughout its transient existence, it carries out functions that are later performed by various distinct organs, such as the liver, intestines, lungs, kidneys, and endocrine glands (1). Although its primary role is to provide the fetus with essential oxygen and nourishment, the placenta plays other different functions including the production of metabolites and hormones that are important for fetal growth and development (2). The functions of the placenta are impacted by maternal environments, influencing to the end the development and programming of the offspring. Maternal inflammation throughout pregnancy has an impact on placental function and is linked to a higher risk of neurodevelopmental and psychiatric disorders in children (3). Thoroughly, prenatal inflammation is strongly associated with a spectrum of neurodevelopmental disorders in exposed offspring, including motor-sensory deficits, delayed learning, and various neurological diseases, such as schizophrenia, autism spectrum disorder and epilepsy (4, 5). Furthermore, a longitudinal study of a cohort of women from early pregnancy through motherhood showed that the degrees of inflammation they experienced during pregnancy were related to the functional organization of their children's brain immediately after birth and the abilities of working memory abilities at two years of age (6).

Although epidemiological data provide robust evidence for a connection between maternal inflammation and fetal neurodevelopmental disorders, the underlying mechanisms that link these phenomena remain unidentified. Tryptophan (TRP) metabolism has been identified as plausible pathways through which maternal inflammation during pregnancy can affect the development and programming of the fetal brain, as several neuroactive properties are attributed to various TRP metabolites (7, 8). TRP is a fundamental amino acid involved in various physiological functions. Apart from its role in protein synthesis, TRP plays a crucial part in supporting proper placental and fetal development (9). In several organs, including the placenta, two primary metabolic pathways of TRP have been recognized: the kynurenine (KYN) pathway and the serotonin (5-HT) pathway.

TRP metabolism along the 5-HT pathway generates biologically active metabolites, such as 5-HT and melatonin (10). This pathway relies on two key enzymes: tryptophan hydroxylase (TPH), which is the initial and rate-limiting enzyme along the 5-HT pathway, and monoamine oxidase (MAO), which is responsible for metabolizing 5-HT (11, 12). 5-HT is vital for fetal

brain development (13). As the placenta serves as a crucial source of 5-HT for the fetal brain, any disruptions in placental TRP metabolism could potentially interfere with 5-HT signaling in specific regions of the developing fetal brain, leading to abnormal programming and fetal brain development (12).

3. BACKGROUND

3.1 Placenta: development, structure, and functions.

The placenta is a multifunctional and ephemeral organ that acts as a vital link between the mother and the baby during pregnancy (14). Throughout this period, the placenta takes on the roles of various essential organs for the fetus, facilitating the supply of nutrients and oxygen while eliminating waste and harmful substances. Moreover, it offers immune protection and endocrine support for fetal development by producing endocrine and paracrine hormones (15).

Following fertilization, the blastocyst undergoes implantation within the maternal decidua, and the trophoblast initiates superficial invasion into the myometrium (16). The development of the human placenta requires a harmonious interaction between the trophoblast lineage of the placenta and the maternal endometrium. The human placentation process begins with the attachment of the polar trophoectoderm of the blastocyst to the receptive endometrial epithelium, which occurs approximately 5-6 days after fertilization. Subsequently, trophoectoderm cells fuse with neighboring cells, giving rise to a primary syncytium. Around 6-8 days after fertilization, a mass of syncytiotrophoblast starts to penetrate between the endometrial cells, inducing the transformation of the endometrium into a specialized tissue known as the decidua (2).

During the initial week of development, the embryo acquires nutrients and eliminates waste substances through simple diffusion. The first instance of maternal-fetal transport takes place when the maternal decidua fulfills the nutritional requirements of the blastocyst. The feto-placental circulation begins after implantation. During invasion, blastocyst cells, at the level of the embryonic pole, show an increase in proliferation, which results in a double layer of trophoblast. The innermost cell layer, known as the cytotrophoblast (CTB), consists of well-defined, undifferentiated, and highly proliferative cells that function as stem cells. These cells ensure trophoblast growth through continuous proliferation and subsequent fusion. On the other hand, the outermost layer of the trophoblast, which directly interfaces with the uterine tissue, differentiates into villous and extravillous trophoblasts. The villous trophoblast undergoes a transformation into syncytiotrophoblast (STB) through fusion with neighboring cells. The SCT forms a continuous multinucleated layer of epithelial cells that lose their generative capacity after fusion, relying on the proliferation and subsequent fusion of the CTB for their growth (2).

During the first trimester of pregnancy, the extravillous endovascular trophoblast forms a plug that obstructs the terminal portion of the spiral uterine arteries. This obstruction restricts blood flow, including oxygen, to the intervillous space. Consequently, maternal blood flow to the developing placenta is not established until the 12th week of gestation, and nourishment during this period relies on histotrophic processes (17). Low oxygen tension within the intervillous space is critical for normal embryogenesis and organogenesis in the developing fetus. It supports cellular proliferation and angiogenesis within the placenta, while also preventing excessive production reactive oxygen species (ROS). Following the completion of embryogenesis at the 12th week of gestation, the dissolution of extravillous trophoblast (EVT) plugs commences, establishing a continuous low flow perfusion of oxygenated blood into the placental intervillous space (18, 19). At this point, the early human placenta undergoes a significant transition, shifting from histotrophic to hemotrophic nutrition. This transition is accompanied by a three-fold increase in intraplacental oxygen tension. This increase in oxygen levels promotes trophoblast differentiation as well as maturation of the placenta into an exchange organ (20, 21).

In terms of its macroscopic structure, the placenta is a disc-shaped organ weighing nearly 500 g at term, with a diameter of approximately 15–20 cm, a thickness of 2–3 cm, and a surface area of nearly 15 m^2 (22). It consists of two discoid surfaces: one attached to the maternal side (myometrium), known as the decidual plate, and the other attached to the fetal side (amniotic cavity), known as the chorionic plate. These two plates are fused together at their edges, forming the spongy parenchyma filled with maternal blood. From the chorionic plate, chorionic villi (fetal blood vessels) emerge, while perpendicular septa called placental septa emerge from the decidual plate. Although these septa do not make direct contact with the chorionic plate, they divide the placenta into several compartments known as cotyledons (23). Each cotyledon represents the functional unit of the placenta, composed of chorionic villi immersed in maternal blood lacunae (Figure 1).

At the microscopic level, the fundamental building block of the placenta is the chorionic villi (Figure 2), which comprises vascular projections of fetal tissue enveloped by the chorion (22). The chorion is composed of two cellular layers: the outer STB, which directly contacts maternal blood within the intervillous space, and the inner CTB. The CTB and SCT layers of the human placenta are dynamic throughout pregnancy and are responsible for the secretion of hormones, as well as the exchange of nutrients in the feto-maternal unit.
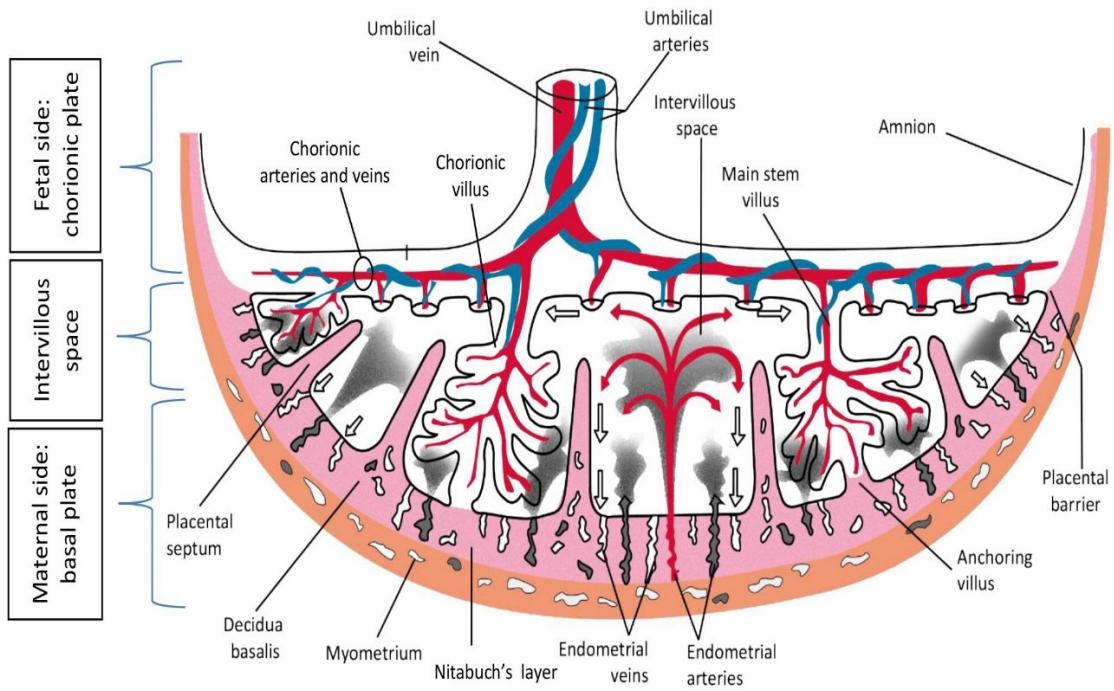
Figure 1. Structure of human placenta.

Adopted from (23).
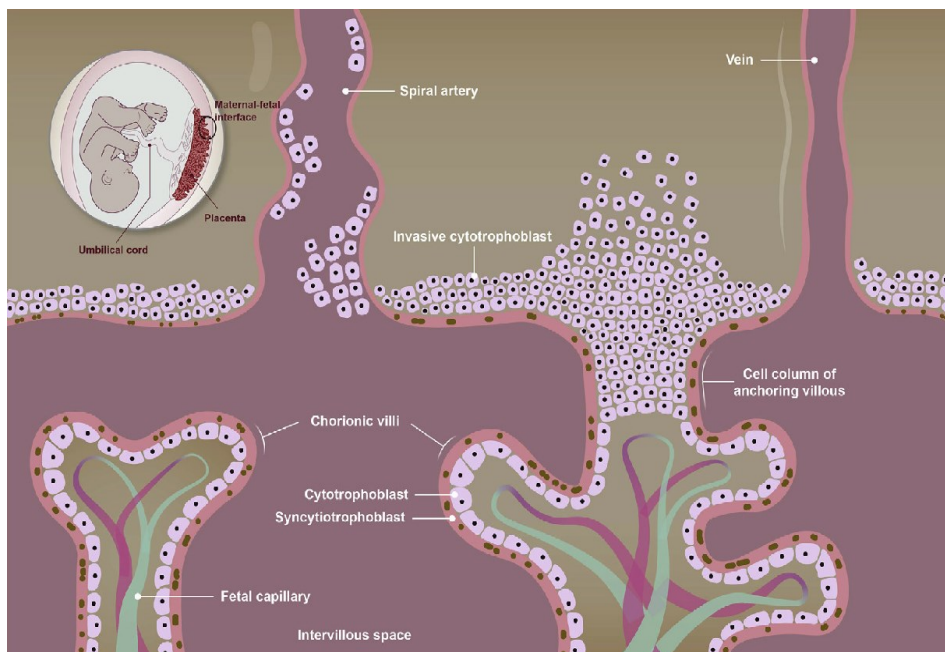
Figure 2. Chorionic Villi, as functional unit of human placenta

Adopted from (24)

The placenta, which serves as the central regulatory organ during pregnancy, is a highly specialized organ. Its functions are diverse and essential for maintaining a healthy pregnancy and ensuring the well-being of both the mother and the unborn. The main functions of the human placenta are:

- Gas exchange: While the fetal lungs are not yet functional for gas exchange during intrauterine life, the placenta takes on the vital role of transferring oxygen and carbon dioxide to and from the developing fetus (22, 25).

- Metabolic transfer: One of the primary functions of the placenta is to facilitate the exchange of nutrients between the maternal and fetal circulation. It acts as a barrier and selectively allows essential nutrients such as glucose, amino acids, fatty acids, electrolytes, vitamins and water from the mother's blood to pass through to the developing fetus (22, 25, 26).

- Waste Removal: In addition to facilitating nutrient and gas exchange, the placenta also plays a role in disposing of waste products produced by the developing fetus. These waste substances are transported from the fetal bloodstream to the maternal bloodstream, where they can be excreted by the mother's excretory organs (22).

- Endocrine functions: The placenta produces a variety of hormones and metabolites that are essential for maintaining pregnancy and supporting fetal development. Some of these hormones include human chorionic gonadotropin (hCG), human placental lactogen (hPL), human growth hormones variant, leptin, estrogens, and progesterone (25, 26).

- Immune Protection: The placental barrier generally prevents large proteins from crossing, but maternal IgG antibodies can pass from mother to fetus through pinocytosis, providing passive immunity during the initial months of life. This transfer begins early in pregnancy and increases significantly in the third trimester(22).

3.2. Fetal programming and DOHaD hypothesis.

The hypothesis known as the Developmental Origins of Health and Disease (DOHaD), which emerged from the concept initially referred to as the Fetal Origins of Adult Disease

proposed by David Baker in the 1990s, suggests that exposure to specific environmental factors during crucial stages of development and growth can profoundly impact an individual's short- and long-term health (27). According to this idea, when the developing fetus is subjected to an unfavorable uterine environment, resulting from factors like inadequate nutrition, infections, inflammation, chemicals, or hormonal disturbances, it reacts by undergoing adaptations that not only promote its immediate viability but also enhance its ability to survive if confronted with a similar environment later in life (28). Certain adaptations made by the fetus involve the reduction of endocrine or metabolic activity and/or the suppression of specific organ functions to moderate its growth rate in response to limited nutrient availability within the deprived uterine environment. Nevertheless, these adaptations, termed 'mismatch,' could subsequently increase their susceptibility to certain metabolic diseases during postnatal life (27, 28).

Numerous studies have established a relationship between low fetal birth weight and various maternal factors such as smoking (29), stress, alcohol consumption, obesity, or malnutrition during pregnancy, leading to related disorders such as metabolic, cardiovascular, and kidney-related dysfunctions (30) in the adult life. Furthermore, prenatal challenges, such as infections, inflammation, or maternal stress experiences, are believed to influence epigenetics in fetal brain programming and may contribute to the occurrence of neurodevelopmental disorders or intellectual impairments later in the offspring's life (31).

3.3 Inflammation during pregnancy

Inflammation is the natural reaction of the body to harmful stimuli, which can include pathogens, injured or deceased cells, and irritants (32). Moreover, inflammation plays a significant role in reproductive function and during pregnancy, where immune-mediated mechanisms are vital for tissue growth, remodeling, and differentiation, all essential components to sustain a successful pregnancy (33). Each phase of pregnancy exhibits a distinctive inflammatory milieu. The first and third trimesters are marked by a proinflammatory state (TH1), while the second trimester corresponds to an anti-inflammatory phase, commonly referred to as the TH2 environment (34).

Maintaining a delicate balance between proinflammatory and anti-inflammatory responses is crucial for the successful progression of pregnancy. Nevertheless, any disruption or imbalance in the inflammatory response during pregnancy can result in unfavorable outcomes. Experiencing acute or chronic inflammation may elevate the risk of pregnancy

complications, including preeclampsia, preterm birth, or intrauterine growth restriction. Additionally, multiple sources of evidence, including epidemiological data and findings from animal studies, strongly suggest connections between maternal inflammation during pregnancy and heightened chances of neurodevelopmental and psychiatric disorders in the offspring (35).

3.3.1. Impact of maternal infection on neurodevelopment

Several studies have highlighted the negative impact of infectious diseases during pregnancy on the subsequent neurodevelopment disease of the offspring. Early epidemiological investigations established a correlation between infections occurring during pregnancy and increased susceptibility to schizophrenia and autism spectrum disorders in children. In this context, a large prospective cohort study involving US women revealed that exposure to respiratory infections during the second trimester of pregnancy was associated with a significantly elevated risk of schizophrenia spectrum disorders (SSD) (36). Similarly, research conducted during influenza pandemics demonstrated a higher incidence of mental disorders such as autism spectrum disorder, schizophrenia, and attention deficit hyperactivity disorder (ADHD) in children after following influenza infection during pregnancy (37, 38). Intriguingly, even in cases of malaria infection in pregnant women, where the pathogen did not reach the placenta, an increased risk of neurodevelopment disorders in the offspring was found (39). In this regard, the recent COVID-19 pandemic has also been explored. A small Chinese study showed a defective social-emotional development in newborns within the first 3 months of life after maternal exposure to SARS-CoV-2 (40). Additionally, two separate studies linked COVID-19 infection during pregnancy with the diagnosis of neurodevelopmental disorders at 12 months of age children (41, 42).

Interestingly, studies examining various infections in pregnant women have revealed that the risk of neurodevelopmental disorders in their offspring remains consistent, indicating that the association is likely related to the maternal immune response or inflammation rather than the specific pathogen itself (43). The presence of intrauterine infection is typically detected by the maternal innate immune system, leading to the synthesis of pro-inflammatory cytokines such as interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 1 beta (IL-1β), tumor necrosis factor (TNF), and macrophage inflammatory proteins, which are then circulated in higher levels within the maternal system (44). Furthermore, elevated levels of cytokines IL-6 and IL-1β in amniotic fluid and placental inflammation have been identified as predictors of brain injury in premature infants (5, 45). Thus, it is hypothesized that maternal acute inflammation during

pregnancy contributes significantly to the development of neurobehavioral and psychiatric disorders in the offspring.

3.4 Tryptophan

TRP is an indispensable amino acid that assumes a critical role in a multitude of physiological processes within the human body. Its discovery, attributed to a fortunate scientific incident in 1901 by Hopkins and Cole, emerged from a pancreatic digest of casein (46). The name "tryptophan" likely stems from the observation that the enzyme trypsin liberates this amino acid during protein digestion, the sole means of acquiring TRP given its inability to be synthesized by the human body (47, 48). TRP is chemically composed of α-carboxylic acid and α-amino groups, and its side chain features an indole moiety that is abundant in non-polar aromatic beta carbon (Figure 3). The recommended dietary intake of TRP for an average-weight adult is approximately 250 to 425 mg per day, equivalent to 3.5-6 mg per kg of body weight. Higher demands for TRP, along with other essential amino acids, are particularly pronounced during infancy and childhood. Newborns and young children typically require around 12 mg of TRP per kg of body weight (49). TRP-rich foods, such as bananas, oats, dairy products, poultry meat, peanuts, and tuna fish, are well-known sources of this essential amino acid (50). As a fundamental building block of proteins, TRP is integral to protein synthesis, aiding in the formation of essential cellular structures. Beyond its role in protein synthesis, TRP plays diverse physiological roles, particularly in immune activation, central nervous system (CNS) functions, maintaining intestinal mucosal balance, and promoting cell growth. During pregnancy, TRP requirements are notably elevated, primarily to support protein synthesis and fetal development. Therefore, TRP is crucial for appropriate placental and fetal development (9). Additionally, TRP is essential for generating signaling molecules like serotonin and kynurenic acid, which contribute to neuronal communication and protection. Moreover, TRP is involved in the synthesis of nicotinamide adenine dinucleotide (NAD+), a vital coenzyme (47).
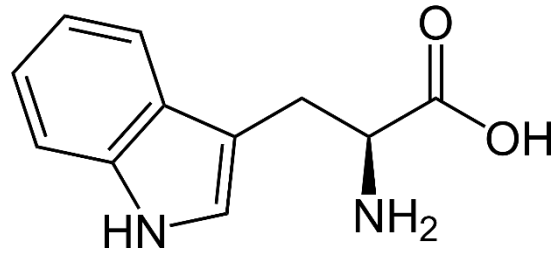
Figure 3. L-Tryptophan
Adopted from (49)

3.5 Metabolism of tryptophan

TRP metabolism constitutes a vital biochemical process with several implications for human health. This amino acid serves as a precursor for various biologically active compounds crucial to diverse physiological functions. In several organs, including the placenta, TRP undergoes complex metabolic transformations through 2 main pathways. The 5-HT and KYN pathways, and the relative flux through these pathways depends on the physiological stage of pregnancy [10], figure 4 provides an overview of the TRP metabolism in the human placenta.
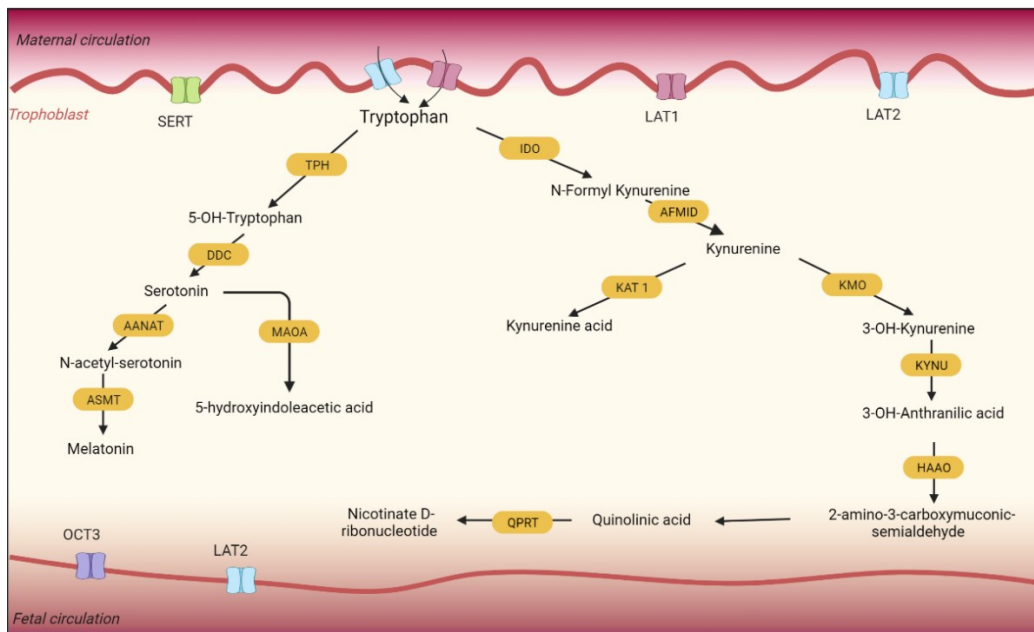
Figure 4. TRP metabolism in Trophoblast cells.

Created in BioRender.com.

3.5.1 Kynurenine pathway

The initiation of tryptophan degradation along the KYN pathway is governed by two principal enzymes. First, tryptophan 2,3-dioxygenase (TDO) exhibits a higher specificity towards TRP. Second, the heme-containing enzyme indoleamine 2,3-dioxygenase (IDO1), found more ubiquitously, acts as a metabolic agent for any indole ring-containing structures. The human placenta demonstrates functional expression of IDO, which serves as the initial and rate limiting enzyme within the KYN pathway (51). Furthermore, gene expression of all enzymes involved in the KYN pathway has been documented (52). IDO expression enhances at both the genetic and protein levels in the placenta throughout pregnancy (53, 54) and is notably induced by various pro-inflammatory cytokines, including interferon-γ (IFN-γ) (55). The catabolism of TRP via the KYN pathway gives rise to various metabolites that have neuroactive, antioxidant, and immunoregulatory functions (56). Subsequent to the conversion of TRP to KYN, the downstream catabolic process bifurcates into two primary branches: the aminotransferase enzyme (KAT) pathway, leading to the generation of neuroprotective kynurenic acid (KYNA), and the KYN monooxygenase (KMO) enzyme pathway, resulting in 3-hydroxy-kynurenine (3-OH-KYN) and neurotoxic quinolinic acid (QUIN) (Figure 4) (57). Furthermore, certain KYN metabolites like 3-hydroxykynurenine, anthranilic acid, and 3-hydroxyanthranilic acid have demonstrated cytotoxic effects (58). Various end products of KYN metabolism could potentially be released into the fetal circulation (59), influencing fetal development. Overall, aberrations in KYN pathways have been correlated with adverse pregnancy outcomes in rodent models (9, 60), and it has been postulated that KYN metabolites originating in the placenta could contribute to the origins of prenatal brain damage (60).

3.5.2 Serotonin pathway

TRP metabolism along the 5-HT pathway produces active metabolites, such as 5-HT and melatonin (Figure 4) (10). As depicted in Figure 5, the first and rate-limiting enzyme within the 5-HT pathway is TPH, which triggers the hydroxylation of TRP, yielding the intermediate 5-hydroxytryptophan (5-OH-TRP). This intermediate is rapidly subjected to decarboxylation by aromatic amino acid decarboxylase (AADC) to generate 5-HT (5-hydroxytryptamine). TPH exists in two isoforms, with TPH2 being predominantly expressed in the brain, while TPH1 expression prevails in peripheral (48, 61, 62). The production of 5-HT within the placenta plays a vital role in ensuring effective blastocyst implantation, placental development, and the process of decidualization during the initial phases of pregnancy (63, 64). Furthermore, 5-HT holds

significant importance in the developmental processes of the fetal brain (13). The enzyme MAO-A is responsible for the metabolism of 5-HT to 5-hydroxy indoleacetic acid (5-HIAA). Inside the placenta, a portion of 5-HT can undergo additional conversion into melatonin (65), a molecule that contributes to the regulation of fetal growth and placental functionality (66, 67). Given that the placenta serves as a crucial supplier of 5-HT to the fetal brain, any disruptions in the metabolic processing of TRP within the placenta could potentially interfere with 5-HT signaling in specific areas of the developing fetal brain. This interference may consequently result in atypical development of the key axonal pathways (12).

The equilibrium of 5-HT within the placenta is a subject of debate; current insights indicate that in the initial phases of pregnancy, maternal 5-HT plays a crucial role in embryonic development (68). Nevertheless, this responsibility shifts to the placenta's innate 5-HT production capacity at E10.5 in mice (13) and around 11 weeks of human gestation (69). As pregnancy progresses, the fetus gains the ability to synthesize its own 5-HT from maternally supplied TRP (70). Our recent findings also highlight the swift uptake of 5-HT from fetal circulation into trophoblast cells within rat and human term placentas through the organic cation transporter (OCT3/SLC22A3), where it is subsequently broken down by MAO-A (71). Considering that both elevated and reduced serotonemia are harmful to fetal development (72), stringent regulation of the critical enzymes and transporters involved in 5-HT balance within the fetoplacental unit throughout gestation is imperative for the placenta and fetal organs.

The TRP metabolism by 5-HT pathway is subject to modulation during inflammatory episodes. In fact, experimental data demonstrates that the expression of numerous enzymes in the tryptophan metabolism are affected by pro-inflammatory cytokines (73). Additionally, our recent research has provided evidence that preterm birth is connected to substantial alterations in the gene expression of placental TRP metabolism and, this phenomenon is notably correlated with intraamniotic and maternal inflammatory indicators (74).
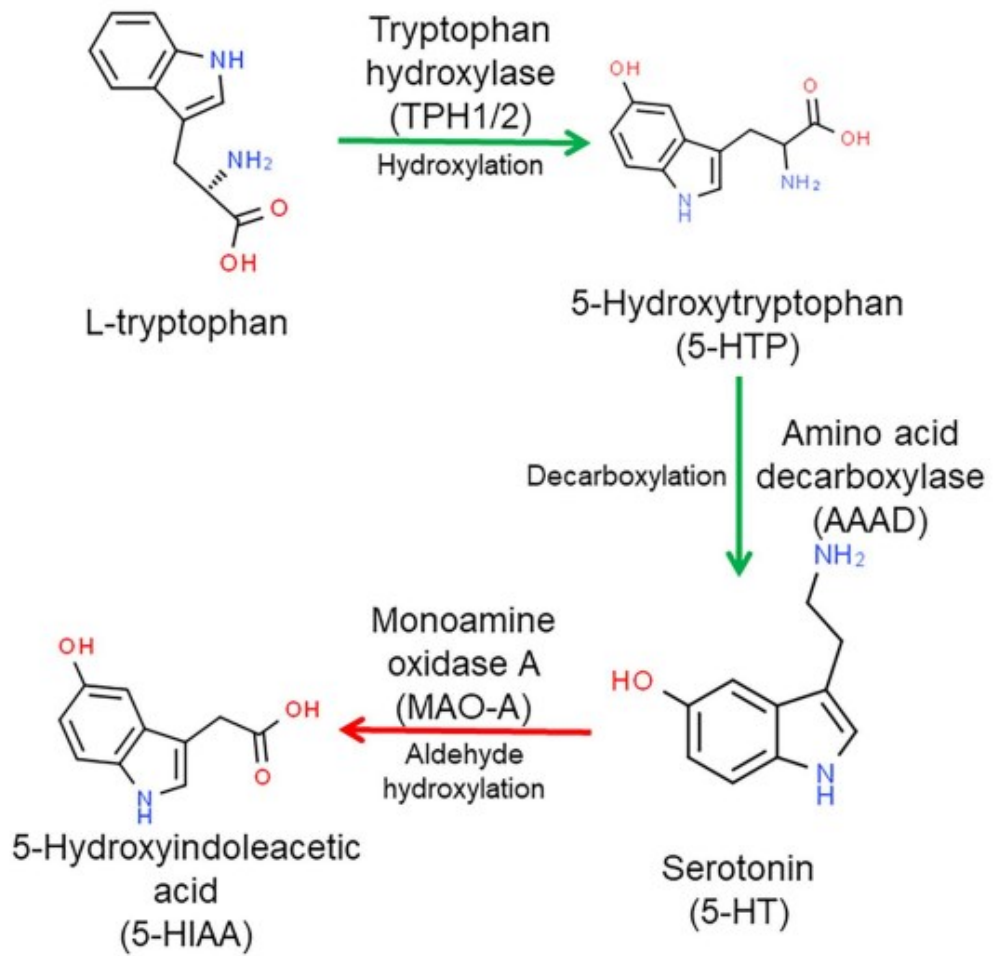
Figure 5. 5-HT pathway of TRP metabolism
Adopted from (75).

4. AIM OF STUDY

Using an ex vivo model of human placenta explants, this thesis aimed to examine the impact of bacterial (LPS) and viral (poly I:C) infections on TRP metabolism by 5-HT pathway in the human placenta. By employing a comprehensive analysis of gene expression, protein expression, enzymatic activity analysis of the key enzymes of 5-HT pathways together with metabolites release of TRP metabolism, we pursued to elucidate the relationship between maternal/intrauterine inflammation and the increased risk of neurodevelopmental disorders in offspring.

5. METHODOLOGY

5.1 Chemicals and reagents

LPS from Escherichia coli O111:B4, Poly I:C and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bicinchoninic acid assay (BCA assay) reagents were purchased from Thermo Scientific (Rockford, IL, USA). Tri Reagent solution was obtained from the Molecular Research Centre (Cincinnati, OH, USA). All other chemicals were of analytical grade.

5.2 Villous samples isolation and cultivation of human term placenta explants.

In this study, term human placentas, with gestational ages between 38 and 40 weeks, were used and obtained from the University Hospital Hradec Králové, Czech Republic. Placentas were collected after planned cesarean sections, involving no-complicated pregnancies. The entire collection process was carried out with the explicit informed consent of the mothers and received approval from the University Hospital Research Ethics Committee (201006S15P). The study also adhered to the ethical principles outlined in the Declaration of Helsinki.

Initially, the umbilical cord, decidual and chorionic membranes of the placenta were carefully removed. Subsequently, cotyledon fragments from various parts of the placenta were meticulously isolated and then randomly cut into villous tissue samples. These samples were cleaned to remove any blood clots or large vessels. Villous tissue was further dissected into explants of approximately 30 mg. The explants were then rinsed in a sterile cold saline solution and placed in 12-well plates containing 2 ml of DMEM-F12 medium and 10% foetal calf serum as culture medium, enriched with penicillin 100 U/ml, streptomycin 0.1 mg / ml and amphotericin B 2.5 μg/ml (76). Each well contained 3 explants in a proportion of approximately 100 mg of villous explants/well. The villous explants were incubated under 8% O_2, 5% CO_2, 87 % N_2 at 37°C in a sterile incubator for 4 hours to equilibrate the cultures and allow recovery from the isolation procedure. After this time, the culture medium was replaced and the explants were kept in the same conditions for an additional 24 to 36 hours before the experiment began. After this period, the explants were incubated with LPS at concentrations of 0.1 or 1 µg/mL, poly I:C at concentrations of 10 or 50 µg/mL, for 4 or 18 hours. After incubation, the cell medium (supernatant) was collected for further analysis, while tissue samples were

homogenized or frozen for the next steps of the experiment. An overview of the experimental design is shown in figure 6.
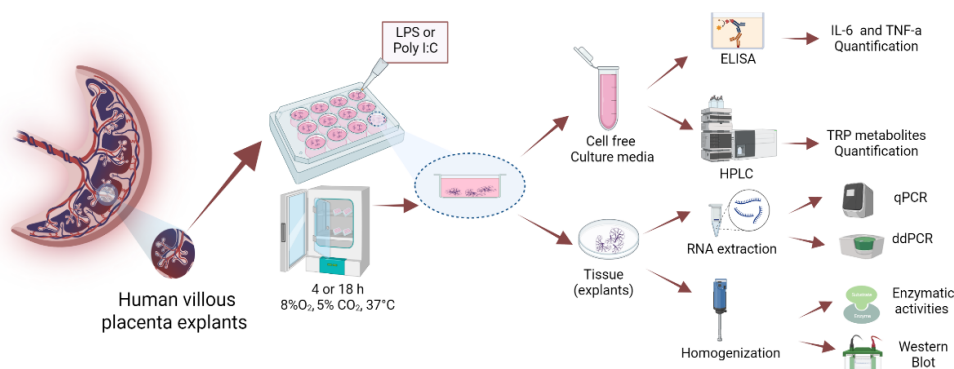
Figure 6. Experimental design used in this study

Healthy term placenta explants were prepared and cultured in 12-well plates for a stabilization period of 24-36 hours. The explants were then incubated with either LPS (0.1 or 1 µg/ml) or Poly I:C (10 or 50 µg/ml) for 4 or 18 hours. Cell-free culture media (supernatant) was collected to quantify pro-inflammatory cytokines using ELISA and TRP and its metabolites using HPLC. Explant tissue was also used for qPCR, ddPCR, and homogenates for Western blot and functional analysis. Adopted from (77).

5.3 Evaluation of viability of term human placenta explants exposed to LPS or Poly I:C.

The viability of the explants was evaluated after 4 or 18 hours of treatment with LPS or Poly I:C employing two different protocols to assess metabolic activity and integrity of the plasma membranes.

The metabolic activity was evaluated by the MTT reduction assay. In this assay, viable cells convert the MTT reagent into formazan crystals by NADPH-dependent mitochondrial oxidoreductase, resulting in a color change that can be quantified using a spectrophotometer. The intensity of the color is directly proportional to the number of metabolically active and viable cells (78). To assess MTT incorporation into tissue, placental explants were subjected to the following procedure: First, the explants were washed with Opti-MEM^{TM} and then incubated with a 0.5 mg/ml MTT solution at 37°C for 1 hour. After the incubation period, the explants were transferred to a new well containing 1 ml of DMSO and shaken for 5 minutes at room temperature to dissolve the formazan crystals. The amount of formazan was quantified by measuring relative absorbance (Abs) in the supernatant at wavelengths 570 nm and 690 nm.

As a positive control, some explants were cultured with 40% DMSO for 18 hours. The results are presented as the difference between Abs 570 and Abs 690 per gramme of tissue.

The integrity of the explants was verified by measuring levels of lactate dehydrogenase (LDH). LDH is an intracellular oxidoreductase enzyme that facilitates the interconversion of pyruvate to lactate. Its activity serves as a commonly used indicator of cell viability and cytotoxicity. When cells experience damage or lysis, LDH is released into the cell culture medium or supernatant, indicating compromised cell membrane integrity. LDH activity was assessed by monitoring the reduction of nicotinamide adenine dinucleotide (NAD) to NADH, which is specifically detected by a colorimetric assay (450 nm) assay, using a Sigma-Aldrich colorimetric LDH activity assay kit (St. Louis, MO, USA), following the manufacturer's instructions (79). The enzymatic activity of LDH in the culture medium was then normalized to the amount of explants (in milligrams), and the results were expressed as nanomoles of NADH produced per milliliter per minute per milligram of tissue. Additionally, to quantify the maximal release of LDH into the medium, explants were cultured with a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 12.7 mM EDTA, 1 mM EGTA, 4 mM Na4P2O7, 1 mM Na3VO4, 1% Triton X-100 and protease inhibitor cocktail, pH 6.8) for 15 minutes at 37°C, serving as a positive control.

5.4 Cytokine analysis

The concentration of specific pro-inflammatory cytokines, IL-6 and TNF-α, was assessed using an enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's guidelines. ELISA kits were obtained from Thermo Fisher Scientific (Rockford, IL, USA). The ELISA method employed a solid phase sandwich technique. Initially, samples and standards were added to the wells, which contained immobilising a capture antibody specific to the target cytokine in a solid phase, to bind to the present antigen. The IL-6/TNF-α biotin conjugate was then introduced, which binds to the antigen. Subsequently, streptavidin-horseradish peroxidase (HRP) was added to bind to the biotin conjugate. After each incubation, unbound fractions were aspirated, and the wells were washed to eliminate nonspecific binding. After adding the substrate (Stabilized Chromogen, Tetramethylbenzidine - TMB), the Abs were read at 450 nm, and the intensity of the color was proportional to the concentration of IL-6/ TNF-α in the samples. A standard curve was constructed, establishing the correlation between color intensity and cytokine concentration. Cytokine release to the culture media was

normalized according to the weight of the tissue and the results were expressed as ng/ml cytokines/g of tissue.

5.5 Ribonucleic acid isolation

Total ribonucleic acid (RNA) was isolated from 100mg of placental villous tissue along with 1ml of TRIzol reagent, according to the manufacturer's instructions. The tissue was homogenized for a duration of 20 seconds. Subsequently, chloroform was utilized to segregate the phases, where the protein located into the organic phase, DNA located at the interface, and RNA persisted within the aqueous phase, as depicted in figure 7. The aqueous phase was removed and 500 µl of isopropanol (2-propanol) was added to precipitate the RNA and allowed to stay at room temperature for 10 minutes. To facilitate co-precipitation and recovery, 1 µl of glycogen was introduced (80). To enable the formation of an RNA pellet, the tubes were subjected to centrifugation at 12,000 g for 15 minutes at a temperature of 4°C. Subsequently, the supernatant was carefully decanted, and the RNA pellet underwent two washes with 75% ethanol, followed by centrifugation under the identical conditions as mentioned earlier. The resultant pellet was then separated from the supernatant and reconstituted in 20 µl of aqua pro injectione (API).

The RNA's purity was assessed through examination of the Abs 260/280 ratio, while potential contamination from organic solvents was evaluated using the Abs 260/230 ratio. The Abs were quantified using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The Abs at a wavelength of 260 was utilized to determine the overall RNA concentration. Finally, the RNA stock solutions were diluted to the work concentration of 500 ng/µl, and the samples were stored at -80 ºC until reverse transcription was performed.
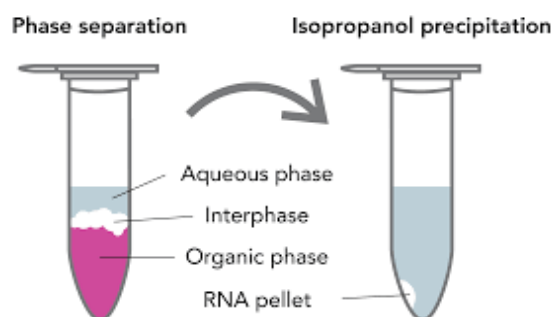

Figure 7. Separated phases and precipitated RNA
(Adopted from *Optimizing RNA Extraction from Cells and Tissues with TRIzol® (quartzy.com)*).

5.6 Reverse transcription

To facilitate subsequent quantitative PCR (qPCR) analysis, the synthesis of complementary DNA (cDNA) was imperative. For this purpose, iScript Advanced cDNA Synthesis Kit (Bio-Rad, USA) was employed. 5.0 µg of isolated RNA was transcribed within a 20 µl reaction employing the T100TM Thermal Cycler (BioRad, Hercules, CA, USA). This kit encompasses a master mix containing an oligo(dT) primer, which binds to the polyA tail of RNA, coupled with reverse transcriptase to generate a complementary cDNA strand. Reverse transcription was executed at 46°C for a duration of 20 minutes, succeeded by a 1-minute phase at 95°C to deactivate the transcriptase enzyme. All procedural steps adhered to the manufacturer's guidelines. Following this reaction, cDNA was obtained at a concentration of 250 ng/µl. For application in qPCR, it was necessary to dilute this concentration by 20 times, resulting in a concentration of 12.5 ng/µl.

5.7 Quantitative PCR analysis

The polymerase chain reaction (PCR) was originally introduced by Mullis in 1985 (81). This technique, highly manageable in nature, employs short-chain sequences of cDNA (or RNA) that are several hundred base pairs in length for the analysis of gene expression. To facilitate this process at elevated temperatures, the method utilizes Taq DNA polymerase derived from the thermophilic bacterium Thermus aquaticus (82).

qPCR analysis for assessing gene expression was executed using the QuantStudioTM 6 instrument (Thermo Fisher Scientific, Waltham, MA, USA). The amplification of cDNA (12.5 ng/µl) was conducted employing the TaqMan® Universal Master Mix II without UNG (Thermo Fisher Scientific, Waltham, MA, USA), within a total reaction volume of 5 µL per well. This was achieved using predesigned TaqMan® Real Time Expression PCR assays (Table 1), while adhering to the thermal conditions stipulated by the manufacturer's guidelines. To establish relative gene expression, normalization was undertaken with respect to the geometric mean of reference genes including Ubiquitin (UBC), DNA Topoisomerase I (TOP1), Eucaryotic Translation Initiation Factor 4A2 (EIF4A2), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), and β2 microglobulin (B2M).

Table 1. List of TaqMan gene assays (Thermo Fisher Scientific, USA).

Gene name	Gene symbol	Assay ID
Reference genes		
Eukaryotic initiation factor-4A	*EIF4A2*	Hs00756996_g1
Ubiquitin	*UBC*	Hs05002522_g1
DNA topoisomerase 1	*TOP1*	Hs00243257_m1
β2 microglobulin	*B2M*	Hs00187842_m1
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	*YWHAZ*	Hs01122445_g1
Target genes		
Interleukin 6	*IL-6*	Hs00174131_m1
Tumor necrosis factor α	*TNF-α*	Hs00174128_m1
Sepiapterin reductase	*SPR*	Hs00268403_m1
6-pyruvoyltetrahydropterin synthase	*PTS*	Hs00609393_m1
Tryptophan hydroxylase 1	*TPH1*	Hs00188220_m1
Tryptophan hydroxylase 2	*TPH2*	Hs00542783_m1
Monoamine oxidase A	*MAO-A*	Hs00165140_m1
Monoamine oxidase B	*MAO-B*	Hs01106246_m1
Solute carrier family 22 member 3 (OCT 3)	*SCL22A3*	Hs01009571_m1
Solute carrier family 6 member 4 (SERT)	*SCL6A4*	Hs00984349_m1
Solute carrier family 7 member 5 (LAT 1)	*SCL7A5*	Hs01001189_m1
Solute carrier family 3 member 2 (4F2HC)	*SCL3A2*	Hs00374243_m1
Solute carrier family 7 member 8 (LAT 2)	*SCL7A8*	Hs00794796_m1

5.8 Digital droplet PCR

Digital droplet PCR (ddPCR) stands as a sophisticated advancement of the PCR technique, initially pioneered by Sykes in 1992 (83). This innovative approach employs the minute partitioning of sub-microliter droplets within an oil medium. Through the encapsulation

of cDNA within these droplets, an enhanced way for the improved amplification and precise quantification of specific target genes is made possible. ddPCR employs identical probes, primers, Taq polymerase, and reaction components as conventional PCR, yet it exhibits elevated levels of repeatability and sensitivity (84, 85).

Duplex ddPCR analysis of TPH, and MAO within the placental explant was executed following previously established methodologies (54). By harnessing the duplex feature, we achieved the simultaneous absolute quantification of both target and reference gene expressions. In essence, the duplex reaction mixture comprised 10 µL of ddPCR™ Supermix for Probes (Bio-Rad, Hercules, CA, USA), along with 1 µL of each predetermined probe assay (FAM and HEX), and 1 µL of cDNA (25 ng/µL), all in a volume of 20 µL. Droplets were generated through a QX200 Droplet Generator and subsequently amplified to an endpoint using a T100™ Thermal Cycler, following thermal conditions in accordance with the manufacturer's guidelines. Subsequent droplet enumeration took place within a QX200™ Droplet Reader, and target gene concentration was deduced through the utilization of QuantaSoft™ Software. For ultimate data assessment, only wells exhibiting droplet counts exceeding 13,000 were considered. Results are presented as transcript numbers per ng of transcribed RNA. Unless otherwise noted, the QX200™ Droplet Digital™ PCR System, T100™ Thermal Cycler, and all related consumables and reagents were procured from BioRad (Hercules, CA, USA).

5.9 Placenta homogenates

Explant tissues were rinsed with 0.9% NaCl solution while maintained at 4°C. Post-weighing, the explants were finely sectioned and homogenized at 4°C within a buffer solution containing 50 mM Tris-Hepes (pH 7.2), 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose for functional analysis or with a lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 12.7 mM EDTA, 1 mM EGTA, 4 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% Triton X-100 and protease inhibitor cocktail, pH 6.8; for Western blot analysis. The homogenized mixtures underwent centrifugation at 10,000 g for 15 minutes. The resulting supernatant was carefully harvested and then preserved in the freezer at a temperature of -80°C, earmarked for subsequent usage. The quantification of protein concentration was accomplished through the application of the Pierce™ BCA protein assay kit, in strict adherence to the manufacturer's directives.

5.10 Tryptophan hydroxylase enzyme activity assessment

To determine the enzymatic activity of TPH, placenta homogenates were fortified with 1mM dithiothreitol (DTT) to reduce the protein and achieve maximal enzymatic activity (86). The TPH activity was estimated using the protocol outlined by Goeden and colleagues (3). The enzymatic reaction was conducted at 37°C under pH 7.5 conditions. The reaction mixture comprised (at final concentrations): 50 mM Tris buffer, 1mM EGTA, 100 units/ml catalase, 0.1 ammonium iron (II) sulphate, and 0.1 tetrahydrobiopterin (BH4), which serves as a cofactor essential for TPH function. This setup was executed both in the absence and presence of 0.25 mM L-tryptophan. In brief, 100 µl of placenta explant homogenate were incubated alongside 400 µl of the reaction mixture for 30 minutes at 37°C. The reactions were halted by adding 100 µl of HClO4 containing 100 µM EDTA. Following this, the samples were subjected to 15 minutes of ice incubation for comprehensive protein denaturation, followed by centrifugation at 21,000 g for 15 minutes. The resulting supernatants were collected for quantification of 5-OH-TRP using high-performance liquid chromatography (HPLC). The outcomes were computed as the disparity between the liberated 5-OH-TRP in tubes containing L-TRP and that in tubes lacking TRP and results are expressed as nanomoles of 5-OH-TRP per milligram of protein per minute.

5.11 Monoamine oxidase enzymatic activity determination

MAO was assessed following the approach outlined by Carrasco and colleagues (87). In essence, 180 µl of placenta homogenate (1.5-2 mg/ml) underwent a preincubation phase of 5 minutes at 37°C, with or without phenelzine (100 µM). The reaction was then instigated by introducing 20 µl of 5-HT (0.5 mM) and left to incubate for 60 minutes. To stop the reaction, 40 µl of HClO4 (3.4 M) was added, and the mixture was placed on ice for 5 minutes. Afterward, the samples were subjected to centrifugation at 5,000 g for 10 minutes, and the ensuing supernatant was gathered for 5-HT quantification via HPLC. The results are presented as the percentage of metabolized 5-HT relative to the initially added 5-HT.

5.12 Western blot analysis

Samples of placental homogenate, containing 35 µg total protein, were combined with loading buffer under reducing conditions (88), heated to 96°C for a duration of 5 minutes, and subjected to separation via SDS-PAGE on either 10% (MAO) or 15% (TPH) polyacrylamide

gels. Electrophoresis was carried out at 120 V, and the proteins were subsequently transferred onto PVDF membranes (BioRad, Hercules, CA, USA). The membranes were blocked in a solution of 20mM Tris-HCl at pH 7.6, 150 mM NaCl, and 0.1% Tween 20 (TBS-T), containing 5% bovine serum albumin (BSA), for a duration of 1 hour at room temperature. Following this, the membranes were rinsed with TBS-T buffer. Incubation with primary antibodies took place overnight at 4°C against MAO-A (Abcam, ab126751, at a dilution of 1:1000) and TPH (Invitrogen, PAI-777, at a dilution of 1:100). After a wash with TBS-T buffer, the membranes were exposed to the respective secondary antibody, namely anti-rabbit horseradish peroxidase-linked antibody (Dako, P0217, at a dilution of 1:20000), for a duration of 1 hour at ambient temperature. Development of the membranes was executed using the Chemiluminescence HRP Substrate Kit (ECL™ Prime Western Blotting System). Visualization and quantification of band intensity were achieved through densitometric analysis employing the ChemiDocTM MP Imaging system (BioRaD, Hercules, CA, USA). In order to ensure uniform loading of proteins, the membranes were probed for β-actin (Abcam, ab 8226, at a dilution of 1:10000), accompanied by the specific secondary antibody, anti-mouse HRP (Dako, P0260, at a dilution of 1:20000).

5.13 Quantification of TRP, 5-OH- TRP, 5-HT, and HIAA

Quantification of TRP and its metabolites in cell-free supernatants was carried out employing a Shimadzu LC20 HPLC chromatograph (Shimadzu, Kyoto, Japan) outfitted with a fluorescence detector. The chromatographic separation employed a Kinetex EVO C18 100 A 150 x 3 mm column (Phenomenex, USA) with a particle size of 5 μm, serving as the stationary phase. The flow rate was meticulously set at 0.5 mL/min, while the operational temperature was maintained at 20°C. For the effective elution of 5-OH-TRP, 5-HT, and TRP, a mobile phase comprised of 3:97 (v/v) methanol: acetic acid (0.1M, pH 4.5, modified with NaOH) was utilized. The fluorescence detector's excitation and emission wavelengths were configured as 276/33 nm. In the case of HIAA analysis, the mobile phase consisted of 7:93 (v/v) methanol: acetic acid (0.2M), and the identical wavelengths were adopted for the fluorescence detector as previously mentioned.

5.14 Statistical analysis

The experimental results were evaluated employing the non-parametric Mann–Whitney test (for pairwise group comparisons) or the Kruskal–Wallis test (for comparisons involving

multiple groups), followed by Dunn's post hoc multiple comparisons test. These statistical analyses were conducted using GraphPad Prism 8.3.1 software (GraphPad Software, Inc., San Diego, USA). Significance levels are denoted in the figures by asterisks: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

6. RESULTS

6.1 Viability and integrity of term placenta explants exposed to LPS and Poly I:C.

Placental explant viability was assessed throughout the experiments by examining the tissue's mitochondrial metabolic function using the MTT assay. Notable alterations in metabolic activity were not discerned in explants subjected to LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) incubation for 4 hours (Figure 8A) or 18 hours (Figure 8C) in comparison to the control. As a measure of cell death control (positive control), explants were cultured for 18 hours with 40% DMSO.

Furthermore, the integrity of the cell membrane was evaluated by quantifying the release of the intracellular enzyme LDH into the culture medium after treatment with LPS or Poly I:C for 4 hours or 18 hours. The LDH activity in the culture supernatant of explants exposed to LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) exhibited no significant deviations from the control (Figure 8A and 8D). To determine the maximal LDH activity in the culture medium, explants were treated with lysis buffer for 15 minutes at 37°C, serving as a positive control for comparison.
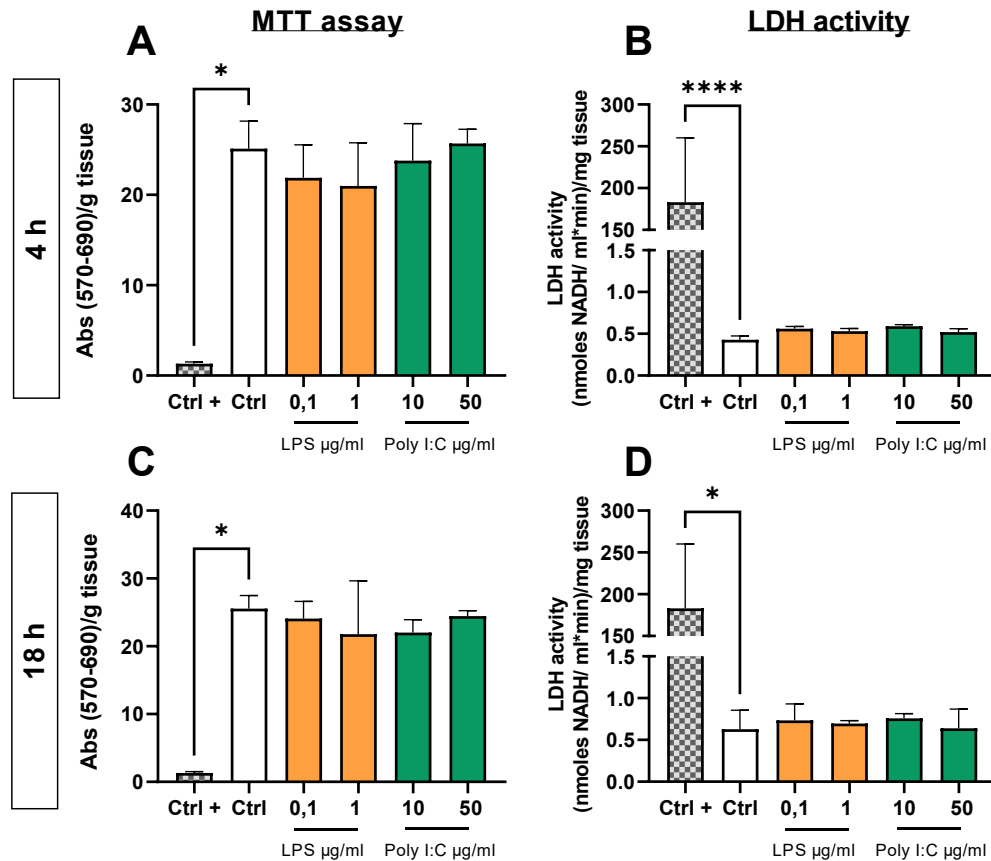
Figure 8. Viability of human term placenta explants.

Explants' viability was assessed via MTT assay, gauging mitochondrial activity colorimetrically. MTT incorporation was evaluated in explants incubated with or without LPS or Poly I:C for 4 hours (B) and 18 hours (D). As a positive control, explants cultured with 40% DMSO for 18 hours (Ctrl+) were included. Additionally, explant integrity was probed by quantifying LDH activity in the culture medium after 4 hours (A) and 18 hours (C) of LPS or Poly I:C treatment. Positive control samples (Ctrl+) consisted of explants treated with lysis buffer for 15 minutes at 37°C. Data, presented as the median with IQR, were derived from five samples (n = 5). Statistical analysis involved the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test; significance levels indicated by * ($p \leq 0.05$) and **** ($p \leq 0.0001$).

6.2 Gene expression and protein release of pro-inflammatory cytokines in response to treatment with LPS or poly I:C.

Incubation of term placenta explants with LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) resulted in notably elevated gene expression levels of the pro-inflammatory cytokines IL-6 and TNF- α at all examined time points (4h and 18h), as depicted in Figure 9A, 9C, 9E, and 9G. Moreover, explants subjected to LPS or Poly I:C exposure exhibited a marked increase in the release of IL-6 and TNF-α cytokines into the culture medium, providing confirmation of the successful induction of inflammatory conditions (Figure 9B, 9D, 9F, 9H).
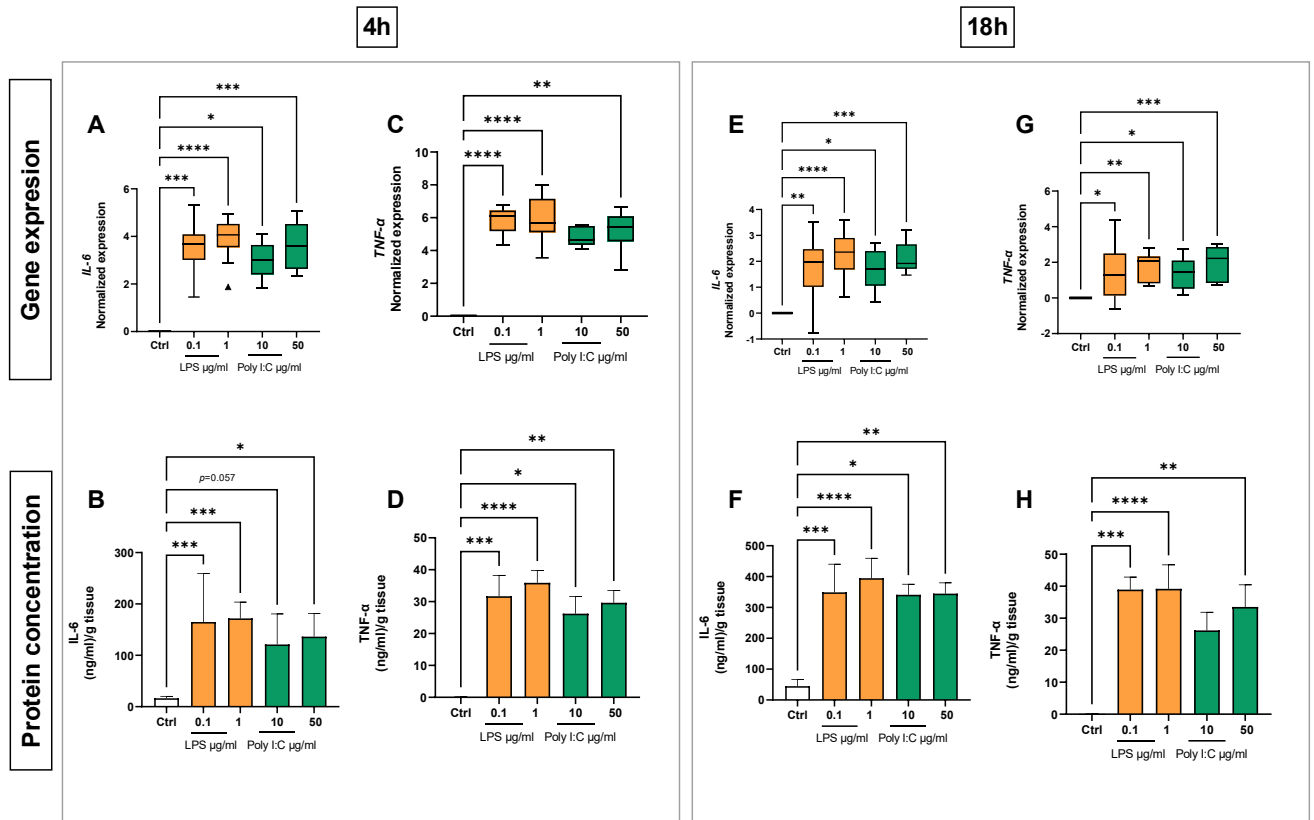
Figure 9. Gene expression and protein release of pro-inflammatory cytokines in human term placenta explants exposed to LPS and Poly I:C.

Gene expression of IL-6 (A, E) and TNF-α (C, G) was examined through qPCR analysis in explant cultures for either 4 hours (A, C) or 18 hours (E, G), in the presence of LPS or Poly I:C. Concurrently, the production of IL-6 (B, F) and TNF-α (D, H) by villous explants during 4-hour (B, D) or 18-hour (F, H) culture, with or without LPS or Poly I:C, was quantified in the culture medium using ELISA. Cytokine concentrations in the conditioned media were adjusted based on the wet weight of the explant tissue. The data are depicted as Tukey boxplots (1.5-times IQR) for qPCR or as mean ± SD for cytokine concentrations; with a sample size of n ≥ 8. The statistical significance was evaluated employing the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test, where * (p ≤ 0.05), ** (p ≤ 0.01), ***(p ≤ 0.001), and ****(p ≤ 0.0001) denote the levels of significance.

6.3 TRP, 5-HT and HIAA metabolites level in culture media from placenta explants under inflamed conditions.

We assessed the impact of bacterial endotoxin and viral mimic infection on the production and release of TRP and its metabolites, 5-HT and 5-HIAA, from placenta explants into the culture media. The concentration of TRP notably decreased upon treatment with LPS or Poly I:C, regardless of the time and concentration used (Figure 10A and 10F). This observation suggests active TRP consumption by placenta explants from the available media, as depicted in Figure 10. On the contrary, placenta explants exposed to LPS or Poly I: C exhibited a significant reduction in 5-HT and 5-HIAA production (Figure 10B, 10C, and 10G) compared to untreated control cultures. This decrease implies a diminished conversion of TRP along the

5-HT pathway in the presence of inflammation. In particular, after 18 hours of culture, 5-HT was undetectable in the medium, implying its metabolism by MAO during the extended incubation period. Interestingly, the media from explants cultured for 4 hours under inflammatory conditions displayed a notable reduction in the 5-HT/TRP ratio compared to the control, while the HIAA/5-HT ratio remained unaffected (Figure 10D, 10E).
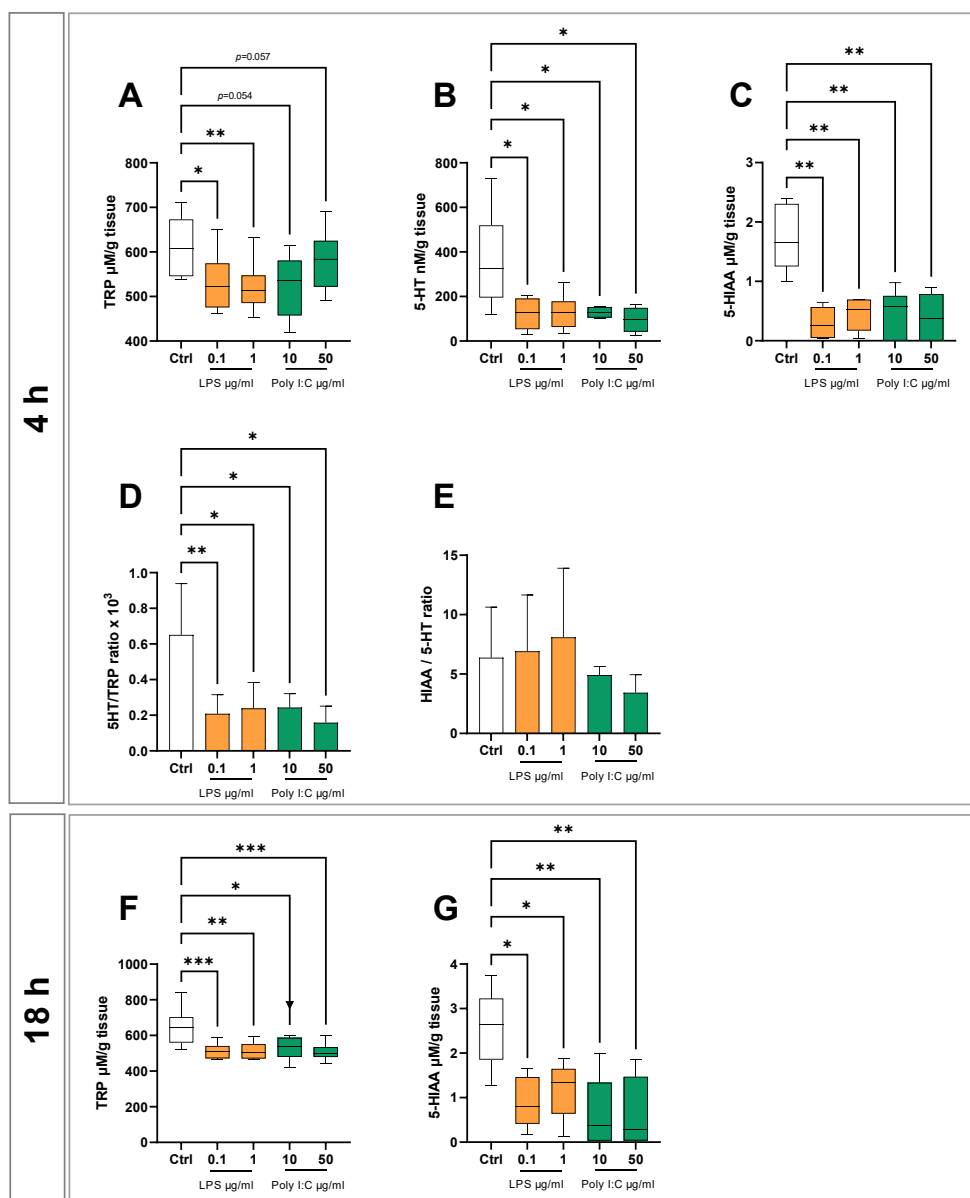
Figure 10. TRP metabolites in culture media from placenta explants under inflamed conditions. Concentration of TRP (A, G) and its main metabolites, 5-HT (C) and HIAA were assayed on culture media from placenta explants exposure to LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) for 4 or 18 h. The results are displayed as Tukey boxplots (1.5-times IQR) of metabolite concentrations normalized to wet weight of the explant tissue n ≥8. Ratio of 5-HT/TRP and HIIA/5-HT are presented as mean ± SD. Statistical significance was evaluated using the non-parametric Mann-Whitney test; * (p ≤ 0.05), ** (p ≤ 0.01) and ***(p ≤ 0.001).

6.4 Relative gene expression of enzymes and transporters involved in TRP metabolism by 5-HT pathway in the human term placenta explants exposed to inflammation.

A comprehensive examination was performed using qPCR on a panel of 13 genes associated with tryptophan metabolism through the 5-HT pathway, in addition to transport mechanisms, within human term placenta explants. These explants were cultured in the presence of LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) for 4 or 18 hours. The obtained results are illustrated in Figures 11, 12, and 13. According to the Kruskal-Wallis analysis, differences were observed in the expression of four genes from 5-HT pathway when comparing control explants to those treated with LPS or Poly I:C. Specifically, the relative expression of two genes in the serotonin pathway, MAOB and THP2, exhibited significant upregulation following a 4-hour exposure to Poly I:C (Figure 11). Moreover, the relative expression of three genes, namely PTS, MAOA, and MAOB, demonstrated significant upregulation after an 18-hour exposure to either LPS or Poly I:C (Figure 12). Furthermore, within the spectrum of assessed transport proteins, SLC7A8 (LAT2) exhibited a downregulation in expression among explants treated with LPS for 4 hours, while SLC6A4 (SERT) showed a downregulation after an 18-hour exposure to Poly I:C, both in comparison to the control (Figure 13).
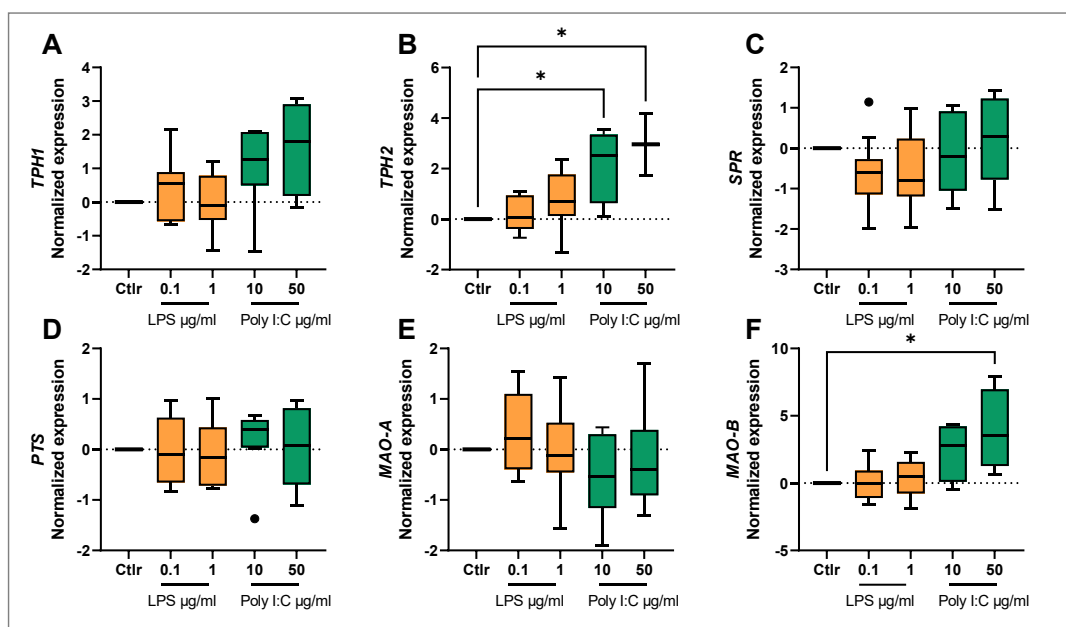
Figure 11. Gene expression of proteins involved in 5-HT pathway exposed to inflammation for 4 hours.
Gene expression analysis in placenta explants incubated for 4 with and without LPS or Poly I:C were analyzed by qPCR. The results are presented as Tukey boxplots (1.5-times IQR) n ≥8. Statistical significance was evaluated employing the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test, * (p ≤ 0.05).
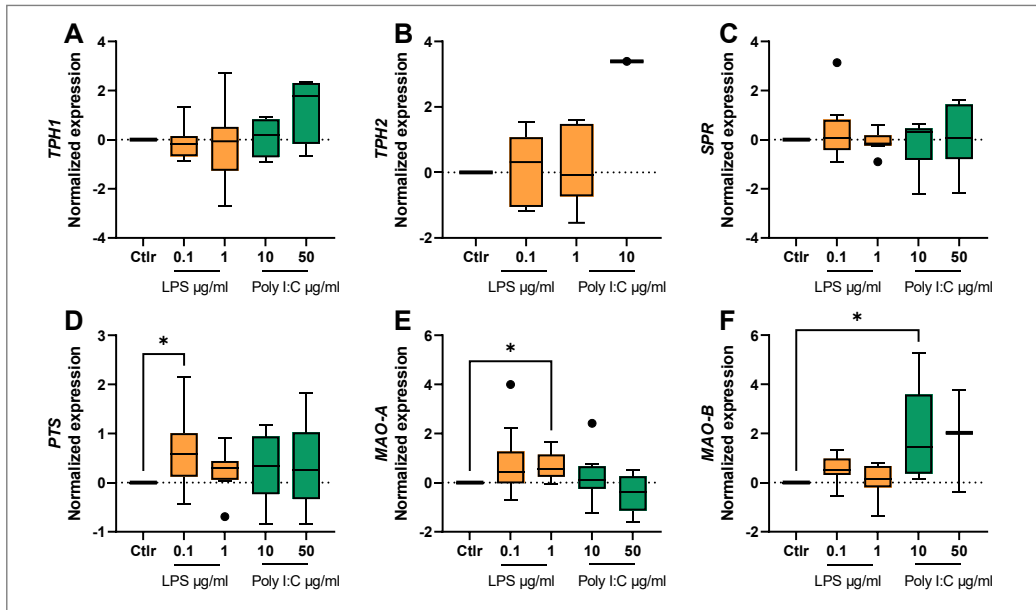
Figure 12. Gene expression of proteins involved in 5-HT pathway exposed to inflammation for 4 hours.

Gene expression analysis in placenta explants incubated for 8 with and without LPS or Poly I:C were analyzed by qPCR. The results are presented as Tukey boxplots (1.5-times IQR) n ≥8. Statistical significance was evaluated employing the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test, * (p ≤ 0.05).

Figure 13. Gene expression of transports protein for TRP and 5-HT.

Gene expression analysis in placenta explants incubated for 8 with and without LPS or Poly I:C were analyzed by qPCR. The results are presented as Tukey boxplots (1.5-times IQR) n ≥8. Statistical significance was evaluated employing the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test, * (p ≤ 0.05).
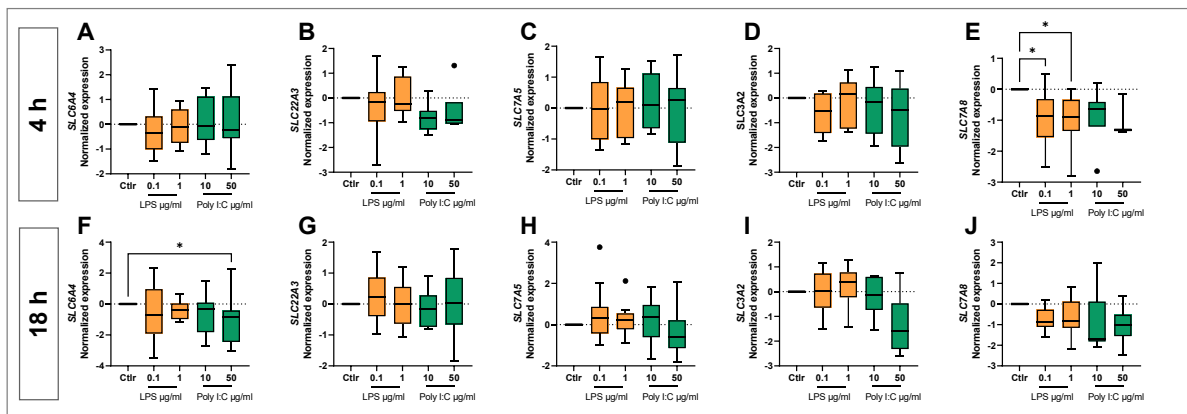
6.5 Gene/protein expression and functional analyses of the main enzymes of TRP metabolism by 5-HT pathway under inflammation.

A noteworthy reduction in metabolites stemming from the 5-HT pathway was noted in explants subjected to LPS or Poly I:C treatment in comparison to the control group. This observation implies potential alterations in the expression and/or activity of key enzymes implicated in the 5-HT pathway. To gain a comprehensive understanding of these alterations, an in-depth assessment encompassing gene expression, protein expression, and functional analysis of the principal enzymes involved in the 5-HT pathway (TPH and MAO) was executed utilizing ddPCR, Western blotting (WB), and enzymatic activity analysis.

We performed precise TPH and MAOA transcript quantification through ddPCR analysis in placenta explants cultured under different conditions: with and without LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) for 4 or 18 hours. Intriguingly, a statistically significant decrease in THP1 expression, the initial and rate-limiting enzyme of the 5-HT pathway, was observed in placenta explants exposed to LPS (0.1 and 1µg/ml) for 4 hours and to either LPS or Poly I:C for 18 hours (Figure 14A and 14G). On the contrary, MAO-A expression exhibited an up-regulation after 4 or 18 hours of treatment with LPS or Poly I:C (Figure 14D and 14J).

For a comprehensive exploration of protein expression, a quantitative WB analysis was conducted utilizing specific antibodies targeting TPH and MAOA. This analysis was performed on homogenates derived from placenta explants cultured under inflamed conditions: with and without LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) for time periods of 4 or 18 hours. Comparable to the gene expression findings, TPH protein expression displayed a reduction in placenta explants exposed to LPS (0.1 and 1µg/ml) for both the 4-hour and 18-hour treatment (Figure 7B and 7H). Conversely, MAO protein expression exhibited an elevation subsequent to Poly I:C treatment for either 4 or 18 hours (Figure 14E and 14K).

The concluding phase of our investigation involved the examination of enzymatic activity for both proteins. Regarding this aspect, the enzymatic activity of TPH was notably diminished in explants subjected to LPS or Poly I:C treatment, across various concentrations and time frames, when compared to the untreated control (Figure 14C and 14I). In contrast, the enzymatic activity of MAO remained unaffected by the treatment with LPS or Poly I:C (Figure 14F and 14L).
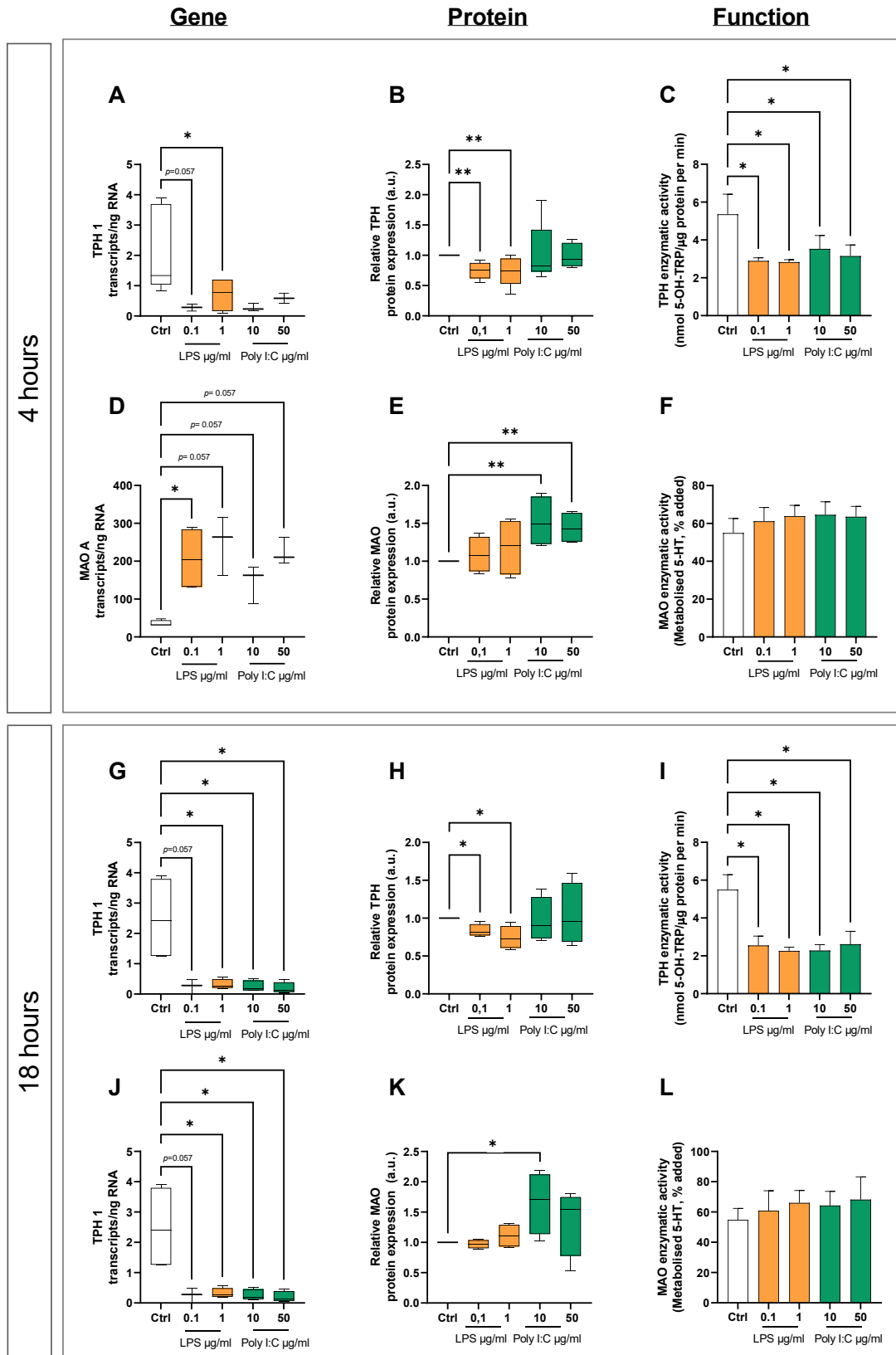
Figure 14. Gene/protein expression and functional analyses of the main enzymes of TRP metabolism by 5-HT pathway under inflammation.

Human term explants were cultured by 4 h or 18 h in the presence of LPS or Poly I:C. Absolute quantification of the number of transcripts was evaluated by digital droplet PCR (A, D, G, J), and protein expression was evaluated

by western blot analysis (B,E,H,K). Protein expression was normalized to β-actin. Enzymatic activity of TPH (C, I) and MAO (F, L) was evaluated as described in the methods section. Data are presented as Tukey boxplots (1.5-times IQR) or the mean ± SD; n = 5. Statistical significance was evaluated using the non-parametric Mann-Whitney test; * ($p \leq 0.05$) and ** ($p \leq 0.01$).

7. DISCUSSION

A comprehensive collection of epidemiological, preclinical, and prospective observational studies substantiates the notion that the embryonic/fetal phase of brain development is particularly vulnerable to disruptive factors (89). A prominent factor that has garnered extensive attention and research in recent times is maternal inflammation. Indeed, the correlation between maternal inflammation during pregnancy and the susceptibility of offspring to neuropsychiatric disorders has gained growing acceptance in recent years. Moreover, inflammatory processes in mothers during pregnancy influence placental functions and are linked to an elevated probability of neurodevelopmental disorders in their offspring (3). Nevertheless, the precise molecular pathways that connect placental malfunction to atypical fetal neurodevelopment remain unknown. Since 5-HT is crucial for fetal brain development, this work aimed to investigate alterations in the serotonin pathway of TRP metabolism induced by inflammatory effects within the placenta.

Human placenta explants form healthy pregnancies were treated with LPS (0.1 and 1µg/ml) or Poly I:C (10 and 50 µg/ml) for 4 or 18 h to induce inflammation, as outlined in our results. This treatment led to a significant increase in gene expression and the release of pro-inflammatory cytokines IL-6 and TNF-α from the placenta explants. Human placenta expresses toll-like receptors 4 and 3, which recognize the LPS and Poly I:C respectively, leading to activation of the transcription factors NF-κB and IRFs (90) which results in the upregulation, synthesis, and release of numerous pro-inflammatory cytokines, including IL-6 and TNF-α. Other studies employing LPS as a treatment have demonstrated elevated concentrations of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, across diverse placental models (91).

Evaluating cellular viability following treatment holds significant importance in tissue culture simulations. Our investigation focused on viability indicators within explants, encompassing LDH activity in the culture medium and mitochondrial activity assessed via MTT. These parameters displayed no changes during the treatment phases in comparison to the control conditions. Our findings indicate that, within the employed concentration and exposure times, neither LPS nor Poly I:C induces any harm to placental explants. Consequently, any subsequent tissue responses observed in our experiments are solely attributed to the proinflammatory mechanisms instigated by LPS or Poly I:C.

TRP metabolism is crucial for the regulation of immune response, generation of oxidative radicals, and generation of neuroregulatory substances (73, 92). Furthermore, TRP metabolism is particularly sensitive to proinflammatory cytokines (93). Certainly, in our model, explants treated with LPS, or Poly I:C exhibited a decline in TRP levels in culture media compared to control, implying an increase in TRP utilization under inflamed conditions. Nonetheless, during the assessment of TRP metabolite release via the 5-HT pathway (5-HT and HIAA) into the culture medium, we identified a reduction in 5-HT production, subsequently leading to diminished HIAA concentrations in explants exposed to LPS or Poly I:C for 4 hours. Suggesting that TRP consumption in the culture media by inflamed conditions may be related to TRP metabolism by KYN pathway.

5-HT has been associated with a broad spectrum of cerebral functions, covering autonomic control, sensory interpretation, nourishment, motor operations, as well as emotional equilibrium and cognitive processes (94). Notably, 5-HT actively regulates neurodevelopment, and alterations in its concentrations throughout the developmental period can wield lasting implications across one's lifespan (75). In the course of normal pregnancy and normal developmental progression, 5-HT is produced within the placenta through the conversion of maternal TRP. This synthesized 5-HT then crosses to the fetal brain, where it exerts regulatory effects on pivotal neurodevelopmental mechanisms (3). Experimental findings indicate that disruptions in gestational 5-HT regulation, including both low and high serotonin levels during the perinatal period can lead to behavioral deficits in adulthood and have been linked to the etiology of neurodevelopmental disorders (95, 96).

Explants subjected to inflammatory stimuli exhibited a reduction in 5-HT concentrations and the 5-HT/TRP ratio, indicating a decrease in TRP metabolism within the 5-HT pathway. Additionally, our model revealed a decrease in TPH expression and enzymatic activity suggesting a direct impact of inflammation on TPH. Reduced TPH levels have been documented and linked to inflammation triggered by acute ischemic stroke and carotid stenosis (97). Furthermore, a decreased in 5-HT/TPH, melatonin/TRP and melatonin/5-HT ratios were found in sequential inflammatory response in the brain, after traumatic brain injury (TBI) in a rabbit pediatric TBI model (98). Consistent with prior studies, intrauterine inflammation was found to reduce 5-HT levels in the placenta and neonatal rabbit brain (99, 100), while maternal inflammation increased 5-HT synthesis within the mouse placenta (3). Intriguingly, the enzyme MAO, responsible for serotonin metabolism, displayed increased activity in placental explants

exposed to inflammation, implying an elevated serotonin breakdown alongside diminished synthesis.

Our prior research has demonstrated that within normal physiological conditions, the metabolic activity of TRP in the placenta is a dynamic process that undergoes changes over gestation in response to the needs of the fetus (53, 54). Identifying the precise timing of the inflammatory challenge holds significant importance in comprehending its repercussions on fetal development (101). During the initial phases of pregnancy, the development of 5-HT-dependent organs, including the brain, relies on placental 5-HT synthesis. A reduction in 5-HT synthesis due to inflammatory influences might lead to adverse effects on the fetal brain's maturation. Conversely, during the later stages of pregnancy, the fetus becomes capable of generating its own 5-HT using maternal TRP. However, maternal inflammation disrupts TRP levels, affecting the availability of placenta-derived TRP to the fetus, consequently impacting 5-HT production in the fetal brain.

The decline in placental 5-HT synthesis induced by inflammatory triggers can potentially influence melatonin levels, which is a subsequent product within the 5-HT pathway known for its antioxidative and anti-inflammatory attributes. Studies have indicated that melatonin has the capability to decrease cytokine production linked to inflammation (102). Consequently, a reduction in melatonin levels may contribute to sustaining the pro-inflammatory environment within the placenta.

8. Conclusions

In summary, our findings indicate that inflammatory processes within the placenta disrupt the equilibrium of TRP, potentially influencing fetal neurodevelopmental programming. This perturbation is primarily orchestrated through the reduction of 5-HT production during intrauterine stages, which can detrimentally impact the maturation of the fetal brain. These outcomes shed light on the intricate interplay between maternal/intrauterine inflammation and the heightened susceptibility of offspring to neurodevelopmental disorders.

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