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# RÉGULATION DE LA BIOSYNTHÈSE DES ESTROGÈNES PAR LA SÉROTONINE DANS LE TROPHOBLASTE HUMAIN : EFFETS DES ANTIDÉPRESSEURS INHIBITEURS DE LA RECAPTURE DE LA SÉROTONINE

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# RÉSUMÉ

La dépression affecte jusqu'à 20% des femmes enceintes et les principaux traitements pharmacologiques agissent sur le transporteur de la sérotonine (ISRS; inhibiteurs sélectifs de la recapture de la sérotonine). Il a été démontré que l'enzyme de synthèse des estrogènes, aromatase (cytochrome P450 19; CYP19), est régulée par la sérotonine dans une lignée cellulaire de choriocarcinome humain (BeWo). Par contre, cet effet n'a pas été confirmé dans des primocultures de trophoblastes villeux et le potentiel de perturbation endocrinienne des ISRS n'a jamais été étudié. Les hypothèses de recherche de ce projet doctoral sont (1) que la sérotonine stimule la production des estrogènes par le CYP19 dans le placenta humain et (2) que les ISRS altèrent la régulation du CYP19 ainsi que la synthèse des estrogènes par l'unité fœto-placentaire. Les objectifs spécifiques sont de 1) développer une co-culture de lignées cellulaires de trophoblastes villeux et de surrénales fœtale pour évaluer la stéroïdogenèse fœto-placentaire; 2) caractériser le CYP19 pendant la syncytialisation des primocultures de trophoblastes villeux *in vitro*; 3) déterminer si la sérotonine, par le biais de l'activation de son récepteur 5-$HT_{2A}$, régule l'activité du CYP19 dans les cellules trophoblastiques et, le cas échéant, déterminer le mécanisme d'action et 4) déterminer l'effet des ISRS sur l'activité du CYP19 et sur la production des estrogènes par la co-culture développée à l'objectif 2 et par les primocultures de trophoblastes villeux. La co-culture BeWo/H295R a été développée et caractérisée comme un modèle représentatif de la stéroïdogenèse fœto-placentaire en raison de sa production synergique de 17β-estradiol et d'estriol. Nous avons caractérisé que l'activité du CYP19 et le taux d'ARNm du CYP19*A1* augmente jusqu'à 48 h au cours de la différenciation des primocultures de trophoblaste villeux *in vitro*. Lorsque traités avec de la sérotonine ou avec du 2,5-dimethoxy-4-iodoamphetamine (DOI, agoniste du récepteur 5-$HT_{2A}$), une augmentation de l'activité du CYP19 est observée, mais le mode d'action est différent de celui des cellules BeWo. Dans la co-culture BeWo/H295R, la fluoxétine n'affecte pas la production d'estrogènes, alors que la norfluoxétine réduit la production d'estrone et de 17β-estradiol. Ces résultats nous ont mené à comparer les effets de différents ISRS sur le CYP19. Dans les cellules BeWo, l'activité du CYP19 est augmentée par la fluoxétine, la paroxétine, la sertraline, alors qu'elle est diminuée par la norfluoxétine. Dans les primocultures de trophoblastes villeux, la paroxétine, venlafaxine, sertraline et fluoxétine diminuent l'activité du CYP19. Nos résultats mettent en exergue les différences entre les ISRS ainsi que les différences entre la lignée cellulaire BeWo et les primocultures de trophoblastes villeux. Dans une revue de littérature, nous avons également montré que les systèmes de la sérotonine et des estrogènes interagissent ensemble dans le contexte de différentes pathologies et que ces interactions sont particulièrement importantes pendant la grossesse puisque le placenta exprime les composantes de ces deux systèmes. Ainsi, ce projet permet de mieux comprendre l'interaction sérotonine-estrogène dans le placenta humain, et ouvre la porte vers l'étude des mécanismes d'action impliqués dans les altérations du développement fœtal et placentaire associés avec la dépression et/ou les antidépresseurs.

Mots clés : Co-culture, stéroïdogenèse, unité fœto-placentaire, sérotonine, estrogènes, antidépresseurs, inhibiteurs sélectifs de la recapture de la sérotonine (ISRS), dépression, trophoblaste, placenta, grossesse.

# ABSTRACT

Depression affects up to 20 % of pregnant women and the main pharmacological treatment target the serotonin system, especially by inhibiting the serotonin transporter (SSRI; selective serotonin reuptake inhibitors). It was shown that the estrogen synthesis enzyme, aromatase (cytochrome P450 19; CYP19), is regulated by serotonin in a human choriocarcinoma cell line (BeWo). However, the effect of serotonin on estrogen synthesis has not been confirmed in primary culture of villous trophoblast and the potential endocrine disruption by SSRI is not known. Research hypotheses of this doctoral project are (1) that serotonin stimulates estrogen production by CYP19 in the human placenta and (2) that SSRIs alter this CYP19 regulation as well as estrogen synthesis by the feto-placental unit. Our specific objectives are to 1) develop a co-culture of villous trophoblast and fetal adrenocortical cell lines to evaluate feto-placental steroidogenesis; 2) characterize CYP19 during syncytialization of primary culture of villous trophoblast *in vitro*; 3) determine if serotonin, via its receptor 5-$HT_{2A}$, regulates CYP19 activity in trophoblast cell lines and, if so, determine the mechanism of action and 4) determine the effects of SSRIs on CYP19 activity and on estrogen production by the co-culture developed in objective 2 and by primary culture of villous trophoblasts. We have developed and characterized the BeWo/H295R co-culture as a representative model of feto-placental steroidogenesis because of its synergistic 17β-estradiol and estriol production. We have also characterized that activity of CYP19 and mRNA level of *CYP19A1* increased until 48 h during *in vitro* differentiation of primary culture of villous trophoblasts. When treated with serotonin or with 2,5-dimethoxy-4-iodoamphetamine (DOI, 5-$HT_{2A}$ receptor agonist), an increase of CYP19 activity is observed, but the mode of action is different than in BeWo cell line. In the BeWo/H295R co-culture, fluoxetine does not affect estrogen production, while norfluoxetine reduces estrone and 17β-estradiol production. These results led us to compare the effects of different SSRIs on CYP19. In BeWo cells, CYP19 activity in increased by fluoxetine, paroxetine, sertraline, while it is decreased by norfluoxetine. In primary culture of villous trophoblast, paroxetine, venlafaxine, sertraline and fluoxetine decrease CYP19 activity. Our results highlight the differences between SSRIs and between the BeWo cell line and primary culture of villous trophoblast. In a literature review, we also showed that serotonin and estrogen systems interact together in the context of different pathologies and that these interactions are especially relevant during pregnancy, where placenta expresses the components of those two systems. Overall, this project allows a better understanding of the serotonin-estrogen interaction in the human placenta, and leads to further study the mechanisms of action involved in fetal and placental development alteration associated with depression and/or antidepressants.

Key words : Co-culture, steroidogenesis, feto-placental unit, serotonin, estrogens, antidepressants, selective serotonin reuptake inhibitors (SSRI), depression, trophoblast, placenta, pregnancy.

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# LISTE DES ABRÉVIATIONS

5-HIAA : acide 5-hydroxyindole acétique

5-HT : 5-hydroxytryptamine

5-HTP : 5-hydroxytryptophane

5-HTR : récepteur de la sérotonine

AADC : L-aromatique amino-acide décarboxylase

ABC : ATP-binding cassette

ACTH : hormone corticotrope

BPA : bisphénol A

BPC : biphényle polychloré

CRH : corticolibérine

CTev : cytotrophoblaste extravilleux

CTv : cytotrophoblaste villeux

CYP : cytochrome P450

CYP19 ou CYP19A1 : aromatase

DES : diethylstilbestrol

DHEA : déhydroépiandrostérone

E1 : estrone

E2 : 17β-estradiol

E3 : estriol

E4 : estetrol

ER : récepteur d'estrogènes

ERE : élément de réponse aux estrogènes

ERO : espèce réactive à l'oxygène

ERK : kinase régulée par signal extracellulaire

ERR : récepteur similaires aux récepteurs des estrogènes

ERRE : élément de réponse aux récepteurs similaires aux récepteurs des estrogènes

GnRH : hormone de relâchement des gonadotrophine

GPER1 : récepteur aux estrogènes couplé aux protéines G

hCG : gonadotrophine chorionique humaine

hPL : lactogène placentaire

IMAO : inhibiteur des monoamines oxydases

IRSN : inhibiteur de la recapture de la sérotonine et de la norépinéphrine

ISRS : inhibiteur sélectif de la recapture de la sérotonine

JAK : janus kinase

JNK : c-jun N-terminal

MAO : monoamine oxydase

MAPK : protéines kinases activées par les agents mitogènes

NET : transporteur de la norépinéphrine

OCT-3 : transporteur aux cations organiques 3

pGH : hormone de croissance placentaire

p-gp : glycoprotéine p 1

PI3K : phosphoinositide-3-kinase

PLC : phospholipase C

PKC : protéine kinase C

RCIU : restriction de croissance intra-utérine

RCPG : récepteur couplés aux protéines G

SERM : Modulateur sélectif des récepteurs d'estrogène

SERT : transporteur de la sérotonine

ST : syncytiotrophoblaste

STAT : transducteur du signal et activateur de transcription

TCA : antidépresseur tricyclique

TPH : tryptophane hydroxylase

VMAT2 : transporteur vésiculaire des monoamines 2

# PREMIÈRE PARTIE : INTRODUCTION GÉNÉRALE

# 1 LE PLACENTA HUMAIN

Le placenta relie les métabolismes du fœtus et de la mère pour les échanges de nutriments, d'oxygène ($O_2$), de dioxyde de carbone ($CO_2$) et de déchets. Il possède également des fonctions de modulation immunitaire afin d'empêcher le rejet du fœtus et une fonction endocrine. Cette dernière sera détaillée dans la **section 1.3**. Le placenta humain, de type hémomonochorial, est un organe dont le développement est limité dans le temps et l'espace. Lors de l'implantation du blastocyste dans la décidue maternelle, des trophoblastes sont présents sous la forme de cytotrophoblastes (CT) et de syncytiotrophoblaste (ST), cellule multinucléée formée à partir de la fusion des CT (Carlson, 2009; Marieb, 2005). À ce stade, le ST est très invasif et migre dans l'endomètre (Evain-Brion, 2001). Le processus d'implantation se déroule sur une période d'environ une semaine (Marieb, 2005). Graduellement, les villosités chorioniques, unité structurale du placenta, croissent par la prolifération du mésenchyme dans les villosités, qui est ensuite envahi par les capillaires fœtaux. Le CT forme la couche intérieure des villosités et le ST est adjacent, directement en contact avec l'espace intervilleux qui sera éventuellement rempli de sang maternel (Carlson, 2009; Evain-Brion, 2001; Marieb, 2005). Les échanges entre le fœtus et la mère sont favorisés par les microvillosités du ST à la membrane maternelle qui augmentent la surface d'échange (Audus, 1999; Prouillac & Lecoeur, 2010). Les structures placentaires définitives, ainsi que la barrière placentaire qui sépare le sang maternel du sang fœtal, sont illustrées à la **figure 1.1**.

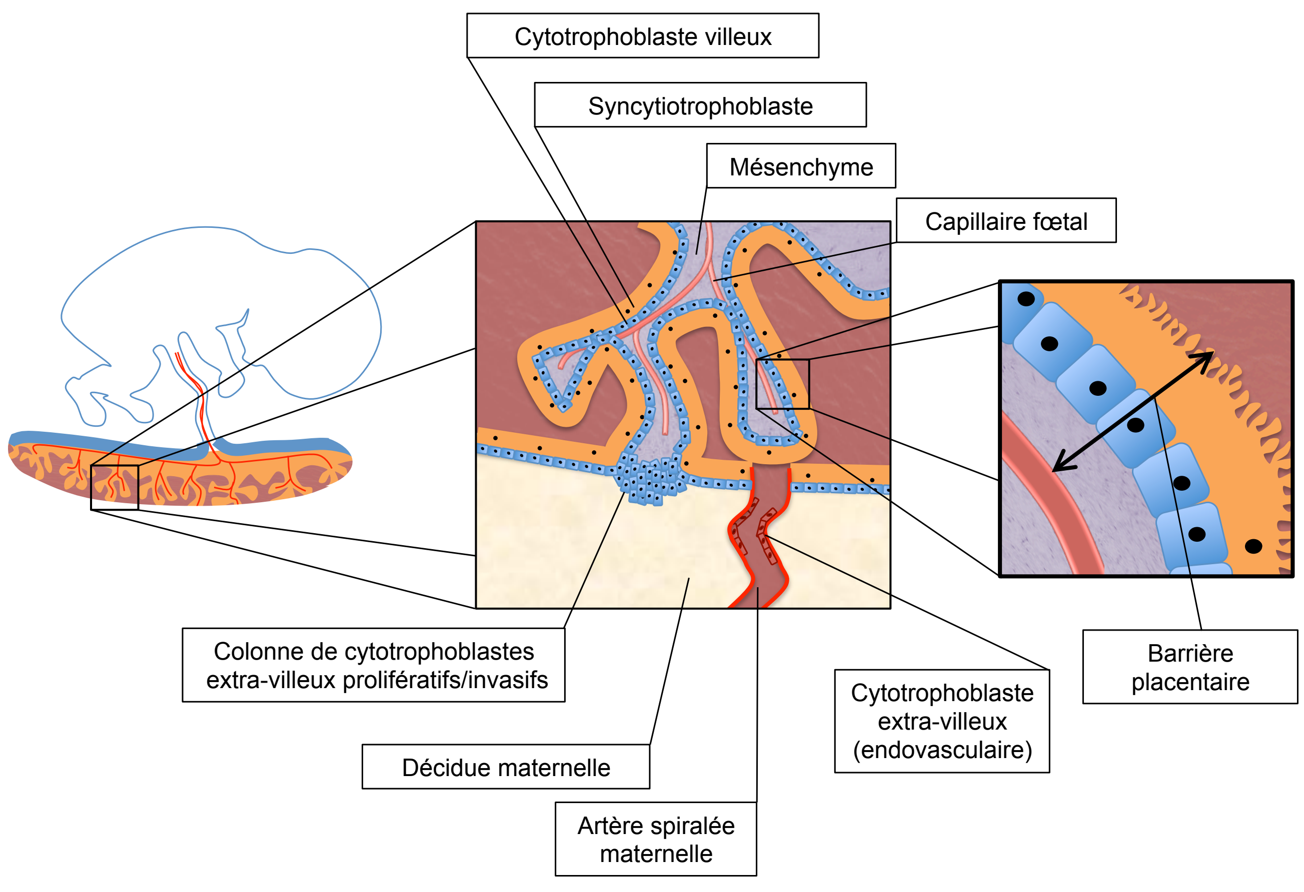


**Figure 1.1 : Anatomie du placenta humain et de la villosité choriale**

**Le placenta humain est composé de villosités choriales, donc la couche externe est une cellule multinucléée, le syncytiotrophoblaste qui est en contact avec le sang maternel. Le syncytiotrophoblaste est produit par la fusion des cytotrophoblastes villeux adjacents. Les cytotrophoblastes à la base des villosités ancrées prolifèrent et envahissent la décidue (ils peuvent former des cellules géantes multinucléées) et les artères spiralées maternelles pour les rendre atones. La barrière placentaire est composée de l'endothélium fœtal, du mésenchyme, d'une couche de cytotrophoblaste villeux et du syncytiotrophoblaste.**

## 1.1 Différenciation des cellules du placenta

Une fois cette structure établie, les cellules du placenta se développent principalement par deux processus : la différenciation des cytotrophoblastes extravilleux (CTev) et la différenciation des cytotrophoblastes villeux (CTv)(Evain-Brion, 2001).

### 1.1.1 Différenciation des cytotrophoblastes extravilleux

Les CTev sont d'abord situés à la base des villosités ancrées, puis envahissent environ le tiers du myomètre et forment des cellules géantes multinucléées dont un des rôles proposés est la synthèse d'hormones protéiques ou envahissent les artères spiralées maternelles (CTev endovasculaires) (**Figure 1.2**) (Burton *et al.*, 1999b; Evain-Brion, 2001; Guibourdenche *et al.*, 2009). En remplaçant la couche musculaire des artères, les CTev rendent la paroi atone, ce qui permet un meilleur apport de sang maternel dans l'espace intervilleux (Evain-Brion, 2001; Guibourdenche *et al.*, 2009; Kaufmann & Castellucci, 1997; Redman, 1997; Zhou *et al.*, 1997). Jusqu'à la douzième semaine de grossesse, le développement du placenta et du fœtus se déroule dans un environnement relativement pauvre en oxygène, avec la présence de bouchons de CTev endovasculaires qui limitent l'apport sanguin (Burton *et al.*, 1999a; Guibourdenche *et al.*, 2009). D'ailleurs, la concentration en oxygène de l'environnement cellulaire régule le processus de migration des CTev (Genbacev & Fisher, 1997; Genbacev *et al.*, 1996).

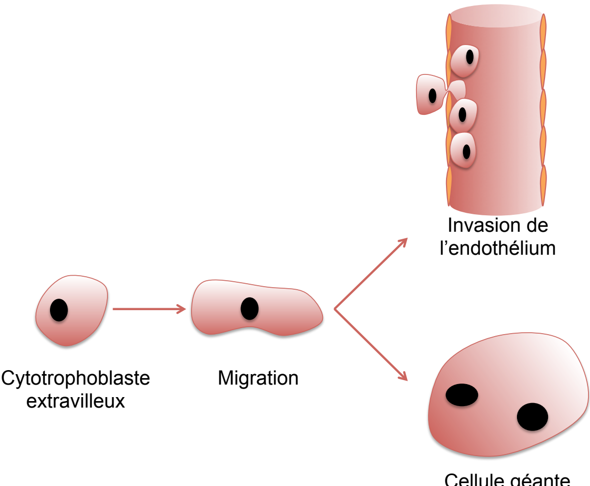


**Figure 1.2 : Voie de différenciation du cytotrophoblaste extravilleux**

**Les cytotrophoblastes extravilleux migrent dans la décidue et peuvent différencier en cellule géante multinucléée ou envahir l'endothélium des artères spiralées maternelles pour les rendre atones.**

### 1.1.2 Différenciation des cytotrophoblastes villeux

La villosité choriale est composée d'une couche de CTv adjacente au ST (Guibourdenche *et al.*, 2009). La différenciation des CTv est caractérisée par une différenciation morphologique (fusion et formation du ST) (Huppertz & Borges, 2008) et par une différenciation fonctionnelle ou biochimique qui correspond à une sécrétion typique d'hormones, de facteurs de croissance et de peptides (Larsen *et al.*, 2002; Malassine & Cronier, 2002; Tsatsaris *et al.*, 2006). Lors de la différenciation, le ST développe aussi sa fonction d'échange qui s'observe par l'expression de protéines de transport actif et de diffusion facilitée (Tsatsaris *et al.*, 2006). L'expression de ces protéines est fortement polarisée. Le ST est constamment régénéré à partir des CTv de la couche adjacente et libère notamment des amas de noyaux syncytiaux (nœuds syncytiaux) dans la circulation sanguine maternelle (**Figure 1.3**) (Malassine & Cronier, 2002; Mayhew, 2014).

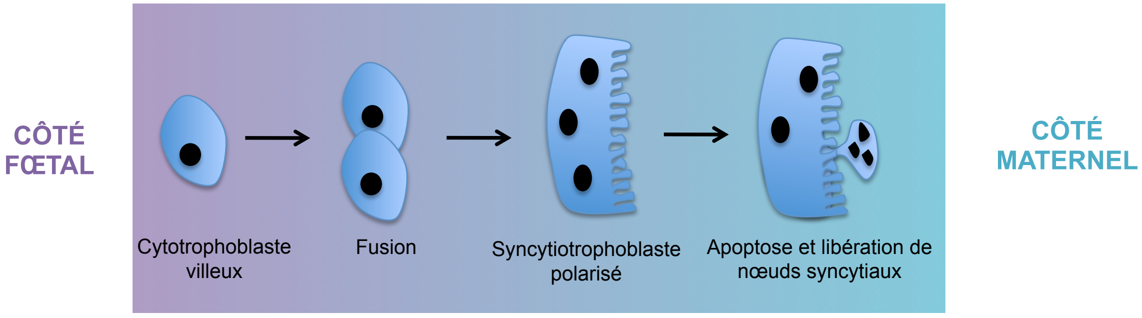


**Figure 1.3 : Voie de différenciation morphologique du cytotrophoblaste villeux**

**Les cytotrophoblastes villeux fusionnent et forment une cellule multinucléée polarisée dont la côté maternel est caractérisé par des microvillosités, le syncytiotrophoblaste. Celui-ci dégénère par apoptose et libère des nœuds syncytiaux dans la circulation sanguine maternelle. Il est regénéré par la fusion des cytotrophoblastes villeux.**

Les deux processus de différenciation (biochimique et morphologique) du CTv sont étroitement reliés (Douglas & King, 1990). En effet, la différenciation du CTv est régulée, entre autres, par une boucle de rétroaction positive impliquant les hormones produites par le ST (gonadotrophine chorionique (hCG) et estrogène) qui stimulent la fusion des CTv afin de produire le ST (Albrecht & Pepe, 1999; Cronier *et al.*, 1999; Évain-Brion & Malassiné, 2010; Yashwanth *et al.*, 2006).

## 1.2 Passage transplacentaire et biotransformation

La barrière placentaire correspond aux couches de cellules qui séparent le sang maternel du sang fœtal. Celle-ci évolue au cours de la grossesse; la couche de ST, qui est en contact direct avec le sang maternel, s'amincissant avec l'avancement de la grossesse, ce qui facilite les échanges entre la mère et le fœtus (Prouillac & Lecoeur, 2010). Contrairement à ce que suggère son nom, la barrière placentaire n'est pas étanche aux différentes molécules auxquelles la femme enceinte est exposée (Minnes *et al.*, 2011). Le transfert transplacentaire peut se faire par diffusion passive (principal mode d'action), par diffusion facilitée, par transport actif, par pinocytose ou par filtration (Myllynen *et al.*, 2005; Poulsen *et al.*, 2009). De nombreux transporteurs peuvent être impliqués dans le passage transplacentaire. D'ailleurs, la présence de transporteurs ABC (pour *ATP binding cassette*) a été démontrée dans le placenta avec une spécialisation de la bordure fœtale et maternelle (revu dans (Gedeon & Koren, 2006; Iqbal *et al.*, 2012; Prouillac & Lecoeur, 2010; Vähäkangas & Myllynen, 2009)). Les mécanismes qui régulent le transfert transplacentaire et l'exposition fœtale à un composé dépendent de l'affinité de celui-ci aux protéines de couplage, de sa lipophilicité, de sa constante d'ionisation (pKa) et des paramètres pharmacocinétiques maternels, tels que le volume de distribution, le taux de métabolisme, l'efflux placentaire et le gradient de pH entre le compartiment maternel et fœtal (Audus, 1999; Gedeon & Koren, 2006; Poulsen *et al.*, 2009). Il est important de considérer ces paramètres dans le développement de nouveaux médicaments. Idéalement, les médicaments devraient avoir une affinité de couplage aux protéines élevée, une demi-vie d'élimination courte et un faible volume de distribution, ce qui limite l'exposition du fœtus (Gedeon & Koren, 2006). En plus des transporteurs, le placenta possède des enzymes de métabolisme de phase I et II qui contribuent à la biotransformation (dégradation ou bioactivation) des composés qui traversent la barrière fœto-placentaire (revu dans (Myllynen *et al.*, 2005; Prouillac & Lecoeur, 2010)). Enfin, les substances peuvent bioaccumuler dans le placenta comme c'est le cas du cadmium (Jolibois Jr *et al.*, 1999). Cette bioaccumulation permet de limiter l'exposition du fœtus, mais peut aussi nuire au développement et au fonctionnement du placenta (Jolibois Jr *et al.*, 1999).

## 1.3 Fonction endocrine

Le placenta humain produit des hormones peptidiques et stéroïdiennes parmi lesquelles certaines sont uniques à la grossesse (par exemple, l'hormone lactogène placentaire (hPL)) (Napso *et al.*, 2018). Soulignons également la production de neurotransmetteurs par le placenta, qui peuvent être qualifiés de neurohormones en raison de leurs fonctions endocrines (Napso *et al.*, 2018).

### 1.3.1 Hormones peptidiques

Parmi les hormones peptidiques produites par le placenta, soulignons la hCG, la hPL, l'hormone de croissance placentaire (pGH) ainsi que des peptides hypothalamiques tels que l'hormone de relâchement des gonadotrophines (GnRH) et la corticolibérine (CRH) (Carlson, 2009; Evain-Brion & Malassiné, 2003; Tsatsaris *et al.*, 2006). Les fonctions des principales hormones peptidiques du placenta sont résumées au **tableau 1.1**.

**Tableau 1.1 : Hormones peptidiques placentaires**

| Hormone | Cellule | Description et profil de production | Fonction |
|---|---|---|---|
| **Gonadotrophine chorionique humaine (hCG)** | ST | Forte homologie de séquence d'acide aminé avec l'hormone lutéinisante (LH), l'hormone folliculo-stimulante (FSH) et la thyréostimuline (TSH)<br>Pic de production entre 8 et 12 semaines de grossesse associé avec une diminution de la TSH maternelle (rétroaction négative) | Prolonge la durée de vie du corps jaune afin d'assurer une production de progestérone adéquate<br>Différenciation du tractus génital masculin (récepteurs LH-hCG sur les cellules de Leydig)<br>Autorégulation de la différenciation morphologique du CT en ST<br>Stimulation de la sécrétion de déhydroépiandrostérone-sulfate (DHEAS) par les surrénales fœtales<br>Immunosuppression<br>Régulation de la diminution du seuil osmotique pour la soif chez la mère |
| **Lactogène placentaire (hPL)** | ST | Homologie d'acides aminés de 85% avec l'hormone de croissance (GH) et de 13% avec la prolactine (13%)<br>Production augmente jusqu'à 34 semaines, puis se stabilise<br>Forte corrélation de la sécrétion avec le poids placentaire et la masse de ST | Effet somatotrope (à terme, responsable de 12% de l'activité somatotrope totale)<br>Faible effet lactotrope<br>Effet diabétogène par la promotion de la résistance à l'insuline : régulation de la production du facteur de croissance ressemblant à l'insuline-1 (IGF1) |
| **Hormone de croissance placentaire (pGH)** | ST | Augmentation progressive au cours de la grossesse<br>Sécrétion continue (contrairement à la GH pituitaire maternelle) | Activité somatotrope (à terme, responsable de 85% de l'activité somatotrope)<br>Stimule la production d'IGF-1, ce qui est responsable de la suppression de la sécrétion de GH maternelle |
| **Gonadolibérine (GnRH)** | CT, ST | Même structure et activité que GnRH hypothalamique<br>Concentration corrélée avec la masse de CT (pic au premier trimestre) | Régulation de la sécrétion d'hCG |
| **Corticolibérine (CRH)** | CT, ST | Identique à la CRH hypothalamique<br>Faibles concentrations jusqu'à 30 semaines, puis augmentation de 20 fois dans les 5 dernières semaines<br>Insensibilité à la rétroaction négative par le cortisol | Stimule la synthèse et le relâchement de prostaglandines pour augmenter la maturation du col de l'utérus<br>Initiation et promotion de l'accouchement<br>Stimulation de la production de l'hormone corticotrope (ACTH) pituitaire fœtale pour augmenter la production de déhydroépiandrostérone (DHEA) |

**ST : syncytiotrophoblaste et CT : cytotrophoblaste. Créé à partir de (Larsen *et al.*, 2002).**

### 1.3.2 Hormones stéroïdiennes

La progestérone et les estrogènes sont les principaux stéroïdes produits par le placenta, majoritairement dans le ST. Au tout début de la grossesse, la progestérone, nécessaire au maintien de la quiescence utérine, est produite par le corps jaune de l'ovaire, puis à la huitième semaine de grossesse, il se produit une transition lutéo-placentaire où le corps jaune dégénère et le placenta assure la sécrétion de progestérone (Braunstein, 2003; Chen *et al.*, 2011; Evain-Brion & Malassiné, 2003). La production placentaire de progestérone est sécrétée à 90% du côté maternel (Braunstein, 2003; Evain-Brion & Malassiné, 2003). La quasi-absence de cytochrome P450 (CYP) 17 (voir section 2.4) empêche la production d’androgènes à partir de pregnénolone et permet le maintien de taux élevés de progestérone dans le placenta (Braunstein, 2003; Evain-Brion & Malassiné, 2003). Cette production limitée d’androgènes par le placenta permet d’éviter les effets délétères de l’exposition à ces stéroïdes sur le développement du fœtus femelle (Xita & Tsatsoulis, 2006). La production des estrogènes requiert donc des précurseurs androgéniques en provenance des surrénales fœtales et maternelles. Les estrogènes, leurs rôles et les différentes étapes qui mènent à leur synthèse sont décrits de manière plus détaillée au **chapitre 2**.

# 2 ESTROGÈNES

## 2.1 Rôles des estrogènes pendant la grossesse

Les estrogènes, en plus d'être reliés à plusieurs fonctions dans le corps humain (système nerveux central, métabolisme des os, système cardiovasculaire) (Napso *et al.*, 2018; Nilsson & Gustafsson, 2002), sont essentiels au bon déroulement de la grossesse tel que présenté au **tableau 2.1**.

**Tableau 2.1 : Effets des estrogènes produits pendant la grossesse**

| Processus régulé par les estrogènes | Description de l'effet | Références |
|---|---|---|
| **Autocrine/Paracrine** | | |
| **Formation du syncytiotrophoblaste** | La gonadotrophine chorionique humaine (hCG) et les estrogènes stimulent la formation du syncytiotrophoblaste qui à son tour sécrète ces hormones (boucle de rétroaction) | (Albrecht & Pepe, 1999; Cronier *et al.*, 1999; Évain-Brion & Malassiné, 2010; Yashwanth *et al.*, 2006) |
| **Prolifération trophoblastique** | Prolifération des trophoblastes suite à la stimulation des récepteurs aux estrogènes | (Albrecht *et al.*, 2006; Gambino *et al.*, 2012a; Jeschke *et al.*, 2007) |
| **Invasion trophoblastique** | Régulation de la production de facteurs de croissance, cytokines, hormones (hCG, progestérone) qui affectent la migration et l'invasion des trophoblastes extravilleux<br>Collaboration des estrogènes et de la concentration d'oxygène pour réguler le flux sanguin utéro-placentaire | (Albrecht & Pepe, 1999; Albrecht *et al.*, 2000; Chen *et al.*, 2012; Chen *et al.*, 2011; Tsatsaris *et al.*, 2006) |
| **Endocrine** | | |
| **Développement du fœtus** | Développement fonctionnel des surrénales fœtales (expression d'enzymes de la stéroïdogenèse) par son action sur la corticolibérine (CRH)<br>Régulation de la différenciation sexuelle et croissance fœtale | (Albrecht *et al.*, 2005; Albrecht *et al.*, 2006; Gambino *et al.*, 2012a; Jeschke *et al.*, 2007; Kaludjerovic & Ward, 2012; Lash *et al.*, 2009; Mastorakos & Ilias, 2003; Olwenn *et al.*, 2008; Toppari *et al.*, 2010; Tsatsaris *et al.*, 2006) |
| **Adaptation de la mère à la grossesse et préparation à la lactation** | Adaptation du système respiratoire, de l'humeur, de la rétention du sel et de l'eau, du myomètre (formation de jonctions serrées, récepteurs d'ocytocine, biodisponibilité des prostaglandines) et des glandes mammaires<br>Accouchement, évènements qui mènent à l'initiation du travail | (Albrecht & Pepe, 2010; Chaim & Mazor, 1998; Kaludjerovic & Ward, 2012; Napso *et al.*, 2018) |

## 2.2 Différents estrogènes : estrone, 17β-estradiol, estriol et estetrol

La sécrétion des différents estrogènes est associée à des stades précis de la reproduction, ce qui suggère des rôles physiologiques différents : l'estrone (E1) est associée à la post-ménopause, le 17β-estradiol (E2) à l'ensemble de la vie reproductive alors que l'estriol (E3), produit par le placenta, et l'estétrol (E4), produite par le foie fœtal à partir d'E2 et d'E3, sont associés à l'état gravidique (Gérard *et al.*, 2015; Newby *et al.*, 2000). Pendant la grossesse, le taux d'E3 est un indicateur du bon développement du bébé (Chard & Macintosh, 1995; Ostergard & Kushinsky, 1971; Shenhav *et al.*, 2003). E4 est d'un intérêt limité comme biomarqueur du déroulement de la grossesse (Holinka *et al.*, 2008). La sécrétion des différents estrogènes pendant la grossesse est présentée à la **figure 2.1**.

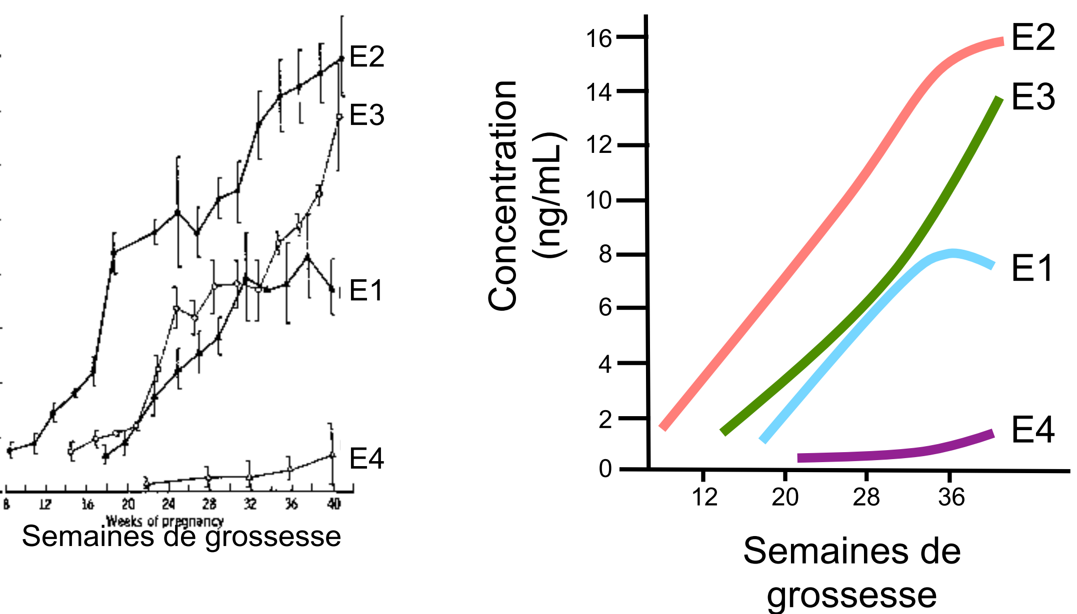


**Figure 2.1 : Dosage plasmatique d'estrogènes au cours de la grossesse.**

**Concentration en estrogènes (ng/mL) selon le nombre de semaines de grossesse. E1 : estrone; E2 : 17β-estradiol; E3 : estriol et E4 : estetrol. Créée à partir de (Levitz & Young, 1977).**

Pour évaluer la puissance des différents estrogènes, des modèles d'étude sensibles aux estrogènes, comme les tissus mammaire et utérin, sont généralement utilisés. Les études montrent toutefois de plus en plus que les effets varient selon le modèle d'étude. Il est généralement reconnu que E2 est l'estrogène avec la plus grande puissance estrogénique (Abot *et al.*, 2014; Caldwell *et al.*, 2012; Gérard *et al.*, 2015; Jozan *et al.*, 1981; Liu *et al.*, 2015; Martucci & Fishman, 1977; Watson *et al.*, 2008). E3 et E4 possèdent tous les deux une plus

faible activité estrogénique par rapport à E2 et une activité estrogénique mixte (agoniste ou antagoniste selon le tissu) (Gérard *et al.*, 2015; Giretti *et al.*, 2014; Son *et al.*, 2002; Tseng & Gurpide, 1976). La présence de E3 et E4 pendant la grossesse a été suggérée comme un mécanisme d'inactivation de E2 afin de protéger le fœtus contre les effets cancérogènes de E2 (Holinka *et al.*, 1980; Melamed *et al.*, 1997). D'ailleurs, Melamed *et al.* (1997) ont suggéré que E3 exerce un effet antagoniste de E2 seulement lorsque le ratio molaire E2 : E3 se rapproche de 1 : 10. Sous ce ratio, E2 agit comme un estrogène puissant et est faiblement antagonisé par E3.

Les rôles de E1, E3 et E4 sur la fonction placentaire ou sur le déroulement de la grossesse ne sont pas connus. Par contre, il a été démontré que la régulation du niveau d'E2 joue un rôle important dans le développement placentaire. Une augmentation prématurée de la concentration d'E2 chez un modèle primate non humain peut mener à des problèmes de placentation, incluant une altération de l'invasion des CTev (Aberdeen *et al.*, 2012; Albrecht *et al.*, 2006). De plus, dans des cas de fertilisation *in vitro*, associés avec des niveaux élevés d'E2, des effets délétères sur l'implantation et des problèmes de grossesse associés à une mauvaise placentation ont été observés (Farhi *et al.*, 2010; Forman *et al.*, 1988; Simón *et al.*, 1995).

## 2.3 Les récepteurs d'estrogènes

Les estrogènes agissent par la voie génomique ou non génomique en impliquant différents récepteurs des estrogènes (ER) : ERα, ERβ ainsi qu'un récepteur membre de la famille des récepteurs couplés aux protéines G (RCPG), le GPER1 (Beato & Klug, 2000; Prossnitz *et al.*, 2008; Revankar *et al.*, 2007). Les affinités différentes des estrogènes pour les ER et le profil de dimérisation influence la puissance estrogénique (Melamed *et al.*, 1997; Sasson, 1991). Il existe également des récepteurs similaires aux récepteurs des estrogènes (ERR pour *estrogen-related receptor*; α, β et γ ) qui sont des récepteurs nucléaires orphelins (Poidatz *et al.*, 2012). Les ERR possèdent une homologie de séquence avec les ER, mais contrairement à ces derniers, ils ne sont pas activés par les estrogènes naturels (Poidatz *et al.*, 2012). Ils ont une certaine affinité pour certains composés estrogéniques comme le diethylstilbestrol (DES) et le bisphénol A (BPA) (Morice *et al.*, 2011; Tremblay *et al.*, 2001) et possèdent une activité constitutive, indépendante de la présence de ligands exogènes (Kallen *et al.*, 2004).

### 2.3.1 Voie génomique

Dans la voie génomique classique, les estrogènes diffusent à travers la membrane plasmique pour atteindre les ER (α et β) qui sont présents sous forme de monomères liés à des chaperones dans le cytoplasme. La liaison des estrogènes aux ER permet la libération des chaperones et l'homo- ou hétéro-dimérisation. Dans le noyau, ces dimères agissent, avec leurs coactivateurs ou corépresseurs, sur les éléments de réponse aux estrogènes (ERE) (Nilsson *et al.*, 2001). Les ER peuvent également exercer leurs effets en interagissant avec des facteurs de transcription qui se lient sur des éléments de réponses de l'ADN. Il s'agit alors d'un mode d'action génomique non classique (Safe & Kim, 2008). Les ERR, quant à eux, se lient aux éléments de réponse aux ERR (ERRE pour *estrogen-related response element*) (Beato & Klug, 2000), mais sont également capables d'activer certains ERE (Vanacker *et al.*, 1999). Soulignons enfin que dans certaines situations les ERα peuvent activer les ERRE (Vanacker *et al.*, 1999).

### 2.3.2 Voie non génomique

Dans la voie non génomique, le temps d'action des estrogènes est beaucoup plus court. Ce processus implique les GPER1 ainsi que les ERα, ERβ, mais sous leur forme membranaire (plasmique ou membranes des organelles) (Levin, 2009; Prossnitz *et al.*, 2008; Watson *et al.*, 2007). Le ERα s'associe avec la cavéoline-1 afin d'être transporté vers la membrane (Levin, 2009). Les ERα membranaires forment rapidement des dimères après la liaison des estrogènes. Cette dimérisation est nécessaire pour l'association avec les sous-unités de protéines G comme $G_{\alpha q}$, $G_{\alpha s}$, $G_{\alpha i}$ et $G_{\beta\gamma}$ (Levin, 2009; Levin *et al.*, 1999; Mineo *et al.*, 2007). De plus, la cavéoline-1 permet l'échafaudage des molécules de signalisation qui sont activées par l'interaction d'E2 avec ERα (Levin, 2009). Le complexe E2-ERα permet notamment l'association avec la protéine adaptatrice shc, la tyrosine kinase (src), la sous-unité p85α dePI3K et avec des récepteurs de facteurs de croissance comme IGF1R et le récepteur du facteur de croissance épidermique (EGFR) (Acconcia & Kumar, 2006; Levin, 2009; Migliaccio *et al.*, 1996; Zhang *et al.*, 2004). GPER1 peut aussi activer plusieurs effecteurs, incluant l'adenylate cyclase (production d'AMPc), src, et la kinase sphingosine (SphK) (Filardo & Thomas, 2012). Src et SphK, par le biais de l'activation de MMPs, sont impliqués dans l'activation d'EGFR (Filardo & Thomas, 2012). L'activation de ces kinases et récepteurs permet la stimulation des voies phospholipase C (PLC) MAPK/ERK et PI3K (Acconcia & Kumar, 2006; Levin, 2009; Migliaccio *et al.*, 1996; Zhang *et al.*, 2004).

### 2.3.3 Expression placentaire

L'expression des différents ER dans le placenta humain a été étudiée dans plusieurs modèles. ERα et ERβ sont exprimés dans la lignée cellulaire HTR-8/SVneo, dérivée de CTev invasif du premier trimestre et dans la lignée BeWo, issue d'un choriocarcinome avec les caractéristiques du CTv (Gambino *et al.*, 2012b; Jiang *et al.*, 1997; Patel *et al.*, 2015). Dans les cellules BeWo, ERα et ERβ sont exprimés au niveau de la membrane plasmique, ce qui suggère un mode d'action non génomique (Gambino *et al.*, 2012b). L'expression d'ERα et ERβ a également été démontrée dans les tissus placentaires avec une plus forte expression d'ERα dans les CTv que dans les ST (Bechi *et al.*, 2006; Bukovsky *et al.*, 2003; Patel *et al.*, 2015), alors que l'expression d'ERβ est plus grande dans le ST que dans le CTv (Bechi *et al.*, 2006; Patel *et al.*, 2015). L'ARNm de *GPER1* a été détecté dans le tissu total placentaire, sans distinction du type cellulaire (Carmeci *et al.*, 1997; Takada *et al.*, 1997) et le taux d'ARNm est diminué dans le placenta de grossesses pré-éclamptiques (Feng *et al.*, 2017). Enfin, l'expression d'ERRγ (ARNm et protéine) augmente au cours de la grossesse et pendant la différenciation trophoblastique (Poidatz *et al.*, 2012).

### 2.3.4 Rôles placentaires

Les ER (α et/ou β) sont impliqués dans la différenciation biochimique du CTv (Pepe & Albrecht, 1999). De plus, la stimulation d'ERα induit des effets apoptotiques et antiprolifératifs sur les cellules HTR8-SVneo (Patel et al., 2015), inhibe l'expression génique de CRH dans les primocultures de CTv (Ni et al., 2004) et régule l'expression de la leptine (ARNm et protéine), une hormone impliquée dans la survie et la prolifération trophoblastique (Gambino et al., 2012b). Il a été démontré que les voies de signalisation des kinases activées par des mitogènes (MEK)-kinases régulées par le signal extracellulaire (ERK) et PI3K sont activées par le ERα membranaire dans les explants placentaires et les cellules BeWo (Gambino et al., 2012b). De plus, dans les cellules BeWo, les voies MAPK p38 et kinases c-jun N-terminal (JNK) sont activées par ERα (Gambino et al., 2012b). Les auteurs n'ont toutefois pas démontré comment ces voies de signalisation sont associées au ERα membranaire. Ceux-ci peuvent toutefois interagir avec des protéines G (voir section 2.3.2) (Guillaume *et al.*, 2017; Levin, 2014; Razandi *et al.*, 2003).Par ailleurs, ERRy est impliqué dans la stimulation de la production hormonale, notamment par la régulation du taux d'ARNm de CYP19A1 et de la fusion cellulaire, ce qui indique qu'il est un facteur de transcription important pour la différenciation des CTv en ST (Kumar & Mendelson, 2011; Luo et al., 2013; Poidatz et al., 2015). Ces observations sont cohérentes avec les différents rôles des estrogènes placentaires (**Tableau 2.1**).

## 2.4 Synthèse des estrogènes par l'unité fœto-placentaire

La production d'estrogènes pendant la grossesse requiert une séquence de conversions à partir du cholestérol. Brièvement, le cholestérol en provenance de la circulation maternelle traverse la membrane cellulaire par les transporteurs de lipoprotéines, exprimés sur la membrane maternelle du ST (Évain-Brion & Malassiné, 2010; Guibourdenche *et al.*, 2009). Le cholestérol est converti en prégnénolone, puis en progestérone qui peuvent toutes les deux être utilisées comme substrat par l'enzyme CYP17.

Le CYP17 possède deux activités enzymatiques, la 17α-hydroxylase et la 17,20-lyase qui s'exercent de manière séquentielle soit dans la voie Δ5 pour produire de la déhydroépiandrostérone (DHEA) à partir de prégnénolone ou dans la voie Δ4 pour produire de l'androstènedione à partir de progestérone (Rainey *et al.*, 2002) (**Figure 2.2**).

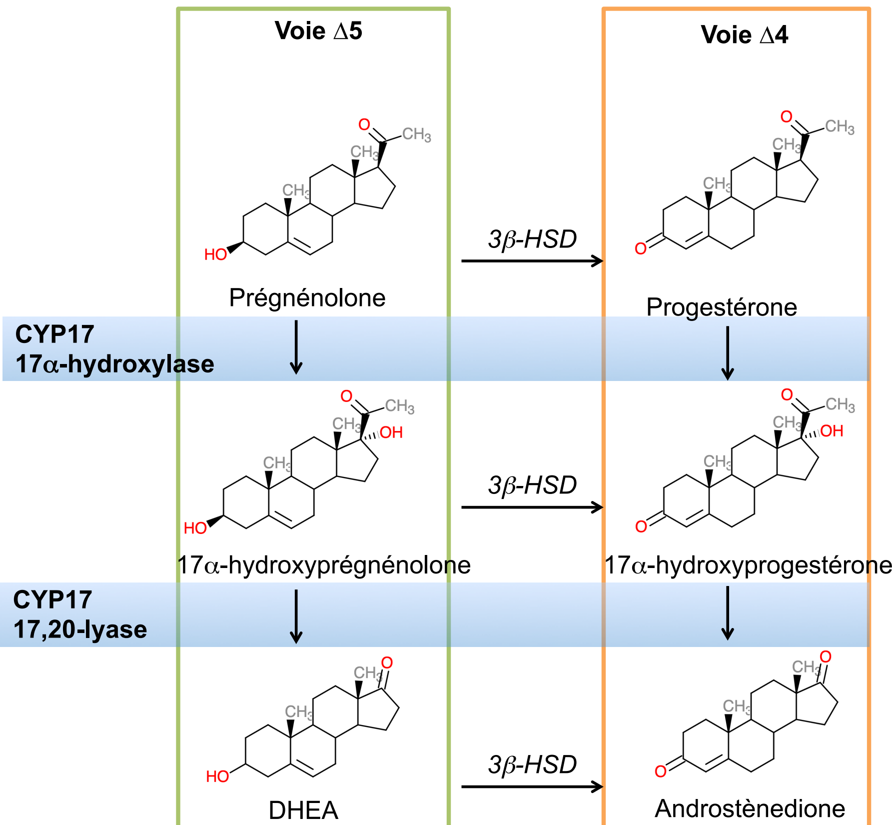


**Figure 2.2 : Activités 17α-hydroxylase et la 17,20-lyase de l'enzyme CYP17**

**La voie Δ5 permet de produire déhydroépiandrostérone (DHEA) à partir de prégnénolone, alors que la voie Δ4 produit de l'androstènedione à partir de progestérone. L'activité 17α-hydroxylase et 17,20-lyase du CYP17 exercent des actions successives dans les deux voies. Les images des molécules ont été produites avec *Chemdoodle 2D sketcher.* Créée à partir de (Rainey et al., 2002).**

Le placenta exprime peu ou pas l'enzyme CYP17 et donc, les précurseurs androgéniques pour produire des estrogènes proviennent principalement des surrénales fœtales ou maternelles (Alsat & Evain-Brion, 1998; Chaim & Mazor, 1998; Evain-Brion & Malassiné, 2003; Guibourdenche *et al.*, 2009). La DHEA des surrénales fœtales est sulfatée par la sulfotransférase (SULT2A1). La DHEAS entre dans la circulation sanguine et atteint le foie fœtal et le placenta. Dans le foie fœtal, la DHEAS est hydroxylée par l'enzyme 16α-hydroxylase (CYP3A7) avant d'atteindre le placenta par la circulation sanguine. Dans le placenta, les groupements sulfates de la DHEAS et de la 16α-OH-DHEAS sont hydrolysés par la sulfatase. La DHEA et la 16α-OH-DHEA sont converties en androstènedione et en 16α-OH-androstènedione respectivement par l'enzyme 3β-hydroxystéroide deshydrogénase 1 (3β-HSD1). L'androstènedione et 16α-OH-androstènedione peuvent ensuite être convertis en testostérone et en 16α-OH-testostérone respectivement par l'enzyme 17 β-hydroxystéroïde deshydrogénase 1 (17β-HSD1). L'androstènedione, la testostérone et la 16α-OH-testostérone sont des substrats de l'aromatase (CYP19; *CYP19A1*) pour la production d'E1, E2 et E3, respectivement (Évain-Brion & Malassiné, 2010; Guibourdenche et al., 2009; Miller & Auchus, 2011; Simpson, 2003; Tsatsaris et al., 2006; Vaillancourt & Lafond, 2009). La conversion des androgènes en estrogènes est donc terminée par le CYP19 dont l'activité et le taux d'ARNm sont beaucoup plus grands dans le placenta que dans les surrénales fœtales (Guibourdenche et al., 2009; Pezzi et al., 2003). Cette enzyme catalyse l'étape limitante de la production des estrogènes (Guibourdenche et al., 2009; Richard et al., 2005; Sanderson et al., 2000; Tsatsaris et al., 2006). Les estrogènes ainsi produits dans le placenta peuvent être transférés dans les circulations maternelles et fœtales (Guibourdenche et al., 2009) ou agir de manière autocrine/paracrine sur les cellules du placenta (Albrecht et al., 2000; Évain-Brion & Malassiné, 2010; Yashwanth et al., 2006). Bien qu'il existe une unité materno-fœto-placentaire, les principales étapes de la production des estrogènes ont lieu dans les surrénales et le foie du fœtus ainsi que le trophoblaste, d'où l'intérêt d'étudier plus particulièrement l'unité fœto-placentaire. Les étapes menant à la production des estrogènes placentaires pendant la grossesse sont schématisées à la **figure 2.3**.

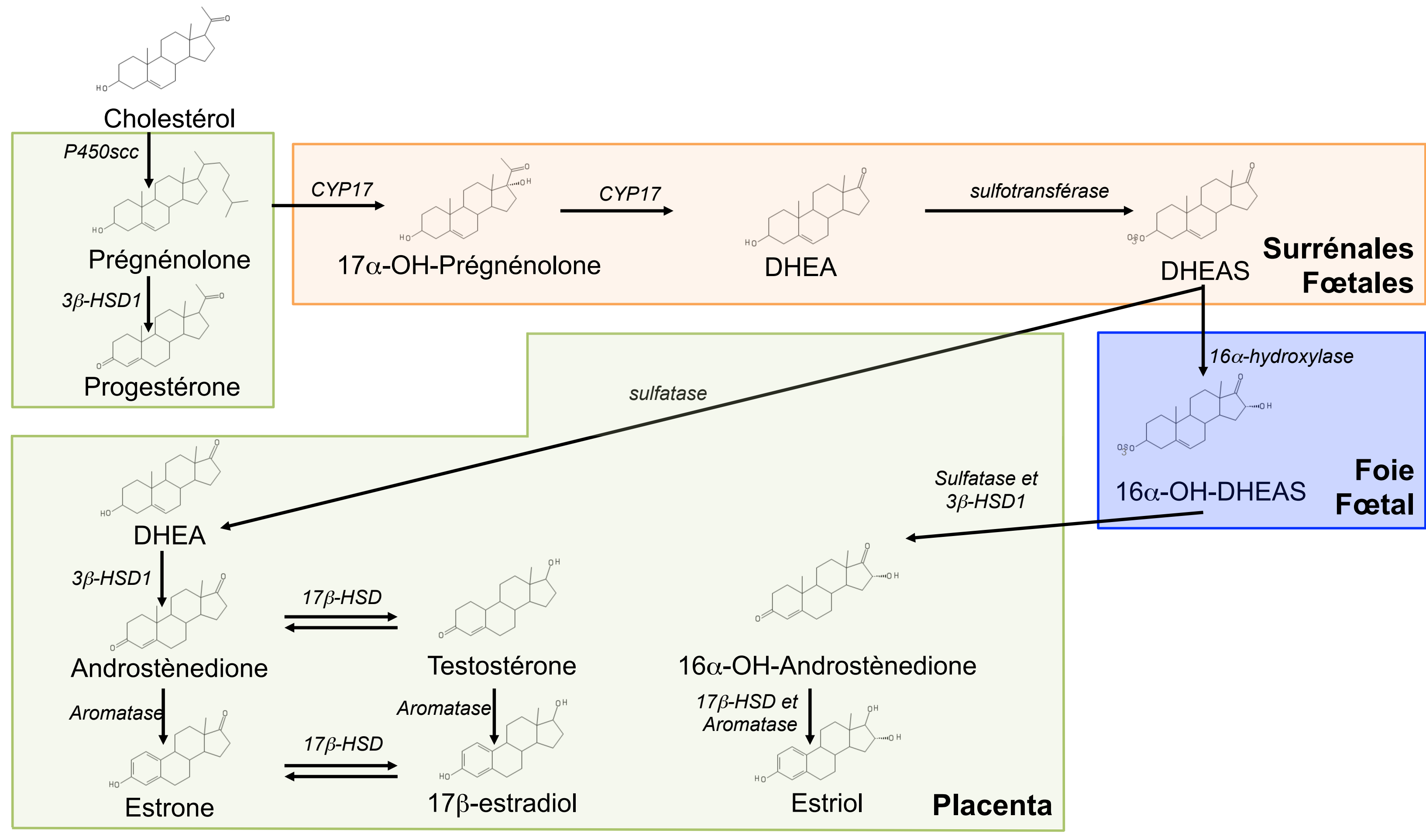


**Figure 2.3 : Stéroïdogenèse de l'unité materno-fœto-placentaire**

**La prégnénolone est synthétisée dans le placenta puis transférée au fœtus afin de produire de la déhydroépiandrostérone sulfatée (DHEAS) dans les surrénales ou de la 16α-hydroxydéhydroépiandrostérone sulfatée (16α-OH-DHEAS) dans le foie. Ces précurseurs androgéniques retournent au placenta où leurs conversions se terminent par l'aromatase (CYP19) afin de produire de l'estrone, du 17β-estradiol et de l'estriol. Vert : placenta; orange : surrénales fœtales et bleu : foie fœtal. P450scc : enzyme de clivage de la chaîne latérale du cholestérol; 3β-HSD : 3β-hydroxystéroïde deshydrogénase; CYP17 : cytochrome P450 17; 17β-HSD : 17β-hydroxystéroïde deshydrogénase.**

**Créée à partir de (Sanderson, 2009). Les dessins des molécules ont été produits à partir de https://pubchem.ncbi.nlm.nih.gov/edit2/index.html.**

## 2.5 Impact d’une perturbation estrogénique pendant la grossesse

Bien que de nombreux rôles des estrogènes pendant la grossesse soient connus (**Tableau 2.1**), les effets d’une perturbation estrogénique sur le fonctionnement placentaire et le déroulement de la grossesse demeurent peu caractérisés. Le terme perturbateur endocrinien regroupe des composés avec différents mécanismes d’action, incluant agonisme et antagonisme des récepteurs stéroïdiens (nucléaires et membranaires), non stéroïdiens et orphelins comme le récepteur d’aryl hydrocarbone (AhR). De plus, ces composés peuvent affecter les voies enzymatiques impliquées dans la biosynthèse des stéroïdes et/ou leur métabolisme (De Coster & van Larebeke, 2012; Stefanidou *et al.*, 2009). Les perturbateurs endocriniens possèdent souvent plus d’un mode d’action et il est difficile de les catégoriser comme « estrogénique » ou « anti-estrogénique » (**Tableau 2.2**). Le **tableau 2.3** présente les principaux effets d’une perturbation de l’homéostasie estrogénique pendant la grossesse. Dans ce tableau, les perturbateurs endocriniens sont comparés au DES qui est un puissant estrogène synthétique, et à des modèles où la synthèse d’estrogène est réduite ou inhibée pendant la grossesse (inactivation du gène *cyp19a1* dans des modèles animaux ou déficience en CYP19 chez l’humain). Notons qu’il est fréquent d’observer, avec les perturbateurs endocriniens, des effets non monotones et à de faibles doses (De Coster & van Larebeke, 2012).

**Tableau 2.2 : Description des modes d'action de certains perturbateurs endocriniens**

| Composé | ER | AR | Stéroïdogenèse | Autres actions |
|---|---|---|---|---|
| **Diethylstilbestrol (DES)**<br>Estrogène synthétique qui a été prescrit aux femmes enceintes jusque dans les années 1970, afin de prévenir les fausses-couches, avortements spontanés et accouchements prématurés (affirmation non fondée) | Agoniste | Antagoniste | | Agoniste inverse des ERR |
| **Bisphénol A (BPA)**<br>Monomère structurellement similaire au DES utilisé comme plastifiant dans les polycarbonates et résine époxy | Agoniste et antagoniste | Antagoniste | Inhibition du CYP19 | Forte affinité pour ERR, agoniste PXR, antagoniste ThR |
| **Phtalates et métabolites**<br>Plastifiants qui augmentent la flexiblitié des plastiques, faiblement attachés au plastique et donc facilement libérés dans l'environnement. Les métabolites ont également une activité de perturbation endocrinienne | Agoniste (faible) | Antagoniste | Augmentation et diminution de l'expression du CYP19 selon la dose | Agoniste de PPAR, perturbateur des hormones thyroïdiennes |
| **Génistéine**<br>Isoflavone de la famille des phytoestrogènes présente en grandes quantités dans le soya | Agoniste | Antagoniste (faible) | Diminution de l'expression /inhibition de 17β-HSD1, P450scc et 3β-HSD | Bloque la synthèse de thyroxine |
| **2,3,7,8-tetrachlorodibenzo-p-dioxine (TCDD)**<br>Produit chimique industriel persistant dans l'environnement | Agoniste (par le biais de l'effet sur AhR) | | | Agoniste des AhR |
| **Prochloraz**<br>Fongicide imidazole utilisé en jardinage et agriculture | Antagoniste | Antagoniste | Inhibition de l'activité du CYP19 | Agoniste |
| **Biphényles polychlorés (BPC)**<br>Contaminant persistant utilisé dans les années 1970 notamment pour ses caractéristiques d'isolant | Couplage faible | Couplage faible | Inhibition de la sulfotransférase | Interaction avec AhR, perturbateur des hormones thyroïdiennes |
| **Atrazine**<br>Pesticide persistant utilisé dans la culture du maïs | | | Augmente l'activité du CYP19 | |

**ER : récepteur d'estrogène; AR : récepteur des androgènes; ERR : récepteur lié aux estrogènes; CYP19 : cytochrome P450 19/aromatase PXR : récepteur pregnane-X; PPAR : récepteur activé par les proliférateurs de peroxysomes; 17β-HSD1: 17β-hydroxystéroïde deshydrogénase 1; P450scc : enzyme de clivage de la chaîne latérale du cholestérol; 3β-HSD : 3β-hydroxystéroïde deshydrogénase. Créé à partir de (Cravedi *et al.*, 2007; De Coster & van Larebeke, 2012; Douglas & Philippa, 2014; Patisaul, 2017; Rosenfeld, 2012; Stefanidou *et al.*, 2009; Troisi *et al.*, 2016; Vinggaard *et al.*, 2006)**

**Tableau 2.3 : Effets de l'exposition à des agents perturbateurs de l'homéostasie estrogéniques pendant la grossesse**

| Effets | Estrogène synthétique | Perturbateurs endocriniens | Diminution des estrogènes |
|---|---|---|---|
| **Fœtus** | Masculinisation du cerveau<br>Malformations du système reproducteur<br>Altération de la fertilité<br>Risque de cancer/néoplasie (du sein, du système reproducteur féminin et de la prostate)<br>Problèmes de grossesse (fausse-couche, grossesse ectopique)<br>Ménopause précoce<br>Augmentation des maladies auto-immunes | Masculinisation du cerveau (génisteine)/ Féminisation du comportement sexuel (TCDD)<br>Féminisation du système reproducteur: diminution de la distance anogénitale (génistéine, phthalates, prochloraz)<br>Malformations du système reproducteur (TCDD, phthalates, prochloraz)<br>Altération de la fertilité (BPC, TCDD, phtalates)<br>Risque de cancer/néoplasie (sein et du système reproducteur) (génisteine, BPA, phtalates, BPC) | Masculinisation des organes génitaux féminins<br>Diminution de la réceptivité sexuelle[1]<br>Féminisation des comportements sexuels[1] |
| **Placenta** | Diminution de la taille du placenta, surabondance de cellules trophoblastiques géantes et absence de cellules diploïdes[1]<br>Mauvaise vascularisation et/ou oxygénation | Diminution de la taille du placenta, réduction de la zone labyrinthe[1] (BPA)<br>Diminution de la prolifération, augmentation de l'apoptose des trophoblastes humains (lignées et cultures primaires) (BPA)<br>Mauvaise vascularisation et/ou oxygénation (BPA, BPC, TCDD) | |
| **Grossesse/ Mère** | Avortement spontané<br>Naissance prématurée<br>Cancer du sein chez la mère | Naissance prématurée (atrazine, BPC)<br>Restriction de croissance intra-utérine, faible poids de naissance (atrazine, BPC) | Avortements spontanés<br>Masculinisation de la mère |

**[1]Les effets sont uniquement pertinents et observés dans des modèles murins**

**TCDD : 2,3,7,8-tetrachlorodibenzo-p-dioxine; BPC : Biphényles polychlorés ; BPA : Bisphénol A**

**Créé à partir de (Albrecht & Pepe, 1999; Bakker *et al.*, 2002; Chevrier *et al.*, 2011; Colborn *et al.*, 1993; Conlon, 2017; Cravedi *et al.*, 2007; De Coster & van Larebeke, 2012; Ehrlich *et al.*, 2016; Kanova & Bicikova, 2011; Miller *et al.*, 2004; Noriega *et al.*, 2005; Rinsky *et al.*, 2012; Robins *et al.*, 2011; Troisi *et al.*, 2016; Vinggaard *et al.*, 2005; Vrooman *et al.*, 2016)**

# 3 AROMATASE

Le CYP19 est un membre de la famille des CYP qui sont des hémoprotéines impliquées notamment dans le métabolisme de phase I et la stéroïdogenèse. Le CYP19 est situé sur le réticulum endoplasmique lisse (Conley & Hinshelwood, 2001; Perez-Sepulveda *et al.*, 2013; Simpson *et al.*, 2002). La protéine de 503 acides aminés est incluse dans un complexe enzymatique, nommé aromatase, qui contient une NADPH-P450 réductase. Le site catalytique du CYP19 contient [illegible] tisation (**Figure 3.1**). Cette [illegible] niques, mais elle est égalem [illegible] activité est d'ailleurs inhibé [illegible] nité du CYP19 pour les pré [illegible] 10 fois plus grande que pour E2 (Osawa *et al.*, 1993).

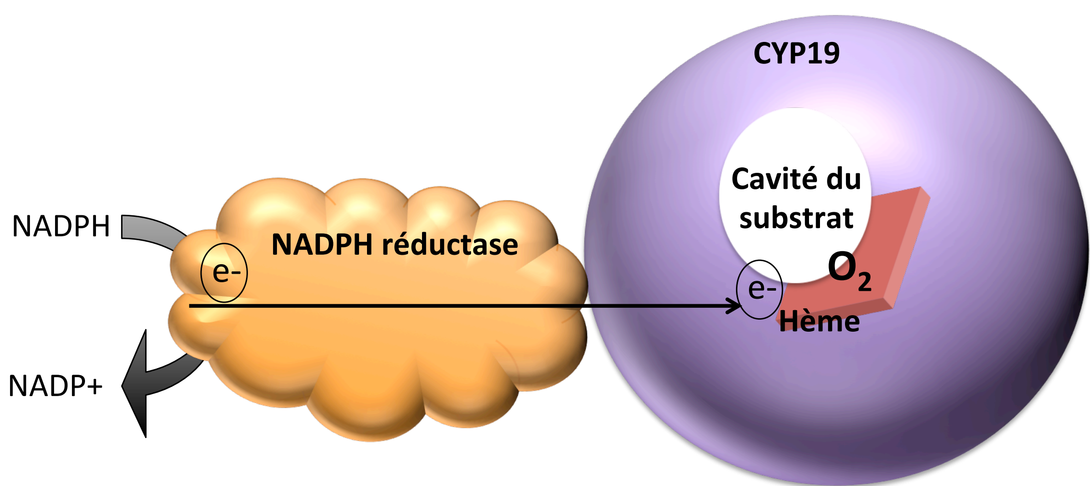


**Figure 3.1 : Complexe enzymatique de l'aromatase**

**Lors de la synthèse d'estrogènes, le précurseur androgénique est amené dans la cavité du substrat du cytochrome P450 19 (CYP19). Cette cavité est adjacente à un groupe hème qui permet d'apporter une molécule d'$O_2$ à la réaction d'aromatisation. Cette réaction requiert également la nicotinamide adénine dinucléotide phosphate (NADPH) réductase comme co-facteur qui utilise la NADPH afin de lui fournir un électron (e-).**

Le gène codant CYP19 (*CYP19A1*) est localisé sur le chromosome 15q21.2 et est constitué d'une région de 30 kb contenant 9 exons (exons II à X) (Bulun *et al.*, 2003; Demura *et al.*, 2008; Sebastian & Bulun, 2001), en amont desquels sont situés différents promoteurs qui régulent l'expression de plusieurs exons I non codants (région de 93 kb) (Sebastian & Bulun, 2001). Cette organisation permet de réguler le taux d'ARNm de *CYP19A1* d'une manière spécifique

selon les tissus qui expriment différents exons I régulés par différents facteurs de transcription (Bulun *et al.*, 2003; Demura *et al.*, 2008; Kumar & Mendelson, 2011; Mendelson & Kamat, 2007; Simpson *et al.*, 2002; Zhou *et al.*, 2009a). Tous les exons I non traduits sont épissés sur une jonction d'épissage commune située en amont du codon d'initiation de l'exon II (Demura *et al.*, 2008) (**Figure 3.2**). La protéine du CYP19 est donc identique, peu importe le promoteur activé (Demura *et al.*, 2008; Simpson *et al.*, 2002).

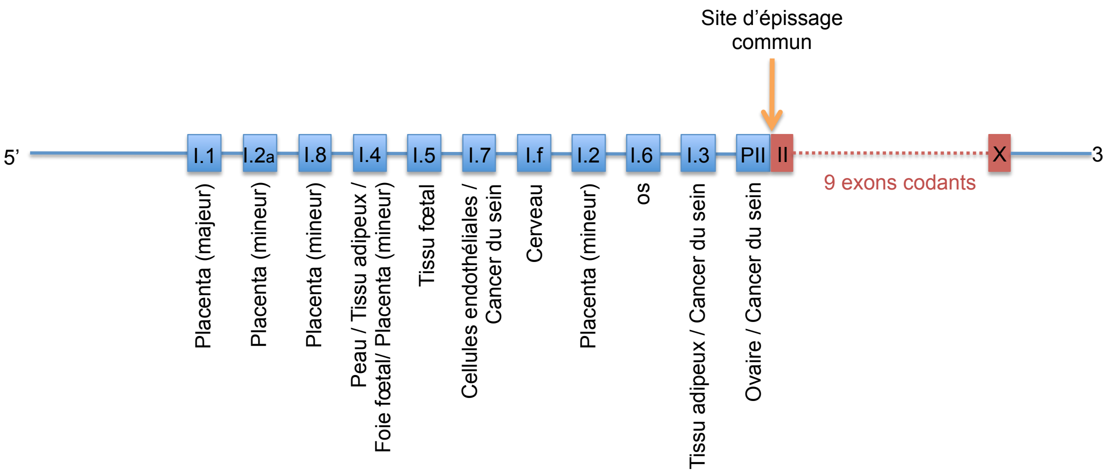


**Figure 3.2 : Représentation schématique du gène humain *CYP19A1***

**Le gène humain *CYP19A1* est composé de 11 exons I non-codants connus à ce jour et exprimés selon les tissus. Ce sont les exons II à X qui codent pour la protéine du CYP19. Figure créée à partir de (Bulun & Simpson, 1994; Demura *et al.*, 2008).**

## 3.1 Régulation de *CYP19A1* dans le placenta et le fœtus

Au niveau placentaire, le principal transcrit est dérivé de l'exon I.1, qui est le plus en amont des exons codants (Bulun & Simpson, 1994; Demura *et al.*, 2008; Kamat & Mendelson, 2001; Mahendroo *et al.*, 1993; Sebastian & Bulun, 2001). Plusieurs éléments de réponse à différents facteurs de transcription ont été trouvés en amont du promoteur I.1 (**Figure 3.3**). La grande quantité de CYP19 dans le placenta humain en comparaison aux autres espèces s'expliquerait d'ailleurs, d'un point de vue évolutif, par l'acquisition du promoteur et de l'exon I.1 (Sebastian & Bulun, 2001). D'autres promoteurs mineurs peuvent aussi guider l'expression du *CYP19A1* placentaire, au niveau des exons PII, I.3, I.2, I.4, I.8 et 1.2a (Bulun *et al.*, 2003; Demura *et al.*, 2008).

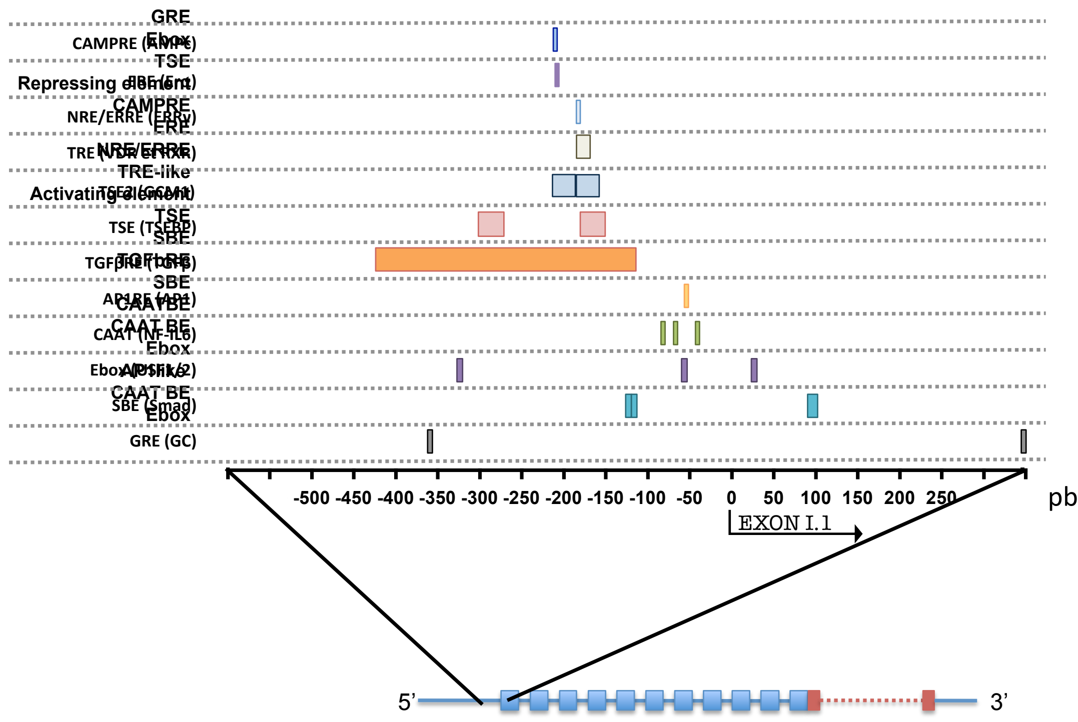


**Figure 3.3 : Éléments de régulation de l'exon I.1 du gène *CYP19A1***

**Le principal exon I placentaire est le I.1, situé le plus en amont du gène. Plusieurs éléments de réponses à des facteurs de transcription ont été rapportés dans la littérature. CAMPRE : Élément de réponse à l'adénosine monophosphate cyclique (AMPc); ERE : Élément de réponse aux récepteurs d'estrogènes (ER); NRE/ERRE : Élément de réponse aux récepteurs nucléaires/ Élément de réponse aux récepteurs reliés aux estrogènes (ERR); TRE : Élément de réponse aux hormones thyroïdiennes; TSE2 : Élément de réponse spécifique aux trophoblastes-2; TSE : Élément de réponse spécifique aux trophoblastes; TSEBP : Protéine de couplage au TSE; TGFβRE : Élément de réponse au facteur de croissance de transformation β (TGF β); AP1RE : Élément de réponse à la protéine d'activation 1; NF-IL6 : Facteur nucléaire pour l'expression d'interleukine 6; USF1/2 : Facteur de stimulation en amont 1/2; Ebox : Boîte d'amplification; SBE : Élément de couplage à Smad; GRE : Élément de réponse aux glucocorticoïdes; GC : Glucocorticoïdes. Créée à partir de (Bulun *et al.*, 2003; Kumar & Mendelson, 2011; Means *et al.*, 1989; Mendelson *et al.*, 2005; Simpson *et al.*, 2002; Sun *et al.*, 1998; Yamada *et al.*, 1999; Zhou *et al.*, 2009a).**

## 3.2 Régulation post-transcriptionnelle

La régulation transcriptionnelle de *CYP19A1* a été étudiée de manière détaillée, notamment en ce qui a trait à sa régulation tissu-spécifique. Certains auteurs ont trouvé des modes de régulation post-transcriptionnels de *CYP19A1*. Wang *et al.* (2009) ont observé que l'exon I.4 contient des éléments agissants en cis, responsables de l'altération de la stabilité de l'ARNm et de la traduction de la protéine, résultant ainsi en une diminution du taux d'ARNm de *CYP19A1* (Wang *et al.*, 2009). *CYP19A1* est également régulée par des miARN dans les cellules de granulosa et dans les CTv (Kumar *et al.*, 2013; Xu *et al.*, 2011).

## 3.3 Régulation post-traductionnelle

Des conclusions sont souvent émises concernant la synthèse d'estrogènes basées seulement sur le taux d'ARNm de *CYP19A1* (Bulun *et al.*, 2003). Par contre, le taux d'ARNm n'est pas toujours le reflet de l'activité de l'enzyme (Hudon Thibeault *et al.*, 2018). La glycosylation peut affecter le CYP19 en augmentant son activité d'environ 40% (Sethumadhavan *et al.*, 1991). De plus, des études se sont intéressées à la phosphorylation du CYP19. Il est connu que les différents CYP peuvent être phosphorylés (Redlich *et al.*, 2008). Par exemple, le CYP17 qui agit également au niveau de la stéroïdogenèse, est régulé par la phosphorylation par des kinases dépendantes de l'AMPc qui augmente l'activité 17,20-lyase de l'enzyme (Zhang *et al.*, 1995). Deux principaux effets de la phosphorylation du CYP19 ont été identifiés, ceux-ci n'étant pas mutuellement exclusifs.

### 3.3.1 Régulation de l'activité catalytique par la phosphorylation du CYP19

La phosphorylation du CYP19 peut en augmenter l'activité, comme dans la lignée cellulaire de cancer du sein MCF7 transfectée avec le *CYP19* humain (*hCYP19)* (Catalano *et al.*, 2009). Plus précisément, dans ce modèle, la phosphorylation de la tyrosine 361 est catalysée par la tyrosine kinase c-SRC suite au couplage des estrogènes avec son récepteur ERα (Catalano *et al.*, 2009). Cette phosphorylation est réversible, notamment par l'action de la tyrosine phosphatase PTP1B (Barone *et al.*, 2012). D'autres études ont montré que la phosphorylation du CYP19 est associée à une diminution de son activité (Charlier *et al.*, 2015; Remage-Healey *et al.*, 2011). Cette phosphorylation (sérine/thréonine/tyrosine) est rapide et réversible et elle a été observée chez la caille japonaise (cerveau, ovaires et follicules ovariens) et dans les cellules HEK293 transfectées avec *hCYP19* (Charlier *et al.*, 2011). Différents inhibiteurs de kinases ont été testés en combinaison avec des conditions phosphorylantes (ATP/Mg/Ca) et suggèrent l'implication de sérine et thréonine kinases comme la PKC et la PKA ainsi que la calmoduline dans les effets observés (Balthazart *et al.*, 2003; Charlier *et al.*, 2015). L'identification d'acides aminés spécifiquement phosphorylés par mutagenèse dirigée suggère que l'effet d'inhibition de l'activité par la phosphorylation requiert une combinaison complexe de différents sites de phosphorylation (Charlier *et al.*, 2015; Charlier *et al.*, 2011). L'effet de ces phosphorylations n'a pas été caractérisé, mais elles pourraient affecter l'affinité du CYP19 pour son substrat ou pour ses cofacteurs. De plus, le CYP19 possède une activité hydroxylase des estrogènes et il est possible que le statut de phosphorylation affecte l'équilibre entre l'activité hydroxylase et d'aromatisation (Osawa *et al.*, 1993).

### 3.3.2 Régulation de la stabilité de la protéine CYP19 par la phosphorylation

La phosphorylation d'une protéine peut stimuler sa dégradation par ubiquitination (Lecker *et al.*, 2006). Dans un système cellulaire de microsome combiné avec la fraction cytosolique de cellules JEG3, l'ajout d'ATP/Mg/Ca induit la phosphorylation (Sérine/Thréonine/Tyrosine), mais contrairement aux études de Balthazar *et al.*, cette phosphorylation mène à une dégradation protéique du CYP19 (Hayashi & Harada, 2014). Cette phosphorylation impliquerait des processus régulés par la protéine kinase dépendante de $Ca^{2+/}$calmoduline II (CaMKII) et une déphosphorylation par la calcineurine (Hayashi & Harada, 2014). De plus, la phosphorylation de la sérine 118 est nécessaire pour assurer l'activité basale du CYP19 et une mutation empêchant la phosphorylation de cette sérine diminue la quantité de CYP19, suggérant un effet sur la stabilité de la protéine (Miller *et al.*, 2008).

## 3.4 Régulation de la cinétique catalytique de l'aromatase

L'activité du CYP19 est également dépendante de l'apport en substrat. En effet, il existe un effet de saturation où, lorsque le substrat est présent en quantité importance, la vitesse de réaction atteint un maximum (Vmax, pour vitesse maximale) (**Figure 3.4**).

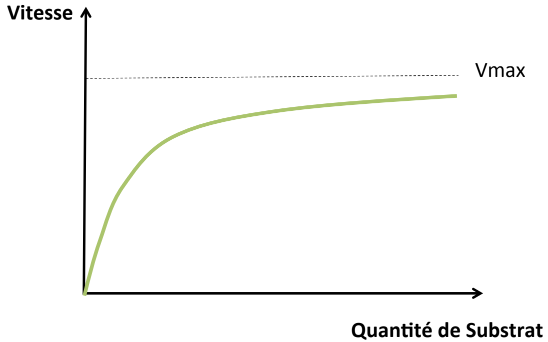


**Figure 3.4 : Influence de la quantité de substrat sur la vitesse de réaction d'une enzyme**

**La vitesse de réaction d'une enzyme est fonction de la quantité de substrat disponible. Plus la quantité de substrat augmente et plus la vitesse tend vers une vitesse de réaction maximale (Vmax). Créée à partir de (Voet & Voet, 2016).**

La vitesse de réaction diminue lorsque la quantité de produits augmente, afin d'atteindre un équilibre. Ainsi, il est important de considérer le type d'approche expérimentale utilisée pour évaluer l'activité du CYP19. S'il s'agit d'un modèle complexe *in vitro* ou *in vivo* où la production d'hormones est dosée, il est possible que l'apport en substrat soit altéré et affecte ainsi la production finale d'estrogènes.

Comme toute enzyme, le CYP19 peut être soumis à une inhibition (compétitive ou non compétitive) par une substance exogène. Celle-ci peut prendre différentes formes telles qu'illustrées à la **figure 3.5**.

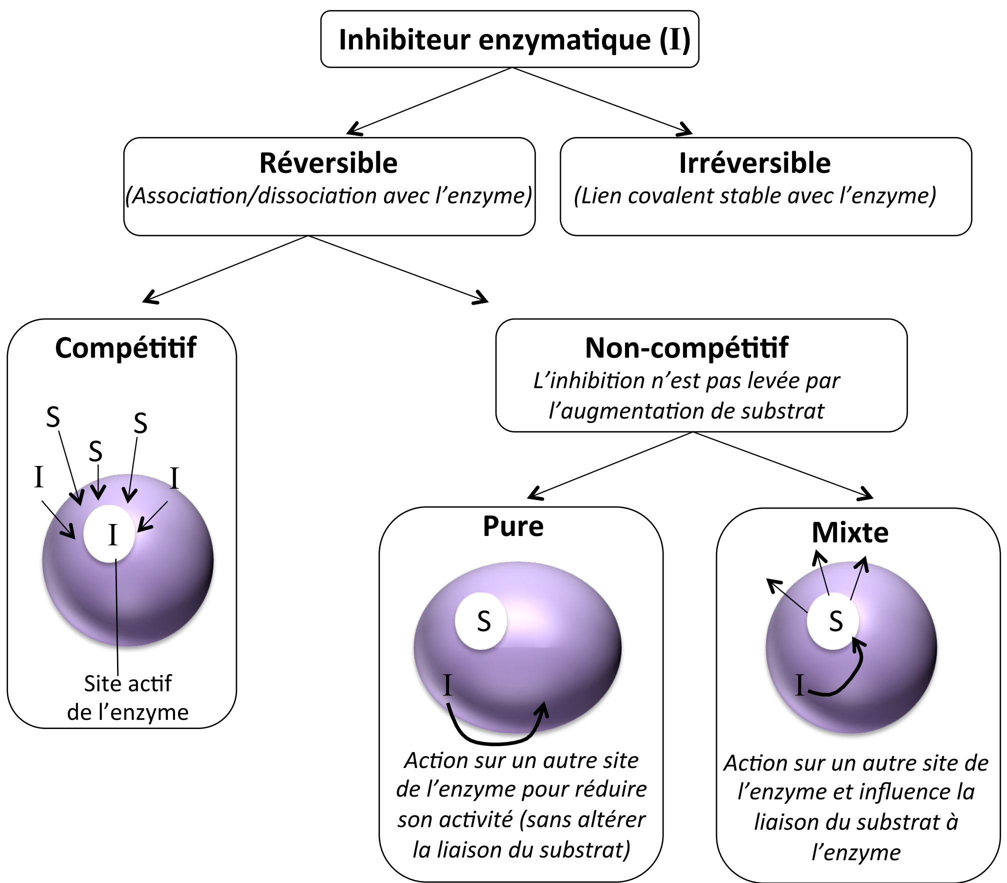


**Figure 3.5 : Catégories d'inhibiteurs enzymatiques**

**Les inhibiteurs enzymatiques (I) peuvent agir de manière réversible ou irréversible. Dans le cas d'un inhibiteur réversible, il peut être compétitif avec le substrat (S) (dans le site actif de l'enzyme) ou non-compétitif, c'est-à-dire qu'il agit ailleurs que sur le site actif du substrat. L'inhibition non-compétitive peut être pure et affecter l'activité de l'enzyme sans agir sur la liaison du substrat ou mixte, avec une action sur la liaison du substrat à l'enzyme. Créée à partir de (Voet & Voet, 2016).**

Les inhibiteurs de CYP19 sont utilisés dans le traitement des cancers du sein hormono-dépendants (Petkov *et al.*, 2009; Stephen Paul *et al.*, 2018). Il en existe deux catégories. Les premiers, inhibiteurs stéroïdiens, sont similaires aux substrats naturels, l'androstènedione ou la testostérone, et interagissent de manière irréversible avec la poche de couplage au substrat du CYP19 (ex. : examestane). Les seconds, inhibiteurs non-stéroïdiens, se lient au CYP19 par des

interactions non covalentes résultant en un processus de compétition réversible (ex. : anatrozole et letrozole) (Kang *et al.*, 2018). Ils agissent sur le cofacteur hème et interfèrent ainsi avec l'hydroxylation du stéroïde (Petkov *et al.*, 2009). Par ailleurs, certains métabolites du tamoxifène, utilisé également dans le traitement de cancer du sein hormono-dépendant, mais pour son action modulatrice des récepteurs d'estrogènes (SERM pour *selective estrogen receptor modulator*), inhibent CYP19 par un mécanisme allostérique. Il s'agit d'une inhibition non compétitive (Sgrignani *et al.*, 2014).

# 4 SÉROTONINE

## 4.1 Synthèse, dégradation et mode d'action de la sérotonine

La sérotonine (5-HT) est présente de manière ubiquitaire et est impliquée dans plusieurs processus physiologiques dont la régulation de l'appétit, les fonctions gastro-intestinales, l'agrégation plaquettaire, la vasoconstriction, la fonction cardiaque, la régulation de la glycémie et la régulation du rythme circadien (Brenner *et al.*, 2007; Coates *et al.*, 2004; Côté *et al.*, 2003; Ellwood & Curtis, 1997; Greenwood-van Meerveld, 2007; Kuemmerle *et al.*, 1995; Morin *et al.*, 1990; Rosen, 2009; Villalon & Centurion, 2007; Yamada *et al.*, 1997; Yamada *et al.*, 1995). Des altérations du système sérotoninergique sont d'ailleurs associées à diverses pathologies telles que la migraine, le syndrome du côlon irritable, les maladies cardiovasculaires, l'autisme, la dépression, ainsi qu'à des pathologies typiques de la grossesse, le diabète gestationnel mellitus et la pré-éclampsie (Anderson *et al.*, 1990; Bolte *et al.*, 2001a; Bolte *et al.*, 2001b; Breum *et al.*, 1995; Côté *et al.*, 2003; Gomez *et al.*, 2001; Humphrey *et al.*, 1990; Kaumann & Levy, 2006; Levinson, 2006; Mawe *et al.*, 2006; Mohammad-Zadeh *et al.*, 2008; Ni & Watts, 2006; Owens & Nemeroff, 1994; Sharp & Cowen, 2011; Sjoerdsma & Palfreyman, 1990; Spiller, 2007).

### 4.1.1 Synthèse et dégradation de la sérotonine

La 5-HT est produite à partir de l'acide aminé L-tryptophane (Odile, 2012). Cet acide aminé doit provenir de l'alimentation et ne peut être synthétisé *de novo* (Parker & Brotchie, 2011). La synthèse de la 5-HT requiert l'action de deux enzymes : 1) la tryptophane hydroxylase (TPH) permet l'hydroxylation du L-tryptophane en 5-hydroxytryptophane (5-HTP) qui traverse les membranes (notamment la barrière hématoencéphalique) par diffusion passive, 2) le 5-HTP est ensuite décarboxylé en 5-hydroxytryptamine (5-HT) par la L-aromatique amino-acide décarboxylase exprimée de manière ubiquitaire dans l'organisme (Boadle-Biber, 1993; Odile, 2012). Ces étapes sont illustrées à la **figure 4.1**.

La TPH catalyse l'étape limitante de la synthèse de 5-HT (Boadle-Biber, 1993; Fernstrom *et al.*, 2013; Tyce, 1990). La concentration de L-tryptophane dans les tissus est généralement inférieure à la constante de Michaelis-Menten ($K_m$) de la TPH, ce qui signifie que l'apport en L-tryptophane sera déterminant pour la production de 5-HTP et ultimement pour la production de 5-HT (Tyce, 1990). D'ailleurs, l'apport alimentaire en acides aminés neutres qui entrent en compétition avec le L-tryptophane est déterminant pour la production de 5-HT (Boadle-Biber, 1993; Fernstrom *et al.*, 2013; Tyce, 1990).

Il existe deux isoformes de TPH, la TPH-1 et la TPH-2 qui possèdent environ 70 % d'homologie de séquence en acides aminés entre eux (Odile, 2012; Sakowski *et al.*, 2006). Ils produisent une molécule de 5-HT identique (Sakowski *et al.*, 2006). La TPH-1, qualifiée de périphérique, est exprimée dans le cerveau fœtal et dans les tissus autres que les neurones sérotoninergiques (Nordlind *et al.*, 2008). La TPH-2, qualifiée de neuronale, est principalement exprimée dans les neurones sérotoninergiques des noyaux raphé (tronc cérébral), mais aussi dans le placenta (Laurent *et al.*, 2017).

La 5-HT circulante est principalement localisée dans les plaquettes sanguines (plus de 90 %), mais elle est également retrouvée dans le plasma (1-2 %) et dans les cellules mononucléées (5-10 %) (Odile, 2012). Dans les cellules, elle est entreposée dans des vésicules (Tamir & Gershon, 1990; Weihe & Eiden, 2000). Le transporteur vésiculaire des monoamines 2 (VMAT2) permet l'entrée de la 5-HT dans les vésicules (Odile, 2012). La 5-HT est ensuite libérée par exocytose ou par efflux par le biais du transporteur de la sérotonine (SERT) (Odile, 2012). La 5-HT ne peut ni traverser la barrière hématoencéphalique ni diffuser à travers le placenta (Jonnakuty & Gragnoli, 2008; Nordlind *et al.*, 2008; Prasad *et al.*, 1996). Afin de traverser les membranes lipidiques, elle doit utiliser des SERT (Blakely *et al.*, 1994; Gershon, 2003; Hardebo & Owman, 1980; Young, 2007). Les SERT sont présents notamment dans les neurones, les plaquettes sanguines, les vaisseaux sanguins, le coeur, les glandes surrénales, le tractus gastro-intestinal et les trophoblastes (Balkovetz *et al.*, 1989; Bottalico *et al.*, 2004; Cool *et al.*, 1990b; Gershon, 2004; Kanner & Zomot, 2008; Ni & Watts, 2006; Prasad *et al.*, 1996; Viau *et al.*, 2009). Ils co-transportent la 5-HT avec un ion sodium ($Na^+$) et un ion chlore ($Cl^-$) vers le compartiment intracellulaire alors qu'un ion potassium ($K^+$) est transporté dans le sens inverse (Gether *et al.*, 2006; Kanner & Zomot, 2008).

La 5-HT intracellulaire est dégradée par désamination par l'enzyme monoamine-oxydase (MAO), suivi de l'action de l'aldéhyde deshydrogénase afin de produire l'acide 5-hydroxyindole acétique (5-HIAA) (Odile, 2012) tel qu'illustré à la **figure 4.1**. Il existe deux isoformes de l'enzyme MAO qui diffèrent par leur activité et leur affinité pour les différents substrats; la MAO-A ayant une meilleure affinité et une activité plus grande pour la 5-HT, la norépinéphrine et l'épinéphrine alors que la MAO-B préfère le neurotransmetteur phényléthylamine (Billett, 2004). Les deux isoformes sont fréquemment co-localisées dans les différents tissus, dont le placenta (Auda *et al.*, 1998; Billett, 2004; Shih *et al.*, 2011).

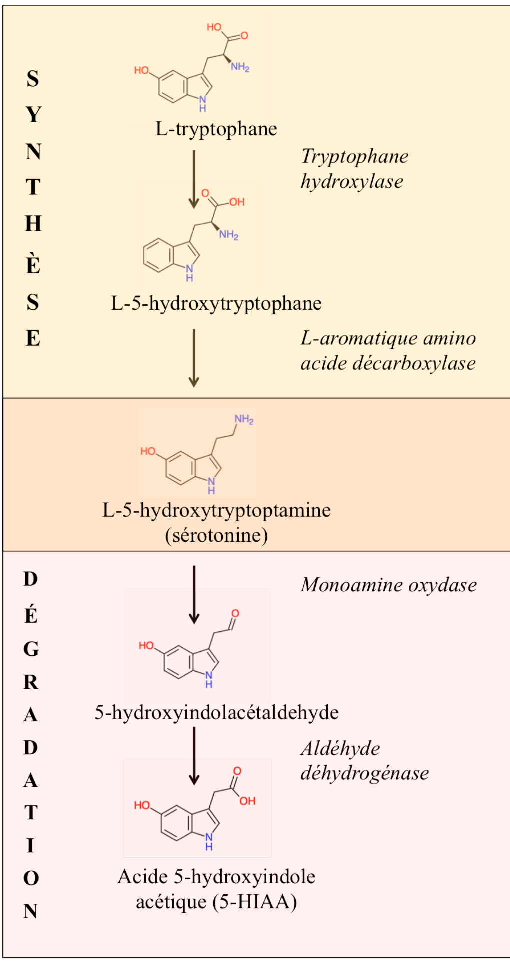


**Figure 4.1 : Étapes de la synthèse et de la dégradation de la sérotonine**

**La sérotonine (L-5-hydroxytryptamine) est produite à partir du précurseur L-tryptophane, par les actions successives de la tryptophane hydroxylase et de la L-aromatique amino acide décarboxylase. La sérotonine est dégradée principalement par l'action de la monoamine oxydase suivie de celle de l'aldhéhyde deshydrogénase afin de produire de l'acide 5-hydroxyindole acétique (5-HIAA). Les images des molécules ont été produites avec *Chemdoodle 2D sketcher.***

### 4.1.2 Récepteurs de la sérotonine

Pour exercer son action, la 5-HT se couple à ses récepteurs sérotoninergiques (5-HTR). Ceux-ci font partie de la famille des RCPG et possèdent 7 domaines transmembranaires, à l'exception du récepteur 5-HT3 qui déclenche une ouverture rapide des canaux cationiques non sélectifs (influx de $Na^+$ et de $Ca^{2+}$ et efflux de $K^+$) (Odile, 2012). Les 5-HTR ont été classés en fonction de leurs caractéristiques opérationnelles (interaction avec différentes molécules), structurelles (séquence du gène et de la protéine) et transductionnelles (effets suite au couplage de la 5-HT) (Hoyer *et al.*, 1994). Il existe 7 grandes catégories de 5-HTR qui sont décrites au **tableau 4.1**.

**Tableau 4.1 : Récepteurs de la sérotonine, modes d'action et localisation**

| Récepteur | Catégorie | Modes d'action principaux | Localisation dans le corps | Localisation synaptique |
|---|---|---|---|---|
| **5-$HT_1$ Sous-types : 5-$HT_{1A}$ 5-$HT_{1B}$ 5-$HT_{1D}$ 5-$HT_{1E}$ 5-$HT_{1F}$** | RCPG ($G_{i/o}$ ) | Inhibe AC (↘AMPc) Autres modes d'action (activation des canaux $K^+$, stimulation de ERK, inhibition de la conductance du $Ca^{2+}$) | Neurones (surtout centraux) et placenta | Pré et Post |
| **5-$HT_2$ Sous-types : 5-$HT_{2A}$ 5-$HT_{2B}$ 5-$HT_{2C}$** | RCPG ($G_{q/11}$ ) et RCPG ($G_{12/13}$ ) | Active PLC, Active la PKC, Stimule ERK Autres modes d'action (activation de PLA2, changements de structures cellulaires) | SNC, muscle vasculaire lisse, plaquettes sanguines, poumons, tractus gastro-intestinal, placenta | Post |
| **5-$HT_3$** | Canal ionique | Ouverture rapide des canaux ioniques non sélectifs (influx de $Na^+$ et $Ca^+$ et efflux de $K^+$) | Neurones centraux et périphériques, tractus gastro-intestinal | Post |
| **5-$HT_4$** | RCPG ($G_s$) | Stimule AC (↗AMPc) | SNC,tractus gastro-intestinal, coeur, vessie, placenta | Post |
| **5-$HT_5$** | RCPG | Inconnu | SNC | Post |
| **5-$HT_6$** | RCPG ($G_s$) | Stimule AC (↗AMPc) | SNC | Post |
| **5-$HT_7$** | RCPG ($G_s$) | Stimule AC (↗AMPc) | SNC, placenta | Post |

**RCPG : récepteurs couplés aux protéines G; AC : Adénylate cyclase; AMPc : adénosine monophosphate cyclique; ERK : kinase régulée par signal extracellulaire ou *extracellular signal-regulated kinase*; PLC : phospholipase C; PKC : protéine kinase C; PLA2 : phospholipase A2; SNC : système nerveux central. Créée à partir de (Barnes & Sharp, 1999; Bonnin & Levitt, 2011; Greenwood-van Meerveld, 2007; Hiroi *et al.*, 2001; Irge *et al.*, 2016; Odile, 2012; Raymond *et al.*, 2001).**

La localisation des différentes composantes du système sérotoninergique dans le système nerveux central est illustrée à la **figure 4.2**.

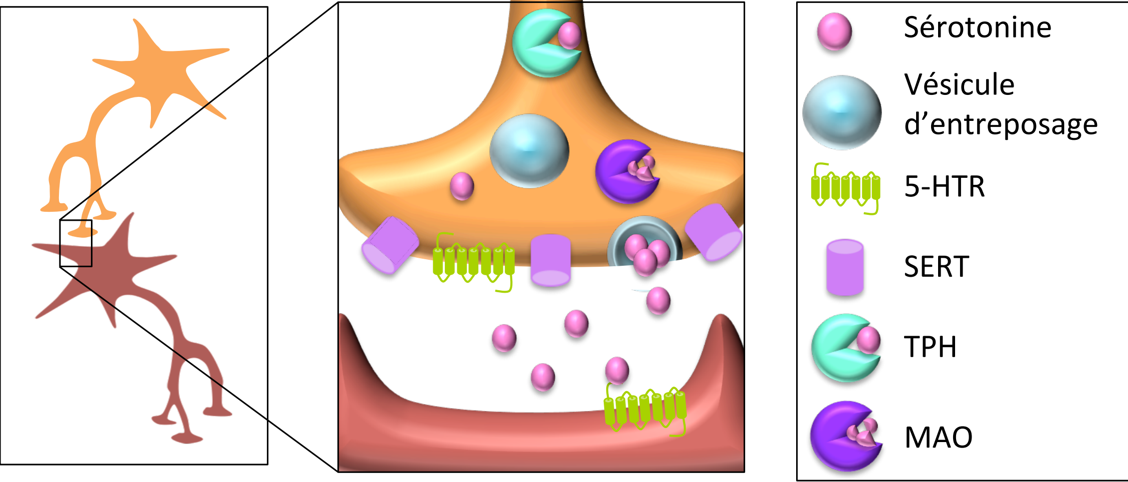


**Figure 4.2 : Composantes du système de la sérotonine dans les neurones**

**La sérotonine (5-HT) est produite par l'enzyme tryptophane hydroxylase (TPH). Elle peut être entreposée dans des vésicules ou libérée dans la fente synaptique. La sérotonine peut ensuite activer ses récepteurs (5-HTR) ou être retourner dans le neurone par les transporteurs de la sérotonine (SERT). L'enzyme monoamine oxydase (MAO) est ensuite responsable de sa dégradation.**

## 4.2 Système de la sérotonine dans le placenta

### 4.2.1 Sérotonine (5-HT)

La 5-HT a été détectée dans le placenta humain (CTv et ST, avec une immunoréactivité plus grande dans le ST) et sécrétée dans les milieux de culture des cellules BeWo et des primocultures de CTv (Bonnin *et al.*, 2011; Huang *et al.*, 1998; Laurent *et al.*, 2017). La 5-HT placentaire pourrait être entreposée dans des vésicules puisque le VMAT2 est exprimé dans les placentas murins et humains (Bottalico *et al.*, 2004).

### 4.2.2 Tryptophane hydroxylase (TPH)

Le système de transport du L-tryptophane est présent sur les membranes maternelle et fœtale du ST (Kudo & Boyd, 2001). De plus, l'expression protéique des TPH-1 et TPH-2 a également été démontrée dans les CTv, ST, CTev prolifératifs et endovasculaires, cellules déciduales ainsi que dans les cellules stromales mésenchymateuses (Laurent *et al.*, 2017).

### 4.2.3 Transporteur de la sérotonine (SERT)

Le SERT est exprimé sur le CTv, le ST et l'endothélium des capillaires fœtaux (Balkovetz *et al.*, 1989; Bottalico *et al.*, 2004; Cool *et al.*, 1990a; Ganapathy *et al.*, 1993; Prasad *et al.*, 1996; Viau, 2008; Viau *et al.*, 2009). Il a été suggéré que, dans le placenta, le SERT, en combinaison avec la MAO, pourrait jouer un rôle dans la protection contre l'effet vasoconstricteur de la 5-HT sur les vaisseaux sanguins utérins et/ou contre le transfert de la 5-HT maternelle vers le fœtus (Balkovetz *et al.*, 1989; Bonnin *et al.*, 2011; Bottalico *et al.*, 2004; Cool *et al.*, 1990a; Prasad *et al.*, 1996; Viau, 2008; Viau *et al.*, 2009). La 5-HT peut également être transportée par d'autres transporteurs qui joueraient ensemble différents rôles dans l'influx et l'efflux de 5-HT au niveau des différentes cellules du placenta (Kliman *et al.*, 2018). Ainsi, le SERT, surtout exprimé à la surface maternelle du ST et du CTv, permettrait de capter la 5-HT maternelle (Kliman *et al.*, 2018). Les autres formes de transporteurs par lesquels la 5-HT peut être transportée et qui sont exprimés dans la villosité placentaire sont illustrées à la **figure 4.3**.

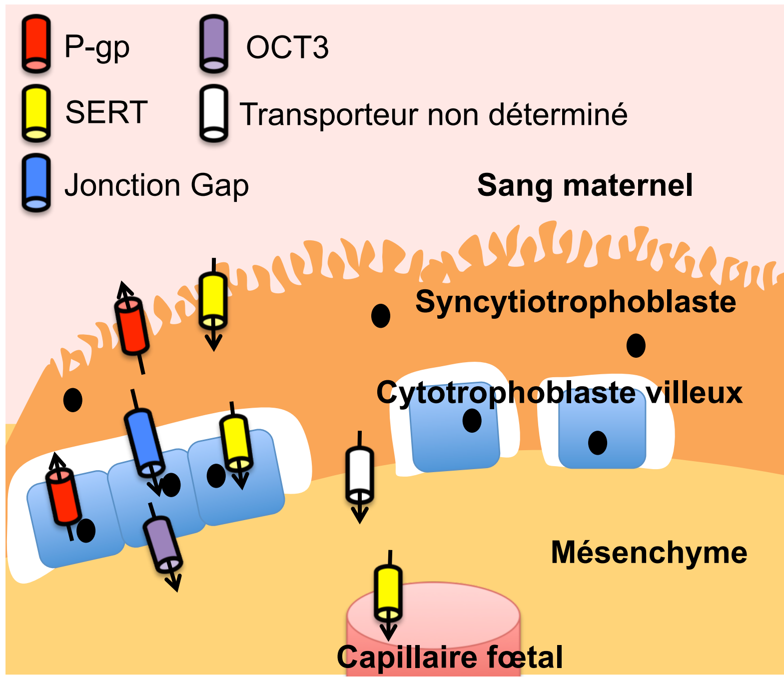


**Figure 4.3 : Transporteurs de la sérotonine dans le placenta humain**

**Selon Kliman *et al.* (2018), le transporteur de la sérotonine (SERT) permet l'entrée de sérotonine maternelle dans le syncytiotrophoblaste. La sérotonine peut alors retourner à la circulation sanguine maternelle par la glycoprotéine p1 (p-gp), être transférée aux cytotrophoblastes villeux par les jonctions gap ou être transférée à l'espace interépithélial par un transporteur non caractérisé. La sérotonine interépithéliale peut retourner au cytotrophoblaste villeux par le SERT, puis vers le mésenchyme par un transporteur aux cations organiques 3 (OCT-3) ou encore retourner à l'espace interépithélial par p-gp. La sérotonine peut traverser les capillaires fœtaux par les SERT. Créée à partir de (Kliman *et al.*, 2018).**

### 4.2.4 Monoamine oxydase (MAO)

Les deux isoformes de la MAO ont été détectées par hybridation *in situ* dans le placenta humain, la MAO-A étant principalement située dans le ST, alors que la MAO-B a été détectée dans le CTv, dans le ST et dans l'endothélium fœtal (Auda *et al.*, 1998). Par contre, au niveau protéique, seule la MAO-A a été détectée par immunohistochimie dans le CTv et le ST humain (Billett, 2004).

### 4.2.5 Récepteurs de la sérotonine (5-HTR)

À ce jour, seuls les récepteurs $5\text{-HT}_1$ et $5\text{-HT}_2$ ont été détectés dans le CTv, ST, endothélium du capillaire fœtal et cellules stromales des villosités choriales du placenta humain (Huang *et al.*, 1998; Klempan *et al.*, 2011; Oufkir *et al.*, 2010; Sonier *et al.*, 2005; Vaillancourt *et al.*, 1994b; Viau *et al.*, 2009). L'ARNm de $5\text{-HT}_7$ et $5\text{-HT}_4$ ont également été détectés dans le tissu total de placenta humain et dans la lignée cellulaire de choriocarcinome T3M-3 respectivement (Hiroi *et al.*, 2001; Irge *et al.*, 2016). De plus, il a été démontré que le $5\text{-HT}_{2A}\text{R}$ est exprimé et fonctionnel dans les lignées cellulaires de choriocarcinome placentaire humain BeWo et JEG-3 (Huang *et al.*, 1998; Sonier *et al.*, 2005; Vaillancourt *et al.*, 1994b; Viau *et al.*, 2009).

## 4.3 Système de la sérotonine dans le fœtus

Les sources de 5-HT fœtales incluent le transfert placentaire de la 5-HT maternelle et placentaire ainsi que la production par l'intestin du fœtus (Bonnin *et al.*, 2011; Bonnin & Levitt, 2011). Les cellules entérochromaffines fœtales qui sécrètent de la 5-HT sont identifiables à partir de 7 semaines de grossesse (Facer *et al.*, 1989; Lolova *et al.*, 1998). Cette observation suggère que le fœtus humain pourrait synthétiser de la 5-HT dès le premier trimestre de grossesse (Fecteau & Eiler, 2001). Par exemple, chez la souris, le profil d'expression des récepteurs 5-HT dans le cerveau change selon le stade de développement, ce qui suggère une régulation séquentielle du développement par différents récepteurs (Bonnin *et al.*, 2006).

## 4.4 Rôles de la sérotonine pendant la grossesse

La présence du système de la 5-HT dans plusieurs composantes de l'unité fœto-placentaire suggère une implication dans le déroulement de la grossesse et dans le développement fœtal. La souris a été utilisée afin de démontrer l'importance de la 5-HT au niveau du fœtus pour la régulation du développement craniofacial, cardiaque, du système nerveux et de la crête neurale (Buznikov *et al.*, 2001; Choi *et al.*, 1997; Côté *et al.*, 2007; Lauder *et al.*, 1988; Moiseiwitsch,

2000; Nebigil *et al.*, 2000; Nebigil *et al.*, 2001; Nguyen *et al.*, 2001; Whitaker-Azmitia *et al.*, 1995). Ainsi, la 5-HT régule la prolifération, la migration et la différenciation des cellules dans ces différents tissus (Buznikov *et al.*, 2001; Choi *et al.*, 1997). Bonnin *et al.* (2011) ont utilisé un modèle murin afin d'étudier les rôles de la 5-HT produite par le placenta. Leurs résultats montrent que le placenta est une source importante de 5-HT pour le prosencéphale du fœtus au cours de la neurogenèse corticale, la migration et le guidage axonal initial (Bonnin & Levitt, 2011). Il y a ensuite un déplacement vers une provenance de la 5-HT endogène (produite par le cerveau fœtal) (Bonnin & Levitt, 2011). Cette période de dépendance de la source placentaire de 5-HT correspond aux premier et deuxième trimestres de grossesse chez l'humain (Bonnin & Levitt, 2011). Leurs résultats soulèvent notamment des questions sur les effets des altérations d'une production placentaire de 5-HT sur le développement du cerveau fœtal.

Au niveau mécanistique, plusieurs effets de la 5-HT dans des lignées cellulaires de choriocarcinome humain sont régulés par la stimulation du 5-$HT_{2A}$R (Klempan *et al.*, 2011; Oufkir *et al.*, 2010; Oufkir & Vaillancourt, 2011; Sonier *et al.*, 2005). Plus particulièrement, le 5-$HT_{2A}$R est impliqué dans la prolifération des lignées cellulaires BeWo et JEG-3 (Oufkir *et al.*, 2010; Oufkir & Vaillancourt, 2011; Sonier *et al.*, 2005). Nous avons aussi démontré que la 5-HT, par son action sur 5-$HT_{2A}$R, dans les cellules BeWo, régule l'activité du CYP19 et le taux d'ARNm de *CYP19A1* (Klempan *et al.*, 2011). Cet effet de la 5-HT sur le CYP19 n'avait jamais été observé dans d'autres modèles cellulaires humains. Les principales voies de signalisations connues pour être activées suite à la stimulation du 5-$HT_{2A}$R dans les lignées cellulaires placentaires sont JAK 2-STAT3 (transducteur de signal et activateur de transcription 3) et PLCβ-PKCβ-ERK1/2 (phospholipase C β-protéine kinase C β-kinase régulée par signal extracellulaire 1/2) (Oufkir *et al.*, 2010; Oufkir & Vaillancourt, 2011). Contrairement à la stimulation des voies JAK/STAT par les récepteurs de cytokines, l'activation par les RCPG est beaucoup moins bien caractérisée. L'association entre 5-$HT_{2A}$R et JAK2 a été observée dans des cellules de trophoblastes et de myoblastes (Guillet-Deniau *et al.*, 1997; Oufkir & Vaillancourt, 2011). Il existe toutefois peu d'information sur les mécanismes moléculaires et cellulaires par lesquels JAK2 est activé par les RCPG. D'autres voies ont également été associées au 5-$HT_{2A}$R dans d'autres modèles d'étude (**Figure 4.4**).

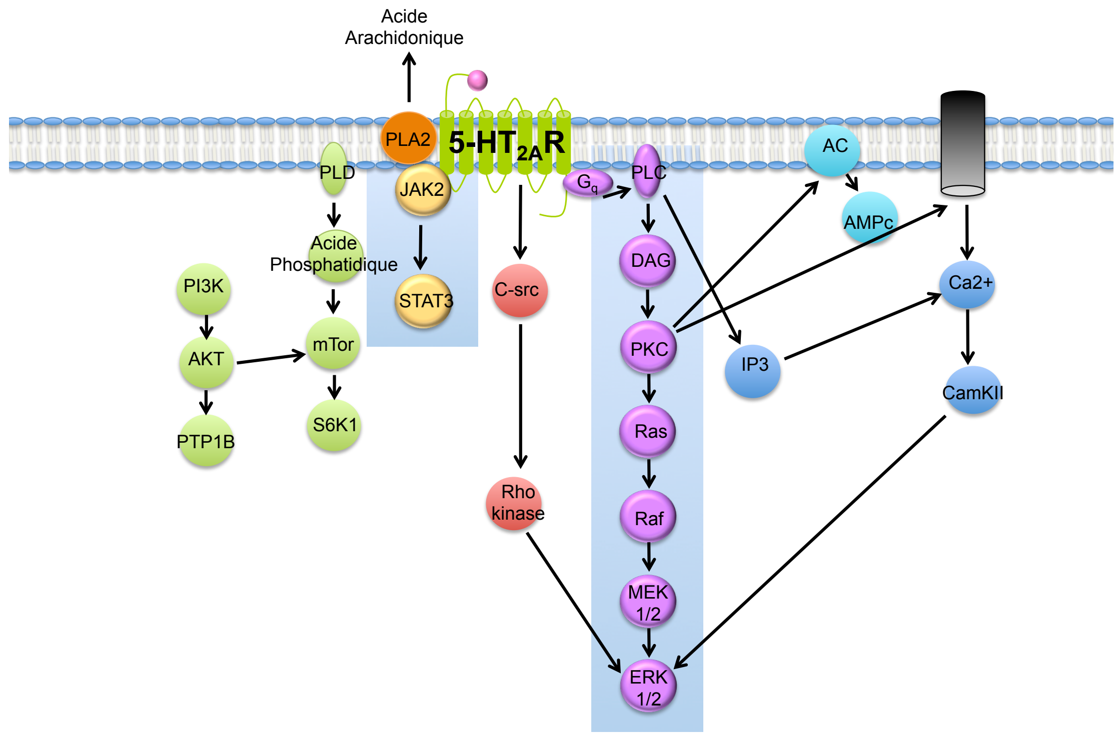


**Figure 4.4 : Voies de signalisation du récepteur 5-HT$_{2A}$ dans le trophoblaste villeux**

**Les voies de signalisations qui ont été démontrées dans le placenta sont mises en évidence par un rectangle bleu. 5-HT$_{2A}$ : récepteur de la sérotonine de type 2A; JAK2 : janus kinase 2; STAT3 : transducteur de signal et activateur de transcription 3; G$_q$ : protéine G$_q$; PLC : phospholipase C; DAG : diacylglycérol; PKC : protéine kinase C; Ras : proto-oncogène Ras; Raf : kinase fibrosarcome rapidement accéléré; MEK 1/2 : protéine kinase activée par les agents mitogènes 1/2; ERK 1/2 : kinase régulée par signal extracellulaire 1/2; c-scr : proto-oncogène tyrosine-protéine kinase Src; Rho kinase : kinase de la protéine GTPase Rho; AC : adénylate cyclase; AMPc : adénosine monophosphoate cyclique; IP3 : inositol triphosphate; Ca2+ : calcium; CmaKII : Protéine kinase Ca2+/calmoduline-dépendante; PLA2 : phospholipase A2; PLD : phospholipase D; mTor : cible mécanistique de la rapamycine; S6K1 : S6 kinase 1; PI3K : phosphoinositide 3-kinase; AKT : protéine kinase B et PTP1B : protein-tyrosine phosphatase 1B. Créée à partir de (Klempan et al., 2011; Masson et al., 2012; Oufkir & Vaillancourt, 2011).**

Oberlander *et al.* (2009) ont émis l'hypothèse que lorsque le système sérotoninergique est modifié par rapport à la normale pendant la grossesse (par exemple, suite à une exposition aux antidépresseurs inhibiteurs sélectifs de la recapture de la sérotonine -ISRS), la maturation du système 5-HT fœtal est affectée, ce qui prédisposerait à certaines psychopathologies (Oberlander *et al.*, 2009). L'étude des effets neurodéveloppementaux de la perturbation des niveaux de 5-HT est souvent biaisée par la difficulté d'éliminer la condition maternelle en soi (ex. : dépression) (Oberlander *et al.*, 2009). De plus, le stress maternel pendant la grossesse est associé à des perturbations du comportement chez l'enfant et avec certains problèmes qui impliquent le système sérotoninergique tel que l'hyperactivité avec déficit de l'attention, l'anxiété

et l’humeur dépressive (Oberlander *et al.*, 2009). Ainsi, des modifications du taux de 5-HT induites par la dépression et/ou par les antidépresseurs pourraient altérer le fonctionnement du placenta, le développement du fœtus et le déroulement de la grossesse et ces effets pourraient être régulés par une modulation du taux d’estrogènes. Bien que les deux systèmes de la 5-HT et des estrogènes co-existent dans le placenta (**Figure 4.5**), les mécanismes détaillés par lesquels la 5-HT régule le CYP19 et la biosynthèse des estrogènes placentaires n’ont jamais été étudiés.

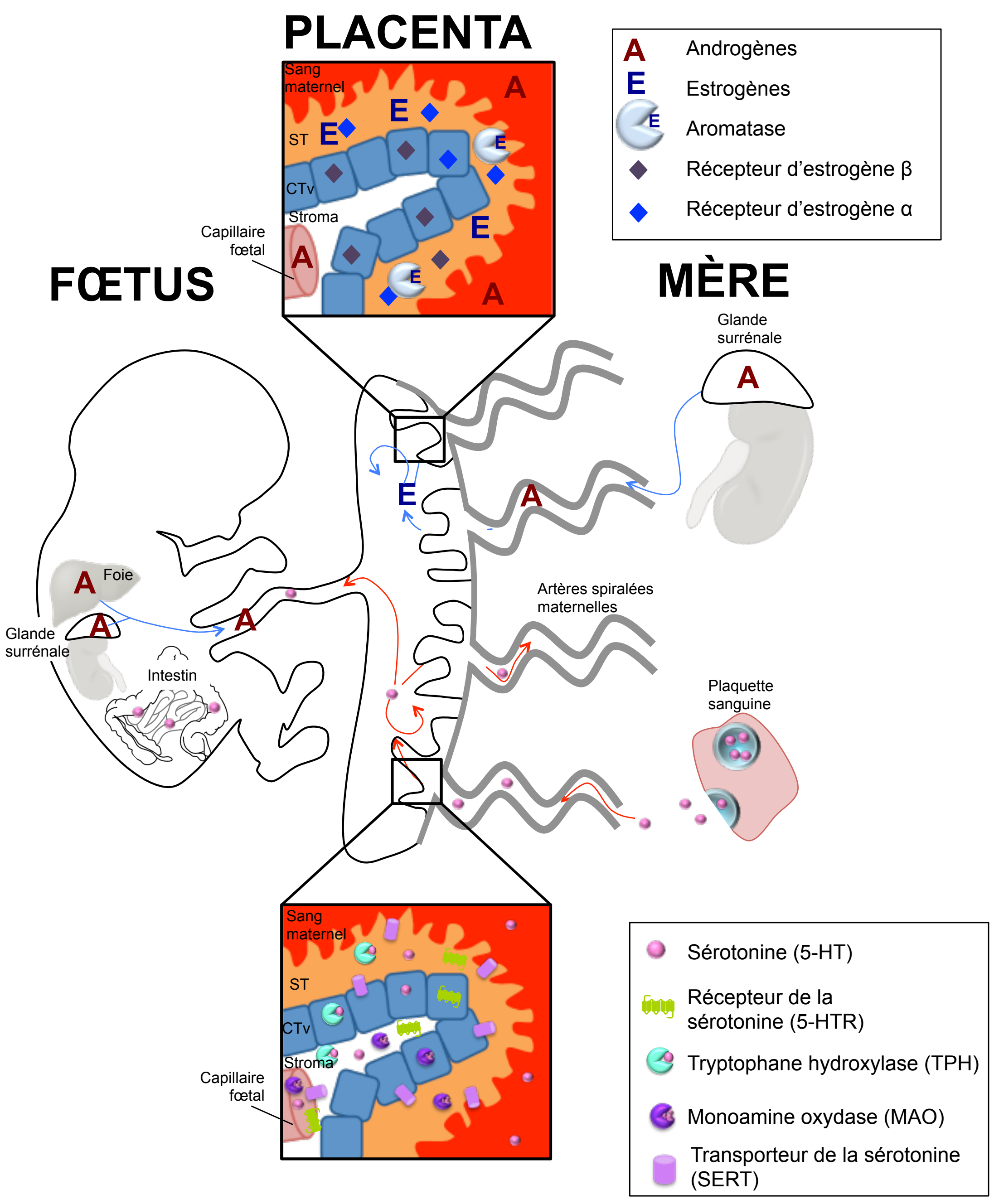


**Figure 4.5 : Systèmes de la sérotonine et des estrogènes dans l'unité materno-fœto-placentaire**

**Système des estrogènes (flèches bleues): Le placenta produit des estrogènes à partir d'androgènes en provenance des surrénales maternelles et fœtales et du foie fœtal. Les estrogènes peuvent exercer des effets sur le placenta, par leur action sur les récepteurs d'estrogènes ERα, ERβ, ainsi que GPER1 (non illustré). Système de la sérotonine (flèches rouges) : La sérotonine est synthétisée par le placenta et par les cellules entérochromaffines de l'intestin fœtal et elle peut également provenir du sang maternel dans lequel elle est entreposée dans les plaquettes sanguines. La 5-HT placentaire peut exercer des effets sur les cellules du placenta où les récepteurs sont exprimés (leur localisation du côté maternel ou fœtal n'est pas entièrement caractérisée). ST : syncytiotrophoblastes; CTv : cytrophoblaste villeux. Image des organes tirée de Pixabay.**

# 5 DÉPRESSION ET ANTIDÉPRESSEURS PENDANT LA GROSSESSE

## 5.1 Dépression pendant la grossesse

Au Canada, selon une enquête effectuée en 2006, la dépression affecte plus de 10% des individus au cours de leur vie (Patten *et al.*, 2006). Une étude publiée en 2018 indique que la prévalence de dépression majeure au cours de la vie a même atteint 20% aux États-Unis (Hasin *et al.*, 2018). Les femmes en âge de procréer sont deux fois plus à risque d'en souffrir que les hommes (Bethea *et al.*, 2002; Fishell, 2010; Holden, 2005; Marcus *et al.*, 2008). D'ailleurs, 10 à 25 % des femmes enceintes présentent des symptômes dépressifs (Bennett *et al.*, 2004; Bérard & Sheehy, 2014; Bonari *et al.*, 2004a; Marcus *et al.*, 2003).

### 5.1.1 Altération du système de la sérotonine et dépression

L'implication des neurotransmetteurs dopamine, norépinéphrine et 5-HT dans la dépression a été mise en évidence par l'efficacité des traitements qui agissent sur ces systèmes pour traiter la dépression (Blier, 2013; El Mansari *et al.*, 2010). De plus, l'hyperactivité de l'axe hypothalamo-hypophyso-surrénalien, similaire à la réponse au stress, est caractéristique de la pathogenèse de la dépression (Kalia, 2005; Pariante, 2003). La 5-HT est une cible à privilégier dans le traitement de la dépression, qui permet même d'agir indirectement sur d'autres systèmes monoaminergiques. La dépression est associée avec une modification du taux de 5-HT dans le SNC entrainant une diminution de la stimulation des récepteurs 5-HT neuronaux (Field *et al.*, 2004; Meltzer, 1990). En outre, la dépression est associée à des polymorphismes (SNP pour *single nucleotide polymorphism)* dans le gène du *TPH-2*, ce qui pourrait suggérer une altération de la synthèse de 5-HT au niveau du système nerveux central (Fasching *et al.*, 2012). Ainsi, les processus régulés par la 5-HT tels que le cycle veille-sommeil, la prise alimentaire et l'humeur sont perturbés chez les patients dépressif (Gorman, 2006; Mohammad-Zadeh *et al.*, 2008; Odile, 2012). Une modification des composantes du système 5-HT est également associée à la dépression. Par exemple, une augmentation de la densité de 5-HT$_2$R ainsi qu'une diminution du nombre de SERT au niveau des plaquettes sanguines a été décrit dans les cas de dépression majeure et particulièrement associée au suicide (Müller-Oerlinghausen *et al.*, 2004; Pandey *et al.*, 1995; Risch & Nemeroff, 1992; Serebruany *et al.*, 2003; Ziegelstein *et al.*, 2009). Par contre au niveau placentaire, la quantité d'ARNm de SERT est plutôt augmentée chez les femmes souffrant de troubles de l'humeur (dépression et anxiété) et cette augmentation est maintenue

malgré un traitement avec des ISRS (Ponder *et al.*, 2011). Chez les femmes enceintes qui souffrent de dépression, le taux d'ARNm de *MAOA* est diminué dans le placenta (Blakeley *et al.*, 2013).

### 5.1.2 Effets de la dépression pendant la grossesse

La dépression chez la femme enceinte est associée à des complications obstétricales telles que l'accouchement prématuré, la pré-éclampsie, la restriction de croissance intra-utérine, un faible poids à la naissance pour l'âge gestationnel, un décollement placentaire ainsi qu'à des facteurs de comorbidité chez la mère (comportements psychotiques, impulsifs et suicidaires) (Bonari *et al.*, 2004a; Bonari *et al.*, 2004b; Carrasco *et al.*, 1998; Carrasco *et al.*, 2000; de Paz *et al.*, 2011; Kurki *et al.*, 2000). De plus, la dépression a été associée avec une modification de la morphologie placentaire, plus précisément à une diminution de l'hétérogénéité de l'épaisseur de la barrière placentaire, ce qui suggère que cette pathologie affecte le développement du placenta (Lahti-Pulkkinen *et al.*, 2018).

## 5.2 Antidépresseurs pendant la grossesse

Bien que les thérapies comportementales aient montré leur efficacité dans le traitement de la dépression périnatale avec un taux de succès jusqu'à 80% (Lavender *et al.*, 2016; Van Lieshout *et al.*, 2017), certaines femmes doivent recourir à la médication. D'ailleurs, l'importance de la médication est mise en évidence par le fait que 67,7% des femmes qui ont cessé leur traitement antidépresseur pendant la grossesse ont fait une rechute de dépression (Austin, 2006).

Les principaux traitements de la dépression utilisés au Québec (2005-2009) sont, dans l'ordre, les ISRS, les antidépresseurs tricycliques (TCA), les inhibiteurs de la recapture de la 5-HT et de la norépinéphrine (IRSN), le trazodone (inhibiteur de la recapture de la sérotonine et agoniste de 5-$HT_{2C}$), la mirtazapine (antidépresseur tétracyclique) et le bupropion (inhibiteur de la recapture de la norépinéphrine et de la dopamine) (Tremblay *et al.*, 2011). L'utilisation d'inhibiteurs des monoamines-oxydases (IMAO) est inférieure à 1% (Tremblay *et al.*, 2011). Chez les femmes enceintes, comme dans la population générale, les ISRS demeurent les principaux antidépresseurs prescrits (Andrade *et al.*, 2008; Petersen *et al.*, 2018). Cette catégorie d'antidépresseurs est préférée aux TCA en raison de ses effets secondaires moins nombreux (Joshi, 2018). En effet, la sélectivité des ISRS pour la 5-HT limite les effets sur d'autres neurotransmetteurs contrairement aux TCA qui ont plusieurs effets secondaires dus à leur affinité pour différents récepteurs des neurotransmetteurs (récepteurs adrénergiques,

histaminiques, muscariniques) (Artigas, 2013). L'utilisation d'ISRS chez les femmes enceintes a d'ailleurs augmenté de plus de 400% entre 1996 et 2005, atteignant une utilisation de 6,2% en 2005 aux États-Unis (Andrade *et al.*, 2008). Les TCA sont toutefois préférés chez les femmes enceintes qui ont des saignements pendant le premier trimestre puisque les ISRS et IRSN ont un plus grand risque d'augmenter ces saignements (Fishell, 2010). Les principaux ISRS/IRSN prescrits, ainsi que leurs caractéristiques sont présentées au **tableau 5.1**.

**Tableau 5.1 : Utilisation d'antidépresseurs pendant la grossesse**

| ISRS/IRSN | % Utilisation population générale Québec 2005-2009 | % Utilisation grossesse Québec 1998-2009 | % Utilisation grossesse États-Unis 2008 | Passage placentaire (concentration cordon ombilical: sang maternel) | Catégorie de risque |
|---|---|---|---|---|---|
| **Fluoxétine (Prozac[MD])** | 0,1 | 1,0 | 2,1 | 0,72 | C |
| **Sertraline (Zoloft[MD])** | 0,5 | 2,0 | 1,8 | 0,33 | C |
| **Paroxetine (Paxil[MD])** | 1,1 | 6,1 | 1,2 | 0,15 | D |
| **Citalopram (Celexa[MD])** | 3,4 | 3,2 | 0,7 | 0,83 | C |
| **Escitalopram (Cipralex[MD])** | N.D. | N.D. | 0,2 | 0,73 | C |
| **Venlafaxine (Effexor[MD])** | 2,4 | 4,0 | N.D. | 0,72 | C |
| **Fluvoxamine (Luvox[MD])** | N.D. | 0,3 | 0 | 0,78 | C |

**Interprétation de la catégorie de risque selon l'Agence fédérale américaine des produits alimentaires et médicamenteux (FDA) : C : Le risque ne peut être exclu; D : Preuve positive de risque. N.D. : non déterminé. Les concentrations sanguines de ces ISRS retrouvées dans le sang maternel et de cordon sont présentées en annexe I. Le pourcentage d'utilisation dans la population générale au Québec, chez les femmes enceintes au Québec et aux États-Unis sont tirés respectivement de (Tremblay e*t al.*, 2011), (Bérard *et al.*, 2017) et (Andrade *et al.*, 2008). Les valeurs de passage placentaire sont tirées de (Rampono *et al.*, 2009). La catégorie de risque est tirée de (Fishell, 2010).**

Le centre Info-Médicaments en Allaitement et Grossesse (IMAGe) du centre hospitalier universitaire (CHU)-Ste Justine offre un service de renseignements sur l'innocuité des médicaments aux professionnels de la santé. Les pharmaciens et pharmaciennes qui travaillent au centre IMAGe présentent aux professionnels un résumé de l'ensemble des données sur les traitements antidépresseurs durant la grossesse et dirigent les professionnels vers quelques références reconnues pour leur permettre de bien comprendre les enjeux de la prescription durant la grossesse, comme les recommandations du rapport CANMAT (Canadian network for

mood and anxiety treatments) 2016 et les avis d'experts du Collège américain des obstétriciens et gynécologues (gynecologists, 2008; Kennedy et al., 2016) (Communication personnelle, Brigitte Martin, pharmacienne, MSc, responsable du centre IMAGe). Les pharmaciens et pharmaciennes du centre IMAGe présentent de manière générale les recommandations actuelles de considérer comme premier recours les ISRS, dont le citalopram, l'escitalopram et la sertraline, mais ils insistent également sur la nécessité d'individualiser la décision et les choix de traitement. Les antidépresseurs souvent cités en premier recours sont ceux pour lesquels le recul clinique est le plus important et pour lesquels les éléments de pharmacocinétique sont plus favorables pour minimiser l'exposition à l'enfant (Communication personnelle, Brigitte Martin, pharmacienne, MSc, responsable du centre IMAGe). De plus, malgré ces précautions, aux États-Unis et au Canada, la moitié des grossesses ne sont pas planifiées, ce qui pourrait être associé à des expositions à des médicaments non conseillés, du moins en début de grossesse (Corbett *et al.*, 2011; Fishell, 2010). Soulignons que bien que cette exposition soit plus marginale par rapport à l'exposition par prise de médicament, les femmes enceintes peuvent également être exposées aux antidépresseurs par la contamination environnementale (Kwon & Armbrust, 2006; Lajeunesse *et al.*, 2008; Silva *et al.*, 2012). Effectivement à Montréal, des antidépresseurs ont été trouvés dans les eaux traitées des usines de traitement des eaux (Lajeunesse *et al.*, 2008).

### 5.2.1 Caractéristiques pharmacocinétiques

La structure chimique des ISRS se caractérise par la présence d'au moins un groupe benzène et d'atomes de chlore ou de fluor. Le groupe halogène est d'ailleurs responsable de la spécificité des ISRS pour le SERT (ils se couplent à la poche de couplage aux halogènes du SERT) (Zhou *et al.*, 2009b). Le rapport entre le $K_i$ du transporteur de la norépinéphrine (NET) et le $K_i$ de SERT permet d'établir la sélectivité d'un composé pour la 5-HT. Le citalopram (ratio NET : SERT de 1500) se démarque des autres ISRS pour sa forte sélectivité pour le SERT (Hiemke & Hartter, 2000; van Harten, 1993). Certaines molécules ont également la capacité d'inhiber l'activité des transporteurs glycoprotéines p 1 (p-gp) impliqués dans la protection du fœtus contre les substances qui lui sont étrangères (xénobiotiques), mais également dans le transport de la 5-HT (Bhuiyan *et al.*, 2012; Weiss *et al.*, 2003) (**Figure 4.3**).

Les ISRS sont couplés aux protéines de transport lorsqu'ils sont dans la circulation sanguine (Ciraulo *et al.*, 2011; van Harten, 1993), ce qui implique qu'ils peuvent entrer en compétition avec d'autres molécules comme le cortisol qui se couple également à l'albumine (Rezaei-Tavirani *et al.*, 2012).

Les ISRS sont des molécules lipophiles et le métabolisme hépatique vise à les rendre plus solubles pour faciliter leur élimination urinaire, notamment par déméthylation, glucuroconjugaison ou sulfoconjugaison (Hiemke & Hartter, 2000; Silva *et al.*, 2012). Les ISRS affectent également l'activité des CYP hépatiques qui les métabolisent. Le CYP2D6 est la principale cible de la fluoxétine et de la paroxétine qui inhibent son activité (Ciraulo *et al.*, 2011; Hiemke & Hartter, 2000). Seuls les métabolites de la fluoxétine et de la venlafaxatine, la norfluoxétine et le desmethylvenlafaxine respectivement, conservent une activité inhibitrice du SERT et contribuent significativement à l'effet de l'antidépresseur (métabolites actifs). En plus d'inhiber la recapture de la 5-HT, les ISRS peuvent également augmenter sa synthèse (augmentation de l'activité et de l'expression de TPH) (Baik *et al.*, 2005; Kim *et al.*, 2002) ou se coupler à des récepteurs (récepteur de la dopamine D1 et D2, $5HT_{1A}$, $5HT_{2A}$, récepteurs adrénergiques α1, α2 et β, récepteur histaminique H1, récepteur nicotinique de l'acétylcholine) (Cusack *et al.*, 1994; Hyttel, 1993; Messa *et al.*, 2003; Meyer *et al.*, 2001).

Dans le projet de recherche présenté dans cette thèse, nous avons étudié les principaux ISRS prescrits aux femmes enceintes (fluoxétine, sertraline, paroxétine et citalopram). Nous incluons dans cette catégorie la venlafaxine qui est un IRSN, mais dont l'affinité pour le SERT est beaucoup plus élevée par rapport au NET (Montgomery, 2008). Nous avons également évalué les effets du métabolite actif de la fluoxétine, la norfluoxétine. Bien que tous les ISRS aient un mode d'action similaire, ils se distinguent par certaines caractéristiques pharmacodynamiques, telles que présentées au **tableau 5.2**.

**Tableau 5.2 : Caractéristiques des ISRS étudiés dans les travaux présentés dans cette thèse**

| Antidépresseur (Principal métabolite) | Structure chimique | Activité du métabolite | Temps pour atteindre l'état d'équilibre | Demi-vie d'élimination (Métabolite) | Couplage aux protéines de transport (%) | Inhibition de la recapture de la sérotonine (Ki en nM) | Inhibition de la recapture de la noradrénaline (Ki en nM) | Inhibition de p-gp (IC50 en µM) | Inhibition de CYP |
|---|---|---|---|---|---|---|---|---|---|
| **Fluoxétine (Norfluoxétine)** | | Oui | > 4 semaines | 1-4 jours (7-15 jours) | 95 | 14-25 (25) | 143-500 (416) | 115.5 +/- 11.7 | CYP2D6 CYP2C6 CYP2C19 CYP2C9 CYP3A |
| **Sertraline (Desmethyl-sertraline)** | | Non/faible | 5-7 jours | 26 h (62-104 h) | 98 | 3,4-7,3 (76) | 220 (420) | 31,8+/-2,8 | CYP2C19 |
| **Paroxétine** | | Non | 7-14 jours | 15-20 h | 93-95 | 0,7-1,1 | 33-350 | 29,8+/-11,1 | CYP2D6 CYP2B6 |
| **Citalopram (Desmethyl-citalopram)** | | Non | 6-10 jours | 33-36 h | 50 | 2,6 (14) | 3900 (740) | Très élevé (impossible à déterminer) | CYP1A2 CYP2C19 CYP2D6 CYP3A4 |
| **Venlafaxine (Desmethyl-venlafaxine)** | | Oui | < 5 jours | 5 h (11 h) | faible | modéré | faible | Très élevé (impossible à déterminer) | Aucune inhibition importante |

**Ki : Constante d'inhibition; N.D. : Non déterminé et CYP : cytochrome P450. Créé à partir de (Canada, 2012; Ciraulo *et al.*, 2011; Ereshefsky & Dugan, 2000; GlaxoSmithKline Inc., 2012; Hiemke & Hartter, 2000; Lundbeck, 2012; van Harten, 1993). Les images des molécules ont été produites avec https://pubchem.ncbi.nlm.nih.gov/edit2/index.html.**

### 5.2.2 Passage transplacentaire

Des ISRS ont été détectés dans le compartiment fœtal dans un modèle de perfusion placentaire (Heikkinen *et al.*, 2002; Loughhead *et al.*, 2006). Le passage varie toutefois selon le type d'ISRS (Loughhead *et al.*, 2006). La concentration plasmatique maternelle en ISRS prédit faiblement la concentration dans le liquide amniotique (Heikkinen *et al.*, 2002; Loughhead *et al.*, 2006) ou le sang fœtal (Heikkinen *et al.*, 2002). Ces constats démontrent bien la complexité de l'évaluation du passage transplacentaire qui doit notamment intégrer les variations individuelles en enzymes métabolisant les ISRS (Heikkinen *et al.*, 2002). Certains polymorphismes affectent l'activité des enzymes du métabolisme des ISRS comme CYP2D6, CYP2C9 et CYP2C19 (Gong *et al.*, 2017; Koren & Ornoy, 2018). Ces variations interindividuelles contribuent à limiter la généralisation des données sur le passage transplacentaire des ISRS et de leurs métabolites. De plus, le métabolisme des ISRS varie pendant la grossesse avec un métabolisme augmenté en fin de grossesse (Koren & Ornoy, 2018).

Compte tenu des données limitées au niveau placentaire, les paramètres utilisés pour évaluer la capacité de différents ISRS à traverser la barrière hématoencéphalique sert de référence pour de mettre en évidence les différences entre les molécules. Ceux-ci sont résumés au **tableau 5.3** pour les ISRS étudiés dans le projet de recherche présenté dans cette thèse. Selon le système de classification biopharmaceutique de la distribution d'un médicament (BDDCS), seule la distribution du citalopram ferait intervenir des transporteurs de manière significative. Par contre, les résultats avec le test de perméabilité sur membrane artificielle en parallèle de la barrière hématoencéphalique (PAMPA-BBB), suggèrent que seule la sertraline ferait intervenir d'autres mécanismes que la perméabilité passive. Pour ce paramètre, des résultats contradictoires ont été trouvés pour la fluoxétine. De plus, le p-gp, en favorisant le retour des ISRS vers la circulation sanguine, limite l'exposition des cellules qui expriment p-gp aux ISRS. Dans des tests *in vitro* Weiss et al. (2003) ont trouvé que les ISRS/IRSN sont des inhibiteurs de p-gp, la sertraline et la paroxétine sont les plus puissants inhibiteurs, la fluoxétine et la norfluoxétine des inhibiteurs intermédiaires, alors que citalopram et venlafaxine de faibles inhibiteurs (Weiss *et al.*, 2003). Bien que plusieurs parallèles puissent être faits entre la barrière hématoencéphalique et la barrière placentaire, notamment au niveau de l'expression de p-gp, le transfert placentaire des ISRS/IRSN pourrait être différent (Ni & Mao, 2011). En effet, il a été démontré que la sertraline augmente l'efflux régulé par p-gp dans le placenta, alors qu'elle a l'effet opposé dans la barrière hématoencéphalique (Bhuiyan *et al.*, 2012).

**Tableau 5.3 : Capacité des antidépresseurs à traverser la barrière hématoencéphalique**

| Molécules étudiées | Classe BDDCS[a] | PAMPA-BBB[b] (cm/sec x $10^{-6}$) | Substrat pour le p-gp[c] | $K_{p,uu,brain}$[d] |
|---|---|---|---|---|
| **Fluoxétine** | 1 | 0,1/7,4[e] | Non | 1,36 |
| **Sertraline** | 1 | 2,8 | Oui | 1,60 |
| **Paroxétine** | 1 | 5,7 | Oui | 1,83 |
| **Citalopram** | 2 | 9 | Non | 1,29/2,30[e] |
| **Venlafaxine** | 1 | 8,2 | Non | 1,80 |

**[a] Système de classification biopharmaceutique de la distribution d'un médicament. Interprétation : Classe 1 : Effet minimal (non significatif) des transporteurs dans l'intestin, le foie et le cerveau et classe 2 : Les transporteurs peuvent affecter la pénétration de la molécule dans le cerveau et le foie.**

**[b] Test de perméabilité sur membrane artificielle parallèle-barrière hématoencéphalique. Interprétation > 4 cm/sec x 10-6 : perméabilité passive**

**[c] Déterminé à partir d'une lignée cellulaire qui surexprime p-gp (MDCK-MDR1)**

**[d] Ratio de concentration du composé non couplé dans le cerveau sur le plasma à l'état d'équilibre déterminé dans des souris avec délétion du gène MDR1 (p-gp). Interprétation : < 1 : restriction de la distribution dans le système nerveux central par efflux actif**

**[e] Les deux valeurs proviennent de deux études différentes**

**Créé à partir de (Zheng et al., 2016).**

### 5.2.3 Inhibiteurs sélectifs de la recapture de la sérotonine et grossesse

Les ISRS peuvent agir directement sur le placenta et/ou le fœtus ou indirectement, par la modulation des taux de 5-HT maternels (Banga *et al.*, 2011; Bhuiyan *et al.*, 2012). La prise d'ISRS pendant la grossesse a été associée à des perturbations telles que des naissances prématurées, des nouveau-nés avec faible poids à la naissance pour l'âge gestationnel et des mortalités fœtales (Rahimi *et al.*, 2006; Wen *et al.*, 2006). Soulignons d'ailleurs que la prise d'ISRS a été associée avec une augmentation du poids du placenta, ce qui suggère un effet sur son développement et fonctionnement (Frayne *et al.*, 2018). De plus, les ISRS affectent différents systèmes du fœtus et sont associés à des malformations cardiaques (Bérard *et al.*, 2007; Ellfolk & Malm, 2010; Gao *et al.*, 2018; Källén & Otterblad Olausson, 2007; Moses-Kolko *et al.*, 2005), hypertension pulmonaire (Chambers *et al.*, 2006) et syndrome sérotoninergique de sevrage aussi nommé "mauvaise adaptation néonatale" (tremblements, agitation, rigidité, irritabilité, pleurs continus, frissons, difficulté de sommeil et d'alimentation et convulsions chez le nouveau-né) (Ellfolk & Malm, 2010; Laine *et al.*, 2003; Moses-Kolko *et al.*, 2005; Nordeng *et al.*,

2001; Sanz *et al.*, 2005). Des effets à plus long terme, comme une perturbation du développement du système nerveux, sont également associés à une telle exposition *in utero* (Ansorge *et al.*, 2004; Oberlander *et al.*, 2009; Reznikov & Nosenko, 1996). De plus en plus d'études s'intéressent à la programmation fœtale de l'autisme et des troubles de déficit de l'attention avec hyperactivité (Brown *et al.*, 2017; Figueroa, 2010; Harrington *et al.*, 2013; Malm *et al.*, 2016; Montgomery *et al.*, 2018; Yang *et al.*, 2014). Soulignons toutefois que dans les études épidémiologiques, il est difficile de contrôler pour le facteur de confusion de la dépression. D'autres études montrent une absence d'association entre l'utilisation d'antidépresseurs et les observations décrites précédemment (Einarson *et al.*, 2011; Einarson & Einarson, 2005). Ces divergences peuvent s'expliquer par des différences dans l'intégration des paramètres tels que la dose, le type de traitement ainsi que le trimestre d'exposition (Oberlander *et al.*, 2008). L'étude des ISRS en tant que groupe de médicaments plutôt que séparément pourrait expliquer certains résultats controversés. En effet, en ce qui concerne la santé fœtale, seules la paroxétine et la fluoxétine ont été associées à des malformations cardiaques (Dubé, 2012; Ellfolk & Malm, 2010; Källén & Otterblad Olausson, 2007). Le moment d'exposition aux ISRS lors de la grossesse peut également influencer les effets observés. L'exposition pendant le troisième trimestre est associée à un plus grand nombre d'effets sur le développement du bébé comme la détresse respiratoire et le syndrome de sevrage néonatal du bébé (Laine *et al.*, 2003; Moses-Kolko *et al.*, 2005; Nordeng *et al.*, 2001; Oberlander *et al.*, 2008). Par contre, l'incidence de malformations cardiaques chez le nouveau-né a été associée à une exposition pendant le premier trimestre (Bérard *et al.*, 2007; Ellfolk & Malm, 2010). La dépression des femmes exposées tardivement en grossesse aux ISRS est toutefois généralement plus profonde, ce qui est un facteur de confusion (Oberlander *et al.*, 2008).

# 6 MODÈLES EXPÉRIMENTAUX D'ÉTUDE DE LA STÉROÏDOGENÈSE FŒTO-PLACENTAIRE HUMAINE

Les voies d'exposition aux perturbateurs endocriniens sont nombreuses pour les femmes enceintes. Notons entre autres la prise de médicaments, l'exposition due au mode de vie (drogues, cigarette...) et l'exposition occupationnelle ou environnementale (Myllynen *et al.*, 2005). Les études épidémiologiques ont identifié, chez la femme enceinte, des effets délétères de l'exposition à des perturbateurs endocriniens, dont les métaux lourds, les BPC, les dioxines et les pesticides (Lo *et al.*, 2003; Siddiqui *et al.*, 2003; Stasenko *et al.*, 2010; Weselak *et al.*, 2008; Woodruff *et al.*, 2010). Parmi les problèmes observés, notons la diminution du poids du bébé à la naissance, une augmentation du risque de naissance prématurée, des avortements spontanés, des malformations congénitales, une induction de l'hypertension chez la mère et une altération neurodéveloppementale associée à une diminution du quotient intellectuel (Lo *et al.*, 2003; Stasenko *et al.*, 2010; Weselak *et al.*, 2008; Woodruff *et al.*, 2010). D'ailleurs, plusieurs contaminants, comme les phtalates, présents dans les plastiques, traversent le placenta et sont retrouvés dans le liquide amniotique et dans le sang fœtal (Li *et al.*, 2018; Mose *et al.*, 2007). De plus, l'exposition aux perturbateurs endocriniens pendant la grossesse peut contribuer à une altération de la fonction placentaire pouvant mener à des mortinatalités et à des restrictions de croissance intra-utérines (Crain *et al.*, 2008; Roncati *et al.*, 2016; Yu *et al.*, 2018).

L'unité fœto-placentaire humaine est particulièrement complexe à étudier puisque les approches expérimentales sont majoritairement invasives et impossibles en raison de considérations éthiques. Les études antérieures ont surtout été axées sur l'analyse des concentrations d'hormones en circulation dans le sang maternel et fœtal (cordon ombilical), sur la production d'hormones surrénales placentaires et fœtales *in vitro* et sur l'examen de grossesses anormales (Albrecht & Pepe, 1999). Les modèles animaux complètent ces études en permettant l'analyse de l'effet de xénobiotiques sur l'ensemble d'un organisme. Par contre, les différences de structure placentaire limitent les extrapolations au niveau des trophoblastes humains (Malassiné *et al.*, 2003). En outre, lorsque nous nous intéressons à la stéroïdogenèse fœto-placentaire, il est important de considérer l'ensemble des interactions qui sont beaucoup plus complexes que le simple apport de précurseurs androgéniques par les surrénales fœtales et leur conversion dans le compartiment placentaire. Les principales interactions hormonales fœto-placentaires sont présentées au **tableau 6.1**.

**Tableau 6.1 : Interactions hormonales entre le fœtus et le placenta**

| Hormones ou facteurs placentaires | Effets sur le compartiment fœtal | Référence |
|---|---|---|
| **Hormone de croissance placentaire (pGH) ou Lactogène placentaire (hPL)** | Stimulation de l'axe du facteur de croissance semblable à l'insuline (IGF) dans le fœtus, impliqué dans la croissance des cellules surrénales fœtales et dans la régulation de l'hormone corticotrope (ACTH) | (Myatt & Sun, 2010) |
| **17β-estradiol et gonadotrophine chorionique (hCG)** | Stimulation de la production de DHEAS dans la lignée cellulaire H295R (lignée cellulaire de carcinome surrénalien avec des caractéristiques fœtales) | (Gell *et al.*, 1998; Rao *et al.*, 2004) |
| **17β-estradiol, progestérone et prostaglandine** | Régulation de l'expression de l'enzyme 11β-hydroxystéroide deshydrogénase de type 2 (11β-HSD2) qui joue un rôle dans la régulation de l'exposition fœtale aux glucocorticoïdes. Ce type d'interaction est particulièrement importante pour la maturation des poumons du fœtus et pour le développement de la zone fœtale des glandes surrénales | (Gell *et al.*, 1998; Kaludjerovic & Ward, 2012; Myatt & Sun, 2010; Rao *et al.*, 2004) |
| **Corticolibérine (CRH)** | Stimulation de la production de cortisol et de DHEA | (Sirianni *et al.*, 2005; Smith *et al.*, 1998) |
| **Facteur de croissance épidermique (EGF)** | Stimulation de la prolifération des surrénales fœtales | (Riopel *et al.*, 1989) |
| **Hormones fœtales (surrénaliennes)** | **Effets sur le compartiment placentaire** | **Référence** |
| **Aldostérone** | Stimulation de la prolifération des cellules JEG-3, lignée cellulaire de trophoblaste extravilleux | (Gennari-Moser *et al.*, 2011) |
| **Cortisol** | Inhibition de la prolifération des cellules JEG-3, lignée cellulaire de trophoblaste extravilleux | (Gennari-Moser *et al.*, 2011) |
| **Déhydroépiandrostérone (DHEA)** | Régulation de l'implantation de l'embryon murin | (Frolova *et al.*, 2011) |

**Adapté de (Thibeault *et al.*, 2018).**

## 6.1 Modèles *in vivo*

Plusieurs modèles animaux non mammifères sont utilisés pour étudier la perturbation endocrinienne : les invertébrés, les amphibiens, les poissons et les oiseaux principalement (Stokes, 2004). Les systèmes endocriniens de ces modèles possèdent certaines similarités avec celui des humains et ils sont principalement utilisés comme sentinelle pour les effets perturbateurs endocriniens des contaminants environnementaux (Iguchi *et al.*, 2001; Kloas & Lutz, 2006; Kloas *et al.*, 2009; Smith *et al.*, 2012; Stokes, 2004). Les modèles de mammifères, comme les rongeurs possèdent une fonction endocrinienne reproductive hautement conservée au niveau cellulaire et moléculaire (Gray *et al.*, 2004). Par contre, pendant la grossesse, la stéroïdogenèse chez l'humain est passablement différente de la production hormonale dans les modèles du rat et de la souris. En effet, dans ces deux modèles, la production des estrogènes pendant la gestation est principalement ovarienne (corps jaune). Bien que le CYP19 soit présent

dans le placenta murin, son importance dans la production des estrogènes est incertaine (Chow *et al.*, 2009; Gray *et al.*, 2004; Malassiné *et al.*, 2003; Soares *et al.*, 2012). Ces modèles animaux sont toutefois utiles grâce aux techniques d'inactivation de gènes qui permettent d'étudier des fonctions spécifiques de certains gènes, comme les ER, par exemple (Stokes, 2004). En outre, le modèle primate non humain est celui qui possède une production hormonale fœto-placentaire la plus proche de celle de l'humain, mais qui implique plusieurs contraintes relativement à son utilisation (Albrecht *et al.*, 2000; Albrecht *et al.*, 2006).

## 6.2 Modèles *in vitro* et *ex vivo*

Les modèles *in vitro* (lignées cellulaires ou primocultures) et *ex vivo* (explants) possèdent l'avantage de permettre notamment d'étudier l'activité enzymatique, le taux d'ARNm/protéines d'enzymes et le couplage des hormones aux récepteurs (Charles, 2004; Lephart & Simpson, 1991). Les tests *in vitro* seuls ne peuvent pas analyser l'absorption, la distribution, le métabolisme et l'excrétion et ne sont pas efficaces lorsque le composé testé doit être métabolisé pour exercer son effet (Gray *et al.*, 2004).

La lignée cellulaire BeWo issue de choriocarcinome est la principale lignée utilisée comme modèle de CTv, puisqu'elle est capable de différenciation (biochimique et morphologique) suite à une stimulation notamment avec de la forskoline (Taylor *et al.*, 1991). Avec ce type de lignée cellulaire, il est possible d'utiliser des techniques de caractérisation en temps réel du comportement cellulaire pour évaluer la cytotoxicité telle que la technologie xCELLigence (**annexes II et III**). Par contre, les primocultures isolées de placenta humain sont considérées comme étant un modèle plus physiologique (non pathologique). Les trophoblastes peuvent être isolés de placenta de différents trimestres, les placentas de premiers trimestres étant surtout utilisés pour obtenir des CTev, alors que les placentas de grossesse à terme, obtenu suite à un accouchement vaginal ou césarienne, permettent d'obtenir des CTv (James *et al.*, 2007; Jankovic-Karasoulos *et al.*, 2018; Sagrillo-Fagundes *et al.*, 2016). Les primocultures peuvent être maintenues en culture sur une période d'environ 96 h avec une différenciation spontanée pendant la période de culture. À environ 72 h de culture, les cellules sécrètent un maximum de hCG et sont principalement fusionnées en ST, qui ensuite dégénère progressivement par apoptose.

Enfin, soulignons que les microsomes placentaires humains sont également utilisés pour étudier l'activité du CYP19 (Canton *et al.*, 2008). Les effets cytotoxiques sont ainsi évités et la

procédure expérimentale est de plus courte durée qu'avec les lignées cellulaires (Canton *et al.*, 2008). Toutefois, les effets transcriptionnels ne peuvent être détectés (Canton *et al.*, 2008).

### 6.3 Modèles de co-culture

Pour tenir compte des interactions entre différents types de cellules dans des études *in vitro*, des modèles de co-culture ont été développés. Par exemple, il est possible d'étudier l'attachement et l'implantation du blastocyste sur une couche de cellules stromales ou d'étudier les interactions materno-fœtales avec une co-culture de cytotrophoblastes de premier trimestre (invasifs) et de cellules endothéliales déciduales (Cohen & Bischof, 2009). Ces modèles *in vitro* impliquent un contact direct entre les différentes cellules. Des inserts de culture sont également disponibles pour étudier des interactions cellulaires sans contact entre les deux compartiments (Cai *et al.*, 2010; Tyan *et al.*, 2011). Ce type de co-culture est approprié notamment dans les cas où des facteurs diffusent dans le milieu de culture pour atteindre le second type cellulaire.

Les modèles de co-culture sur insert ont notamment servi à l'étude de la relation paracrine entre différents types de cellules comme entre les cellules déciduales et les trophoblastes afin de produire des prostaglandines par exemple (Cai *et al.*, 2010; Nayeem *et al.*, 2015; Tyan *et al.*, 2011). Plus récemment, des systèmes microfluidiques multicouches ont été développés afin de mettre en co-culture des trophoblastes et des cellules endothéliales fœtales (Blundell *et al.*, 2016; Lee *et al.*, 2016). Ces systèmes permettent de reproduire l'architecture de la barrière placentaire (Blundell *et al.*, 2016; Lee *et al.*, 2016). D'autres auteurs utilisent plutôt une co-culture 3D composée d'un cœur de fibroblastes entouré d'une couche de trophoblaste (Nayeem *et al.*, 2015).

# 7 HYPOTHÈSES ET OBJECTIFS

## 7.1 Problématique

La dépression et son traitement, principalement par les ISRS, pendant la grossesse sont un réel enjeu sociétal. La dépression affecte jusqu'à 20% des femmes enceintes et est associée à des complications obstétricales (Bennett *et al.*, 2004; Bérard & Sheehy, 2014; Bonari *et al.*, 2004a; Marcus *et al.*, 2003). La diminution du taux de 5-HT est un acteur majeur dans l'étiologie de cette maladie et les ISRS sont les principales molécules prescrites, avec une utilisation estimée à 6,2% des femmes enceintes en 2005 (Andrade *et al.*, 2008). La prise d'ISRS durant la grossesse a été associée chez le fœtus à des malformations pulmonaires et cardiaques, au syndrome de sevrage néonatal et à des altérations du développement du système nerveux. Par contre, les mécanismes moléculaires pour expliquer certaines associations entre des perturbations du déroulement de la grossesse ou du développement fœtal avec la dépression et les antidépresseurs ne sont pas bien caractérisés. Il existe une vaste littérature sur les interactions entre les systèmes 5-HT et estrogène, principalement au niveau du système nerveux central. Ces deux systèmes sont présents dans le placenta humain (**Figure 4.5**) et nous avons d'ailleurs déjà démontré dans la lignée cellulaire trophoblastique de choriocarcinome placentaire BeWo, que la 5-HT, par l'activation du récepteur 5-$HT_{2A}$, augmente l'activité du CYP19 et le taux d'ARNm du *CYP19A1*, enzyme clé de la production d'estrogènes (Klempan *et al.*, 2011). Ces résultats n'ont toutefois jamais été validés dans un modèle d'étude du trophoblaste non cancéreux. De plus, l'effet des ISRS sur le système de la 5-HT et la production des estrogènes pendant la grossesse n'a jamais été étudié. Les estrogènes sont produits en grande quantité par le placenta pendant la grossesse et ils sont essentiels au bon fonctionnement placentaire ainsi qu'à l'adaptation de la physiologie maternelle et au développement fœtal. Il s'avère donc crucial de mieux comprendre l'interaction 5-HT-estrogène dans le placenta humain, puisque des modifications du taux de 5-HT induites par la dépression et/ou par les antidépresseurs pourraient altérer la production d'estrogènes placentaires. Ainsi il est essentiel de caractériser le rôle de la 5-HT et des ISRS sur la biosynthèse d'estrogènes par le placenta afin de bien évaluer le type et la dose de traitement à favoriser chez les femmes enceintes atteintes de dépression.

## 7.2 Hypothèses de recherche

Les hypothèses de recherche du présent projet doctoral sont que : (1) la 5-HT stimule la production des estrogènes par le CYP19 dans le placenta humain et (2) les ISRS altèrent la régulation du CYP19 ainsi que la synthèse des estrogènes par l'unité fœto-placentaire.

## 7.3 Objectifs

L'**objectif général** est de déterminer les mécanismes d'action par lesquels la 5-HT et l'activation de son récepteur 5-$HT_{2A}$ régulent la production des estrogènes par le CYP19 placentaire et de déterminer si les ISRS modifient cette régulation. Les **objectifs spécifiques** sont :

1) Développer une co-culture de lignées cellulaires de trophoblaste villeux et de surrénale fœtale pour évaluer la stéroïdogenèse fœto-placentaire.
2) Caractériser le taux d'ARNm de *CYP19A1* et l'activité du CYP19 pendant la syncytialisation des primocultures de trophoblastes villeux *in vitro*.
3) Déterminer si la 5-HT, par le biais de l'activation du récepteur 5-$HT_{2A}$, régule l'activité du CYP19 dans les cellules trophoblastiques et, le cas échéant, déterminer le mécanisme d'action.
4) Déterminer l'effet des ISRS sur l'activité du CYP19 et sur la production des estrogènes par la co-culture développée à l'objectif 2 et par les primocultures de trophoblastes villeux.

# DEUXIÈME PARTIE : RÉSULTATS

# 8 UN MODÈLE D'ÉTUDE DE LA STÉROÏDOGENÈSE FŒTO-PLACENTAIRE HUMAINE

Titre complet : A unique co-culture model for fundamental and applied studies of human fetoplacental steroidogenesis and interference by environmental chemicals

Titre en français : Un modèle de co-culture unique pour les études fondamentales et appliquées de la stéroïdogenèse fœto-placentaire et son interférence par des produits chimiques environnementaux

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**Contribution de l'étudiante :** L'étudiante a participé à l'élaboration de l'étude, complété toutes les expériences de caractérisation de la co-culture ainsi que sur la perturbation endocrinienne. Elle a également analysé les résultats, rédigé l'article, effectué les révisions des correcteurs.

**Contribution des coauteurs** : Kathy Deroy a participé aux expériences initiales de mise au point du milieu de co-culture. Cathy Vaillancourt et J. Thomas Sanderson ont participé à l'élaboration de l'étude incluant la planification des expériences, l'analyse des résultats et la correction du manuscrit.

## 8.1 Résumé de l'article en français

**Mise en contexte :** Les outils expérimentaux pour étudier les interactions stéroïdogéniques complexes qui surviennent entre le placenta et le fœtus pendant la grossesse sont extrêmement limités.

**Objectifs :** Développer un modèle de co-culture pour étudier la stéroïdogenèse de l'unité fœto-placentaire humaine et sa perturbation par des expositions à des contaminants environnementaux.

**Méthodologie :** Les cellules de choriocarcinome humain BeWo, représentant les cytotrophoblastes villeux, et les cellules de carcinome corticosurrénalien humain H295R, représentant l'unité fœtale, ont été cultivées dans un milieu de co-culture adapté. Les cellules H295R ont été cultivées dans des plaques de 24 puits et les cellules BeWo sur des inserts *transwell* avec ou sans traitement à des pesticides (atrazine ou prochloraz). L'activité du CYP19 et la production hormonale après 24 h de co-culture a été évaluée par dosage du relâchement de l'eau tritiée et dosage immunoenzymatique respectivement.

**Résultats :** La co-culture a démontré le profile stéroïdogénique de l'unité fœto-placentaire, permettant une production synergique d'estradiol et d'estriol (mais pas d'estrone) de 133,3 ± 11,3 pg/mL et 440,8 ± 44,0 pg/mL, respectivement. L'atrazine et le prochloraz ont des effets sur l'activité du CYP19 et la production d'estrogènes dans la co-culture qui sont spécifiques au type cellulaire. L'atrazine a induit l'activité du CYP19 et la production d'estrogènes dans les cellules H295R seulement, mais n'a pas affecté la production globale d'estrogènes dans la co-culture, alors que le prochloraz a inhibé l'activité du CYP19 exclusivement dans les cellules BeWo et réduit la production d'estrogènes dans la co-culture d'environ 90%. En revanche, le prochloraz n'a pas affecté la production d'estradiol ou d'estrone dans les cellules BeWo en monoculture. Ces effets différentiels soulignent la pertinence de notre approche de co-culture afin de modéliser la stéroïdogenèse fœto-placentaire.

**Conclusions :** La co-culture de cellules H295R et BeWo crée un modèle *in vitro* unique pour reproduire la coopération stéroïdogénique entre le fœtus et le placenta pendant la grossesse et peut être utilisée pour étudier les effets perturbateurs endocriniens des produits chimiques environnementaux.

## 8.2 Article

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# A Unique Co-culture Model for Fundamental and Applied Studies of Human Fetoplacental Steroidogenesis and Interference by Environmental Chemicals

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**Background:** Experimental tools for studying the complex steroidogenic interactions that occur between placenta and fetus during human pregnancy are extremely limited.

**Objectives:** We aimed to develop a co-culture model to study steroidogenesis by the human fetoplacental unit and its disruption by exposure to environmental contaminants.

**Methods:** We cultured BeWo human choriocarcinoma cells, representing the villous cytotrophoblast, and H295R human adrenocortical carcinoma cells, representing the fetal unit, in a carefully adapted co-culture medium. We placed H295R cells in 24-well plates and BeWo cells on transwell inserts with or without pesticide treatment (atrazine or prochloraz) and assessed CYP19 activity and hormonal production after 24 hr of co-culture.

**Results:** The co-culture exhibited the steroidogenic profile of the fetoplacental unit, allowing a synergistic production of estradiol and estriol (but not of estrone) of 133.3 ± 11.3 pg/mL and 440.8 ± 44.0 pg/mL, respectively. Atrazine and prochloraz had cell-type specific effects on CYP19 activity and estrogen production in co-culture. Atrazine induced CYP19 activity and estrogen production in H295R cells only, but did not affect overall estrogen production in co-culture, whereas prochloraz inhibited CYP19 activity exclusively in BeWo cells and reduced estrogen production in co-culture by almost 90%. In contrast, prochloraz did not affect estradiol or estrone production in BeWo cells in monoculture. These differential effects underline the relevance of our co-culture approach to model fetoplacental steroidogenesis.

**Conclusions:** The co-culture of H295R and BeWo cells creates a unique *in vitro* model to reproduce the steroidogenic cooperation between fetus and placenta during pregnancy and can be used to study the endocrine-disrupting effects of environmental chemicals.

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## Introduction

Appropriate fetoplacental communication is required for healthy pregnancy, and sex steroid hormones play an essential role in maintenance of pregnancy and fetal development. Pregnant women are exposed to various potential endocrine-disrupting chemicals through diet, medication use, occupational or environmental activities, and other lifestyle factors (Myllynen et al. 2005). Risks posed by chemical exposures are a focus of the Inter-Organization Programme for the Sound Management of Chemicals as stated in its 2012 report (World Health Organization/United Nations Environment Programme 2013). Most of these chemicals will pass through the placental barrier and enter the placenta and fetus, as evident from the presence of contaminants in placental tissues, amniotic fluid, and/or fetal blood (Foster et al. 2000; Ikezuki et al. 2002; Leino et al. 2013). Epidemiological studies have associated altered pregnancy and fetal outcomes with exposure to contaminants such as heavy metals, polychlorinated biphenyls, dioxins, and pesticides (Siddiqui et al. 2003; Stasenko et al. 2010; Weselak et al. 2008). Adverse effects include reduced birth weight, altered fetal cognitive and reproductive tract development, and increased risk of premature birth and spontaneous abortion. Some of these adverse effects may be a consequence of chemical-induced alterations in estrogen production by the syncytiotrophoblast, the functional endocrine unit of the placenta (Albrecht and Pepe 1999; Siddiqui et al. 2003; Stasenko et al. 2010; Weselak et al. 2008). Several processes regulated by estrogens, such as uteroplacental blood flow, trophoblast invasion, and syncytialization are necessary for healthy pregnancy (Albrecht and Pepe 1999; Cronier et al. 1999; Yashwanth et al. 2006). Disruptions of these functions are associated with serious obstetric complications, including altered fetal development, preterm birth, preeclampsia, and intrauterine growth restriction (Albrecht et al. 2005; Kaufmann et al. 2003). The importance of regulation of local estrogen levels during pregnancy was notably underlined by toxicological studies of the well-known estrogenic compound diethylstilbestrol (DES). Exposure *in utero* to DES resulted in severe malformations and malfunctioning of male and female reproductive organs (Norgil Damgaard et al. 2002; Toppari et al. 2010).

Crucially, the human placenta is not in itself capable of producing androgens *de novo* because it lacks significant steroid 17α-hydroxylase/17,20-lyase activity catalyzed by the cytochrome P450 enzyme CYP17 (Braunstein 2003). Therefore, estrogen production by the trophoblast relies on sufficient quantities of fetal and maternal androgen precursors (Rainey et al. 2004), which act as substrates for placental aromatase (CYP19). Among estrogens, estriol, which is uniquely produced by the fetoplacental unit, predominates during pregnancy and is used as a diagnostic marker of fetal well-being (Mucci et al. 2003). Thus, a finely tuned cooperation between placenta and fetus is essential for a healthy pregnancy.

Unfortunately, experimental tools for studying the complex steroidogenic interactions that occur during human pregnancy are extremely limited. Invasive experimental approaches using humans are not possible for obvious ethical reasons. Although *in vivo* rodent models may be useful for specific gene inactivation studies (Stokes 2004), human steroidogenesis during pregnancy differs vastly, making rodent models irrelevant for human studies. In contrast to human pregnancy, the rodent placenta does not synthesize estrogens because it does not express CYP19 or display aromatase activity (Malassine et al. 2003). *In vitro* models have been used to assess hormonal secretion from placenta or fetal cells, but they can provide only partial information because they do not take into consideration the steroidogenic interactions

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between placenta and fetus. To study those interactions, we developed an *in vitro* co-culture model using H295R human adrenocortical carcinoma (fetal compartment) and BeWo human choriocarcinoma (villous trophoblast compartment) cells. H295R cells possess all the enzymatic capacities of the undifferentiated or fetal-like adrenal gland (Gazdar et al. 1990; Montanaro et al. 2005; Sanderson 2009; Staels et al. 1993) and they produce 16α-hydroxylated androgens (Gazdar et al. 1990), suggesting they can provide the uniquely fetal precursors for the human pregnancy estrogen, estriol. BeWo cells have a high degree of similarity to the villous trophoblast and can, for example, be induced to fuse and form syncytiotrophoblasts that behave like the human syncytium (Nampoothiri et al. 2007). Also, as in syncytiotrophoblasts, basal CYP19 activity in BeWo cells is relatively high. We evaluated the co-culture of H295R and BeWo cells as a model of steroidogenesis and, specifically, of estrogen production in the fetoplacental unit and its disruption by chemical exposures.

## Materials and Methods

***Cells and co-culture conditions.*** We cultured BeWo human placental choriocarcinoma cells [catalog no. CCL-98; ATCC, Manasses, VA, USA) in Dulbecco's modified Eagle's medium (DMEM)/F-12 without phenol red, supplemented with 0.6 g/L sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Oakville, Ontario, Canada) and 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ, USA). We cultured H295R human adrenocortical carcinoma cells (catalog no. CRL-2128; ATCC) in DMEM/F-12 without phenol red, supplemented with 1.2 g/L $NaHCO_3$ (Sigma-Aldrich), 2.5% NuSerum (BD Biosciences, Mississauga, Ontario, Canada), 2 mg/L pyridoxine·HCl (Sigma-Aldrich), and 1% ITS + Premix (BD Biosciences). Experiments were performed using cells between passages 7 and 25.

We cultured cells in 75-$cm^2$ filter-cap culture flasks (Techno Plastic Products, MIDSCI, St. Louis, MO, USA) in a humidified atmosphere containing 5% carbon dioxide ($CO_2$) at 37°C. At 90% confluence, cells were trypsinized [0.5% trypsin (Sigma-Aldrich)] and transferred to new 75-$cm^2$ flasks. We added suspensions of H295R cells ($2.5 \times 10^4$ cells/well) to the wells of one set of 24-well plates, and we added BeWo cells ($1.25 \times 10^4$ cells/insert) to transwell (Corning Life Sciences, Corning, NY, USA) clear polycarbonate membrane inserts with 0.4-μm pores of another set of 24-well plates, with each cell type in its respective regular growth medium. We removed the regular media 24 hr after seeding, assembled the co-culture (placing inserts with BeWo cells into the wells with H295R cells), and added co-culture medium (0.8 mL/well; 0.2 mL/insert). The co-culture medium was based on ATCC-recommended H295R medium but was supplemented with 1% stripped FBS. For full protocol, see Supplemental Material, Figure S1.

***Chemicals.*** Phorbol-12-myristate-13-acetate (PMA), forskolin, formestane, atrazine, and prochloraz were obtained from Sigma-Aldrich. PMA and forskolin are inducers of CYP19 via protein kinase C (PKC) and protein kinase A (PKA) pathways, respectively; formestane is an irreversible inhibitor of CYP19. We dissolved each compound in DMSO to make 1,000-fold concentrated stock solutions. We exposed the cells to various concentrations of each compound in culture medium with a final DMSO concentration of 0.1%. Inserts and wells always contained treated medium from the same solution.

***Cell proliferation.*** We monitored cell proliferation quantitatively and in real time in a humidified atmosphere with 5% $CO_2$ at 37°C using an xCELLigence™ RTCA DP instrument (ACEA Biosciences, San Diego, CA, USA). This instrument measures changes in impedance detected by gold electrode microarrays at the bottom of each well of a 16-well E-plate (ACEA Biosciences) to which the cells are attached. Before each experiment, we corrected cell impedance for background signals, which corresponded to the cell index measured after equilibrating the E-plate for 30 min with 100 μL appropriate culture medium. We added BeWo and H295R cells to 16-well E-plates in 100 μL at optimized densities of $1 \times 10^4$ and $2 \times 10^4$ cells/well, respectively. We normalized cell index after cell adherence, which took 3 hr for H295R and 6 hr for BeWo cells. In co-culture experiments, we seeded cells in co-culture medium in E-plates or in E-plate inserts with 0.4 μm pores (ACEA Biosciences) at the above-mentioned cell densities. We assembled the co-culture 24 hr later and refreshed the co-culture medium with the treatments (130 μL/well; 70 μL/insert) (for details, see Supplemental Material, Figure S1). Using ACEA Biosciences RTCA software, version 1.2.1 (http://www.aceabio.com/product_info.aspx?id=187), we collected cell impedance data every 10 min to calculate doubling times from the slope of the linear phase of the proliferation curves.

***CYP19 catalytic activity.*** We determined CYP19 catalytic activity by tritiated water-release assay according to the method of Lephart and Simpson (1991) adapted by our laboratory (Sanderson et al. 2000). Briefly, we cultured BeWo ($2.5 \times 10^4$ cells/well) or H295R ($5 \times 10^4$ cells/well) cells in 24-well plates in their regular media or in co-culture medium for 24 hr. Cells were then exposed to 54 nM 1β-$^3$H-androstenedione (PerkinElmer, Wellesley, MA, USA) in serum-free culture medium for 1.5 hr at 37°C. The conversion of substrate was linear over this time. For co-culture experiments, we assembled and treated the co-culture as described above (see Supplemental Material, Figure S1). Then, we separated the inserts from the wells and placed them directly in the bottoms of the wells of a 12-well plate. We measured CYP19 activity in the wells and inserts separately. We preserved the culture media (insert and well were pooled) at –80°C for subsequent analysis of hormone production.

***Hormone quantification.*** We determined hormone production by ELISA using assay kits from DRG Diagnostics (Marburg, Germany) and Abnova (Taipei City, Taiwan) (for details, see Supplemental Material, Table S1).

***Statistical analysis.*** We performed experiments at least three times using different cell passages; treatments were performed in triplicate per experiment. We determined statistically significant ($p < 0.05$) differences by two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test or one-way ANOVA followed by a Tukey post hoc test or Student's *t*-test, depending on the experimental design, using GraphPad Prism (version 5.04; GraphPad Software, San Diego, CA, USA).

## Results

***Characterization of each individual cell-type under co-culture conditions.*** The co-culture medium did not alter the proliferation rate of either cell type compared with those in their regular recommended media (see Supplemental Material, Figure S2A,B). However, we observed that after plating, regardless of the culture medium, BeWo cells required an adaptation period before proliferating, whereas H295R cells proliferated without delay (see Supplemental Material, Figure S2A,B). Doubling times determined from the linear sections (24–72 hr) of the proliferation curves were not significantly different whether H295R or BeWo cells were grown in co-culture medium (35.9 ± 2.3 hr and 25.2 ± 3.6 hr, respectively) or their respective regular media (30.9 ± 1.9 hr and 22.9 ± 2.7 hr, respectively). When placed in co-culture with BeWo cells, H295R cell proliferation over a period of 72 hr was reduced, although this effect was not observed until > 24 hr of co-culture (see Supplemental Material, Figure S2C). When cultured together for < 24 hr, H295R cell doubling time (39.3 ± 6.1 hr) in the presence of BeWo cells was not significantly different from that of cells in monoculture using co-culture medium (34.1 ± 5.3 hr). In contrast, BeWo cell proliferation was not affected by the presence of H295R cells in co-culture (see Supplemental Material, Figure S2D).

Under our co-culture conditions, basal CYP19 activity in BeWo cells (32.5 ± 7.0 fmol/hr) was about 15 times greater than that in H295R cells (2.2 ± 0.4 fmol/hr) and CYP19 activity was unaffected by the presence of the other cell type (data not shown). In each cell line, PMA and forskolin induced CYP19 activity, although induction was more pronounced in H295R than BeWo cells (see Supplemental Material, Figure S3). CYP19 activity and its inducibility were not different in either H295R or BeWo cells whether we cultured the cells in their respective regular media or in the co-culture medium (see Supplemental Material, Figure S3).

Basal production of β-human chorionic gonadotropin (β-hCG), a biochemical indicator of trophoblast health, was 7.7 ± 1.8, 40.5 ± 9.3, and 89.1 ± 14.1 mIU for BeWo cells after 24, 48, and 72 hr in regular medium; in the co-culture medium, it was 10.9 ± 3.2, 80.5 ± 17.8, and 88.5 ± 19.6 mIU (see Supplemental Material, Table S2). Forskolin, a known stimulant of the fusion and biochemical differentiation of BeWo cells, increased β-hCG production markedly. After 48 hr, basal and forskolin-induced β-hCG production were greater in co-culture medium than in regular medium (see Supplemental Material, Table S2), although this increase was not observed after 24 or 72 hr of culture. Basal and forskolin-stimulated β-hCG production by BeWo cells (over a 24-hr period) was not affected when co-cultured with H295R cells (see Supplemental Material, Table S2).

***Steroidogenesis in the H295R/BeWo co-culture model.*** BeWo cells, representing the placental compartment, mainly produced progesterone after 24 hr in co-culture (Figure 1A); whereas H295R cells, representing the fetal compartment, exclusively produced dehydroepiandrosterone (DHEA) and androstenedione (Figure 1B,C). Testosterone production was not detected (see Supplemental Material, Table S1). Basal estrogen production over a 24 hr period (Figure 1D–F) was relatively low in H295R and BeWo cells in monoculture, with estriol production being greater in BeWo cells than in H295R cells (173 ± 22 vs. 35 ± 35 pg/mL), whereas the opposite was seen for estrone (11.7 ± 3.2 vs. 34.7 ± 1.7 pg/mL). The production of estradiol (133 ± 11 pg/mL) and estriol (441 ± 44 pg/mL) increased synergistically when we placed the two cell types in co-culture (Figure 1D,E), whereas the increase in estrone production (55.1 ± 3.9 pg/mL) was additive. Estrogen production was not saturated because PMA- and forskolin-treated cells in co-culture produced estradiol levels of 422 ± 137 and 954 ± 264 pg/mL, respectively (data not shown).

***Effects of atrazine and prochloraz in the co-culture model.*** Treatment with atrazine (30 μM) did not alter the proliferation rate of either cell type in co-culture over a 72-hr period compared with vehicle control (Figure 2A, 2B). However, atrazine, after a 24-hr exposure, induced CYP19 activity to a statistically significant extent in H295R, but not in BeWo cells (Figure 2C). The 3-fold greater CYP19 activity that atrazine produced in the H295R compartment did not result in an increased production of estradiol, estriol, or estrone by the co-culture (112 ± 30, 459 ± 224, and 53.5 ± 7.4 pg/mL, respectively) (Figure 2D–F). Treatment with prochloraz (1 and 3 μM) did not alter the proliferation rate of either cell type in co-culture during the first 24 hr (Figure 3A,B). However, during this period, prochloraz decreased CYP19 activity concentration-dependently in BeWo, but not in H295R cells (Figure 3C). The 2.5- and 6.8-fold inhibition (to 39% and 15% of control, respectively) of CYP19 activity in BeWo cells by 1 and 3 μM prochloraz, respectively, translated into a > 90% inhibition of estradiol and estriol, and 80% inhibition of estrone production by the co-culture, with 1 μM prochloraz reducing estradiol, estriol, and estrone concentrations to 5.3 ± 4.3, 34.6 ± 9.3, and 15.0 ± 4.5 pg/mL, respectively; and 3 μM prochloraz, to 4.1 ± 2.8, 38.2 ± 11.8, and 13.9 ± 4.9 pg/mL, respectively. (Figure 3D–F). Neither atrazine nor prochloraz affected the production of β-hCG in co-culture after a 24-hr exposure (data not shown).

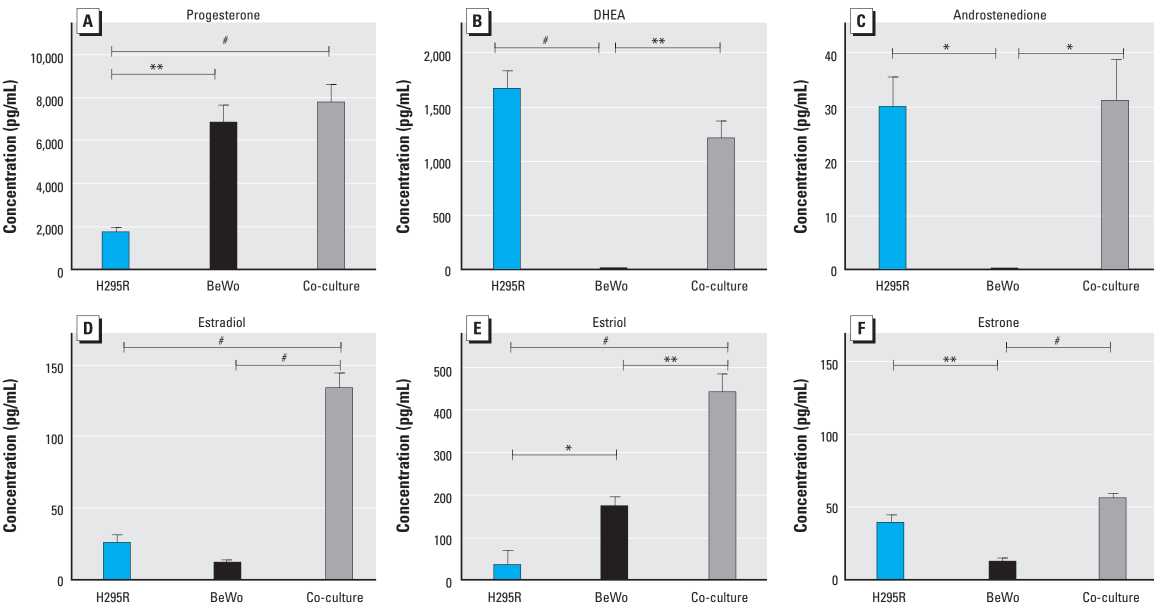


**Figure 1.** Progesterone (*A*), DHEA (*B*), androstenedione (*C*), estradiol (*D*), estriol (*E*), and estrone (*F*) production by H295R and BeWo cells in monoculture or in co-culture over a 24-hr period. Hormone concentrations (pg/mL) are presented as mean ± SE (progesterone, $n = 4$; DHEA, androstenedione, estriol, and estrone, $n = 3$; estradiol, $n = 5$).
$^{*}p < 0.05$. $^{**}p < 0.01$. $^{\#}p < 0.001$.

## Discussion

***Establishing the H295R/BeWo co-culture model.*** We have succeeded in developing a co-culture of H295R human adrenocortical carcinoma cells with characteristics of the fetal adrenal and BeWo human choriocarcinoma cells with characteristics of the villous trophoblast that exhibits the steroidogenic functionality of the human fetoplacental unit.

A major challenge in the development of our co-culture model was to maintain the unique characteristic of each individual cell line in the co-culture medium, which we adapted to accommodate the culture requirements of both cell lines. In the co-culture medium, the concentration of FBS, which is required for BeWo cells, was reduced to 1% and was charcoal-stripped to remove steroids

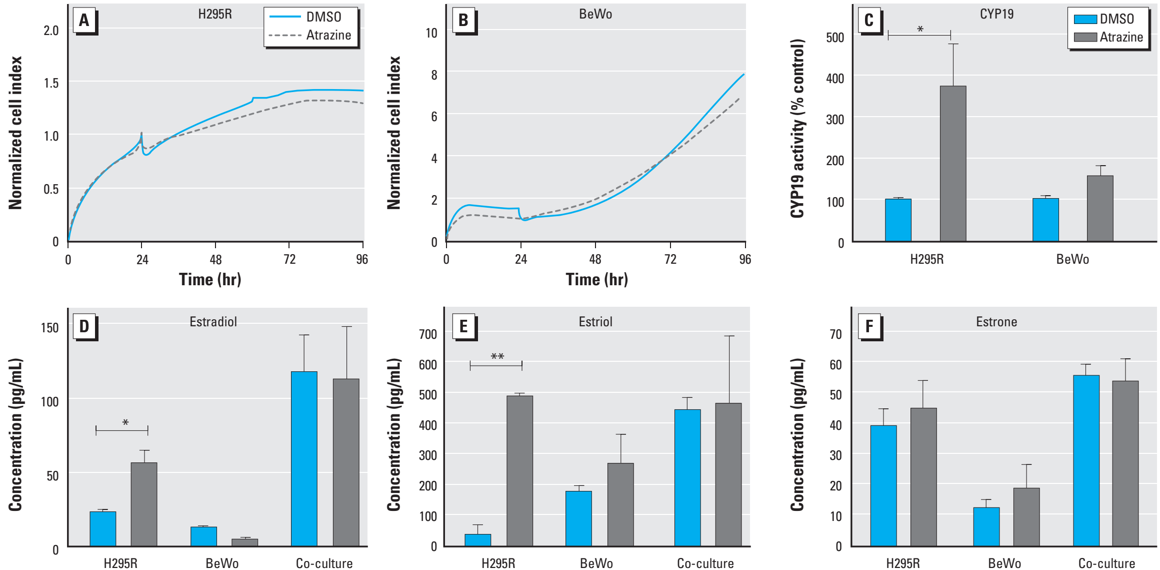


**Figure 2.** Effects of atrazine (30 μM) on the proliferation of H295R (*A*) and BeWo (*B*) cells in co-culture monitored in real time and its effects on CYP19 activity in each cell line after 24 hr of co-culture (*C*). The effects of atrazine on estradiol (*D*), estriol (*E*), and estrone (*F*) production by H295R and BeWo cells in monoculture or co-culture (24-hr exposure). Concentrations are presented as mean ± SE; $n = 3$.
*$p < 0.05$, **$p < 0.01$, compared with DMSO control.

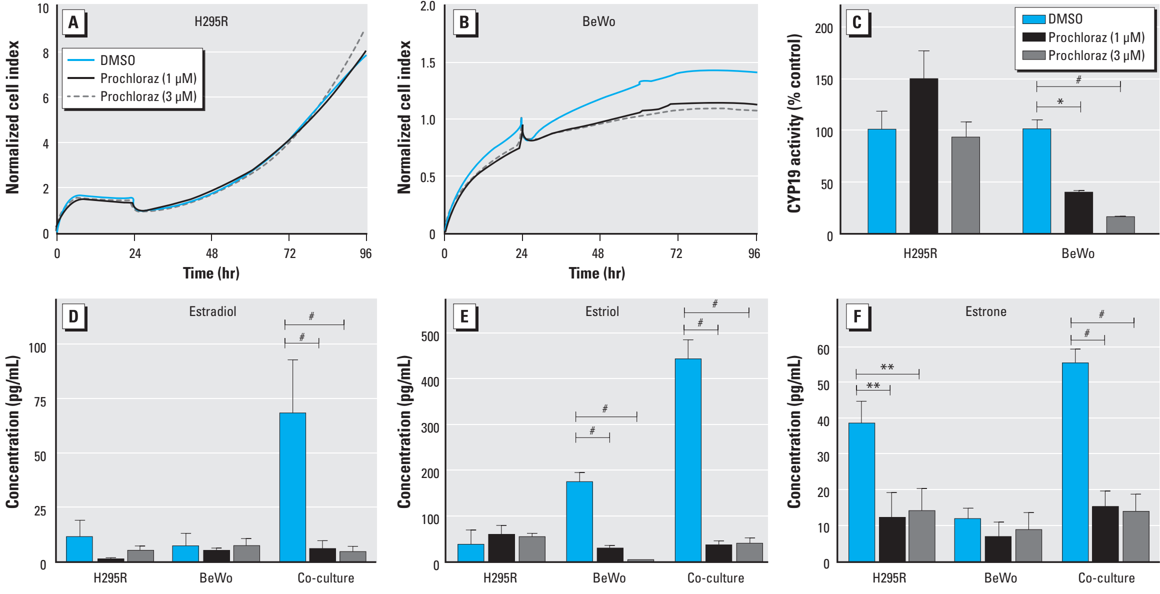


**Figure 3.** Effects of prochloraz (1 and 3 μM) on the proliferation of H295R (*A*) and BeWo (*B*) cells in co-culture monitored in real time and its effects on CYP19 activity in each cell line after 24 hr of co-culture (*C*). The effects of prochloraz on estradiol (*D*), estriol (*E*), and estrone (*F*) production by H295R and BeWo cells in monoculture or co-culture (24-hr exposure). Concentrations are presented as mean ± SE; $n = 3$.
*$p < 0.05$, **$p < 0.01$, and $^{\#}p < 0.001$, compared with DMSO control.

and limit interference with endogenous steroid hormone production by the co-culture. H295R cells do not tolerate high concentrations of FBS but were not affected by the presence of 1% stripped FBS. Although we always completed our experiments within 24 hr, we observed a decrease in proliferation of H295R cell after 36–48 hr of co-culture with BeWo cells (see Supplemental Material, Figure S2C), suggesting that the presence of BeWo cells in the inserts affects the long-term proliferation of H295R cells in co-culture. This may be attributable to the far higher levels of total estrogens produced by BeWo cells in co-culture because greater concentrations of estradiol ($10^{-6}$ M) are known to inhibit H295R cell proliferation (Jaroenporn et al. 2008). Various other hormones uniquely secreted by BeWo cells, such as β-hCG, could also be contributing to altered H295R cell proliferation because H295R cells are known to express functional LH (luteinizing hormone)/hCG receptors (Rao et al. 2004). Whether β-hCG affects fetal adrenal cell proliferation or function *in utero* remains to be investigated, but in the present study we used β-hCG secretion levels as an established indicator of normal trophoblast function to confirm that biochemical differentiation of BeWo cells occurred appropriately in our co-culture model. We observed a β-hCG secretion rate that was somewhat greater in co-culture medium than under regular culture conditions (see Supplemental Material, Table S2). This increase, evident after 48 hr, but no longer apparent after 72 hr, may have been attributable to the insulin in the co-culture medium because insulin is known to increase β-hCG production in villous trophoblast cells (Ren and Braunstein 1991).

***Steroidogenesis in the co-culture model.*** Consistent with the functional steroidogenic fetoplacental unit during human pregnancy, our co-culture model is capable of progesterone, androgen, and estrogen biosynthesis *de novo* (Figure 1). In our co-culture, progesterone production occurs predominantly in BeWo cells, consistent with the progesterone biosynthetic function of the trophoblast (Braunstein 2003), which is essential for maintenance of pregnancy (Wetendorf and DeMayo 2012). Moreover, precursors for estrogens are produced *de novo* predominantly by H295R cells (DHEA and androstenedione, but no detectable testosterone).

CYP19 is expressed and active in H295R and BeWo cells, but different tissue-specific promoters are involved in its expression in each cell type (Klempan et al. 2011; Sanderson et al. 2004). In the fetoplacental unit, CYP19 is mainly regulated via the PKC pathway through the major placental I.1-promoter of *CYP19* (Harada et al. 2003). Although human fetal tissues possibly contain CYP19 activity and/or *CYP19* transcripts (Pezzi et al. 2003), CYP19 levels are low and its regulation is not understood. Fetal aromatase transcript appears to be mainly derived from the gonadal pII-promoter of *CYP19*, which is regulated by gonadotropins, including hCG, via the Gs-protein-coupled follicle stimulating hormone (FSH) and LH/hCG receptors that activate cyclic adenosine monophosphate (cAMP)/PKA signaling, suggesting that this pathway is involved in fetal CYP19 regulation (Bulun et al. 1994). Our co-culture system responded to stimulation of the PKC and PKA signaling pathways with increased CYP19 activity (see Supplemental Material, Figure S3). The relative contribution of basal or induced CYP19 activity was considerably (15 times) greater in BeWo than H295R cells (data not shown), which is consistent with evidence that *CYP19* gene expression and CYP19 catalytic activity are far greater in placental tissue than fetal adrenal or fetal liver tissue (Pezzi et al. 2003).

A relevant *in vitro* steroidogenic model of the fetoplacental unit requires the *de novo* production of estrogens, including the pregnancy-specific hormone estriol, which is an indicator of fetal well-being (Mucci et al. 2003). We found basal estrogen production by H295R and BeWo cells in monoculture to be very low; however, in co-culture, estradiol and estriol production increased synergistically. Estriol production is almost uniquely (> 90%) dependent on the fetal precursor 16α-hydroxyandrostenedione produced by fetal hepatic CYP3A7 (Kitada et al. 1987). The synergistic production of estriol by our co-culture indicates that H295R cells are also acting as a suitable (steroidogenic) model for the fetal liver by providing the 16α-hydroxyandrostenedione precursor. Estrone production during pregnancy is lower than that of estriol and estradiol, and its levels do not correlate with the other estrogens; neither is its function during pregnancy well understood (Braunstein 2003). Estrone was produced by H295R and BeWo cells in monoculture; in co-culture, estrone production was increased additively, not synergistically, which is again consistent with the observed kinetics of estrogens during human pregnancy (Tulchinsky et al. 1972).

Taken together, the steroidogenic profile of our co-culture model—given the lack of quantifiable testosterone production but great production of DHEA and, to a lesser extent, androstenedione—indicates that estradiol is produced mainly via the aromatization of androstenedione to estrone and its subsequent rapid conversion to estradiol by 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1). Estradiol production would thus be achieved without the requirement for the synthesis of large quantities of the potent androgen testosterone, which could cause inappropriate masculizing/defeminizing of the fetus. This explanation is plausible because HSD17B1 is known to be highly expressed in BeWo cells (Lewintre et al. 1994), as it is in human trophoblast cells (Brown et al. 2003). Although H295R cells are known to express *HSD17B1* (Hilscherova et al. 2004), this isoform is not effective at converting androstenedione to testosterone (Poirier 2010). On the other hand, H295R cells express *HSD17B4*, which has dehydrogenase (oxidative) activity and would favor the conversion of testosterone to androstenedione (Poirier 2010). Although testosterone production in H295R cells has been reported, our inability to detect significant quantities of testosterone are consistent with the original studies that characterized the steroidogenic profile of H295R and its parent line NCI-H295 (Gazdar et al. 1990; Rainey et al. 1994).

***Disruption of fetoplacental steroidogenesis by endocrine-disrupting pesticides.*** Atrazine is a member of the triazine herbicide family and is suspected to have long-term adverse environmental effects (Jablonowski et al. 2011). Adverse birth outcomes (fetal growth restriction and preterm birth) have been associated with atrazine exposure (Chevrier et al. 2011; Rinsky et al. 2012). The endocrine-disruptive effect of atrazine on CYP19 has been studied in several cell models (Fan et al. 2007; Sanderson et al. 2000). Atrazine induces *CYP19* expression in H295R cells via the I.3 and pII promoters by increasing the intracellular levels of cAMP (Sanderson et al. 2002). In addition, an interaction of atrazine with steroidogenic factor 1, a transcription factor required for activation of the pII promoter of *CYP19*, may be involved (Fan et al. 2007).

In our co-culture, atrazine increased CYP19 activity in H295R cells only (Figure 2), which is consistent with the importance of the PKA pathway in the pII promoter-driven regulation of *CYP19* in these cells, whereas in placental cells, *CYP19* is under the control of the PKC-responsive I.1 promoter (Watanabe and Nakajin 2004). Atrazine did not modify aromatase activity in BeWo cells, although forskolin induced CYP19 activity in this cell line. We suggest that forskolin increases aromatase activity indirectly—as a result of its known stimulatory effect on BeWo cell syncytialization, which is normally associated with increased CYP19 expression (Taylor et al. 1991). Atrazine does not have this effect and it did not affect β-hCG levels (data not shown). Despite the induction by atrazine of CYP19 activity in H295R cells (the fetal compartment), estradiol and estriol production by the co-culture (the cooperative fetoplacental unit) was not altered, indicating that the contribution of "fetal" CYP19 to overall

estrogen production is small, if not negligible, in our co-culture model, as it is in the human fetoplacental unit *in vivo*. This furthermore emphasizes the relevance of the tissue-specific and condition-specific (pregnancy) nature of the regulation of CYP19 in humans. Chemicals that induce aromatase expression and activity in selected *in vitro* cell systems may have very different, if any, effects *in vivo* if the tissue-specific mechanisms of such observed induction is not taken into consideration.

Prochloraz, a fungicide with anti-androgenic properties, has a range of actions on cytochrome P450 enzymes (Vinggaard et al. 2002, 2005). Perinatal exposure of rats to prochloraz resulted in feminization of the male pups, which was associated with reduced testosterone levels, likely due to inhibition of CYP17 (Vinggaard et al. 2005). In rats exposed prenatally to prochloraz, malformations of the male reproductive tract were observed (Noriega et al. 2005). Our laboratory previously showed prochloraz to be a mixed-type catalytic inhibitor of CYP19 activity in H295R cells (Sanderson et al. 2002). However, in the present study we did not observe such inhibition in H295R cells in our co-culture (Figure 3). This discrepancy may be attributed to different experimental conditions, including our lower cell densities and the presence of 1% (stripped) FBS in the co-culture medium. Prochloraz clearly inhibited CYP19 activity in BeWo cells (Figure 3). Consistent with the behavior of the human fetoplacental unit in which estrogen production is predominantly dependent on placental CYP19, prochloraz reduced estrogen production by the co-culture to background levels despite its lack of inhibition of "fetal" aromatase. The observation that estrogen production by the co-culture was already decreased by 90% at a prochloraz concentration of 1 μM that only partially inhibited CYP19 activity in BeWo cells (and not at all in H295R cells) may be explained by the known inhibitory effect of prochloraz on CYP17 activity, which would reduce the essential supply of precursors androgens from the H295R cells. Indeed, prochloraz (1 μM) inhibited DHEA production in co-culture by 97% (data not shown). Because the estrogen receptor is involved in H295R cell proliferation and antiestrogens inhibit H295R cell proliferation (Montanaro et al. 2005), it is not surprising to observe a decreased proliferation of H295R but not BeWo cells in the co-culture treated with prochloraz. This endocrine-disrupting effect of prochloraz could affect pregnancy outcome because estrogen deprivation is associated with a spontaneous abortion rate of 50% in the baboon, a species commonly used as a model for primate/human pregnancy (Albrecht and Pepe 1999).

***A new tool to study steroidogenesis.*** We developed our model to respond to the demand for noninvasive *in vitro* research tools for studying the effects of chemical exposures during pregnancy on placental and fetal health. Our co-culture of BeWo and H295R cells not only allows the study of the complete fetoplacental steroidogenesis pathway, it also takes into consideration the impacts of numerous fetoplacental interactions (Figure 4), which occur in real time, affecting the behavior of both cell types. For instance, placental β-hCG appears to be involved in the regulation of DHEA sulfation via LH/hCG receptors present in the H295R cells by stimulating sulfotransferases (Rao et al. 2004) and could affect availability of androgen precursors for BeWo cells in the co-culture. Moreover, several steroid hormones produced in the placenta, such as estradiol and progesterone, also regulate the expression of the 11β-hydroxysteroid dehydrogenases (HSD11B), which play a role in the regulation of fetal growth and development of the fetal adrenal zone (Beaudoin et al. 1997; Kaludjerovic and Ward 2012; Myatt and Sun 2010). The ability of the co-culture to produce mineralo- and glucocorticoids also allows for the study of stress responses and homeostasis.

**Figure 4.** Schematic representation of the fetoplacental interactions in our co-culture model of H295R human (fetal-like) adrenocortical carcinoma and BeWo human (trophoblast-like) choriocarcinoma cells. The fetal unit expresses CYP17 (steroid 17α-hydroxylase/17,20-lyase) and produces androgen precursors, which are converted to estrogens by the placental aromatase (CYP19). Placental estrogens and β-hCG act in autocrine, paracrine, and endocrine manners on the trophoblast and fetal unit. Enzymes/hormones in bold type indicate relatively greater activities/levels.

## Conclusions

The co-culture of H295R and BeWo cells is a unique *in vitro* model that reproduces the steroidogenic cooperation between the fetal adrenal/liver and the villous trophoblast during pregnancy. The model provides a versatile tool to study the impact of potential endocrine-disrupting chemicals (e.g., environmental contaminants, drugs) to which pregnant women may be exposed.

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## 8.3 Données supplémentaires

*Environmental Health Perspectives* doi :10.1289/ehp.1307518

# Supplemental Material

# A Unique Co-culture Model for Fundamental and Applied Studies of Human Fetoplacental Steroidogenesis and Interference by Environmental Chemicals

Andrée-Anne Hudon Thibeault, Kathy Deroy, Cathy Vaillancourt, and J. Thomas Sanderson

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**Figure S3.** Relative CYP19 activity in H295R (A) and BeWo (B) cells cultured in their respective regular media or in co-culture medium after 24 h exposure to formestane (1 μM), phorbol-12-myristate-13-acetate (PMA; 1 μM) or forskolin (10 μM). **4**

**Table S1.** Sensitivity of the ELISA kits used to detect and quantify the cellular production of β−hCG and steroid hormones. **5**

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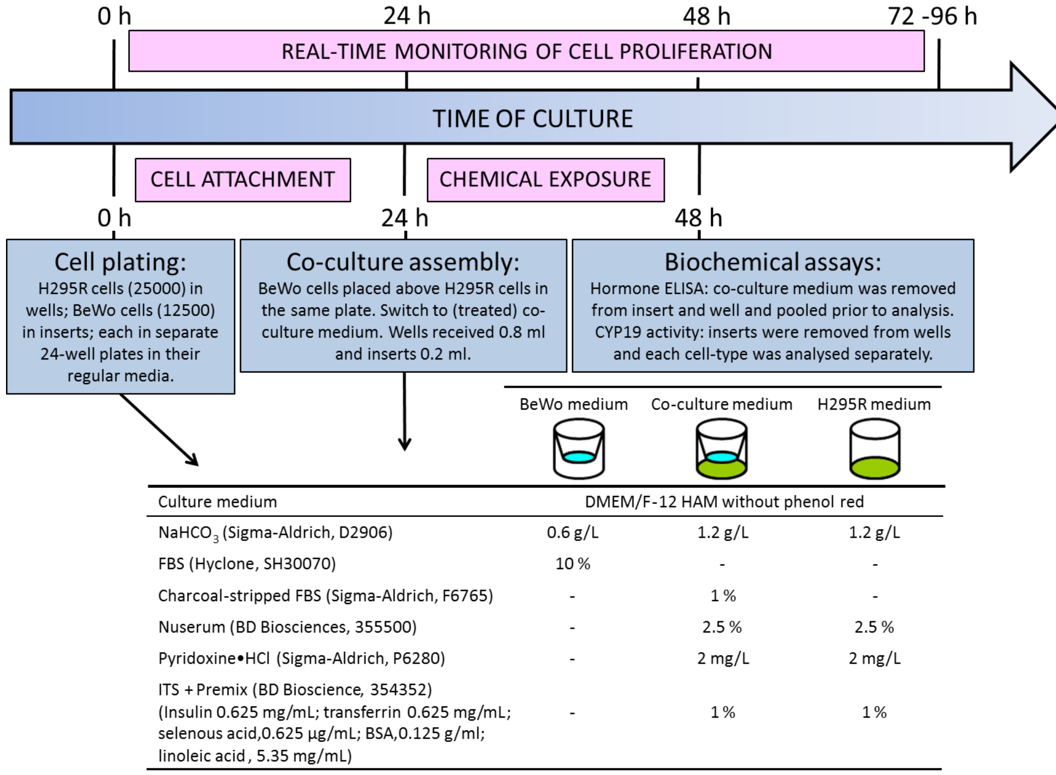


| Culture medium | DMEM/F-12 HAM without phenol red | | |
|---|---|---|---|
| | BeWo medium | Co-culture medium | H295R medium |
| $NaHCO_3$ (Sigma-Aldrich, D2906) | 0.6 g/L | 1.2 g/L | 1.2 g/L |
| FBS (Hyclone, SH30070) | 10 % | - | - |
| Charcoal-stripped FBS (Sigma-Aldrich, F6765) | - | 1 % | - |
| Nuserum (BD Biosciences, 355500) | - | 2.5 % | 2.5 % |
| Pyridoxine•HCl (Sigma-Aldrich, P6280) | - | 2 mg/L | 2 mg/L |
| ITS + Premix (BD Bioscience, 354352) (Insulin 0.625 mg/mL; transferrin 0.625 mg/mL; selenous acid,0.625 µg/mL; BSA,0.125 g/ml; linoleic acid , 5.35 mg/mL) | - | 1 % | 1 % |

**Figure S1**. Experimental design of the co-culture experiments with description of the composition of the co-culture medium.

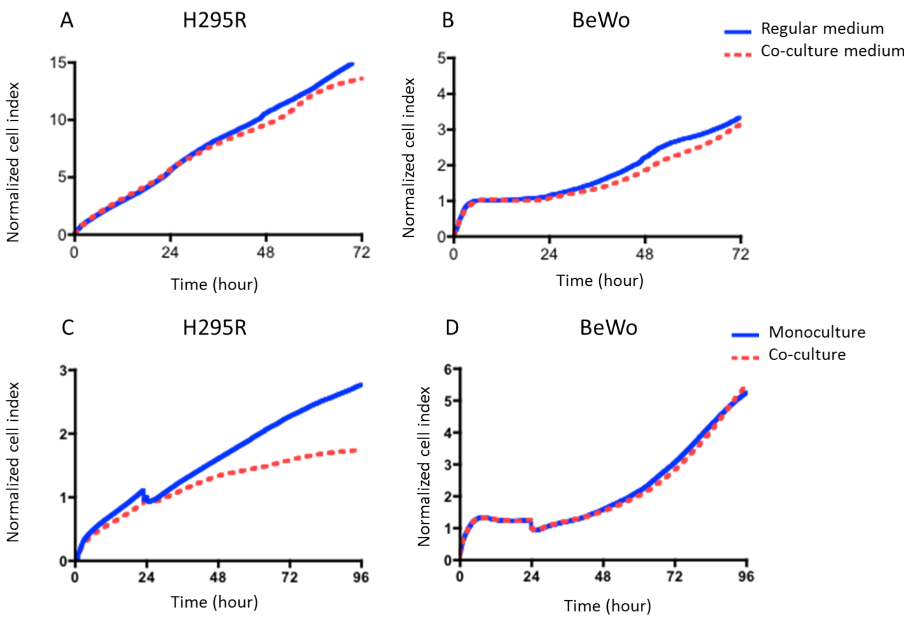


**Figure S2.** H295R **(A)** and BeWo (**B**) cell proliferation in regular (ATCC-recommended) medium or in co-culture medium. H295R **(C)** and BeWo (**D**) cell proliferation in co-culture medium either as monocultures or in co-culture with the other cell line. We monitored cell proliferation in real-time using an impedance-based xCELLigence™ RTCA DP instrument (ACEA Biosciences, San Diego, CA). We normalized cell index after complete adhesion of H295R (at 3 h, **A**) or BeWo (at 6 h, **B**) cells or, in the case of co-culture, 24 h after plating and immediately after co-culture assembly (**C, D**). Each trace is the average of three measurements.

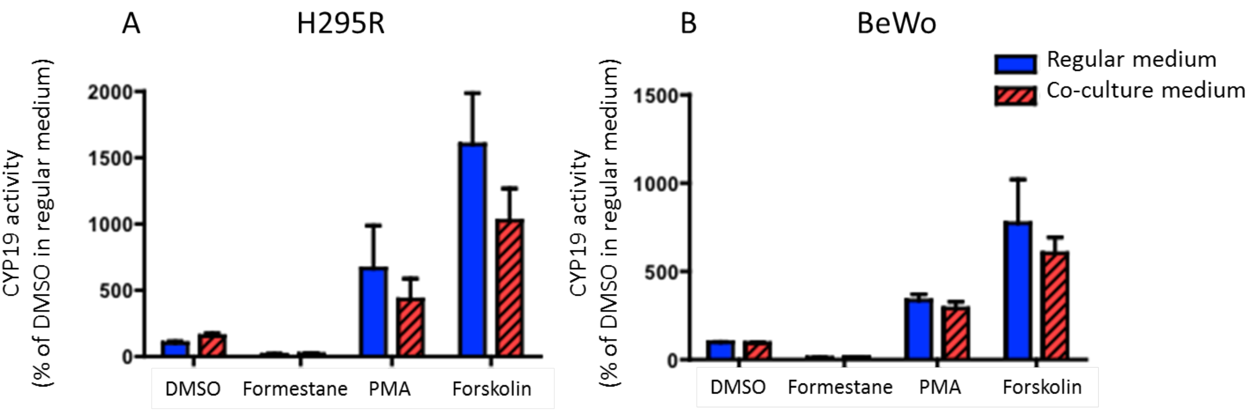


**Figure S3.** Relative CYP19 activity in H295R (**A**) and BeWo (**B**) cells cultured in their respective regular media or in co-culture medium after a 24 h exposure to formestane (1 µM), phorbol-12-myristate-13-acetate (PMA; 1 µM) or forskolin (10 µM). Activities were expressed as percentage (mean ± SEM; $n = 3$) of the activities in cells exposed to vehicle control (DMSO; 0.1% v/v) in their respective regular culture media. Two-way ANOVA did not detect a statistically significant ($P < 0.05$) effect of the co-culture medium on basal or induced CYP19 activities. Regardless of medium, inducibility by PMA ($P < 0.05$) and forskolin ($P < 0.001$) was statistically significantly greater in H295R than in BeWo cells.

**Table S1.** Sensitivity of the ELISA kits used to detect and quantify the cellular production of β−hCG and steroid hormones.

| Hormone | ELISA kit Company, catalogue number | Sensitivity of the kit (β−hCG: mIU/mL) (steroids: pg/mL) | Lowest concentration detected in cell culture (basal 24 h production) (β−hCG: mIU/mL) (steroids: pg/mL) |
|---|---|---|---|
| β−hCG | DRG Diagnostics EIA-1911 | 1.0 | 7.7 ± 1.8 [a] |
| Progesterone | DRG Diagnostics EIA-1561 | 45 | 1697 ± 257 |
| Dehydroepiandrosterone (DHEA) | DRG Diagnostics EIA-3415 | 108 | < 108 [b] |
| Androstenedione | DRG Diagnostics EIA-3265 | 19 | < 19 [b] |
| Testosterone | DRG Diagnostics EIA-1559 | 83 | ≤ 83 [b, c] |
| Estradiol | DRG Diagnostics EIA-2693 | 9.7 | 11.0 ± 2.0 |
| Estriol | DRG Diagnostics EIA-3717 | 40 | (35 ± 35) [c] |
| Estrone | Abnova KA-1908 | 10.0 | 11.7 ± 3.2 |

[a]β-hCG was detectable in BeWo cells only. [b]Levels were below the limit of detection in BeWo cells. [c]Levels were at or below the limit of detection in H295R cells.

**Table S2.** Basal and forskolin-stimulated (10 μM) β-hCG production (mIU/mL) by BeWo cells in regular or in co-culture medium over a 24, 48 or 72 h period of monoculture or after 24 h in co-culture with H295R cells.

| Treatment | Regular medium | Co-culture medium | In co-culture with H295R cells |
|---|---|---|---|
| **Basal** | | | |
| 24 h | 7.7 ± 1.8 | 10.9 ± 3.2 | 36.0 ± 8.7[a] |
| 48 h | 40.5 ± 9.3 | 80.5 ± 17.8* | - |
| 72 h | 89.1 ± 14.1 | 88.5 ± 19.6 | - |
| **Forskolin** | | | |
| 24 h | 156.7 ± 93.5 | 177.4 ± 18.1 | 241.8 ± 41.8[a] |
| 48 h | 1126.7 ± 409.7 | 3626.1 ± 444.7* | - |
| 72 h | 4918.1 ± 2178.3 | 4952.1 ± 617.7 | - |

*Statistically significant difference from corresponding production in regular medium over the same period determined by two-way ANOVA ($p < 0.05$) and Bonferroni post-hoc test.

[a]Note that in co-culture the exposure regime was as described in Supplemental Material, Figure S1 and is not directly comparable to the β-hCG production levels in monoculture.

## 8.4 Erratum

**Erratum:** A Unique Co-culture Model for Fundamental and Applied Studies of Human Fetoplacental Steroidogenesis and Interference by Environmental Chemicals

Andrée-Anne Hudon Thibeault, Kathy Deroy, Cathy Vaillancourt, and J. Thomas Sanderson

In Figure 3, the H295R and BeWo cell-type labels are inverted between graphs A and B. This does not alter the conclusion that treatment with prochloraz (1 and 3 μM) did not affect the proliferation of either cell type in co-culture during the first 24 hr. The corrected figure is included in this erratum. The authors regret the error.

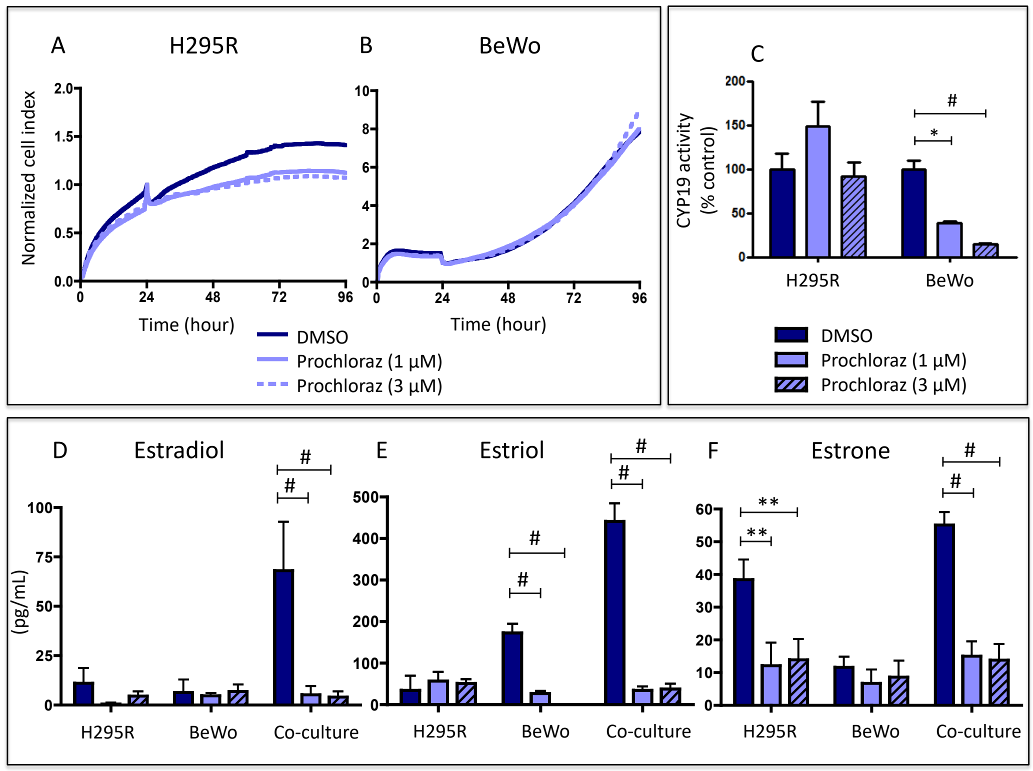


Figure 3. Effects of prochloraz (1 and 3 μM) on the proliferation of H295R (A) and BeWo (B) cells in co-culture monitored in real time and its effects on CYP19 activity in each cell line after 24 hr of co-culture (C). The effects of prochloraz on estradiol (D), estriol (E), and estrone (F) production by H295R and BeWo cells in monoculture or co-culture (24-hr exposure). Concentrations are presented as mean ± SE; $n = 3$. *$p < 0.05$, **$p < 0.01$, and #$p < 0.001$, compared with DMSO control.

# 9 AROMATASE ET DIFFÉRENCIATION DES PRIMOCULTURES DE TROPHOBLASTES VILLEUX

Titre complet : Profile of *CYP19A1* mRNA expression and aromatase activity during syncytialization of primary human villous trophoblast cells at term

Titre en français : Profil d'expression d'ARNm de *CYP19A1* et d'activité de l'aromatase pendant la syncytialisation des primocultures de trophoblastes villeux de placenta à terme.

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**Contribution de l'étudiante** : L'étudiante a participé à l'élaboration de l'étude, complété toutes les expériences. Elle a également analysé les résultats, rédigé l'article, effectué les révisions des correcteurs.

**Contribution des coauteurs** : Cathy Vaillancourt et J. Thomas Sanderson ont participé à l'élaboration de l'étude incluant la planification des expériences, l'analyse des résultats et la correction du manuscrit.

## 9.1 Résumé de l'article en français

La production d'estrogènes par le trophoblaste villeux humain est dépendante de l'enzyme de biosynthèse aromatase (CYP19; *CYP19A1*) et est cruciale pour le bon développement placentaire et l'issue de la grossesse. En utilisant des cellules de cytotrophoblastes villeux (CTv) fraichement isolées de placenta normal à terme, nous avons caractérisé le taux d'ARNm promoteur-spécifique de *CYP19A1* (dérivé des promoteurs I.1, I.4, I.8 ou transcrit total) ainsi que l'activité du CYP19 lors de la syncytialisation du trophoblaste villeux. Le niveau d'ARNm de CYP19A1 et l'activité du CYP19 des CTv ont atteint un maximum après 48 h de culture. L'inducteur d'AMPc forskoline (10 mM) et l'activateur de la protéine kinase C phorbol myristate acétate (PMA; 1 mM) a augmenté le niveau d'ARNm de *CYP19A1* par 1,8- et 1,6-fois, respectivement, et ils ont induit l'activité du CYP19 Le dexaméthasone (100 nM) et le facteur de croissance de l'endothélium vasculaire (VEGF; 5 ng/mL) ont diminué le niveau d'ARNm de *CYP19A1*, sans avoir d'effet sur l'activité du CYP19. Nos résultats mettent l'emphase sur l'importance de ne pas seulement étudier la régulation et le fonctionnement de *CYP19A1* au niveau de l'ARNm, mais d'également considérer des mécanismes post-traductionnels qui peuvent altérer l'activité finale du CYP19.

## 9.2 Article

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Short communication

# Profile of *CYP19A1* mRNA expression and aromatase activity during syncytialization of primary human villous trophoblast cells at term

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A B S T R A C T

Estrogen production by the human villous trophoblast is dependent on the biosynthetic enzyme aromatase (CYP19; *CYP19A1*) and is crucial for successful placental development and pregnancy outcome. Using villous cytotrophoblast cells (vCTs) freshly isolated from normal term placenta, we characterized the promoter-specific expression of *CYP19A1* mRNA (derived from promoters I.1, I.4, I.8 or total transcript) and aromatase activity during villous trophoblast syncytialization. *CYP19A1* mRNA levels and aromatase activity in vCTs reached a maximum after about 48 h of culture. The cAMP inducer forskolin (10 μM) and protein kinase C stimulant phorbol myristate acetate (1 μM) increased *CYP19A1* mRNA levels by 1.8- and 1.6-fold, respectively, as well as inducing aromatase catalytic activity. Dexamethasone (100 nM) and vascular endothelial growth factor (5 ng/mL) decreased *CYP19A1* mRNA levels, while having no effect on aromatase activity. Our results emphasize the importance of not solely studying *CYP19A1* regulation and function at the mRNA level but also considering posttranslational mechanisms that alter the final catalytic activity of aromatase.

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## 1. Introduction

Villous trophoblast syncytialization in the human placenta is regulated by a positive feedback mechanism where the hormones secreted by the syncytiotrophoblast cells (STs) stimulate the fusion of villous cytotrophoblast cells (vCTs) into the adjacent syncytium [1]. Thus, well-regulated estrogen synthesis by the enzyme CYP19 (aromatase), which is mainly expressed and catalytically active in the STs [2], is crucial for proper placental syncytialization and function. The *CYP19* gene (*CYP19A1*) is composed of 9 coding exons (II to X) [3,4]. Various promoters upstream of non-coding exons I regulate the tissue-specific expression of *CYP19A1* [3,4]. Each active promoter produces *CYP19A1* transcript with a unique 5'-untranslated region, but the translated protein is identical regardless of the promoter used [3,4]. The CYP19 protein undergoes posttranslational modifications including heme insertion and complexation with NADPH-dependent cytochrome P450 reductase before it becomes a catalytically active cytochrome P450 enzyme capable of aromatizing androgens to estrogens [5,6]. In the human placenta, specific response elements for various transcription factors are found upstream of exon I.1 of the *CYP19A1* gene [7–10]. Transcripts derived from other *CYP19A1* promoters are also expressed, including PII, I.3, I.2, I.4, I.8 and 1.2a [3,4]. In this study, we focused on the major placental *CYP19A1* promoter I.1 as well as on promoter I.8 because of the presence of upstream glucocorticoid response elements [3], which are important regulators of trophoblast function [11] and the I.4 promoter, which is also under glucocorticoid control in adipose tissue [12], but of which its regulation is unknown in the placenta. Moreover, promoter I.4 has an interesting negative effect on *CYP19A1* mRNA stability and protein translation [13]. We tested forskolin and phorbol ester PMA because of their known effects on *CYP19* expression in various cell lines and primary trophoblasts [14–17]. Vascular

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endothelial growth factor (VEGF) was included given its important role in placental function [18–20] and its known action on aromatase in breast cancer cell lines [21]. Finally, we studied dexamethasone because of the importance of glucocorticoid response elements in the regulation of *CYP19* via several specific promoters, including I.1, I.4 and I.8 [3,22–24].

The aim of this study was to compare the profiles of *CYP19A1* gene expression and catalytic activity during *in vitro* syncytialization of human vCTs in primary culture isolated from term placentas and to determine the effects of pharmacological stimulants of cell signaling pathways on CYP19 regulation.

## 2. Materials and methods

### 2.1. Isolation and purification of human villous cytotrophoblast cells (vCTs)

The vCT population from placentas at term (37–41 weeks) of uncomplicated pregnancies were isolated and purified using a trypsin-DNase/Percoll method as previously described [25] and cultured in a humidified atmosphere containing 5% $CO_2$ at 37 °C. Cells were seeded in 96-well plates ($1.25 \times 10^5$ cells/well) and maintained as previously described in culture medium containing 10% FBS [25]. Culture medium was changed 4 h after seeding to remove unattached cells and debris and then every 24 h. Placental collection was approved by the ethical committee of CHUM-St-Luc Hospital (Montreal, QC, Canada).

### 2.2. Treatments

Forskolin (Sigma-Aldrich, Oakville, ON, Canada), a cAMP/protein kinase A pathway activator, phorbol myristate acetate (PMA; Sigma-Aldrich), a protein kinase C activator, vascular endothelial growth factor (VEGF; ATCC, Rockville, MD), an agonist of VEGF receptors and dexamethasone (Sigma-Aldrich), a glucocorticoid receptor agonist, were prepared in dimethyl sulfoxide (DMSO) as 1000-fold stock solution, which resulted in a final concentration of 10 μM (forskolin), 1 μM (PMA), 5 ng/mL (VEGF) and 100 nM (dexamethasone) and 0.1% DMSO in culture medium. The concentrations of the pharmacological stimulants were chosen based on the available literature [17,21,26–33]. Cells were exposed to each compound for a 24 h period, which started at different stages of differentiation. Primary culture was considered to have started ($t = 0$) after the initial 4 h of culture to eliminate non-adherent cells.

### 2.3. Aromatase activity

The catalytic activity of aromatase was determined by tritiated water-release assay as previously described [34] except that cells were exposed to 150 nM 1β-$^3$H-androstenedione (Perkin Elmer, Wellesley, MA) for 1 h.

### 2.4. Real-time quantitative PCR

RNA extraction, RNA purity and concentration determination, reverse transcription, quantitative PCR (qPCR) and reference gene selection were all performed as described previously [34]. Reference genes selected for qPCR normalization (ΔΔCT) were peptidylprolyl isomerase A (*PPIA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein, zeta polypeptide (*YWHAZ*). We used the following primer pair sequences (forward and reverse respectively) for *CYP19A1* exon I.1: 5′-GGA TCT TCC AGA CGT CGC GA-3′ and 5′-CAT GGC TTC AGG CAC GAT GC-3'; Exon I.4: 5′-GGC TCC AAG TAG AAC GTG ACC AAC TG-3′ and 5′-CAG CCC AAG TTT GCT GCC GAA-3'; Exon I.8: 5′-TGA TCT CCC TCT TCC CAG TG- 3′ and 5′-GGG GCA ATT TAG AGT CCT TAA GTC-3'; Coding exons (total *CYP19A1* mRNA): 5′-TGT CTC TTT GTT CTT CAT GCT ATT TCT C-3′ and 5′-TCA CCA ATA ACA GTC TGG ATT TCC-3'; *CGB* (human chorionic gonadotropin or β-hCG transcript): 5′-GCT ACT GCC CAC CAT GAC C-3′ and 5′-ATG GAC TCG AAG CGC ACA TC-3'; PPIA: 5′-GTT TGC AGA CAA GGT CCC A-3′ and 5′-ACC CGT ATG CTT TAG GAT G-3'; YWHAZ: 5′-GGC AAC CTA AGA ACA AAT G-3′ and 5′-CAT GTT AGG CAA GTA TCA AA-3′

### 2.5. Statistical analysis

All experiments were performed using 3 to 6 placentas. Per experiment, measurements were made in triplicate. Statistically significant differences ($P < 0.05$) were determined using a Kruskal-Wallis test followed by Dunn's *post-hoc* test. All analyses were performed using GraphPad Prism (version 5.04; GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. CYP19A1 mRNA levels and aromatase activity profile during biochemical differentiation of vCTs in vitro

Aromatase activity in vCTs increased during the first 48 h of primary culture, after which it declined until 96 h when the measurements were ended (Fig. 1A). Expression levels of I.1 and I.8 promoter-derived transcripts of *CYP19A1* (Fig. 1B–C) followed a similar pattern, whereas levels of I.4 promoter-derived transcript (Fig. 1D) reached a maximum between 24 and 48 h of culture. The expression profile of total *CYP19A1* transcript (coding exons) (Fig. 1E) reflects that of the various promoter-specific transcripts, and reached a plateau between 24 and 72 h *CYP19A1* transcripts reached maximum levels at least 24 h before those of β-hCG (*CGB*), a biomarker of vCT differentiation (Fig. 1F).

### 3.2. Effects of pharmacological treatments on CYP19A1 mRNA levels and aromatase activity during syncytialization of vCTs in vitro

Exposure of vCTs during the first 24 h of culture to forskolin (10 μM) or PMA (1 μM) increased total *CYP19A1* mRNA levels by 1.8- and 1.6-fold, respectively (Fig. 2A). Dexamethasone (100 nM) and VEGF (5 ng/mL) decreased total *CYP19A1* mRNA levels by 76% and 56%, respectively (Fig. 2A). We observed similar effects in vCT cultured for 48 h and 72 h, although these were not statistically significant. Only forskolin induced the catalytic activity of aromatase in vCTs exposed during the first 24 h of primary culture (2.4-fold; Fig. 2B). A similar effect of a 24-h exposure to forskolin was observed in vCTs that had been in culture for either 48 or 72 h (in other words, in cells exposed from 24 to 48 or 48 to 72 h of culture, respectively), with an average increase of 2-fold (Fig. 2C–D). PMA induced aromatase activity at a more advanced stage of vCT differentiation (1.5-fold in cells that were exposed from 48 to 72 h of primary culture; Fig. 2D). Neither dexamethasone nor VEGF affected aromatase activity after a 24 h exposure of vCTs at 24, 48 or 72 h of primary culture (Fig. 2B–D).

## 4. Discussion

We characterized the profile of *CYP19A1* mRNA expression and aromatase activity during the differentiation in primary culture of vCTs isolated from normal term placentas. A maximum increase of aromatase activity and *CYP19A1* mRNA levels was observed at 48 h of primary culture, which was 24–48 h earlier than maximal release of β-hCG (a marker of biochemical differentiation) by these

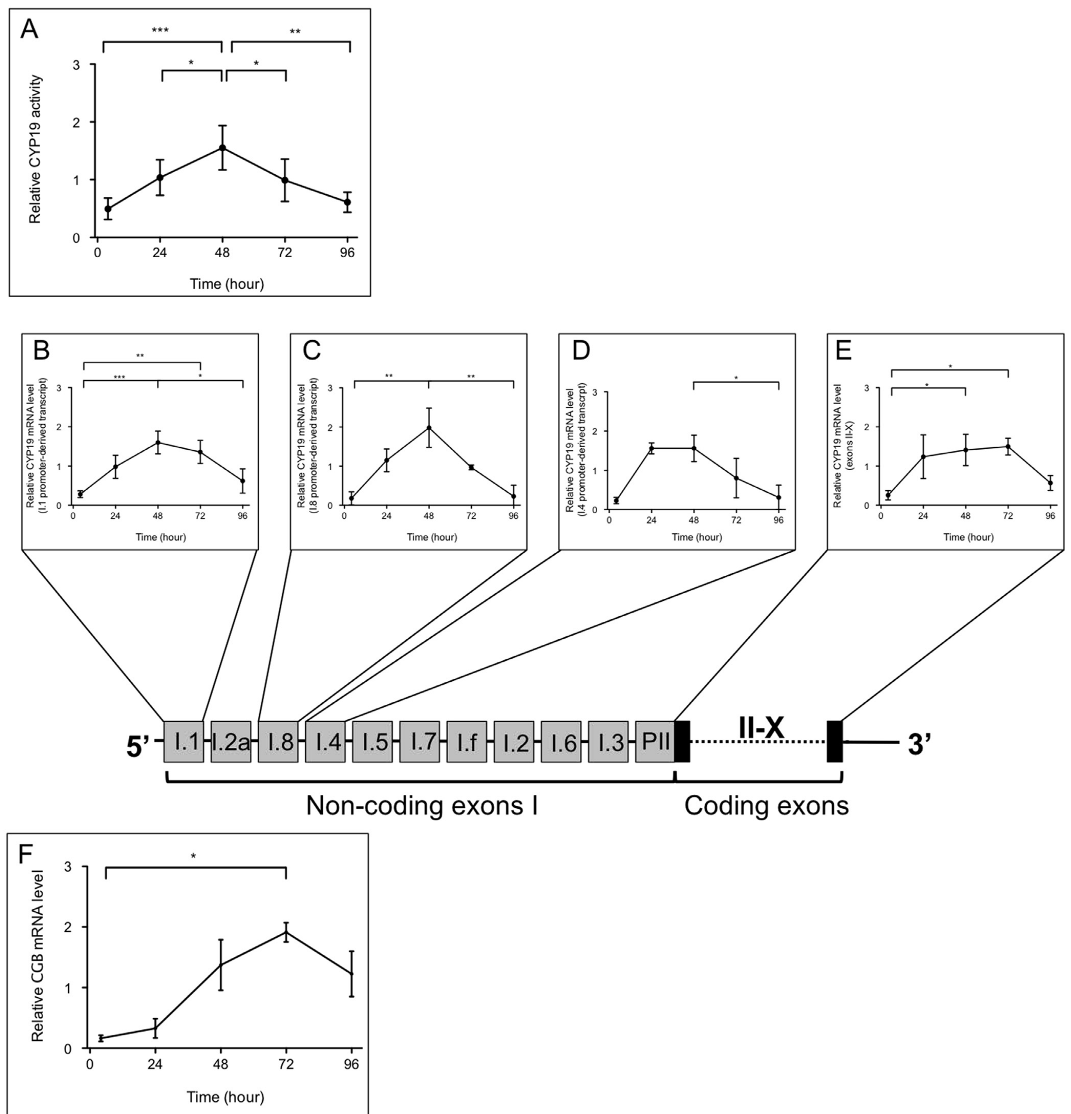


**Fig. 1. Relative aromatase activity and mRNA levels of *CYP19A1* and *CGB* in human villous trophoblasts throughout differentiation in primary culture**. (A) CYP19 (aromatase) activity was expressed relative to the average aromatase activity of each placenta ±SD. Statistically significant differences among time-points are indicated with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) as determined by Kruskal-Wallis and Dunn's post-hoc test, $n \geq 3$. Relative levels of *CYP19A1* transcript derived from (B) promoter I.1, (C) promoter I.8, (D) promoter I.4 and (E) total coding exons (total or non-promoter-specific); (F) levels of *CGB* (human chorionic gonadotropin) transcript. Levels were normalized using reference genes peptidylprolyl isomerase A (*PPIA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein-zeta polypeptide (*YWHAZ*) and are shown as means ± SD. The schematic representation of the *CYP19A1* gene is adapted from Demura et al. (2008) [3]. Statistically significant differences among time-points are indicated with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) as determined by Kruskal-Wallis and Dunn's post-hoc test, $n \geq 3$.

cells, as consistently found in our previous studies [35,36]. This study is the first to describe the expression pattern of I.4 and I.8 promoter-derived transcripts of *CYP19A1* in the primary villous trophoblast. Our results are consistent with others who observed increased aromatase activity and promoter I.1-derived *CYP19A1* mRNA levels throughout the culture of primary trophoblast cells (until 72 h of cell culture) [7,14,37,38]. It has been shown that, as pregnancy progresses, increased placental aromatase activity is associated with increased differentiation into syncytium [39]. Our chronological observations are consistent with the fact that one of the products of aromatase, 17β-estradiol, is required for stimulation of β-hCG release by STs [40] and that β-hCG contributes to CYP19

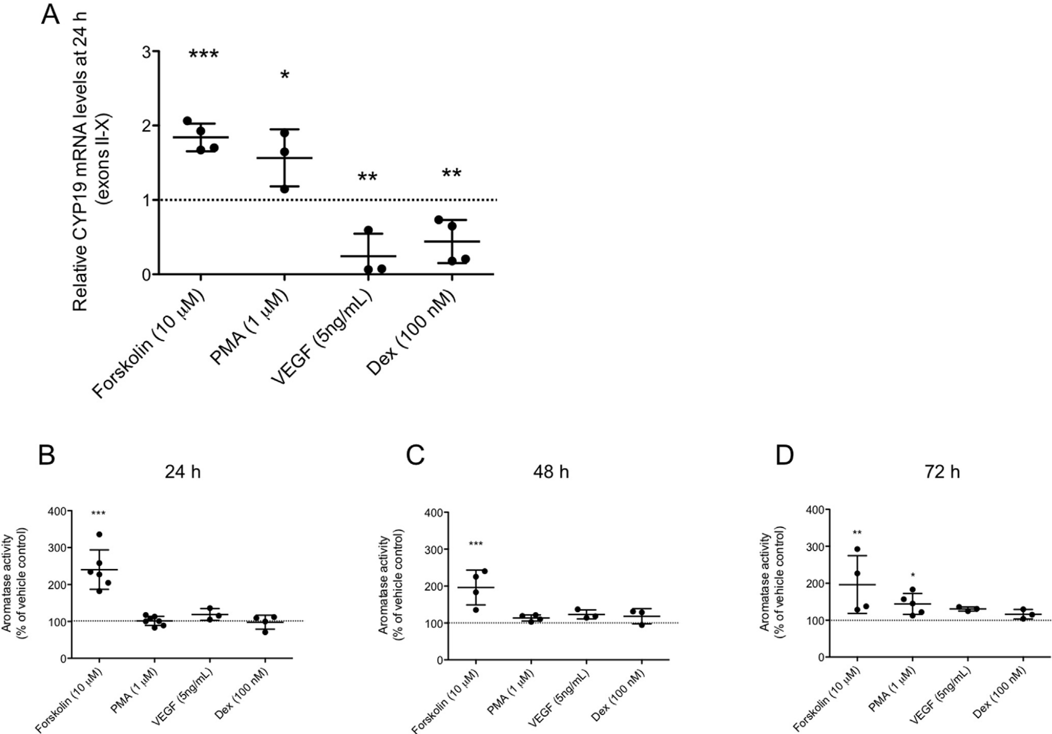


**Fig. 2. Effects of stimulation of various cell signaling pathways on *CYP19A1* expression and aromatase activity in human villous trophoblasts in primary culture.** (A) Relative levels (mean ± SD) of total (non-promoter-specific) *CYP19A1* transcript in cells treated with forskolin (10 μM), phorbol-2-myristate-3-acetate (PMA; 1 μM), vascular endothelial growth factor (VEGF; 5 ng/mL), dexamethasone (Dex; 100 nM) or vehicle-control (0.1% DMSO) during the first 24 h of primary culture. Levels were normalized using reference genes peptidylprolyl isomerase A (*PPIA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein-zeta polypeptide (*YWHAZ*) and are shown as means ± SD. Aromatase activity (mean ± SD) in cells treated for 24 h with above compounds at (B) 24 h, (C) 48 h and (D) 72 h of culture expressed as a percentage of vehicle-control (0.1% DMSO). Each individual data point represents an individual placenta. Per placenta all treatments were performed in triplicate. Statistically significant differences from control are indicated with asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) as determined by Kruskal-Wallis and Dunn's post-hoc test, $n \geq 3$. Dashed lines indicate the 100% level of *CYP19A1* mRNA or aromatase activity in DMSO control cells.

expression in a positive feedback manner [41]. The post-maximal decline in aromatase activity and *CYP19A1* mRNA levels at 96 h may be explained by the well-described apoptosis of the differentiated STs, which cannot be regenerated in primary cell culture [43].

Inferences concerning the functional (catalytic) behavior of aromatase and the biosynthesis of estrogens are often made based solely on levels of *CYP19A1* mRNA transcript [4]. However, changes in levels of *CYP19A1* mRNA are not always consistent with those observed in the catalytic activity of aromatase [34]. Others have also reported that posttranslational regulation of CYP19 through phosphorylation [42] or by alteration of RNA or protein stability [13] affects catalytic activity of the enzyme. To illustrate that measuring catalytic activity of aromatase should always complement mRNA analysis, we determined the effects of the stimulation of several cell signaling pathways on *CYP19A1* mRNA levels and aromatase activity.

Forskolin increased *CYP19A1* mRNA during the first 24 h of exposure and induced aromatase activity at 24, 48 and 72 h in primary vCTs, which is consistent with the observations by Lobo and Bellino (1989) using $(Bu)_2$cAMP mixed with theophylline [14]. The regulation by forskolin of *CYP19A1* expression via the I.1 promoter may involve the transcription factor glial cell missing 1 (GCM1) [10,43] or estrogens, which can act in a positive feedback manner via estrogen receptor-alpha (ERα) as suggested by Kumar et al. [44]. In addition, forskolin may also act indirectly to increase *CYP19A1* expression through its stimulating effect on vCT syncytialization [45].

The PKC activator PMA also increased *CYP19A1* mRNA levels during the first 24 h of exposure, but aromatase activity was only induced in vCTs exposed between 48 and 72 h of primary culture. We have previously shown that PMA also induces aromatase activity in BeWo choriocarcinoma cells [17], indicating that BeWo cells, which are often used as models of undifferentiated vCTs, respond more similarly to later-stage differentiating vCTs in primary culture. In the human placenta, VEGF has been studied mainly in the invading extravillous trophoblast cells [20], but our present observations suggest that VEGF may also play a role in the villous trophoblast. Here, we show for the first time that VEGF decreases total *CYP19A1* transcript levels in normal vCTs without affecting aromatase activity. Similarly, dexamethasone decreased *CYP19A1* mRNA levels, while not altering aromatase activity. The action of glucocorticoids on I.1 and I.8 promoter-mediated regulation of *CYP19A1* expression has never been studied, although it is known that in adipose stromal cells and fetal hepatocytes

glucocorticoids are required for I.4 promoter-mediated expression of *CYP19A1* [24]. It is possible that the I.4 promoter mediates the downregulation of *CYP19A1* by VEGF and dexamethasone via alteration of RNA stability and protein translation as suggested by Wang et al. [13]. The absence of effects on aromatase activity despite a decrease in *CYP19A1* mRNA levels associated with VEGF and dexamethasone is likely due to the fact that the aromatase enzyme is more stable than its mRNA. Other posttranslational mechanisms required for the formation or breakdown of the catalytically active enzyme may also be altered by VEGF and dexamethasone in a way to maintain aromatase activity, at least over the 24 h exposure period used in our experiments.

## 5. Conclusions

This study provides a better understanding of the time-dependent expression of *CYP19A1* mRNA and aromatase activity, as well as its response to stimulants of specific signaling pathways, during the differentiation of normal term human vCTs into STs in primary culture. We have shown that changes in promoter-specific *CYP19A1* mRNA levels after stimulation of various cell signaling pathways do not always affect aromatase catalytic activity. This emphasizes our point that *CYP19A1* mRNA levels cannot be used as a reliable surrogate for effects on aromatase activity or to draw any conclusions on subsequent estrogens biosynthesis.

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# 10 LA FLUOXÉTINE ET LA NORFLUOXÉTINE ALTÈRENT LA STÉROÏDOGENÈSE FŒTO-PLACENTAIRE

Titre complet : Fluoxetine and its active metabolite norfluoxetine disrupt estrogen synthesis in a co-culture model of the feto-placental unit

Titre en français : La fluoxétine et son métabolite actif norfluoxétine perturbent la synthèse d'estrogènes dans un modèle de co-culture de l'unité fœto-placentaire

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**Contribution de l'étudiante** : L'étudiante a participé à l'élaboration de l'étude, complété toutes les expériences de culture cellulaire, d'activité du CYP19, de RT-qPCR et de quantification d'hormone par ELISA. Elle a également analysé les résultats, participé au choix du journal, rédigé l'article et effectué les révisions des correcteurs.

**Contribution des coauteurs** : Laetitia Laurent a effectué les expériences qui relèvent de l'effet de E2 sur le SERT, en collaboration avec l'étudiante. Elle a réalisé les expériences d'immunobuvardage de type western et d'activité du SERT. Sung Vo Duy a réalisé les expériences de dosage de la fluoxétine et de la norfluoxétine par LC-MS/MS sous la supervision de Sébastien Sauvé. Patrick Caron a réalisé les dosages d'hormones par LC-MS/MS et GC-MS/MS pour valider les résultats des dosages par ELISA sous la supervision de Chantal

Guillemette. Chaque coauteur a participé à l'analyse et à la rédaction des résultats des expériences qu'il a réalisées. Cathy Vaillancourt et J. Thomas Sanderson ont travaillé à l'élaboration de l'étude incluant la planification des expériences, l'analyse des résultats, la rédaction et la correction du manuscrit.

## 10.1 Résumé de l'article en français

Les effets de la fluoxétine, un des inhibiteurs sélectifs de la recapture de la sérotonine (ISRS) les plus prescrits pendant la grossesse, et de son métabolite actif norfluoxétine ont été étudiés sur l'aromatase (CYP19) placentaire et la stéroïdogenèse fœto-placentaire. La fluoxétine n'a pas altéré la sécrétion d'estrogènes dans la co-culture de cellules surrénaliennes fœtales (H295R) et trophoblastiques (BeWo) utilisée comme modèle de l'unité fœto-placentaire. L'activité du CYP19 était induite, par le récepteur 5-$HT_{2A}$ et par la voie de signalisation de la PKC. La norfluoxétine a réduit la sécrétion d'estrogènes dans la co-culture fœto-placentaire et a inhibé compétitivement l'activité du CYP19 dans les cellules BeWo. L'activité du transporteur de la sérotonine (SERT) réduite dans la co-culture était comparable à celle d'un traitement au 17β-estradiol des cellules BeWo. Cette étude démontre que les interactions complexes de la fluoxétine et de la norfluoxétine avec la production d'estrogènes placentaire impliquent des mécanismes dépendants et indépendants de la sérotonine. Considérant le rôle crucial des estrogènes pendant la grossesse, nos résultats soulèvent des inquiétudes quant à l'impact des traitements ISRS sur la fonction placentaire et la santé fœtale.

## 10.2 Article

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# Fluoxetine and its active metabolite norfluoxetine disrupt estrogen synthesis in a co-culture model of the feto-placental unit

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ABSTRACT

The effects of fluoxetine, one of the most prescribed selective serotonin-reuptake inhibitors (SSRIs) during pregnancy, and its active metabolite norfluoxetine were studied on placental aromatase (CYP19) and feto-placental steroidogenesis. Fluoxetine did not alter estrogen secretion in co-culture of fetal-like adrenocortical (H295R) and trophoblast-like (BeWo) cells used as a model of the feto-placental unit, although it induced CYP19 activity, apparently mediated by the serotonin $(5\text{-}HT)_{2A}$ receptor/PKC signaling pathway. Norfluoxetine decreased estrogen secretion in the feto-placental co-culture and competitively inhibited catalytic CYP19 activity in BeWo cells. Decreased serotonin transporter (SERT) activity in the co-culture was comparable to 17β-estradiol treatment of BeWo cells. This work shows that the complex interaction of fluoxetine and norfluoxetine with placental estrogen production, involves 5-HT-dependent and -independent mechanisms. Considering the crucial role of estrogens during pregnancy, our results raise concern about the impact of SSRI treatment on placental function and fetal health.

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## 1. Introduction

Depressive disorders, which are associated with serotonin (5-hydroxytryptamine, 5-HT) dysfunction, affect 10%–20% of pregnant women (Marcus et al., 2003; Bennett et al., 2004). Untreated depression is associated with increased risks of obstetric complications such as preterm birth, pre-eclampsia, and low birth weight (Bonari et al., 2004; Olivier et al., 2013). Over the last decade, maternal use of antidepressants has increased more than 3-fold (Andrade et al., 2008; Alwan et al., 2011). This increase is mainly attributable to selective serotonin reuptake inhibitors (SSRIs), with Prozac[MD] (fluoxetine) being one of the most prescribed (Andrade et al., 2008; Wemakor et al., 2015; De Vera and Bérard, 2012). SSRIs have proven their efficiency against depression, but the impact of the exposure to SSRI during pregnancy remains controversial. Several studies have shown that SSRIs are associated with alterations of the respiratory, gastrointestinal, cardiac and

*ABBREVIATIONS:* 5-HT, 5-hydroxytryptamine, serotonin; $5\text{-}HT_{2A}$, 5-HT receptor type 2A; ANOVA, one-way analysis of variance; CYP19, cytochrome P450 19 (aromatase); DHEA, dehydroepiandrosterone; DOI, 2,5-dimethoxy-4-iodoamphetamine; JAK2, janus kinase 2; $K_i$, inhibition constant; $K_m$, Michaelis constant; LC-MS/MS, liquid-chromatography-tandem mass spectrometry; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PPIA, peptidylprolyl isomerase A; SDHA, succinate dehydrogenase complex subunit A; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; STAT3, signal transducer and activator of transcription 3; TOP1, topoisomerase 1; $V_{max}$, maximum reaction rate.

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neurologic development of the fetus (Olivier et al., 2013; Moses-Kolko et al., 2005; Ellfolk and Malm, 2010; Berard et al., 2007; Boukhris et al., 2016; Ramos et al., 2008) and with pregnancy complications such as low birth weight or premature delivery (Wen et al., 2006; Rahimi et al., 2006; Nakhai-Pour et al., 2010; Ramos et al., 2010), whereas other studies have shown no effect of SSRI treatment on fetal and child outcome (Einarson and Einarson, 2005; Alwan et al., 2016). In animal studies, fluoxetine was associated with altered reproductive phenotype and behavioral change in the offspring (Moore et al., 2015; Morrison et al., 2005; Monteiro Filho et al., 2014).

SSRIs block the reuptake of 5-HT by the serotonin transporter (SERT, *SLC6A4*) and increase 5-HT level in the extracellular domain. The serotonergic system, including SERT and 5-HT receptor type 2A ($5\text{-HT}_{2A}$), is expressed and functional in the trophoblast (Bottalico et al., 2004; Viau et al., 2009). SERT are located on the villous trophoblasts (Viau et al., 2009; Ganapathy, 2011), which are exposed to maternal blood containing SSRIs (Loughhead et al., 2006; Hendrick et al., 2003). Using BeWo human choriocarcinoma cell line as study model of the human villous trophoblast, we have previously shown that 5-HT and the $5\text{-HT}_{2A}$ agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) induce the estrogen biosynthetic enzyme aromatase (cytochrome P450 19; CYP19, *CYP19A1*) (Klempan et al., 2011). Thus, the use of SSRIs is of particular concern for their impact on the placental 5-HT and estrogen systems, which are crucial for fetal health.

Placental estrogen synthesis requires feto-placental communication, as the placenta cannot produce estrogens *de novo*. Therefore, we recently developed a co-culture of fetal-like H295R adrenocortical cells and trophoblast-like BeWo cells that reproduces the steroidogenic interactions within the feto-placental unit (Hudon Thibeault et al., 2014). H295R cells represent the fetal compartment by providing androgen precursors, including 16α-hydroxylated androgens that are converted to estrogens in BeWo cells, which express relatively high levels of placental CYP19 (Hudon Thibeault et al., 2014). Disruption of estrogen synthesis by SSRIs may affect several processes during pregnancy, including remodeling of utero-placental arteries, trophoblast invasion and syncytialization of villous trophoblast (Albrecht et al., 2000; Cronier et al., 1999). Indeed, normal pregnancy is characterized by continuous estrogen secretion increase throughout pregnancy and estriol secretion can be used to assess pregnancy health and fetal well-being (Braunstein et al., 2003). However, the effects of fluoxetine in human trophoblast cells have never been studied.

This study aims to characterize the effects of fluoxetine and norfluoxetine on placental estrogen synthesis by CYP19. We hypothesize that the SSRI fluoxetine and its active metabolite norfluoxetine, through their known mechanisms of action on SERT, which results in increased 5-HT levels, induce placental CYP19 and estrogen production.

## 2. Materials and methods

### 2.1. Treatments

Fluoxetine hydrochloride, norfluoxetine hydrochloride, 17β-estradiol, the PKC activator phorbol-12-myristate-13-acetate (PMA) and the irreversible CYP19 inhibitor 4-hydroxyandrostenedione (formestane) were obtained from Sigma Aldrich (Oakville, ON, Canada). Selective PKC inhibitors GF109203X and GÖ6976, as well as the $5\text{-HT}_{2A}$ antagonists, ritanserin and ketanserin tartrate were obtained from Tocris Bioscience (Ellisville, MO). Drugs were dissolved in DMSO except for norfluoxetine, which was dissolved in water. All treatments were adjusted for final DMSO concentrations in culture medium of 0.1% or 0.2% for co-treatments. Physiologically relevant concentrations of fluoxetine and norfluoxetine were used in this study (Hendrick et al., 2003; Sit et al., 2011).

### 2.2. Cell culture

BeWo (CCL-98; ATCC, Rockville, MD) and H295R (CRL-2128; ATCC) cells were cultured as previously described (Hudon Thibeault et al., 2014). Experiments were performed using cells between passages 7 and 25. For the co-culture experiments, we seeded $2.5 \times 10^4$ H295R cells in 0.8 mL in 24-well plates and $1.25 \times 10^4$ BeWo cells in 0.2 mL in transwell inserts (polycarbonate membrane with 0.4 μm pores, Corning Life Sciences, Corning, NY). Cells were maintained for 24 h in their regular culture medium after which the co-cultures were assembled in a co-culture medium (H295R medium supplemented with 1% stripped FBS; Sigma-Aldrich). Co-cultures were treated for 24 h with fluoxetine or norfluoxetine. The monocultures were maintained in the same co-culture medium for the duration of the treatments. The culture media were harvested and kept at −80 °C until analysis. CYP19 activity was determined in 96-well plates using $1.25 \times 10^4$ BeWo cells/well or $2.5 \times 10^4$ H295R cells/well. For protein and RNA extraction, $3.5 \times 10^5$ BeWo cells/well in 6-well plates were used.

### 2.3. Aromatase catalytic activity

CYP19 catalytic activity was determined by tritiated water-release assay according to the method of Lephart and Simpson (1991) adapted by our laboratory (Sanderson et al., 2000). Briefly, after a treatment of 24 h, cells were exposed to 54 nM of 1β-[$^3$H]-androstenedione (Perkin Elmer, Wellesley, MA) in serum-free culture medium (50 μL) for 1.5 h at 37 °C followed by chloroform (40 μL of supernatant + 100 μL of chloroform) and dextran-coated charcoal (20 μL of supernatant + 20 μL of dextran-coated charcoal) extractions. The supernatants (20 μL) were transferred to 96-well flexible microplates (Perkin Elmer) containing 100 μL of Scinti-Verse liquid scintillation cocktail (Thermo Fisher Scientific, Waltham, MA). Tritiated water-release was determined using a Microbeta Trilux counter (Perkin Elmer). The conversion of substrate was linear over time. Specificity of tritiated water-release for the aromatization reaction was assessed using formestane (1 μM) as a positive control for inhibition of CYP19 activity. PMA was used as positive control for CYP19 induction via the protein kinase C (PKC) pathway. For kinetic experiments, cells were exposed to norfluoxetine (1, 3 and 10 μM) for 1.5 h with increasing concentrations of 1β-[$^3$H]-androstenedione (12.5, 25, 50, 100, 250 and 500 nM). Maximum reaction rate ($V_{max}$), Michaelis constant ($K_m$) and inhibition constant ($K_i$) were calculated by non-linear regression using GraphPad Prism (version 5.04; GraphPad Software, San Diego, CA).

### 2.4. RT-qPCR

Total RNA was isolated following a 24 h SSRI treatment using the AllPrep RNA/Protein extraction kit (Qiagen, Toronto, ON, Canada) according to manufacturer instructions. RNA purity ($OD_{260/280}$ ratio ≥ 1.8) and concentration in ng/μL ($OD_{260} \times 40$) were determined using a Nanodrop instrument (Thermo Fisher 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). For SERT mRNA expression (*SLC6A4*), cDNA was preamplified for 12 cycles using SsoAdvanced PreAmp Supermix (Bio-Rad). Reference genes were selected using geNorm software (BioGazelle, Zwijnaarde, Belgium) as previously

described (Lanoix et al., 2012a). Stable reference genes used for qPCR normalization (ΔΔCT), determined with geNorm software, were topoisomerase 1 (*TOP1*), peptidylprolyl isomerase A (*PPIA*) and succinate dehydrogenase complex subunit A (*SDHA*). An annealing temperature of 60 °C was used. Primer sequences are presented in Table 1.

### 2.5. Western blot

Total proteins were extracted as described previously (Lanoix et al., 2012b). Denatured proteins (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis followed by transfer onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% skimmed milk (1 h, room temperature) followed by incubation with a 1/1000 dilution of SERT antibody (1.5 h, room temperature; Abcam, ab181034) and a 1/10,000 dilution of goat anti-rabbit HRP-conjugated antibody (1 h, room temperature; Millipore, AP307P). The band intensities were quantified by densitometry using Image Lab software 5.1 (Bio-Rad). Protein levels were expressed as a ratio of the target band density and total protein stained using MemCode Staining Solution (Thermo Fisher Scientific) as previously described (Lanoix et al., 2012b).

### 2.6. SERT activity

The protocol was adapted from (Qian et al., 1997). Briefly, after 24 h of treatment with fluoxetine, norfluoxetine or 17β-estradiol, the co-culture medium was removed and BeWo cells were washed thrice with phosphate-buffered saline (PBS). Cells were incubated for 2 h at 37 °C in 1 mL (800 μL/well; 200 μL/insert) of Krebs–Ringer-HEPES (KRH) buffer containing an inhibitor of monoamine oxidase, phenelzine sulphate (100 μM; Sigma-Aldrich) and ascorbic acid (100 μM; Thermo Fisher Scientific), with or without fluoxetine (50 μM). Assays were initiated by adding 50 nM tritiated 5-HT creatinine sulphate ([$^3$H]-5-HT, Perkin Elmer) to the insert and terminated after 20 min at 37 °C by three rapid washes with cold PBS. Membranes of the inserts were cut and transferred to 24-well plates containing 200 μL/well of radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL, pH 7.4, 1% nonidet-40, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM EDTA; 3 min; −80 °C). Cell lysates (100 μL) were transferred to 24-well flexible microplates (Perkin Elmer) with 1 mL/well of Scinti-Verse liquid scintillation cocktail. Microplates were incubated overnight at room temperature and tritiated 5-HT accumulation was evaluated using a Microbeta Trilux liquid scintillation counter (Perkin Elmer). Specific 5-HT uptake via SERT was determined by subtracting the amount of [$^3$H]-5-HT accumulated with 50 μM fluoxetine from the amount accumulated without 50 μM fluoxetine.

### 2.7. Hormone quantification

We determined hormone secretion using enzyme-linked immunosorbent assay (ELISA) kits from DRG Diagnostics (Marburg, Germany). Steroids content was also assessed using an independent approach consisting of gas chromatography-mass spectrometry for androstenedione (4-Dione), dehydroepiandrosterone (DHEA), progesterone, estrone and 17β-estradiol and using liquid-chromatography-tandem mass spectrometry (LC-MS/MS) for estriol, as previously described (Caron et al., 2015; Falk et al., 2008).

### 2.8. Statistical analysis

All experiments were performed at least three times. Statistically significant differences ($p < 0.05$) were determined by one-way analysis of variance (ANOVA) followed by a Dunnett *post hoc* test or by two-way ANOVA and Bonferroni *post-hoc* test, depending on the experimental design. If parametric conditions were not met, we used 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. Fluoxetine induces CYP19 activity, but not estrogen secretion by the co-culture

Fluoxetine induced CYP19 activity in H295R cells (1.4-fold at 1 μM) and in BeWo cells (1.6 and 2.3-fold at 1 and 3 μM respectively) (Fig. 1 A, C). Fluoxetine treatment did not modify estrone and 17β-estradiol secretion by H295R and BeWo cells in monoculture neither did it in co-culture, whereas estriol was only increased in H295R monoculture (> 5-fold at 0.3, 1 and 3 μM; Fig. 2A–C). β-hCG and progesterone, markers of trophoblast function (Fig. S1 A, B), as well as DHEA and androstenedione, androgen precursors for estrogen synthesis (Fig. S1 C, D), were not affected by fluoxetine.

### 3.2. Norfluoxetine alters CYP19 activity and decreases estrogen secretion by the co-culture

Norfluoxetine treatment (3 μM) increased CYP19 activity by 1.8-fold in H295R cells (Fig. 1 B) whereas it decreased its activity by by 54% in BeWo cells (Fig. 1 D). In the co-culture, norfluoxetine decreased levels of estrone by 58% and 62% at 1 and 3 μM, respectively, and 17β-estradiol by 51%, 44% and 70%, respectively, compared to vehicle control (Fig. 2D–F). A decrease in DHEA secretion in H295R cells by 35% and 36% at 1 and 3 μM, respectively, was also observed (Fig S1 G). β-hCG, progesterone and androstenedione secretion were not modified after exposure to norfluoxetine (Fig. S1 E, F, H).

### 3.3. Norfluoxetine inhibits CYP19 catalytic activity and mRNA expression in BeWo cells

To better understand the mechanism involved in the opposite effects of fluoxetine and norfluoxetine on placental CYP19 activity and estrogen production, we analyzed whether these SSRIs could

**Table 1**
Primers used in RT-qPCR experiments.

| Gene | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| *CYP19A1* (exon I.1) | GGA TCT TCC AGA CGT CGC GA | CAT GGC TTC AGG CAC GAT GC |
| *CYP19A1* (exons II-X) | TGT CTC TTT GTT CTT CAT GCT ATT TCT C | TCA CCA ATA ACA GTC TGG ATT TCC |
| *SLC6A4* (SERT) | CCC GCC ACA ACT ACG ACT T | CTG TTG GTG TTT CTG GGG TAA T |
| *PPIA* | ACC CGT ATG CTT TAG GAT G | GTT TGC AGA CAA GGT CCC A |
| *SDHA* | TAC AAG GTG CGG ATT GAT G | CGA TCA CGG GTC TAT ATT CAA |
| *TOP1* | CTT AAA GGG TAC AGC GAA TG | GGC GAG TGA ATC ATC TAA GG |

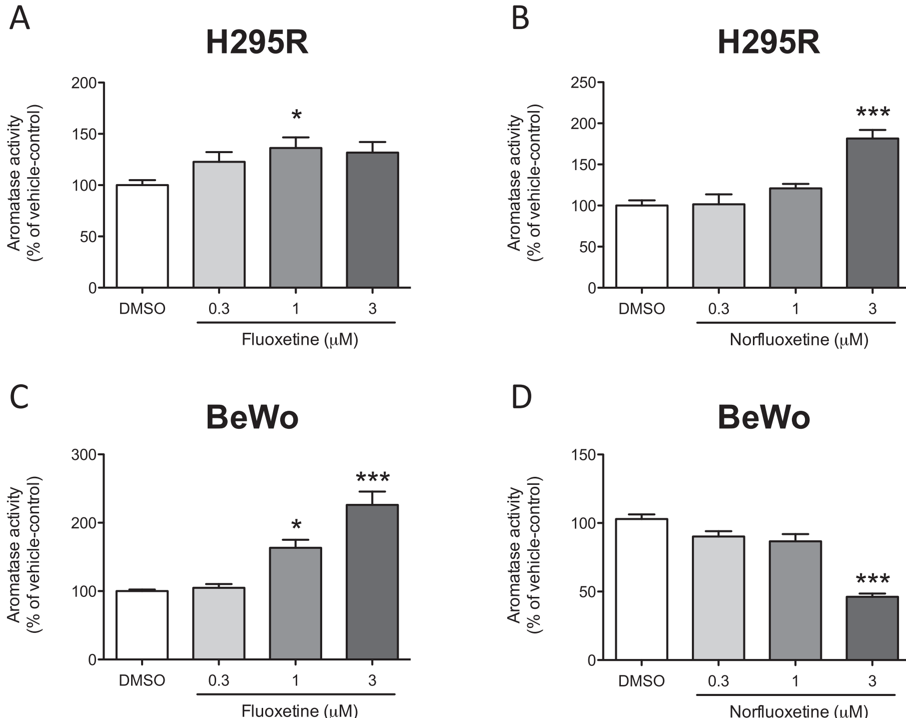



**Fig. 1. Effects of fluoxetine and norfluoxetine on CYP19 activity.** Relative CYP19 activity in (A, B) H295R and (C, D) BeWo cells treated with fluoxetine (0.3, 1 and 3 μM) or norfluoxetine (0.3, 1 and 3 μM) for 24 h. Activities are expressed as percentage of vehicle-control (mean ± SEM). Significant differences compared to vehicle-control (DMSO) are indicated with asterisks (*$P < 0.05$ and ***$P < 0.001$) as determined by a Kruskal-Wallis and Dunn's *post-hoc* test, n = 3–5.

act directly on the catalytic activity of CYP19 in BeWo cells. Norfluoxetine was found to be a catalytic competitive inhibitor of CYP19 with a Ki value of 2.1 μM (Fig. 3 A), while fluoxetine had no catalytic effect (data not shown). Norfluoxetine also decreased *CYP19A1* mRNA expression (via placental promoter I.1) by 40% at 0.3, 1 and 3 μM (Fig. 3 B), while fluoxetine had no effect.

### *3.4. Fluoxetine induces CYP19 activity via activation of the 5-HT$_{2A}$/PKC pathway in BeWo cells*

When BeWo cells were co-treated with the selective 5-HT$_{2A}$ receptor antagonists ritanserin or ketanserin, fluoxetine (3 μM) no longer caused a statistically significant increase of CYP19 activity (Fig. 4 A). The 5-HT$_{2A}$ is a G$_q$ protein-coupled receptor that activates the PLC-PKC signaling pathway, and the PKC inhibitors GF109203X (100 nM) or GÖ6976 (100 nM) also decreased fluoxetine-induced CYP19 activity in BeWo cells (Fig. 4 B).

### *3.5. The co-culture and 17β-estradiol inhibit SERT activity in BeWo cells*

As expected, in BeWo monoculture, fluoxetine and norfluoxetine decreased SERT activity by 70% and 86%, respectively (Fig. S2). Fluoxetine also increased *SLC6A4* mRNA (1.4-fold at 0.3 and 1 μM) and protein (2.6-fold at 0.3 μM) levels compared to control (Fig. S3 A, B), whereas norfluoxetine had no statistically significant effect (Fig. S3 C, D). Interestingly, in co-culture, SERT activity in BeWo cells was 67% lower than in monoculture (Fig. S2). Considering the high levels of estrogens in the co-culture medium, we determined the effect of 17β-estradiol in BeWo monocultures and observed a significant 82% decrease in SERT activity at 1 nM of 17β-estradiol compared to control (Fig. 5 A). SERT protein levels in BeWo cells treated with 17β-estradiol (3 nM) were significantly higher than control (2.7-fold; Fig. 5 C), whereas no effect on *SLC6A4* mRNA transcript levels was observed (Fig. 5 B).

## 4. Discussion

Fluoxetine and its active metabolite norfluoxetine are known to cross the placental barrier (Rampono et al., 2009), exposing the placenta and the fetus to these molecules. In this study we determined the effects of fluoxetine and norfluoxetine on placental CYP19, estrogen secretion and SERT in a co-culture system that represents steroidogenesis of the feto-placental unit.

### *4.1. Effects of fluoxetine and norfluoxetine on CYP19 activity and estrogen production in the feto-placental co-culture*

In both H295R and BeWo cells, fluoxetine induced CYP19 activity, with no statistically significant effect on levels of estrogen secretion. In the co-culture system, estrogen synthesis is relatively high, which may explain why further induction of CYP19 activity by fluoxetine in BeWo cells no longer results in increased estrogen levels. Fluoxetine is converted to norfluoxetine by hepatic

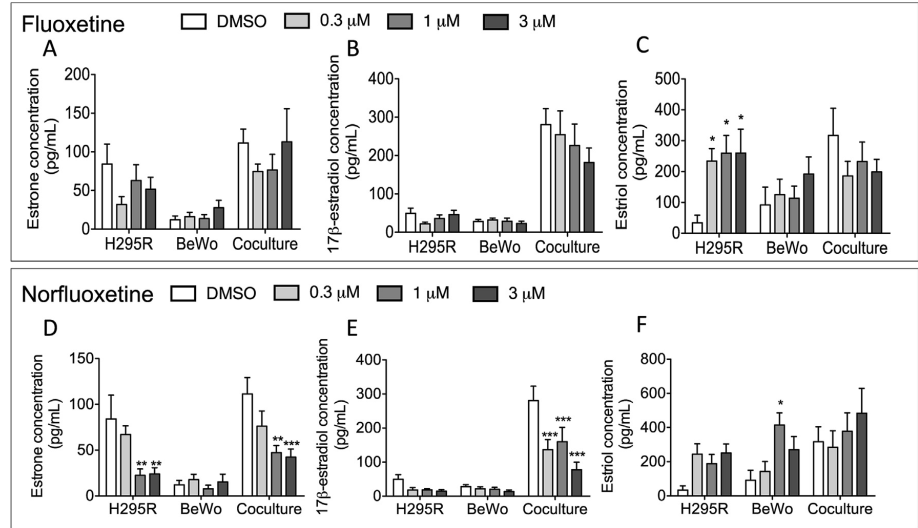
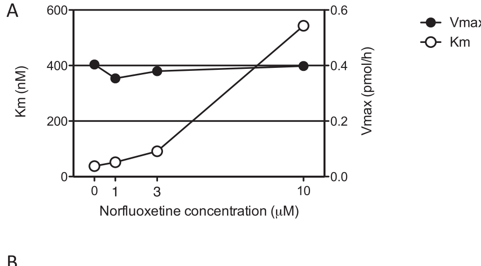



**Fig. 2. Fluoxetine and norfluoxetine disrupt estrogen secretion**. Secretion of (A, D) estrone, (B, E) 17β-estradiol and (C, F) estriol by H295R cells, BeWo cells and by the H295R/BeWo co-culture following a 24 h exposure to various concentrations of fluoxetine (A, B, C) or norfluoxetine (D, E, F). Concentrations (pg/mL) are shown as means ± SEM. Statistically significant differences compared to vehicle-controls (DMSO) are indicated with asterisks (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$) as determined by two-way ANOVA and Bonferroni *post-hoc* test, n = 3–5.



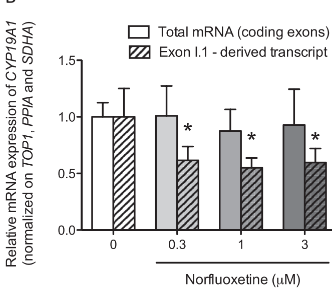




**Fig. 3. Norfluoxetine inhibits the catalytic activity and mRNA expression of CYP19 in BeWo cells**. Michaelis constant (Km, nM) and maximum reaction rate (Vmax, pmol/h) (A) in BeWo cells following 1.5 h of treatment with norfluoxetine (1, 3 and 10 μM) and with different concentration of 1β-[$^3$H]-androstenedione substrate (12.5, 25, 50, 100, 250 and 500 nM), n = 3. mRNA expression of the coding exons II-X and non-coding exon I.1 (B) in BeWo cells treated with norfluoxetine (0.3, 1 and 3 μM). Results are normalized for reference genes *TOP1*, *PPIA* and *SDHA* (mean ± SEM). Significant differences compared to control are indicated with asterisks. (* $P < 0.05$. one-way ANOVA and Dunnett's *post-hoc* test, n = 3–5.) *TOP1*: topoisomerase (DNA) I, *PPIA*: peptidylprolyl isomerase A and *SDHA*: succinate dehydrogenase complex flavoprotein subunit A.

cytochrome P450 enzymes, mainly CYP2D6. CYP2D6 is expressed at the mRNA level in human placenta tissue, as well as in the fetal liver, but given the low levels of its catalytic activity, it would contribute only slightly to overall metabolism (Hakkola et al., 1998). It is not known if CYP2D6 is catalytically active in BeWo and H295R cells, but we have observed that the enzyme is expressed at the mRNA level in both cell lines (data not shown). In our co-culture model, fluoxetine is weakly converted to norfluoxetine (0.2%), suggesting that the observed effects are caused by fluoxetine and not norfluoxetine (Fig. S4). For this reason, we also studied the effect of norfluoxetine separately. In the feto-placental co-culture system, norfluoxetine decreased estrone and 17β-estradiol secretion, without affecting estriol secretion. Norfluoxetine decreased DHEA secretion by H295R cells in monoculture, but did not significantly alter DHEA levels in the co-culture, suggesting that altered DHEA production is unlikely to be a contributing factor in the observed decrease in estrogen production in the co-culture. Since estriol is not affected by norfluoxetine, it is possible that norfluoxetine stimulates the 16α-hydroxylation of androgen precursors, which would occur uniquely in the H295R compartment. Indeed, norfluoxetine treatment resulted in a greater ratio of estriol to total estrogens in co-culture. The implications of this increased ratio remain to be studied in our model. Each estrogen has a different affinity for estrogen receptors and changes in the ratio of estriol to the other estrogens would alter the profile of dimerized estrogen receptors that are produced (more dimerized estrogen receptors containing estriol), which may result in different 'estrogenic' potencies and actions (Sasson, 1991).

Interestingly, norfluoxetine decreased CYP19 activity in BeWo cells, but increased it in H295R cells in monoculture. In the co-culture system, we have previously shown that the level of estrogen production is mainly dependent on CYP19 activity in BeWo cells, which is about 15 times greater than that in H295R cells (Hudon Thibeault et al., 2014). The dominance of CYP19 activity in BeWo cells likely explains why we observe a net decrease of estrone

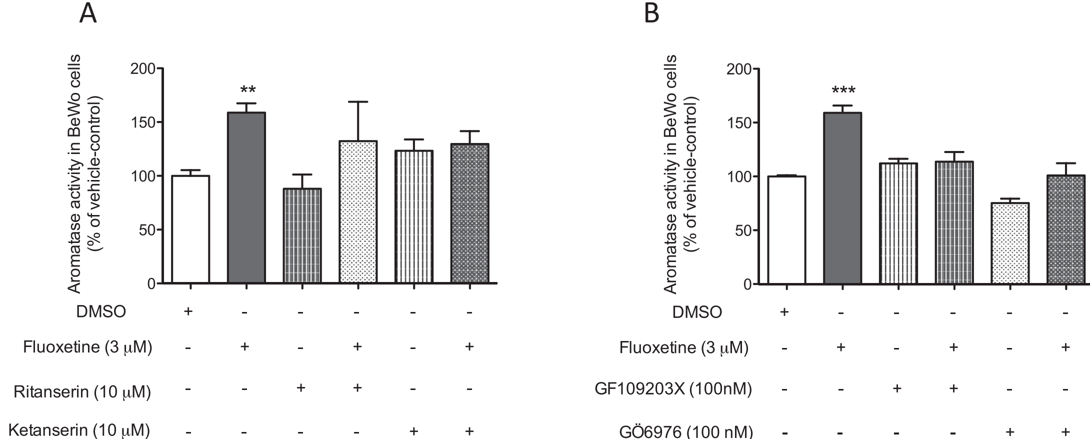



**Fig. 4. Fluoxetine-induced CYP19 activity involves 5-HT$_{2A}$ receptor and PKC pathway**. Relative CYP19 activity in BeWo cells treated with fluoxetine (3 μM) with or without ritanserin (10 μM) and ketanserin (10 μM) (A) or with or without GF109203X (100 nM) and GÖ6976 (100 nM) (B). Results are shown as mean percentage of vehicle-control (DMSO) ± SEM. Significant differences compared to control are indicated with asterisks (**$P < 0.01$, ***$P < 0.001$) as determined by one-way ANOVA and Dunnett's *post-hoc* test, n = 5–6.

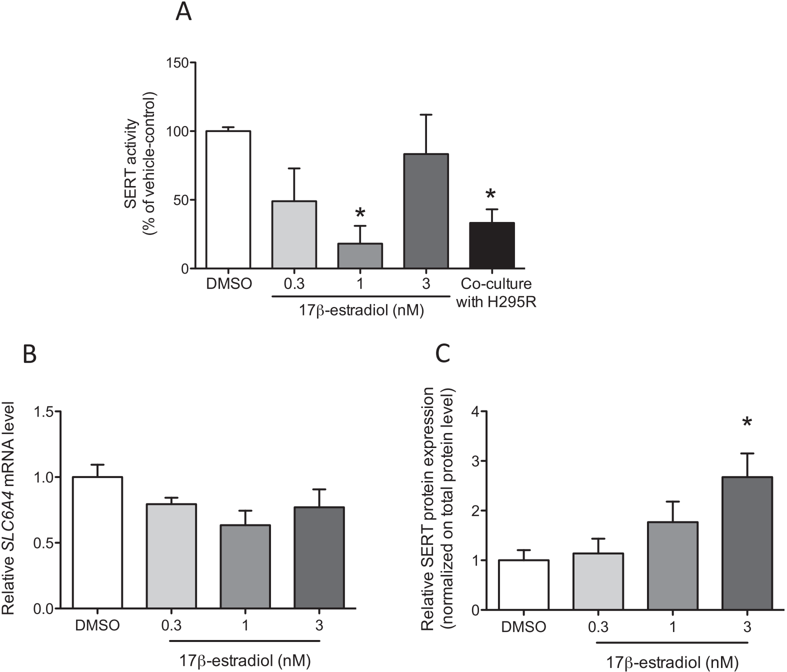



**Fig. 5. 17β-estradiol decreases SERT activity, while increasing protein expression in BeWo cells**. SERT activity in BeWo monoculture treated with 17β-estradiol (0.3, 1 and 3 nM) or in co-culture with H295R cells (A). Results are shown as percentage of basal SERT activity in BeWo cells in monoculture (mean ± SEM). *SLC6A4* mRNA (B) and protein (C) expression in BeWo cells treated with 17β-estradiol (0.3, 1 and 3 nM). Results are shown as means ± SEM. Significant differences compared to control are indicated with asterisks (*$P < 0.05$) as determined by ANOVA and Dunnett's *post-hoc* test or Kruskal-Wallis and Dunn's *post-hoc* test, n = 3–6.

and 17β-estradiol secretion in the co-culture. The difference in the effect of norfluoxetine on CYP19 activity in BeWo and H295R cells is somewhat surprising, since the CYP19 protein and its catalytic activity are considered to be identical, regardless of the promoter utilized for its expression (Demura et al., 2008). However, certain post-translational steps in the production of catalytically active

CYP19, such as phosphorylation (Hayashi and Harada, 2014) or heme synthesis and insertion of the iron-containing catalytic center, could be altered in a cell-specific manner, thus affecting final CYP19 activity. This remains to be further studied in our model.

### *4.2. Effects of fluoxetine and norfluoxetine on catalytic activity and mRNA expression of CYP19 in BeWo cells*

To better understand the complex effects of fluoxetine and norfluoxetine in BeWo cells, we explored *CYP19A1* at the level of transcription and catalytic activity. We found that norfluoxetine is a competitive inhibitor of CYP19 activity, whereas fluoxetine had no effect. The transcriptional regulation of *CYP19A1* is complex with several alternate promoters in the non-coding exon I region involved in its tissue-specific expression (Demura et al., 2008). In placenta, *CYP19A1* transcripts are mainly derived from the promoter associated with the non-coding exon I.1, which is activated by various signaling pathways including PKC (Demura et al., 2008; Kamat and Mendelson, 2001). In H295R cells, *CYP19A1* transcripts are derived from promoters PII and I.3 (Watanabe and Nakajin, 2004). Thus, changes in *CYP19A1* expression by fluoxetine and norfluoxetine could involve several different mechanisms. Fluoxetine did not affect *CYP19A1* mRNA expression. This suggests that fluoxetine may affect CYP19 activity by acting on a promoter located elsewhere on the multi-promoter-containing *CYP19A1* non-coding region. In contrast, a decrease in exon I.1-derived transcript of *CYP19A1* with norfluoxetine treatment was observed, whereas total mRNA (coding region) was not affected. Hence, norfluoxetine has a double mode of action: a direct inhibition of catalytic activity and a longer-term downregulation of mRNA expression.

### *4.3. 5-HT$_{2A}$/PKC pathway and the effect of fluoxetine on CYP19 activity*

We have previously shown that activation of the 5-HT$_{2A}$ receptor induces CYP19 activity and mRNA expression in BeWo cells (Klempan et al., 2011a). Our findings confirm that induction of CYP19 activity by fluoxetine involves activation of the 5-HT$_{2A}$ receptor and the subsequent downstream PKC signaling pathway. This is consistent with the fact that PKC is a major regulator of placental *CYP19A1* expression via its I.1 promoter (Harada et al., 2003), which we have previously reported to be involved in the downstream signaling of 5-HT$_{2A}$ in trophoblast cells (Oufkir and Vaillancourt, 2011). This suggests that 5-HT$_{2A}$ is either stimulated directly by fluoxetine, as binding of fluoxetine to the 5-HT$_{2A}$ receptor was previously observed (Owens et al., 1997) or indirectly by an increase of 5-HT, possibly via SERT inhibition (Fig. S5A). This should be further investigated, as well as the role of the janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway, which we have shown is also associated with 5-HT$_{2A}$ receptor activation in BeWo cells (Oufkir and Vaillancourt, 2011).

### *4.4. 17β-estradiol inhibits placental SERT activity*

Fluoxetine and norfluoxetine do not further reduce SERT activity in BeWo cells in co-culture with H295R cells, because SERT activity is already reduced under the co-culture conditions relative to BeWo cells in monoculture (Fig. S2). Considering the synergistic production of estrogens in the co-culture, especially of 17β-estradiol, we tested whether 17β-estradiol would reduce SERT activity in BeWo cells in monoculture, which was the case. In contrast, SERT protein levels were increased. An increase in peripheral SERT protein levels following 17β-estradiol treatment was also reported in a human pulmonary arterial smooth muscle cell line and in rat and primate brain (White et al., 2011; Attali et al., 1997; Sanchez et al., 2013). The discrepancy between the effect of 17β-estradiol on SERT activity (decrease) and on protein level (increase) may be explained by disruption of "transporter trafficking" from the intracellular compartment to the cell membrane, which is notably regulated by PKC (reviewed in Lau and Schloss, 2012). Moreover, it has been shown in a rat brain cell line (RN46A) that estrogens can act via two modes of action: (1) the genomic pathway, involving estrogen receptors, which could affect SERT expression and (2) the non-genomic pathway, which allows rapid effects on the regulation of SERT activity by increasing intracellular calcium levels (Koldzic-Zivanovic et al., 2004). Time- and dose-response experiments will improve our understanding of these processes.

Together our findings point at several mechanisms of action whereby fluoxetine and norfluoxetine may disrupt the regulation of estrogen synthesis in the trophoblast (Fig. S5). This portrait is more complex than what we had first hypothesized; we observed that even though fluoxetine induces CYP19 via the 5-HT$_{2A}$ and PKC pathway, it does not alter estrogen synthesis in the co-culture. In contrast, norfluoxetine inhibits CYP19 activity as well as estrogen synthesis. Our results lead us to question whether 5-HT is involved in the observed effects on CYP19 activity and how 17β-estradiol can act on 5-HT levels.

## 5. Conclusion

The present study shows that fluoxetine induces whereas its active metabolite norfluoxetine inhibits placental CYP19 in BeWo cells and that norfluoxetine alters estrogen production in a co-culture model of feto-placental steroidogenesis. Estrogens, in turn, regulate SERT (expression and activity) resulting in the same effects as those of SSRIs on 5-HT transport in a co-culture model of feto-placental steroidogenesis. These data will need to be validated using primary culture of villous trophoblast. Since SSRIs, such as fluoxetine, are commonly prescribed for depression during pregnancy, it is essential to better understand the prenatal effects of these compounds, while also taking into account the deleterious effects of depression *per se* during pregnancy. Considering the importance of estrogens throughout the progress of pregnancy, it is crucial to better understand the interaction between estrogens and SERT in the placenta.

## 6. Funding and disclosure

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.11.021.

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## 10.3 Résultats supplémentaires

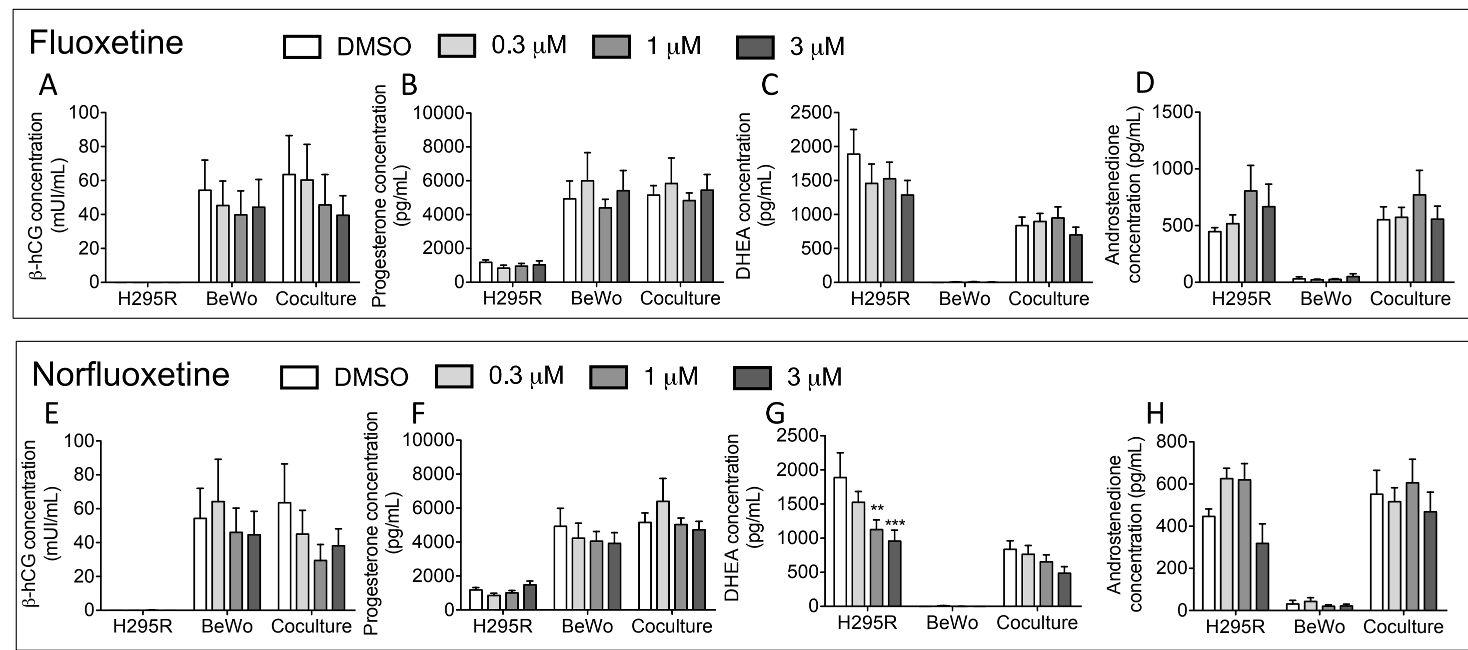



**Figure S1. Effects of fluoxetine and norfluoxetine on β-hCG, progesterone and androgen precursors secretion.** Secretion of (A, E) β-hCG, (B, F) progesterone, (C, G) dehydroepiandrosterone (DHEA) and (D, H) androstenedione in the H295R cells, BeWo cells and in the H295R/ BeWo co-culture following (A, B, C, D) fluoxetine (0.3, 1 and 3 $\mu$M) or (E, F, G, H) norfluoxetine treatments (0.3, 1 and 3 $\mu$M). Results are shown as mean concentration (pg/mL) ± SEM. Significant differences compared to vehicle-control (DMSO) are noted * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Two-way ANOVA followed by Bonferroni post-hoc test, n = 3-5.

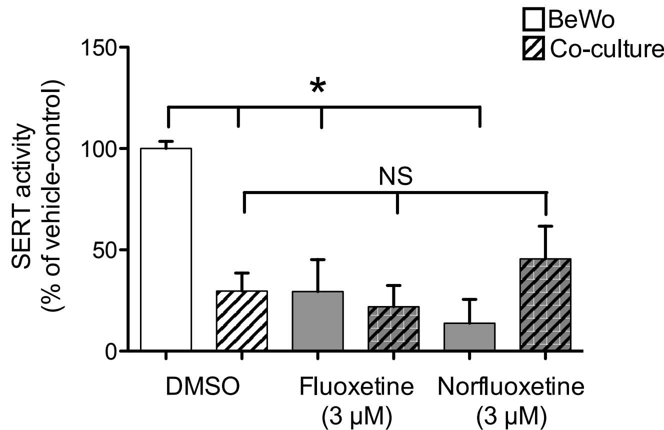



**Figure S2. SERT activity in the H295R/BeWo co-culture is not affected by fluoxetine and norfluoxetine.** SERT activity in BeWo (unhashed) or in the co-culture (hashed) treated with fluoxetine (3 $\mu$M) and norfluoxetine (3 $\mu$M). Results are shown as percentage of control (BeWo monoculture) ± SEM. Significant differences compared to BeWo monoculture vehicle-control (DMSO) are noted * $P < 0.05$. Kruskal-Wallis and Dunn's *post-hoc* test, n=3-9.

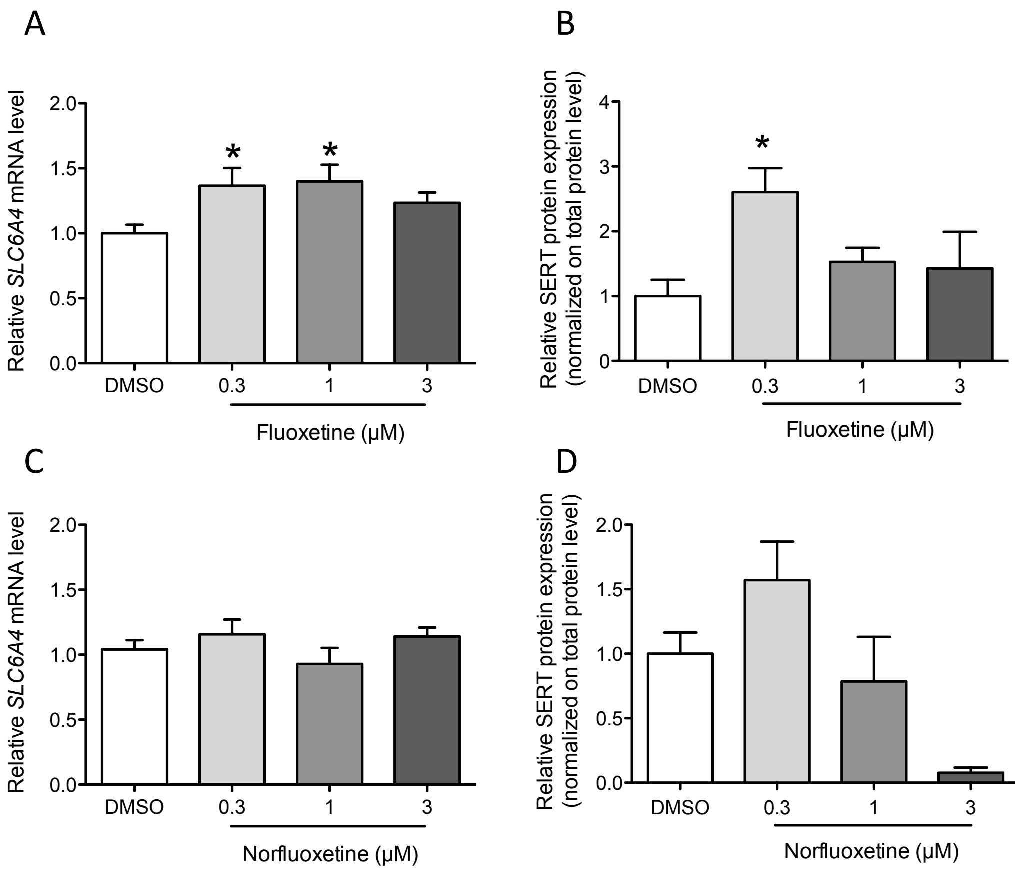



**Figure S3. Fluoxetine, but not its metabolite norfluoxetine, increases SERT expression in BeWo cells.** SERT (*SLC6A4*) (A, C) mRNA and (B, D) protein levels in BeWo cells treated with (A, B) fluoxetine (0.3, 1 and 3 $\mu$M) and (C, D) norfluoxetine (0.3, 1 and 3 $\mu$M). Results are shown as means ± SEM. Significant differences compared to vehicle-control (DMSO) are noted * $P < 0.05$. ANOVA and Dunnett's *post-hoc* test or Kruskal-Wallis and Dunn's *post-hoc* test, n=4-11.

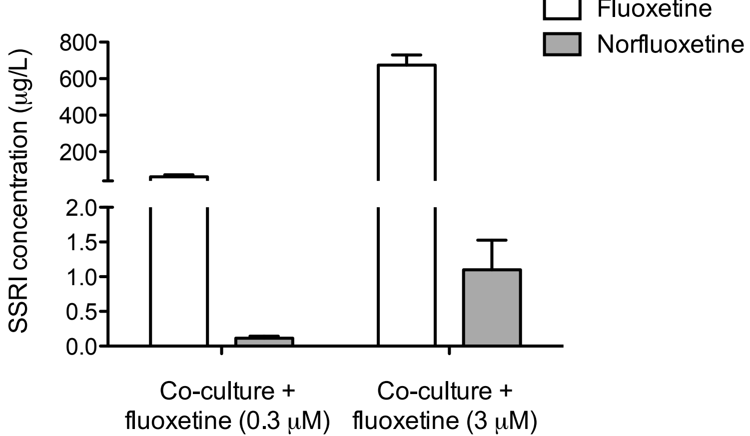



**Figure S4. Fluoxetine is weakly converted to norfluoxetine in the co-culture model.** Fluoxetine and norfluoxetine concentrations were determined in the co-culture medium after 24 h of treatment with 0.3 or 3 $\mu$M fluoxetine using LC-MS/MS analysis. Briefly, the system consisted of an HTC thermopal autosampler (CTC analytics AG, Zwingen, Switzerland) with a 50 $\mu$L loop and a liquid chromatography tandem mass spectroctrometry system. The elution was achieved using a quaternary pump Accela 1250 (Thermo Fisher Scientific, San Jose, CA) and the chromatographic separation performed with a Hypersil Gold column (100 mm x 2.1 mm, 1.9 $\mu$m particle size, Thermo Fisher Scientific) kept at 50 °C in a thermostated column compartment. The aqueous mobile phase (A) consisted of HPLC-water and the organic mobile phase (B) of methanol with 0.1% formic acid in both. Details on chromatographic gradient elution conditions are described in Table S1. A Triple Quadrupole TSQ Quantiva Mass Spectrometer (Thermo Fisher Scientific) with a Heated Electrospray Ionisation (HESI) source was used for detection and quantification. The mass spectrometer was operated in selected reaction monitoring (SRM) mode and the ionization achieved in positive mode. The initial compound-dependent parameters for MS and MS/MS optimization conditions are presented in Table S2. The source-dependent parameters were as follows: spray voltage (2100 V), vaporizer temperature (400 °C), sheath gas pressure (45 arbitrary units), auxiliary gas (15 arbitrary units), ion sweep gas pressure (0 arbitrary units) and capillary temperature (350 °C). The dwell time was set at 20ms. The first and third quadrupole were operated at 0.7 Da FWHM and the collision gas pressure of the second quadrupole was set at 1.5 mTorr. Results are shown as mean concentration ($\mu$g/L) ± SEM. n=3

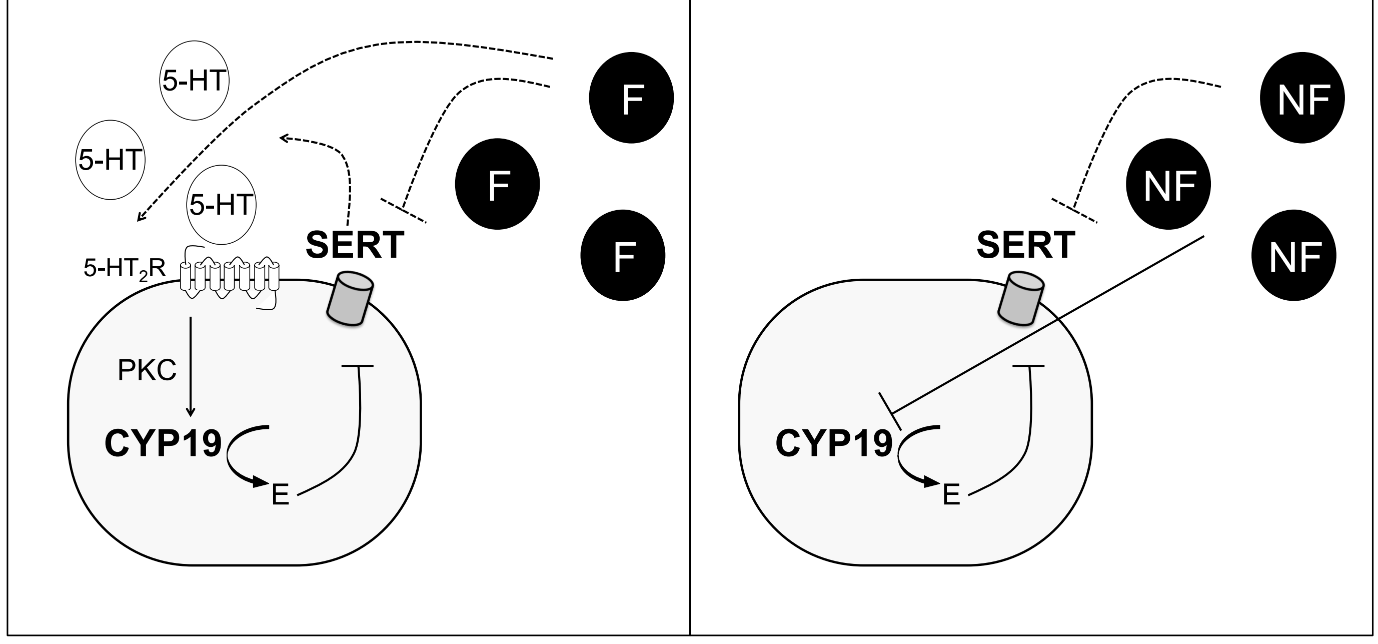



**Figure S5. Schematic representation of a proposed mode of action of fluoxetine and norfluoxetine on CYP19, estrogen production and SERT activity in BeWo cells.** (A) Fluoxetine stimulates 5-$HT_{2A}$ either by inhibiting SERT and increasing serotonin (5-HT) extracellular levels or by stimulating directly 5-$HT_{2A}$ and then PKC pathway is stimulated to induce CYP19 activity. In turn, estrogens inhibit SERT activity. (B) Norfluoxetine inhibits CYP19 catalytic activity, inducing a decrease in estrogen secretion. Solid lines represent the observations from this study. F: fluoxetine, NF: norfluoxetine, SERT: serotonin transporter, 5-HT: serotonin, 5-$HT_{2A}$: serotonin receptor type 2, PKC: protein kinase C, CYP19: aromatase, E: estrogens.

**Table S1: Chromatographic gradient conditions**

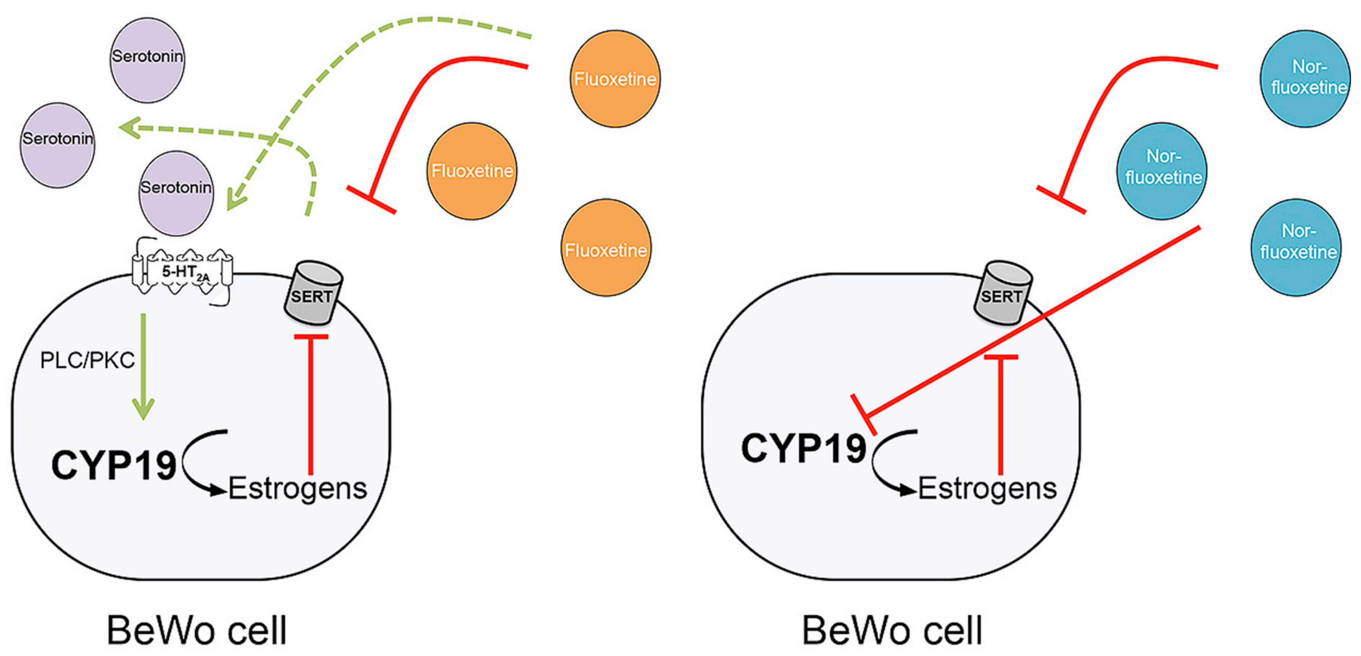
| Time (min) | % A | % B | Flow rate (µL/min) |
|---|---|---|---|
| 0 | 90 | 10 | 500 |
| 5 | 0 | 100 | 500 |
| 6 | 0 | 100 | 500 |
| 6.1 | 90 | 10 | 500 |
| 8 | 90 | 10 | 500 |

**Table S2 : MS/MS optimized parameters for selected compounds**

| Compound | Precursor ion (m/z) | Product ion (m/z) | Intensity ratio (%) | RF Lens (V) | CE (V) |
|---|---|---|---|---|---|
| Fluoxetine | 310<br>$[M+H]^+$ | 44<br>148 | 100<br>24 ± 3 | 43<br>43 | 12<br>10 |
| Norfluoxetine | 296<br>$[M+H]^+$ | 134<br>30 | 100<br>15 ± 2 | 30<br>30 | 10<br>10 |
| CBZ* | 247 | 204 | - | 54 | 23 |

## 10.4 Résumé graphique

Proposed mode of action of the antidepressant fluoxetine on estrogen synthesis in human placental BeWo cells. Fluoxetine stimulates serotonin 5-$HT_{2A}$ receptor either by inhibiting serotonin transporter (SERT) and increasing serotonin (5-HT) extracellular levels or by stimulating directly 5-$HT_{2A}$ and then phospholipase C/protein kinase C (PLC/PKC) pathway to induce aromatase activity. In turn, estrogens inhibit SERT activity. Norfluoxetine inhibits aromatase catalytic activity, decreasing estrogen secretion.



# 11 PERTURBATION DE L'AROMATASE PLACENTAIRE PAR LES INHIBITEURS SÉLECTIFS DE LA RECAPTURE DE LA SÉROTONINE

Titre complet : Serotonin reuptake inhibitors alter placental aromatase

Titre en français : Les inhibiteurs de la recapture de la sérotonine altèrent l'aromatase placentaire

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**Contribution de l'étudiante** : L'étudiante a participé à l'élaboration de l'étude, complété toutes les expériences *in vitro*. Elle a également analysé les résultats et rédigé l'article.

**Contribution des coauteurs** : Yossef Lopez de Los Santos a réalisé les simulations moléculaires sous la supervision de Nicolas Doucet. Cathy Vaillancourt et Thomas Sanderson ont participé à l'élaboration de l'étude incluant la planification des expériences, l'analyse des résultats et la correction du manuscrit.

## 11.1 Résumé de l'article en français

Les inhibiteurs de la recapture de la sérotonine (IRS) sont actuellement les principales molécules prescrites aux femmes enceintes qui souffrent de dépression. Les cellules placentaires sont exposées aux IRS par le sang maternel et nous avons précédemment démontré qu'ils peuvent altérer la stéroïdogenèse fœto-placentaire dans un modèle de co-culture *in vitro*. Plus précisément, la sérotonine (5-HT) régule l'enzyme de synthèse des estrogènes aromatase (cytochrome p450 19; CYP19), qui est perturbée par la fluoxétine et son métabolite actif norfluoxétine dans les cellules de choriocarcinome BeWo. Basé sur des simulations moléculaires, cette étude démontre que les IRS fluoxétine, norfluoxétine, paroxétine, sertraline, citalopram et venlafaxine possèdent un affinité de couplage pour le site actif du CYP19, suggérant une possible inhibition compétitive. En utilisant la lignée cellulaire BeWo et des primocultures de trophoblastes villeux isolés de placentas normaux à terme, nous avons comparé les effets de différents IRS sur l'activité du CYP19. Nous avons observé que l'altération de l'activité du CYP19 par les IRS dépend de la structure de la molécule, de la concentration et du modèle d'étude. Nous avons aussi démontré que la 5-HT et l'agoniste du récepteur 5-$HT_{2A}$, 2,5-dimethoxy-4-iodoamphétamine (DOI), induisent l'activité du CYP19. Une augmentation de la phosphorylation des sérines et tyrosines et une diminution de la phosphorylation des thréonines de CYP19 ont aussi été associées avec un traitement au DOI. Nos résultats contribuent à mieux comprendre comment la 5-HT et les IRS interagissent avec le CYP19 et peuvent affecter la production d'estrogènes. De plus, cette étude suggère que l'altération de la 5-HT placentaire par la dépression et/ou les traitements aux IRS pendant la grossesse pourrait être associée avec une perturbation de la synthèse des estrogènes placentaires.

## 11.2 Article

Serotonin reuptake inhibitors alter placental aromatase

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**Running title:** *Antidepressants disrupt pregnancy hormones*

** These authors share joint senior authorship and contributed equally to this work*

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**Keywords:** Antidepressant; estrogen; trophoblast; CYP19; phosphorylation

## 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 feto-placental 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 the effects of the SRIs on CYP19 activity depend on their molecular structure, concentration and the study model. 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-HT levels due to depression and/or SRI treatment during pregnancy may be associated with disruption of placental estrogen production.


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, which is specifically associated with estrogen withdrawal following delivery (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 behaviours (12-15). Doctors 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 steroidogenesic 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. Understanding the underlying molecular mechanisms for these differences could contribute to a better decision making process for using the appropriate SRI(s) during pregnancy (27).

In this study, we compared the effects of several SRIs and a 5-$HT_{2A}$ receptor agonist on placental

CYP19 activity. We also compared how effects on CYP19 activity in BeWo cells relates to the more physiologically relevant model of primary villous trophoblast cells. We showed that SRIs all had a binding affinity for the active-site pocket of CYP19, but with different effects on CYP19 activity, dependent on the molecule, the concentration and the study model. Moreover, we validated that 5-HT, throughout activation of the 5-$HT_{2A}$ receptor, increased CYP19 activity in primary villous trophoblast cells.

## RESULTS

### *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 (kcal*$mol^{-1}$) 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 S1). 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).

### *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 mM 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).

### *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 S2). 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).

***Functional effects of putative phosphorylation sites in human CYP19***

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 (28). 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 (29,30). 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).

## DISCUSSION

***SRIs are potential competitive inhibitors 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, consistent with previous observations obtained with distinct SRIs in a CYP19 inhibition assay using supersomes (order of inhibition: sertraline > paroxetine > fluoxetine > citalopram) (31). However, these assays do not take into consideration the different capacities of SRIs to penetrate the cell and access the CYP19 enzyme. In our *in vitro* assay, competitive inhibition in only allowed when the SRI reaches the cytoplasm. It was suggested that SRIs could be transported using membrane transporters (32), which could explain some difference of effects between SRIs and between cell models.

***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 (31,33). We found no effect of citalopram on CYP19 activity in BeWo cells, although others found an induction of CYP19 activity in H295R cells (31,33). This could be due to the higher concentrations used in these assays (10-50 μM) (31,33) or by the 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 (34). These effects are consistent with the observed affinity of SRIs for the active-site pocket of CYP19.

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 (35).

Our group has studied the effects of SRIs on various parameters of villous cytotrophoblasts differentiation in BeWo and primary trophoblast cells (36). We observed that SRIs increase cell fusion and markers of villous cytotrophoblasts differentiation (36). The increased CYP19 activity by fluoxetine, paroxetine, and sertraline observed in BeWo cells is consistent with an increase in syncytialization (36). We chose to work with villous cytotrophoblasts in primary culture for 24 h, when they are mainly undifferentiated (37). This allows for a better comparison with BeWo cells, which do not spontaneously differentiate in culture (38). 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 (36).

Consistent with our current findings, overwhelming evidence now advocates for improved scrutiny when cell lines are being used to study normal trophoblast function (39). The response of BeWo cells to various treatments are often not reproduced in primary villous trophoblast cells (36,40,41). 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 (42)). 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 (43,44). Moreover, estrogen receptors (ERs) and miRNAs regulate *CYP19A1* and may have altered expression in the BeWo choriocarcinoma cell line (44-46).

***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 (47-50) or stimulate ER (51,52). 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 (25,53). This suggests that the observed differences in effects among the SRIs may be attributable to secondary effects rather than the inhibition of SERT.

***Serotonin and DOI induce aromatase activity in primary villous trophoblast cells***

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 (29,54,55). 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. 6), which was previously shown to regulate its activity (30,56-59). Our molecular modelling of previously confirmed phosphorylation sites S118 and Y361 (29,30) 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 (60-62). 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 (57), 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).

## CONCLUSION

In conclusion, we show that SRIs have a dual effect on CYP19, *i.e.* a direct inhibitory potential and an indirect effect on the regulation of CYP19 activity. 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.

## EXPERIMENTAL PROCEDURES

### *Cell culture*

BeWo cells were obtained from American Type Culture Collection (CCL-98, Manasses, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 without phenol red, supplemented with 0.6 g/L sodium bicarbonate ($NaHCO_3$) (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 ($CO_2$) at 37°C. Cells were seeded in 96-well plate (1.25 x $10^4$ cells/well in 200 μL).

### *Isolation of human villous cytotrophoblast cells*

The cytotrophoblast cell population from placentas at term (37-41 weeks) of uncomplicated pregnancies was isolated using trypsin-DNase/Percoll and purified as previously described (63) from an adapted protocol of Kliman *et al.* (64). Cells were then cultured in DMEM-high glucose (ThermoFisher Scientific, Burlington, ON) 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% $CO_2$ at 37°C. Cells were seeded in 96-well plates (1.25 x $10^5$ cells/well) for CYP19 activity assay and in 24-well plates (1 x $10^6$ 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.

### *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. 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.

***Treatments***

Serotonin (5-HT hydrochloride), 2,5-dimethoxy-4-iodoamphetamine (DOI), a selective $5\text{-HT}_{2A}$ 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 (18,65). Concentrations used are based on pharmacologically relevant concentrations found in maternal and cord blood of women treated with SRIs (17,19). Concentrations used were non-cytotoxic, as determined by measuring cell impedance with xCELLigence™ RTCA SP instrument (ACEA Biosciences, San Diego, CA) in BeWo cells (36) 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%.

***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 (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 1β-[$^3$H]-androstenedione (Perkin Elmer, Wellesley, MA) in serum-free culture medium (50 μL) for 1 h at 37°C followed by chloroform (40 μL of supernatant + 100 μL of chloroform) and dextran-coated charcoal (20 μL of supernatant + 20 μL of dextran-coated charcoal) extractions. The supernatants (20 μL) were transferred to 96-well flexible microplates (Perkin Elmer) with 100 μL 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 μM) as a positive control for CYP19 inhibition.

***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 ≥ 1.8) and concentration in ng/μL ($OD_{260}$ x 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) (66), were used for qPCR normalization ($^{\Delta\Delta}$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.

***Protein extraction and immunoprecipitation***

Total proteins were extracted from frozen placental explants and quantified using a BCA protein assay (ThermoFisher Scientific), as described previously (67). 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).

***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-phosphotysorine 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 (67).

***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 (68). 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 (69). 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 S1). 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 (70).

***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 (28). 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 (29,30). The Vienna-PTM web server was used to build CYP19 models that mimic single amino acid phosphorylation

states (71). 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.

***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).

**CONFLICT OF INTEREST:** The authors declare that they have no conflicts of interest with the contents of this article.

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**FOOTNOTES**

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Table 1. Primer pair sequences used for the amplification of *CYP19A1* transcript and reference genes.

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

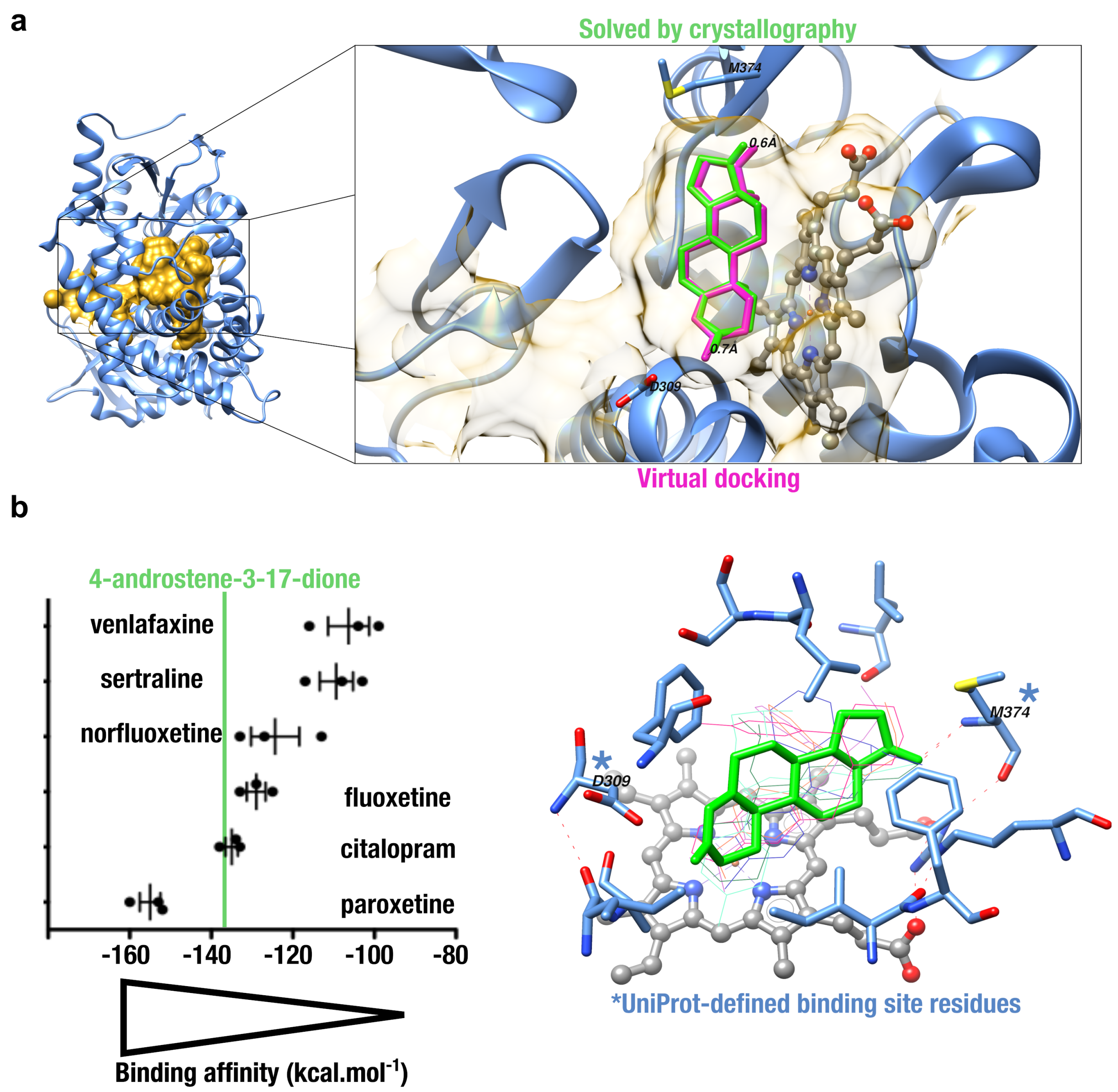
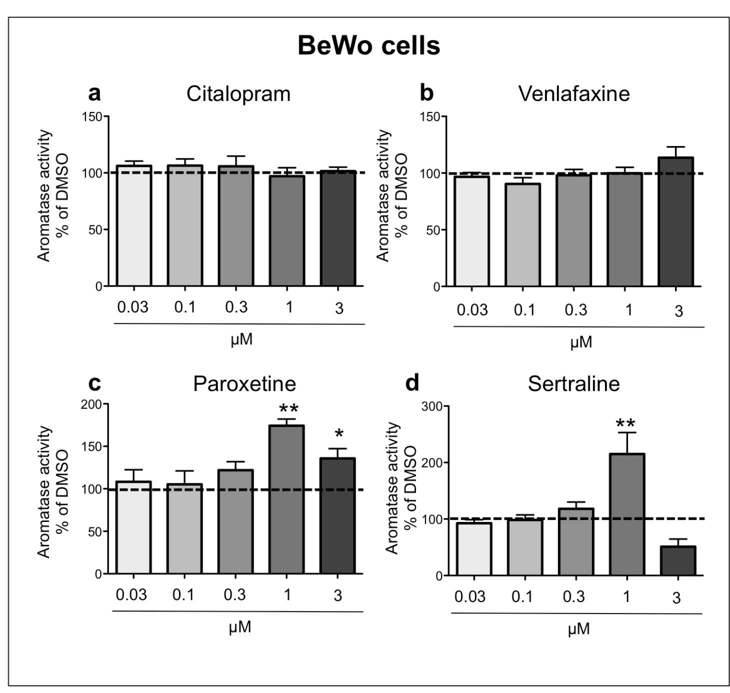



**Figure 1** Molecular docking simulations of serotonin reuptake inhibitors (SRIs) inside the active-site pocket of CYP19 human aromatase. (a) Comparison between the crystallographically resolved aromatase (CYP19)-androstenedione enzyme-substrate complex (PDB 5JL6) and molecular docking predictions. 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 crystalographically resolved androstenedione substrate is shown in green. UniProt-defined CYP19 binding site residues D309 and M374 are highlighted by asterisks (UniProt entry P11511)



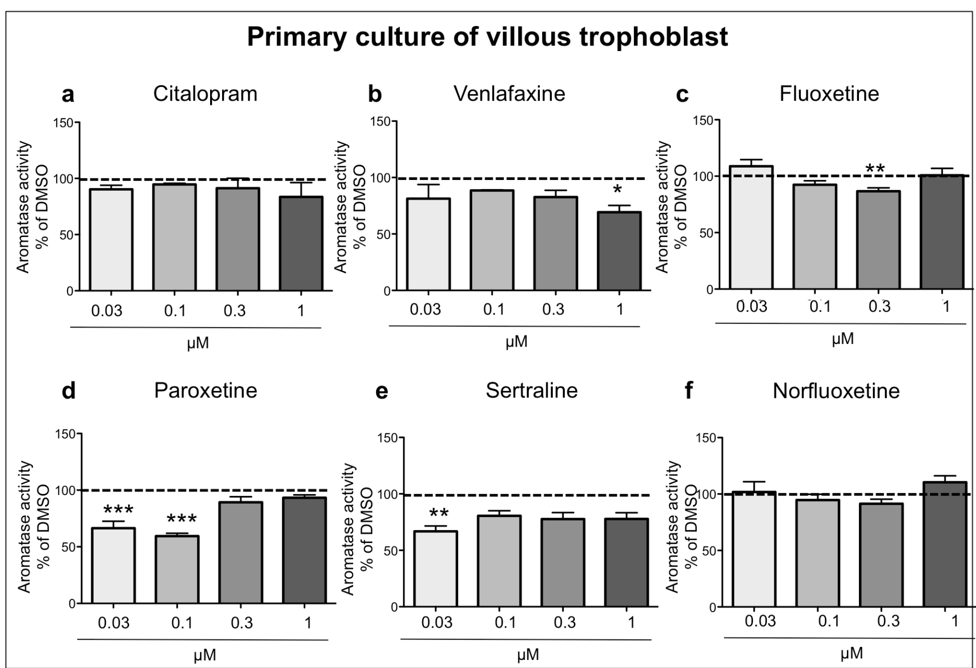




**Fig 2** Effects of serotonin reuptake inhibitors on aromatase activity in BeWo and primary villous trophoblasts. Relative aromatase (CYP19) activity in BeWo cells treated 24 h with (a) citalopram, (b) venlafaxine, (c) paroxetine and (d) sertraline (0.03, 0.1, 0.3 and 1 μM) and in in primary villous 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

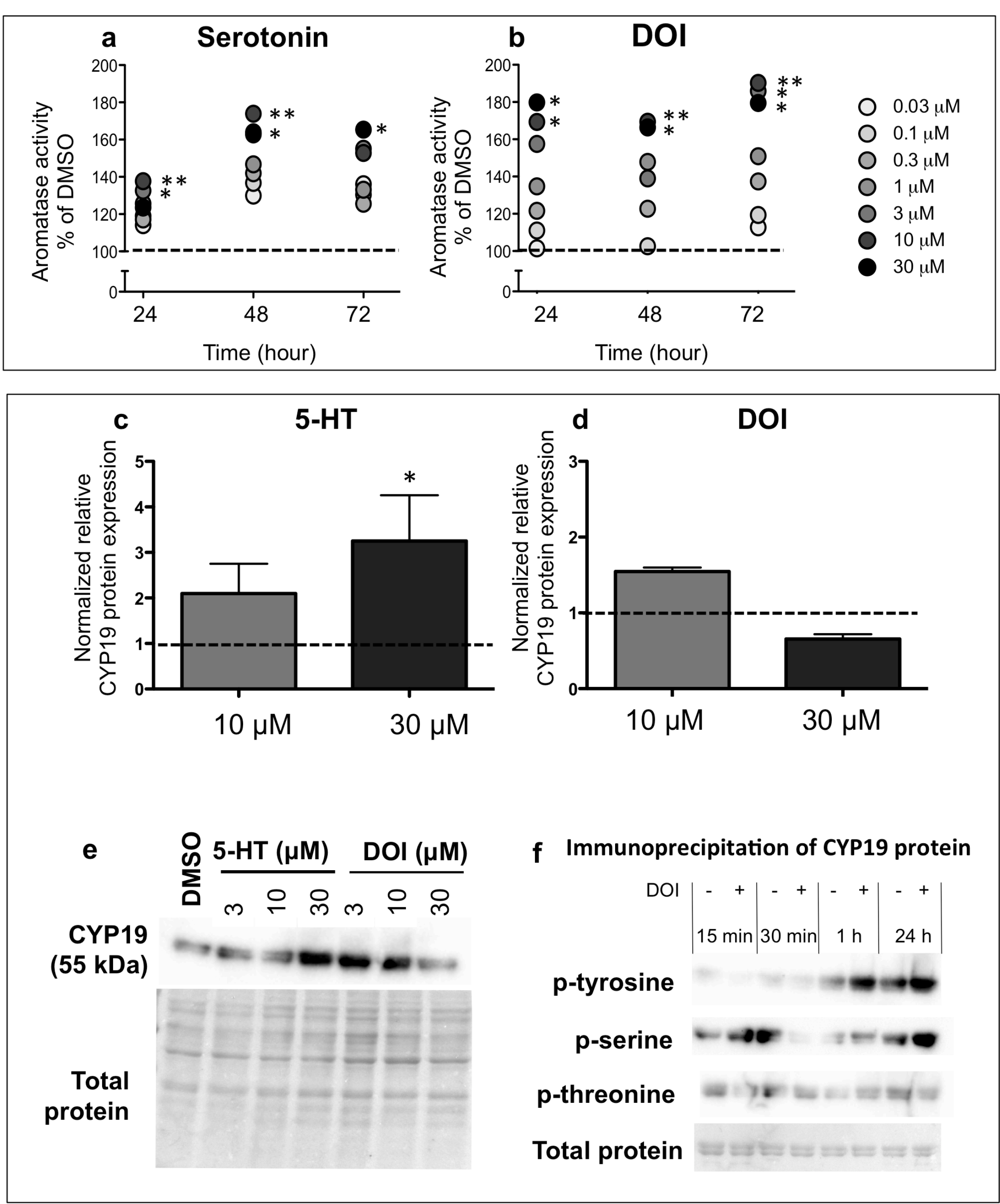



**Fig 3** Effect of serotonin and 2,5-dimethoxy-4-iodoamphetamine (DOI) on aromatase in villous trophoblasts. Relative aromatase (CYP19) activity in primary villous trophoblast cells were treated for 24h with (a) serotonin or (b) DOI (5-$HT_{2A}$ agonist) at different time points. CYP19 activity is expressed in percentage of vehicle-control ± SEM. Protein expression of CYP19 in placental explants treated with (c) serotonin or (d) 2,5-dimethoxy-4-iodoamphetamine (DOI, 10 and 30 μM). CYP19 protein expression is expressed relative to vehicle-control ± 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, $n \geq 3$. (e) Representative blot of CYP19 with total protein used to normalize protein expression. (f) Representative blots of phosphorylated tyrosine, serine and threonine residues from immunoprecipitated CYP19 in placental explants treated or not with DOI (30 μM) for 15 min, 30 min, 60 min or 24 h

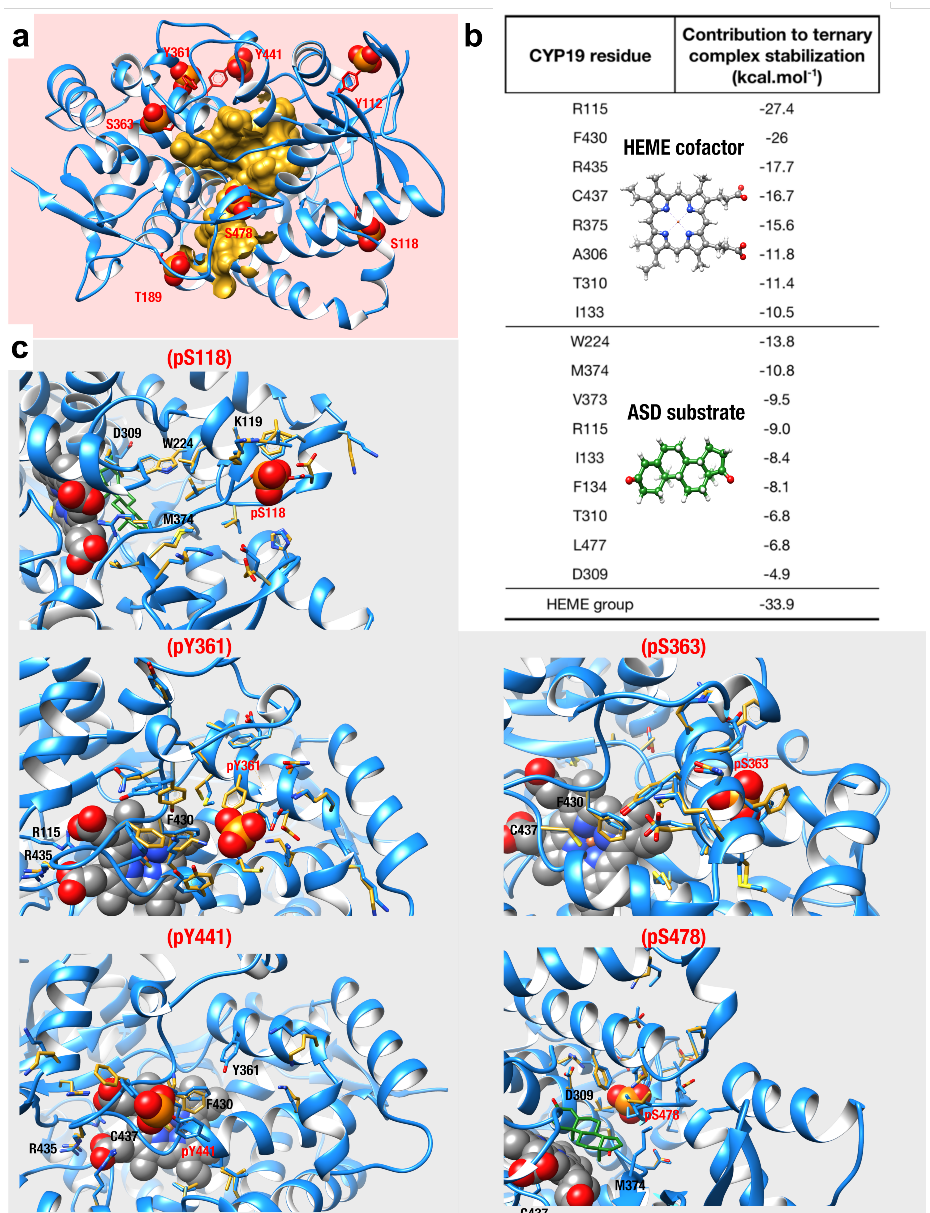



**Fig 4** Predicted conformational changes induced by single-site phosphorylation of human CYP19 aromatase. (a) Single-site phosphorylation of seven target residues predicted in human CYP19 aromatase (see Experimental procedures for details). The active-site pocket is depicted as a gold surface and the 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 kcal*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

## 11.3 Données supplémentaires

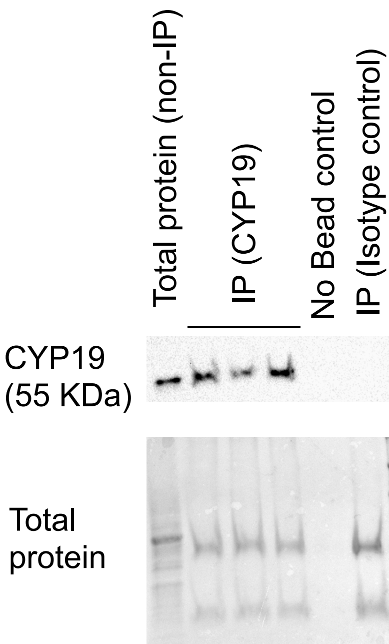
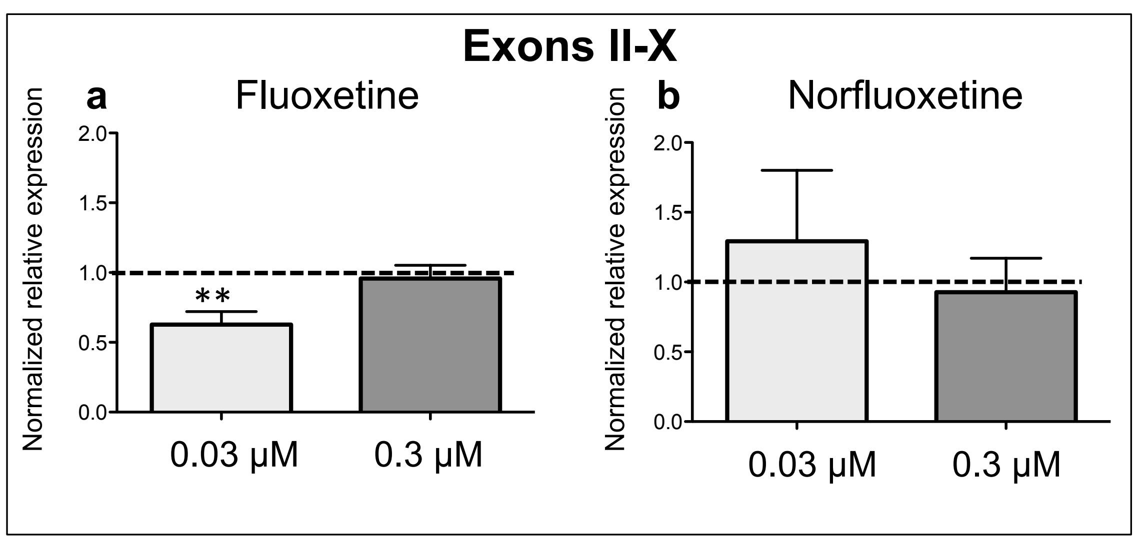



**Fig. S1** Controls for the immunoprecipitation assays. CYP19 detection for total protein, immunoprecipitation for CYP19, no bead control and isotype control



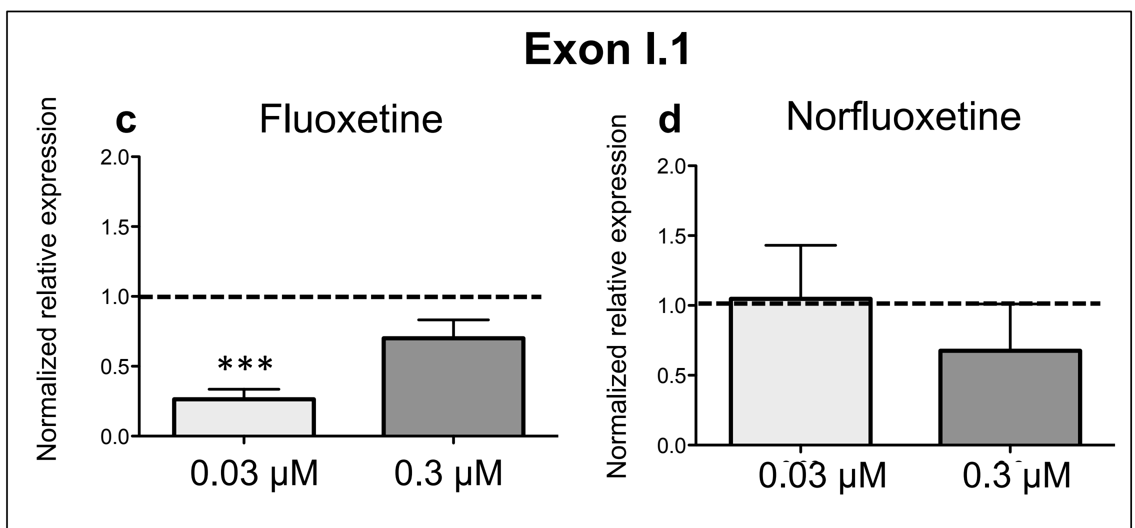




**Fig. S2.** Relative mRNA expression of *CYP19A1* in 24 h primary villous trophoblast cells treated with (a, c) fluoxetine or (b, d) norfluoxetine for 24 h. Relative mRNA expression of *CYP19A1* transcript derived from (a, b) total coding exons and (c, d) exon-I.1. Dashed line represents DMSO-vehicle control. Results are normalized for reference genes (peptidylprolyl isomerase A (*PPIA*) and tyrosine 3-monooxygenase/tryptophane 5-monooxygenase-activation protein, zeta polypeptide (*YWHAZ*)) ± SEM. Significant difference compared to control is indicated with asterisks (***P < 0.001) as determined by a Kruskal Wallis and Dunn's post-hoc test, n=3-4

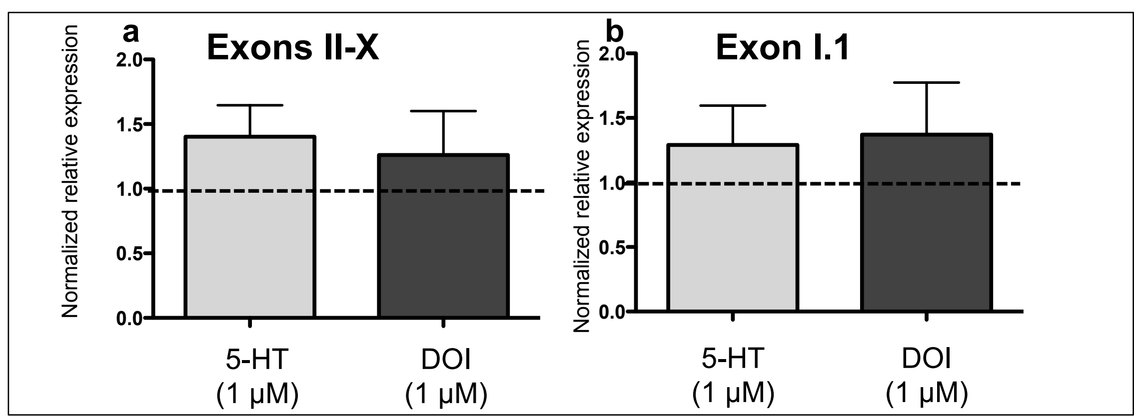



**Fig. S3.** Relative mRNA expression of *CYP19A1* transcript derived from (a) total coding exons and (b) exon I.1 in primary villous trophoblast cells treated with serotonin (5-HT, 30 uM) or 2,5-dimethoxy-4-iodoamphetamine (DOI, 30 uM) for 24h. Dashed line represents DMSO-vehicle control. Results are normalized for reference genes peptidylprolyl isomerase A (*PPIA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase-activation protein, zeta polypeptide (*YWHAZ*) ± SEM. Kruskal Wallis test, n = 3

**Table S1.** Binding affinity of SRIs and natural ligands to aromatase

| | Ligand | Pose | Binding affinity (kcal/mol) |
|---|---|---|---|
| **Natural ligand** | 16a-OH androstedione | 00 | -139 |
| | | 01 | -132 |
| | | 02 | -118 |
| | Testosterone | 00 | -137 |
| | | 01 | -127 |
| | | 04 | -114 |
| **Steroidal aromatase inhibitor** | Formestane | 00 | -137 |
| | | 02 | -117 |
| | | 04 | -114 |
| **SRIs** | Citalopram | 00 | -138 |
| | | 02 | -134 |
| | | 04 | -133 |
| | Fluoxetine | 00 | -129 |
| | | 01 | -133 |
| | | 02 | -125 |
| | Norfluoxetine | 00 | -133 |
| | | 01 | -127 |
| | | 04 | -113 |
| | Paroxetine | 00 | -160 |
| | | 01 | -158 |
| | | 04 | -152 |
| | Sertraline | 00 | -117 |
| | | 02 | -108 |
| | | 03 | -103 |
| | Venlafaxine | 00 | -116 |
| | | 01 | -104 |
| | | 02 | -99 |

**Table S2.** Significant fold-increase in aromatase activity following serotonin and DOI treatment in primary villous trophoblast cells

| | **5-HT** | **DOI** |
|---|---|---|
| **24h** | 3 μM: 129.9 %<br>10 μM: 136.2 % | 10 μM: 192.2 %<br>30 μM: 206.3% |
| **48h** | 10 μM: 173.9 %<br>30 μM: 163.9 % | 10 μM: 201.5 %<br>30 μM: 193.8 % |
| **72h** | 30 μM: 165.3 % | 3 μM: 188.0 %<br>10 μM: 195.7 %<br>30 μM: 182.0 % |

Aromatase activity is expressed in percentage of DMSO vehicle-control.

# TROISIÈME PARTIE : DISCUSSION ET CONCLUSION GÉNÉRALE

# 12 DISCUSSION GÉNÉRALE

La 5-HT et les estrogènes sont impliqués dans la physiopathologie de la dépression et sont essentiels au bon déroulement de la grossesse et au développement du fœtus. Bien que la dépression affecte près 20% des femmes enceintes et que les ISRS soient utilisés comme première ligne de traitement, les effets des ISRS sur les fonctions placentaires n'ont jamais été étudiés. Dans ce projet de recherche, nous avons évalué les effets de la 5-HT sur la synthèse d'estrogènes par le CYP19 dans le trophoblaste humain. Pour ce faire, nous avons utilisé différents modèles : la lignée cellulaire BeWo, modèle de trophoblaste villeux, et les primocultures de trophoblastes villeux isolées de placentas de grossesses normales à terme. De plus, pour mimer l'interaction essentielle entre le placenta et le compartiment fœtal pour la synthèse d'estrogènes, un nouveau modèle de co-culture a été développé. Les travaux de cette thèse démontrent que (1) la co-culture de cellules BeWo et H295R mime la stéroïdogenèse fœto-placentaire avec une production d'estrogènes représentative de celle de la grossesse (**chapitre 8 et annexe IV**); (2) l'activité du CYP19 et le taux d'ARNm de *CYP19A1* varient au cours de la syncytialisation avec un pic à 48 h de culture dans les primocultures de CTv (**chapitre 9**); (3) l'expression d'ARNm de *CYP19A1* dans les primocultures de CTv implique notamment les exons I.1, I.8, l'exon I.4 (**chapitre 9**); (4) la 5-HT et la stimulation de son récepteur $5\text{-HT}_{2A}$ induit l'activité du CYP19 dans les primocultures de CTv (**chapitre 11**) et (5) les ISRS ont une affinité de couplage pour la poche de couplage au substrat du CYP19 et perturbent l'activité de l'enzyme et la synthèse des estrogènes par des mécanismes structures- et doses-dépendants (**chapitres 10 et 11**).

## 12.1 Une avancée dans l'étude de la stéroïdogenèse fœto-placentaire

### 12.1.1 Polyvalence du modèle de co-culture de cellules BeWo/H295R

Les femmes enceintes étant considérées comme une population vulnérable (Canada, 2011), il est important d'être en mesure d'évaluer la toxicité, incluant la perturbation endocrinienne, des différents composés auxquels elles peuvent être exposées, que ce soit par la prise de médicaments ou par des contaminants environnementaux. Afin de compléter des évaluations toxicologiques de l'impact de différents composés, les modèles d'étude doivent reproduire l'ensemble des étapes de la stéroïdogenèse.

La lignée cellulaire H295R utilisée dans notre co-culture, est un modèle reconnu pour l'évaluation de perturbateurs endocriniens par l'organisation pour la coopération économique et le développement (OECD) (Hecker *et al.*, 2011). Nous avons toutefois observé différents effets d'un même composé entre les deux lignées cellulaires qui composent la co-culture (Hudon Thibeault *et al.*, 2017; Hudon Thibeault *et al.*, 2014), ce qui signifie que certains effets pourraient être difficilement extrapolés au contexte de la grossesse si les évaluations se basent uniquement sur les cellules H295R en monoculture. La co-culture cellulaire BeWo/H295R développée dans les travaux rapportés dans cette thèse permet de déterminer les effets de perturbateurs endocriniens qui ont plusieurs mécanismes d'action simultanés, par le dosage des hormones qui représente l'effet global sur la stéroïdogenèse. Ces résultats permettent ainsi d'orienter les études sur les mécanismes d'action pertinents d'un point de vue physiologique. Par exemple, le BPA peut agir sur les récepteurs d'estrogènes, d'androgènes, des hormones thyroïdiennes et des glucocorticoïdes ainsi que sur le CYP19 (Chu *et al.*, 2018; MacKay & Abizaid, 2017). Ainsi, le dosage des hormones dans notre modèle de co-culture permet d'établir quel(s) effet(s) se traduisent réellement par une stéroïdogenèse altérée. Enfin, les modèles que nous avons utilisés et notamment la co-culture, permettent d'appliquer différentes techniques *in vitro* afin de déterminer les effets sur la stéroïdogenèse fœto-placentaire, tel que l'utilisation de chambres à hypoxie, la transfection des cellules ou encore appliquer une contrainte de cisaillement (*shear stress*).

Notre groupe a utilisé ce modèle pour caractériser les effets des pesticides néonicotinoïdes (Caron-Beaudoin *et al.*, 2017). Ces composés augmentent la sécrétion de DHEA, E1 et E2 alors que la production d'E3 est réduite (Caron-Beaudoin *et al.*, 2017). Ces travaux ont permis de démontrer que les néonicotinoïdes entrent en compétition avec la DHEA pour l'enzyme CYP3A7, réduisant la production de 16$\alpha$OH-DHEAS, précurseur de l'E3 (Caron-Beaudoin *et al.*, 2017). Ces résultats confirment la pertinence de notre modèle de co-culture comme outil toxicologie pour l'étude de la stéroïdogenèse fœto-placentaire.

La co-culture possède également un potentiel pour l'étude des interactions fœto-placentaires comme les transporteurs et les enzymes de métabolisme de la barrière placentaire. En effet, la polarisation des membranes basales et apicales des cellules BeWo lorsqu'elles sont mises en culture dans un insert, permet d'évaluer les différents transporteurs de la barrière placentaire (**Annexe IV**) (Li *et al.*, 2013; Thibeault *et al.*, 2018). Ce modèle a donc été utilisé pour étudier l'effet des estrogènes sur l'activité de SERT des cellules BeWo (**Chapitre 11**). Nous avons montré que l'activité de SERT est diminuée dans la co-culture et que cette modification serait

attribuable à une plus grande quantité d'estrogènes dans la co-culture (Hudon Thibeault *et al.*, 2017). Drwal *et al.* (2017) ont quant à eux caractérisé le profil de métabolisme de la co-culture et ont développé différentes variantes de notre modèle, en remplaçant les cellules BeWo par des BeWo syncytialisées (stimulées à la forskoline) ou par des cellules JEG-3 (Drwal *et al.*, 2017). Ils ont conclu que la co-culture de cellules JEG-3/H295R représente davantage la stéroïdogenèse au premier trimestre comparativement à la co-culture BeWo/H295R qui est plutôt représentative de la stéroïdogenèse au troisième trimestre (Drwal *et al.*, 2017). Par ailleurs, la co-culture avec des BeWo syncytialisées est un outil pertinent pour l'étude des enzymes du métabolisme (Drwal *et al.*, 2017).

#### 12.1.2 Limites du modèle de co-culture de cellules BeWo/H295R

De plus en plus d'études montrent des différences entre la physiologie des lignées cellulaires trophoblastiques et des primocultures de CTv (Bilban *et al.*, 2010; Clabault *et al.*, 2018; Hudon Thibeault *et al.*, 2017; Hudon Thibeault *et al.*, 2014; Lanoix *et al.*, 2012; Novakovic *et al.*, 2011; Sagrillo-Fagundes *et al.*, 2019). La lignée cellulaire BeWo est dérivée d'un choriocarcinome placentaire mâle, alors que la lignée H295R provient d'un carcinome corticosurrénalien femelle ce qui signifie que des régulateurs de voies de signalisation liées au cancer, tel que l'oncoprotéine c-myc, pourraient être augmentées (Agosta *et al.*, 2018; Sarkar *et al.*, 1986). D'ailleurs, c-myc est connu pour réguler le *CYP19A1* dans le placenta humain (Kumar et al., 2013). De plus, les ER, qui peuvent réguler le taux d'ARNm de *CYP19A1*, sont liés à des voies de signalisation différentes dans les explants de placenta normaux et dans les cellules BeWo (Gambino et al., 2012b). Dans les cellules BeWo, les estrogènes activent la phosphorylation de p38-MAPK et JNK contrairement aux explants issus de tissus normaux où cet effet n'est pas observé (Gambino et al., 2012b). En outre, le *CYP19A1* peut être régulé par les miARN (Kumar et al., 2013). Il est donc possible que certaines différences entre la lignée cellulaire et les primocultures résident dans les profils d'expression des miARN de ces deux modèles qui ne sont pas encore caractérisés. Soulignons ici qu'il est bien connu que les taux d'ARNm de *CYP19A1* sont dérégulés dans le tissu de cancer du sein par rapport au tissu normal (revu dans (Zhao et al., 2016)). Ce phénomène pourrait également s'observer dans les lignées cellulaires issues de choriocarcinomes. Ainsi, les primocultures de CTv sont un modèle plus représentatif d'un point de vue physiologique (non pathologique). Nos résultats préliminaires indiquent que la co-culture des cellules H295R avec des primocultures de CTv produit aussi de l'E2 de manière synergique et donc modélise l'interaction fœto-placentaire humaine (**Annexe V, figure V.1**). Cette avenue devra être approfondie afin d'améliorer la pertinence physiologique du modèle de

co-culture. Un tel modèle demeure un outil accessible puisqu'il est relativement facile d'obtenir du tissu de placenta (généralement considéré comme un déchet biologique) pour en isoler les CTv ou même de les acheter commercialement (ScienCell Research Laboratories Inc.), ce qui assurerait une certaine uniformité de la technique d'isolation entre les différents laboratoires.

De plus, bien que le modèle de co-culture comporte des composantes placentaire et fœtale physiquement séparées par un insert, le modèle développé dans les travaux rapportés au **chapitre 8** n'est pas optimisé pour étudier le transport transplacentaire. L'étude du passage placentaire requiert un dispositif expérimental qui reproduit l'ensemble des couches de cellules du placenta, comme dans le modèle de perfusion placentaire (Heikkinen et al., 2002; Nagai et al., 2013). Dans notre modèle *in vitro,* nous pourrions seulement évaluer le passage et le transport à travers la couche de trophoblastes. D'ailleurs, le clone b30 des cellules BeWo a déjà été utilisé afin de former un modèle de transport *in vitro* (Cartwright *et al.*, 2012; Li *et al.*, 2013). Ce clone est favorisé en raison de sa capacité à former une monocouche (Cartwright *et al.*, 2012; Li *et al.*, 2013). Nous avons toutefois déjà montré qu'il est possible d'obtenir une monocouche confluente de cellules BeWo sur l'insert en mesurant la résistance transépithéliale (**Annexe IV**).

## 12.2 Régulation de l'expression et de l'activité de l'aromatase dans les primocultures de trophoblastes villeux

La plupart des études sur la régulation de l'ARNm et les éléments de réponses de *CYP19A1* dans le placenta humain ont été réalisées sur les lignées cellulaires de choriocarcinome (BeWo ou JEG-3), dans le tissu total, avec des construits de gènes ou par l'analyse de la séquence de l'ARNm (Means *et al.*, 1989; Sun *et al.*, 1998; Yamada *et al.*, 1999; Zhou *et al.*, 2009a). Pour la première fois, dans cette étude, nous avons caractérisé le CYP19 (ARNm de *CYP19A1* et activité catalytique) dans les primocultures de CTv isolés de placentas de grossesses normales à terme. Nous avons observé que l'activité du CYP19 et le taux d'ARNm de *CYP19A1* atteint un pic à 48 h de culture, ce qui précède le pic de hCG qui survient à 72 h. Nous avons également déterminé que le transcrit dérivé de l'exon I.4 de *CYP19A1* est exprimé dans les primocultures de CTv. De plus, le VEGF et le dexamethasone ont tous les deux diminué le taux d'ARNm de *CYP19A1* (exons II-X), mais sans affecter l'activité enzymatique (**Chapitre 9**). Ces résultats, en combinaison avec ceux obtenus avec les traitements aux ISRS, à la 5-HT et au DOI (**Chapitres 10 et 11**), mettent en évidence différents modes de régulation du CYP19, incluant notamment l'inhibition catalytique, la régulation transcriptionnelle et les modifications post-traductionnelles. En résumé, ces travaux démontrent que, pour expliquer un effet sur l'activité du CYP19, l'étude

des niveaux d'ARNm gagne à être complétée par l'étude des autres modes de régulation du CYP19 tels que résumés à la **figure 12.1**.

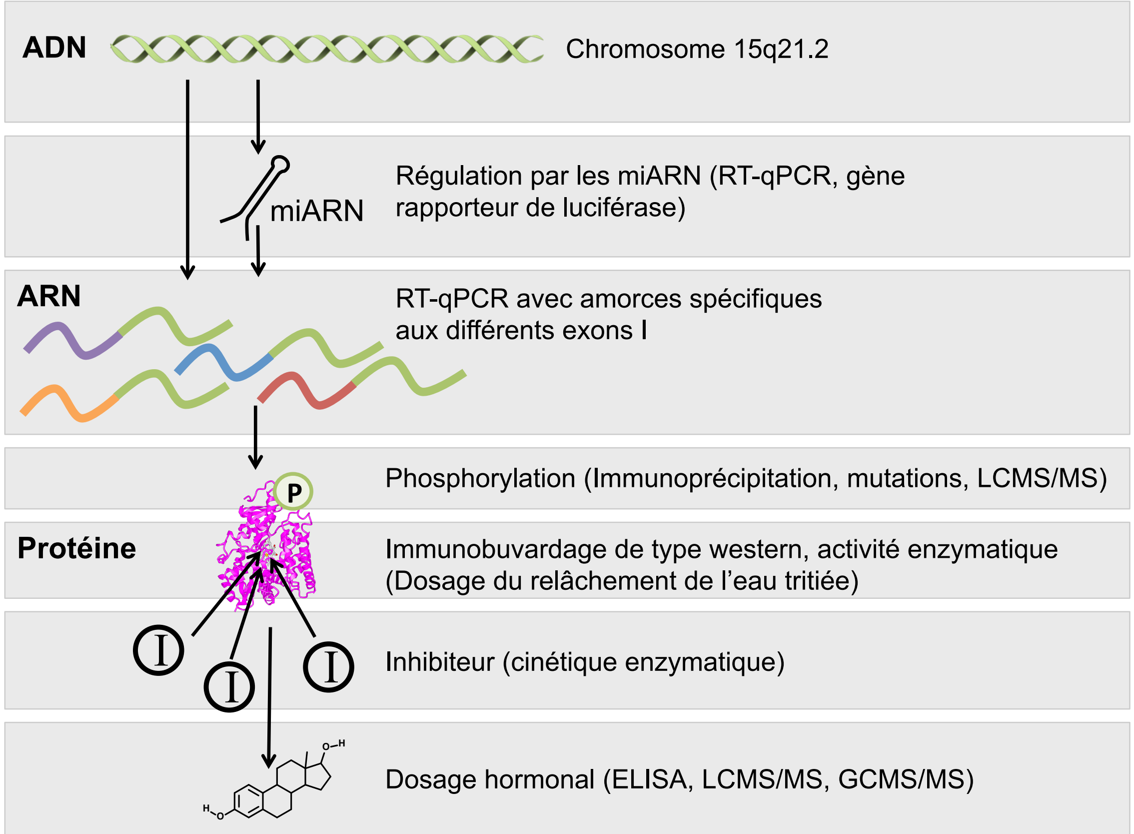



**Figure 12.1 : Principales approches pour l'étude de l'aromatase**

**La transcription du gène de *CYP19A1* est affectée par les miARNs et peut être évaluée par transcription inverse suivie d'une réaction par polymérase en chaîne quantitative (RT-qPCR). L'ARNm produit est ensuite traduit. La protéine du CYP19 peut subir des phosphorylations et son activité peut être affectée par la présence d'inhibiteurs. Ces modifications de l'activité de l'enzyme se traduiront par une modification de la synthèse des estrogènes évaluée par dosage immunoenzymatique (ELISA), chromatographie liquide couplée à un spectromètre de masse en tandem (LCMS/MS) ou chromatographie en phase gazeuse couplée à un spectromètre de masse en tandem (GCMS/MS).**

Dans le CTv, le CYP19 peut également être affecté par la syncytialisation, un processus qui peut lui-même être influencé par plusieurs facteurs, dont le GCM1 (*glial cell missing 1*) (Kumar et al., 2013; Yu et al., 2002). Il existe d'ailleurs des éléments de réponse à GCM1 sur l'exon I.1 du *CYP19A1* (Yamada *et al.*, 1999). Dans ce sens, nos données préliminaires montrent que la 5-HT et le DOI diminuent les taux d'ARNm de *GCM1*, ce qui suggère un effet indirect ou parallèle à l'effet sur le CYP19 de la 5-HT et du 5-$HT_{2A}$R (**Annexe V, figure V.2**). De plus, il est démontré que GCM1 agit en coopération avec le facteur de transcription placentaire Distal-less

3 (DLX3) (Li & Roberson, 2017) il serait donc intéressant d'évaluer si la 5-HT et la stimulation de son récepteur 5-$HT_{2A}$ influence également l'expression de DLX3.

## 12.3 Mise en évidence d'interactions sérotonine-estrogène au niveau placentaire

Les travaux Oufkir *et al*. et Klempan *et al.* avaient déjà démontré que la stimulation du récepteur 5-$HT_{2A}$ régule la migration, l'invasion, la viabilité ainsi que l'activité du CYP19, en utilisant des lignées cellulaires de choriocarcinome (JEG-3 et BeWo)(Klempan *et al.*, 2011; Oufkir *et al.*, 2010; Oufkir & Vaillancourt, 2011). Dans les travaux rapportés dans cette thèse, nous avons observé que la 5-HT et la stimulation du récepteur 5-$HT_{2A}$ augmentent l'activité du CYP19 dans les primocultures de CTv. Cet effet serait attribuable notamment à une modification de la phosphorylation du CYP19 (**chapitre 11**). Il serait intéressant de poursuivre la caractérisation de la ou des kinases impliquées dans la régulation de la phosphorylation du CYP19. De plus, nous avons également constaté que les estrogènes pouvaient exercer une rétroaction sur le SERT (**chapitre 10**). Nos résultats s'insèrent directement dans les études qui visent à comprendre les pathologies liées à la 5-HT en fonction des différents statuts estrogéniques (ex. : grossesse, ménopause, puberté...) (**Annexe VI**). L'évolution de la prévalence et des symptômes des pathologies liées à la 5-HT (ex. : dépression, migraine, syndrome du côlon irritable et troubles alimentaires) selon le statut estrogénique, notamment pendant la grossesse, suggère l'implication d'une interaction entre la 5-HT et les estrogènes. De plus, des maladies spécifiques à la grossesse comme l'hyperémèse gravidarum, le diabète gestationnel mellitus et la pré-éclampsie sont associées à un état hypersérotoninergique et à une perturbation des niveaux d'estrogènes. Ainsi, dans les pathologies liées à la 5-HT, il est possible que le système 5-HT soit hypersensible à la stimulation aux estrogènes. Ces interactions sont illustrées à la **figure 12.2**.

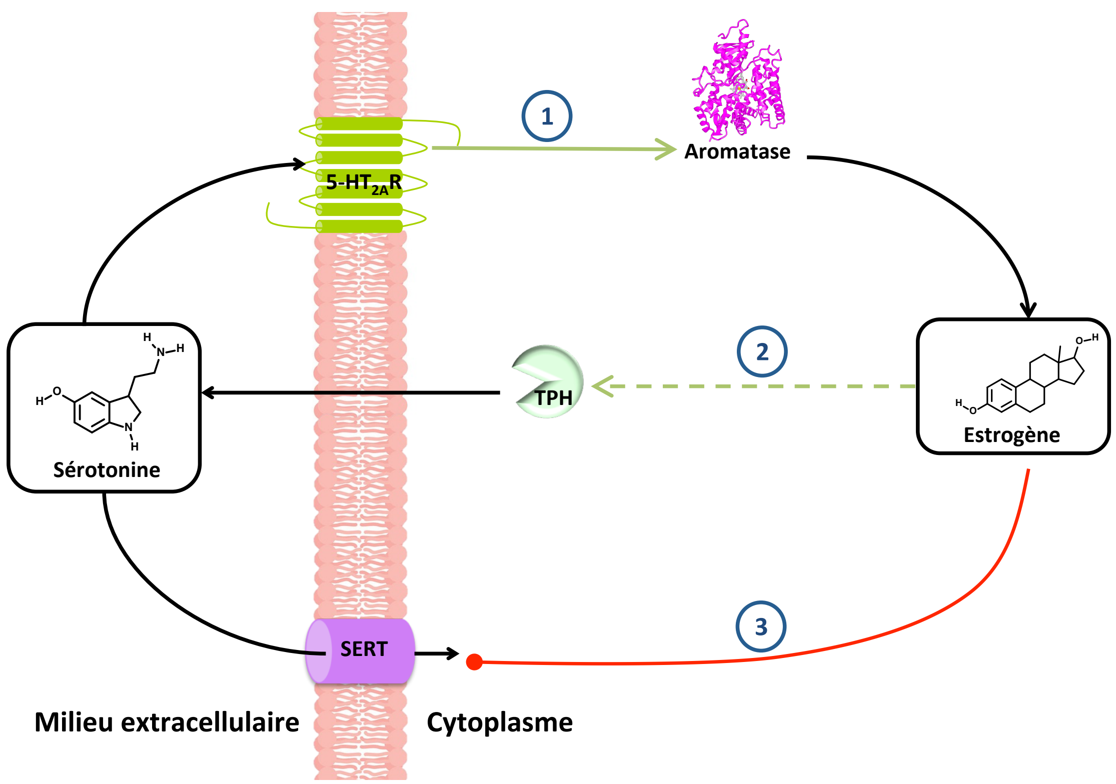



**Figure 12.2 : Interactions sérotonine/estrogènes observées dans les travaux présentés dans cette thèse et dans la littérature**

**La sérotonine disponible pour stimuler les récepteurs (5-HTR) est influencée par le transporteur de la sérotonine (SERT), qui recapture la sérotonine menant à sa dégradation. 1) La stimulation du 5-HT$_{2A}$R par la sérotonine augmente l'activité de l'aromatase et la production des estrogènes; 2) Les estrogènes régulent l'enzyme de synthèse de la sérotonine (TPH); 3) Les estrogènes inhibent le SERT qui influence la quantité de sérotonine disponible pour stimuler les 5-HTR. Les effets des estrogènes sont possiblement régulés par l'interaction avec leurs récepteurs membranaires et/ou cytoplasmiques (non illustré). Lignes vertes : stimulation; Ligne rouge : inhibition; ligne pleine : observation dans les travaux présentés dans cette thèse; ligne pointillée : hypothèse basée sur la littérature. L'estrogène illustré (17β-estradiol) est l'estrogène le plus étudié, les effets des autres estrogènes ne sont pas exclus. Les images des molécules ont été produites avec *Chemdoodle 2D sketcher,* l'illustration du CYP19 est tirée de https://pubchem.ncbi.nlm.nih.gov/edit2/index.html et les membranes tirées de https://pixabay.com/fr/cellulaire-membrane-cellulaire-1740543. Adapté de (Hudon Thibeault *et al.*, 2019).**

## 12.4 Perturbation de la stéroïdogenèse par les inhibiteurs sélectifs de la recapture de la sérotonine

Pour évaluer les effets des antidépresseurs les plus prescrits chez les femmes enceintes, les ISRS, nous avons utilisé la lignée cellulaire BeWo en monoculture ou en co-culture avec les cellules H295R ainsi que des primocultures de CTv. Les concentrations d'ISRS utilisées correspondent aux concentrations retrouvées dans le sang maternel et le sang de cordon (**annexe I**). Nous avons comparé les résultats obtenus dans les travaux rapportés dans cette

thèse à ceux d'équipes qui travaillent sur le modèle H295R pour étudier la stéroïdogenèse (**Figure 12.3**).

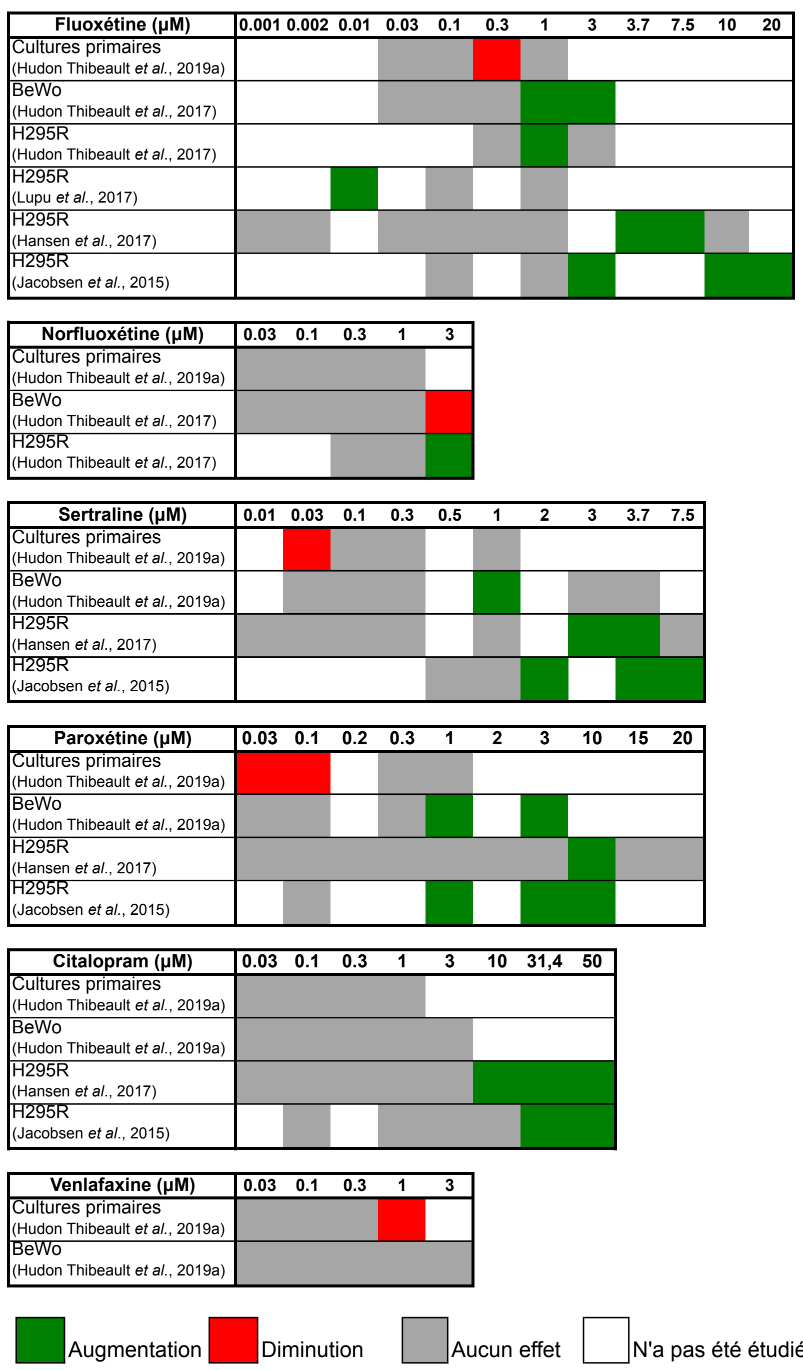



**Figure 12.3 : Résumé des résultats d'activité d'aromatase obtenus avec différents ISRS présentés dans cette thèse et dans la littérature**

**Les études réalisées par Lupu *et al.*, Jacobsen *et al.* et Hansen *et al.*, utilisent des cellules H295R traitées 48 h avec les ISRS et l'activité du CYP19 (aromatase) a été évaluée à partir du dosage d'hormones dans le milieu de culture (Hansen *et al.*, 2017; Jacobsen *et al.*, 2015; Lupu *et al.*, 2017), contrairement à nos expériences où le traitement était de 24 h et l'activité du CYP19 a été évaluée par dosage du relâchement de l'eau tritiée (chapitre 11 et (Hudon Thibeault *et al.*, 2017)).**

Nos résulats montrent que les ISRS altèrent la relation entre le système 5-HT et les estrogènes (**Figure 12.4**). Il serait notamment intéressant de caractériser plus en détail le mode d'action des estrogènes sur le SERT.

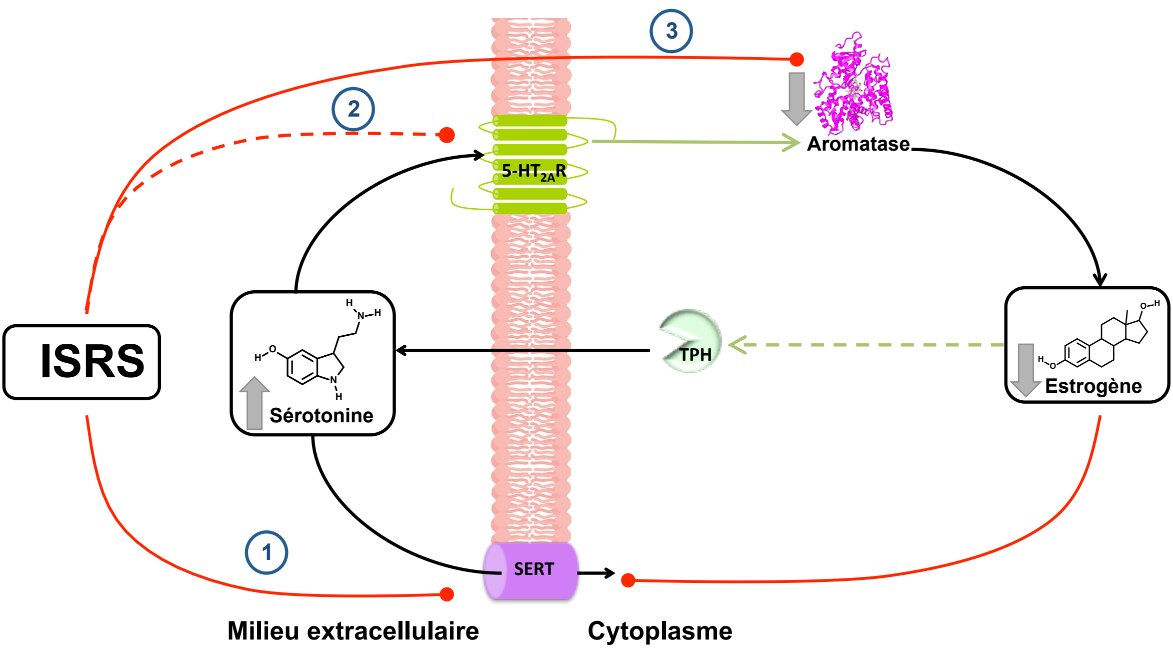



**Figure 12.4. Effets des ISRS sur les interactions entre les systèmes sérotonine et estrogène**

**Les inhibiteurs sélectifs de la recapture de la sérotonine (ISRS) agissent à la fois sur le système de la sérotonine et sur la synthèse des estrogènes. 1) Les ISRS inhibent le transporteur de la sérotonine (SERT), augmentant la quantité de sérotonine extracellulaire. 2) Certains ISRS ont une affinité de couplage pour le récepteur 5-HT$_{2A}$ (impliqué dans la régulation de l'activité du CYP19). 3) Les ISRS peuvent également agir comme inhibiteurs compétitifs du CYP19 résultant en une diminution de la quantité d'estrogènes produite. Lignes vertes : stimulation; Ligne rouge : inhibition; Ligne pleine : observation dans les travaux présentés dans cette thèse; Ligne pointillée : hypothèse basée sur la littérature. L'estrogène illustré (17β-estradiol) est l'estrogène le plus étudié, les effets des autres estrogènes ne sont pas exclus. Les images des molécules ont été produites avec *Chemdoodle 2D sketcher,* l'illustration du CYP19 est tirée de https://pubchem.ncbi.nlm.nih.gov/edit2/index.html et les membranes tirées de https://pixabay.com/fr/cellulaire-membrane-cellulaire-1740543.**

### 12.4.1 Différents effets des inhibiteurs sélectifs de la recapture de la sérotonine selon les modèles d'étude

De manière générale, dans la littérature, une augmentation de l'activité du CYP19 est observée aux plus grandes doses d'ISRS, alors que seulement notre groupe a montré une diminution de l'activité du CYP19 aux faibles doses et ce, dans les primocultures de CTv. Jacobsen *et al.* (2015), utilisant des modèles d'étude d'inhibition catalytique du CYP19 (supersomes), ont trouvé que tous les ISRS testés étaient des inhibiteurs du CYP19 (Jacobsen *et al.*, 2015), ce qui confirme nos résultats de simulation moléculaire (**chapitre 11**). Ces résultats sont en accord avec les primocultures de CTv dans lesquelles nous observons des diminutions d'activité du

CYP19. Une hypothèse est que les ISRS pénètreraient plus facilement les CTv pour entrer en compétition avec le substrat du CYP19, alors dans les cellules BeWo, les ISRS agiraient sur la régulation du CYP19 en augmentant son activité. Dans ce sens, une étude a montré que l'effet des ISRS sur p-gp est différent selon les tissus étudiés et notamment selon le caractère cancéreux ou non du tissu (Bhuiyan et al., 2012). De plus, le profil d'expression des transporteurs membranaires diffère entre les cellules BeWo et les cultures primaires (Kallol et al., 2018).

L'activité du CYP19 est étroitement associée avec la différenciation des CTv en ST. Nous avons comparé nos résultats avec ceux de Clabault *et al.* (2018) où notre groupe a étudié les mêmes ISRS sur des marqueurs de la syncytialisation dans les cellules BeWo et dans les primocultures de CTv (Clabault *et al.*, 2018). Les résultats suggèrent que les cellules BeWo sont plus sensibles aux effets des ISRS, puisqu'un plus grand nombre d'ISRS augmentent les marqueurs de différenciation trophoblastique (Clabault *et al.*, 2018), ce qui est cohérent avec ce que nous observons également.

Enfin, nos résultats suggèrent que l'utilisation d'ISRS pourrait augmenter la synthèse des estrogènes dans un tissu cancéreux, tel que les cellules BeWo. Cet aspect est particulièrement préoccupant et mériterait d'être approfondi dans le contexte de cancer hormono-dépendant comme certains cancers du sein (Waks & Winer, 2019).

### 12.4.2 Différents effets entre les inhibiteurs sélectifs de la recapture de la sérotonine

Même si les ISRS possèdent plusieurs similarités dans leurs structures ainsi que dans leurs modes d'action, ils affectent différemment l'activité du CYP19 dans un même modèle d'étude. Ces molécules ont différentes affinités pour les récepteurs des neurotransmetteurs, ce qui pourrait expliquer certaines de nos observations. La fluoxétine et la norfluoxétine ont une plus grande affinité pour les récepteurs 5-$HT_2$ et possiblement histaminique 1 (H1) en comparaison aux autres ISRS (Cusack *et al.*, 1994; Owens *et al.*, 1997). De plus, la sertraline possède une plus grande affinité pour les récepteurs adrénergiques α1 et α2 et la paroxétine pour les récepteurs muscariniques en comparaison aux autres ISRS (Cusack *et al.*, 1994; Owens *et al.*, 1997). Le citalopram n'a pas été étudié alors que la venlafaxine, pour l'ensemble des récepteurs présentés, possède un Ki trop élevé pour être comparé aux autres ISRS (Cusack *et al.*, 1994; Owens *et al.*, 1997). Ces récepteurs ont tous été détectés dans le placenta (Falkay & Kovács, 1994; Huang *et al.*, 1998; Khosrow Tayebati *et al.*, 1997; Matsuyama *et al.*, 2006; Resch *et al.*, 2003; Vaillancourt *et al.*, 1994a). De plus, la fluoxétine et la norfluoxétine ont démontré des

effets estrogéniques par la stimulation des ER *in vitro* (Lupu *et al.*, 2015; Müller *et al.*, 2012). Par contre, à des doses plus élevées de norfluoxétine (>10 µM), une diminution de la stimulation des ER est observée (Lupu *et al.*, 2015). Cette différence sur la stimulation des ER entre la fluoxétine et la norfluoxétine pourrait expliquer en partie, pourquoi la fluoxétine augmente l'activité du CYP19 alors que la norfluoxétine la diminue dans les cellules BeWo.

Les transporteurs permettant à la fluoxétine et à la norfluoxétine de pénétrer dans les trophoblastes ne sont pas entièrement caractérisés et il est possible que ceux-ci diffèrent entre les deux molécules. Par exemple, le transporteur OCTN1 (organic cation transporter, novel, type 1/*SLC22A4* est impliqué dans le passage de la fluoxétine du sang maternel vers le syncytiotrophoblaste (Evseenko *et al.*, 2006)). Cette différence dans l'accès au cytoplasme du trophoblaste influencera la capacité de la molécule à agir comme inhibiteur compétitif.

Les travaux présentés dans cette thèse ont également mis en évidence l'importance d'étudier séparément la molécule mère et son métabolite actif par l'étude de la fluoxétine et de son métabolite actif, la norfluoxétine. Ces deux molécules sont présentes dans l'unité fœto-placentaire (Heikkinen *et al.*, 2003; Rampono *et al.*, 2009), mais la conversion de la fluoxétine en norfluoxétine est faible dans notre système *in vitro* de co-culture de cellules BeWo/H295R. Étudier uniquement l'effet de la fluoxétine pourrait suggérer une augmentation de la synthèse des estrogènes, mais paradoxalement nous avons observé que la norfluoxétine est un inhibiteur catalytique du CYP19 (Hudon Thibeault *et al.*, 2017). Des analyses supplémentaires combinant un co-traitement de fluoxétine et de norfluoxétine permettraient de déterminer l'effet dominant sur l'activité du CYP19 placentaire.

### 12.4.3. Effets perturbateur endocrinien des ISRS

L'exposition *in utero* à des ISRS a été associée à des perturbations qui suggèrent une altération de la production d'estrogènes par le placenta, tel que des altérations du développement du cerveau et des comportements sexuels (Gemmel *et al.*, 2018; Rayen *et al.*, 2013), du développement testiculaire (diminution du volume des tubules séminifères) (de Oliveira et al., 2013) et une diminution de la distance anogénitale chez les mâles (Rayen et al., 2013). Nous avons montré que les ISRS perturbent le CYP19 et de la stéroïdogenèse fœto-placentaire, mais avant de pouvoir suggérer des effets possibles chez une femme enceinte, il est nécessaire de connaître les effets des ISRS sur les ER.

Les ISRS ont été testés dans des modèles *in vitro* de lignées cellulaires de cancer du sein (T47D-KBlur ou MCF7) transfectées avec un gène rapporteur de luciférase afin de déterminer

leur interaction avec les récepteurs d'estrogènes (Lupu *et al.*, 2015; Müller *et al.*, 2012; Pop *et al.*, 2015). Des effets agonistes des récepteurs d'estrogènes ont été observés avec la fluoxétine (1 µM), sertraline (0,1 µM) et norfluoxétine (10 µM), mais pas avec la paroxétine (Lupu *et al.*, 2015; Müller *et al.*, 2012; Pop *et al.*, 2015). En co-traitement avec E2, les ISRS agissent principalement comme agonistes à de faibles doses (fluoxétine 1 µM, sertraline 0,01µM, paroxétine 0,03 et 0,01 µM et norfluoxétine 0,01, 0,03 et 0,1 µM) (Lupu *et al.*, 2015; Müller *et al.*, 2012; Pop *et al.*, 2015). Des effets antagonistes des ER sont observés à des doses plus élevées de fluoxétine (10 et 15 µM), sertraline (10 µM) et norfluoxétine (10 µM) (Lupu *et al.*, 2015; Müller *et al.*, 2012; Pop *et al.*, 2015). De plus, dans un modèle classique d'étude de la perturbation endocrinienne *in vivo*, le test utérotrophique chez le rat immature qui permet d'évaluer les effets (anti)estrogéniques d'une molécule, la fluoxétine possède un effet estrogénique (Müller *et al.*, 2012), alors que la sertraline n'a aucun effet (Montagnini *et al.*, 2013). Ainsi, les ISRS pourraient avoir un effet agoniste des ER aux mêmes doses où nous avons observé des diminutions de la synthèse des estrogènes. Les interactions des ISRS avec les ER placentaires devront aussi être caractérisées dans nos modèles d'étude et comparées pour les différents ISRS afin d'obtenir un portrait plus complet de leur mode d'action.

## 12.5 Limites du projet

Les cellules trophoblastiques sont capables de synthétiser la 5-HT *de novo* (5-HT placentaire) (Laurent *et al.*, 2017), mais les modèles d'étude que nous avons utilisés ne tiennent pas compte de la 5-HT d'origine maternelle et fœtale. Par contre, la 5-HT est présente dans le sérum bovin fœtal (FBS) du milieu de culture. D'un point de vue technique, il serait intéressant de comparer les traitements de 5-HT avec une perte de fonction de la TPH et dans un milieu de culture qui ne contient pas de 5-HT afin d'évaluer le rôle de la 5-HT endogène.

De plus, dans nos modèles *in vitro*, les informations sur le transport et le métabolisme des ISRS sont limitées. Les connaissances du métabolisme des ISRS ont principalement été déterminées à partir d'un organisme complet où les ISRS subissent le passage hépatique, alors que nos systèmes *in vitro* sont plus simples et dépendent du métabolisme par les CYP placentaires. Il s'agit d'une limite de l'utilisation de modèles *in vitro* où il est difficile de reproduire le métabolisme complexe qui se produit dans un organisme complet, mais il s'agit également d'une force qui nous permet d'isoler plus facilement l'effet d'une molécule, sans qu'elle ne soit biotransformée.

## 12.6 Perspectives

La caractérisation des effets de la 5-HT, du récepteur 5-$HT_{2A}$ et des différents ISRS en utilisant plusieurs modèles d'étude (co-culture, BeWo, cultures primaires, explants) amène plusieurs questions de recherche comme :

- Pourquoi les ISRS ont-ils des effets différents dans les cellules de choriocarcinome par rapport aux primocultures de CTv? Quelle est la signification pour les patients avec un cancer et qui prennent des ISRS ?
- Compte tenu des différences entre les modèles d'étude, comment faire pour considérer plusieurs modèles complémentaires dans l'évaluation du potentiel toxique d'une substance ?
- Est-ce que la dépression *per se*, altère la stéroïdogenèse fœto-placentaire ? En cas de dépression, est-ce que la perturbation endocrinienne associée avec les ISRS est bénéfique ou délétère pour la mère, le placenta et le fœtus ? Dans quelle mesure contribue-t-elle aux pathologies associées avec la dépression et son traitement ?
- Est-ce que des effets similaires aux ISRS peuvent être observés avec les traitements d'autres pathologies liées à la 5-HT (ex. migraines et triptans) ?

Un nombre grandissant d'études suggère que la 5-HT placentaire jouerait un rôle auto/paracrine (Klempan *et al.*, 2011; Oufkir *et al.*, 2010; Oufkir & Vaillancourt, 2011). Les résultats présentés dans cette thèse permettent d'ajouter la régulation de l'activité du CYP19 aux autres effets de la 5-HT dans le placenta humain. En démontrant la régulation d'une fonction cruciale placentaire par la 5-HT, nous soulevons des questionnements par rapport à l'impact de maladies liées à la 5-HT pendant la grossesse, telles que la dépression. Face à une augmentation de l'utilisation des ISRS qui est associée à une augmentation du risque de problèmes neurodéveloppementaux chez les enfants (Rotem - Kohavi & Oberlander Tim, 2017), il est important de mieux comprendre les effets de l'exposition aux ISRS pendant la vie utérine (Andalib *et al.*, 2017; Gemmel *et al.*, 2018).

Nos résultats ont montré que les ISRS affectent la stéroïdogenèse, mais le projet ne visait pas à comprendre les effets de la dépression *per se* sur la stéroïdogenèse. En perspective, l'effet de la dépression sur la 5-HT placentaire et la perturbation endocrinienne pourrait être déterminé par une étude de cohorte ou l'utilisation d'un modèle animal de dépression. La dépression est toutefois une pathologie complexe qui implique différents systèmes qui sont eux-mêmes affectés par des facteurs génétiques et environnementaux (Malhi & Mann, 2018). La 5-HT est le

principal neurotransmetteur qui a été étudié pour le développement d'antidépresseurs, basé sur l'hypothèse monoaminergique (Malhi & Mann, 2018). Par contre, la dopamine, la norépinéphrine, le glutamate et l'acide gamma-aminobutyrique (GABA) sont également altérés dans la dépression (Dean & Keshavan, 2017; Hasler, 2010). D'autres hypothèses sur la pathophysiologie de la dépression sont émises, telles que l'hyperactivité de l'axe hypothalamo-hypophyso-surrénalien, l'inflammation, une réduction de la neuroplasticité, une perte du volume de l'hippocampe et l'altération de la connectivité du système limbique (Dean & Keshavan, 2017; Hasler, 2010; Malhi & Mann, 2018). Ces différentes hypothèses ne sont pas mutuellement exclusives et pourraient expliquer les différents sous-types de dépression ainsi que l'absence d'efficacité des ISRS chez certains patients (Dean & Keshavan, 2017). D'ailleurs, dans une cohorte de stress maternel prénatal (The QF2011 Queensland Flood Study), le taux d'ARNm placentaire de *CYP19A1* a été corrélé avec celui de *CRH*, *HTR2A* et *TPH2*, suggérant ainsi une relation entre le *CYP19A1* et les systèmes des glucocorticoïdes et de la sérotonine (St-Pierre, 2017). Dans le cadre d'une éventuelle étude de l'effet de la dépression sur la fonction placentaire, il serait intéressant de prendre en considération les sous-types de dépression, les effets sur les différents systèmes monoaminergiques et les hormones du système du cortisol placentaire. Enfin, soulignons que bien que nous ayons étudié le CYP19 placentaire, celui-ci est également exprimé dans le cerveau où des perturbations du système de la 5-HT ont été observées dans des cas de dépression (Bethea *et al.*, 2002; Fishell, 2010; Holden, 2005; Marcus *et al.*, 2008). Ainsi, nos résultats ouvrent ègalement la porte à l'étude des interactions 5-HT/estrogènes au niveau du système nerveux central.

# 13 CONCLUSION GÉNÉRALE

Ce projet de doctorat a permis de développer et caractériser une nouvelle co-culture cellulaire (BeWo/H295R) comme modèle pour étudier la stéroïdogenèse fœto-placentaire et pour analyser la régulation de l'activité du SERT par les stéroïdes. Nos résultats préliminaires montrent la faisabilité de remplacer la lignée cellulaire BeWo par des primocultures de trophoblaste villeux afin de mieux représenter la physiologie normale. Les résultats présentés dans cette thèse montrent également que la régulation de la transcription et de l'activité du CYP19 pendant de la différenciation des trophoblastes villeux *in vitro* est un processus complexe, qui implique notamment les exons I.1, I.8 et I.4. De plus, notre étude montre que les effets observés au niveau de la transcription ne reflètent pas nécessairement un effet catalytique. Les différences observées dans la régulation du CYP19 entre les cellules BeWo et les primocultures de CTv soulignent également l'importance de ne pas transposer directement les résultats obtenus dans les lignées cellulaires. Entre autres, contrairement à ce qui a été observé dans les cellules BeWo, dans les primocultures de trophoblastes villeux, la stimulation du récepteur 5-$HT_{2A}$, régule l'activité du CYP19 par la phosphorylation, plutôt que par la transcription. Nous avons également démontré que les ISRS peuvent avoir des effets perturbateurs endocriniens sur le CYP19 : (1) inhibition par compétition avec le substrat du CYP19 et (2) régulation de l'expression du CYP19 (ARNm, protéine) et de l'activité du CYP19. De plus, les différences structures- et doses-dépendantes (non-monotones) observées soulignent les effets complexes des ISRS. Il est donc crucial de mieux caractériser les effets perturbateurs endocriniens des ISRS particulièrement sur le déroulement de la grossesse et le développement fœtal. Enfin, l'ensemble de ces travaux s'insère dans une plus vaste littérature sur les interactions entre les systèmes sérotoninergique et estrogénique. Une meilleure compréhension de ces interactions permettra de développer de nouvelles approches préventives et thérapeutiques pour les pathologies de grossesse comme la dépression.

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# ANNEXES

# ANNEXE I PASSAGE DANS LE SANG DE CORDON DES PRINCIPAUX ISRS

**Concentrations d'ISRS dans le sang maternel et dans le sang de cordon**

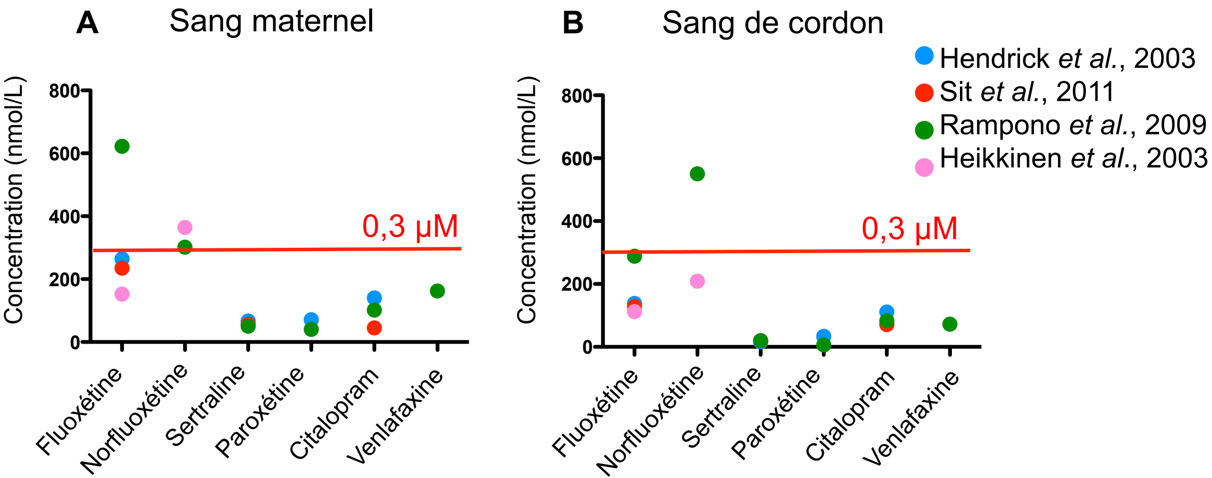


**Figure I.1 : Concentrations d'ISRS/ISRN moyennes retrouvée dans le sang maternel et dans le sang de cordon**

**(A) Concentrations dans le sang maternel et (B) concentrations dans le sang de cordon, tirées de (Heikkinen *et al.*, 2003; Hendrick *et al.*, 2003; Rampono *et al.*, 2009; Sit *et al.*, 2011). Pour Heikkinen *et al.*, 2003, les moyennes n'étant pas disponibles, les médianes ont été utilisées. La ligne rouge identifie la concentration médiane utilisée dans nos *expériences in vitro*.**

# ANNEXE II ÉVALUATION DES CELLULES PLACENTAIRES AVEC LE XCELLIGENCE

Titre complet : An electrical impedence-based assay to examine functions of various placental cell types in vitro

Titre en français : Une expérience basée sur l'impédance électrique pour examiner les fonctions de différents types de cellules placentaires *in vitro*.

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**Contribution de l'étudiante** : L'étudiante a contribué aux expériences et à la rédaction du chapitre de façon égale avec Tejasvy Chollangi et Hélène Clabault.

**Contribution des coauteurs** : Hannah E. J. Yong, Shagun Narula et Ellen Menkhorst ont contribué à la mise au point des protocoles et à la révision de l'article. J. Thomas Sanderson, Cathy Vaillancourt et Padma Murthi ont supervisé la réalisation des expériences et la rédaction de l'article.

## II.1 Résumé de l'article en français

Les analyses fonctionnelles in vitro des cellules sont largement utilisées pour investiguer les mécanismes moléculaires impliqués dans la pré-éclampsie. Les fonctions cellulaires communément étudiées incluent l'adhésion, l'apoptose, la prolifération, la migration et l'invasion. Actuellement, la plupart des chercheurs utilisent des expériences à un temps précis qui permettent seulement la détermination de la fonction cellulaire à un seul temps, avec la nécessité de répéter l'expérience pour un temps alternatif. Ici, nous décrivons un outil basé sur l'impédance électrique qui permet le contrôle en temps réel, afin d'obtenir efficacement des données à de multiples temps pendant toute la durée d'une expérience.

## II.2 Article

**This is an accepted manuscript of an article published by Springer in *Preeclampsia : Methods and protocols. Methods in Molecular Biology* on December 2, 2017. The final publication is available at Springer via http://dx.doi.org/978-1-4939-7498-620**

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# Chapter 20

# An Electrical Impedance-Based Assay to Examine Functions of Various Placental Cell Types In Vitro

**Tejasvy Chollangi, Hélène Clabault, Andrée-Anne Hudon Thibeault, Hannah E.J. Yong, Shagun Narula, Ellen Menkhorst, J. Thomas Sanderson, Cathy Vaillancourt, and Padma Murthi**

## Abstract

In vitro functional analyses of cells are widely used to investigate the molecular mechanisms involved in preeclampsia. Common cellular functions studied include adhesion, apoptosis, proliferation, migration, and invasion. At present, most researchers will use endpoint experimental assays that only allow the determination of cell function at a single time point, with the need to repeat the experiment for an alternate time point. Here, we describe an electrical impedance-based tool that allows real-time monitoring of cells, which enables the efficient assessment of multiple time points over the duration of a single experiment.

**Key words** Functional assays, Adhesion, Proliferation, Apoptosis, Migration, Invasion, xCELLigence® RTCA systems

## 1 Introduction

A large variety of cell types are involved in placentation. Understanding how these cells function is imperative to further the current knowledge of placental disorders such as preeclampsia. For example, much research performed has focused on the migration and invasion of extravillous trophoblast cells into the decidua. Another example would be determining apoptosis of endothelial cells in the context of endothelial dysfunction. Current assays that are commonly used, such as migration scratch assays and cell viability assays, are tedious and require multiple experiments to measure different time points. In this chapter, we describe the use of the xCELLigence® real-time cell analysis (RTCA) systems (ACEA

Cathy Vaillancourt and Padma Murthi share joint senior authorship.
Tejasvy Chollangi, Hélène Clabault, Andrée-Anne Hudon Thibeault, and Hannah E.J. Yong, contributed equally to this study.

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Biosciences Inc., San Diego, CA), which offer the opportunity to continuously monitor cellular behavior and explore multiple cell functions in real time [1]. The xCELLigence® RTCA systems can be used to study basic cell behaviors, such as adhesion, apoptosis, migration, proliferation, and invasion, as well as be customized to determine specialized cell functions. These systems enable cell activity and kinesis to be measured and quantified as changes in the impedance of an electric current (Fig. 1). Gold microelectrodes embedded in the base of the wells of a cell culture plate transmit the current at specific intervals. The presence of adherent cells impedes the flow of electrons when current is applied. This impedance in the conduction of electricity is measured as a quantifiable signal referred to as the cell index (Fig. 1a). The cell index is an arbitrary unit of measurement defined as $\frac{R(t)-R(t_0)}{15}$. $R(t_0)$ represents the background impedance in the well with only medium, while $R(t)$ reflects the impedance at any given time point when the cells are present [2, 3]. The cell index accounts for background impedance through the use of an initial "blanking" step, which enables the system to correct for the impedance caused by medium alone. The cell index rises as cells spread across the electrodes (Fig. 1b). Eventually, when the cells reach confluence in the well, the cell index will plateau for a period of time. Any subsequent decline will reflect the cell death or detachment. Therefore, the real-time monitoring of cell activity allows users to select the optimal time points for other endpoint experiments.

There are seven different xCELLigence® RTCA models, with different plate compatibilities. For placental studies, the most popular models are the xCELLigence® dual plate (DP), single plate (SP), and multiple plate (MP) (Table 1). Customized plates allow various functions to be tested. The E-plate can contain anywhere from 8 to 384 wells depending on the xCELLigence® RTCA model with the most popular plates having 16 or 96 wells. E-plates can be used to measure events such as cell adhesion, proliferation, and apoptosis (Table 1). E-Plates are similar to regular cell culture plates except that the base of each well contains electrodes. The alternative cell invasion/migration (CIM)-plate, which is only available in 16-well format, is used to determine cell migration or invasion in wells that are pre-coated with a gel matrix. The CIM-plate utilizes a modified Boyden chamber, and each well consists of an upper and lower chamber separated by a microporous membrane (8 μm pore). The microelectrodes are present at the underside of the upper chamber to detect electrical impedance change caused by migratory and invasive cells that have passed through the membrane. E-Plate inserts, which are to be used for co-culture assays, are also available can be added on top of a 16- or 96-well E-plate. There is no impedance monitoring in the insert. E-plate

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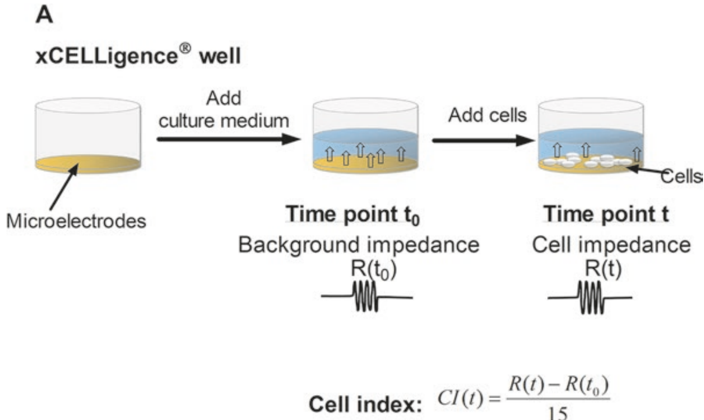


$$CI(t) = \frac{R(t) - R(t_0)}{15}$$

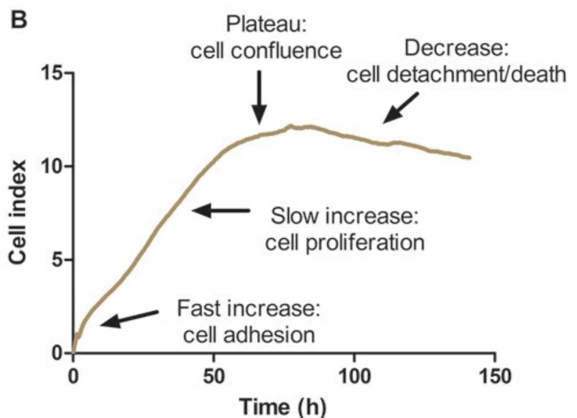


**Fig. 1** Basic principles of the xCELLigence® RTCA systems. (**a**) Impedance and cell index measurements. The xCELLigence® RTCA systems utilize differences in electrical impedance caused by cell attachment to provide a measurement of cell function expressed as a cell index. The background impedance ($R(t_0)$) is determined at the start of the experiment, with its cell index always rendered to a value of 0. Any subsequent measurements of impedance caused by cell attachment are then reflected in positive cell index values ($R(t)$). (**b**) A typical real-time impedance curve

inserts are rather used to evaluate the influence of another cell type on the cells in the E-plate wells.

The use of xCELLigence® RTCA instruments can be challenging when setting up experiments with non-adherent or non-proliferative cells. Cell impedance monitoring is also sensitive to any change in the atmospheric conditions, and experiments can be affected by humidity and temperature changes in the incubator due to door opening, for instance. Consumables can also be expensive (*see* **Note 1**). Nevertheless, xCELLigence® RTCA systems have

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**Table 1**
**Description of the most commonly used xCELLigence instruments to study placental cell lines**

| Instrument | Assays: – Cell characterization – Proliferation and cytotoxicity – Adhesion – Receptor signaling – Cell interaction: co-culture – Hypoxia studies – Phenotypic screening (mode of action of drugs) | Assays: – Invasion – Migration | Format | Compatible plate |
|---|---|---|---|---|
| RTCA DP | Yes | Yes | 3 × 16 wells | – CIM-plate 16<br>– E-plate 16 (±View and ±PET)<br>– E-plate insert |
| RTCA SP | Yes | No | 1 × 96 wells | – E-plate 96 (±View and ±PET)<br>– E-plate insert |
| RTCA MP | Yes | No | 3 × 96 wells | |

*RTCA* real-time cell analyzers, *DP* dual plate, *SP* single plate, *MP* multiple plates, *CIM* cell invasion/migration, *View* wells have a clear inspection window to visualize cells (no electrode in this window), *PET* bottomed plates, polyethylene terephthalate is an alternative to more expensive glass-bottomed plates. Offered only for E-plate 16 and 96 VIEW

numerous advantages over conventional end-point-based cell assays. The benefits of xCELLigence® RTCA systems include considerable time savings and the ability to continuously measure cellular events without the use of invasive cell labels. Furthermore, xCELLigence® RTCA systems have the capacity to adapt a running protocol in real-time response to the collected data. It is also possible to harvest the conditioned medium and the cells after xCELLigence monitoring to perform other analyses such as the staining of cell markers or measurement of cell secretions, such as human chorionic gonadotropin (hCG), human placental lactogen (hPL), or steroid hormones.

## 2 Material

In the present chapter, we will focus on three xCELLigence® models: DP, SP, and MP.

### 2.1 Cell Passage and Counting

1. Cells of interest.
2. Phosphate-buffered saline (PBS); pH 7.4.

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3. Trypsin or other cell dissociation buffers.
4. Cell culture medium.
5. Treatment (optional).
6. Manual or automated cytometer.
7. Trypan blue (optional).

### 2.2 The xCELLigence Assays

1. The xCELLigence® RTCA instrument: dual plate (DP, 16 wells × 3 cradles), single plate (SP, 96 wells × 1 cradle), or multiple plate (MP, 96 wells × 6 cradles) (ACEA Biosciences).
2. E-Plates 16 or 96 for adhesion, proliferation, and apoptosis assays (ACEA Biosciences).
3. CIM-plates 16 for migration and invasion assays (ACEA Biosciences).
4. Extracellular matrix (e.g., Matrigel™) for invasion assays.

## 3 Methods

Handle all materials in a sterile hood. The xCELLigence instrument® RTCA should be placed in an incubator and maintained at standard humidified conditions (37 °C/5% $CO_2$). Ensure that the instrument is properly connected to the computer and functional before starting the experiment (*see* **Note 2**). Optimal time points and cell densities must be determined for each cell type to be used. The following steps are a guide only; the xCELLigence user manual should be consulted before use.

### 3.1 Adhesion/Proliferation Assay

1. Add 100 μL of medium to each well (*see* **Notes 3–5**). Leave E-plate to equilibrate for 30 min at room temperature.
2. During plate equilibration, trypsinize cells and resuspend in culture medium to obtain the appropriate dilution of cell suspension (100 μL per well will be necessary). The ideal cell density has to be optimized for each cell type studied (Fig. 2 and Table 2).
3. Once the plate is equilibrated, do a background measurement in the xCELLigence instrument (*see* Subheading 3.3).
4. Add 100 μL of cell suspension and allow cells to settle on the base of the well for 30 min at room temperature (*see* **Note 6**).
5. Place plate into the instrument cradle and begin the experiment (*see* **Notes 7–9**). If performing a co-culture experiment, *see* Subheading 3.2 in Chapter 23 for a detailed protocol.

### 3.2 Migration/Invasion Assay

1. When performing an invasion assay, pre-coat the upper chamber with Matrigel™ or another desired gel matrix (*see* **Note 10**).

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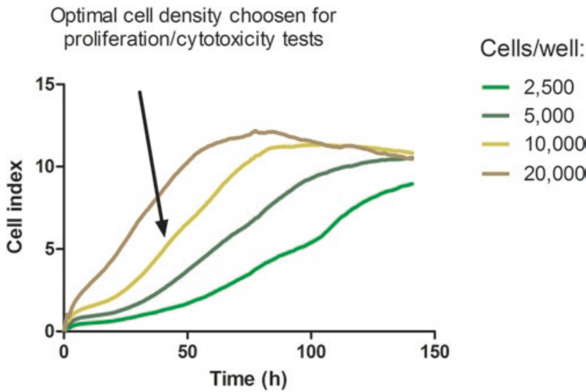


**Fig. 2** The xCELLigence® RTCA system can be used to produce typical cell titration curves to determine optimal cell densities and time points for other experiments (an example is given for the proliferation of BeWo cells, 200 μL of medium by well)

**Table 2**
**Optimal cell densities for placental cell lines using an xCELLigence® RTCA system**

| Cell line | Plate type | Cell concentration (cells/well) | References |
|---|---|---|---|
| *Proliferation tests* | | | |
| BeWo | E-plate 16 | $1 \times 10^4$ | [5] |
| JEG-3 | E-plate 96 | $2.5 \times 10^3$ and $5 \times 10^3$ | [6] (Clabault data not published) |
| HIPEC | E-plate 96 | $2.5 \times 10^3$ | (Clabault data not published) |
| HTR8/SVneo | E-plate 16 | $4 \times 10^3$–$4 \times 10^4$ | [3, 7] |
| SGHPL-4 | E-plate 16 | $1.25 \times 10^3$–$4 \times 10^4$ | [3] |
| BeWo/H295R co-culture | E-plate 16 and E-plate insert | H295R (well): $2 \times 10^4$<br>BeWo (insert): $1 \times 10^4$ | [5] |
| *Migration/invasion tests* | | | |
| HTR8/SVneo migration | CIM-plate 16 | $4 \times 10^4$–$2 \times 10^5$ | [3, 7–9] |
| HTR8/SVneo invasion | CIM-plate 16 | $4 \times 10^4$ | [8] |

2. Add 165 μL of culture medium to the bottom chamber of each well. Attach the upper chamber and add a further 50 μL of culture medium to the upper chamber of each well (*see* **Note 11**). Leave CIM-plate to equilibrate for 1 h in the xCELLigence instrument.
3. During plate equilibration, trypsinize cells and resuspend in culture medium to obtain the appropriate cell density. The ideal number of cells has to be optimized for each cell type studied (Table 2).

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4. Once the plate is equilibrated, do a background measurement of medium alone in the xCELLigence instrument (*see* Subheading 3.3).
5. After the background check, add 100 μL of cell suspension and allow cells to settle on the base of the upper chamber of each well for 30 min at room temperature. This is a critical step as convection currents from the warm medium (37 °C) may affect cell attachment and introduce variations into the experimental results.
6. Place plate into the instrument cradle and begin the experiment.

#### 3.3 Using the xCELLigence Software

1. Create a new experiment.
2. Enter the relevant information such as treatment conditions, cell types, cell density, and number of replicates in the "Layout" tab.
3. In the "Schedule" tab, add at least two steps. The first step is the background measurement step and the default settings should not be modified. The second step is the start of the experiment and can be split into sub-steps if desired to, for example, require readings to be taken every 5 min in the first hour (12 sweeps during 1 h) and once an hour for the next 48 h (48 sweeps during 48 h). Experimental protocols can be set up and saved in the computer software beforehand.
4. After placing the plate into the instrument cradle, check the "Message" tab to ensure that all connections are good. The message should say "Plate scanned. Connections ok." Usually at this step, pressing the "Play" button will prompt the software to ask if you wish to save the experiment. Click "Yes" and the background measurement will be recorded and accounted for. When completed, remove the plate and proceed to add the cells.
5. After cells have been added to the wells and allowed to settle, place the plate back into the cradle to begin the experiment. Before pressing the "Play" button, ensure that there are no connection errors in the "Message" tab as above. When the plate has to be removed from the instrument cradle (e.g., for treatments), press the "Pause" button.
6. When the experiment is finished (either at the end of the last step or after abortion of the experiment), problematic curves may be repaired using the curve adjustment tool (shift or line fit) under the "Plot" tab in the xCELLigence software.
7. Data can be normalized relative to a given time point, usually at the beginning of the experiment after the cells have settled down. Normalization corrects for well-to-well variation in initial cell densities that are not due to experimental conditions/treatments. It is also possible to express cell index as the

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difference between an experimental condition and a baseline at each given time point, which is called delta cell index.

8. Analyze data using the data analysis tool.

## 4 Notes

1. Plates may potentially be reused to minimize costs [4]. It is up to the end user to decide if they wish to do so. However, we personally would not recommend it as there is a substantial risk of contamination once the plate is placed in the incubator and exposed to a non-sterile environment. Additionally, the process of removing the cells and spent medium may damage the sensitive electrodes within the wells, which would affect subsequent experimental results.
2. The instrument must be regularly maintained as per manufacturer's instructions. Routinely check for any corrosion of cradle pins as this will likely affect experimental results. Ensure that control plates (supplied with the instrument) are run to identify any problems if the instrument has not been used for a significant period of time.
3. It is possible to add PBS in the gaps between the wells to minimize evaporation from the wells.
4. The incubator housing the xCELLigence instrument should have sufficient water in the water tray to ensure high humidity, as the electrodes are highly sensitive to fluctuations in surrounding humidity. Care should be taken to recognize a potential "edge effect" in the plate, as wells on the periphery of the plate are not exposed to the same humidity level as the wells within, which may affect cell behavior depending on the cell type studied.
5. If not all the wells are used in an experiment, the unused wells can be kept sterile with a sealing adhesive film (e.g., PCR sealing film).
6. Ensure that no air bubbles are present after pipetting the culture medium into the wells. The air bubbles can affect the electrical impedance detected in the well and give an inaccurate background measurement. Be extra careful when pipetting medium containing fetal calf serum, which can produce a lot of bubbles. One method to avoid air bubbles is to perform reverse pipetting, which will allow the target volume to be released without introducing any air.
7. If you need to treat the cells, pause the cell impedance monitoring step, remove the plate from the station and add the test compounds to the culture medium or replace the culture

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medium in the wells with pretreated culture medium. Another possibility is to plate cells directly in pretreated culture medium at the beginning of the experiment, but be aware that treatments may affect cell adhesion.

8. If at any time point of the experiment, the culture medium needs to be removed to either renew the medium or to add pretreated culture medium to the wells, avoid touching the bottom of the wells with the pipet tip as it could detach cells and introduce additional variability to the experiment. This can be done by leaving a small amount of culture medium (10 μL) in each well. Fresh culture medium should be added carefully, ideally by pipetting down slowly against the inside wall of the well.
9. Using E-Plate View, it is possible to visually inspect the cells (e.g., morphology, proliferative state), while pausing the experiment.
10. When coating the wells with Matrigel™ for the invasion assays, ensure that ice-cold medium is used to dilute the Matrigel™. It is also imperative that there are no air bubbles in the Matrigel™ coating, as these will interfere with cell movement through the matrix. From our experience, the best technique to ensure this is by reverse pipetting. The coated plate can then be incubated for 30 min at 37 °C or left in the cell culture cabinet overnight at room temperature to set. If using the incubator, do not leave the gel to set for more than 2 h as the coating may dry out.
11. There should be a clear meniscus on top of the wells in the lower chamber. This comes into direct contact with the electrodes. A "click" sound should be heard when attaching the upper and lower chambers together.

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# ANNEXE III DÉVELOPEMENT D'INSERTS DE CO-CULTURE POUR LE SYSTÈME XCELLIGENCE

Titre complet : Roche applied science launches the E-plate insert for an[illegible]f cell-cell interactions using the xCELLigence system

Titre en français : Roche applied science lance les inserts E-plate pour l'analyse d'interactions cellules-cellules en utilisant le système xCELLigence

## Trade News

Penzberg, Germany, June 13, 2012

**Roche Applied Science Launches the E-Plate Insert for Analysis of Cell-Cell Interactions using the xCELLigence System**

The co-cultivation of two different cell types is an important way to investigate cell-cell interactions and communication.

Co-cultivation can be used in a wide variety of applications, including cell signalling and toxicology testing. A key goal in drug discovery is to identify how to most effectively monitor cell-cell interactions when cells show pathology. Roche Applied Science is now providing the new E-Plate Insert 16 and E-Plate Insert 96, permitting continual impedance-based monitoring of indirect cell-cell interactions/communication using xCELLigence Instruments.

In a recent study at the INRS-Institut Armand-Frappier (Laval, Quebec, Canada) the E-Plate Insert had been used to test the behaviour of BeWo human placental choriocarcinoma cells in co-culture with H295R human adrenocortical carcinoma cells. "Using the E-Plate Insert in our co-culture experiments requires fewer manipulations and allows us to perform several experiments simultaneously - hormone measurements, signalling pathway analysis, real-time monitoring of biochemical and morphological differentiation of the cells," summarized Prof. Thomas Sanderson and Prof. Cathy Vaillancourt, who are co-investigators on this project and co-supervisors of Andrée-Anne Hudon Thibeault, the Masters student who realised the study. The Canadian group plans to use the unique abilities of the co-culture-compatible E-Plate Insert and xCELLigence cell response monitoring technology, to determine the effect of co-culture on primary villous trophoblast homeostasis (polarization, differentiation/syncytialization, fusion, apoptosis) and on H295R cells (proliferation, viability, apoptosis) as well as expression and activity levels of key placental and fetal adrenal steroidogenic enzymes.

The membrane-containing E-Plate Inserts enable sensitive, reproducible measurement of indirect cell-to-cell interactions in real-time, and in a label-free environment using the xCELLigence Instruments (RTCA DP, SP, and MP Analyzers). Combined with microscopy-compatible E-Plates VIEW, E-Plate Inserts 16 and 96 are available for low to high throughput applications. Impedance-based measurements are particularly advantageous when co-culturing cells for pinpointing when and how cells respond to each other, thus identifying the best time points for further experiments.

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# ANNEXE IV ÉTUDE D'INTERACTIONS FŒTO-PLACENTAIRES AVEC LA CO-CULTURE BEWO/H295R

Titre complet : Co-culture of H295R adrenocortical carcinoma and BeWo choriocarcinoma cells to study feto-placental interactions : Focus on estrogen biosynthesis

Titre en français : Co-culture de cellules de carcinome cortico-surrénalien H295R et de choriocarcinome BeWo pour étudier les interactions fœto-placentaires : accent sur la biosynthèse d'estrogènes

Andrée-Anne Hudon Thibeault [a, b], J. Thomas Sanderson [a, b] * et Cathy Vaillancourt [a, b] *

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*Auteur-ressource

L'article a été publié en décembre 2017 dans Methods in Molecular Biology pour le livre Preeclampsia : Methods and protocols (DOI : 10.1007/978-1-4939-7498-623)

**Contribution de l'étudiante** : L'étudiante a participé à l'élaboration de la co-culture et des protocoles et complété les expériences de caractérisation de la co-culture. Elle a également analysé les résultats, rédigé le chapitre, préparé les figures et effectué les révisions des correcteurs.

**Contribution des coauteurs** : Cathy Vaillancourt et J. Thomas Sanderson ont participé à l'élaboration de l'étude incluant la planification des expériences, l'analyse des résultats et la correction du manuscrit.

## IV.1 Résumé de l'article en français

Les estrogènes sont produits en grande quantité pendant la grossesse, suite à la coopération finement régulée entre les cortex surrénaux maternel et fœtal qui produisent les précurseurs androgènes, et le trophoblaste villeux placentaire, qui transforme ces précurseurs en estrogènes. Ces estrogènes jouent un rôle important dans le fonctionnement normal placentaire, dans l'adaptation de la mère à la grossesse, ainsi que dans le développement fœtal adéquat. La perturbation de la production des estrogènes est associée avec de mauvaises issues de grossesse et des malformations fœtales ou une altération de la programmation fœtale. Les femmes enceintes peuvent être exposées à des perturbateurs endocriniens de sources environnementales ou par la médication, et il est crucial d'étudier les effets de tels composés sur la stéroïdogenèse fœto-placentaire. Le modèle de co-culture BeWo/H295R permet d'étudier ces interactions, en rendant possible l'évaluation des expositions chimiques sur la biosynthèse d'androgènes et d'estrogènes, ainsi que sur plusieurs autres aspects de la communication fœto-placentaire.

## IV.2 Article

**This is an accepted manuscript of an article published by Springer in *Preeclampsia : Methods and protocols. Methods in Molecular Biology* on December 2, 2017. The final publication is available at Springer via http://dx.doi.org/978-1-4939-7498-623**

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# Chapter 23

# Co-culture of H295R Adrenocortical Carcinoma and BeWo Choriocarcinoma Cells to Study Feto-placental Interactions: Focus on Estrogen Biosynthesis

Andrée-Anne Hudon Thibeault, J. Thomas Sanderson, and Cathy Vaillancourt

## Abstract

Estrogens are produced in large amounts during pregnancy, as a result of a tightly regulated cooperation between the maternal and fetal adrenal cortex, which produce androgen precursors, and the placental villous trophoblast, which transforms these precursors into estrogens. These estrogens play an important role in proper placental function, in adaptation of the mother to pregnancy, as well as in adequate fetal development. Disruption of estrogen production is associated with poor pregnancy outcomes and fetal malformation or altered fetal programming. Pregnant women may be exposed to endocrine disruptors from environmental sources or medications, and it is crucial to study the effects of such compounds on feto-placental steroidogenesis. The H295R/BeWo co-culture model offers the opportunity to study these interactions, by making it possible to evaluate the effects of chemical exposures on androgen and estrogen biosynthesis, as well as on various other aspects of feto-placental communication.

**Key words** Steroidogenesis, Feto-placental unit, Estrogen, Co-culture, Trophoblast, Fetal adrenocortical

## 1 Introduction

In the human placenta, the villous trophoblast produces large amount of estrogens, including pregnancy-specific estriol. This synthesis is dependent on precursors produced by the adrenal cortex of the mother and that of the developing fetus. We have previously shown that the H295R/BeWo co-culture is a relevant model to study estrogen biosynthesis and fulfills an essential need as research tool considering the lack of suitable in vivo or ex vivo human models. In the co-culture model, H295R cells possess fetal adrenocortical characteristics, including the ability to synthetize

J. Thomas Sanderson and Cathy Vaillancourt share joint senior authorship and contributed equally to this work.

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16-α-hydroxylated androgens [1]. BeWo cells are used as a villous trophoblast model since they possess high aromatase (CYP19) activity, which confer them the capacity of transforming androgens to estrogens in great amounts.

The H295R/BeWo co-culture model was first characterized for its capacity to reproduce the feto-placental estrogen biosynthetic profile with interactions between the two cell types occurring in real time. Moreover, culturing BeWo cells on porous inserts allows the cell to adopt a polarized phenotype with differential expression of proteins on the fetal membrane and maternal membrane [2–4], which opens up the possibility of studying feto-placental transport. The co-culture model also offers the possibility to study several other feto-placental interactions (stress hormones, IGF axis, drug metabolism, etc.) as summarized in Table 1.

**Table 1**
**Major human feto-placental interactions**

| Placenta hormone/factor | Effect on fetal compartment | Reference |
|---|---|---|
| Placental growth hormone (pGH) or human placenta lactogen (hPL) | Stimulation of insulin-like growth factor (IGF) axis, which stimulates growth of the fetal adrenal cortex | [6] |
| Estradiol, progesterone, and prostaglandins | Regulation of the expression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) which oxidizes cortisol to cortisone, a major regulator of fetal organ maturation | [6–9] |
| Estradiol and β-human chorionic gonadotropin (β-hCG) | Regulation of DHEA sulfate synthesis, a precursor for estrogen production, in the H295R cells (REF) | [7, 8] |
| Estrogens | Development and maturation of fetal adrenal gland (expression of steroidogenesis enzymes)<br>Sexual differentiation<br>Fetal growth | [9–19] |
| Corticotropin-releasing hormone (CRH) | Stimulation of cortisol and DHEA production | [20–22] |
| Epidermal growth factor (EGF) | Stimulation of fetal adrenal cell proliferation | [23] |

| Fetal adrenocortical hormone | Effects on placental compartment | Reference |
|---|---|---|
| Aldosterone | Stimulation of proliferation of trophoblast | [24] |
| Cortisol | Inhibition proliferation of trophoblast | [24] |
| Dehydroepiandrosterone (DHEA) | Regulation of implantation (mouse) | [25] |

## 2 Materials

### 2.1 Cell Culture

1. H295R medium: Dulbecco's Modified Eagle's Medium (DMEM)/F12 without phenol red supplemented with 1.2 g/L sodium bicarbonate ($NaHCO_3$), 2 mg/L pyridoxine·HCl, 2.5% Nu-serum, 1% insulin, transferrin, selenium (ITS) + premix.
2. BeWo medium: Dulbecco's Modified Eagle's Medium (DMEM)/F12 without phenol red supplemented with 0.6 g/L sodium bicarbonate ($NaHCO_3$) and 10% fetal bovine serum (FBS).
3. Co-culture medium: Dulbecco's Modified Eagle's Medium (DMEM)/F12 without phenol red supplemented with 1.2 g/L sodium bicarbonate ($NaHCO_3$), 2 mg/L pyridoxine·HCl, 2.5% Nu-serum, 1% insulin, transferrin, selenium (ITS) + premix, and 1% stripped FBS.
4. 24-well cell culture plate and polycarbonate transwell permeable support with 0.4 M pores.
5. Phosphate-buffered saline (PBS).
6. TrypLE Express.

### 2.2 Real-Time Monitoring of Cell Proliferation

1. The xCELLigence Real-Time Cell Analyser Single Plate (RTCA-SP, *see* **Note 1**) (ACEA Biosciences).
2. Eplate and Eplate inserts (ACEA).

### 2.3 Aromatase Catalytic Activity

1. Dulbecco's Modified Eagle's Medium (DMEM)/F12 without phenol red supplemented with 54 nM [1β-3H(N)] androst-4-ene-3,17-dione.
2. 12-well plate.
3. Chloroform.
4. Dextran-coated charcoal (5% charcoal + 0.5% dextran (w/v)) (*see* **Note 2**).
5. Scintiverse BD scintillation cocktail.
6. MicroBeta TriLux liquid scintillation counter.
7. Liquid scintillation counting flexible microplates in 96-well and 24-well format.
8. Plate seal.
9. Uncompleted culture medium: Dulbecco's Modified Eagle's Medium (DMEM)/F12 without phenol red.
10. Radioimmunoprecipitation assay buffer (RIPA): 150 mM NaCl, 50 mM Tris–HCl PH 7.1, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate.
11. Pierce BCA Protein Assay.

12. Powder weighing spatula (for cell scraping).
13. 70% ethanol.

#### 2.4 Hormone Assay

Commercial enzyme-linked immunosorbent assays (ELISA) kits for β-hCG, dehydroepiandrosterone, androstenedione, estrone, estradiol, estriol, and progesterone.

#### 2.5 Transepithelial Resistance

1. Epithelial voltohmmeter (World Precision Instruments).
2. Bleach.
3. 1.5 M KCl solution.

## 3 Methods

#### 3.1 Cell Co-culture (See Note 3)

1. Use 90% confluent 75 $cm^2$ flasks of BeWo and H295R cells. Proceed one cell line at a time.
2. Discard the culture medium and rinse with 5 mL of PBS.
3. Trypsinize with 2 mL of TrypLE, until cells are detached.
4. Complete with 8 mL of regular culture medium for each cell type, respectively.
5. Mix by pipetting up and down and determine cell concentration.
6. Seed H295R cells in a 24-well plate at 25,000 cells/well density, 1 mL/well.
7. In another plate, seed BeWo cells in transwell inserts at 12,500 cells/well density, 0.2 mL/insert. Add 0.8 mL culture medium to the wells underneath the inserts.
8. Incubate at 37 °C for 24 h for cell adhesion.
9. Remove BeWo culture medium from the transwell inserts and wells and rinse cells twice with co-culture medium to remove FBS (*see* **Note 4**).
10. Remove H295R culture medium from the wells containing H295R cells. Rinsing is not necessary since H295R medium does not contain FBS.
11. Assemble the co-culture: 0.8 mL of co-culture medium in the wells and 0.2 mL in the transwell inserts (*see* **Note 5**).
12. Incubate at 37 °C for 24 h or longer according to the type of experiment (*see* **Note 6**).

#### 3.2 Real-Time Monitoring of Cell Proliferation

1. Set the xCELLigence software schedule in two steps: **step 1**, background measurement (default), and **step 2**, 10 min sweeps for at least 96 h.
2. Add 50 μL of regular culture medium/well in an Eplate (96 well for SP instrument) and measure background (**step 1**).

3. To the wells of the Eplate, add 100 μL of either BeWo (10,000 cells/well) or H295R (20,000 cells/well) cells.
4. Let the cells settle for 30 min at room temperature.
5. To the Eplate inserts, add 50 μL of BeWo (10,000 cells/insert) or H295R (20,000 cells/insert) cells. Add 130 μL of culture medium to the receiver plate.
6. Monitor cell proliferation in the Eplate (**step 2**) and incubate the receiver plate with the inserts containing cells for 24 h at 37 °C.
7. Pause **step 2** and remove the Eplate from the instrument.
8. Rinse wells and inserts containing BeWo medium twice with co-culture medium, before adding the co-culture medium containing the test compound.
9. Assemble the co-culture by placing the inserts containing BeWo cells above the H295R cells in wells or vice versa.
10. Continue monitoring cell proliferation (resume **step 2**).

### 3.3 Aromatase Catalytic Activity (Tritiated Water-Release Assay)

1. After the treatment period (*see* **Note 7**), remove co-culture medium from the co-culture (*see* **Note 8**).
2. Place the inserts in a 12-well plate so that the bottom of the insert is in direct contact with the well.
3. Rinse inserts and wells twice with PBS.
4. Add 50 μL/insert or 250 μL/well of 54 nM [1β-3H(N)] androst-4-ene-3,17-dione in uncompleted culture medium (Table 2).
5. Incubate 1.5 h at 37 °C.

**Table 2**
**Comparison of the protocol for inserts and wells**

| | Insert | | Well | |
|---|---|---|---|---|
| | Volume | Dilution factor | Volume | Dilution factor |
| Working solution of [1β-3H(N)] androst-4-ene-3,17-dione (54 nM) | 50 μL | 50/40 | 250 μL | 250/200 |
| Volume of supernatant + chloroform | 40 μL + 100 μL | 100/40 | 200 μL + 500 μL | 500/200 |
| Volume of supernatant + dextran-coated charcoal | 20 μL + 20 μL | 40/20 | 100 μL + 100 μL | 200/100 |
| Volume of supernatant + scintillation cocktail | 20 μL + 100 μL | 120/20 | 100 μL + 1000 μL | 1100/100 |
| Counting microplate | 96 well | | 24 well | |
| Final dilution factor | | 37.5 | | 68.75 |

6. Take 40 μL/insert or 200 μL/well of supernatant and place in a microtube containing 100 μL or 500 μL chloroform, respectively (Table 2).
7. Vortex and centrifuge at 12,000 × *g* for 5 min.
8. Take 20 μL/insert or 100 μL/well of supernatant and place in microtubes containing 20 μL or 100 μL dextran-coated charcoal, respectively (Table 2).
9. Vortex and incubate 5 min at room temperature and centrifuge at 12,000 × *g* for 15 min.
10. Take 20 μL/insert or 100 μL/well of supernatant and place in a 96-well or 24-well liquid scintillation counting microplate, respectively (Table 2).
11. Add 10 μL of 54 nM [1β-3H(N)]androst-4-ene-3,17-dione in uncompleted culture medium in a well of the liquid scintillation counting microplate to determine the specific activity in the co-culture medium (Table 2).
12. Add 100 μL/insert or 1000 μL/well of liquid Scintiverse BD scintillation cocktail.
13. Count each well for 2 min in a MicroBeta TriLux liquid scintillation counter.
14. To assess protein content, rinse cells in the wells and inserts twice with cold PBS. Scrape cells in the wells and inserts (50 μL of RIPA/well) with powder weighing spatulas. Wash spatulas with 70% ethanol between samples. Vortex every 5 min during 30 min. Centrifuge 10 min at 21,000 × *g*. Use the supernatant for protein quantification following manufacturer's instruction (Pierce BCA Protein Assay).
15. Results are either expressed as percentage of control or in pmoles of androstenedione converted/hour (Fig. 1). This expression can be normalized to mg protein content of the well or cell number.

### 3.4 Hormone Assay

1. Use the supernatant from the co-cultures and monocultures (*see* **Note 9**).
2. Keep the supernatants at −80 °C until use in the commercial kits, following manufacturer's instructions (*see* **Note 10**).

### 3.5 Transepithelial Resistance

1. Rinse the electrodes with 70% ethanol in a 15 mL tube for 10 min.
2. Remove the electrodes from the ethanol and let dry for 15 s.
3. Place the electrodes in tubes containing pre-warmed (37 °C) culture medium for at least 5 min.
4. Remove the BeWo culture medium and change to co-culture medium in the co-culture and BeWo monoculture, and place

i) Conversion of count per minute (CPM) to disintegration per minute (DPM)

$$Considering\ a\ quenching\ of\ 15\%, \quad DPM = CPM\ x\ 1.15$$

ii) Conversion of DPM to pmol

This conversion will depend on the specific activity of your [1β-3H(N)] androst-4-ene-3,17-dione.

*Example of conversion of [1β-3H(N)] androst-4-ene-3,17-dione at 26.3 Ci/mmol to DPM/mmol*

$$\frac{26.3\ Ci}{mmol}\ x\ \frac{3.7\ x\ 10^{10}\ Disintegration\ per\ second\ (DPS)}{Ci}\ x\ \frac{60\ sec}{min}\ x\ \frac{1\ mmol}{10^{9}\ pmol} = \frac{58\ 386\ DPM}{pmol}$$

$$\frac{DPM}{\frac{58\ 386 DPM}{pmol}} = pmol$$

iii) Expression considering the 3H position on androst-4-ene-3,17-dione

74.2% of tritiated-hydrogen (3H) is situated in β position in the substrate [1β-3H(N)] androst-4-ene-3,17-dione, which will lead to tritiated water release when converted.

Tritiated-water

[1β-3H(N)] androst-4-ene-3,17-dione

Estrone

$$\frac{pmol}{0.742} = pmol_{corrected}$$

iv) Dilution factor during the extraction

The dilution factors are presented in table 1.

$$For\ inserts, pmol_{corrected}\ x\ 37.5$$

$$For\ wells, pmol_{corrected}\ x\ 68.75$$

v) Correction for time of incubation with substrate [1β-3H(N)] androst-4-ene-3,17-dione

$$\frac{pmol_{corrected}}{1.5\ hour} = pmol_{corrected}/hour$$

vi) Correction for protein content of the well or number of cells

$$\frac{pmol_{corrected}/hour}{Cell\ number\ or\ protein\ content} = pmol/hour/cell\ number\ or\ pmol/hour/\ mg\ protein$$

**Fig. 1** Conversion of CYP19 activity in count per minute (CPM) to pmol of androstenedione converted/hour

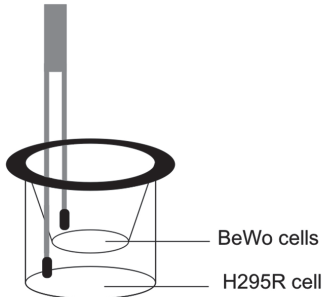


**Fig. 2** Measuring transepithelial resistance in the co-culture with a voltmeter (epithelial voltohmmeter—World Precision Instruments)

the electrodes in the well and insert. The longest electrode has to touch the bottom of the well, passing through the side hole of the insert. The electrodes should stay immobile during the measurement (Fig. 2).

5. Measure 4 h after seeding the cells, directly after assembling the co-culture and every 24 h after assembly (*see* **Note 11**).
6. Place the electrode in co-culture medium between each measurement. At the end, place the electrodes in bleach for 3 min.
7. Rinse with water.
8. Between experiments, leave the electrodes in a KCl solution.

## 4 Notes

1. The instrument RTCA dual plate (DP) could also be used with the Eplate16. The well formats are identical, but with the DP instrument, there are only 16 wells/plate instead of 96 wells/plate with the SP.
2. Prepare and mix the solution overnight on a magnetic stir plate and mix by inverting the bottle five times before using.
3. We recommend comparing the responses of the co-culture with those of BeWo and H295R cells in monoculture.
4. To rinse the inserts, prepare wells with 0.8 mL of co-culture medium. Remove the medium from each insert as well as under the insert, where a droplet often forms, and place it in the well containing co-culture medium. Add co-culture medium to the insert and repeat.
5. In order to have an even exposure of the cells to the test compound, it should be dissolved in culture medium before treatment instead of adding the compound directly to the culture medium in the well and insert.

6. We recommend a 24-h incubation of the co-culture, since a decrease in H295R cell proliferation is observed after 24 h under untreated circumstances [5].
7. To determine specificity of the tritiated water-release assay for aromatization, an irreversible inhibitor of the catalytic activity of aromatase, formestane (4-hydroxyandrostenedione), (1 μM) should be used. Positive controls (CYP19 inducers), such as forskolin (10 μM) or phorbol-12-myristate-13-acetate (1 μM), should also be included to determine the responsiveness of the cells.
8. The culture medium from the insert and well can be placed in microtubes at −80 °C for later hormone assay. Medium from the insert and well may be mixed together (total volume of 1 mL) or harvested separately, depending on the experimental requirements.
9. If too much cell debris is present, the culture medium may be centrifuged for 5 min at 10,000 × *g*.
10. Estrogen production by the untreated co-culture should be determined as a quality control. A synergistic production of estrogens and the presence of estriol should be observed in the co-culture compared to BeWo and H295R cells in monoculture.
11. Measurements will vary depending on the experiment. Values should always be compared within the same experiment. We consider that cells form a confluent monolayer when transepithelial resistance reaches a plateau.

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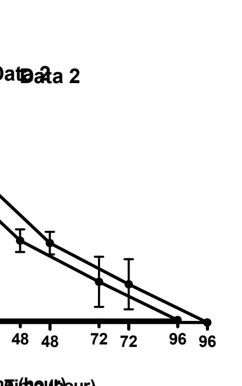
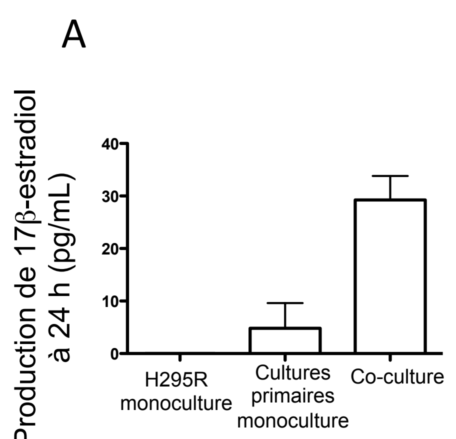
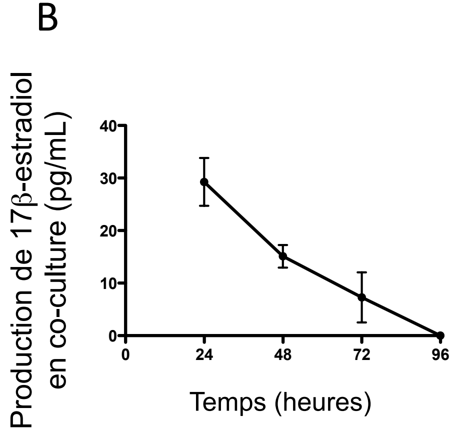
# ANNEXE V RÉSULTATS PRÉLIMINAIRES





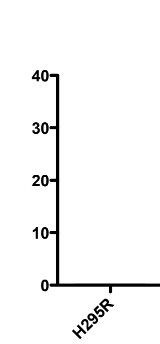



**Figure V.1 : Production de 17β-estradiol par une co-culture de cellules H295R et de primocultures de trophoblastes villeux**

**Production de 17β-estradiol par les cellules en monoculture ou en co-culture (H295R et primocultures de trophoblastes villeux mis en culture pendant 24 h (A) Résultats préliminaires de sécrétion de 17β-estradiol par une co-culture de H295R et de primocultures de trophoblastes villeux isolés d'un placenta à terme mis en culture pendant 24 h, 48 h ou 72 h (B). Les trophoblastes ont été mis en plaque dans des inserts transwell (7,7 x $10^5$ cellules par insert). Le milieu de culture a été changé quotidiennement. Les cellules H295R (2,5 x $10^4$/puit) ont été mises en culture dans une plaque de 24 puits. Les co-cultures ont été assemblées pendant les 24 h qui précèdent la fin de l'expérience. Pour la période de co-culture, le milieu utilisé était le milieu régulier H295R avec 1% de FBS dont les stéroïdes ont été extraits par traitement au charbon et 2.5% HEPES 1M.**

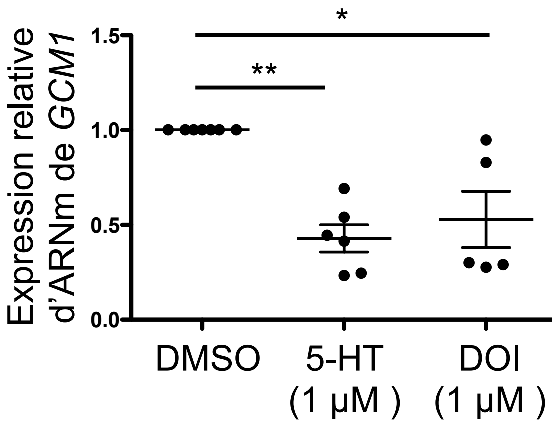



**Figure V.2 : Taux d'ARNm de *GCM1* dans les primocultures de trophoblastes villeux**

**Taux d'ARNm de *GCM1* dans les primocultures de trophoblastes villeux isolés de placenta à terme traités avec de la sérotonine (5-HT, 1 µM) ou un agoniste du $5HT_{2A}R$, le 2,5-dimethoxy-4-iodoamphetamine (DOI, 1 µM) pendant les premiers 24 h de culture. Les résultats sont normalisés sur les gènes de référence YWHAZ et PPIA. Les différences significatives (* $p< 0.05$ et ** $p<0.01$) ont été déterminées par un test de Kruskal wallis suivi d'un post-test de Dunn's.**

# ANNEXE VI LES INTERACTIONS SÉROTONINE-ESTROGÈNE

Titre complet : Serotonin-estrogen interactions : what can we learn from pregnancy?

Titre en français : Interactions sérotonine-estrogènes : Que pouvons-nous apprendre de la grossesse ?

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**Contribution de l'étudiante** : L'étudiante a effectué la revue de littérature, rédigé l'article, préparé les figures et effectué les révisions.

**Contribution des coauteurs :** Cathy Vaillancourt et J. Thomas Sanderson ont participé à l'élaboration du plan de la revue et à la correction du manuscrit.

## VII.1 Résumé de l’article en français

Nous avons révisé la littérature scientifique en lien avec quatre maladies dans lesquelles la sérotonine (5-HT) est impliquée dans l’étiologie, nommées ici maladies liées à la 5-HT, et dont la prévalence est influencée par le statut estrogénique : la dépression, la migraine, le syndrome du côlon irritable et les troubles alimentaires. Ces maladies ont toutes en commun une prévalence sexuellement dimorphique; les femmes étant plus affectées que les hommes.

La co-occurence entre ces maladies liées à la 5-HT suggère qu’il existe des mécanismes physiopathologiques communs. Dans la plupart des maladies liées à la 5-HT (à l’exception de l’anorexie nerveuse et du syndrome du côlon irritable), une diminution du tonus sérotoninergique est observée et les estrogènes contribueraient à l’amélioration des symptômes en stimulant le système sérotoninergique. La grossesse humaine est caractérisée par une synthèse unique de 5-HT et d’estrogènes par le placenta. Des maladies spécifiques de la grossesse, telles que l’hyperemesis gravidarum, le diabète gestationnel mellitus et la pré-éclampsie, sont associées avec des états hypersérotoninertiques et un niveau d’estrogènes diminué. La programmation fœtale des maladies liées à la 5-HT est un phénomène complexe qui implique notamment des différences liées au sexe fœtal, ce qui suggère l’implication des stéroïdes sexuels.

D’un point de vue mécanistique, nous avons émis l’hypothèse que les estrogènes régulent le système sérotoninergique, résultant en un effet protecteur contre les maladies liées à la 5-HT, mais qu’en retour, la 5-HT affecte la synthèse d’estrogènes dans une tentative d’atteindre l’homéostasie. Ces deux processus (synthèse de 5-HT et d’estrogènes) sont cruciaux pour une bonne issue de grossesse et donc, une perturbation de cette relation 5-HT-estrogène pourrait expliquer des pathologies spécifiques à la grossesse ou des complications de grossesse associées à des maladies liées à la 5-HT.

## VII.2 Revue de littérature

Biochimie 161 (2019) 88–108

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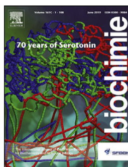

Review

# Serotonin-estrogen interactions: What can we learn from pregnancy?

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Pregnancy complications
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A B S T R A C T

We have reviewed the scientific literature related to four diseases in which to serotonin (5-HT) is involved in the etiology, herein named 5-HT-linked diseases, and whose prevalence is influenced by estrogenic status: depression, migraine, irritable bowel syndrome and eating disorders. These diseases all have in common a sex-dimorphic prevalence, with women more frequently affected than men. The co-occurrence between these 5-HT-linked diseases suggests that they have common physiopathological mechanisms. In most 5-HT-linked diseases (except for anorexia nervosa and irritable bowel syndrome), a decrease in the serotonergic tone is observed and estrogens are thought to contribute to the improvement of symptoms by stimulating the serotonergic system. Human pregnancy is characterized by a unique 5-HT and estrogen synthesis by the placenta. Pregnancy-specific disorders, such as hyperemesis gravidarum, gestational diabetes mellitus and pre-eclampsia, are associated with a hyperserotonergic state and decreased estrogen levels. Fetal programming of 5-HT-linked diseases is a complex phenomenon that involves notably fetal-sex differences, which suggest the implication of sex steroids. From a mechanistic point of view, we hypothesize that estrogens regulate the serotonergic system, resulting in a protective effect against 5-HT-linked diseases, but that, in turn, 5-HT affects estrogen synthesis in an attempt to retrieve homeostasis. These two processes (5-HT and estrogen biosynthesis) are crucial for successful pregnancy outcomes, and thus, a disruption of this 5-HT-estrogen relationship may explain pregnancy-specific pathologies or pregnancy complications associated with 5-HT-linked diseases.

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## 1. Introduction

In this review, the interaction between serotonin (5-HT) and estrogens is addressed in the context of pregnancy, a physiological state that is characterized by high estrogen levels as well as a 5-HT synthesis by the placenta. Most studies have used sex-dimorphic differences to evaluate the role of sex steroids on diseases and treatments, including estrogens. In this review, we are particularly interested in the specific cellular mechanisms that underlie those observations. We have identified four 5-HT-linked diseases: depression, migraine, irritable bowel syndrome and eating disorders, which are relevant in the context of pregnancy. Interactions between 5-HT and estrogens are also reviewed in the context of pregnancy-specific pathologies such as hyperemesis gravidarum, gestational diabetes mellitus (GDM) and pre-eclampsia.

## 2. Molecular mechanisms of 5-ht-Estrogen interaction

Most of the studies have focused on the central nervous system (CNS) of different animal models and especially in the dorsal raphe nucleus. The co-localization of the 5-HT and estrogen system supports the interactions between the two systems [1,2]. The 5-HT system is composed of the rate-limiting enzyme tryptophan hydroxylase (TPH-1: peripheral and TPH-2: central), serotonin transporters (SERTs) to allow 5-HT crossing lipid membranes [3–6] and 5-HT receptors (5-HTRs), which are G protein-coupled receptor (GPCR), with the exception of the ion-channel receptor 5-$HT_3$ [7]. Finally, 5-HT can be degraded by the enzyme monoamine oxidase (MAO) to produce 5-hydroxyindole-3-acetic acid (5-HIAA) [7]. The estrogen system includes the rate-limiting enzyme aromatase (CYP19), which converts androgen precursors to estrogens. There are four different estrogens of which secretion is typically associated with different stages of reproduction, suggesting different physiological roles: estrone (E1) is associated with the post-menopausal period, 17β-estradiol (E2) with women of childbearing age, and estriol (E3), produced by the placenta and estetrol (E4), produced by the fetal liver (from placental estradiol and estriol), are almost exclusively produced during pregnancy [8,9]. We point out that both latter estrogens can only be produced in significant quantities due to the complementary steroidogenic interactions between fetus and placenta. Since it is generally recognized that E2 has the highest estrogenic potency [9–15], most of the studies have focused on this estrogen. However, the purported high potency of E2 was not confirmed under all conditions [16] and other estrogens might have specific roles considering different affinities for estrogen receptors (ER) resulting in modification of the dimerization profile of estrogen receptors [17,18]. Estrogens can act on genomic or non-genomic pathways via different ERs: ERα, ERβ as well as GPER1, a G-protein-coupled member of the estrogen receptor family [19–21]. GPER1 was notably shown to colocalize with 5-$HT_{1A}$R in the rat hypothalamus and with 5-$HT_3$R in the rat dorsal root ganglion [22–24].

### 2.1. Effects of estrogen on 5-HT system

The effects of estrogen on the different components of the 5-HT system were studied using two ovariectomized (OVX) animal models, the rat and the primate treated or not with 17β-estradiol (E2). Hildebrandt et al. have suggested a summary of the effects of estrogens on the serotonergic system in the brain in which estrogens regulate gene expression of serotonin transporter (SERT; *SLC6A4;* decrease), monoamine oxidase A (MAOA; *MAOA*; decrease), tryptophan hydroxylase (TPH; *TPH*; increase) and serotonin receptor 2A (5-$HT_{2A}$R; *HTR2A*; increase) [25]. A compilation of published studies shows diverging effects of E2 on SERT (Table 1). Most studies have observed that E2 increases SERT expression in many brain regions. However, in HEK-293 cells that express recombinant human SERT (hSERT), E2 concentration-dependently inhibits 5-HT reuptake [26]. We have also previously shown that E2 treatment of human placental BeWo cell line decreased SERT activity, while increasing its protein expression, which could be a compensatory mechanism [27]. SERT regulation is dependent on the study model and the local 5-HT system.

Activation of 5-$HT_{1A}$R decreases the firing activity of 5-HT neurons [28]. E2 suppress 5-$HT_{1A}$R signaling, by triggering the uncoupling of the 5-$HT_{1A}$R to its associated G protein [29,30] suggesting that E2 increases the activity of the 5-HT neurons. Interestingly, in *sl6a4*$^{-/-}$ mice, where brain 5-HT concentrations are

**Table 1**
Effects of 17β–estradiol (E2) treatment in ovariectomized animals on the serotonin system in the central nervous system.

| Component of the 5-HT system in the CNS (mRNA and/or protein) | Effect of E2 treatment in OVX animals | Reference |
|---|---|---|
| 5-HT concentration | Decreased | [388] |
| | Increased | [389] |
| TPH1 (*tph1*) | Increased | [152,390–396] |
| SERT (*slc6A4*) | Increased | [152,153,155,277,389,391,397–401] [a] |
| | Decreased | [154,402–404] |
| MAOA (*maoa*) | Decreased | [405–407] |
| | Increased | [391] |
| MAOB (*maob*) | Decreased | [405–407] |
| | No effect | [391] |
| $5\text{-HT}_{2A}$ (*htr2a*) | Increased | [153,155,408] |
| | No effect | [391,400,409] |
| $5\text{-HT}_{2C}$ (*htr2c*) | Decreased | [409] |
| | No effect | [400] |
| $5\text{-HT}_{1B}$ (*htr1b*) | Decreased | [392] |
| $5\text{-HT}_{1A}$ (*htr1a*) | Decreased | [144,154,408,410,411] |
| | No effect | [392,395,400,409] |

[a] In Krajnak, 2003, increased number of binding sites in most brain regions (except suprachiasmatic nucleus where it is decreased).

decreased, the density of $5\text{-HT}_{1A}$R receptors is decreased to a greater extent in females than males [31], suggesting that female hormones might promote the effects of 5-HT on $5\text{-HT}_{1A}$R expression. However, in COS-1 cells transfected with the promoter for the $5\text{-HT}_{1A}$R and the estrogen receptor α (ERα; *ESR1*)-promoter, E2 upregulates $5\text{-HT}_{1A}$R levels via activation of ERα and NF-κB [32]. The authors suggest that NF-κB cooperates with ERα to recruit cofactors that together stimulate *HTR1A* gene promoter activity [32].

Post-synaptic $5\text{-HT}_{2A}$R are excitatory neuron receptors. E2 upregulates $5\text{-HT}_{2A}$R level in the brain of post-menopaused women [33,34]. Activation of $5\text{-HT}_{2A}$R induces transient desensitization of $5\text{-HT}_{1A}$R signaling [35]. It was postulated that following $5\text{-HT}_{2A}$R activation, $5\text{-HT}_{1A}$R becomes unable to reduce 5-HT production and so, 5-HT concentrations increase [36]. Indeed, E2 treatment of OVX rats increases the firing rate of serotonergic neurons in the dorsal raphe nucleus [37]. Rybaczyk et al. suggested that the balance between $5\text{-HT}_2$R and $5\text{-HT}_1$R receptor signaling activity contribute to different pathologies depending on the affected system [36].

Several effects of E2 on the serotonergic system in the brain are mediated by ERβ (*ESR2*) [38]. In *esr2*$^{-/-}$ mice, lower 5-HT levels were observed in several brain regions [39]. Consistent with this, in murine dorsal raphe nucleus, *tph1* level was increased by E2 and this effect was regulated by ERβ [40,41]. In a rat serotonergic neuronal cell line expressing SERT and ERβ, but not ERα, E2 treatment decreased SERT activity [42]. Moreover, E2-induced antidepressant effects in mice were also mediated via ERβ [43].

Progesterone (P4) is a widely studied ovarian and placental hormone that interacts with estrogens. Rybaczyk et al. have proposed that, in the CNS, E2 in the presence of P4 (mimicking pre-menopause), increases density of $5\text{-HT}_{2A}$R, whereas E2 without P4 (mimicking post-menopause) stimulates $5\text{-HT}_{1A}$R signaling [36]. Steroid hormones can also affect the levels of tryptophan, the precursor for serotonin synthesis. For instance, in male-to-female transsexuals, free tryptophan levels were decreased with E2 and anti-androgen treatment, whereas in female-to-male transsexuals, testosterone treatment increased tryptophan levels [44].

### 2.2. Effects of 5-HT on estrogens system

If the neuroprotective effects of estrogens on the serotonergic system in the brain have been relatively well characterized [45], the reciprocal relationship (effect of 5-HT on estrogen system) has been less studied. Associations between 5-HT and steroid hormones were observed in the rat testis, where 5-HT reduced the secretion of testosterone, and in the human adrenal cortex where it increased that of aldosterone [46,47]. In our laboratory, we have studied this relationship in the human placenta. Several effects of 5-HT in human placenta are regulated via $5\text{-HT}_{2A}$R [48–51] and notably the expression and catalytic activity of aromatase [48] (Fig. 1). This effect on feto-placental steroidogenesis suggests an important role for 5-HT in placental function and in the course of pregnancy since placental estrogens are involved in autocrine, paracrine and endocrine processes affecting placental development as well as maternal and fetal function.

The 5-HT-estrogen relationship was also indirectly studied by looking at the disruption of E2 levels by the selective-serotonin reuptake inhibitor (SSRI). SSRI treatments, which are known to increase 5-HT levels at the synaptic cleft, are associated with decreased circulating estrogen levels in the rat [52,53]. Exposure to SSRIs was also associated with altered reproductive function in fish, mollusks [54–59] and in human [60]. In fish and mussels, exposure to these antidepressants was associated with altered E2 levels (increase or decrease depending on the study model and exposure

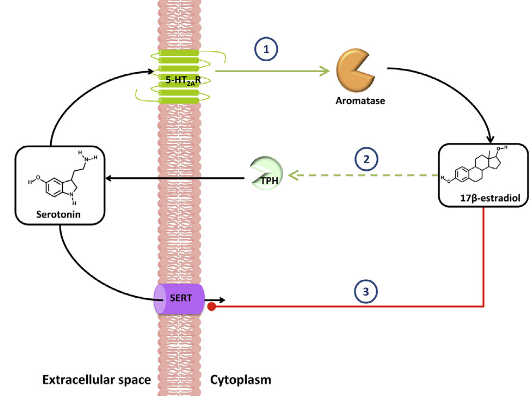


**Fig. 1. Serotonin/estrogen interactions observed in the human trophoblast and in the literature.** The amount of serotonin (5-HT) available to stimulate 5-HT receptors (5-HTR) is influenced by the 5-HT transporter (SERT). 1) 5-HT stimulates $5\text{-HT}_{2A}$R, which increases aromatase activity to produce estrogens; 2) 17β-estradiol regulates the synthesis enzyme of 5-HT, tryptophan hydroxylase (TPH); 3) 17β-estradiol inhibits SERT. Green lines: stimulation; Red line: inhibition; Full line: our observation in the human trophoblast; dotted line: observation in the literature.

duration) [59,61–63]. In certain fish species, *cyp19a1a* expression was modified by SSRI exposure in ovarian tissue [63] and brain [64]. The expression of *CYP19* was also affected differently depending on the time of exposure (a short-term decrease and longer-term increase) [64]. Jacobsen et al. studied the effects of five SSRIs on aromatase catalytic activity using microsomes and hormone secretion by the adrenocortical carcinoma cell line H295R. They observed that all tested SSRIs (fluvoxamine, citalopram, paroxetine, sertraline and fluoxetine) inhibited CYP19 catalytic activity in the microsome assay, whereas various effects were seen on hormonal secretion by H295R cells [65]. For example, they showed that E2 secretion was decreased by fluvoxamine, whereas it was increased by fluoxetine, paroxetine and sertraline [65]. In another study, Hansen et al. found that fluoxetine, paroxetine, citalopram, escitalopram, sertraline and fluvoxamine all increase the estrogen/androgen ratio, which is an indicator of aromatase activity [66]. Using a co-culture model of feto-placental steroidogenesis developed by our laboratory (BeWo/H295R cell co-culture), we also observed that fluoxetine induced aromatase activity in BeWo and H295R cell lines without affecting overall estrogen secretion (E1, E2 and E3) by the co-culture, while its metabolite, norfluoxetine, inhibited aromatase activity in BeWo cell line, resulting in decreased estrogen secretion by the co-culture [27]. Hence, the effects of SSRIs on CYP19 and hormone secretion depend on the type of SSRI and on the study model used. The expression of the different components of the 5-HT system should be better characterized in the various study models to better understand these effects.

### 2.3. Sex differences in basal 5-HT and estrogen levels

Little is known about basal (non-pathological) sex differences in the interaction between 5-HT and estrogen systems and its implications. Serotonin concentrations are higher in multiple brain regions of female compared to male rats [67]. In women, 5-HT levels are higher in plasma and whole blood [68], whereas it is lower in blood platelets compared to men [69]. Consistent with this, healthy women have lower SERT activity in platelets than men [70], which could contribute to higher extra-platelet 5-HT levels (plasma and whole blood). Moreover, plasma 5-HIAA, the main 5-HT metabolite, is lower in women, suggesting a lower metabolism of 5-HT in women compared to men [68]. In women's brain, $5\text{-HT}_{1A}R$ binding potential is lower than in men, whereas $5\text{-HT}_{2A}R$ binding potential is not affected by sex [71]. Considering the inhibitory action of $5\text{-HT}_{1A}R$ on 5-HT firing, this may also contribute to the increased 5-HT tone in women compared to men.

Regarding the estrogen system, the distribution of ERα- and ERβ-immunoreactive neurons is similar in males and female mouse brainstem and spinal cord [72], while only ERα-immunoreactive neurons display a higher staining density in males than females in most brain regions as well as in OVX female treated with vehicle control compared to OVX + E2 [72]. This suggests that ERα is upregulated by low estrogen levels and/or that competition between estrogens and the antibody used for ER binding occurred [72]. Moreover, in primary cultures of serotonergic neurons from rat embryo, E2 inhibits neurite growth suggesting that this could be involved in sexual dimorphic serotonergic innervation [73]. To date, no data is available on sex-difference in GPER1 expression and function.

Altogether these findings show that 5-HT levels are higher and neuronal ERα expression is lower in females than males, suggesting that females might be more responsive to lower doses of estrogens. Considering this, we postulate that a greater response to estrogens could contribute to the observed increased 5-HT levels in females.

## 3. Serotonin-linked diseases

Several 5-HT-linked diseases co-occur with each other. For instance, irritable bowel syndrome (IBS) overlaps in occurrence with migraine [74,75], anxiety, and depression [76,77]. Eating disorders are associated with other comorbidities linked to 5-HT, such as depressive symptoms, anxiety and obsessive-compulsive disorder [78]. Pregnant migraine-sufferers are at increased risk of depression [79]. Moreover, having depressive symptoms prior to pregnancy increases the risk of suffering from pre-eclampsia, a pregnancy-specific disease, which involves 5-HT, in a bidirectional relationship (see section 7.3) [80]. Hyperemesis gravidarum was linked to migraine based on the common olfactory sensibility that triggers nausea and vomiting [81]. Interestingly, pregnancy with a female fetus shows higher rates of depression and of cardiovascular disease-related mortality [80], which suggest an impact of fetal sex on the co-occurrence of those two pathologies.

In animal models, there is a co-occurrence of symptoms that are linked to 5-HT disruption. In *slc6a4*$^{-/-}$ mice, anxiety behaviors are observed [31] as well as altered gastrointestinal motility [82]. In *tph1*$^{-/-}$ mice, gastrointestinal and cardiac functions are altered [83], while in *tph2*$^{-/-}$ mouse, increased anxiety- and depression-like behaviors are observed, with sex-dimorphic effects [84]. Males *tph2*$^{-/-}$ had increased impulsive and aggressive behavior, while females had increased anxiety and depressive-like behaviors [84]. Alteration of behaviors (anxiety and depression-like) were observed in central 5-HT-deficient mice (deletion of *tph2* or *Lmx1b*, a transcription factor involved in the development of serotonergic neurons) [85]. These behaviors are affected by pancreatic islet cell depletion, underlying the interaction of CNS with peripheral organs for mental diseases.

### 3.1. Sex differences in the lifetime prevalence of 5-HT-linked diseases

It is well known that the prevalence of depression, migraine, IBS and eating disorders is influenced by the estrogenic state. Sex differences, especially those observable during higher levels of sex steroids periods are an indicator of the possible etiology of diseases. Women of childbearing age (from menarche to menopause) compared to men are twice as likely to suffer from depression [2,86–88] and with greater severity [87,89,90], three times as likely to suffer from migraine [91–94], with more symptoms and associated disability [94], twice as likely to suffer from IBS [95], and three times as likely to suffer from eating disorders [25]. The efficacy of antidepressant treatments also varies between men and women [96–99]. For instance, women appear to respond better to the SSRI citalopram than men [100]. These sex-dimorphisms are likely attributable to sex steroids, although the possibility that other gender-related variables play a role, such as differences in healthcare-seeking behavior and sociocultural characteristics between men and women cannot be excluded [101]. For example, the predominant occurrence of IBS in women is not observed in Africa and Asia [102,103]. For all 5-HT-linked diseases studied, the female predominance becomes evident after the onset of puberty, suggesting that female hormones are permissive for the development of 5-HT-linked diseases and/or that male hormones are protective.

Several reproductive milestones (menstruation, pregnancy, the postpartum period and menopause) associated with large changes in estrogen levels are associated with changes in prevalence of 5-HT-linked disease and the intensity of their symptoms (Table 2). For example, the aura associated with migraines, vary with estrogen levels (increase with high estrogen) [93]. During pregnancy and when a woman is taking the contraceptive pill continuously, estrogen levels are high and prevalence is decreased for most 5-HT-

**Table 2**
Prevalence of depression, migraine, irritable bowel syndrome and eating disorders in women, according to the reproductive stage and estrogen level.

| Reproductive stage/contraception | Estrogen level | Depression [412–415] | Migraine [92,93,201,210] | Irritable bowel syndrome [108,238,242,253,416] | Eating disorders [25,78,417] |
|---|---|---|---|---|---|
| Puberty/Menarche | ↑ | ↑ | ↑ | ↑ | ↑ |
| Follicular phase of the menstrual cycle | ↑ | - | ↓ | ↓ | - |
| Luteal phase of the menstrual cycle | ↓ | ↑ Premenstrual dysphoric disorder | ↑ | ↑ | - |
| Pregnancy | ↑ | → | ↓ | ↓ Many IBS symptoms ↑ Constipation | ↑↓ |
| Postpartum | ↓ | ↑ | ↑ | - | - |
| Peri menopause | ↓ | ↑ | ↑ | ↑ | ↑ |
| Post menopause | ↓→ | ↓ | ↓ | ↓ IBS symptoms ↑Constipation | ↓ |
| Estrogen-containing hormonal contraception (extended use) | ↑→ | ↑→ mood symptoms | ↓ | ↓ | - |
| Estrogen-containing hormonal contraception (week of withdrawal) | ↓ | - | ↑ | - | - |

↑: High level/Increased prevalence; ↓ Low level/Decreased prevalence.
→ Stable level/Unchanged prevalence.

linked diseases. In situations where estrogen levels decrease such as the luteal phase of the menstrual cycle, postpartum and peri-menopause periods, a worsening of 5-HT-linked diseases occurrence is the most frequent scenario, while in the post-menopause period, when estrogen levels are low but stable, an improvement is observed. In accordance with this, estrogen withdrawal is a recurrent hypothesis to explain several of those pathologies [92–94,104–109].

## 4. Serotonin-linked diseases: are they related to altered estrogen system?

Alterations of the 5-HT system are associated with several diseases, such as depression, migraine, IBS, eating disorders and pregnancy complications such as GDM and preeclampsia [110–122]. Insufficient information exists to establish a formal link between 5-HT and estrogen in these 5-HT-related diseases, but this area of research raises interest since selective estrogen receptor modulators (SERMs) have been suggested to be further explored for the treatment of some 5-HT-linked diseases [25]. In this section, we review the alteration of the 5-HT and estrogens system in 5-HT-linked diseases in general population and pregnant woman.

### *4.1. Depression*

Depression affects more than 300 million people and is the largest cause of disability worldwide [123]. Use of antidepressants has increased more than 2-fold since 2000 in the organization for economic co-operation and development (OECD) countries, with an average consumption of antidepressants of 6.7% in 2017 [124]. However, these data do not include the United States, where, between 2005 and 2008, the use of antidepressants reached 10% [125].

#### *4.1.1. Alteration of the 5-HT system associated with depression*

Depression is associated with a decrease of 5-HT levels in the CNS, leading to a decreased stimulation of neuronal 5-HTRs [126,127], disrupting function regulated by 5-HT such as sleep-wake cycle, food intake and mood, which explain some symptoms of depression [121,128,129]. Depression has been associated with single nucleotide polymorphisms (SNPs) in the *TPH2* gene, which suggests altered 5-HT biosynthesis in the CNS [130]. Moreover, the p11 protein is a member of the S100 EF-hand protein family, which increases the localization of 5-$HT_{1B}$R to the cell surface, is decreased in the brain of human and animal models of depression, suggesting a decrease in 5-$HT_{1B}$R at the cell membrane. Reciprocally, *p11−/−* mice have depression-like behavior [131]. It was also observed that the pre-synaptic 5-$HT_{1D}$ autoreceptor is increased in the brain of female patients with major depression compared to match female control, but not in male [132], suggesting a role of sex hormones in the etiology of this mental illness.

In platelets from depressive patients, 5-HT content is decreased [133]. Most studies have shown a decrease in SERT density in platelets associated with depression [133–137]. Other studies have reported inconsistent results [138–140]. As for 5-$HT_{2A}$R in platelets, an increased density was associated with depression, mainly in women [134,138–142]. The observed inconsistencies in 5-HT system parameters in platelets were suggested to be due to methodological flaws between the different studies [143], such as patient's age which influence serotonergic function in platelets [137].

#### *4.1.2. Alteration of the estrogen system associated with depression: what can we learn from treatments?*

Knowledge of the disturbance of estrogenic function in patients with depression is limited. Difference in *ESR* gene level between depressive and non-depressive people has been observed. For example, *ESR2* mRNA is decreased in the brains of suicidal people, a condition highly associated with depression [144]. In a stress-sensitive monkey model, E2 and P4 levels were lower and associated with higher mRNA level of *HTR2A* and *HTR2C* in the brain compared to normal animal, while *HTR1A* was not affected [145].

Interestingly, studies have shown an antidepressant effect of E2 suggesting again a role of this hormone in the etiology of depression. Indeed, hormone replacement therapy in postmenopausal women was shown to enhance the antidepressant effect of SSRIs and/or to be effective antidepressant *per se* [146–150]. It was also demonstrated that E2 induced antidepressant effects in rodent models of depression [151,152]. The mode of action of estrogens likely involves ER signaling [90,153–155] and/or coupling to sigma-1 receptors, which are receptors expressed in the CNS and known to modulate neurotransmitter systems [151]. In the brain of rodent, ERβ stimulation increases 5-HT synthesis [156], while GPER1 stimulation is involved in desensitization of 5-$HT_{1A}$R associated with antidepressant effects [22,156,157]. Indeed, it was suggested that E2 could exert an antidepressant effect by increasing levels of 5-HT and 5-$HT_{2A}$R signaling [36]. In the Flinders-sensitive-line (FSL) rat, which was genetically selected for its features that resemble human depression, a reduced *htr2a* mRNA level was observed in the brain and restored by E2 treatment [144], suggesting regulation of this receptor by estrogen. Moreover, when administered with

SSRI, E2 accelerates the 5-$HT_{1A}R$ desensitization [158] and blocks the inhibitory effect of SSRI on SERT by a mechanism involving ERα [159–161].

*4.1.3. Depression during pregnancy*

Depression affects around 10–15% of pregnant women worldwide and has been associated with obstetrical complications such as premature birth, pre-eclampsia, intrauterine growth restriction (IUGR), reduced birth weights and premature placental detachment, as well as co-morbidity factors, such as psychotic, impulsive and suicidal behaviors [162–167]. Maternal use of antidepressants during pregnancy has increased more than 3-fold over the last decade, with 6–8% of pregnant women in 2005 under antidepressant treatment [168,169]. Some pregnancy problems associated with depression and/or antidepressant treatment, such as intrauterine growth restriction (IUGR) and pre-eclampsia, are also related to placental dysfunction and altered 5-HT and estrogen systems [170,171] (see section 7). Alterations in the placental 5-HT system could explain a certain vulnerability to pre-eclampsia in pregnant women with depression considering the role of 5-HT in vasoconstriction (see section 7.3) [172,173]. For example, an increase of placental *SLC6A4* mRNA [172] and a decrease of *MAOA* mRNA levels [173] have been associated with depression and anxiety.

SSRI treatment remains one of the main treatment prescribed to pregnant women [168,169]. SSRI use during pregnancy is associated with obstetric complications such as small for gestational age, premature birth, and fetal morbidity [174,175]. SSRIs may affect fetal development resulting in cardiac malformations [176–179], pulmonary hypertension [180] and serotonergic weaning syndrome, also called altered neonatal adaptation (tremors, restlessness, stiffness, irritability, continuous crying, chills, difficulty sleeping and feeding and convulsions) [177,179,181–183]. Other studies report no association between SSRI antidepressant use and alteration of pregnancy/fetal development [184,185]. Those inconsistencies can be explained by differences in the methodological parameters such as the dose, type of treatment and trimester of exposition [186]. Studying SSRIs as a group of drugs instead of separately could explain some controversial results. Indeed, concerning fetal health, paroxetine and fluoxetine were associated with cardiac malformation, while this association was not observed for other SSRIs [176,177,187]. The effects of the SSRIs also vary according to the trimester of pregnancy. Exposure to SSRIs during the third trimester was associated with an increased incidence of serotonin weaning syndrome, premature birth, minor anomalies, increased need in specialized care and poor neonatal adaptation, which include respiratory difficulties and small for gestational age [179,181,183,186,188]. Cardiac malformations were associated with exposure during the first trimester [177,178]. Pregnant women with depression that have a later gestational exposure to SSRIs, however, generally end up suffering from deeper depression, which is a confounding factor [186]. Moreover, in human studies, it is not possible to exclude that the observed effects are due to depression *per se*.

*4.2. Migraine*

Migraine affects about 15% of the population and is one of the most frequent causes of disability [189]. Migraine is a neurovascular disorder, in which there is a dilatation of blood vessels, sterile inflammation and further nerve activation [190,191]. Serotonin is thought to play a role in the sensitization of sensory neurons [190].

*4.2.1. Alteration of the 5-HT system associated with migraine*

Reduced serotonergic tone have been described in migraine [192]. Serotonin depletion from blood platelets is thought to cause cranial extra-cerebral vasodilatation [193] and the measure of the 5-HT released by platelets have been suggested to monitor the development of the attack [194]. Migraine attacks are associated with high levels of urinary 5-HIAA and low levels of platelet 5-HT [190]. In migraine patients with aura (a visual symptom that precedes or coexist with migraine), during the headache-free interval, increased serum 5-HT levels cause downregulation of 5-$HT_2R$ in platelets [195], but the role of this receptor in migraine remains unclear. The 5-$HT_{1B}R$ and 5-$HT_{1D}R$ are known to play a role in migraine via the regulation of vascular smooth muscle constriction and inhibition of vasoactive peptide synthesis [104]. Indeed, during migraine attacks, 5-$HT_{1A}R$ expression in the brainstem is increased [196], which could contribute to the decreased serotonergic tone.

Changes in platelet 5-HT levels during menstrual cycle correlate with migraine prevalence (increased 5-HT in high estrogen-follicular phase and decreased 5-HT in lower estrogen-luteal phase) [190]. Martin and Behbehani reviewed the influence of the menstrual cycle phase on the serotonergic system in migraine patients [107]. They concluded that menstrual migraines could be characterized by decreased serotonergic tone during late luteal and early follicular phases, which are low estrogen phases [107]. Indeed, a decrease in platelet 5-HT and an increase in MAO-B and platelet 5-HIAA levels during the luteal phase is associated with an increased frequency of migraine attacks [197,198]. Consistently, during the high estrogen state of the estrus cycle (proestrus), *tph1* mRNA and protein levels are higher in mice trigeminal ganglia, a brain region involved in migraine [199]. However, in primary cultures of trigeminal cells from rats, tph1 protein levels were not affected by E2 treatment [199]. In a small cohort (4 women with migraine and 6 matched healthy women), oral contraceptive discontinuation was associated with a decreased response of cortisol and prolactin to m-chlorophenylpiperazine, a 5-HT agonist with high affinity for 5-$HT_1R$ and 5-$HT_2R$. The response was restored by supplementation with transdermal $E_2$, as was the duration and severity of the migraines [200]. This suggests that in migraine patients, estrogens are crucial to stimulate the serotonergic system.

*4.2.2. Alteration of the estrogen system associated with migraine: what can we learn from treatments?*

Serum levels of estrogen are not modified in women with menstrual migraines [107,201]. However, polymorphisms in *CYP19A1* gene were associated with migraine risk (rs10046) or protective effect (rs4646) [202,203]. Some polymorphisms in the *ESR1* gene, which codes for ERα, could also contribute to the pathogenesis of migraine [204], but no association of polymorphisms in *ESR2*, which codes for ERβ, were observed [202]. The role of such polymorphism in the heritability of migraine needs to further be explored [93]. Moreover, ERα and GPER1 (but not ERβ) were shown to be involved in trigeminal sensitization in the rat, but the role in relation to migraine remains to be studied [205]. Several authors have proposed a mechanism of the 5-HT-estrogen interaction in the physiopathology of migraine. Gupta et al. proposed that estrogen regulates the serotonergic system in migraine patients and has a protective role by enhancing 5-HT-mediated actions [38]. Fioroni et al. suggested that menstrual migraine patients have a serotonergic system hypersensitive to hormonal stimulation [198]. However, considering that estrogen levels are not altered in migraine patients, but that *CYP19A1* polymorphisms are associated with migraine, it is hard to postulate if migraine is a result of an abnormal response to estrogens (via ER notably) or of an abnormal synthesis of estrogens.

Oral contraceptive pills have shown some effectiveness in migraine treatment. By eliminating menstrual cycling (especially if taken without interruption to avoid the withdrawal period), they

help reduce hormonal migraines [92,206]. They act, in a way, to mimic pregnancy by avoiding the hormonal fluctuations that normally occur during the menstrual cycle. Consistent with this, E2 gels administered percutaneously or E2 transdermal patches showed some efficiency in preventing the frequency, duration and severity of migraines [207,208]. However, others found no effects [209]. Shuster et al. suggested that therapy for migraine should aim to stabilize estrogens levels [210].

#### 4.2.3. Migraines during pregnancy

A reduced frequency of migraine, especially without aura, from the second trimester of pregnancy, is the most observed scenario [94,106,211–213]. Preceding this improvement, a transient worsening of migraine symptoms may occur during the first trimester [92,214]. Factors that may contribute to migraines in the first trimester of pregnancy are hormonal fluctuations, morning sickness and sleep deprivation [214]. A proposed explanation for the evolution of migraine prevalence during pregnancy is the shifting ratio of maternal estrogen to progesterone as pregnancy progresses [215]. However, for some women, their condition is worsened as pregnancy progresses [216].

Women with migraines are at increased risk of adverse pregnancy outcomes or altered fetal development such as low birth weight, preterm birth, hypertensive disorders such as pre-eclampsia, adverse cardiometabolic risk profile, delivery by Cesarean and increased risk of congenital malformations [94,217–223]. There are also maternal concerns associated with migraines in pregnancy due to the increased risk of ischemic stroke, acute myocardial infarction, heart disease and thromboembolic events [224].

The options to treat migraine during pregnancy are limited and women often prefer avoiding any treatment. A favored option is to delay pharmacological treatment until the end of the first trimester as risks for pregnancy outcome and fetus are lower after this period, but this is also when women often experience migraine relief [212]. Prophylactic treatment might be prescribed if migraine crisis is frequent [94,225,226]. For example, non-pharmacological treatments of migraine have shown some efficiency and could be beneficial to pregnant women, such as biofeedback, in which the patient develops a physiologic response associated with measurement of physiologic systems based on operant conditioning, and relaxation [94].

Usual treatments of migraine for non-pregnant women are category D and X (positive evidence of fetal abnormalities and/or fetal risk) according to the Food and Drug Administration (FDA) classification during pregnancy and should be avoided [92,94]. This includes aspirin, nonsteroidal anti-inflammatory drugs, and ergot derivatives such as ergotamine, methysergide and dihydroergotamine [92,94]. Triptans, which are the main medication prescribed in the non-pregnant population, lack data on teratogenicity [94]. Triptans are defined as 5-$HT_{1B/1D}$R agonists but they also, to a lesser extent, stimulate 5-$HT_{1A}$ or $_{1F}$ receptors [227]. Triptans produce selective cranial extra-cerebral vasoconstriction (via 5-$HT_{1B}$) and inhibition of the trigeminovascular system (via 5-$HT_{1D}$ and $_{1F}$) [193]. To reduce side effects associated with triptans, other receptors are currently being studied as potential targets for migraine treatment, including 5-$HT_{1B/1D/1F}$R agonists combined or not with antagonists of 5-$HT_{2B/2C}$R, 5-$HT_3$R and 5-$HT_7$R [193]. Development of new drugs to treat migraine that activate 5-HT receptors more selectively (especially 5-$HT_{1F}$R) should target central receptors over peripheral receptors [192]. Most of the data on triptan use during pregnancy arises from sumatriptan. Sumatriptan is classified as category C (animal studies showed adverse effects on the fetus, but no adequate study in human) according to the FDA classification [228]. Studies are either insufficient to rule out the risk of birth defects [229] or they show no evidence to suggest an adverse effect on fetal development [218,230]. Risks of birth defects, postpartum hemorrhage and spontaneous abortions have been associated with triptans [212]. However, triptan treatment redemption previous to pregnancy was associated with a higher risk of major congenital malformation compared to redemption during pregnancy [231]. Hence, considering the effects of migraines *per se* during pregnancy, the benefits of using triptans during pregnancy remain to be explored.

Based on these FDA classifications of medications, acetaminophen and metoclopramide are the recommended first-line treatments for migraine and nonsteroidal anti-inflammatory drugs can be used in the first and second trimesters. In a letter to the editor of the journal Headache, Maggioni et al. question the current therapeutic options offered to pregnant women, who are often left to treat their migraines with acetaminophen and rely on the expectation that their situation will improve with the progress of pregnancy [232]. They highlight the importance of research on the effects of triptans during pregnancy [232]. In a study of the Norwegian population, a shift in the type of medication (favoring acetaminophen) and a decrease in the medication use were observed during pregnancy [216]. This shows a real concern of pregnant women about the safety of migraine treatments during pregnancy.

### 4.3. Irritable bowel syndrome

IBS is a gastrointestinal disorder associated with abdominal pain or discomfort [233]. There are two main subtypes of IBS, with constipation (IBS–C, decreased gastrointestinal motility) or with diarrhea (IBS-D, increased gastrointestinal motility) [233]. In the following section, we will present the nuances in the 5-HT-estrogen relationship between those two subtypes of IBS.

#### 4.3.1. Alteration of the 5-HT system associated with IBS

Serotonin is known to be involved in the motor-sensory function of the gastrointestinal tract and colonic motility [234–236]. The 5-$HT_3$R is involved in colonic motility and visceral pain in the gut [237] and is expressed in neurons that excite the gastrointestinal muscle [238], which makes it a target of interest in IBS treatment. Regarding 5-HT concentrations in colonic mucosa, the literature reports contradictory results. In rectal tissue from IBS-C and IBS-D patients, mucosal 5-HT content, *TPH1* and *SLC6A4* mRNA levels and SERT immunoreactivity were decreased, although 5-HT release by these tissues did not differ [239]. However, some have found that colonic mucosal 5-HT concentrations from IBS-C patients were higher [240] and others that in IBS-C or IBS-D patients, *SLC6A4* mRNA levels remained unchanged, whereas p11 was increased, which suggest increased 5-$HT_1$R levels to the cell membrane [241].

Platelet and platelet-depleted plasma 5-HT concentrations in IBS patients are influenced by sex, menstrual status and the subtype of IBS [70,242]. Indeed, IBS-D patients exhibit higher postprandial platelet-depleted plasma 5-HT concentrations compared with healthy volunteers, while IBS-C patients have reduced postprandial platelet-depleted plasma 5-HT concentrations [242,243]. A greater brain 5-HT synthesis in female IBS patients compared to controls was also observed [244]. It was suggested by Franke et al. that the assessment of SERT function in platelets could be used as a screening tool for detecting IBS under certain conditions [70]. Sex would have to be taken into account as men IBS patients have greater SERT activity in platelets compared to control men, without affecting platelet 5-HT concentration, while in women, 5-HT uptake rate in platelet is unchanged, but 5-HT concentrations are lower compared to control women [70]. This data suggests that 5-HT is less available in the women bloodstream than in men [70]. While

the mechanisms by which 5-HT levels are affected in IBS remain unknown, a decreased 5-HIAA/5-HT ratio in platelet-depleted plasma and rectal mucosal tissue suggests that IBS patients have an altered metabolism and/or reuptake rather than synthesis and/or release of 5-HT [242,243,245].

A meta-analysis showed no genetic polymorphism in *SLC6A4* associated with IBS-D [246]. However, in a study where the sex of the IBS patient was taken into account, men with IBS-D had a reduced frequency of a *SLC6A4* short variant (S) with lower transcriptional activity than for the long variant [247]. Distribution of long/short genotype varies greatly among various populations and this may explain certain inconsistencies regarding the observed association of SERT polymorphisms with IBS [248]. Taken together these data show a role of 5-HT with sex-dimorphic effects that suggest a role for sex hormone in the IBS etiology.

#### 4.3.2. Alteration of the estrogen system associated with IBS: what can we learn from treatments?

Estrogen receptors (ERβ and to a lesser extent ERα, as well as GPER1) are found in the gastrointestinal tract in the rat and primate tissue [249,250] and it has been postulated that estrogens may affect IBS symptoms by influencing inflammation or via their effects on the peripheral and central nervous system [67]. However, it is not clear whether female or male sex hormones are deleterious or protective [251]. Interestingly, in an animal model of IBS (restraint stress-induced bowel dysfunction of rats), treatment of OVX females with E2 and progesterone improved colon contractility to restraint stress [238]. Ovariectomized rats have increased *htr3* mRNA levels in the colon which is also restored to control level by hormone replacement therapy [238].

Several drugs have been tested to treat IBS symptoms. This includes selective $5\text{-HT}_4$R agonists and $5\text{-HT}_3$R antagonists [234,237]. $5\text{-HT}_3$R antagonists improve the symptoms of IBS-D patients, whereas $5\text{-HT}_4$R agonists are more effective in IBS-C patients [234,243]. Interestingly, the $5\text{-HT}_3$R antagonist alosetron is more effective in women than men [108,252], suggesting that its effectiveness could be associated with sex differences in $5\text{-HT}_3$R expression, alosetron clearance, 5-HT synthesis, adipose tissue compartment distribution and/or hormonal levels [108]. Treatments used for other 5-HT-linked diseases also exert positive effects on IBS symptoms, such as SSRIs, sumatriptan, and buspirone ($5\text{-HT}_1$R agonist) [234]. Moreover, novel agonists for IBS treatment target $5\text{-HT}_2$R [234]. There is still a lot of work to be done to understand the involvement of these receptors in IBS and the role of the sex hormones in their different effectiveness.

Therapeutic approaches for IBS have also targeted suppression of ovarian steroidogenesis [253]. Leuprolide a gonadotropin-releasing hormone agonist induces a hypoestrogenic state and was successful in relieving female IBS patients with menstrual cycle-related symptoms [254]. Oral contraception is also associated with a reduction of symptoms of IBS at menses, whereas hormone replacement therapy during menopause has the opposite effect [108]. This is a complex portrait that requires further investigation in order to understand the potential role of estrogenic treatments of IBS patients.

#### 4.3.3. Irritable bowel syndrome during pregnancy

Normal changes in gastrointestinal function occur in pregnant women, such as a delayed orocecal transit time in the third trimester [255], decreases intestinal transit and increases GI symptoms [238,253]. Pregnancy may increase or decrease IBS symptoms (Table 2) and IBS has been associated with increased risk of miscarriage, ectopic pregnancy, recurrent fetal loss, IUGR, preterm delivery and low-birth weight independently from associated depression/anxiety [256,257]. However, the mechanism of action involved has never been studied.

### 4.4. Eating disorders

It is difficult to understand the biological processes underlying eating disorders (notably anorexia, bulimia nervosa and binge-eating disorder) considering the multitude of interacting factors including socio-psychological, stress, gender, genetic, socio-cultural pressures and gonadal steroid secretion that may exacerbate 5-HT deregulation [258].

#### 4.4.1. Alteration of the 5-HT system associated with eating disorders

Changes in the 5-HT system are known to influence feeding behaviors in animal models and humans [25], but reciprocally the restricted diet associated with eating disorder could also contribute to the decrease in brain 5-HT synthesis, due to a decrease in tryptophan availability and should be considered as a confounding factor [122,258,259].

Alterations of the 5-HT system in the cerebrospinal fluid (CSF) are associated with eating disorder, even after recovery (reviewed in Ref. [258]). Interestingly, women with long-term recovery from eating disorder have elevated concentration of 5-HIAA in the CSF, which would be associated with behavioral inhibition, whereas levels are normal in eating disorder patients [122]. The $5\text{-HT}_{1A}$R and $5\text{-HT}_{2A}$R are the two subtypes of receptors that were mainly studied and linked to eating disorders symptoms [25]. Balance in $5\text{-HT}_{2A}$/$5\text{-HT}_{1A}$R activation could be associated with the behaviors observed in eating disorder patients since, in the brain, $5\text{-HT}_{2A}$R binding potential in anorexia nervosa is decreased, while $5\text{-HT}_{1A}$R binding potential is increased in bulimia nervosa [258,260,261]. Low SERT binding potential has also been associated with greater $5\text{-HT}_{1A}$R binding potential in the brain [262,263]. A reduced SERT expression was observed in the brain of remitted bulimia-type anorexia nervosa patients (binging and purging behavior), whereas greater SERT expression was observed in restricting-type anorexia nervosa (restriction of food intake) [262]. In the brain and platelets of bulimia nervosa patients (remitted or not), SERT was reduced [264–266]. Södersten and Bergh have questioned whether divergent alterations of SERT levels in the brain among different subtypes of eating disorders and whether these differences are a cause or a consequence of the pathology [267]. Indeed, most studies indicate that platelets from patients with eating disorders have decreased SERT density, but the duration of the pathology and/or the age of the patient are important factors that influence SERT density [268]. In platelets, $5\text{-HT}_{2A}$R density is increased in older patients with eating disorders [269] and decreased in younger (adolescent) patients [268]. Interestingly, this is also observed in co-morbidities associated with eating disorders such as depression and anxiety [258].

Polymorphisms have also been described in eating disorders patient. The S allele or SS genotype for the *SLC6A4* gene is more frequent in anorexia nervosa and/or bulimia nervosa [270,271] but others have found no significant association [272,273]. A $5\text{-HT}_{2A}$R polymorphism (−1438A allele) is also considered a risk factor in anorexia nervosa [274].

Serotonin is the main therapeutic target for eating disorders with SSRI as the treatment of choice, even though they have controversial effects [25,258]. Indeed, Kaye et al. have suggested the possibility that decreased SERT function could be related to poor response to SSRI medication in some individuals [258]. Moreover, by reducing dietary intake, anorexia nervosa patients could affect tryptophan availability and thus modulate brain 5-HT function which could also explain why SSRI administration is sometimes ineffective [258].

#### 4.4.2. Alteration of the estrogen system associated with eating disorders: what can we learn from treatments?

It is well known that sex hormones are involved in appetite control, food intake and weight gain, as reviewed in Hirschberg and Hildebrandt et al. [25,275]. Sex hormones interact with gastrointestinal peptides and neurotransmitters to achieve this control [276]. For example, OVX rats treated with E2, compared to OVX + vehicle control, have decreased food intake (anorexigenic), increased pheochromocytoma 12 E26 transformation-specific (ETS) domain transcription factor (Pet-1), a transcription factor with a critical role in the development and regulation of the 5-HT system, and increased *slc6a4* mRNA level in the brain [277]. In OVX rat, E2 also increases the anorectic effect of 5-HT [278,279], while decreased 5-HT stimulation, via the 5-$HT_{1A}$R, is associated with hyperphagia that is further increased by E2 treatment [280,281]. Little is known about how estrogens may specifically contribute to eating disorders. However, polymorphisms in *ESR1* and *ESR2* genes were associated with restrictive anorexia nervosa and bulimia nervosa [275]. GPER1 was not associated with eating disorders but was shown to be involved in the anorectic effect of estrogen in the hypothalamus [282].

Hildebrandt et al. have reviewed and highlighted 5-HT-estrogen interactions in bulimia nervosa [25]. The most robust neurobiological model of bulimia nervosa presented involves deregulation of the 5-HT system, which would notably be responsible for impulsive and compulsive traits [25]. Hildebrandt et al. address the etiology of bulimia nervosa with a developmental approach, which includes genetic factors from conception, prenatal exposure to estrogens and environmental endocrine disruptors, which affect brain development. This model also includes societal factors (“thin ideal”), puberty and menstrual cycle onset, which affect mood and behavior [25], and suggest that E2 plays an activational role for genetic factors involved in bulimia nervosa [25]. Altogether these data suggest that estrogens contribute to a vulnerability to eating disorders.

#### 4.4.3. Eating disorders during pregnancy

Eating disorders have been associated with several adverse pregnancy outcomes such as miscarriage, preterm birth, maternal hypertension, fetal growth restriction and small for gestational age babies (reviewed in Kimmel et al. [78]). However, having a history of eating disorders would not predispose to adverse perinatal outcomes [78].

Pregnancy is a period of important biological changes, notably in hormones that regulate appetite such as leptin and cortisol. Studies have shown improvement of symptoms of eating disorders during pregnancy [78]. Remission of anorexia nervosa during pregnancy is thought to be due to psychological, social and biological influence [283]. On the biological point of view, it was hypothesized that hormone production by the placenta, such as dehydroepiandrosterone (DHEA), a precursor for estrogen, contribute to this effect, but the mode of action remains to be studied [283]. On the other hand, other studies have suggested that pregnancy can trigger relapse of eating disorders, but it was not associated with a physiological change that occurs during pregnancy [78]. This highlight the complexity of eating disorders since pregnancy can be a vulnerable period because of physiologic changes that are particularly triggering or it can be perceived as a heightened motivation to address eating disorders in relation to the health of the baby [78].

### 4.5. Common and divergent aspects between 5-HT-linked diseases and their relation to the estrogen system

Different features characterize the 5-HT-linked diseases that were previously presented. For instance, while depression, migraine and bulimia nervosa are associated with a decrease in serotonergic tone in the CNS, anorexia nervosa and IBS are rather associated with increased serotonergic tone. As shown in Fig. 2, overall, low estrogen levels are associated with low 5-HT levels while high estrogen levels are associated with high 5-HT levels. Hence, estrogens could stimulate the 5-HT system in order to improve the symptoms of the 5-HT-linked diseases, except in anorexia nervosa and IBS where another mechanism remains to be characterized. Moreover, drugs that act on estrogen levels were efficient to treat depression, bulimia and migraine [207,208,284].

Studies suggest that TPH and MAO enzymes are regulated in a pattern that is consistent with the observed 5-HT concentration in the CNS. It is hard to interpret the regulation of the various 5-HTR as studies have not consistently characterized the subtypes of receptors present in the study models. In some cases, it appears that downregulation of inhibitory receptors, such as 5-$HT_1$R, is associated with decreased 5-HT levels (Fig. 2).

Finally, studies on platelets 5-HT have gained interest, especially because of the easy access to biological material. It is still unclear how the 5-HT system in platelets can be compared to central or peripheral 5-HT systems. Fig. 3 shows that data on 5-HT and estrogen in blood platelets do not allow generalizations to CNS. Indeed, the levels of 5-HT and estrogen are not always consistent with observations in the CNS (Fig. 2). We postulate that platelet 5-HT might be subjected to more influencing factors and/or be regulated in a different manner. Studying the 5-HT system in blood platelets requires making a parallel assessment of altered 5-HT function in the target organ to validate that extrapolations from data in blood platelets are relevant.

## 5. Why using pregnancy to study 5-ht-estrogen interactions?

Pregnancy is a reproductive state that can be very informative when studying the 5-HT-estrogen relationship. This is mainly due to the placenta, a unique transient organ that is essential for a healthy pregnancy and fetal development. The human placenta is composed of trophoblast cells, which can undergo villous or extravillous differentiation [285]. In the former, which compose the chorionic villi, cells fuse and form an endocrine–active multinucleated syncytium, the syncytiotrophoblast cells while in the latter, cells invade the maternal decidua to regulate blood flow [285–288]. The human villous trophoblasts produce a large quantity of estrogens, as well as 5-HT, making it an interesting model to study 5-HT-estrogen interaction [289–291].

### 5.1. Pregnancy a hyperestrogenic state

Pregnancy has been known for a long time to be a hyperestrogenic state [289]. Indeed, CYP19 is highly active in the human placenta [292–295] and estrogen production requires perfect cooperation between mother, placenta and fetus. Estrogens produced by the placenta can be transferred to the maternal and fetal circulation where they have endocrine actions [295] or act as autocrine/paracrine factors on placenta cells [296–298]. For example, estrogen activation of ERs are involved in villous cytotrophoblast syncytialization [299], growth [300] and endocrine regulation [301,302]. They also play a role in trophoblast invasion [296,303,304], and fetal development [304,305] and good pregnancy outcome [306]. Considering the high secretion levels of estrogens and their crucial role in pregnancy, concerns have been raised about endocrine disruption during pregnancy. Such endocrine disruptors can affect placental function as well as fetal development [307–310].

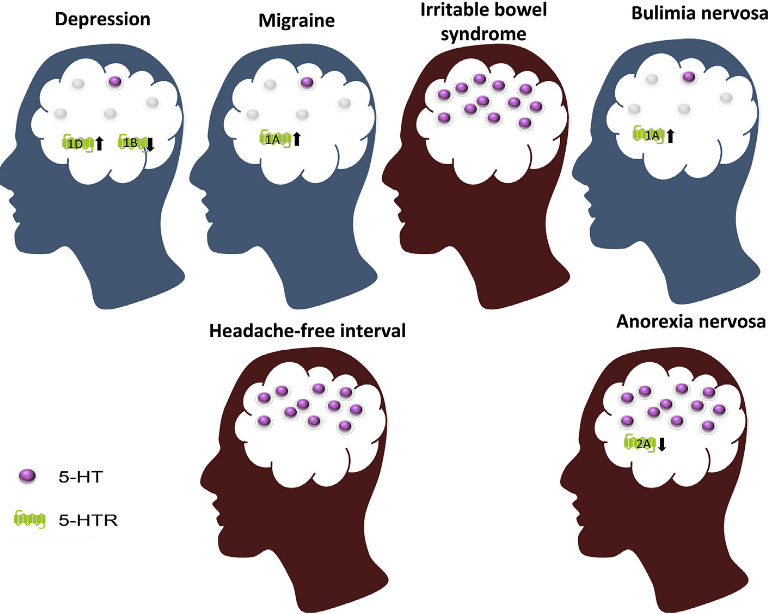


**Fig. 2. Summary of the impact of four 5-HT-linked diseases: depression, migraine, irritable bowel syndrome and eating disorders on serotonin and serotonin receptors in the central nervous system.** Low serotonergic tone is illustrated by grey dots (absents) replacing the pink ones. The silhouette is colored blue when estrogen levels are low and red when they are high. 5-HT: serotonin; 5HTR: serotonin receptor.

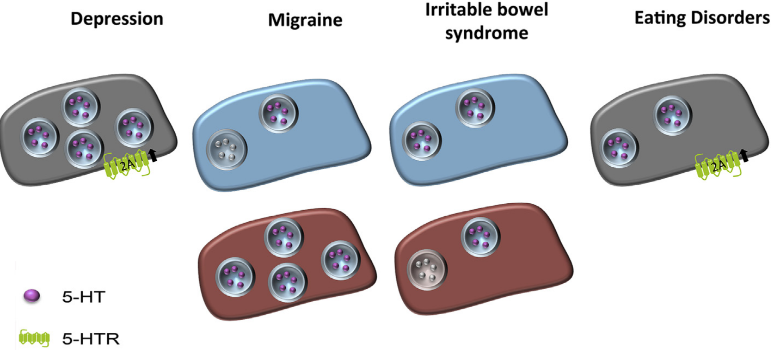


**Fig. 3. Summary of the impact of four 5-HT-linked diseases: depression, migraine, irritable bowel syndrome and eating disorders on serotonin and serotonin receptors in blood platelets.** Serotonin is contained in vesicles. The platelet is colored blue when estrogen levels are low and red when they are high. 5-HT: serotonin; 5HTR: serotonin receptor.

### *5.2. Serotonin system during pregnancy*

The placenta as a source of peripheral 5-HT is a relatively new concept. Serotonin has been detected in human placental tissue (syncytiotrophoblast and to a lesser extent villous trophoblast) and in trophoblast cells isolated and cultured under serum deprivation condition, which suggests a placental production of 5-HT [311]. The placental *de novo* production of 5-HT was confirmed by our group in primary trophoblast cells [312]. This suggests that placental production of 5-HT could be a contributor to the observed increased platelet 5-HT concentrations during pregnancy [313]. In fact, the whole 5-HT system is expressed in the human placenta (Fig. 4), including a transport system for L-tryptophan [314], the serotonin biosynthetic enzymes TPH1 and TPH2 and aromatic L-amino acid decarboxylase (AADC) [315], SERT [3,4,316], the 5-HT degrading enzyme MAO [128,317,318], 5-HTRs [48,49,51,311,319–321], and the vesicle membrane transporter (VMAT) [322]. Other transporters such as the organic cation transporter 3 (OCT-3), p-glycoprotein (p-gp) and gap junction are also involved in 5-HT transport across different cells of the placenta [323].

The physiological role of placental 5-HT has only been characterized to a slight extent. Interestingly, 5-HT induces the expression *CYP19A1* gene in the placenta by the activation of the $5HT_{2A}R$ [48]. Genetic polymorphisms in placental *MAO* and *SLC6A4* that influence their expression and activity [103] are associated with poor pregnancy outcomes and impaired fetal development. Moreover, 5-HT synthesized by the placenta directly modulates fetal mouse forebrain development [291].

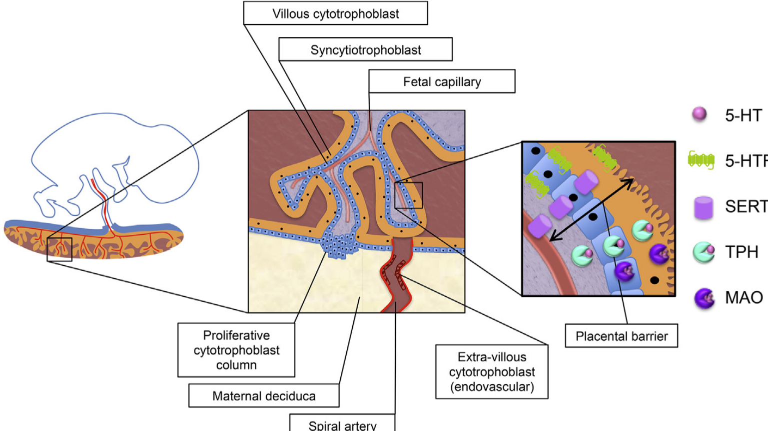


**Fig. 4. Human placental structure and serotonin system**. The placental villi are composed of the syncytiotrophoblast, villous cytotrophoblasts, mesenchyme and the endothelium of fetal capillaries. Those cell layers separate maternal and fetal blood. Serotonin (5-HT) is synthesized by trophoblast cells, serotonin receptors (5-HTR), serotonin transporter (SERT), 5-HT synthesis enzyme tryptophane hydroxylase (TPH) and degradation enzyme monoamine oxidase (MAO) are all expressed in the human placenta.

The sources of fetal 5-HT include placental 5-HT, transfer of maternal 5-HT and intestinal production by the fetus from the 7th week of pregnancy [99–102]. Serotonin is crucial for the embryo/fetal development, including left/right axis modeling, neuronal development and cardiac morphogenesis and growth (reviewed in Ref. [324]). Moreover, it has been suggested that prenatal 5-HT disruption may be associated with metabolic changes in the offspring increasing the risk of type 2 diabetes, obesity and dyslipidemia [325]. In addition, 5-HT-specific pharmacological agents interfere with mice embryonic development, suggesting that early embryos use 5-HT before the onset of neurogenesis to regulate cell proliferation and morphogenetic movements [326,327]. A decreased proliferation of myocardium, cardiac mesenchyme, and endothelium was also reported in embryos cultured in the presence of either a high concentration of 5-HT or an SSRI, indicating that serotonin regulates embryonic heart development [328–331]. Inactivation of the *htr2b* gene leads to embryonic and neonatal death caused by heart defects; all surviving newborn display severe ventricular hypoplasia caused by an impaired proliferative capacity of myocytes [331]. Hence, regulation of the components of the 5-HT system is crucial for a healthy pregnancy and fetal development and programming.

### 5.3. *In utero programming and the 5-HT-estrogens relationship*

Fetal programming is a complex phenomenon that involves fetal-sex differences, suggesting the involvement of sex steroids. Reviewing this topic is beyond the scope of this article, but we did aim to identify sex-related relationship with 5-HT linked diseases that may originate from *in utero* programming and which could be explored in further studies with a 5-HT-estrogen relationship approach. It is well-known that there is a bias in the male–to-female conception and birth ratio (primary and secondary sex ratio, respectively) in favor of males [332]. Fetal programming is thought to contribute to this observation [332]. Sex differences in pregnancy complications have been suggested to be mediated by the sex-specific characteristics of the human placenta [333]. It was proposed that male offspring respond to adverse maternal environment with a continued growth and adapt less to *in utero* conditions, which is associated with greater risks of adverse pregnancy outcomes and poorer neonatal and infant health outcomes, while female fetuses adapt their growth and development according to their impaired environment [332,333]. It is not clear if and how steroids are involved in this since steroid profiles for E2 and E3 were not significantly different between male and female fetal cord blood [334].

## 6. Pregnancy-specific disorders associated with alteration in serotonin-estrogen systems

Pregnancy is subject to development of specific pathologies, of which the occurrence is usually limited to the pregnancy period. In this section, we explore three of these pathologies in which 5-HT and estrogen levels are altered: hyperemesis gravidarum, gestational diabetes mellitus (GDM) and preeclampsia.

### 6.1. *Hyperemesis gravidarum*

Hyperemesis gravidarum is characterized by nausea and vomiting that results in weight loss, disturbance of electrolyte balance, ketonuria, and dehydration often severe enough to require hospitalization [335]. It typically occurs during the first trimester, but in some cases, persists throughout pregnancy [336]. The peak of hyperemesis gravidarum corresponds to high levels of progesterone and human chorionic gonadotropin (hCG) combined with low levels of thyroid-stimulating hormone (TSH) [337].

Serotonin was thought to be involved in this pathology, as 5-HT plasma levels are increased in hyperemesis gravidarum [338], without difference in the urinary excretion of 5-HIAA, compared to unaffected pregnant women [339]. 5-HT$_3$R antagonists have been used successfully for hyperemesis gravidarum [338,340,341], which also suggests that 5-HT plays a role in this pathology.

It has been proposed that estrogens are involved in the pathogenesis of hyperemesis gravidarum for several reasons, including their action on the gastrointestinal transit [342,343] and the greater prevalence of hyperemesis gravidarum in pregnancy conditions associated with high estrogen levels [337]. Inconsistent results were obtained concerning E2 levels in hyperemesis gravidarum women; some studies have observed an increase of E2 levels, while others have observed no effect [337,344]. E3 levels were not

associated with symptoms of hyperemesis gravidarum [345]. There is a lack of studies that relate severity of hyperemesis gravidarum to estrogen levels [337]. Considering that estrogen levels rise progressively during pregnancy, but that symptoms of hyperemesis gravidarum are mainly associated with the first trimester, it is unlikely that high estrogen level is the unique contributor to hyperemesis gravidarum [337]. Interestingly, the female to male sex ratio in pregnancies complicated by severe hyperemesis gravidarum is increased [346]. This may be due to male fetuses being more vulnerable during pregnancy accompanied by severe hyperemesis gravidarum, with greater rates of miscarriage [346].

### 6.2. Gestational diabetes mellitus

One in seven births is affected by gestational diabetes mellitus (GDM), which is commonly defined as glucose intolerance that begins during pregnancy [347–349]. Serotonin and 5-HT$_{2A}$R were shown to modulate glucose homeostasis (glucose uptake, plasma membrane content of glucose transporters (GLUTs) and interference with insulin-induced capillary recruitment) in different cell models [350]. The contractile effect of 5-HT on human umbilical arteries, which involves activation of a mixed population of 5-HT$_1$R and 5-HT$_{2A}$R, is reduced in GDM [351]. In JAR placental choriocarcinoma cell line, a model of human trophoblast cells, SERT expression is downregulated following glucose treatment and restored by insulin treatment [352]. In placentas from pregnancies complicated by GDM, SERT and 5-HT$_{2A}$R expression is decreased compared to non-GDM pregnancies [353]. This effect could be direct or indirect via other pathologies associated with GDM, such as pre-eclampsia [319]. Others have found that, in GDM, free plasma 5-HT levels were elevated, whereas 5-HT uptake rates by trophoblasts were decreased because of impaired translocation of SERT to the plasma membrane, which is regulated by insulin signaling [354]. All of this suggests that 5-HT uptake by trophoblasts is decreased during a high glycemic state, possibly increasing extracellular 5-HT levels and stimulating 5-HT signaling.

Estrogens also modulate glucose homeostasis. In normal pregnancy, it is thought that insulin sensitivity declines with advancing gestation due to placental factors, notably estrogens [355,356]. Indeed, E2 is involved in β-cell function (increase of insulin biosynthesis) and in the decreasing expression of GLUT4, insulin-sensitive membrane transporters, in muscle and adipose tissue [357–360]. Disruption of these processes in GDM could contribute to insulin resistance. Estrogen levels and estrogen/testosterone ratio are lower in GDM compared to control, which suggests a decrease in aromatase activity during pregnancy [361].

The expression of ERα is also upregulated in placentas from women with GDM, except in the decidual vessels where it is decreased but only when the fetus is a male [362,363]. The regulation of ERα in GDM could involve epigenetic mechanism (hypomethylation of the ERα gene promoter) [362] and changes in miRNA regulation [357]. Interestingly, women carrying male fetuses which tend to have lower estrogen concentration in the maternal and cord blood [364], have an increased risk of suffering from GDM [365,366].

### 6.3. Pre-eclampsia

Approximately 5–8% of pregnancies are affected by pre-eclampsia, which is defined as persistent hypertension that develops after the 20th week of pregnancy and is associated with a metabolic abnormality, proteinuria, decreased platelets, and kidney, liver, lung and brain disorders [367]. Preeclampsia is the leading cause of maternal and perinatal morbidity and mortality in developing countries. It is often associated with IUGR and premature delivery [368,369].

The involvement of 5-HT has been suggested in the etiology of pre-eclampsia since 5-HT stimulates *in vitro* arteries vasoconstriction, an important phenomenon in pre-eclampsia [370]. It is known that 5-HT, via its 5-HT$_{2A}$R, 5-HT$_1$R and/or 5-HT$_7$R receptors, regulates contraction of placental vessels [321,371]. Moreover, pre-eclampsia is associated with a hyperserotonergic state. Serotonin concentrations in platelet-poor plasma, which indicate the free circulating 5-HT, are higher in pre-eclamptic than in normotensive women [372,373]. Platelet uptake of 5-HT is increased in pre-eclamptic compared to normotensive women, but the effect on platelet 5-HT concentration is not as clear [373,374]. Urinary excretion of 5-HT and 5-HIAA was also increased in pre-eclamptic patients [374,375]. Consistent with a hyperserotonergic state, the use of SSRIs is associated with an increased risk of pre-eclampsia [80]. It was suggested that this association is due to antagonist effects of SSRIs on 5-HT$_2$R, which are involved in vasoconstriction/dilatation [80,376] and to increased 5-HT levels.

Alteration of platelet 5-HT levels in hypertensive pregnant women would not be associated with *SLC6A4* or *HTR2A* polymorphism [377]. It would rather result from platelet activation following endothelial damage [377]. Pre-eclampsia placentas have lower 5-HT metabolism and MAOA expression, while SERT activity and expression are not affected [378–380] and resulting placental 5-HT concentrations are increased [380]. In pre-eclampsia, 5-HT$_7$R expression, a G protein-coupled receptor (Gs) involved notably in anxiety, mood and vasoconstriction, is increased about 8 times in the placenta [321,376], but the significance of this important increase remains to be characterized. No difference in 5-HT$_{2A}$R expression (protein or mRNA) was observed in chorionic vessels of pre-eclamptic women [371], but ketanserin, a 5-HT$_{2A}$R antagonist with an antihypertensive effect in pre-eclampsia, was explored as a potential treatment for this pathology [117,381]. This is somewhat contradictory with the potential antagonist effect of SSRI on 5-HT$_{2A}$R which was suggested earlier to increase the risk of pre-eclampsia. We suggest that 5-HT$_{2A}$R antagonism has beneficial effects under pathological condition (pre-eclampsia), while in placentas from normotensives pregnancies, it would rather have deleterious effect with an increased risk or pre-eclampsia. It is possible that basal expression of 5-HT$_{2A}$R in the trophoblast of pre-eclampsia placentas is disrupted and reestablished with ketanserin, which is not observed in normotensives pregnancies. This could also involve differences in estrogen system between pathological and non-pathological placenta.

Precursors of estrogen, testosterone and androstenedione, are increased in pre-eclampsia compared to normotensive pregnancies [382], whereas E2 and E1 are decreased [383]. The resulting estrogen/testosterone ratio, a measure of functionality of the CYP19 enzyme, is also decreased [382,384]. Consistent with this, placental CYP19 expression is decreased in pre-eclampsia [384]. Reduced *ESR1* expression may also play a role in pre-eclampsia etiology [385].

2-Methoxyestradiol (2-ME) is a metabolite of estrogen with low estrogenic activity and produced by catechol-O-methyltransferase (COMT). It can induce differentiation of cytotrophoblasts into an invasive phenotype under hypoxic condition, a crucially regulated process in pre-eclampsia [386]. Pregnant mice deficient in *comt* have multiple features of pre-eclampsia-like phenotype and administration of 2-ME prevented the pre-eclampsia-like symptoms [387]. 2-ME levels increase during normal pregnancy and are lower in women with pre-eclampsia than in controls [387]. Interestingly, this difference is observable before clinical manifestations of pre-eclampsia [382]. The role of 2-ME in the etiology of pre-eclampsia is an important aspect to explore.

**Table 3**
Areas of research to prioritize in the field of serotonin and estrogen research.

| Areas of research to be prioritized |
|---|
| Characterize the basal differences in 5-HT and estrogen systems between men and women as well as among different organs/study models (e.g. platelet-depleted plasma, blood platelets, brain regions, placenta) to understand how observations from one sex or organ can extrapolate to the other. |
| Assess the role of placental 5-HT to distinguish its effects on placental function from its effects on fetal development. |
| Determine the role and mode of action of 5-HT and estrogens in altered pregnancy associated with 5-HT-linked diseases. |
| Evaluate the common and divergent aspects of the physiopathology of subtypes of 5-HT-linked diseases such as eating disorders (anorexia nervosa, bulimia nervosa and binge-eating disorder), IBS (IBS–C, IBS-D), migraine (with or without an aura) to better adapt the treatments specifically for these subtypes of pathology. |
| Evaluate the effects of estrogens on the serotonin system, the differential effects of E1, E2, E3 and E4 and the involvement of different ERs as well as genomic versus non-genomic ER signaling in the estrogens-5-HT interaction. |

### *6.4. Common and divergent aspects of pregnancy disorders and their relation to the estrogen system*

Hyperemesis gravidarum, GDM and pre-eclampsia are all associated with hyperserotonergic states. While the role of 5-HT and estrogens in hyperemesis gravidarum is controversial, it has been shown that both are involved in the etiology of GDM and pre-eclampsia. Indeed, via their action on placental GLUTs, 5-HT and estrogens affect glycemic state and glucose transfer to the fetus. In pre-eclampsia, the increased level of 5-HT may be involved in the regulation of vessel constriction [376], while the role of estrogens remains to be studied. However, data are lacking on the interactions between 5-HT and estrogen systems that are involved in the etiology of these pathologies.

## 7. Conclusion

This review has shown that 5-HT-linked diseases have in common a sex-dimorphic prevalence with women being at greater risk of suffering from these diseases. The co-occurrence of the 5-HT-linked diseases with each other suggests certain common physiopathological mechanisms. Estrogens, mainly E2, were shown to be an effective treatment in several of these diseases. In most 5-HT-linked diseases, a decrease in the serotonergic tone is observed and estrogens contribute to the improvement of the symptoms by stimulating the serotonergic system. During pregnancy, a highly estrogenic state, an improvement of the symptoms is often observed. Pregnancy-specific disorders, which are associated with alterations in 5-HT and estrogen systems are rather associated with hyperserotonergic state and decreased estrogen levels. We postulate in these cases that the 5-HT system is hypersensitive to estrogen stimulation.

From a mechanistic point of view, a lot of attention has been given to the neuroprotective effects of estrogens on the 5-HT system, but the reciprocal relationship has received much less attention and could contribute to a better understanding of the etiology 5-HT-linked diseases. We propose that estrogens stimulate the serotonergic system, but that in turn, serotonin affects estrogen synthesis in an attempt to reach homeostasis. These two processes (5-HT and estrogen synthesis) are crucial for successful pregnancy and thus, pregnancy problems associated with 5-HT-linked diseases or pregnancy-specific pathologies may find some explanation in a disruption of this complex 5-HT-estrogen relationship. Considering this, we have identified areas of research that should be prioritized (Table 3).

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## Authors contribution

All co-authors contributed to the decision of each section of the manuscript. AHT, was actively involved in the writing of the manuscript and created both figures and tables of the manuscript. CV and JTS assisted in drafting the manuscript. Finally, all co-authors provided critical revisions and provided their final approval for the manuscript's publication in Biochimie. All authors have approved the final article. This manuscript is not under review elsewhere and all authors are in agreement with the contents of this manuscript.

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