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Serotonin and serotonin reuptake inhibitors alter placental aromatase

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Abstract

Serotonin reuptake inhibitors (SRIs) are currently the main molecules prescribed to pregnant women that suffer from depression. Placental cells are exposed to SRIs via maternal blood, and we have previously shown that SRIs alter fetoplacental steroidogenesis in an *in vitro* co-culture model. More specifically, serotonin (5-HT) regulates the estrogen biosynthetic enzyme aromatase (cytochrome P450 19; CYP19), which is disrupted by fluoxetine and its active metabolite norfluoxetine in BeWo choriocarcinoma cells. Based on molecular simulations, the present study illustrates that the SRIs fluoxetine, norfluoxetine, paroxetine, sertraline, citalopram and venlafaxine exhibit binding affinity for the active-site pocket of CYP19, suggesting potential competitive inhibition. Using BeWo cells and primary villous trophoblast cells isolated from normal term placentas, we compared the effects of the SRIs on CYP19 activity. We observed that paroxetine and sertraline induce aromatase activity in BeWo cells, while venlafaxine, fluoxetine, paroxetine and sertraline decrease aromatase activity in primary villous trophoblast. The effects of the paroxetine and sertraline in primary villous trophoblasts were observed at the lower doses tested. We also showed that 5-HT and the 5-HT_{2A} receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) induced CYP19 activity. An increase in phosphorylation of serine and tyrosine and a decrease in threonine phosphorylation of CYP19 was also associated with DOI treatment. Our results contribute to better understanding how 5-HT and SRIs interact with CYP19 and may affect estrogen production. Moreover, this study suggests that alteration of placental 5-

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5-HT: serotonin; CYP19: cytochrome P450 19/aromatase; DOI: 2,5-dimethoxy-4-iodoamphetamine; SERT: serotonin transporter; SRI: serotonin reuptake inhibitor

HT levels due to depression and/or SRI treatment during pregnancy may be associated with disruption of placental estrogen production.

Keywords

antidepressant; estrogen; trophoblast; CYP19; phosphorylation

1. INTRODUCTION

Depression is a societal burden, affecting about 15% of the population over their lifetime, and is associated with significant treatment costs and work disabilities [1, 2]. Women of childbearing age are twice as likely to suffer from depression than men of similar age [3, 4]. Pregnancy has no effect on depression prevalence, other than indirectly through post-partum depression, where withdrawal of placental hormones following delivery are suggested to contribute to this pathology [5–7]. Drugs that target the serotonin transporter (named SRIs for serotonin reuptake inhibitors) are currently the main compounds prescribed in the general population, including pregnant women [8, 9]. Various SRIs are used to treat depression, with different efficacies and side effects [10, 11]. Although concerns have been raised about the safety of using SRIs during pregnancy, cessation of treatment is not necessarily recommended since relapse rates are high and depression can lead to adverse maternal behaviors [12–15]. Clinicians are thus faced with making the decision whether or not to treat depression during pregnancy based on the risk-benefit balance. Detailed information on the molecular effects of SRIs during pregnancy is lacking in order to make scientifically advised decision.

SRIs are shown to cross the placental villi, the main endocrine structure of the placenta [16–19]. Hence, villous trophoblasts (cytotrophoblasts and syncytiotrophoblast), which produce serotonin (5-HT) and express the serotonin transporter (SERT) [20–22], but also the estrogen biosynthetic enzyme cytochrome P450 (CYP19 or aromatase) are exposed to these drugs. Alterations of 5-HT levels in the placenta by SRIs could affect estrogen synthesis, since we have previously shown that 5-HT regulates the mRNA expression of the *CYP19A1* gene, which encodes the CYP19 enzyme, *via* stimulation of the 5-HT_{2A} receptor in BeWo choriocarcinoma cells, a commonly used as a model of the villous trophoblasts [23]. We also previously assessed the effects of the SRI fluoxetine and its active metabolite norfluoxetine in a co-culture model of the feto-placental steroidogenesis unit [24]. Surprisingly, we observed that fluoxetine and norfluoxetine had opposite endocrine disruptive effects [24], despite the fact that they display similar inhibition potencies against SERT [25, 26]. We hypothesized that SRIs differentially alter CYP19 activity in the villous trophoblasts, which could explain some of the adverse pregnancy outcomes associated with specific SRIs, such as preterm birth, low birth weight for gestational age and fetal mortalities [27, 28]. Understanding the underlying molecular mechanisms for these differences could contribute to a better decision making process for using the appropriate SRI(s) during pregnancy [29].

In this study, we compared the effects of several SRIs, 5-HT and a 5-HT_{2A} receptor agonist on placental CYP19 activity. We showed that all SRIs exhibit binding affinity for the active-

site pocket of CYP19, resulting in different effects on CYP19 activity and subjected to compound concentration and study model. We also compared how effects on CYP19 activity in BeWo cells relate to the more physiologically relevant model of primary villous trophoblast cells. Moreover, we validated that 5-HT, throughout activation of the 5-HT_{2A} receptor, increased CYP19 activity in primary villous trophoblast cells.

2. MATERIAL AND METHODS

2.1. Cell culture

BeWo cells were obtained from American Type Culture Collection (CCL-98, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 without phenol red, supplemented with 0.6 g/L sodium bicarbonate (NaHCO₃) (Sigma Aldrich, Oakville, ON, Canada) and 10% fetal bovine serum (FBS, Hyclone, Tempe, AZ). Experiments were performed with cells between passages 7 and 25. Cells were cultured in a humidified atmosphere containing 5% carbon dioxide (CO₂) at 37°C. Cells were seeded in 96-well plate (1.25 x 10⁴ cells/well in 200 µL).

2.2. Isolation of human villous cytotrophoblast cells

The cytotrophoblast cell population from placentas at term (37-41 weeks) of uncomplicated pregnancies with vaginal delivery (no pregnancy complication at birth such as diabetes gestational mellitus, pre-eclampsia, etc.) was isolated using trypsin-DNase/Percoll and purified as previously described [30] from an adapted protocol of Kliman *et al.* [31]. Cells were then cultured in DMEM-high glucose (ThermoFisher Scientific, Burlington, ON, Canada) with 10% FBS (Corning Life Science, Corning, NY), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ThermoFisher Scientific) 1% antibiotic (10,000 units/ml penicillin G, 100 mg/ml streptomycin sulfate) (Hyclone) in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were seeded in 96-well plates (1.25 x 10⁵ cells/well) for CYP19 activity assay and in 24-well plates (1 x 10⁶ cells/well) for RNA extraction. Culture medium was changed 4 h after seeding to remove unattached cells and debris and every 24 h thereafter. Placental collection was approved by the ethical committee of CHUM-St-Luc Hospital and Sainte-Justine Hospital Ethics Board (Montreal, QC, Canada). This study abides by the Declaration of Helsinki principles.

2.3. Explants

Placental explants were used for protein analysis due to the limited amount of protein available in primary villous trophoblast cells. Explants were isolated from term placentas of uncomplicated pregnancies from elective C-sections. Explants (2-3 mm diameter) were dissected from the maternal side of the placenta after removing basal membrane. They were rinsed three times in warm 0.9% saline, 3 times in warm HBSS and then kept in Opti-MEM culture medium without phenol red with penicillin/streptomycin (ThermoFisher Scientific) in 24-well plates (3 explants/well). At the end of the culture, explants were snap frozen in liquid nitrogen and kept at -80°C.

2.4. Treatments

Serotonin (5-HT hydrochloride), 2,5-dimethoxy-4-iodoamphetamine (DOI), a selective 5-HT_{2A} agonist and SRIs were obtained from Sigma-Aldrich. Fluoxetine, citalopram, paroxetine and sertraline are all selective serotonin reuptake inhibitors (SSRI), whereas venlafaxine is a serotonin and norepinephrine reuptake inhibitor (SNRI). Norfluoxetine is a metabolite of fluoxetine that is equally active to its parent compound and although it is not a prescribed molecule, it was included as SRI in our study because of its similar mode of action and presence in maternal and cord blood [18, 32]. Concentrations used (0.03, 0.1, 0.3, 1 and 3 μ M) are based on pharmacologically relevant concentrations found in maternal and cord blood of women treated with SRIs (Table S1) [17–19, 32]. Concentrations used were non-cytotoxic, as determined by measuring cell impedance with xCELLigence™ RTCA SP instrument (ACEA Biosciences, San Diego, CA) in BeWo cells [33] and by measuring lactate dehydrogenase release with a cytotoxicity detection kit (Sigma Aldrich) in primary villous trophoblast cells. Drugs were dissolved in DMSO, except for norfluoxetine and venlafaxine, which were dissolved in water. All treatments were adjusted for final DMSO concentrations in culture medium of 0.1%.

2.5. Aromatase activity

The catalytic activity of CYP19 was determined by a tritiated water-release assay according to the method of Lephart and Simpson (1991) and adapted by our laboratory for primary culture of villous trophoblast cells [34, 35]. Briefly, after a 24-h treatment with SRIs, cells were incubated with 150 nM (for primary culture) or 54 nM (for BeWo cells) of 1 β -[³H]-androstenedione (Perkin Elmer, Wellesley, MA) in serum-free culture medium (50 μ L) for 1 h at 37°C followed by chloroform (40 μ L of supernatant + 100 μ L of chloroform) and dextran-coated charcoal (20 μ L of supernatant + 20 μ L of dextran-coated charcoal) extractions. The supernatants (20 μ L) were transferred to 96-well flexible microplates (Perkin Elmer) with 100 μ L of ScintiVerse cocktail (ThermoFisher Scientific). Tritiated water release was determined using a Microbeta Trilux counter (Perkin Elmer). The conversion of the substrate was linear over time. Specificity of tritiated water release for the aromatization reaction was assessed using formestane (1 μ M) as a positive control for CYP19 inhibition.

2.6. RT-qPCR

Total RNA was isolated using an AllPrep RNA/Protein extraction kit (QIAGEN, Toronto, ON) according to manufacturer instructions. RNA purity (OD_{260/280} ratio $\geq$ 1.8) and concentration in ng/ μ L (OD₂₆₀ $\times$ 40) were determined using a NanoDrop instrument (ThermoFisher Scientific). Reverse transcription of total RNA was performed using an iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). Amplification of cDNA (40 cycles) was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Stable reference genes, determined using geNorm software (BioGazelle, Zwijnaarde, Belgium) [36], were used for qPCR normalization (–CT): peptidylprolyl isomerase A (PPIA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein, zeta polypeptide

(YWHAZ). An annealing temperature of 60°C was used. Primer sequences are presented in Table 1.

2.7. Protein extraction and immunoprecipitation

Total proteins were extracted from frozen placental explants and quantified using a BCA protein assay (ThermoFisher Scientific), as described previously [37]. CYP19 was immunoprecipitated using SureBeads protein G magnetic beads (Bio-Rad) according to the manufacturer's protocol. Briefly, SureBeads were incubated for 10 min at room temperature with 5 µg of anti-CYP19 antibody (ab124766, Abcam, Toronto, ON, Canada). SureBeads were incubated with 150 µg total protein overnight at 4 °C and eluted with 40 µL of Laemmli buffer (Bio-Rad) for 10 min at 70°C. Specificity of immunoprecipitation was validated using isotype control (ab172730, Abcam) and with a no-bead control (Fig. S1).

2.8. Western blot

Total eluted immunoprecipitated proteins or 30 µg of total proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 10% gels followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature with either 5% skimmed milk (for CYP19 protein detection) or bovine serum albumin (BSA, for phospho amino-acid detection) followed by incubation with primary antibody (overnight, 4°C). Anti-CYP19 antibody (1/10 000; EPR4524(2)) was obtained from Abcam, anti-phospho-serine (1/500; AB1603) and anti-phospho-threonine (1/500; AB1607) antibodies were obtained from Millipore (Etobicoke, ON, Canada) and anti-phosphotyrosine antibody (1/500; sc-7020) was from Santa Cruz (Dallas, TX). Membranes were incubated for 1 h at room temperature with either goat anti-rabbit HRP-conjugated antibodies (1/10000 dilution; ab181034, Abcam) for total protein detection or with Clean-Blot IP detection reagent HRP-conjugated antibodies (1/10000 dilution; ThermoFisher Scientific) for detection of immunoprecipitated proteins to avoid interference with denatured immunoprecipitation antibody fragments. CYP19 antibody specificity was confirmed using a blocking peptide (ab186919, Abcam) according to the instructions of the manufacturer. Chemiluminescent detection was performed using Clarity Max Western ECL blotting substrate (Bio-Rad) and band intensities were quantified by densitometry using Image Lab software 5.1 (Bio-Rad). For total protein blots, protein concentration was expressed as a ratio of a specific band density to total protein stained using MemCode Staining Solution (Thermo Fisher), as previously described [37].

2.9. Molecular docking simulations

Molecular docking simulations were performed to evaluate whether human CYP19 can productively bind SRIs and to understand the potential mechanism governing competitive inhibition with CYP19 substrates. Virtual docking was initiated from an energy-minimized and solvated structure of crystallized human placental aromatase cytochrome P450 (PDB entry 5JL6), to which the androstenedione (androst-4-ene-3,17-dione, androstenedione) substrate was first removed. To obtain a stable protein complex (*i.e.* structure with the lowest energy), energy minimization was performed with the YASARA force field [38]. All docking simulations were performed with the Molegro Virtual Docker 6.0 and the MolDock docking scoring function, combined with a cavity prediction algorithm to identify the best

ligand conformations [39]. A guided differential evolution search method was used to complete 20 independent rounds of searching and scoring for every target molecule. This allowed up to 4,000,000 CYP19-ligand complexes to be analyzed per ligand molecule. The 3 most energetically favorable docked CYP19-ligand conformations were sampled and reported with the scoring values expressed in kcal/mol (Table S2). PubChem IDs of ligands used for virtual docking simulations were CID_2771 (citalopram), CID_3386 (fluoxetine), CID_4541 (norfluoxetine), CID_43815 (paroxetine), CID_68617 (sertraline) and CID_5656 (venlafaxine). All molecular structure preparations and analyses were performed using the UCSF Chimera 1.1 platform [40].

2.10. Identification of putative phosphorylation sites on CYP19

An artificial neural network provided by the NetPhos 3.1 server was used to identify putative phosphorylation sites on human CYP19 [41]. From 31 potential phosphorylation sites, 7 primary targets were prioritized based on relative residue proximity to the cofactor and active-site pocket, in addition to surface exposed residues in direct contact with the solvent. These particular sites could play important roles in mediating protein-protein interactions between CYP19 and its cognate kinase partners. Finally, two experimentally confirmed phosphorylation sites were also included in the analysis, *i.e.* Ser118 and Tyr361 [42, 43]. The Vienna-PTM web server was used to build CYP19 models that mimic single amino acid phosphorylation states [44]. The MVD suite force field was used to minimize side-chain energy for residues surrounding the phosphate group of the target tyrosine, serine and threonine residues.

2.11. Statistical analysis

All experiments were performed at least three times using different cell passages or different placentas; for each experiment, each treatment was tested in triplicate. Statistically significant differences ($p < 0.05$) were determined by a Kruskal-Wallis test followed by Dunn's post-hoc test. Data were analyzed using GraphPad Prism (version 5.04; GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Virtual docking of SRIs inside the active-site pocket of human CYP19

To validate the molecular docking procedure, we tested whether docking simulations properly accommodated the endogenous androstenedione substrate inside the active-site pocket of CYP19. Since the CYP19-androstenedione complex was previously crystallized and deposited in the Protein Data Bank (PDB 5JL6), this served as a positive control to qualify our best predictions. We found that 20 runs of 2000 iterations with a starting population size of 50 conformations using the evolution search method and 15 Å search diameter reproduced crystallographic results of the 5JL6 complex with a precision of ≈ 1 Å (Fig. 1a). We observed less than 1 Å difference between the predicted and experimental positions for each androstenedione atom, further validating standard initial parameters used for all subsequent SRI docking procedures.

Molecular docking simulations suggest that the active-site pocket of CYP19 can easily accommodate all SRIs tested in this study (Fig. 1b). Additionally, since negative binding energy values ($\text{kcal}\cdot\text{mol}^{-1}$) denote favorable and/or spontaneous binding events, docking results suggest that all SRIs can potentially bind CYP19 with similar affinities as the natural substrate androstenedione (Fig. 1b and Table S2). These results also suggest that paroxetine may exhibit stronger affinity than androstenedione toward human placental CYP19, with decreasing binding affinities for citalopram, fluoxetine, norfluoxetine, sertraline and venlafaxine (Fig. 1b).

3.2. SRIs alter aromatase activity in BeWo cell line and in primary villous trophoblast cells

In BeWo cells, CYP19 activity was induced by paroxetine (1.7- and 1.4-fold at 1 and 3 μM) and sertraline (2.1-fold at 1 μM), and unaffected by citalopram and venlafaxine (Fig. 2 a–d). In primary villous trophoblast cells, the highest venlafaxine and fluoxetine doses decreased CYP19 activity by 13% at 0.3 μM and by 31% at 1 μM , while the same effect was observed with lower doses of paroxetine (by 33.6% at 0.03 μM and 41% at 0.1 μM) and sertraline (by 33% at 0.03 μM) (Fig. 2 f–i). The decrease in CYP19 activity observed at 10 μM sertraline was attributable to cytotoxicity, as previously described (30). Citalopram and norfluoxetine did not affect CYP19 activity in primary villous trophoblast cells (Fig. 2 e and j).

We further assessed the effect of fluoxetine and norfluoxetine on *CYP19A1* mRNA expression in primary villous trophoblast cells. We observed that fluoxetine decreased total coding exon and promoter I.1-derived transcript of *CYP19A1* by 37% and 71%, respectively, at 0.03 μM (Fig. S2 a and c). Norfluoxetine had no effect on either total *CYP19A1* mRNA transcript or promoter I.1-derived transcript (Fig. S2 b and d).

3.3. Serotonin and its 5-HT_{2A} receptor induce aromatase activity in primary villous trophoblast cells

Given the discrepancies of the effects of SRIs between our two cellular models, we questioned whether the previously published regulation of *CYP19A1* by 5-HT and its receptor 5-HT_{2A} in BeWo cell line [23] was also observable in primary villous trophoblast cells. 5-HT and DOI induced CYP19 activity after a 24h treatment at different stages of *in vitro* villous trophoblasts differentiation (24 h, 48 h and 72 h) (Fig. 3 a and b). The highest concentrations of 5-HT and DOI significantly increased CYP19 activity (Table S3). Neither 1 μM 5-HT nor DOI had significant effect on *CYP19A1* mRNA levels in primary villous trophoblast cells (total coding exons and I.1-derived transcript) (Fig S3 a and b), whereas only 5-HT at 30 μM induced CYP19 protein expression in villous explants (Fig. 3 c–e). To explain the effects of DOI on CYP19, we assessed the global phosphorylation of CYP19 protein. We observed that 30 μM DOI induced tyrosine phosphorylation 2.5- and 1.5-fold after a 1 and 24 h treatments, respectively and serine phosphorylation 3.4-fold after a 24 h treatment, whereas threonine phosphorylation was decreased by 47% after 24h (Fig. 3 f).

3.4. Functional effects of putative phosphorylation sites in human CYP19

Considering the effect of DOI on CYP19 phosphorylation, we further investigated the potential sites of phosphorylation that could affect CYP19 activity. To investigate the

molecular effects of phosphorylation on the enzymatic activity of CYP19, we simulated potential phosphorylation events for 7 of the 31 potential target sites suggested by the neural network NetPhos 3.1 server [41]. The positions analyzed were Y112, S118, T189, Y361, S363, Y441 and T478 (Fig. 4), based on relative residue proximity to the cofactor/active-site pocket, in addition to surface exposed residues in direct contact with the solvent. Prior reports have highlighted both increases and decreases in CYP19 activity upon phosphorylation. Since many phosphorylation sites are too far away from the substrate and/or cofactor to be in direct contact, we hypothesized that site-specific phosphorylation could allosterically regulate CYP19 activity [42, 43]. To provide additional information on the molecular mechanisms governing these functional effects, we dissected residue networks that either stabilize the substrate molecule or heme cofactor inside the active-site pocket. We applied the MolDock scoring function and force field to identify the most relevant residues that stabilize the ternary complex between CYP19, the heme cofactor and androstenedione substrate (Fig. 4). Our simulations suggest that phosphorylation at 5 of the 7 explored positions could alter CYP19 activity by perturbing side-chain conformation of residues essential to ternary complex formation and stabilization. For instance, phosphorylation of residues Y361, Y441 or S363 will likely alter side-chain conformation of F430, R345 and C437, all important contributors to stabilization of the heme cofactor (Fig. 4). In a similar fashion, phosphorylation of S118 and S478 could affect androstenedione substrate binding through conformational changes of residues W224, M374 and D309 (Fig. 4).

4. DISCUSSION

4.1. SRIs have a binding affinity for the active-site pocket of CYP19

We have previously shown that, in BeWo cells, fluoxetine induces CYP19 activity, whereas its active metabolite norfluoxetine decreases it [24]. Moreover, we observed a competitive inhibition of CYP19 by norfluoxetine, which was not observed with fluoxetine [24]. As a result, we used molecular docking to compare the binding affinities of different SRIs to CYP19. We observed that all SRIs can productively bind to the active-site pocket of CYP19, showing binding affinities similar to the androstenedione and the control inhibitor. These results suggest that SRIs can act as competitive inhibitors of the natural CYP19 substrates, in accord with previous observations obtained with distinct SRIs in a CYP19 inhibition assay using supersomes (order of inhibition: sertraline > paroxetine > fluoxetine > citalopram) [45]. However, these assays do not take into consideration the different capacities of SRIs to penetrate the cell and access the CYP19 enzyme.

4.2. SRIs distinctively disrupt aromatase activity in different cell models

Two cell models (BeWo and villous trophoblast cells) were used to assess the effects of SRIs on the regulation of CYP19 activity. In BeWo cells, paroxetine and sertraline induced CYP19 activity, consistent with data obtained in H295R cells treated for 48 h with SRIs [45, 46]. We found no effect of citalopram on CYP19 activity in BeWo cells, although others found an induction of CYP19 activity in H295R cells [45, 46]. This could be due to the higher concentrations used in these assays (10-50 μ M) [45, 46] or intrinsic differences between in CYP19 regulation between these cell lines. In primary villous trophoblasts, we observed a decrease of CYP19 activity with venlafaxine, fluoxetine, paroxetine and sertraline,

and no effect with norfluoxetine and citalopram. Low-dose effect for paroxetine, sertraline and fluoxetine suggest a concentration-dependent action of SRIs on CYP19, which is commonly observed with endocrine disruptors [47]. These effects are consistent with the observed affinity of SRIs for the active-site pocket of CYP19 described in section 4.1. In our *in vitro* assay, competitive inhibition is only allowed when the SRI reaches the cytoplasm. It was suggested that SRIs could be transported using membrane transporters [48], which could explain effect differences between SRIs and between cell models. Hence, while molecular docking results show that SRIs exhibit high binding affinity for the active-site pocket, this action is limited by their capacity to reach the active-site pocket of the enzyme. Characterization of the transport of SRIs in our two cell models as well as in conditions that can affect this passage is an interesting perspective as it could result in alteration of estrogen production in specific conditions, such as intrauterine growth restriction, where placental transport is altered [49].

We have previously shown that norfluoxetine decreases promoter I.1-derived transcript of *CYP19A1* in BeWo cell line, whereas fluoxetine had no effect [24]. In primary villous trophoblast cells, fluoxetine decreases while norfluoxetine does not affect total and I.1-derived transcript of *CYP19A1*. These observations are consistent with the effects on CYP19 activity in primary villous trophoblast cells. CYP19 activity is closely related to villous cytotrophoblasts differentiation and fusion into syncytiotrophoblast [34].

Aromatase activity and expression is tightly related to villous cytotrophoblast differentiation in syncytiotrophoblast and one can influence each other [34]. Our group has studied the effects of SRIs on various parameters of villous cytotrophoblasts differentiation in BeWo and primary trophoblast cells [33]. SRIs increase cell fusion and markers of villous cytotrophoblasts differentiation [33]. The increased CYP19 activity by fluoxetine, paroxetine, and sertraline observed in BeWo cells is consistent with an increase in syncytialization, while in primary trophoblasts, sertraline and venlafaxine stimulated cell fusion only after 48 h of culture [33]. We chose to work with villous cytotrophoblasts in primary culture for 24 h, when they are mainly undifferentiated [50]. This allows for a better comparison with BeWo cells, which do not spontaneously differentiate in culture [51]. Other effects of SRIs on CYP19 could may occur at more advanced stages of differentiation in primary villous trophoblast cells, since effects of SRIs on cell fusion occur at 48 h and 72 h of culture [33].

Consistent with our current findings, overwhelming evidence now advocates for improved scrutiny when cell lines are being used to study normal trophoblast function [52]. The responses of BeWo cells to various treatments are often not reproduced in primary villous trophoblast cells [33, 53, 54]. Our results suggest that the cell environment involved in CYP19 regulation differs between these two cell types. These differences may be explained by the cancerous nature of the BeWo cell line, since CYP19 dysregulation is observed in breast cancer as compared to normal tissue (reviewed in [55]). It is also possible that the proto-oncogene *c-myc*, which is known to regulate *CYP19A1* in villous trophoblasts, is upregulated in cancer cells as opposed to normal trophoblasts [56, 57]. Moreover, estrogen receptors (ERs) and miRNAs regulate *CYP19A1* and may have altered expression in the BeWo choriocarcinoma cell line [57–59].

4.3. Functional differences between SRI molecules

Previous reports have shown that, in addition to inhibiting SERT, SRIs can also increase the expression and activity of a key enzyme in 5-HT biosynthesis, tryptophan hydroxylase (TPH), bind to 5-HT_{2A} receptor [60–63] or stimulate ER [64, 65]. These non-specific SRI effects are likely to contribute to some of the observed effects on CYP19 activity. Citalopram, which is the only SRI exhibiting neutral effect on CYP19 in our two cell models, stands out from other SRIs because of its high selectivity for SERT [25, 66]. This suggests that the observed differences in effects among the SRIs may be attributable to secondary effects rather than the inhibition of SERT. In light of these results, comparing the different signaling pathways activated by these molecules will help understanding these effects. Moreover, transport studies should be performed to decipher how SRI molecules access (or not) the intracellular compartment and whether this results in competitive inhibition (kinetic studies).

4.4. Serotonin and DOI induce aromatase activity in primary villous trophoblast cells

SRIs are designed to increase 5-HT levels in the synaptic cleft but their effects on trophoblastic 5-HT levels are unknown. We aimed to compare the effects of SRIs, which can have multiple modes of action, including 5-HT_{2A} agonist with a more specific treatment, 5-HT and an agonist of a crucial 5-HT receptor (5-HT_{2A}R) in response to SRI treatment. Considering the previous discrepancies in responses between BeWo cells and primary villous trophoblast cells, we assessed whether the effect of 5-HT on CYP19 previously observed in BeWo cell line [23] was representative of what occurs in primary villous trophoblast cells. At every stage of villous trophoblast cell differentiation, we observed that 5-HT and DOI significantly induced CYP19 activity. At the mRNA level, this effect was not significant (not shown). In the villous explant model, 5-HT induced CYP19 protein levels, whereas DOI had no significant effect. The effect of 5-HT on CYP19 protein level may be explained by either an increase in transcription, translation, or protein stability due to post-translational modifications of CYP19 [42, 67, 68]. Although we did not observe a significant increase in *CYP19A1* mRNA in primary villous trophoblast cells, it is possible that the architectural organization of the various cell types influences the regulation of *CYP19A1* expression in the explant model. Since DOI did not affect the protein level of CYP19, we suggest that the effect of 5-HT on protein levels might be receptor-independent or regulated by 5-HT receptors other than 5-HT_{2A}R.

DOI induced CYP19 activity without affecting CYP19 protein levels. However, we observed a modification in CYP19 phosphorylation (Fig. 3), which was previously shown to regulate its activity [43, 69–72]. We observed specific modification of phosphorylation status after a 24 h treatment with DOI. Short-term phosphorylation modifications were also associated with DOI treatment at 15, 30 and 60 min. This type of regulation of CYP19 (rapid and reversible phosphorylation) has already been observed [43, 69, 72], but the relevance of this remains to be characterized. Our molecular modeling of previously confirmed phosphorylation sites S118 and Y361 [42, 43] suggests that both phosphorylation events could perturb residue networks involved in substrate stabilization (W224, D309 and M374) and/or cofactor binding (F430 and R435). We also observed that phosphorylation at residues S363, Y441 and S478 could exert similar effects. These results are consistent with an

extensive list of experimental reports illustrating that phosphorylation leads to local and/or global protein conformational changes, whereby introduction of a phosphate group alters hydrogen bonding networks extending beyond the phosphorylation site [73–75]. Such changes in loops and/or secondary structure elements could easily act as enzyme activity modulators, namely through motional and/or structural alterations of functionally important allosteric transmission events. Although modulation of CYP19 activity through cofactor (de)stabilization was previously suggested [70], to the best of our knowledge the present report provides the first hypothesis of a proposed mechanism of action leading to the modulation of CYP19 activity through heme (de)stabilization (Fig. 4c, pY361, pS363 and pY441).

4.5. Limitations and perspectives

Strength of our study design is the assessment of several molecules with the same known mode of action (SERT inhibition), at different doses and in two different study models. This allows for screening of comparative effects. However, the effect of SRIs that is mediated by modulation of 5-HT levels was not assessed. Moreover, it would be interesting to assess the effect of endogenous 5-HT as compared to exogenous 5-HT, for instance by using siRNA to modulate the synthesis of the TPH enzyme. Several co-treatments with inhibitors of signaling pathways as well as receptor ant/agonists would provide information on the modes of action. This article will contribute to guiding these studies in order to select proper molecules and concentrations to be tested. In the present work, pregnancy complications were excluded, but we did not have access to maternal health condition and treatment. Another interesting perspective for this study resulted in the evaluation of the role of fetal sex on CYP19 activity, since we have previously shown that serotonin and estrogen systems interact together to reach a certain homeostasis [76].

5. CONCLUSION

In conclusion, our study suggests that alteration of placental 5-HT by depression and/or SRI treatment during pregnancy could be associated with placental estrogen disruption. Moreover, the low-dose effects of some SRIs on CYP19 activity in trophoblast cells leads to questions about the appropriate doses to use to treat depressive pregnant women. This study also contributes to better understanding how 5-HT and SRIs interact with CYP19 and promote estrogen production, which are involved in various pathophysiology, including pregnancy-specific diseases such as pre-eclampsia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Molecular docking simulations of serotonin reuptake inhibitors (SRIs) inside the active-site pocket of CYP19 human aromatase. (a) Comparison between the crystallographically resolved aromatase (CYP19)-androstenedione enzyme-substrate complex (PDB 5JL6) and molecular docking predictions. The active-site pocket is depicted as a gold transparent surface, with crystallographically resolved and docked androstenedione substrates shown as green and purple sticks, respectively. The heme cofactor is shown as a ball-and-stick model. (b) Comparative binding energies (kcal*mol⁻¹) for SRIs and androstenedione to CYP19. Binding energies are shown for the three most energetically favorable complexes obtained

by virtual docking. Right panel: wire models showing how distinct SRIs can be accommodated in the active-site pocket of CYP19. The crystallographically resolved androstenedione substrate is shown in green. UniProt-defined CYP19 binding site residues D309 and M374 are highlighted by asterisks (UniProt entry P11511)

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Fig 2.

Effects of serotonin reuptake inhibitors on aromatase activity in BeWo and primary villous trophoblasts. Relative aromatase (CYP19) activity in BeWo cells treated 24 h with (a) citalopram, (b) venlafaxine, (c) paroxetine and (d) sertraline (0.03, 0.1, 0.3 and 1 μM) and in primary villous trophoblast cells treated 24 h with (e) citalopram, (f) venlafaxine, (g) fluoxetine, (h) paroxetine, (i) sertraline and (j) norfluoxetine (0.03, 0.1, 0.3, 1 and 3 μM). Dashed line represents DMSO-vehicle control. Activities are expressed as a percentage of vehicle-control (DMSO, mean ± SEM). Significant differences compared to control are

indicated with asterisks (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$) as determined by a Kruskal Wallis and Dunn's post-hoc test, $n=3-5$

Fig 3.

Effect of serotonin and 2,5-dimethoxy-4-iodoamphetamine (DOI) on aromatase in villous trophoblasts. Relative aromatase (CYP19) activity in primary villous trophoblast cells were treated for 24h with (a) serotonin or (b) DOI (5-HT_{2A} agonist) at different time points. CYP19 activity is expressed in percentage of vehicle-control $\pm$ SEM. Protein expression of CYP19 in placental explants treated for 24h with (c) serotonin or (d) 2,5-dimethoxy-4-iodoamphetamine (DOI, 10 and 30 μ M). CYP19 protein expression is expressed relative to vehicle-control $\pm$ SEM. Dashed line represents DMSO-vehicle control. Significant

differences are indicated with asterisks (* $P < 0.05$; ** $P < 0.01$). Kruskal Wallis and Dunn's post-hoc test, $n = 3$. (e) Representative blot of CYP19 (55 kDa) with total protein used to normalize protein expression. (f) Representative blots (explants isolated from one placenta) of phosphorylated tyrosine, serine, threonine residues and from total protein (at CYP19 molecular weight, 55 kDa) from immunoprecipitated CYP19 in placental explants treated or not with DOI (30 μM) for 15 min, 30 min, 60 min or 24 h.

Fig 4.

Predicted conformational changes induced by single-site phosphorylation of human CYP19 aromatase. (a) Single-site phosphorylation of seven target residues predicted in human CYP19 aromatase (see Experimental procedures for details). The active-site pocket is depicted as a gold surface and the phosphate groups are colored as red-orange spheres. (b) Summary of the most important stabilizing interactions of the CYP19-heme-androstenedione ternary complex, as presented according to the relative individual binding energy contribution of each residue. Residues are sorted based on decreasing order of

stabilizing effects on the heme cofactor (top 8 residues) or androstenedione substrate (bottom 9 residues). Since the heme group acts as an important energy contributor to the androstenedione substrate, its comparative binding energy contribution is also listed ($-33.9 \text{ kcal} \cdot \text{mol}^{-1}$). (c) Energy minimization of the ternary complex showing residue conformational changes induced upon single-site phosphorylation events in CYP19. The heme cofactor is represented as a sphere model with carbon, nitrogen, iron and oxygen atoms shown in gray, blue, orange and red, respectively. The androstenedione substrate is shown as green sticks. Residues affected by phosphorylation are shown as blue (unphosphorylated) and gold (phosphorylated) sticks. Relevant residues are labeled according to single letter code numbering

Table 1.

Primer pair sequences used for the amplification of CYP19A1 transcript and reference genes.

Target	Sequence
Coding exons (total <i>CYP19A1</i>)	Forward: 5'-TGT CTC TTT GTT CTT CAT GCT ATT TCT C-3' Reverse: 5'-TCA CCA ATA ACA GTC TGG ATT TCC-3'
Exon I-derived transcript of <i>CYP19A1</i>	Forward: 5'-GGA TCT TCC AGA CGT CGC GA-3' Reverse: 5'-CAT GGC TTC AGG CAC GAT GC-3'
Peptidylprolyl isomerase A (<i>PPIA</i>)	Forward: 5'-GTT TGC AGA CAA GGT CCC A-3' Reverse: 5'-ACC CGT ATG CTT TAG GAT G-3'
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein, zeta polypeptide (<i>YWHAZ</i>)	Forward: 5'-GGC AAC CTA AGA ACA AAT G-3' Reverse: 5'-CAT GTT AGG CAA GTA TCA AA-3'