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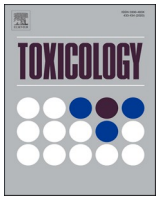

Cannabidiol disrupts tryptophan metabolism in the human term placenta

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ABSTRACT

The increasing use of cannabis during pregnancy raises concerns about its impact on fetal development. While cannabidiol (CBD) shows therapeutic promise, its effects during pregnancy remain uncertain. We investigated CBD's influence on tryptophan (TRP) metabolism in the human placenta. TRP is an essential amino acid that is metabolized via the serotonin and kynurenine (KYN) pathways, which are critical for fetal neurodevelopment. We used human term villous placental explants, an advanced ex vivo model, to study CBD's impact on key TRP metabolic enzymes. In addition, vesicles isolated from the microvillous membrane (MVM) of the human placenta were used to assess CBD's effect on placental serotonin uptake. Explants were exposed to CBD at therapeutic (0.1, 1, 2.5 μg/ml) and non-therapeutic (20 and 40 μg/ml) concentrations to determine its effects on the gene and protein expression of key enzymes in TRP metabolism and metabolite release. CBD upregulated TRP hydroxylase (TPH) and downregulated monoamine oxidase (MAO-A), resulting in reduced levels of 5-hydroxyindoleacetic acid (HIAA). It also downregulated serotonin transporter expression and inhibited serotonin transport across the MVM by up to 60% while simultaneously enhancing TRP metabolism via the kynurenine pathway by upregulating indoleamine-pyrrole 2,3-dioxygenase (IDO-1). Among kynurenine pathway enzymes, kynurenine 3 monooxygenase (KMO) was upregulated while kynurenine aminotransferase 1 (KAT-1) was downregulated; the former is associated with neurotoxic metabolite production, while the latter is linked to reduced neuroprotective metabolite levels. Overall, these results indicate that CBD modulates TRP catabolism in the human placenta, potentially disrupting the tightly regulated homeostasis of the serotonin and KYN pathways.

1. Introduction

Cannabis, alcohol, and tobacco are among the primary drugs of abuse by pregnant women (Cook et al. 2017). Unfortunately, the impact of cannabis on fetal development is poorly characterized, which has contributed to a concerning 170 % rise in cannabis use during pregnancy between 2009 and 2016 (Young-Wolff et al. 2017). In humans, prenatal cannabis exposure has been linked to several negative outcomes, including preterm birth, decreased fetal growth, increased neonatal care requirements, and miscarriage (Gurm et al. 2021). It also negatively impacts fetal neurodevelopment, reducing performance in cognitive domains such as verbal reasoning, memory, and visual function (Gurm et al. 2021; Roncero et al. 2020). However, the limited research on cannabis toxicology and inconsistencies between reported findings present a precarious landscape, especially given the alarming surge in the consumption of cannabis in various forms, concentrations, and public visibility levels (Henschke, 2019; Sohn, 2019).

Cannabis phytocomplex contains over 500 biologically active compounds (Atakan, 2012; Hanus et al. 2016), including the non-psychoactive compound cannabidiol (CBD), which has gained

Abbreviations: BCA, bicinchoninic acid; COVID-19, Coronavirus disease 2019; DMSO, dimethyl sulfoxide; HIAA, 5-hydroxyindoleacetic acid; IDO-1, indoleamine-pyrrole 2,3-dioxygenase; KAT-1, kynurenine aminotransferase; KMO, kynurenine 3 monooxygenase; KYN, kynurenine; KYNA, kynurenic acid; LAT-1, L-type amino acid transporter 1; LAT-2, L-type amino acid transporter 2; LDH, lactate dehydrogenase; MAO-A, monoamine oxidase; MTT, thiazolyl blue tetrazolium bromide; MVM, microvillous membrane; OCT3, organic cation transporter 3; PMSF, phenylmethylsulfonyl fluoride; SERT, serotonin transporter; TDO, tryptophan 2,3-dioxygenase; THC, tetrahydrocannabinol; TPH, tryptophan hydroxylase; TRP, tryptophan.

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recognition for its therapeutic potential. Clinical evidence supports its anti-inflammatory, pain-relieving, and anxiety-reducing effects in the treatment of conditions including chronic pain, multiple sclerosis, epilepsy, Huntington's disease, colorectal cancer, gliomas, and anxiety (Legare et al. 2022). The perception of cannabis as beneficial among pregnant women (Young-Wolff et al. 2022) together with the limited understanding of the physiological effects of cannabis and synthetic cannabinoids poses significant risks to both maternal and fetal health.

Studies in mice have shown that CBD readily crosses the placental barrier (Ochiai et al. 2021), binding to endocannabinoid receptors in the fetal brain (Swenson et al. 2023). Toxic effects resulting from prenatal cannabinoid exposure have, therefore mainly been linked to direct effects on fetal brain development (Hutchings et al. 1989; Richardson et al. 2016; Roberts et al. 2022). However, studies in various species, including humans, monkeys, mice, and rats have reported that cannabinoids disrupt essential placental functions, including nutrient and oxygen transport, hormone production, and immune regulation (Dong et al. 2019; Natale et al. 2020; Rompala et al. 2021). This may adversely affect fetal nourishment and hinder normal organogenesis, with negative long-term implications for fetal growth and development (Benevenuto et al. 2017; De Genna et al. 2022; Grant et al. 2020). Further research is thus needed to clarify the full range of phytocannabinoids' potential effects on fetal health.

The placenta is a transient multifunctional organ that plays an essential role throughout pregnancy (Challier, 1989). Our recent studies using animal and human models have revealed the pivotal significance of maintaining tryptophan (TRP) metabolism homeostasis during placental development (Abad et al. 2020; Karahoda et al. 2020a). TRP is an essential amino acid vital for several physiological processes and a specific pharmacological target (Modoux et al. 2021) that is primarily metabolized via the serotonin and kynurenine (KYN) pathways. TRP metabolism through the serotonin pathway generates active metabolites such as serotonin and melatonin (Perić et al. 2022). Placental serotonin plays a critical role in successful blastocyst implantation and placentation during early pregnancy stages (Mitchell and Hammer, 1983). Additionally, serotonin and melatonin maintain maternal glucose homeostasis, regulate steroid synthesis, and support fetal organ development and programming (Bonnin and Levitt, 2011). Conversely, TRP metabolism through the KYN pathway yields metabolites such as KYN, kynurenic acid (KYNA), 3 hydroxy-kynurenine and quinolinic acid (Muneer, 2020). These metabolites possess immunosuppressive, neurochemical, and redox activities (Modoux et al. 2021). Intensive research over the last decade has demonstrated the importance of the TRP and monoamine system in the placenta (Bonnin et al. 2011; Goeden et al. 2017) for fetal brain development and programming (Staud and Karahoda, 2018). Since the metabolites of both pathways are involved in fetal development and programming, any perturbations in their homeostasis can have serious developmental and functional consequences (Bonnin and Levitt, 2012; Hadden et al. 2017; Marley et al. 1967).

Several studies have suggested interactions between CBD and TRP metabolism in rat and murine brains, and in human peripheral blood mononuclear cells (di Giacomo et al. 2020b; Florensa-Zanuy et al. 2021; Jenny et al. 2009). We, therefore hypothesized that CBD might also modulate TRP homeostasis in placental tissue. To evaluate this hypothesis, we used two advanced models: human placental explants, which are biological models with 3D cellular topography and population genetic heterogeneity (Miller et al. 2005), and isolated syncytiotrophoblast microvillous membrane that enable investigation of mother-to-placenta molecular transport (Illsley et al. 1990).

2. Methods

2.1. Chemicals and reagents

Bicinchoninic Acid (BCA) assay reagents were acquired from Thermo Scientific (Rockford, IL, USA), while thiazolyl blue tetrazolium bromide (MTT) and cannabidiol (CBD) with a purity of $\geq$ 98.5% (HPLC) and concentration of 10 mg/ml in ethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Tri Reagent® solution, which is vital for RNA extraction, was sourced from the renowned Molecular Research Centre (Cincinnati, OH, USA). ^{3}H-serotonin (80 Ci/mmol) was purchased from M.G.P. (Zlín, Czech Republic). All other chemicals used in our experiments were of analytical grade to maximize the accuracy and reproducibility of our results.

2.2. Collection of human placenta samples

The experimental procedures adhered to the principles outlined in the Declaration of Helsinki by the World Medical Association (World Medical, 2013). Healthy human placentas within the gestational age range of 38–40 weeks were procured with written informed consent from pregnant women undergoing elective cesarean section delivery at the University Hospital in Hradec Kralove, Czech Republic. Ethical approval for the study protocol (201006 S15P) was obtained from the University Hospital Research Ethics Committee, ensuring strict compliance with ethical standards and the safeguarding of human subjects. Demographic characteristics of the women participating in the study are shown in supplementary Table 1.

2.3. Human placental explants: culture and CBD treatment

Human placenta cotyledons were isolated by mechanical detachment, excluding the chorionic plate and decidua, as described elsewhere (Karahoda et al. 2020b). The villous tissue was dissected into approximately 30 mg explants measuring 0.5 cm$\times$0.5 cm by random sampling, ensuring exclusion of large vessels and blood clots. The explants were then rinsed with cold sterile saline and placed in 12-well plates. The culture medium consisted of 2 ml DMEM-F12 medium supplemented with 10% fetal bovine serum and antibiotics (penicillin: 100 U/ml, streptomycin: 0.1 mg/ml, and amphotericin B: 2.5 μg/ml) (Chiarello et al. 2014). Each well contained three explants sourced from a single placenta, with a total combined mass of approximately 100 mg. The cultures were then incubated at 37 °C in a sterile environment under an atmosphere of 8 % O_2, 5 % CO_2, and 87 % N_2 for 18–24 h to equilibrate and recover from the isolation process. After equilibration, the cells were treated with CBD at concentrations of 0.1, 2.5, 5, 10, 20, or 40 μg/ml for 48 hours, with medium replenishment and CBD re-dosing after 24 hours. CBD treatments were prepared from a commercial stock solution in ethanol, which was subsequently diluted in DMEM-F12, with ethanol concentrations spanning from 0.0005 % to 0.2 % (v/v). To facilitate a comprehensive assessment of the treatment effects, two control conditions were established: a baseline control comprising solely DMEM-F12, and an ethanol control composed of DMEM-F12 supplemented with 0.2 % ethanol (v/v). The latter was specifically implemented to correspond with the maximum ethanol concentration present in the CBD treatment series. The experiments were conducted in triplicates (as technical replicates) in up to nine different placentas (supplementary Table 1).

The placental explants were then processed to prepare homogenates. This was done by first gently washing the explants with a 0.9 % NaCl solution at 4 °C, after which they were accurately weighed and finely mechanically fragmented before being homogenized by friction in a buffer solution composed of 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) at 4 °C. The resulting homogenates were then centrifuged at 10,000 g for 15 min, after which the supernatant was collected and stored at −80 °C for future utilization. The protein concentration of the homogenates was quantified with the Pierce™ BCA protein assay kit in accordance with the manufacturer's guidelines.

2.4. Evaluating viability of term human placental explants treated with CBD

The metabolic viability of placenta explants was assessed 48 h after treatment using the MTT (thiazolyl blue tetrazolium bromide) reduction assay (Castro-Parodi et al. 2013). Before incubation with 0.5 mg/ml MTT solution at 37°C for 1 hour, explants were washed with Opti--MEM™. The tissue was then transferred to a new well containing 1 ml of DMSO and incubated for 5 min with gentle shaking at room temperature. Formazan production was measured by monitoring the supernatant's absorbance at wavelengths of 570 and 690 nm. As positive controls, explants cultured for 18 h with 40 % DMSO were subjected to the same analysis. Results are expressed as the difference between Abs 570 and Abs 690 per gram of tissue.

To assess the explants' plasma membrane integrity, a Sigma-Aldrich colorimetric LDH activity assay kit (St. Louis, MO, USA) was used in accordance with the manufacturer's instructions (Mirdamadi et al. 2021). LDH enzymatic activity was normalized to milligrams of explants, with results expressed as nanomoles of NADH per milliliter per minute per milligram of tissue. As a positive control, explants were cultured with lysis buffer (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 15 min at 37 °C, and the maximum amount of LDH released to the media was quantified. hCG levels were measured using a highly sensitive enzyme-linked immunosorbent assay (ELISA) from Sigma-Aldrich (St. Louis, MA, USA) according to the manufacturer's protocols. In all assays, we incorporated a control group that contained 0.2 % v/v ethanol (control ethanol), which matches the highest ethanol concentration used in the CBD treatments. This approach was applied to distinguish the effects of CBD from those attributable to the solvent.

2.5. RNA isolation, reverse transcription, and quantitative PCR analysis

Total RNA was extracted from weighed tissue samples using TriReagent™ solution in accordance with the manufacturer's instructions. The purity and quality of the extracted RNA were assessed by measuring the A260/A280 and A260/A230 absorbance ratios, respectively, using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA concentration was then calculated based on the A260 absorbance. Reverse transcription (RT) was done using the iScript Advanced cDNA Synthesis Kit and T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA).

Quantitative gene expression analysis was conducted using TaqMan® Real-Time PCR with pre-designed TaqMan® Real-Time Expression PCR assays. Each reaction was performed in a final volume of 5 μL per well using TaqMan® Universal Master Mix II without UNG (Thermo Fisher Scientific, Waltham, MA, USA) as the PCR master mix. The measured gene expression was normalized against the geometric mean expression of two reference genes: tyrosine 3-monooxygenase/TRP 5-monooxygenase activation protein zeta (*YWHAZ*), and β2 microglobulin (*B2M*).

2.6. Protein profiling of tryptophan metabolism enzymes

Protein expression was assessed by SDS-PAGE using a standard protocol (Laemmli, 1970). Briefly, 35 μg of total protein from a placenta explant homogenate was mixed with loading buffer under reducing conditions and heated at 96°C for 5 min. The samples were then separated on 10 % (MAO-A, SERT and OCT3) or 15 % (IDO, KMO, KAT-1 and TPH) polyacrylamide gels by electrophoresis at 120 V. Proteins were subsequently transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA) that were blocked for 1 h at room temperature in 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1 % Tween 20 (TBS-T) containing 5 % bovine serum albumin (BSA) and washed with TBS-T buffer. Primary antibodies against MAO-A (Abcam, ab126751, dilution 1:1000), IDO (ThermoFisher Scientific, PA5–79437, dilution 1:1000), KMO (Proteintech, 10698–1-AP, dilution 1:1000), KAT-1 (Proteintech, 12156–1-AP, dilution 1:500), OCT3 (Abcam, ab124286, dilution 1:10000), SERT (Sigma-Aldrich, SAB4200039, dilution 1:500) and TPH (Invitrogen, dilution 1:100) were added and incubated overnight at 4°C. The membranes were then washed with TBS-T buffer and incubated with a horseradish peroxidase-linked anti-rabbit antibody as a specific secondary antibody (Dako, P0217, dilution 1:20000) for 1 h at room temperature. Relative protein expression was determined using the Chemiluminescence HRP Substrate Kit (ECL™ Prime Western Blotting System) and band intensity was visualized and quantified by densitometric analysis using the ChemiDoc™ MP imaging system (Bio-Rad, Hercules, CA, USA). To ensure equal loading of proteins, membranes were probed for β-actin (Abcam, ab 8226, dilution 1:10000) or vinculin (Abcam, ab130007, dilution 1:1000) with anti-mouse HRP (Dako, P0260, dilution 1:20000) as the specific secondary antibody.

2.7. HPLC analysis of TRP metabolites in the placenta explants

The concentrations of TRP, 5-hydroxyindoleacetic acid (HIAA), KYN, and kynurenic acid (KYNA) were determined using a Shimadzu LC20 Performance HPLC chromatograph (Shimadzu, JP) with a Kinetex EVO C18 100 A 150 × 3 mm column (Phenomenex, USA) and a guard column. The cell-free supernatant was tested and the column was maintained at 20°C with a flow rate of 0.5 mL/min. The mobile phase for TRP consisted of 3:97 (v/v) methanol:acetic acid (0.1 M, pH 4.5, adjusted with NaOH), while that for HIAA was 7:93 (v/v) methanol:acetic acid (0.2 M), excitation and emission wavelengths of the fluorescence detector were set at 276 and 333 nm, respectively. KYN was determined using a mobile phase of 2:98 (v/v) methanol:acetic acid (0.1 M, pH 6.8, adjusted with NaOH) and UV detection at 289 nm. For KYNA, the mobile phase was 97:3 (v/v) zinc acetate (0.05 M, with 0.025 % acetic acid): acetonitrile. The fluorescence detector's excitation and emission wavelengths were 330/385 nm.

2.8. Influence of CBD on serotonin uptake in microvillous membrane (MVM)

MVM vesicles were prepared from human placentas as described by Illsley (Illsley et al. 1990). All procedures were conducted at 4ºC. Briefly, the maternal decidua and chorionic plate were eliminated and 80–100 g of placental villous tissue was sliced into small fragments. The tissue was then rinsed with 0.9 % NaCl solution to eliminate residual blood. The washed tissue was homogenized with a Kenwood blender for 2 min in a solution (3 ml/g) consisting of 250 mM sucrose, 10 mM Tris-Hepes (pH 7.2), 5 mM EGTA, 5 mM EDTA, and 1 mM PMSF. The isolation procedure involved multiple stages, including differential centrifugation and precipitation of non-microvillous membranes with magnesium ions. MVM membranes were resuspended in an intravesicular buffer (290 mM sucrose, 5 mM Hepes, 5 mM Tris, pH 7.4). To promote vesiculation, the membranes were passed through a 25-gauge needle 15 times. The resulting MVM vesicles were either stored at 4ºC for use in uptake experiments within 3 days of isolation or cryopreserved at −80 ºC and equilibrated to room temperature on the day of the experiments.

The uptake of ^{3}H-serotonin into MVM vesicles was measured at room temperature using the rapid vacuum filtration technique described previously (Karahoda et al. 2020b). Briefly, an MVM (n = 4) suspension was preincubated for 10 min in the presence of CBD at the lowest concentration used in the incubations (0.1 μg/ml), then uptake was initiated by adding 100 nM ^{3}H-serotonin. The reaction was stopped by adding an ice-cold stop solution (130 mM NaCl, 10 mM Na_2HPO_4, 4.2 mM KCl, 1.2 mM $MgSO_4$, 0.75 mM $CaCl_2$; pH 7.4) and filtration through a 0.45 μM mixed cellulose ester filter (MF-Millipore, HAWP00010) under vacuum. Filter-associated radioactivity was determined by liquid scintillation counting. Nonspecific tracer binding to the filter and plasma membranes was accounted for by subtracting the radioactivity

measured in protein-free controls and uptake at time zero, respectively, from the total vesicle uptake measurements.

2.9. Statistical methodology and analysis in the study

Statistical analyses were conducted using the non-parametric Mann–Whitney test (when comparing two groups) or the Kruskal–Wallis test followed by Dunn's multiple comparisons test (when comparing more than two groups). For qPCR and WB studies, statistical analyses were conducted on the data normalized solely to the reference gene/protein. All analyses were performed using GraphPad Prism 8.3.1 (GraphPad Software, Inc.). In the figures, significance levels based on p-values are denoted using asterisks as follows: * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$).

3. Results

3.1. Effects of CBD treatment on viability in human placental explants

The toxicity of CBD towards human placenta explants was evaluated using the MTT assay, LDH detection, and hCG release. At CBD concentrations ranging from 0.1 to 20 μg/ml, the metabolic activity of the explants remained unaffected, as depicted in Fig. 1A. However, a slight decrease was observed at 40 μg/ml. Explants cultured for 18 h in the presence of 40 % DMSO were used as death controls (i.e., positive controls). CBD had no discernible impact on cell membrane integrity at any tested concentration (Fig. 1B). As a positive control, we assessed the maximal LDH activity in the culture media by treating the explants with a lysis buffer for 15 min at 37 °C. hCG secretion, assessed as an endocrine function marker in CBD-treated explants after 48 hours, showed no differences between control and treated groups (Fig. 1C). No toxicity effect of the solvent (ethanol) was observed (Fig. 1); therefore, control-ethanol was excluded from subsequent experiments.

3.2. Effects of CBD on the gene and protein expression of enzymes involved in TRP metabolism

We next studied the gene expression of the main enzymes and transporters involved in TRP metabolism, including the KYN branch enzymes *IDO-1*, *KAT-1*, and *KMO*; the serotonin branch enzymes *TPH* and *MAO-A*; and the transporters *SLC7A5* (LAT1), *SLC7A8* (LAT2), *SLC6A4* (SERT), and *SLC22A3* (OCT3). As shown in Fig. 2, this revealed that several enzymes/transporters were differentially expressed as a result of CBD treatment, suggesting a direct impact of this phytocannabinoid on the modulation of TRP metabolism. The KYN pathway genes *IDO* and *KMO* were upregulated at CBD concentrations of 2.5 μg/ml and above, while *KAT-1* was downregulated. Among the studied serotonin pathway genes, *MAO-A* was downregulated at CBD concentrations of 2.5 μg/ml and below but *TPH* was unaffected. Among the transporters, *SLC6A4* (SERT) was downregulated at CBD concentrations above 2.5 μg/ml, *SLC22A3* (OCT3) was upregulated at a CBD concentration of 40 μg/ml, *SLC7A5* (Lat1) was upregulated at 2.5 μg/ml CBD, and *SLC7A8* (Lat2) was unaffected by CBD treatment.

To investigate changes in protein expression due to CBD treatment (Fig. 3), we performed quantitative Western blotting analyses using specific antibodies for TPH, MAO-A, IDO, KMO, KAT-1, SERT and OCT3 in placenta villous explant homogenates treated with CBD for 48 hours. The MAO-A and KAT-1 proteins exhibited reduced protein expression in explants exposed to CBD at concentrations of 2.5 μg/ml and above, but the expression of TPH increased. CBD also increased the protein-level expression of IDO relative to controls in explants but had no significant effect on KMO, SERT, or OCT3.

3.3. Quantitative profiling of serotonergic and kynurenine metabolite dynamics in human placenta explants during CBD treatment

Our data demonstrate a clear association between CBD treatment and reduced TRP levels, indicating increased TRP metabolic activity within placental tissue in the presence of CBD (Table 1). A notable increase in KYN levels was also seen, suggesting that CBD treatment stimulates TRP metabolism via the kynurenine pathway. This finding was supported by the observed changes in the KYN/TRP ratio. Conversely, CBD exposure reduced levels of KYNA, suggesting a decline in activity along the kynurenine aminotransferase-1 (KAT-1) associated branch. The media samples did not contain detectable quantities of 5-hydroxy-TRP (5-OH-TRP) or serotonin, and CBD exposure had no significant effect on HIAA levels.

3.4. Quantifying the effect of CBD on serotonin uptake in MVM vesicles

Serotonin uptake was studied in ex vivo isolated human maternal placenta-facing membrane vesicles (MVM). The kinetics of ^{3}H-serotonin uptake were measured over time periods ranging from 20 seconds to 60 seconds, with a pH of 7.4 being maintained throughout the experiment. As shown in Fig. 4, our results reveal a significant inhibition of serotonin transport, with a reduction of approximately 60% observed at the lowest CBD concentration tested, when compared with the untreated control. These findings suggest a potential role for CBD in modulating placental serotonin uptake from the maternal circulation.

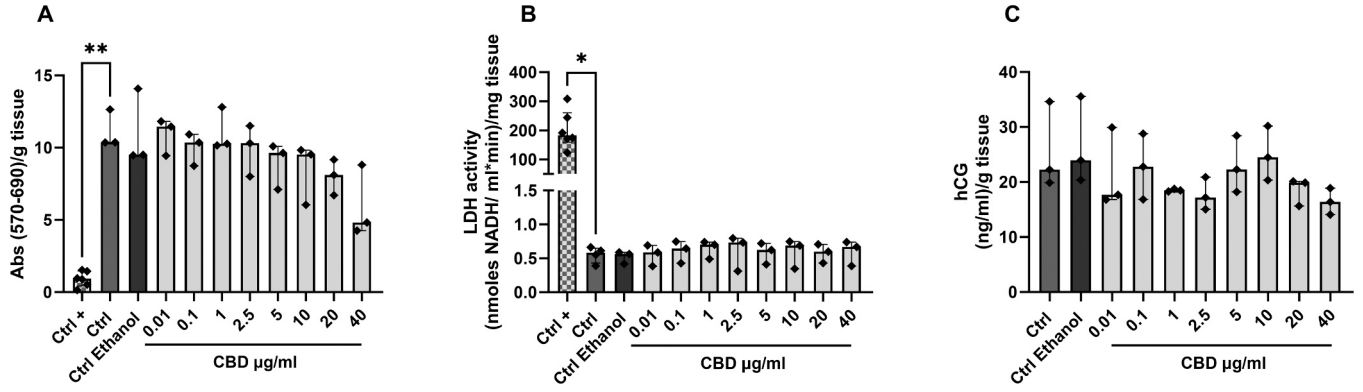

Fig. 1. Viability analysis of term human placenta explants treated with CBD. The metabolic functionality, membrane integrity, and endocrine function of human explants were evaluated using the MTT assay (A), LDH assay (B), and hCG release (C), respectively, with and without CBD treatment at various concentrations. For LDH assay, explants treated with lysis buffer for 15 min at 37 °C were used as positive controls (Ctrl+) and for MTT assay, explants cultured with 40 % DMSO for 18 hours were used as positive controls (Ctrl+). Data are presented as medians with IQRs; n = 3. Statistical significance was evaluated using the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparisons test; * and ** denote results significant at the $p \leq 0.05$ and $p \leq 0.01$ levels, respectively.

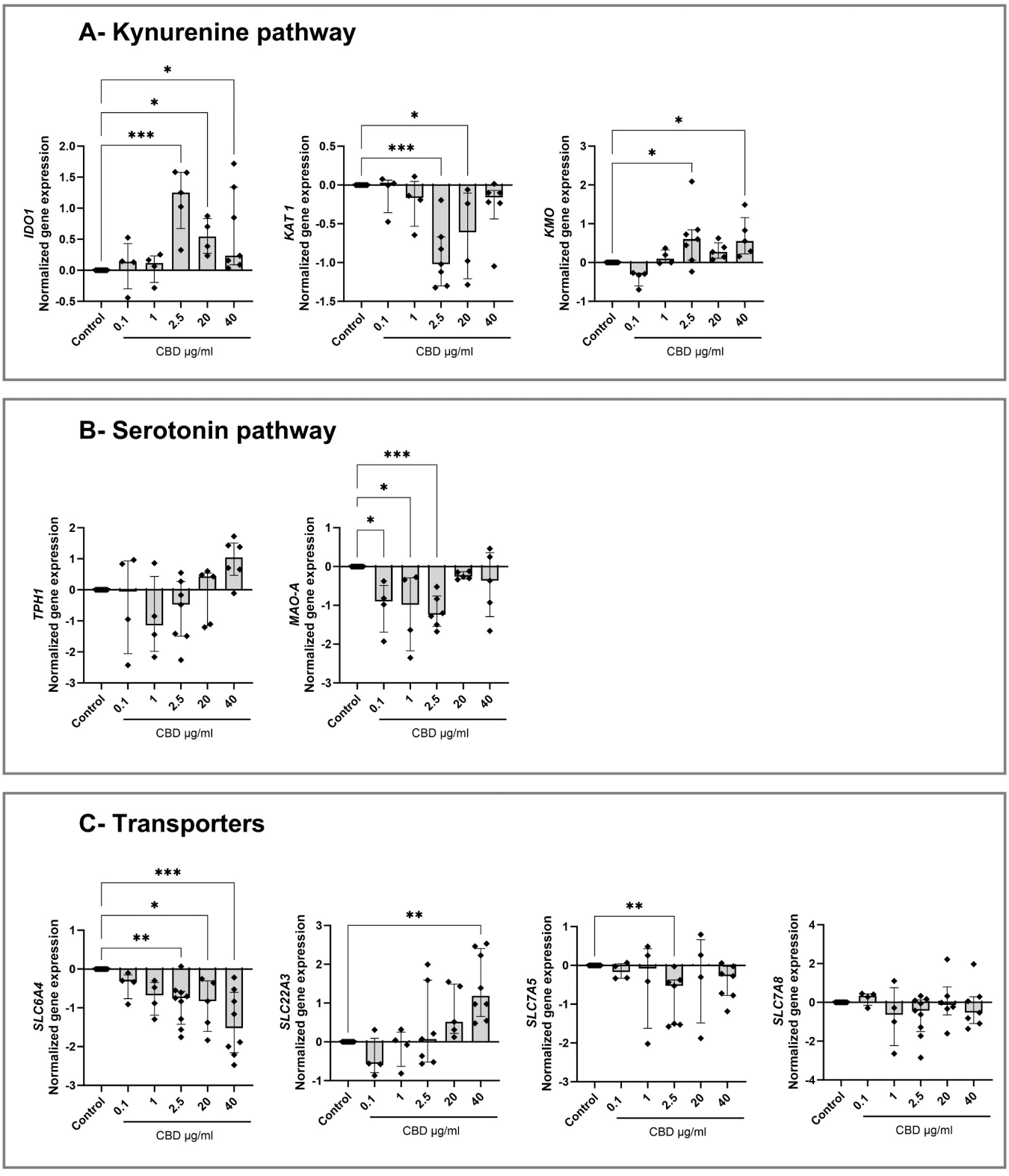

Fig. 2. CBD influence on gene expression of enzymes and transporters related to TRP metabolism in human term placenta explants. Results are shown for the rate-limiting enzymes indoleamine 2,3-dioxygenase (*IDO*), kynurenine aminotransferase 1 (*KAT-1*), kynurenine 3 monooxygenase (*KMO*), TRP hydroxylase (*TPH*), monoamine oxidase (*MAO-A*), as well as the key transporters *SLC6A4*, *SLC22A3*, *SLC7A5* and *SLC7A8*. The measured gene expression was normalized against the geometric mean expression of *YWHAZ* and *B2M*, and subsequently to the untreated control (CTRL). Data are presented as Tukey boxplots (1.5 times IQR); $n \geq 4$. Statistical significance was evaluated using the non-parametric Kruskal-Wallis test followed by the Dunn multiple comparison test; *, **, and *** denote results significant at the $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ levels, respectively.

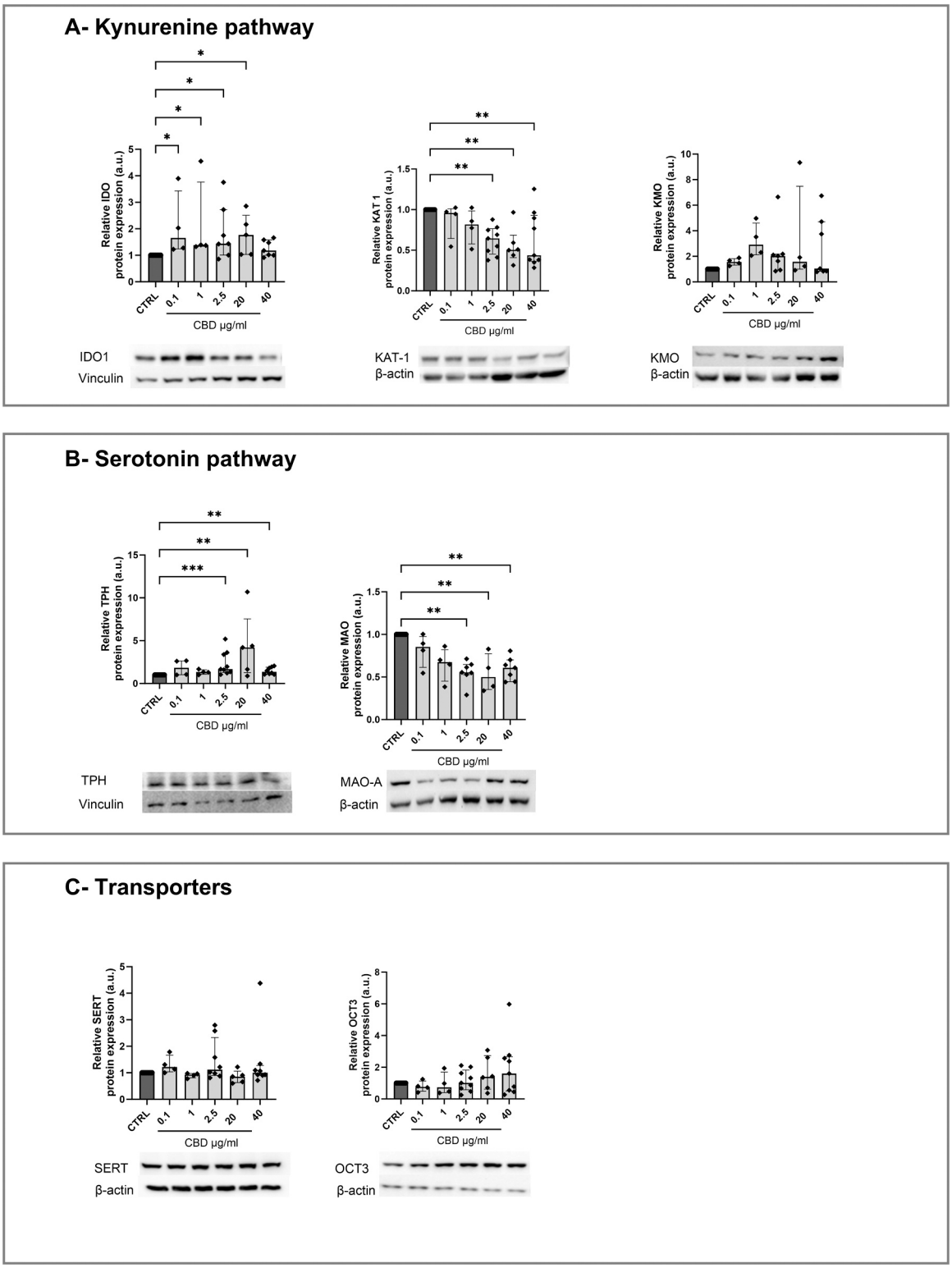

Fig. 3. Protein expression analysis of TRP metabolism enzymes and transporters in CBD-treated human term placenta explants. Villous explants were cultured for 48 hours with various concentrations of CBD, then tissue homogenates were prepared as described above. The protein expression of TPH (A), MAO-A (B), OCT3 (C), KAT-1 (D), and KMO (E) was evaluated by Western blotting. Results were normalized against β-actin and vinculin. Data are presented as medians with IQR, $n \geq 4$. Statistical significance was evaluated using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test; *, **, and *** denote results significant at the $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ levels, respectively.

Table 1
Characterizing TRP metabolites in culture media from human placental explants exposed to various CBD concentrations.

CBD (μg/ml)	TRP (μM/g tissue)	KYN (μM/g tissue)	KYNA (nM/g tissue)	HIAA (nM/g tissue)	Ratio KYN/TRP
0 (Ctrl)	370.2 (362.9–407.7)	34.83 (32.53–35.33)	480.3 (468.2–489.2)	512.6 (292.9–1274.0)	104.3 (89.94–116.3)
0.1	341.0* (326.6–350.9)	48.16** (47.02–52.42)	457.1 (455.3–474.7)	284.0 (173.3–512.4)	140.7* (121.1–155.5)
1	336.0* (331.7–351.1)	60.68** (57.83–62.82)	491.5 (469.5–501.4)	275.7 (154.4–466.1)	186.0** (167.3–195.4)
2.5	345.8** (320.9–351.4)	51.04** (48.92–59.23)	404.9** (385.2–441.9)	285.3 (106.0–743.0)	161.2** (142.1–179.9)
20	349.4 (333.3–380.9)	22.25 (18.76–30.02)	395.8** (374.5–443.1)	228.2 (99.75–554.5)	64.46** (51.71–82.87)
40	356.5 (331.7–370.3)	16.35** (14.36–20.50)	391.7** (366.1–408.3)	352.1 (229.3–578.4)	45.47** (43.42–64.53)

The concentrations of TRP and its main metabolites kynurenine (KYN) and kynurenic acid (KYNA) were evaluated in culture explants treated with CBD for 48 hours; ratios of each metabolite to the precursor are also shown as indirect approximations of enzyme activity. Statistical significance was evaluated using the non-parametric Mann-Whitney test; * and ** denote results significant at the $p \leq 0.05$ and $p \leq 0.01$ levels, respectively.

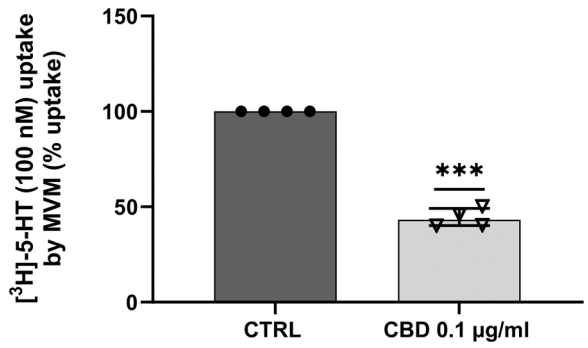

Fig. 4. Analysis of CBD effects on [^{3}H]-serotonin uptake in MVM placenta vesicles. Initial uptake rates (CTRL) were determined in vesicles incubated with 100 nM [^{3}H]-serotonin at room temperature for 1 min. The MVM vesicles of four different placentas were treated with 0.1 μg/ml CBD and incubated for 10 min. After treatment, [^{3}H]-serotonin was added and incubation was continued for 1 min. Results are presented as percent uptake relative to the untreated control (CTRL). Data are shown as medians with IQR, n=4. Statistical analyses were performed using the Wilcoxon signed-rank test; *** denotes significance at the $p \leq 0.001$ level.

4. Discussion

The prevalence of cannabis use during pregnancy has surged in recent times (Chang et al. 2019; Volkow et al. 2019) yet, research in this area is at an early stage. Studies have indicated that CBD exerts modulatory effects on TRP metabolism in various biological models and organs (di Giacomo et al. 2020a; Florensa-Zanuy et al. 2021; Jenny et al. 2010; Sales et al. 2018). It has also been hypothesized that perturbations in placental TRP metabolism during pregnancy may impact fetal brain programming and have long-term consequences in adulthood (Bonnin et al. 2011). This study explored the activity of CBD as an exogenous modulator of placental TRP metabolism by analyzing its impact on protein, genetic, and functional dynamics.

Pregnancy increases the demand for TRP because it is needed to sustain protein synthesis and metabolite formation to enable optimal fetal growth and development (Badawy, 2015). This demand is enhanced by a surge in *de novo* fetal serotonin production during the third trimester (Bonnin and Levitt, 2011). However, we found that exposing placental explants to CBD increased their rate of TRP metabolism, causing the TRP concentration in the culture media to fall. We further showed that CBD modulates TRP metabolism without disrupting its transport because the expression of the *SLC7A5* (LAT-1) and *SLC7A8* (LAT-2) transporter genes was unaffected by any tested CBD concentration. The increased rate of TRP metabolism following CBD exposure can instead be attributed to the upregulation of rate-limiting enzymes in the serotonin and KYN pathways. This conclusion is supported by the observed rates of intermediate metabolite production and TRP consumption at different applied CBD concentrations. CBD exposure could thus reduce the availability of TRP and limit its transport from the uterus to the fetus. Such limitations on amino acid transfer have been linked to babies being small for gestational age (Shibata et al. 2008), a common characteristic of newborns exposed to cannabis during pregnancy (Marchand et al. 2022). Tissue-specific kinetic modeling revealed that TRP catabolism occurs primarily via the dominant kynurenine pathway and is facilitated by IDO/ Tryptophan 2,3-Dioxygenase (TDO) in the brain and liver. Interestingly, IDO and TPH exhibit nearly equimolar utilization of TRP in rodents and humans, with only a slight (10 %) preference for IDO, especially in the brain (Stavrum et al. 2013). This tandem mechanism is absent in the placenta, where TDO is not active. Profound gaps in the research concerning CBD's influence on the rate-limiting enzymes such as IDO and TPH critically limit our comprehension of its implications during pregnancy.

Maintaining serotonin balance is vital during pregnancy because it regulates fetal development and myometrial contractions (Cordeaux et al. 2009; Doherty et al. 2011; Moiseiwitsch, 2000). We have previously demonstrated that interplay between SERT, OCT3, and MAO-A regulates serotonin levels to safeguard the placenta and fetus in rats and humans (Karahoda et al. 2020b; Staud et al. 2023). Here, we have shown that CBD affects the serotonin arm of this system by downregulating MAO-A (at protein and gene level) and upregulating *TPH*, leading to serotonin accumulation. Interestingly, inhibition of MAO-A, which catalyzes serotonin-to-HIAA conversion, has been reported in platelets and the brain following exposure to tetrahydrocannabinol (THC) (Fisar, 2010; Mazor et al. 1982; Schurr et al. 1978). This inhibition of MAO-A reduced production of the intermediate metabolite HIAA, in accordance with our observations of CBD-exposed placental tissues. We have also recently demonstrated that placental HIAA is unidirectionally transported by MRP2 from the placenta to the maternal circulation (Staud et al. 2023). Although this metabolite was traditionally considered inert, recent studies have shown that it can influence the removal and degradation of brain Aβ peptides and the RAS/MAPK signaling pathway (Schmid et al. 2015).

The human placenta primarily derives serotonin from two main sources: 1) TPH-catalyzed endogenous placental synthesis from maternally acquired TRP, and 2) uptake from the maternal circulation via serotonin transporter (SERT) and from the fetal circulation via organic cation transporter 3 (OCT3) (Bonnin et al. 2011; Karahoda et al. 2020b). Having shown that CBD influences placental serotonin synthesis and degradation via its effects on TPH and MAO-A, we next investigated its potential effects on placental serotonin transport. Exposure to CBD had little effect on *OCT3* expression at any of the tested concentrations but

caused pronounced downregulation of *SERT*. In addition, CBD had a significant inhibitory effect on serotonin uptake (reducing it by up to 60%) in MVM vesicles isolated from the human placenta. There is only limited evidence of SERT inhibition by CBD at present, but it has been reported that high concentrations of other phytocannabinoids (THC and WIN 55,212–2) and the endocannabinoid anandamide can inhibit serotonin uptake in platelets (Velenovska and Fisar, 2007). We, therefore speculate that CBD reduces placental serotonin uptake across the MVM by blocking SERT. Considering that during a precise time window early in gestation, maternal serotonin may also be provided to the embryo before and during placentation (Bonnin and Levitt, 2011), our data on SERT inhibition by CBD indicate that serotonin may accumulate on the maternal side of the placenta. Balanced serotonin levels within the fetoplacental unit play a crucial role in placental and fetal development, as well as in regulating the hemodynamic properties of placental vasculature (Karahoda et al. 2020b; Staud et al. 2023). Hence, by observing SERT inhibition by CBD, we can hypothesize potential disruptions in these vital physiological processes.

We have previously shown that under normal physiological conditions, the metabolism of TRP in the placenta is a dynamic process that changes throughout gestation in response to fetal needs (Abad et al. 2020). In particular, the expression and activity of MAO-A increase as pregnancy approaches full term, serving as a detoxification mechanism that safeguards the term placenta and the developing fetus from elevated levels of circulating serotonin (Karahoda et al. 2020b; Staud et al. 2023). A CBD-induced reduction in the expression of MAO-A at the gene and/or protein levels could thus disrupt this natural protective mechanism. It's worth noting that the impact of CBD on TRP metabolism in the placenta might be influenced by the timing of exposure, especially given that our work has focused on the human term placenta. Further experiments are thus needed to clarify the effects of CBD on TRP metabolism during earlier stages of pregnancy.

Our data support the conclusion that CBD promotes serotonin production, which may explain the sense of well-being reported by cannabis users. However, an intriguing dichotomy also emerged: although we found that CBD increased the gene- and protein-level expression of IDO-1 (an enzyme that suppresses inflammatory responses), Jenny et al. reported that CBD reduced IDO-1 expression under proinflammatory conditions (Jenny et al. 2009). We hypothesize that this shift in the response to CBD could bias TRP catabolism towards the serotonin pathway, which would be reflected in intermediate metabolite production. CBD's apparent immune-modulating effects imply that it might influence the pro-inflammatory cytokine cascade that regulates IDO-1 expression (Nichols and Kaplan, 2020) which would explain the reported reduction in IDO-1 activity when facing proinflammatory challenges.

Downstream of the IDO-catalyzed production of KYN, the kynurenine pathway divides into 2 different branches – one proceeding through KAT-1 that yields neuroprotective metabolites and another proceeding through KMO that forms neurotoxic metabolites (Modoux et al. 2021). CBD treatment downregulates KAT-1 at the gene and protein levels, reducing levels of KYNA in the culture media. This is consistent with the recent proposal that inhibiting KMO in the brain to enhance KAT-1-mediated metabolization could be a viable strategy against THC addiction because elevated endogenous KYNA levels counter THC's addictive impact by negatively modulating α7 nicotinic acetylcholine receptors (Justinova et al. 2013). The generalizability of this strategy may be limited because it is based on data originating from neuronal cells and may not be directly applicable in the non-neuronal environment of the placenta. However, the confirmation that CBD induces KAT-1 downregulation (leading to reduced KYNA production) and *KMO* upregulation suggests to us that CBD exposure promotes KYN catabolism via KMO.

The work presented here was motivated by two key considerations. First, the placenta lacks cannabinoid-clearing mechanisms and acts as a reservoir for CBD, resulting in a slow but prolonged distribution to the fetus (Berman et al. 2023). Second, cannabinoids exhibit a dose-dependent biphasic effect (Calabrese and Rubio-Casillas, 2018; Eggers et al. 2019). Consequently, it is important to study their effects across a range of concentrations extending beyond those currently accepted for therapeutic use to fully understand their potential effects on TRP metabolism.

Our study has several strengths that provided new insights into the impact of CBD on placental TRP metabolism. Specifically, we employed human placental explants as an alternative to conventional 2D cultures, owing to their more authentic emulation of the physiological 3D milieu and the intrinsic heterogeneity of cellular composition - attributes that are notably absent in homogeneous cell line models. This methodological choice facilitates a more precise delineation of drug interactions, substantially augmenting the translational applicability of our research outcomes. Our experimental protocol is carefully scheduled within a 2–3 day window, ensuring the analysis of explants at their peak viability just before syncytial regeneration commonly initiates by day four (Siman et al. 2001). This timing ensures healthy and viable explants, as corroborated by the studied viability parameters.

Nonetheless, it is important to acknowledge that placental TRP metabolism undergoes dynamic changes throughout pregnancy (Abad et al. 2020; Karahoda et al. 2020a). Therefore, a limitation of this study is the utilization of term placentas, which only represent the late stage of pregnancy. It is conceivable that the response of placental TRP metabolism to CBD exposure during the early stages of pregnancy may differ from the findings described herein. Further investigations using appropriate experimental models are necessary to explore this potential variability. Moreover, our experimental design represents an acute exposure to CBD. Considering that CBD consumption in real-life scenarios is more likely to be chronic (Ko et al. 2015), further studies involving chronic administration of CBD in animal models are necessary to comprehensively understand the long-term effects of CBD on placental tryptophan homeostasis. Additionally, while there is evidence, primarily from animal studies, indicating that the effects of cannabis may vary based on the fetal sex (Rodríguez de Fonseca et al. 1991), our dataset lacks the size required to perform sex-stratified analyses.

In conclusion, our results show that CBD modulates TRP catabolism in the human placenta (Fig. 5), potentially disrupting the tightly regulated homeostasis of both the serotonin and KYN pathways in ways that may present risks to cognitive health. Given the increasing use of cannabis during pregnancy, these findings indicate a pressing need for in-depth research on the multifaceted impacts of CBD on maternal and fetal well-being.

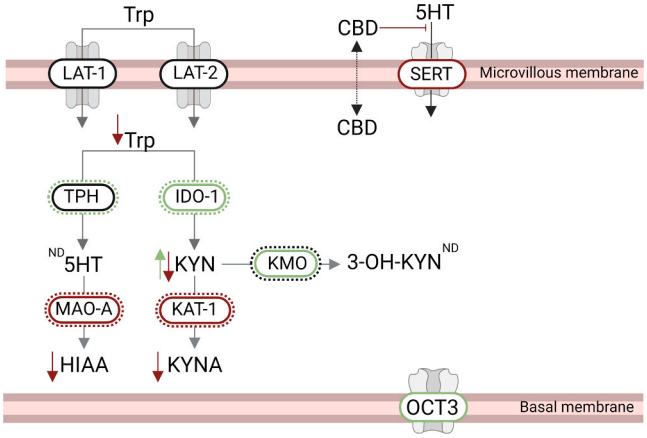

Fig. 5. Effects of CBD on TRP Metabolism in Human Placenta Explants. This diagram summarizes the effects of CBD on key enzymes of TRP metabolism in human placenta explants. Solid and dashed ellipses indicate modulation at the gene and protein levels, respectively, and the colors of the ellipses indicate the nature of the modulation (red for downregulation, green for upregulation, and black for no significant change in expression).

Ethics approval

This manuscript does not include clinical studies or patient data.

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CRediT authorship contribution statement

Tetiana Synova: Writing – original draft, Methodology, Investigation. **Cilia Abad:** Writing – original draft, Methodology, Formal analysis. **Ramon Portillo:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Frantisek Staud:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Daniel Heblik:** Methodology, Investigation. **Petr Kastner:** Methodology, Investigation. **Radim Kucera:** Writing – original draft, Supervision, Methodology, Conceptualization. **Rona Karahoda:** Writing – review & editing, Writing – original draft, Validation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2024.153813.

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