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# Stimulation of serotonergic 5-$HT_{2A}$ receptor signaling increases placental aromatase (CYP19) activity and expression in BeWo and JEG-3 human choriocarcinoma cells

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ABSTRACT

It is known that serotonin can influence the production and function of sex hormones, such as estrogens. Estrogens are critical for maintenance of pregnancy and regulate placental and fetal development. The key enzyme controlling estrogens synthesis during pregnancy is placental aromatase (CYP19). To better understand the regulation of placental aromatase, this study determined whether serotonin is involved in the regulation of this enzyme. BeWo and JEG-3 choriocarcinoma cells were used as models of the human placental trophoblast to evaluate the effects of serotonin and selective 5-$HT_{2A}$ receptor agonists on CYP19 activity and expression. Serotonin and selective 5-$HT_{2A}$ receptor agonists as well as PKC activation increased aromatase activity and expression in BeWo and JEG-3 cells. Dexamethasone, which regulates aromatase expression via JAK/STAT activation in certain tissues, had no effect. Increased *CYP19* gene transcription by 5-$HT_{2A}$ receptor and PKC stimulation was mediated by activation of the placental I.1 aromatase promoter. This study shows that the serotonergic system modulates placental aromatase expression, which would result in altered estrogens biosynthesis in trophoblast cells. Future detailed studies of serotonin–estrogen interactions in placenta are crucial for an improved understanding of the endo-, para- and autocrine role of serotonin during pregnancy and fetal development.



## 1. Introduction

In humans, placental estrogens regulate various functions critical for the maintenance of healthy pregnancy and fetal development, such as trophoblast differentiation/invasion, uterine growth, and progesterone synthesis [1–3]. Placental estrogen synthesis is catalyzed by the rate-limiting enzyme aromatase (CYP19) [4]. *CYP19* is expressed predominantly in differentiated syncytiotrophoblast cells as well as in JEG-3 and BeWo human choriocarcinoma cell lines [5–7]. Human placenta lacks steroid 17α-hydroxylase/17, 20-lyase (CYP17) and cannot produce estrogens *de novo* from cholesterol; it is thus dependent on fetal and maternal androgen precursors to produce estrogens and placental aromatase is key in this process. Aromatase gene expression undergoes complex regulation by a number of discrete promoters found in exon I of the *CYP19* gene which are differentially active in various tissues and cell types, with the I.1 promoter predominantly utilized in placenta, and with a minor contribution from the placenta-specific promoters I.2 [8] and recently discovered 1.8 [9]. The placental I.1 aromatase promoter is localized 93 kb upstream of the coding region exon II-X of the *CYP19* gene and its activation produces *CYP19* transcript with exon I.1 spliced onto a common splice junction directly upstream from exon II [10]. The regulation of placental estrogens synthesis by aromatase in the trophoblast is known to be increased by factors including calcitriol [11], hGCMa, a mammalian homolog of the protein encoded by the *Drosophila* glial cells missing (*gcm*) gene [12], normoxic reduction of inhibitory factor Mash-2 [13], and the AP-2 family of transcription factors (through the second messenger cAMP) [12], as well as activators of the protein kinase C (PKC) signaling pathway [14,15]. The precise role of the PKC signaling pathway in the regulation of *CYP19* gene expression remains to be clarified.

We have recently demonstrated that *de novo* synthesis of serotonin (5-hydroxytryptamine; 5-HT) occurs in the human trophoblast cell [16], suggesting an important endo-, para- and autocrine role for serotonin in placental function. This is supported by our earlier finding that serotonin acts via the 5-$HT_{2A}$ receptor as a key regulator of trophoblast growth, a critical cell process involved in placentation [17]. We observed this mitogenic effect of serotonin in

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BeWo and JEG-3 cells which involved 5-$HT_{2A}$-mediated stimulation of both the JAK2/STAT3 and PLCβ-PKC-β-Ras-ERK1/2 pathways [17]. It has been reported that activation of 5-$HT_{2A}$ receptor signaling stimulates the 17β-estradiol-mediated secretion of prolactin in dopaminergic neurons of ovariectomized rats, an effect likely due to increased local generation of estrogens by brain aromatase [18]. Moreover, a paracrine control of steroidogenesis by serotonin has been observed in the human adrenal cortex, where it stimulates aldosterone release [19]. In Japanese Medaka exposed to the selective serotonin-reuptake inhibitor (SSRI) fluoxetine increased plasma estradiol levels were observed, although a role for serotonin was not directly confirmed [20]. However, the potential effects of serotonin and 5-$HT_{2A}$ signaling on placental aromatase have not been studied.

Nothing is known about the potential interactions between serotonin and estrogens in human placenta, although neuroendocrinological research in brain has demonstrated that the two hormonal systems are intricately interdependent and interactive. Also, serotonin acts via the placental 5-$HT_{2A}$ receptor to stimulate the PKC signaling pathway, which is known to be involved in the regulation of placental aromatase [15,17,21]. Little is known about the molecular mechanism underlying the ability of serotonin to interact with estrogens biosynthesis. Thus, the objective of this study was to determine how serotonergic stimulation affects the expression and catalytic activity of placental aromatase using human BeWo and JEG-3 choriocarcinoma cell lines as trophoblast models.

## 2. Materials and methods

### 2.1. Chemicals

Serotonin (5-HT hydrochloride), the selective 5-$HT_{2A}$ receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) and antagonist ritanserin, dexamethasone (DEX), PKC activator phorbol-12-myristate-13-acetate (PMA), the irreversible aromatase inhibitor 4-hydroxyandrostenedione (formestane), and PKC inhibitor chelerythrine chloride were obtained from Sigma–Aldrich (Oakville, ON). The high affinity 5-$HT_{2A}$ receptor agonist TCB-2 and selective PKC inhibitors GF109203X (also known as Gö6850 or bisindolylmaleimide I) and Gö6976 were obtained from Tocris Bioscience (Ellisville, MO).

### 2.2. Cell culture

The human placental choriocarcinoma cell lines JEG-3 and BeWo were obtained from the American Type Culture Collection (Rockville, MD). BeWo cells were maintained in MEM/Ham's F-12K (50:50 v/v) culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. JEG-3 cells were cultured in MEM Eagle medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were maintained in 75 $cm^2$ filter-cap culture flasks (Techno Plastic Products, MIDSCI, St-Louis, MO) in a humidified atmosphere with 5% $CO_2$ at 37 °C. Cells were trypsinized (0.5% trypsin) and transferred to new 75 $cm^2$ flasks when they reached 90% confluence. For aromatase experiments, cells were treated as described previously [22,23]. Briefly, JEG-3 ($2.5 \times 10^4$ cells/well) and BeWo ($5 \times 10^4$ cells/well) cells were plated in 24-well culture plates containing 1 mL medium per well and exposed to various concentrations of the test compounds in aqueous solution or DMSO (0.2% final concentration in culture medium).

### 2.3. Aromatase catalytic activity

The catalytic activity of aromatase was determined by a tritiated water release assay according to the method of Lephart and Simpson [24] with certain modifications [23,25]. JEG-3 and BeWo cells were exposed to 54 nM 1β-$^3$H-androstenedione (Perkin Elmer, Wellesley, MA) in serum-free culture medium and incubated for 1.5 h at 37 °C in an atmosphere with 5% $CO_2$. 4-Hydroxyandrostenedione was used as a positive control for inhibition of aromatase activity to verify the specificity of the water release assay in both cell lines. A cell-free control using only medium was included in each experiment to correct for enzyme-independent tritiated water release. Further steps were as reported previously [22,23]. Aromatase activities (pmole/min/mg cellular protein) were expressed as percent of control (DMSO) activity.

### 2.4. Expression of CYP19 mRNA

RNA was isolated following 24 h exposures to the test compounds (Table 1) using the High Pure RNA Isolation Kit (Roche Diagnostics, Mississauga, ON) according to manufacturer instructions. Concentration and purity of RNA was determined by measuring the 260/280 nm absorbance ratio using a spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA), with quality assessed visually from 18S and 28S ribosomal bands on agarose gels. Original extracts and 50 ng/μl dilutions in RNAase-free water were stored at −80 °C until RT-PCR analysis.

Primers that recognized either the common coding region (exon II-X) of aromatase [23] or placental I.1-promoter-derived [26,27] *CYP19* transcripts were used for amplification (Access RT-PCR kit, Promega, Madison, WI) of cellular total RNA preparations, using primers for β-actin as reference gene. Conditions for *CYP19* mRNA amplification were: 100 ng RNA, 0.75 mM $Mg^{2+}$, annealing temperature 57 °C (exon II-X) or 61 °C (I.1), for 35 and 30 cycles, respectively. For β-actin amplification conditions were: 10 ng RNA, 2 mM $Mg^{2+}$, annealing temperature 54 °C for 25 cycles. Primer sequences were 5′-TTA-TGA-GAG-CAT-GCG-GTA-CC-3′ (fwd) and 5′-CTT-GCA-ATG-TCT-TCA-CGT-GG-3′ (rev) for the 314 bp exon II-X *CYP19* product, 5′-GGA-TCT-TCC-AGA-CGT-CGC-GA-3′ (fwd) and 5′-CAT-GGC-TTC-AGG-CAC-GAT-GC-3′ (rev) for the 119 bp I.1 *CYP19* product, and 5′-AAA-CTA-CCT-TCA-ACT-CCA-TC-3′ (fwd) and 5′-ATG-ATC-TTG-ATC-TTC-ATT-GT-3′ (rev) for the 163 bp β-actin product. Amplification products were separated and detected on 2% agarose gels stained with ethidium bromide. Densitometry was carried out using AlphaEaseFC Imaging software (version 6.0.0, Alpha Innotech, San Leandro, CA) and normalized to β-actin. The exponential amplification range was determined for each set of primers to optimize the number of cycles in the PCR method for semi-quantitative analysis of gene products, as done previously [23,28]. Exposure experiments were performed three times and amplification reactions were performed in triplicate per experiment.

### 2.5. Statistical analyses

Experiments were performed three times; per experiment each treatment was performed in triplicate. Statistically significant differences ($P < 0.05$) were determined by Student *t*-test or one-way ANOVA followed by Tukey post-hoc test or Dunnett's post-hoc test for multiple comparisons to control (GraphPad Prism v.5.04, GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. Serotonin and 5-$HT_{2A}$ receptor agonists stimulate aromatase activity and expression

Serotonin increased aromatase activity concentration-dependently (1–30 μM) in BeWo, but not JEG-3 cells (Fig. 1A). The selective 5-$HT_{2A}$ receptor agonists DOI (10 μM) and TCB-2 (300 μM) led to a statistically significant increase in aromatase activity in both JEG-3 and BeWo cells (1.2- and 2.0-fold, respectively for DOI, and 1.6 and 2.1-fold, respectively, for TCB-2) (Fig. 1B). These increases were greater than those seen with serotonin. The positive control PMA increased aromatase activity in JEG-3 and BeWo cells by 1.4 and 3.0-fold, respectively (Fig. 1C). Levels of I.1 promoter-derived and promoter non-specific (exon II-X) *CYP19* mRNA were increased statistically significantly by serotonin in BeWo

**Table 1**
Effects of serotonin, selective 5-$HT_{2A}$ receptor agonist (DOI), dexamethasone (DEX) and phorbol-12-myristate-13-acetate (PMA) on semi-quantitative I.1 promoter- or promoter non-specific- (exon II-X) CYP19 expression in BeWo and JEG-3 cells after a 24 h exposure. *Statistically significant difference from DMSO control ($P < 0.05$, one-way ANOVA with Tukey post-hoc test).

| | Treatment | JEG-3 | BeWo |
|---|---|---|---|
| | | Mean ± SD | Mean ± SD |
| I.1 | DMSO | 1.00 ± 0.15 | 1.00 ± 0.21 |
| | 30 μM Serotonin | 1.35 ± 0.20 | 1.46 ± 0.20* |
| | 30 μM DOI | 1.41 ± 0.23* | 1.61 ± 0.24* |
| | 1 μM DEX | 1.13 ± 0.11 | 1.14 ± 0.12 |
| | 1 μM PMA | 1.48 ± 0.17* | 2.53 ± 0.06* |
| Exon II-X | DMSO | 1.00 ± 0.14 | 1.00 ± 0.18 |
| | 30 μM Serotonin | 1.31 ± 0.16 | 1.44 ± 0.22* |
| | 30 μM DOI | 1.43 ± 0.27* | 1.56 ± 0.14* |
| | 1 μM DEX | 1.02 ± 0.12 | 1.27 ± 0.25 |
| | 1 μM PMA | 1.53 ± 0.12* | 2.51 ± 0.10* |