Impacts of selective serotonin reuptake inhibitors on reproductive biology: insights from murine and ovine models

By

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Dedication

This dissertation is dedicated to my family, friends, and mentors who inspired and supported me to pursue my dreams and goals.

Acknowledgments

This dissertation encompasses four years of work; however, it signifies over 13 years of commitment to reproductive biology research. A lot of people contributed tremendously to my personal and professional growth throughout this time.

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Abstract

Maternal use of antidepressants has increased throughout the last decades and selective serotonin reuptake inhibitors (SSRI) are the most prescribed antidepressants. Despite the widespread use of SSRI by women during their reproductive period and pregnancy, an increasing number of studies warns of possible detrimental effects of maternal use of SSRI during pregnancy including low birthweight/small for gestational age and preterm birth. The present thesis sought to investigate the pathophysiology of SSRI-related pregnancy outcomes and to propose measures to mitigate the adverse effects of SSRI. We proposed and tested a physiological model for the occurrence of adverse pregnancy outcomes related to SSRI exposure during gestation: maternal use of SSRI increases free circulating maternal serotonin and serotonin signaling leading to vasoconstriction of the uterine and placental vascular beds with consequent decrease of blood perfusion to the uterus, placenta, and fetus resulting in placental hypoxia and dysfunction which ultimately lead to suboptimal embryonic/fetal development. We demonstrated the effects of two common SSRI, fluoxetine and sertraline, on pregnancy and neonatal outcomes in mice. We also reported and validated the use of serotonin transporter-deficient mice (Sert-/-) to enhance our understanding of the effects of modulating the serotonin transporter, the biological target site for SSRI. Overall, Sert-/- mice have similar pregnancy outcomes compared to wild-type mice treated with SSRI: increased embryonic mortality, decreased offspring weight, increased neonatal mortality. Utilizing strategic breeding schemes between WT, Sert-/-, and Sert+/-, we determined that lack of maternal, rather than embryonic/placental SERT expression, is associated with poor pregnancy outcomes. Histopathological and transcriptomic placental changes associated with maternal SSRI exposure and genotype (Sert-/-) are consistent with the observed pregnancy outcomes. In an ovine model, ultrasonography during late pregnancy allowed us to assess placental alterations

related to SSRI treatment and its impact on pregnancy length (preterm birth) and neonatal morbidity (decreased birthweight, acidemia, hypocalcemia). We were able to prevent the effects of increased serotonin signaling with prevention of some placental alterations and restoration of the number of pups born in SSRI-treated and Sert-/- mice by implementing a therapy that is an antagonist for the serotonin receptors 2A and 2C. An incidental finding in our studies was the endocrine disruption effects of SSRI, particularly fluoxetine, in nonpregnant mice. Short-term fluoxetine treatment has estrogenic action that appears to be associated with increased estrogen synthesis. Importantly, the endocrine disruption action of fluoxetine is not an off-target drug effect but SERT-dependent and serotonin-mediated as demonstrated in Sert-/- and Tph1-/- (no peripheral synthesis of serotonin) mouse models. Taken together, these studies unraveled multiple actions of SSRI on reproductive function. Importantly, our work developed a possible therapeutic measure to mitigate the effects of SSRI on pregnancy outcomes potentially benefiting women and infant worldwide.

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Chapter 1

Maternal serotonin: implications for the use of selective serotonin reuptake inhibitors during gestation

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Abstract

Maternal use of antidepressants has increased throughout the last decades; selective serotonin reuptake inhibitors (SSRI) are the most prescribed antidepressants. Despite the widespread use of SSRI by women during reproductive age and pregnant women, an increasing amount of research warns of possible detrimental effects of maternal use of SSRI during pregnancy including low birthweight/small for gestational age and preterm birth. In this review we revisited the impact of maternal use of SSRI during pregnancy, its impact on serotonin homeostasis in the maternal and fetal circulation and in the placenta, and its impact on pregnancy outcomes – particularly intrauterine growth restriction and preterm birth. Maternal use of SSRI increases maternal and fetal serotonin. The increase in maternal circulating serotonin and serotonin signaling likely promotes vasoconstriction of the uterine and placental vascular beds decreasing blood perfusion to the uterus and consequently to the placenta and fetus with potential impact on placental function and fetal development. Several adverse pregnancy outcomes are similar between women, sheep, and rodents (decreased placental size, decreased birthweight, shorter gestation length/preterm birth, neonatal morbidity and mortality) highlighting the importance of animal studies to assess the impacts of SSRI. Herein, we address the complex interactions between maternal SSRI use during gestation, circulating serotonin, and the regulation of blood perfusion to the uterus and fetoplacental unit, fetal growth, and pregnancy complications.

Introduction

Serotonin is a vasoactive hormone with a multitude of actions throughout the body [1, 2]. Despite the most recognized role for serotonin is related to mood and behavior as a neurotransmitter, >98% of serotonin is present in nonneuronal tissues [3, 4]. Serotonin regulates physiological processes including bone and calcium metabolism, energy homeostasis, gastrointestinal motility, brain development, and vascular resistance, among numerous other functions [1, 2, 4-6]. For decades, altered serotonin signaling has been implicated in the pathophysiology of hypertension, preeclampsia, and neurodevelopment disorders in infants [5, 7-14]. Additionally, serotonin's effects on pregnancy outcomes have been studied for decades; however, more recently, the role of serotonin in processes including embryonic and fetal brain development, placental function, intrauterine growth restriction (IUGR), and neonatal health have been in the spotlight [13, 15-20] given the increasing maternal use of medications that alter serotonin signaling, including selective serotonin reuptake inhibitors (SSRI). The possible detrimental effects of serotonin on pregnancy and neonatal outcomes supports the need for a comprehensive understanding of how serotonin and medications that modulate serotonin signaling are associated with the pathophysiology of multiple conditions such as gestational hypertension, preeclampsia, IUGR, autism spectrum disorder, preterm birth, and sudden infant death syndrome. Of critical importance is the role SSRI may play on reproductive outcomes, placental function, and fetal development.

The SSRI encompass a class of psychotropic medications used to treat several psychological conditions including depression, obsessive compulsive disorder, and panic disorder in adult and pediatric patients [21]. Fluoxetine, the first SSRI introduced to the market, has been commercially available since late-1980's. Since then, other SSRI have become available

and now eight compounds have been approved by the FDA for patient use. Among these, sertraline, fluoxetine, and citalopram are the most commonly prescribed to pregnant women [22]. Because the use of SSRI, particularly by women during reproductive age, has dramatically increased over the last three decades, numerous women are exposed to SSRI during gestation [23, 24]. The frequency of pregnant women taking SSRI during gestation varies from 6 to 13% among studies. Approximately 300,000 women and their infants yearly in the USA are exposed to SSRI during pregnancy, a critical period that is determinant for adequate maternal and fetal/neonatal wellbeing. In a recent report, 92.2% of women that took SSRI during gestation were using SSRI when they became pregnant and only about 38% of women discontinue treatment by week 13 of gestation [22]. Whether these drugs may benefit women under diverse psychological conditions is beyond the scope of this review and has been examined elsewhere [25-27]. Instead, this review will address the impacts of SSRI on serotonin signaling during gestation and its implications on pregnancy outcomes, particularly IUGR and preterm birth, and neonatal health.

Serotonin metabolism during pregnancy

In a nonpregnant state, most of circulating serotonin is synthesized by gastrointestinal enterochromaffin cells whereas other organs minimally contribute to the non-neuronal pool of serotonin [1, 2]. However, during pregnancy and lactation, other organs secrete serotonin into the bloodstream as the demand for serotonin increases. For example, serotonin is involved in regulating maternal glucose homeostasis during pregnancy. Kim et al. [28] demonstrated increased pancreatic synthesis and secretion of serotonin leading to about 2-fold increase in circulating concentrations of the hormone compared to nonpregnant mice. This is critical during

pregnancy as serotonin promotes beta cell expansion increasing insulin secretory capacity. During lactation, mammary gland-derived serotonin is involved in regulation of calcium homeostasis and the mammary gland secretes about 50% of circulating serotonin [29].

In the bloodstream, serotonin is primarily transported inside platelets after uptake by the serotonin transporter (SERT) located on the platelet plasma membrane [1, 3, 4]. Platelets do not synthesize serotonin on their own, therefore, all platelet content of serotonin must be taken up through SERT. Thus, platelet SERT is a key regulator of free (plasma) circulating concentrations of serotonin [4, 30]. A technical caveat of measuring circulating concentrations of serotonin is related to type of blood sample (Figure 1). In whole blood and serum samples, lysis of platelets releases intraplatelet content of serotonin so that total, free and intraplatelet, serotonin is measured. In plasma samples, anticoagulants prevent platelet lysis so that only free (nonplatelet) concentration of serotonin is measured. Accordingly, concentrations of total serotonin in the blood (free serotonin plus platelet content as measured in total blood and serum samples) are much greater than the free (plasma) amounts of the hormone [4, 10]. Inhibition of platelet SERT by SSRI prevents uptake of serotonin resulting in increased free (plasma) concentrations of serotonin, even after greater degradation of free serotonin [30]. In contrast, total (whole blood and serum) concentrations of serotonin are decreased due to depleted amounts of the hormone inside platelets [30]. A similar phenomenon occurs in SERT null mice due to lack of SERT [31].

Before the development of the placenta, maternal-derived serotonin modulates embryo morphogenesis and development. After placenta development, it becomes an important source of serotonin utilized by the fetus, for example for neuronal development [32]. Bonnin and colleges [13] demonstrated that infusion of tryptophan to the uterine artery of a live mouse leads to rapid synthesis and transport of serotonin to the fetus and its detection in the umbilical vein. Towards

the end of gestation, embryonic capacity for serotonin synthesis increases [13, 33, 34] so that fetal serotonin is transported into the placenta for degradation [16, 33]. Organic cation transporter 3 (OCT3) located on the fetal-facing plasma membrane of syncytiotrophoblasts and cytotrophoblasts, and likely other known transporters, promote the transfer of fetal serotonin into the placenta [16, 33]. Furthermore, fetal platelets in late pregnancy express functional SERT [35] so that both fetal platelet SERT and the placenta regulate free serotonin concentrations in the fetal circulation [16, 33, 36]. In a similar manner, placental synthesis of serotonin in humans appears to decrease later in pregnancy as demonstrated by downregulation of the endocrine machinery for synthesis of serotonin in the third trimester compared to the first trimester [33, 37].

Modulation of serotonin signaling by selective serotonin reuptake inhibitors

SSRI inhibition of serotonin transporter

Although the pharmacokinetics of different SSRI vary substantially, all of them inhibit SERT [38]. Inhibition of SERT in the central nervous system prevents serotonin reuptake by the presynaptic neuron resulting in increased serotonin signaling. Additionally, SSRI promote neuroplastic adaptations culminating with its psychotropic actions [39, 40].

In addition to its role in the brain, SSRI also inhibit peripheral SERT modulating serotonin signaling throughout the body [41]. Inhibition of SERT on peripheral tissues prevents serotonin transport into the cell for degradation, thereby, increasing serotonin signaling through its cell surface receptors. Furthermore, inhibition of SERT on platelets prevents the platelets' ability to uptake free serotonin in the blood [30]. Accordingly, SSRI treatment increases the concentrations of free (plasma) circulating serotonin (Figure 1). Because of SSRI's capacity to

increase serotonin signaling in the periphery, recent studies have explored a role for SSRI on several tissues (mammary gland, bone, placenta, etc.) under physiologic and pathologic conditions and their role in altering tissue homeostasis [17, 42-44].

SSRI modulation of serotonin signaling at the uteroplacental unit

Placental SERT is located on the apical region of syncytiotrophoblasts, that is, SERT is in direct contact with maternal blood in the intervillous space [16, 17, 33, 36, 45]. Inhibition of syncytiotrophoblast SERT by SSRI prevents the uptake of maternal serotonin into the placenta increasing serotonin concentrations in the intervillous space [16, 36, 45, 46]. Thus, the decreased SERT-mediated removal of serotonin from the maternal circulation at the placenta due to SSRI treatment, in addition to increased plasma serotonin due to inhibition of platelet SERT, leads to increased serotonin signaling associated with increased vascular resistance of placental vascular beds which compromises blood perfusion to the uteroplacental unit [47]. Interestingly, fluoxetine inhibition of SERT and OCT3 results in increased serotonin on the maternal and fetal sides of the placenta in an ex vivo perfusion system of human term placenta [46] and rodent placenta [36]. In addition to inhibition of SERT, most SSRI decrease the capacity of OCT3 to transport serotonin from fetal circulation into the placenta in an ex vivo model resulting in decreased placental uptake of fetal serotonin [16, 36, 48]. However, the wide variation for in vivo transplacental transfer of each SSRI compound results in different fetal exposure to SSRI [21, 38, 49]. Thereby, the in vivo capacity of each drug to inhibit SERT uptake of fetal serotonin into fetal platelets and to prevent placental uptake of fetal serotonin by OCT3 is not completely defined. Therefore, although all SSRI inhibit SERT on syncytiotrophoblast increasing serotonin concentrations in the intervillous space and generally on the maternal vascular bed, each SSRI

may differently affect plasma content of serotonin in the fetus by modulating fetal platelet SERT function and trophoblast OCT3 fetal-placental transport.

Using in vitro and in situ models, Horackova and collaborators [36] recently demonstrated that SSRI increase serotonin concentrations in the placenta and fetus by inhibiting both SERT and OCT3. Importantly, all tested SSRI were effective at concentrations lower than plasma levels of each drug in humans. Paroxetine has the lowest IC50 for inhibition of SERT and OCT3. Notably, paroxetine appears to be the SSRI with the greatest impairment of fetal development [50, 51], perhaps due to its greater potency to inhibit SERT and OCT3 promoting more drastic alterations of maternal, placental, and fetal concentrations of serotonin. Paroxetine was the only SSRI classified as a pregnancy category D in the previous FDA drug classification which indicated demonstrated risk for adverse fetal reaction. All other SSRI were labeled as category C.

We have recently shown that fluoxetine decreases pregnancy rates and that both fluoxetine and sertraline decrease the number of pups born as well as increase pup mortality despite their differing placental transfer properties and likely different modulation of fetal concentrations of serotonin. Sertraline had the lowest capacity of all SSRI to inhibit OCT3 and had limited placental transfer [23,24] resulting in low concentrations of sertraline in the fetal circulation likely causing minor effects on OCT3. Conversely, placental transfer of fluoxetine is about 70% and it effectively inhibits OCT3. Taken together, the effects of fluoxetine on modulation of fetal serotonin by inhibiting fetal platelet SERT and placental OCT3 is likely greater than that of sertraline. Nevertheless, both fluoxetine and sertraline decrease number of pups born and increase neonatal mortality [52]. The investigation of how each SSRI drug

modulates maternal, fetal, and neonatal concentrations of serotonin will be useful to identify drugs with more limited impact on pregnancy and neonatal outcomes.

Impact of SSRI use during gestation on pregnancy outcomes and neonatal health

General perspectives

All SSRI inhibit SERT uptake of serotonin throughout the body; however, their pharmacokinetics differ quite markedly [21, 38, 49, 53]. For example, following oral ingestion, bioavailability of citalopram is about 95% while it is less than 45% for sertraline [49]. Plasma concentrations of sertraline follow linear kinetics (increased dose promotes proportional increase in systemic concentrations of the drug) [54]. However, fluoxetine follows nonlinear kinetics resulting in a disproportional increase in systemic concentrations after dose augmentation due to inhibition of its own metabolism (inhibition of liver enzymes CYP2D6, CYP2C, CYP3A4) [38]. Also important, the half-life of fluvoxamine is about 15 hours whereas fluoxetine has a half-life of 1–6 days [22]. Furthermore, fluoxetine metabolism produces an active metabolite, norfluoxetine, which has a longer half-life than fluoxetine itself (8–15 days) [38]. The variability in the pharmacokinetics among SSRI, in addition to an individual's capacity to metabolize drugs [55], result in tremendous disparity in systemic concentrations of each SSRI in the maternal circulation and subsequent impact on circulating concentrations of serotonin which may partially account for the apparent differential impact of each SSRI on pregnancy outcomes.

Placental transfer among each SSRI also varies considerably. Although variable between studies (for example fetal exposure of fluvoxamine varies from 7 to 78% compared to maternal exposure in humans), it is generally accepted that placental transfer is greater for fluoxetine, intermediate for sertraline, and lower for paroxetine [38, 49, 56]. Therefore, each SSRI may

result in different fetal exposure of the drug which may account for differences on neonatal outcomes. On the contrary, the effect of SSRI may be related to its efficacy to inhibit placental and fetal SERT and placental OCT3. Horackova et al. demonstrated that all SSRI have the potential to inhibit both placental SERT and OCT3 at systemic concentrations in humans; however, paroxetine appears to have greater potency. These conundrums of the different SSRI add complexity to interpretation of studies that evaluate all SSRI as a single drug category.

Most studies in humans fail to evaluate the effects of each individual SSRI compound, the dose of exposure, and/or the gestational period of exposure. However, these conditions are relevant to understand the impact of SSRI on pregnancy and neonatal outcomes as each of these may differentially affect pregnancy outcomes and neonatal health [57]. For example, it appears that maternal use of paroxetine and citalopram, but not fluoxetine and sertraline, are associated with increased risk for fetal cranial birth defects [50]. In a recent report, neonatal respiratory distress occurred regardless of maternal treatment dose and whether SSRI treatment was discontinued during pregnancy [22]. However, only moderate and high doses of SSRI taken during entire gestation were related to preterm birth while low doses throughout gestation or dose reduction/discontinuation during the first trimester did not impact pregnancy length. Several studies demonstrated that the risk for autism spectrum disorders and congenital cardiovascular malformations are greater upon maternal exposure to SSRI during the first trimester [58]. Conversely, premature birth, low birthweight, and persistent pulmonary hypertension of the newborn occur more frequently when maternal exposure to SSRI takes place during the second and third trimesters [58]. The limited availability of studies investigating each of these specific aspects of SSRI usage in humans obscures a complete assessment of the impact of SSRI on pregnancy and neonatal outcomes. Larger, more comprehensive studies are critical to address the

burden of increasing antidepressant use during gestation and its effects on maternal and infant health.

Although interpretation of reproductive and neonatal outcomes in animals can be challenging due to difference in number of offspring per gestation, gestation length, and stage of fetal development at birth, animal models are crucial for understanding the effects of drugs in humans. Multiple animal models have been used to investigate the impact of SSRI on pregnancy outcomes and neonatal health: rodents [52, 59-62], sheep [63-66], and fish [67, 68]. Additionally, mutant mouse models with altered SERT expression and/or function have been useful to shed light on a role for SERT on pregnancy outcomes and neonatal health [12, 31, 69, 70]. The utilization of animal models allows prospective evaluation of the effects of SSRI on pregnancy outcomes which is limited in humans as most studies in humans are retrospective. The limited availability of studies on drug pharmacology in pregnant women further support the use of animal models [71]. Additionally, animal models allow compliance and uniformity in dose of exposure and interval between treatments in contrast to studies in humans that often rely on data availability on prescription drugs and on patient adherence to treatment.

Among animal models, rodents have been the most explored. Nevertheless, there are a few studies using the ovine model [63, 64]. Sheep have been long recognized as a great model for translational pregnancy studies due to similarities to human pregnancy (number of fetuses per gestation, fetal intrauterine development, and stage of fetal organ maturation at birth) in contrast to mice [72-74]. We recently used sheep to investigate the effects of SSRI on pregnancy and neonatal outcomes [63]. In our study, fluoxetine treatment during late pregnancy recapitulated several findings associated with SSRI exposure during pregnancy in women (decreased placentome [functional unit of placenta in ruminants], shorter gestation length, decreased

birthweight, neonatal morbidity). The similarities between women and sheep treated with SSRI emphasizes the power of the ovine model to investigate the mechanistic effects of SSRI on pregnancy and neonatal outcomes. Specifically, our experiment further supports the use of the ovine model in translational pregnancy studies to investigate the impact of SSRI on the regulation of placental function and fetal development and to explore preclinical implementation of preventive therapies to overcome the adverse effects. Interestingly, the adverse pregnancy outcomes in women, sheep, and rodents (decreased placental size, decreased birthweight, shorter gestation length/preterm birth, neonatal morbidity and mortality) highlight the conserved pregnancy effects of altered SERT function and serotonin signaling among species.

Placental alterations

Cardiovascular adaptations occur during pregnancy to increase blood flow to the uterus to sustain adequate embryonic/fetal needs for oxygen, nutrients, and waste exchange that increase as pregnancy progresses [75-77]. To provide continuous increase of blood supply to the uterus and placenta as fetal demand rises throughout gestation, sustained uterine vasodilation is maintained by increased production of endothelial cell vasodilators and altered responsiveness to vasoactive hormones, for example, serotonin, angiotensin II, and epinephrine [75, 76, 78, 79]. For example, increased serotonin promotes vasoconstriction of arterial beds leading to decreased downstream blood perfusion primarily by binding to serotonin receptor 2 subtypes [47, 80]. The effects of serotonin on uterine/placental vascular perfusion have been demonstrated in rodents [81-83] and sheep [47]. Serotonin treatment to pregnant ewes during the third trimester increases uterine artery vascular resistance by 363% reducing uterine blood flow by 71% [47] and, in rats, serotonin reduces uterine blood blow flow by ~50% and the number of live fetuses by ~85%

[83]. Two studies in sheep [63, 64] reported SSRI-induced placental alterations consistent with decreased uterine/placental blood perfusion. In third trimester pregnant sheep, an intravenous bolus of fluoxetine rapidly decreased blood perfusion to the uterus which was associated with decreased fetal oxygen saturation, partial pressure of oxygen, and blood pH while partial pressure of carbon dioxide and lactate were increased [64]. In another study, pregnant sheep had decreased placentome growth when treated with fluoxetine during the last month of gestation [63]. Similarly, in humans, changes in fetal heart rate and brain blood flow at week 36 of gestation was associated with maternal SSRI use consistent with fetal hypoxia [84] likely due to reduction in blood perfusion. These placental alterations in sheep, and likely in women, appear to be due to fluoxetine inhibition of SERT leading to increased circulating serotonin producing vasoconstriction of uteroplacental vessels and ultimately restricted blood perfusion to the uterus and placenta (Figure 3). Other authors have also suggested a similar mechanism associating SSRI with increased serotonin altering blood perfusion at the uteroplacental unit affecting placental function and pregnancy outcomes [19, 59, 63-65, 84-86].

To fully understand the impact of SSRI on pregnancy and neonatal outcomes, we must understand how the placenta is affected by SSRI treatment as placental dysfunction might be associated with adverse pregnancy outcomes [19, 58, 85-88]. However, to the best of our knowledge, only one study evaluated placental histopathological alterations in women taking SSRI during gestation. Levy et al. [44] reported multiple placental pathologies in women undergoing SSRI treatment during gestation. Placental vascular lesions of maternal malperfusion were increased 7.4-fold, fetal vascular lesions consistent with fetal thrombo-occlusive disease were increased 3.6-fold, and composite fetal vascular malperfusion lesions were increased 2.4-fold in women taking SSRI during gestation compared to an untreated control group.

Importantly, these findings were independent of possible confounders such as maternal age, gestational age (before vs after 37 weeks), smoking status, diabetes, hypertensive disorders, and neonatal birthweight. Limited studies reported placental weight in patients taking SSRI and results are inconsistent. While Levy et al. [44] reported decreased placental weight in women taking SSRI, placental weight was greater in women taking antidepressants in two other studies, although multiple drugs were combined (SSRI, alpha-2 receptor antagonists, serotonin-norepinephrine reuptake inhibitors) [86, 87].

There are limited reports about molecular changes in the placenta associated with maternal use of antidepressants. Kaihola et al. [89] reported increased protein expression of neurotrophic growth factor (NGF). Additionally, the downstream effector of NGF, Rho-associated coiled-coil containing protein kinase 2 (ROCK2) was also increased [89, 90]. Olivier et al. [90] suggested that altered ROCK2 expression in the placenta of women taking SSRI could be related to cardiovascular effects on fetal development although no direct causal relationship was established in their study. Placental NGF is implicated in placenta development and pregnancy maintenance, and has been associated with miscarriage and preterm birth [91, 92]. In a recent report [93], SSRI decreased enzyme activity of aromatase and cytochrome P4501A1 in human term placenta. In vivo and intro studies have also indicated that SSRI may modulate steroidogenic enzymes and steroid synthesis with a shift towards increased estrogen synthesis [94-97]. A recent study reported no effect of maternal SSRI exposure on placental profile of DNA methylation [98]. Although these studies provided useful information concerning maternal exposure to antidepressants, the implications on placental function and clinical outcomes have not been confirmed. Further studies in humans and animal models are needed to fully understand the impact of SSRI on placental blood perfusion, homeostasis, pathology, and transport function

to aid in understanding the pathophysiology of pregnancy complications associated with maternal use of SSRI.

Studies using mutant mouse models with genetic ablation of SERT have shed light on a role for SERT on pregnancy and neonatal outcomes. Hadden and collaborators [31] reported the occurrence of large areas of necrosis, hemorrhage, and fibrosis in the placenta from SERT null mice on embryonic day 18 (day before delivery). Additionally, placenta cell death was 49-fold greater in SERT null mice compared to wild-type although cellular proliferation and DNA repair were unchanged. Similar to animals and humans treated with SSRI, SERT null mice have increased plasma concentrations of serotonin due to lack of platelet uptake of serotonin [30, 31]. Therefore, it seems likely that the detrimental effects of altered SERT function, either due to SSRI inhibition or genetic ablation of SERT (SERT null mice), on placental structural soundness and function are mediated by the increased serotonin effect on placental blood perfusion as serotonin decreases blood flow to the uterus. Noteworthy, the findings in the placenta of SERT null mice are similar to findings in the placenta of pregnancies complicated by maternal hypertension, that is, with decreased placental vascular perfusion [31, 99]. In a recent report, we described pregnancy complications in a SERT null mouse model: increased pregnancy loss after embryonic day 10.5, shorter gestation, dystocia, decreased litter size, increased neonatal mortality, and fetal malformations [69]. After embryonic day 10.5, mouse embryos becomes fully dependent on the placenta [100]; therefore, the increased pregnancy loss in SERT null mice implicates a vital role for a functional SERT in the regulation of placenta function with impact on embryonic development and maintenance. Taken together, the placental pathology in SERT null mice reported by Hadden et al. [31] are consistent with the pregnancy and neonatal outcomes observed in our study [69]. Consistent with the decreased placental vascular perfusion and

function, whole transcriptome sequencing data in SERT null mice suggest abnormal placental uptake and metabolism of nutrients [70]. Abnormal fetal neurodevelopment has also been reported in mice with dysfunctional maternal SERT [12]. Altogether, the altered placental homeostasis observed in women and animal models treated with SSRI [16, 36, 44, 63, 64], and in SERT null mice [31, 69], are consistent with increased serotonin leading to reduction of placenta blood perfusion resulting in decreased placental growth and efficiency with consequent impairment of fetal development [47, 81, 101]. Further studies are needed to define and confirm these mechanistic pathways from the molecular to whole-body physiological levels.

Although it seems likely that the effects of SSRI on the placenta are mediated by restricting blood perfusion to the organ, a direct effect of SSRI on trophoblasts has been suggested. Protein expression of serotonin receptor 1A was greater in the placenta from SSRI-treated women [102]. Sertraline, paroxetine, and fluvoxamine decreased BeWo cell (choriocarcinoma cell line) viability and increased lactate dehydrogenase. Additionally, sertraline increased BeWo cell synthesis of reactive oxygen species, caspase 3/7 activity, and apoptosis while decreasing cellular ATP content and mitochondrial membrane potential [103]. Similarly, in JEG-3 and HIPEC cell models of extravillous trophoblasts, fluoxetine and sertraline were cytotoxic decreasing cell viability at therapeutic levels [104]. In contrast, in another study from the same group, sertraline and fluoxetine did not affect viability of BeWo cell nor human placental trophoblast cells in primary culture [105]. Sertraline and fluoxetine also did not alter expression of biomarkers of syncytialization (chorionic gonadotropin beta and gap junction protein alpha 1) in primary trophoblasts. These contradictory reports emphasize the need for more research in the area to provide more conclusive interpretations of possible direct effects of SSRI on various placental cell types.

Pregnancy outcomes – focus on IUGR and preterm birth

For decades, SSRI have been associated with adverse pregnancy outcomes in humans and animal models. It has been reported that up to 30% of infants may display some clinical manifestation related to maternal SSRI use [106]. We will focus on the adverse pregnancy outcomes that are more frequently associated with maternal SSRI use and may be encountered more often by clinicians (1) decreased birthweight/small for gestational age and (2) preterm birth. Nevertheless, dozens of other pregnancy and neonatal adverse effects have been reported (postpartum hemorrhage, birth defects, persistent pulmonary hypertension of the newborn, neonatal cardiac issues, increased NICU admissions, neonatal abstinence/toxicity, postnatal adaptation syndrome, developmental delays, autism spectrum disorder, neonatal jitteriness, increased hospital admission up to two years of birth, neonatal death, seizures, endocrine disruption, infant obesity, respiratory distress) [107-118]. Numerous reports have investigated the role of maternal SSRI use on the occurrence of persistent pulmonary hypertension of the newborn as it appears to be one of the main neonatal side effects related to in utero exposure to SSRI [119, 120]. Noteworthy, persistent pulmonary hypertension of the newborn seems to be related to increased pulmonary vascular resistance associated with increased neonatal circulating serotonin. Additionally, some researchers have claimed that what has been previously described as neonatal withdrawal might actually be serotonin syndrome due to increased neuronal serotonin concentrations and signaling [121-124].

As uterine and umbilical blood flow are closely related to neonatal weight and placenta size [77, 79], experimental reduction of uterine blood perfusion results in decreased placental and fetal growth in multiple animal models [73]. Multiple studies in humans associated the use

of SSRI during gestation with greater risk for low birthweight and/or small for gestational age neonates [57, 107, 125-130] and preterm birth [51, 57, 61, 115, 125-128, 131-136]. Nevertheless, while the increased risk for preterm birth appears to be a consensus among studies, some report no association between SSRI and greater risk for low birthweight and/or small for gestational age [51, 128, 135, 136]. Unfortunately, the incidence of concomitant low birthweight/small for gestational age and preterm birth is often not reported. Low birthweight/small for gestational age are the clinical manifestation of IUGR, a condition in which the fetus does not develop to its expected biological potential before birth [73, 74, 137]. In addition, IUGR is an important cause of prematurity. Both conditions, whether or not due to SSRI exposure, are associated with greater incidence of poor neonatal outcomes, multiple diseases throughout life, and tremendous economic costs [138, 139]. The placental alterations in women and animals treated with SSRI are consistent with decreased fetal growth, likely due to decreased placental blood perfusion, resulting in low birthweight infants. Further studies are needed to confirm a causal relationship between placental alterations due to SSRI, fetal development, and preterm birth.

In animal models, decreased pregnancy rates, shorter gestation length, decreased neonatal weight, and increased neonatal mortality have been reported when dams are treated with SSRI [52, 59-61, 63, 140, 141]. The main adverse pregnancy and neonatal outcomes due to maternal SSRI treatment during gestation are shown in Table 1. Noteworthy, while pregnancy outcomes were reported in several of these studies, they were not the focus of investigation, but maternal and/or neonatal behavioral changes due to SSRI treatment. The reports of these adverse pregnancy outcomes in different animal models, rodent strains, SSRI drugs, dosage, and period of exposure strengthen the association between maternal SSRI treatment and unfavorable pregnancy outcomes. There are no specific guidelines to determine preterm birth and prematurity

in animal models which challenges comparisons among animal studies and its translational applications to understanding pregnancy complications in women [142]. However, decreased neonatal weight and increased mortality has been used to infer prematurity in rodents [142, 143] which has been associated with SSRI treatment [52, 59-61, 140, 141]. Further support for an in utero, rather than neonatal, exposure effect of SSRI on neonatal wellbeing has been provided by Noorlander and colleagues [60]. They cross-fostered litters between control and fluoxetine-treated dams. Only pups exposed to fluoxetine in utero had increased neonatal mortality despite being fostered by control dams while pups from control dams fostered by dams exposed to fluoxetine during gestation had normal survival rates. In sheep, however, a longer pregnancy (152 days) allows a more robust translational implication concerning prematurity [63]. Shorter pregnancy length compared to a control group along with decreased neonatal weight and increased neonatal morbidity in sheep, as observed in premature babies and neonates exposed to SSRI in utero, implicates a role for maternal SSRI treatment during late pregnancy on fetal development and preterm birth [63]. Additionally, the apparent greater impact of SSRI on the occurrence of low birthweight/small for gestational age neonates and preterm birth upon treatment during late pregnancy [22, 58, 63] is consistent with restriction of fetal growth during the period of greater fetal growth, the last trimester of gestation [144].

Future directions

In this review we addressed the complex interactions among maternal SSRI use during gestation, its modulation of serotonin concentrations and signaling, regulation of blood perfusion to the uterus and fetoplacental unit, fetal growth, and pregnancy complications. The similar pregnancy outcomes in women, rodents, and sheep studies suggest a similar pathophysiological

mechanism among these species despite their different placental structure. Nevertheless, in all these species, increased maternal serotonin cause decreased uterine/placental vascular perfusion. The increase in maternal free (plasma) circulating serotonin due to SSRI use is likely associated with decreased blood flow to the uterus, placenta, and fetus. The decreased vascular perfusion limits placental and fetal growth causing placental pathology and increasing the risk for low birthweight/small for gestational age and preterm birth which are associated with neonatal morbidity.

Despite the numerous studies demonstrating the increased risk for pregnancy complications in women taking SSRI during gestation, more studies investigating the molecular and physiological mechanisms that lead to pregnancy complications are critical to improve pregnancy outcomes in women with diverse psychological conditions. Animal studies are critical in delineating the pathways of SSRI and serotonin-induced vascular changes, placental alterations, and the consequent impact on fetal development and neonatal outcomes. Specifically, sheep appear to be a particularly useful animal model for translational studies as it allows multiple sampling, maternal and fetal instrumentation, longer period of SSRI exposure similarly to humans (in contrast to mice with shorter gestation), and assessment of in utero fetal development.

Understanding the pathophysiology of SSRI-induced adverse pregnancy outcomes is essential to optimize strategies for treatment of psychiatric conditions. As physicians become more aware of the possible detrimental pregnancy effects of SSRI given the increasing amount of research in the area, we expect that better assessment of risk/benefit will be implemented likely reducing the number of women and infants exposed to SSRI. Alternatively, the development of therapies to mitigate the effects of SSRI could allow women to benefit from its neuronal effects

without the off-target effects likely limiting its impact on pregnancy outcomes. Additionally, as alternative therapies become available, other drugs with limited side effects will benefit women and infants. In the meantime, further studies in humans and animal models should address the effects of each SSRI compound, periods of exposure, dose of each SSRI drug to possibly identify an SSRI with less detrimental effects to maternal and fetal/neonatal wellbeing.

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Tables

Table 1. Effects of SSRI treatment during gestation on pregnancy and neonatal outcomes in animal models.

Animal model	Drug	Main pregnancy and neonatal outcomes	Ref.
Mouse	Fluoxetine	Decreased pregnancy rate, decreased maternal weight gain during gestation, decreased litter size, increased neonatal mortality, dystocia	**[52]**
		Decreased pregnancy rate, decreased litter size	**[59]**
		Increased neonatal mortality	**[60]**
		Decreased neonatal weight	**[145]**
	Sertraline	Increased embryonic resorption, decreased maternal weight gain, decreased birthweight, increased fetal malformations	**[141]**
		Decreased pregnancy rate, decreased maternal weight gain during gestation, decreased litter size, increased neonatal mortality, dystocia	**[52]**
Rat	Fluoxetine	Decreased maternal weight gain during gestation, increased estrogen and progestogen metabolites, decreased uterine weight, decreased birthweight, increased neonatal mortality	**[61]**
		Shorter gestation length, decreased birthweight	**[140]**
		Decreased maternal weight gain during gestation, reduced litter size, decreased birthweight, increased neonatal mortality	**[146]**
		Decreased birthweight and weight gain before weaning	**[147]**

		Increased stillbirth, decreased birthweight, increased neonatal mortality, decreased neonatal weight gain	**[148]**
	Paroxetine	Decreased neonatal weight	**[149]**
		Shorter gestation length, decreased birthweight, increased neonatal mortality	**[150]**
Sheep	Fluoxetine	Decreased placentome size, shorter pregnancy length, decreased birthweight, neonatal morbidity (acidemia, increased lactate, hypocalcemia)	**[63]**
		Transient decreased fetal blood pH, partial pressure of oxygen and oxygen saturation, increased partial pressure of carbon dioxide, higher maternal and fetal blood pressure	**[64]**

Figures

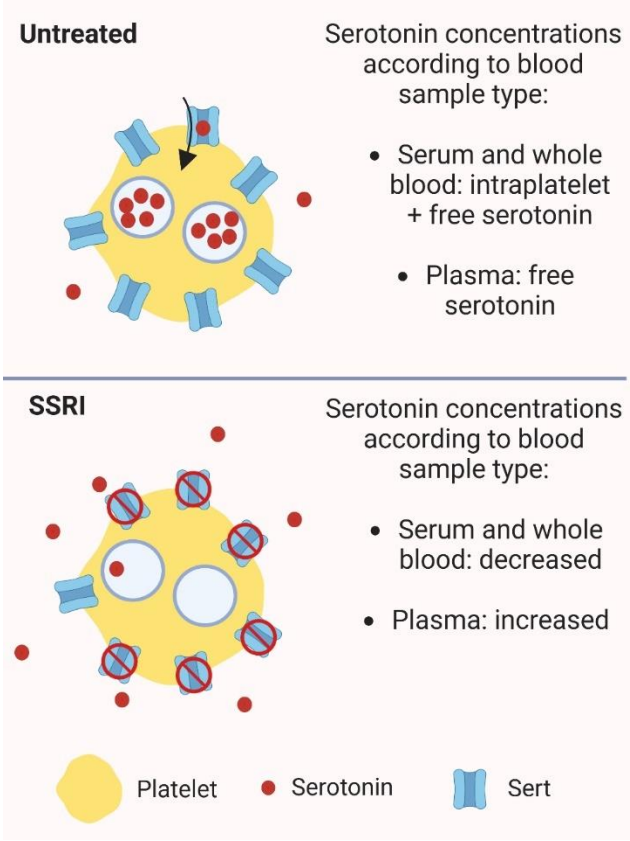

Figure 1. Dynamics of circulating serotonin and role of SSRI inhibition of serotonin transporter (SERT) according to blood sample type. In non-treated individuals, circulating serotonin is mostly transported inside platelets. Platelet lysis in serum and whole blood samples releases intraplatelet serotonin content so that total serotonin (intraplatelet + free) is measured. In plasma samples, anticoagulants prevent platelet lysis so only free serotonin is measured resulting in lower concentrations compared to serum or whole blood samples. Inhibition of platelet SERT in SSRI-treated individuals prevents platelet serotonin uptake. Despite the intense degradation of free serotonin, concentrations of the hormone in plasma samples are increased compared to untreated individuals. On the contrary, circulating serotonin is decreased in serum or whole blood samples due to decreased intraplatelet serotonin content. Created with BioRender.com.

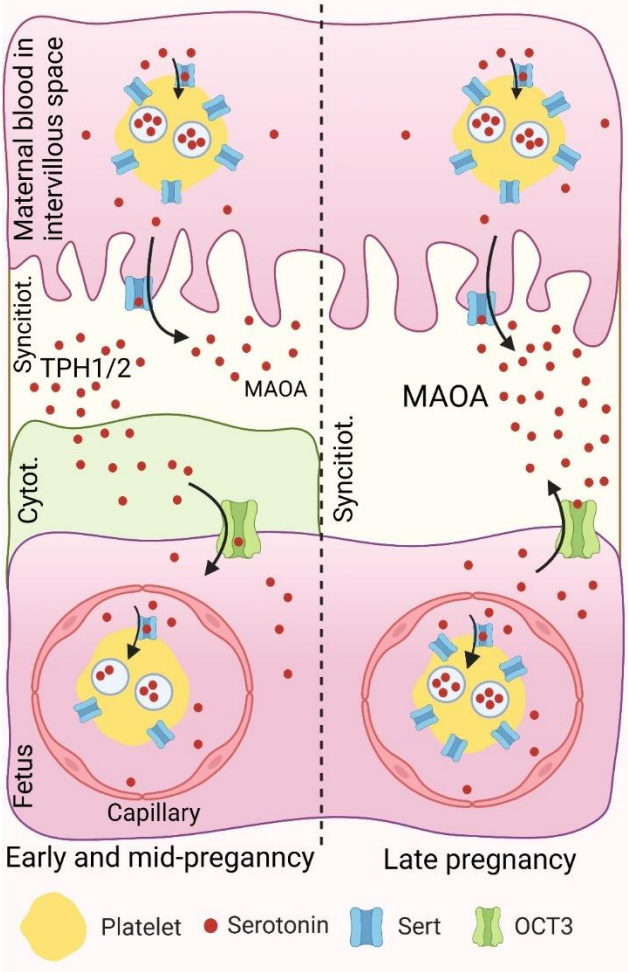

Figure 2. Regulation of serotonin at the maternal-fetal interface. Circulating maternal serotonin in the intervillous space is taken up by serotonin transporter (SERT) on the apical border of syncytiotrophoblast and degraded by placental MAOA. During early and mid-pregnancy, placental-derived serotonin is transported to the placental villous core by OCT3 and, in the fetal blood vessels, taken up into platelets through SERT. During late pregnancy, placental synthesis of serotonin diminishes as fetal serotonin synthesis increases so that fetal serotonin is transported into the placenta by OCT3 and degraded by placental MAOA. Created with BioRender.com.

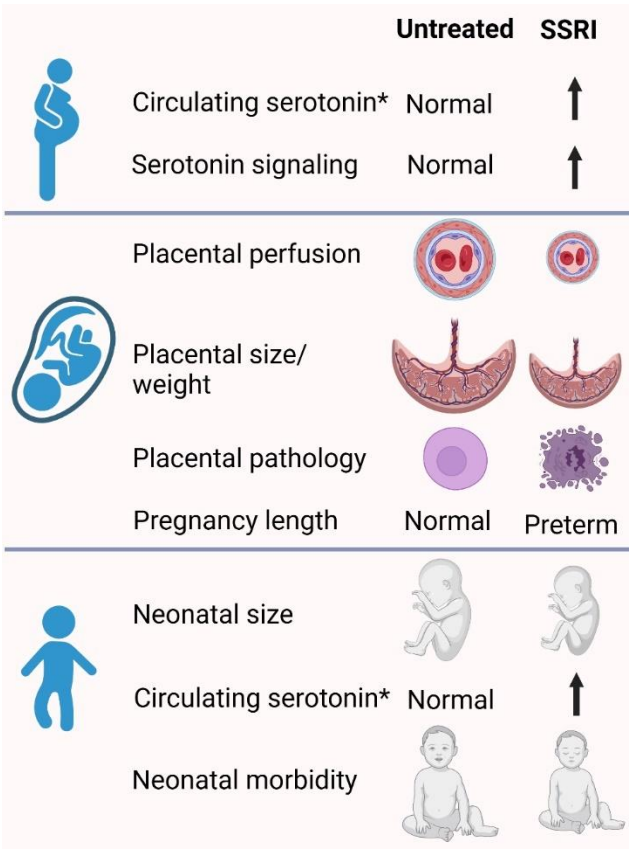

Figure 3. Proposed physiological model for the impact of SSRI on pregnancy outcomes. Free circulating serotonin and serotonin signaling are increased due to SSRI treatment leading to vasoconstriction of uterine and placental blood vessels. The decreased vascular perfusion of fetoplacental unit is likely associated with the placental alterations observed in SSRI-treated women: increased placental pathology, decreased placental size/weight, and shorter gestation length (preterm birth). These placental alterations are consistent with adverse fetal and neonatal outcomes associated with maternal SSRI use: low birthweight/small for gestational age and increased neonatal morbidity. * Free (plasma) circulating serotonin. Created with BioRender.com.

Chapter 2

Effect of low and high doses of two selective serotonin reuptake inhibitors on pregnancy outcomes and neonatal mortality

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Abstract

Selective serotonin reuptake inhibitors (SSRI) are the most common antidepressant used by pregnant women; however, they have been associated with adverse pregnancy outcomes and perinatal morbidity in pregnant women and animal models. We investigated the effects of two SSRI, fluoxetine and sertraline, on pregnancy and neonatal outcomes in mice. Wild-type mice were treated daily with low and high doses of fluoxetine (2 and 20 mg/kg) and sertraline (10 and 20 mg/kg) from the day of detection of a vaginal plug until the end of lactation (21 days postpartum). Pregnancy rate was decreased only in the high dose of fluoxetine group. Maternal weight gain was reduced in the groups receiving the high dose of each drug. Number of pups born was decreased in the high dose of fluoxetine and low and high doses of sertraline while the number of pups weaned was decreased in all SSRI-treated groups corresponding to increased neonatal mortality in all SSRI-treated groups. In conclusion, there was a dose-dependent effect of SSRI on pregnancy and neonatal outcomes in a non-depressed mouse model. However, the distinct placental transfer of each drug suggests that the effects of SSRI on pup mortality may be mediated by SSRI-induced placental insufficiency rather than a direct toxic effect on neonatal development and mortality.

Introduction

Psychotropic medications that are taken during pregnancy can pose risks of toxic effects to both mother and fetus [1,2]. About 8-12% of pregnant women take antidepressants [2-4] and selective serotonin reuptake inhibitors (SSRI) are the most commonly used antidepressant [4,5]. Among SSRI, fluoxetine was the first clinically available and remains one of the most popular while sertraline is currently the most prescribed SSRI to pregnant women [6]. Although teratogenic effects of some SSRI (i.e. paroxetine) are well recognized [7], other SSRI (sertraline, citalopram, fluoxetine) continue to be commonly prescribed to pregnant women [6]. Nevertheless, in the past decades multiple studies highlighted the association between SSRI use during gestation and adverse maternal, fetal, and neonatal health outcomes including decreased birthweight, preterm birth, and increased perinatal morbidity and mortality [3,6,8,9].

In addition to its role as a neurotransmitter, serotonin is a hormone with vasoactive properties [10] so that increased serotonin signaling selectively increases vascular resistance in the uterus causing reduced uterine vascular perfusion [11]. SSRI increase free (plasma) serotonin content by inhibiting serotonin uptake into platelets [12], thereby also decreasing uterine vascular perfusion [13]. Reduced uteroplacental blood flow leads to placental dysfunction/insufficiency, the main cause of fetal growth restriction [14,15]. The role of serotonin and SSRI on fetal growth restriction have been reviewed [10,15-18]. Fetal growth restriction is an important cause of prematurity, perinatal morbidity, and lifelong health impairment in addition to being the second leading cause of perinatal mortality [19,20].

Although fluoxetine and sertraline inhibit the serotonin transporter (SERT), their pharmacokinetics differ quite markedly [21]. Following oral ingestion, fluoxetine exhibits greater bioavailability compared to sertraline (80 vs 44%, respectively) [21]. Plasma concentrations of

sertraline follow linear kinetics (increased dose promotes proportional increase in systemic concentrations of the drug) [22]. However, fluoxetine follows nonlinear kinetics resulting in a disproportional increase in systemic concentrations after dose augmentation. Additionally, while sertraline has a half-life of 22 to 36 hours, fluoxetine has a half-life of 1-6 days [22]. Furthermore, fluoxetine metabolism produces an active metabolite, norfluoxetine, which has a longer half-life than fluoxetine itself (8-15 days) while sertraline's metabolites are essentially inactive. Lastly, placental transfer of fluoxetine is greater compared to sertraline (70 vs 25%) [23,24]. It is unclear whether these two popular SSRI with distinct kinetics similarly affect pregnancy outcomes and neonatal morbidity/mortality, particularly given their distinct placental transfer.

Because of the widespread use of SSRI during gestation and their possible detrimental effects on pregnancy and neonatal outcomes, we aimed to compare the effects of low and high doses of fluoxetine and sertraline on pregnancy and neonatal outcomes. Pregnant mice were treated with SSRI during the second half to pregnancy. Altogether, both low and high doses of sertraline and fluoxetine adversely affected neonatal outcomes; however, only the high dose of fluoxetine resulted in decreased pregnancy rate.

Materials and Methods

Animals

All experiments were approved by the Research Animal Care and Use Committee at the University of Wisconsin-Madison and were performed under protocol number A005789-A01. Mice were housed in a controlled environmental facility for biological research in the Biochemistry Department vivarium (fluoxetine study) and the Animal and Dairy Sciences Department vivarium (sertraline study) at the University of Wisconsin-Madison. Animal facilities

were maintained at a temperature of 25°C and a humidity of 50% to 60%, with a 12:12 hour light-dark cycle with *ad libitum* water and food (LabDiet 5015, TestDiet, Richmond, IN). Wild-type C57BL/6J mice were obtained from Jackson Laboratories (stock # 000664, Jackson Laboratories, Bar Harbor, ME). Females included in our study either originated from Jackson Laboratories or were F1 offspring from our breeding colony. Beginning at 6 weeks of age, female mice were bred with a male overnight.

Experimental design

After detection of a vaginal plug (day post coitum [DPC] 0.5), dams were individually housed and randomly assigned to treatment groups. All mice received a daily intraperitoneal injection between the hours of 0800 and 0900 of either vehicle, low and high dose of SSRI (either fluoxetine or sertraline) from DPC 0.5 until the end of lactation (21 days postpartum). Virgin females (unmated; 2-6 per group) were treated with vehicle, low and high dose of SSRI (either of fluoxetine or sertraline) for evaluation of the effect of SSRI on weight in nonpregnant mice. Virgin mice received 7 treatements, equivalent to pregnant dams treated from DPC0.5 to 6.5. All mice were weighed daily at time of injection. A successful pregnancy was determined by weight gain between DPC 0.5 and 7.5 [25] and confirmed by parturition. Pregnancy length and number of pups born were recorded on the day of parturition. The number of live pups was recorded daily during lactation. Litters were not standardized.

For the fluoxetine study, fluoxetine hydrochloride (F312; Sigma-Aldrich, St. Louis, MO) was reconstituted in saline. Mice were treated with vehicle (saline; n = 28), low dose of fluoxetine (2 mg/kg; n = 32), or high dose of fluoxetine (20 mg/kg; n = 127).

For the sertraline study, sertraline hydrochloride (S6319; Sigma-Aldrich, St. Louis, MO) was reconstituted in 8.3% dimethyl sulfoxide (DMSO) diluted in saline for the low dose group (10 mg/kg; n = 32) and in 15% DMSO diluted in saline for the high dose group (20 mg/kg; n = 16). To account for the different concentrations of DMSO, we had two vehicle groups: 8.3% DMSO diluted in saline (vehicle group for the low dose sertraline; n = 32) and in 15% DMSO diluted in saline (vehicle group for the high dose sertraline; n = 11).

The high dose of fluoxetine (20 mg/kg) has been extensively used in rodent studies [26,27], including reports from our laboratory [28]. However, the systemic concentrations of the drug in mice from previous studies from our laboratory were higher than in humans. Therefore, we selected a low dose (2 mg/kg) that we anticipated would be within expected systemic concentrations of fluoxetine in humans. The low dose of sertraline (10 mg/kg) was based on other reports and is expected to be within normal range of human systemic concentrations [29]. However, we had issues with drug solubility to develop a model with the higher dose of sertraline. Because we did not want to dramatically increase DMSO concentrations nor alter injection volume between studies, we were only able to treat mice at 20 mg/kg.

Blood collection and fluoxetine assay

Blood samples were collected from the dams on DPC 0.75 and DPC 17.75. Mice were fasted for 6-8 hours after the morning treatment until blood collection. Blood was collected from the submandibular vein using a 5.5 mm lancet, placed on ice for 20 minutes, and centrifuged at 846 *g* for 20 minutes at 4˚C; serum was stored at -80˚C until assayed. Serum fluoxetine and norfluoxetine concentrations were measured with a forensic fluoxetine ELISA kit (catalog no. 107619; Neogen, Lexington, KY) according to manufacturer's instructions, samples were diluted

1:100. A displacement curve was prepared from fluoxetine hydrochloride (S6319; Sigma-Aldrich, St. Louis, MO) to create a standard curve for quantification. The cross-reactivity is 100% for fluoxetine and 67% for norfluoxetine; therefore, data is presented as fluoxetine plus norfluoxetine concentrations.

Statistical analysis

All statistical analyses were performed on SAS (version 9.4; SAS Institute Inc., Cary, North Carolina, USA). Data were analyzed with PROC MIXED procedure using one-way ANOVA and two-way ANOVA for repeated measures. Tukey HSH was used for post hoc comparisons. Studentized residuals with deviations from assumptions of normality and/or homogeneity of variance were transformed into square root, logarithms, or ranks. Survival analysis was done with PROC LIFETEST using Wilcoxon test. For the fluoxetine study, comparisons were performed among all groups. For the sertraline studies, comparisons between vehicle and treated group were performed separately for the low and high dose. A probability of ≤ 0.05 indicated a difference was significant and a probability between > 0.05 and ≤ 0.1 indicated significance was approached. Data are presented as the mean ± standard error of mean (SEM).

Results

Systemic fluoxetine concentrations

Fluoxetine was undetected in vehicle-treated mice (Table 1). In fluoxetine-treated mice, there was a dose-dependent effect of treatment on systemic concentrations of fluoxetine. At 6 hours after the first treatment, fluoxetine concentrations were 24-fold greater in mice treated with the high than low dose. At 6 hours after the 18^{th} treatment, fluoxetine concentrations had increased

2.4-fold with the low dose and 4.2-fold with the high dose, producing a 43-fold greater fluoxetine in the high than low dose animals.

Maternal weight

Weight gain after the onset of treatment was evaluated between DPC 0.5 to 6.5 and DPC 7.5 to 18.5. Until DPC 6.5 there is little to no effect of embryonic weight on total maternal weight [25]; therefore, pregnant, nonpregnant, and virgin mice were included in the analysis for more robust analysis of the effect of SSRI on mouse weight. In the fluoxetine study, overall weight gain during DPC 0.5 to 6.5 was decreased in the high dose group (Figure 1). Although all groups in the fluoxetine study lost weight after the first day of treatment, the high dose of fluoxetine caused greater weight loss after a single treatment compared to vehicle and low dose. Additionally, the group receiving the high dose of fluoxetine did not recuperate weight to pretreatment levels until DPC 6.5 while the vehicle and low dose groups reached pretreatment weight on DPC 2.5. Maternal weight gain was overall reduced in the high dose of fluoxetine from DPC 7.5 to 18.5. Maternal weight on the day before parturition was greatest ($p = 0.005$) in the vehicle group (32.7 ± 0.5 g), intermediate in the low dose (32.1 ± 0.7 g), and lowest in the high dose group (30.5 ± 0.4 g).

In the sertraline study, the high dose group had greater weight loss after onset of treatment and overall weight gain between DPC 0.5 to 6.5 and between DPC 7.5 to 18.5 was lower in the high dose group than vehicle. However, maternal weight on the day before parturition was not different between groups ($p = 0.2$, 32.8 ± 0.8 vs 31.6 ± 0.7 g for the low dose; $p = 0.6$, 34.2 ± 0.5 vs 33.5 ± 1.1 g for the high dose).

Pregnancy establishment and maintenance

In the fluoxetine study, the high dose significantly reduced pregnancy establishment (pregnancy per plug; Table 2). Sertraline treatment (low and high doses) had no significant effect on number of pregnant mice. Gestation length was not affected by fluoxetine treatment, but sertraline extended mean gestation length.

Neonatal outcomes and pup survival

The low dose of fluoxetine did not significantly affect the number of pups born compared to the vehicle; however, the low dose of sertraline and the high dose of both fluoxetine and sertraline caused a reduction in the number of pups born (Figure 2).

In the fluoxetine study, there was a dose-dependent increase in pup mortality during the 21 days postpartum resulting in less pups weaned per litter (Fig. 2). The percentage of litters in which all pups died was greater ($p = 0.0008$) for the high dose fluoxetine group (62.5%) compared to the control (20.8%) and low dose (16.0%) groups. The mean number of pups weaned per litter was also reduced by sertraline treatment (low and high doses). However, the number of litters in which all pups died were not different between groups ($p = 0.6$, 5.9 vs 13.6% for the low dose; $p = 0.6$, 28.6 vs 72.7% for the high dose).

To further investigate the effect of in utero SSRI exposure on neonatal mortality, we analyzed pup mortality using survival curves. Besides a clear effect of SSRI on neonatal survival, pup mortality occurred primarily before DPP 4.5 independent of treatment (vehicle vs SSRI) and dose.

Pregnancy complications

Some of the SSRI-treated dams that gave birth to an unusually reduced number of pups appeared to still have unborn pups due to visually large abdominal size. A similar finding was not observed in vehicle-treated dams. Four SSRI-treated dams that had all pups die a few days postpartum were euthanized for necropsy on postpartum days 2.5 to 5.5. These dams had 3 to 5 fully developed dead pups still in the uterus. Additionally, one dam from the high dose sertraline group euthanized on postpartum day 21.5 had 3 dead pups in the uterus that appeared to be mummified. Because our experimental design did not anticipate these issues, only a few (4) dams were euthanized and inspected for unborn pups. Consequently, precise quantification of this finding was not possible. However, reporting this finding is important for designing future studies. To gain insight into the incidence of unborn pups in SSRI-treated dams in the present study we examined the maternal weight change between the day before and the day of parturition (Figure 3). Because maternal weight was not different between groups on the day before parturition (except for high dose fluoxetine), a reduced weight loss suggests that fewer pups were born and is indicative of unborn pups. The high dose of both fluoxetine and sertraline had overall less mean weight loss between the last day of pregnancy and the day of parturition. Based on individual maternal weight loss, it seems likely that other SSRI-treated dams with unsually small litter sizes that were not necropsied also had unborn pups after parturition.

Discussion

Understanding the effects of maternal medication on pregnancy complications and neonatal outcomes is vital to comprehensively assess the risk of perinatal exposure to psychotropic medication on maternal and newborn wellbeing. Herein, we report the effects of two popular antidepressants on pregnancy and neonatal outcomes in a mouse model highlighting a dose-

dependent effect of SSRI, particularly fluoxetine, on neonatal outcomes. Interestingly, fluoxetine and sertraline caused comparable reductions in the number of pups born and pup survival despite the distinct placental transfer of each drug; therefore, exposing fetuses to distinct amounts of each drug. This suggests that these adverse neonatal outcomes are likely to be related to the effect of SSRI on the dam and placenta rather than a direct toxic effect of each drug on pup development.

Because SSRI treatments began on DPC 0.5 in the present study, ovulation and fertilization were expected to take place before the onset of treatments and, therefore, to be similar among vehicle and SSRI-treated groups. Additionally, fluoxetine has little to no effect on embryo development in vitro [30] so a direct effect of fluoxetine on embryo development is unlikely. Therefore, the decreased pregnancy per plug in the high dose fluoxetine group (initially observed on DPC 7.5) is likely due to implantation failure. The high dose of fluoxetine could cause implantation failure via multiple mechanisms including: (1) induction of maternal weight loss that could alter ovarian function [31] disrupting the endocrine environment required for embryo implantation, (2) direct or indirect modulation of estrogen signaling [32-34] with consequent disruption of uterine receptivity, or (3) decreased uterine vascular perfusion [10,11,13,18] disrupting uterine vascular remodeling [35]. Previous studies have also indicated an effect of SSRI on embryo implantation and early pregnancy loss in humans [36,37] and animal models [38,39], although the mechanism remains to be elucidated. Since none of the other doses of SSRI had an effect on embryo implantation, the decreased pregnancy rate in the group receiving the high dose of fluoxetine in the present study may be related to a toxic effect of fluoxetine, as discussed later, rather than an expected effect of the drug at therapeutic concentrations in humans. Further studies are needed to confirm this finding and to define the mechanisms and critical period of fluoxetine

exposure on impaired embryo implantation to establish the safety of fluoxetine in early pregnancy development.

The reduced maternal weight gain after DPC 7.5 in the high dose of fluoxetine and sertraline-treated groups suggests smaller litter size, reduced embryonic/fetal growth, or both. Indeed, maternal SSRI treatment during gestation has been linked to intrauterine growth restriction in humans [3,6,8,17] and in animal models [38,40]. The mechanisms of SSRI-induced fetal growth restriction have been a prominent area of research worldwide [10]. The SSRI-induced increase in serotonin signaling has been associated with decreased uterine vascular perfusion [13] and vascular lesions on the maternal and fetal sides of the placenta in women [18]. Therefore, maternal exposure to SSRI may compromise placenta function leading to inadequate nutrient exchange between mother and fetus which can result in fetal growth restriction [10]. On the other hand, the number of pups born was reduced in dams exposed to the high dose of fluoxetine and the low and high doses of sertraline suggesting a role for SSRI on embryo implantation and embryonic/fetal survival. However, the unexpected finding of fully developed dead pups still in the uterus days after parturition in SSRI-treated mice further clouds our interpretation of the effects of SSRI on pregnancy establishment and fetal survival. Furthermore, it is not known whether the intrauterine pup death was a cause or a consequence of fetal retention. Although dystocia has not been reported in women taking SSRI during gestation and fluoxetine does not affect uterine contractions [41], it was an unexpected but critical finding in our study and warrants further investigation. In light of this finding, reduced litter size in rodent models treated with SSRI in previous reports and future studies should be interpreted with caution.

Neonatal mortality, primarily during early postnatal period, was increased in all groups exposed to SSRI in the present study. Previous rodent studies have also shown that fluoxetine and

sertraline exposure during the perinatal period increase neonatal mortality [23,40,42]. Fetal developmental malformations (primarily cardiac, respiratory, and neurodevelopmental disorders) have been reported as possible causes of neonatal mortality associated with perinatal SSRI exposure [3,23,43-45]. However, neonatal mortality may be due to placental insufficiency caused by SSRI disruption of uterine/placental vascular perfusion and structure [3,10,13,18] rather than a direct effect of SSRI on fetal development. Placental insufficiency is generally regarded as the major cause of fetal growth restriction which is associated with perinatal morbidity and mortality. Fetal growth restriction (unrelated to SSRI exposure) leads to several fetal adaptations to restricted nutrient availability including morphological heart changes, increased cardiac workload, and cardiac function issues resembling dilated cardiomyopathy [14]. Interestingly, rodents exposed to SSRI perinatally have altered cardiac morphology [43,46] and dilated cardiomyopathy [23]. Because both drugs promote comparable neonatal mortality and increase serotonin concentrations in the maternal side of the placenta altering placental homeostasis [18,47] but have distinct placental transfer (70% fluoxetine [23] vs 25% sertraline [24]), placental insufficiency is likely to be the underlying mechanism of SSRI-related pup mortality rather than a direct toxic effect of SSRI on fetal development. Nevertheless, these results do not exclude a direct role of fluoxetine (highest placental transfer) on fetal organogenesis.

The placenta regulates maternal and fetal serotonin homeostasis during gestation [3,48-50]. Because embryonic production of sertonin is limited until day 14.5 of gestation in mice, extraembryonic sources of serotonin are required to maintain fetal brain development [48]. SERT located on the apical region of syncytiotrophoblast (maternal side of the placenta) transports maternal-derived serotonin into the placenta during early and mid gestation regulating serotonin content (signaling) on the maternal side of the placenta and providing maternal-derived serotonin

needed for fetal development [48,49]. However, during late pregnany serotonin is no longer transported from mother to fetus; instead, organic cation transporter 3 (OCT3) located on the fetal side of the placenta transports fetal serotonin into trophoblasts for degradation [47,49,50]. SSRI inhibition of placental SERT prevents the transport of maternal serotonin into the placenta increasing serotonin signaling on the maternal side of the placenta [47]. Interestingly, OCT3 is inhbited by glucocorticoids [51] and exogenous drugs such as SSRI [47,50]. However, fluoxetine, but not sertraline, decreases the capacity of OCT3 to transport serotonin from fetal circulation into the placenta in a in situ model [47]. Therefore, added to the decreased placental transfer of sertraline [23,24] resulting in lower concentrations of the drug in the fetal circulation, it seems likely that the similar effects of the two drugs on neonatal outcomes are mediated by their common capacity to inhibit SERT on the maternal side of the placenta [47] leading to increased serotonin signaling with consequent compromise of placental vascular perfusion and function [11,15].

Systemic concentrations of fluoxetine + norfluoxetine increase after onset of treatment ranging from 160 to 560 ng/mL in humans [21]. In overdosed patients, fluoxetine + norfluoxetine concentrations may reach 1490 ng/mL [52]. In rodent models, although the dose and route of administration of fluoxetine vary among studies, the dose of 20 mg/kg/day via intraperitoneal injection has been widely used [26,27]. Our results clearly demonstrate that this dose produces systemic concentrations that are many fold greater than clinically relevant doses in humans. On the contrary, the low dose of fluoxetine used in our experiment (2 mg/kg/day) resulted in systemic concentrations similar to expected concentrations in humans. Unfortunately, we were unable to measure sertraline concentrations in our study. The overall effects of sertraline (low and high doses) were intermediate compared to the low and high doses of fluoxetine. However, the limitation of sertraline solubility that required increased DMSO concentration and the lack of

systemic drug concentrations clouded our full interpretation of the effects of the high dose of sertraline on pregnancy and neonatal outcomes. Although we recognize that extreme dosage treatments are often important for delineating physiologic pathways and investigating possible toxic effects of drugs in animal models, our study highlights the importance of using therapeutic dosages to more accurately evaluate the risk of maternal drug exposure on pregnancy and neonatal outcomes, particularly in translational studies for more direct relevance to human medicine.

Most mice (vehicle and SSRI-treated) in our studies lost weight in the first 24 hours after the first treatment. This is expected due to the stress of handling and changing cages for mating, individual housing, and initiation of treatments. However, mice exposed to the high dose of each drug experienced greater weight loss. Nevertheless, mice receiving the high dose of sertraline and low dose of fluoxetine reestablished pretreatment weight by day 2 of treatment (similar to vehicle) while mice receiving the high dose of fluoxetine took 6 days to reestablish pretreatment weight. In animal models, SSRI-induced weight loss has been reported [31,39,40,53]. In humans, short-term fluoxetine treatment is also known to cause weight loss [54]. However, with prolonged treatment, weight gain is most commonly observed. Although the SSRI-induced weight loss in mice may resemble the weight loss observed in short-term fluoxetine treatment in humans, the weight loss in rodents exposed to high doses of SSRI seems to be due to drug overdose because it has been associated with amenorrhea [31], digestive disorders, and death [39,53].

Conclusions

Overall, our results demonstrate a dose-dependent effect of SSRI exposure during gestation and lactation on pregnancy outcomes and perinatal pup mortality. The comparable neonatal outcomes during treatment with these two drugs that have distinct placental transfer properties make it likely

that SSRI-induced placental insufficiency and fetal growth restriction lead to the observed neonatal morbidity/mortality rather than a direct toxic effect of each drug on perinatal mortality. Lastly, we highlight that some effects of treatments with excessive doses of psychotropic medication in animal models (as for the highest dose of fluoxetine in the present study) may not reflect expected effects in humans due to extreme systemic concentrations of the drug, and therefore, should be interpreted with caution.

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Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. You might choose to exclude this statement if the study did not report any data.

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Tables

Table 1. Serum concentrations of fluoxetine + norfluoxetine in pregnant mice on DPC 0.75 and 17.75 (6 hours after the first and 18th treatments, respectively).

	Vehicle	**Low dose**	**High dose**	**P value**
DPC 0.75, ng/mL	0	195.4 ± 14.6 [b]	4,724.7 ± 354.0 [a]	< 0.0001
(range)	Undetectable	(169.5 - 219.9)	(2,756.3 – 5,828.4)	
DPC 17.75, ng/mL	0	466.8 ± 61.6 [b]	20,059.2 ± 2,176.5 [a]	< 0.0001
(range)	Undetectable	(336.8 – 746.6)	(13,477.8 – 29,815.6)	

a,b indicate significant difference among groups.

Table 2. Effect of low and high doses of fluoxetine and sertraline on pregnancy outcomes.

	Vehicle	Low dose	High dose	P value
Fluoxetine				
Vaginal plug, n	28	32	127	-
Pregnant dams, n	24	25	24	-
Pregnancy per plug, %	85.7 [a]	78.1 [a]	18.9 [b]	< 0.0001
Gestation length, day	19.1 ± 0.1	19.1 ± 0.1	18.9 ± 0.1	0.17
Sertraline low dose				
Vaginal plug, n	32	32	N/A	-
Pregnant dams, n	22	23	N/A	-
Pregnancy per plug, %	68.8	71.9	N/A	0.99
Gestation length, day	18.8 ± 0.1 [B]	19.0 ± 0.1 [A]	N/A	0.096
Sertraline high dose				
Vaginal plug, n	11	N/A	16	-
Pregnant dams, n	7	N/A	11	-
Pregnancy per plug, %	63.6	N/A	68.7	0.9
Gestation length, day	18.9 ± 0.3 [b]	N/A	19.6 ± 0.2 [a]	0.01

a,b indicate significant difference among groups. A,B indicate significance was approached.

Figures

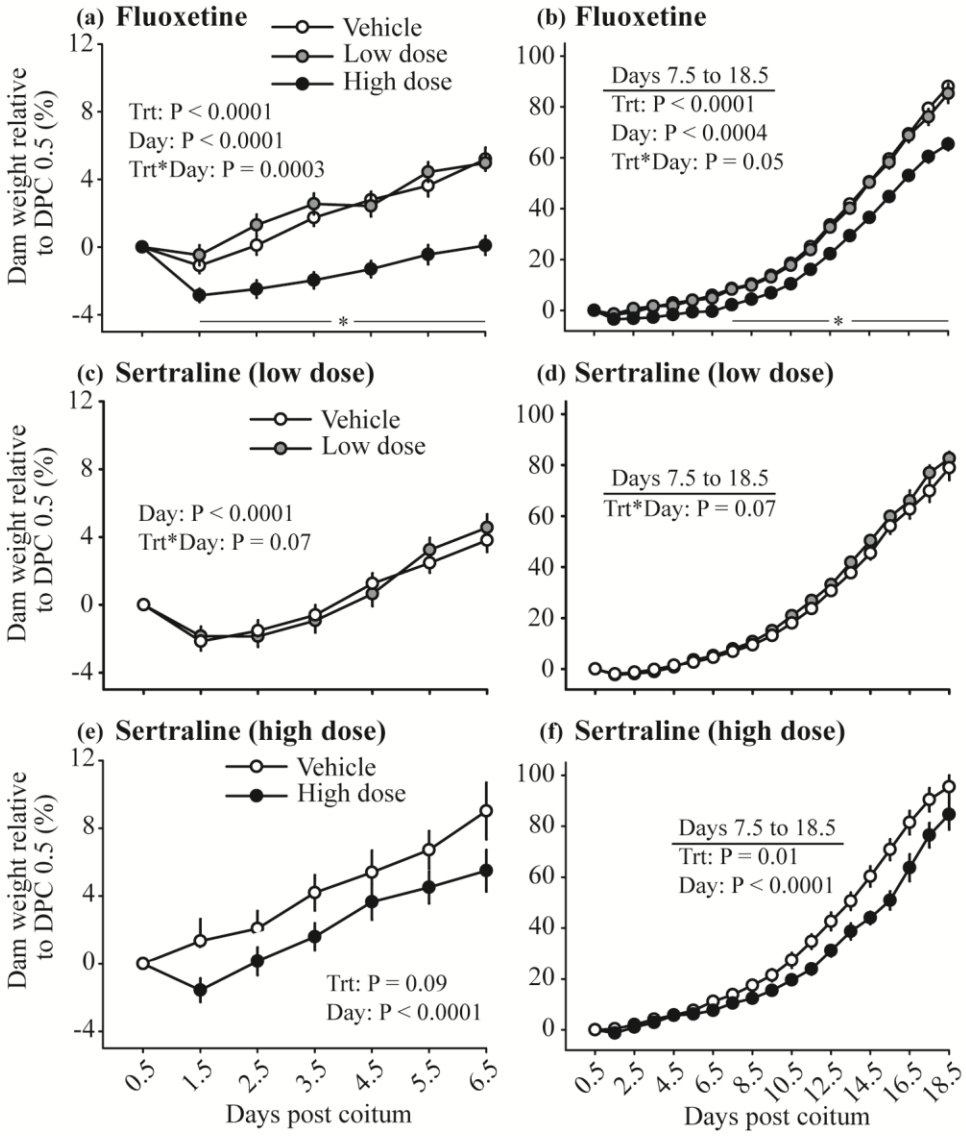

Figure 1. Effect of fluoxetine (a,b) and sertraline (c,d,e,f) on maternal weight gain between days post coitum (DPC) 0.5 to 6.5 (a,c,e) using data from pregnant (before the effect of fetal weight on maternal weight), nonpregnant, and virgin mice. Maternal weight gain (b,d,f) during entire gestation (DPC 0.5 to 18.5) used data from only pregnant mice with data analyzed for DPC 7.5 to 18.5. * indicate significantly decreased weight in the high dose of fluoxetine group.

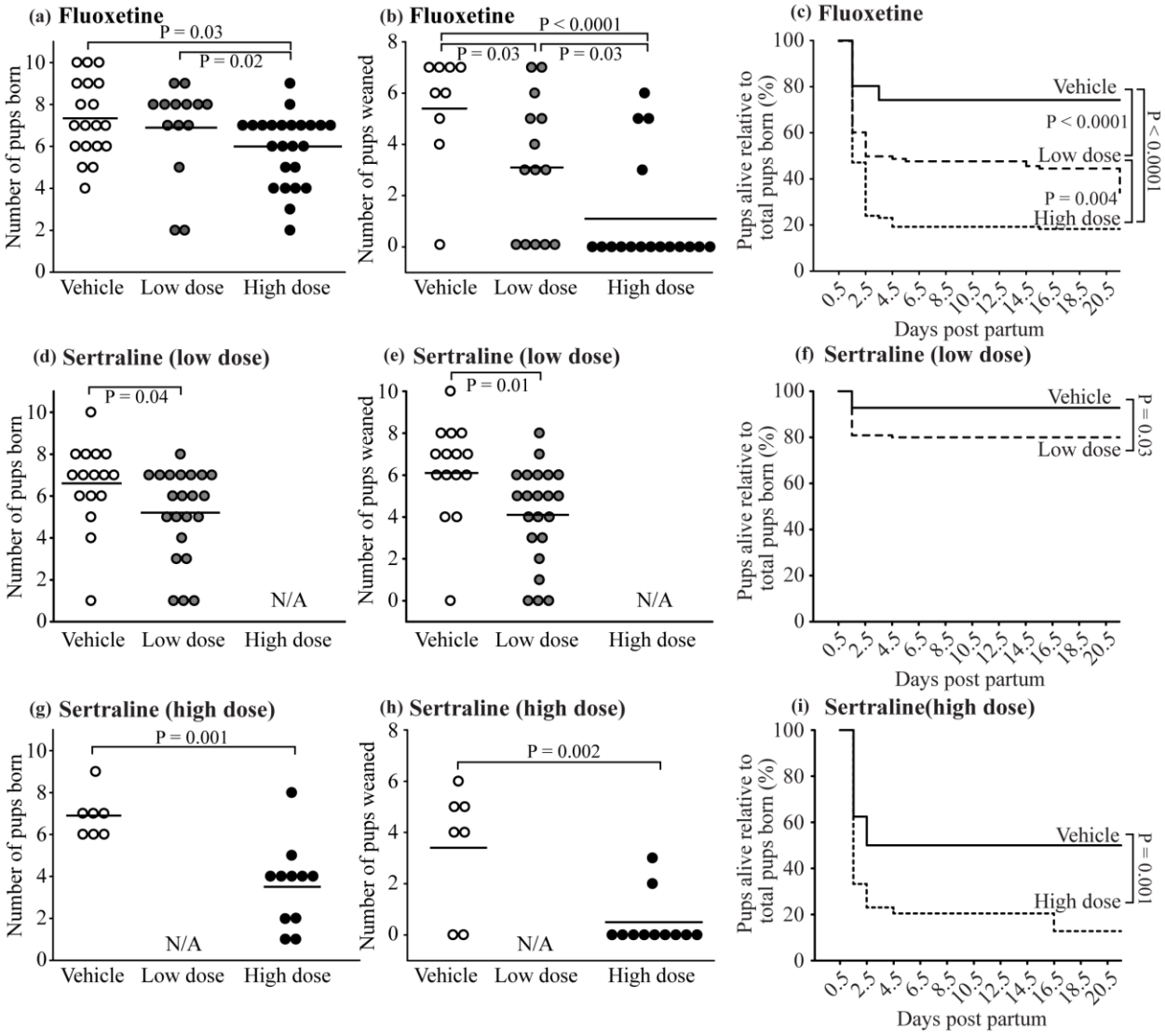

Figure 2. Effect of fluoxetine (a,b,c) and sertraline (d,e,f,g,h,i) on neonatal outcomes: Number of pups born per litter (a,d,g), Number of pups weaned per litter (b,e,h), Survival analysis of pup mortality (c,f,i) during lactation (days postpartum 0.5 to 21.5).

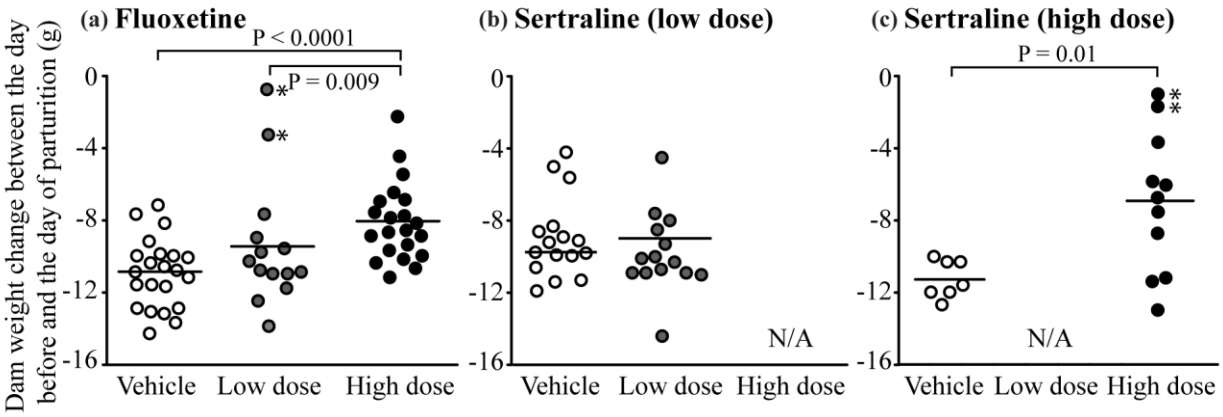

Figure 3. Maternal weight change between the last day of pregnancy and the day of parturition in the fluoxetine (a) and sertraline (b) studies. *Denotes dams that were euthanized after parturition and had fully developed dead pups in the uterus.

Chapter 3

Pregnancy complications and neonatal mortality in a serotonin transporter null mouse model: insight into the use of selective serotonin reuptake inhibitor during pregnancy

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Abstract

Selective serotonin reuptake inhibitors (SSRI) are widely prescribed to pregnant woman. Although some SSRI compounds are known to cause pregnancy loss and fetal malformations, other SSRI continue to be used by pregnant women. However, several studies have associated the use of SSRI with adverse pregnancy outcomes: intrauterine growth restriction, preterm birth, and neonatal morbidity. Nonetheless, interpretation of studies in humans are typically complicated by the adverse pregnancy outcomes caused by depression itself. Therefore, we used a mutant mouse model with genetic ablation of the serotonin transporter, the target site for SSRI, to unravel the role of the serotonin transporter on pregnancy outcomes. The serotonin transporter null mice that increased pregnancy loss (17.5% vs 0%), decreased number of pups born (6.6 ± 0.2 vs 7.5 ± 0.2), and increase in neonatal mortality (2.3-fold). Furthermore, preterm birth, dystocia, and fetal malformations were only observed in serotonin transporter null mice. This mouse model with genetic ablation of the serotonin transporter recapitulates several adverse pregnancy outcomes similar to those in women undergoing SSRI treatment during gestation. Additionally, neonatal loss in the present study reproduced a sudden infant death phenotype as in humans and mice with altered serotonergic signaling. In conclusion, findings from this study demonstrate a role for serotonin transporter in pregnancy maintenance and neonatal health. Additionally, it suggests that the adverse pregnancy outcomes in women taking SSRI during gestation might be due to altered serotonin transporter function caused by SSRI independent of underlying depression. This is a critical finding, given the number of women prescribed SSRI during pregnancy, and provides the framework for critical research in this area.

Introduction

Selective serotonin reuptake inhibitors (SSRI) are the primary class of antidepressants prescribed to pregnant women (1, 2). However, SSRI treatment during gestation has been associated with several adverse pregnancy outcomes – congenital malformations, preterm birth, and increased neonatal morbidity (2-4).Moreover , some SSRI are not indicated for pregnant women (i.e., paroxetine) while others (sertraline, fluoxetine) are still widely used (5). Over the past decades, multiple studies have questioned the safety of SSRI for mother and infant (2, 4, 6-8).

Serotonin is a neurotransmitter and a hormone with a multitude of actions throughout the body ranging from embryo development to regulation of mood and behavior and regulation of vascular resistance (9, 10). A role for serotonin signaling in fetal neurodevelopment (11) and cardiac development (12, 13) has been established. Genetic ablation of serotonin receptors 2B (12) or 3A (14) results in embryonic and neonatal death due to abnormal heart development. Additionally, altered brain and/or peripheral serotonin content and/or signaling have been associated with sudden infant death in humans and mice (15-17). Because SSRI inhibit serotonin transporter (SERT; SLC6A4), it modulates serotonin signaling and also affects fetal heart development (18). Nevertheless, the effect of whole-body genetic ablation of serotonin transporter (Sert-/-) on fetal outcomes is poorly defined. Moreover, reproductive outcomes and pup survival/death rates for the *Sert-/-* mouse model have been inadequately described.

Mouse is the primary animal model used for biomedical research worldwide (19, 20). The development of mutant mouse models has greatly enhanced the understanding of specific genes in health and disease (20, 21). Yet genetic mouse models can represent a challenge in successfully maintaining mouse colonies due to specific fertility/pregnancy issues or decreased

pup survival associated with some genetic lines (22). For example, single gene knockouts are associated with a lethal phenotype in about 25% of mutant mice (21, 23). Aiming to investigate the effects of genetic ablation of *Sert* on reproductive outcomes, we have investigated the reproductive efficiency and occurrence of pregnancy complications in a *Sert-/-* mouse model in light of adverse effects of SSRI treatment during gestation in humans.

Materials and Methods

Animals

All procedures in this study were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin-Madison (protocol A005789-R01-02). Animals were maintained in a constant temperature of 25˚ C, 50-60% humidity, and on a 12/12 hour light/dark cycle. All mice received ad libitum access to water and mouse chow. Male *Sert-/-* (strain 008355; C57BL/6J background) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were bred to wild-type females (WT or *Sert*+/+; C57BL/6J; Jackson Laboratory, strain 000664) to produce SERT+/- offspring. Using successive matings, a colony with *Sert*+/- and *Sert-/-* mice was established. Serotonin reuptake in *Sert*+/- mice is similar to WT while it is almost completely ablated in *Sert-/-* (24). Mice were genotyped by PCR amplification using DNA extracted from tail snips (24).

Animals included in this study were either breeding replacements from our colony or were included in another study (only mice receiving no treatment or saline-treatment were used).

Pregnant mice during late pregnancy were observed daily for detection of parturition and evaluation of neonatal mortality. Dams were euthanized due to pregnancy complications on a case-by-case basis. For euthanasia, dams were anesthetized with isoflurane followed by cervical

dislocation and exsanguination. Immediately after dam's euthanasia, the uterus was excised. Using this euthanasia method, viable pups are still alive inside the uterus.

Animal breeding

All male breeders were *Sert-/-*. For replacement animals from our colony, two or three virgin females (*Sert-/-* or *Sert+/-*; 8-12 weeks old) were placed in a cage with a male. Starting approximately 15 days after exposure to a male, each female was evaluated to detect pregnancy at least three times per week based on visual observation of abdominal size. Once a female was identified pregnant, she was housed individually. Litters were weaned on postnatal day 21. For the females included from other studies, two or three females (*Sert-/-* or *Sert+/-*; 8-12 weeks old) were placed in a cage with a male overnight and were examined for the presence of vaginal plug in the morning. Detection of vaginal plug confirmed copulation (day post coitum, DPC0.5).

Statistical analysis

All statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, North Carolina, USA). Data were analyzed with PROC MIXED procedure using one-way ANOVA. Tukey HSH was used for post hoc comparisons. Studentized residuals with deviations from assumptions of normality and/or homogeneity of variance were transformed into square root, logarithms, or ranks. For probability of frequency of an event Chi-Square test and Goodness of fit test were used. A probability of ≤ 0.05 indicated a difference was significant and a probability between > 0.05 and ≤ 0.1 indicated the presence of a tendency for significance. Data are presented as the mean ± standard error of mean (SEM) unless otherwise indicated.

Results

Pregnancy detection

To reliably identify pregnant mice on DPC10.5 (approximately mid gestation), we conducted a survey with previous data from our laboratory. We evaluated the weight gain of C57BL/6J mice between DPC7.5 and DPC10.5. There were 344 mice with a vaginal plug that were found to be not pregnant and 149 mice with a vaginal plug gave birth. The average weight-gain between DPC7.5 and 10.5 was greater in pregnant mice (Figure 1). Using a cutoff value of 1.5g of weight gain between DPC7.5 and DPC10.5 only 5/149 mice with a vaginal plug were mistakenly identified as non-pregnant, a false-negative rate of 3.4% (96.6% sensitivity). More importantly, no non-pregnant mouse gained more than 1.4g between DPC7.5 and 10.5, resulting in a false-positive rate of 0% (0/344) so that only pregnant mice are selected based on this method (100% specificity). Establishment of a reliable method for pregnancy diagnosis at DPC10.5 was important for our subsequent evaluations of pregnancy loss after DPC10.5.

Pregnancy loss and preterm birth

Pregnancy loss was defined as a female that was deemed pregnant on DPC10.5, based on weight gain, that subsequently did not give birth by DPC19.5 and had no visual indication of still being pregnant, based on abdominal size. As observed in the WT females in our colony analysis, *Sert*+/- females had no pregnancy loss, whereas 17.5% of *Sert*-/- females had pregnancy loss after DPC10.5 (Table 1 and Figure 2).

Preterm birth was defined as any female that gave birth prior to DPC19.5. No pregnant *Sert*+/- had premature birth whereas three *Sert*-/- females gave birth on DPC18.5, a day

before expected parturition. All pups (6 to 8 per dam) from these three dams were found dead the morning of DPC18.5 suggesting the pups were born prematurely (Figure 3).

Prolonged gestation and dystocia

We observed two cases of prolonged gestation, both in *Sert-/-* females. One *Sert-/-* female did not give birth by DPC21 and had a very distended abdomen; it was euthanized. Pups were larger than typical newborn pups and 4 out of 9 pups were already dead. As mentioned above, use of our euthanasia method allows viable pups to still be alive inside the uterus. The other dam (*Sert-/-*) was found with blood in the vagina and a distended abdomen; gestational day was unknown. At euthanasia, all six pups were found dead. Pups were very large (1.78 g per pup and 10.68 g overall) which is much larger than the expected weight of six newborn pups (1.2g per pup and 7.2g for six pups). Hemorrhage was detected around and within the placenta from most pups (Figure 4). Some pups also had abnormal morphology.

All mice (n = 6) that had dystocia (difficult, abnormal birth) were *Sert-/-* breeders from our colony. Three females had dead pups (one to three) in the cage in the morning and 4-6 hours after the dams still appeared to have pups to deliver based on observed large abdominal size. All three dams were euthanized and upon dissection, all retained fetuses were dead (4 or 5 per dam) (Figure 5).

One dam gave birth to one pup that was found dead the afternoon of the same day and another dam gave birth to three pups that were found dead the morning after being born. Because of the unusual circumstances (small litter size, dystocia, all pups died), the dams were euthanized. Each of them had retained dead fetuses (one and two, respectively) in the uterus undergoing resorption/mummification.

Lastly, a *Sert-/-* female gave birth to a single pup in the afternoon and had a dead pup stuck in the birth canal the next morning. Upon euthanasia of the dam, seven pups were found dead in the uterus (Figure 5); some pups presented abnormal morphology.

Litter size and pup survival

To evaluate the effect of maternal genotype on litter size and pup survival before weaning, we followed *Sert-/-* (n = 62) and *Sert+/-* (n = 55) dams from birth until weaning. Litter size was decreased (88%; $p = 0.0009$) in *Sert-/-* dams compared to *Sert+/-* (Table 2). Additionally, pup death from birth until weaning was greater (234%; $p < 0.0001$) for *Sert-/-* than *Sert+/-* dams resulting in fewer (51.7%; $p < 0.0001$) weaned pups per litter. There was no effect of genotype on pup sex or of pup sex on pup survival.

Discussion

A mouse model with genetic ablation of *Sert* was first reported over two decades ago (24); however, detailed reproductive performance has not yet been adequately reported. Although *Sert-/-* mice are fertile, we observed a series of adverse pregnancy outcomes associated with genetic ablation of *Sert* including: pregnancy loss, congenital malformations, premature birth, dystocia, and perinatal mortality. Although most cases of lethal single gene deletion in mice are associated with prenatal mortality/pregnancy loss (~50%), perinatal mortality occurs in about 24% of genetic models carrying lethal mutations (22). Interestingly, *Sert-/-* mice experienced both pregnancy loss and perinatal mortality. Although we do not show a direct effect of SSRI on the occurrence of adverse pregnancy outcomes in the present manuscript, we demonstrate a role for adequate serotonin transporter function for successful pregnancy outcomes and neonatal

health. Of particular importance, our findings for the *Sert-/-* mouse model coincide with the reported effects for SSRI treatment during gestation on pregnancy outcomes in humans. Although not compared in this study, reproductive outcomes in *Sert+/-* and WT dams are similar (Hernandez, unpublished) since serotonin reuptake in *Sert+/-* mice is similar to WT mice (24).

Platelet SERT allows entry of serotonin into platelets, thereby controlling free circulating (plasma) concentrations of serotonin. Therefore, lack of SERT (*Sert-/-*) or pharmacological inhibition of SERT (SSRI treatment) results in increased plasma concentrations of serotonin (9, 25, 26). Previous studies using 5-HTP (serotonin precursor) to elevate circulating serotonin have observed reduced uterine/placental blood perfusion and increased pregnancy loss (27, 28). This is consistent with the decrease in uterine artery blood flow that has been observed during SSRI treatment (29). In addition, the decreased uterine/placental perfusion caused by increased serotonin compromises placental function and results in placental pathology (30). Indeed, placenta collected from *Sert-/-* dams had abnormal hemorrhage, and increased necrosis and fibrosis (25). More recently, abnormal placental morphology and placental gene expression were reported in the placenta of Sert-/- conceptus (31). Similarly, placenta collected from women undergoing SSRI treatment during gestation had increased vascular lesions including hemorrhage and fetal thrombo-occlusive disease (32). Pathology and malperfusion of the placenta result in placental insufficiency, the main cause of fetal growth restriction, a common cause of perinatal mortality and preterm birth (33). Thus, the pregnancy loss and preterm birth observed in the *Sert-/-* dams in the present study are likely to be due to placental malperfusion and insufficiency triggered by the elevated plasma serotonin in mice lacking SERT. Moreover, pregnancy loss in the present study occurred after the fetus became completely dependent on the

placenta (after DPC10.5 in mice) further suggesting a role of placenta function on pregnancy loss (34).

A perinatal lethal phenotype occurs in about 24% of mutant mice (22). The most common causes for neonatal mortality associated with a lethal phenotype in mice are cardiorespiratory, neuromuscular, skeletal, craniofacial, and metabolic defects (22, 35). Additionally, maternal behavior may affect pup survival. In the present study perinatal mortality, either due to dystocia or neonatal death, was clearly increased in *Sert*-/-. Noteworthy, dystocia and neonatal death are increased in mice treated with either fluoxetine or sertraline (two of the most popular SSRI) during gestation (36). Perhaps the lack of reports of dystocia in women and neonatal death in infants exposed to SSRI in utero are due to more prompt medical assistance and interventions, in contrast to laboratory animals. However, other labor-associated complications such as postpartum hemorrhage are increased in women taking SSRI during gestation (37, 38) and neonatal morbidity is increased in babies exposed to SSRI in utero resulting in increased neonatal admission into NICU (1, 2, 4).

Altered serotonin signaling in mutant mouse models (serotonin receptors 2B (12), 3A (14) and *Sert* (25, 39, 40) knockouts) or pharmacological manipulation of SERT (SSRI treatment) during pregnancy in WT mice (18) has been associated with abnormal heart development resulting in pre- and perinatal death. Additionally, a role for SERT on cardiac pathology has been described (25, 39-41). Therefore, not surprisingly, some SSRI cause major cardiac malformations in humans (3). Interestingly, *Sert*-/- mice (25, 31) and women taking SSRI during gestation (32) have several placental pathologies which have also been associated with altered embryonic/fetal cardiovascular development (34). Furthermore, sudden infant death syndrome has been associated with abnormal serotonin signaling such as: increased serum

concentrations of serotonin (17), multiple brain serotonergic abnormalities (42, 43), and polymorphisms in the *Sert* promoter region (39). Therefore, although the cause of neonatal mortality was not investigated in the present study, it reproduced the sudden infant death syndrome phenotype observed in humans and other mouse models (39) and is likely to be associated with similar cardiac pathology as observed in those conditions. However, fetal/neonatal morphological defects were also observed in the present study, and these might underlie at least some of the cases of fetal/neonatal mortality. Further studies are needed to investigate the role of altered SERT function, either by genetic ablation of *Sert* in mice or pharmacological inhibition of SERT, on cardiac development and its role in perinatal morbidity/mortality and sudden infant death syndrome.

Responsible biomedical research involving animals includes reducing and refining animal use in research while still acquiring adequate and useful data. In studies of pregnancy in mice, an accurate method for early pregnancy diagnosis can be particularly useful. Our method for pregnancy diagnosis in mice provided 100% specificity and a sensitivity greater than 96% for identifying pregnant dams by mid gestation (DPC10.5). Implementing this method was invaluable for identifying pregnancy loss after DPC10.5 in *Sert-/-* females, previously overlooked and/or unreported information that may shed light on the role of serotonin in pregnancy maintenance. This simple and accurate pregnancy diagnosis method may be useful for other studies of pregnancy loss in mice.

In conclusion, the similarities between the occurrence of adverse pregnancy outcomes in *Sert-/-* mouse model and women and mice undergoing SSRI treatment during gestation suggest a critical role for SERT in pregnancy maintenance, fetal development, and neonatal health. Hence, *Sert-/-* mice might be a useful model to comprehensively understand how altered serotonin

signaling leads to pregnancy complications and neonatal morbidity/mortality in SSRI-exposed mammals. Additionally, it suggests that the adverse pregnancy outcomes in women undergoing SSRI treatment are due to altered SERT function due to SSRI and are independent of the underlying depression. This is a critical finding given the number of women that are currently prescribed SSRIs during pregnancy (5) and provides the framework for future research in this area.

Contribution to the field statement

Selective serotonin reuptake inhibitors (SSRI) are the primary class of antidepressants prescribed to pregnant women and about 8-13% pf pregnant women are exposed to antidepressants during gestation. Yet, the use of SSRI during gestation has been associated with multiple pregnancy complications such as: pregnancy loss, developmental malformations, intrauterine growth restriction, preterm birth, and neonatal morbidity. Interpretation of studies in humans, however, are typically complicated by the adverse pregnancy outcomes caused by depression itself. The antidepressant effects of SSRI are due to inhibition of serotonin transporter (SERT) in the brain. However, inhibition of extraneuronal SERT gives rise to undesired effects of SSRI. To understand the role of altered SERT function on pregnancy outcomes we used a mouse model with genetic ablation of *Sert* (*Sert-/-*). Interestingly, several pregnancy outcomes observed in *Sert-/-* mice recapitulate reported effects of SSRI on pregnancy outcomes in women: pregnancy loss, fetal malformation, preterm birth, and neonatal morbidity/mortality. Additionally, the increased neonatal mortality in our study add to the growing body of evidence associating SERT function and sudden infant death. Collectively, results from our study suggest that, regardless of

underlying depression, altered SERT function as caused by SSRI affects pregnancy outcomes and pre/perinatal morbidity/mortality.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RRD Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization, Project administration. MCW Conceptualization, Methodology, Resources, Writing – Review & Editing, Visualization, Supervision, Funding acquisition. LLH Conceptualization, Methodology, Resources, Writing – Review & Editing, Visualization, Supervision, Funding acquisition.

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Tables

Table 1. Pregnancy loss in *Sert+/-* and *Sert-/-* females bred to *Sert-/-* male.

	Sert+/-	*Sert-/-*	p value
Number of females with a vaginal plug	34	40	
Pregnancy loss after E10.5, n	0	7	0.01
Pregnancy loss after E10.5, %	0	17.5	0.01

Table 2. Litter size and pup survival in *Sert*+/- and *Sert*-/- dams bred to *Sert*-/- males.

	Sert+/-	*Sert*-/-	p value
Number of dams	55	62	
Pups born, n/litter	7.5 ± 0.2	6.6 ± 0.2	0.0009
Pups dead before weaning, %/litter	23.7 ± 4.4	55.5 ± 5.4	<0.0001
Pups dead before weaning, % of pups born	23.4	53.9	<0.0001
Pups weaned, n/litter	5.8 ± 0.4	3.0 ± 0.4	<0.0001
Pups weaned, %/litter	76.3 ± 4.4	44.6 ± 5.4	<0.0001
Male pups weaned per litter, %	50.3 ± 3.0	50.2 ± 4.8	0.9
Female pups weaned per litter, %	49.7 ± 3.0	49.8 ± 4.8	0.9

Figures

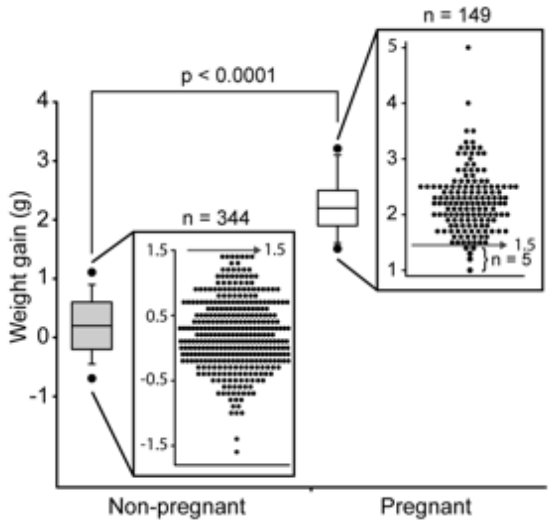

Figure 1. Weight gain between day post coitum 7.5 and 10.5 in C57Bl/6J mice that became pregnant (n = 149) and non-pregnant (n = 344). Boxplot of weight gain and weight gain for individual mice is shown. Boxplot depicts median, 5th, 10th, 25th, 75th, 90th and 95th percentile.

Figure 2. Pregnancy loss in *Sert-/-* mice. (A) Blood was observed in the vagina on unknown day of gestation; the dam was euthanized. A viable pup and a dead fetus were found in the right uterine horn (RUH). In the left uterine horn (LUH) only a pale placenta and the head of a fetus were found. (B) On day post coitum 14, blood was observed in the vagina of a dam that lost 1.2 g in 24 hours. No fetuses were found in the uterus; in the LUH only blood clots and free blood were found indicating pregnancy loss. (C) A pregnant mouse that lost 1.4 g in 24 hours was euthanized; All Fetuses were dead and at different stages of resorption.

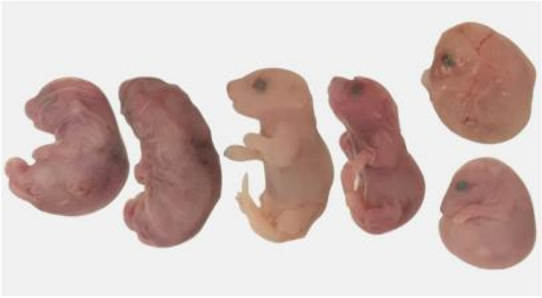

Figure 3. Pregnancy loss in *Sert-/-* mice. A dam gave birth on day post coitum 18.5; all pups were dead at 9 am.

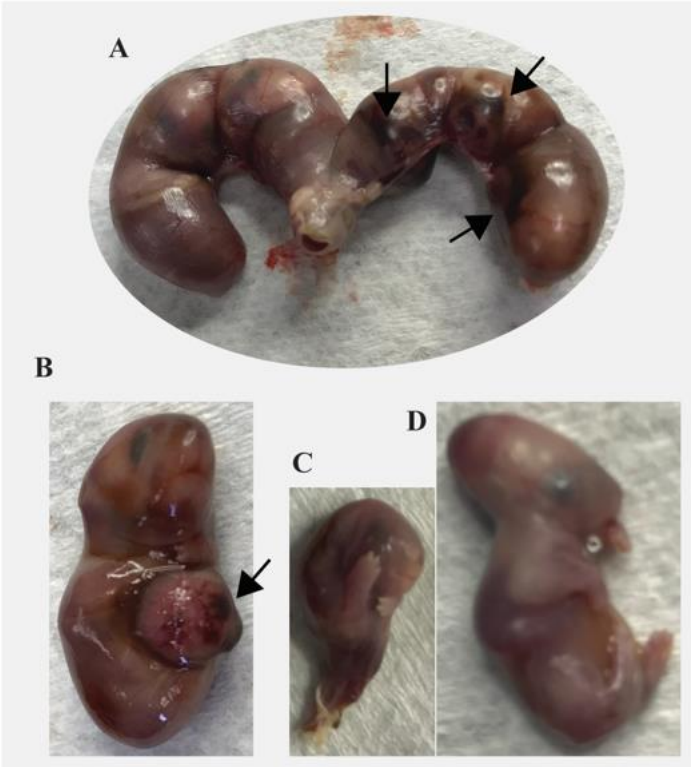

Figure 4. Prolonged gestation in a *Sert-/-* dam. The dam was found with distended abdomen and blood in the vagina on unknown day of gestation. At necropsy of the dam, all fetuses were larger (1.78 g) than average newborn pups (1.3 g) and dead. (A) Excised uterus is shown. (B) A larger fetus, (C) a fetus undergoing resorption, and (D) a fetus with abnormal morphology are shown. Black arrow indicates areas of necrosis in the placenta.

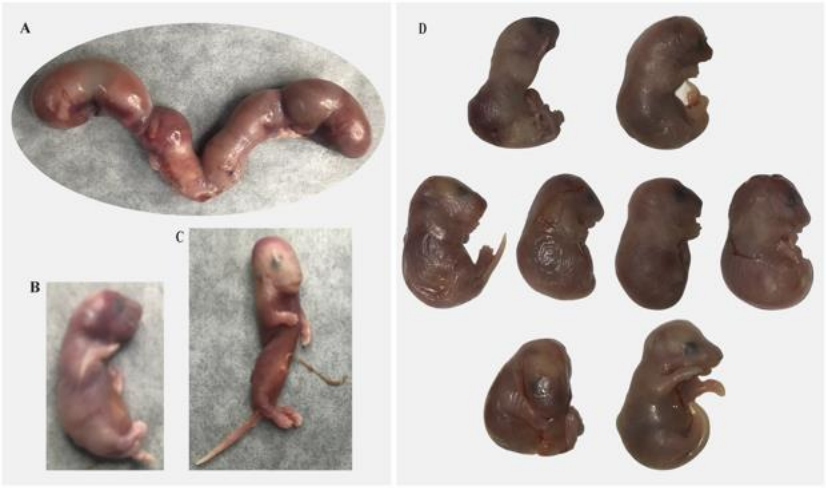

Figure 5. Dystocia in *Sert*-/- mice. (A, B, C) A dam gave birth to three pups that were found dead at 8 am; at 5 pm a large abdominal size indicated delivery was incomplete. Excised uterus with four fetuses is shown (A). Only one fetus was alive (B). One fetus had abnormal morphology (C). (D) A dam was undergoing delivery in the afternoon (one live pup). By the following morning, only one pup has been delivered and was dead and another dead pup was stuck in the birth canal. The dam was euthanized and all retained fetuses were dead (D). Some fetuses presented abnormal morphology.

Chapter 4

Fluoxetine-induced perinatal morbidity in a sheep model

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Abstract

Selective serotonin reuptake inhibitors (SSRI) are the most common antidepressants used by pregnant women. However, adverse pregnancy outcomes have been described in women taking SSRI during pregnancy – placental lesions, premature birth, poor neonatal adaptation. We aimed to investigate the effects of fluoxetine (Prozac®; most commonly used SSRI) treatment during the last month of gestation on pregnancy complications, placental and neonatal health in a non-depressed sheep model. On day 119 ± 1 postbreeding (experimental day 0; E0) of a 151-day expected gestation, Hampshire ewes were randomly assigned to receive fluoxetine (n = 9 ewes, 15 lambs; daily intravenously treatment with 10 mg/kg on E0 and E1 and 5 mg/kg daily thereafter until parturition) or to a control group (n = 10; 14 lambs; vehicle only). Blood samples from ewes were collected throughout the experimental period and postpartum; blood from lambs were collected postpartum. Analysis of variance was used for statistical analysis. Fluoxetine treatment reduced placentome growth during the last month of pregnancy. Gestation length was decreased by 4.5 days in fluoxetine-treated ewes. Birthweight was reduced in lambs exposed to fluoxetine in utero; weights remained decreased until postnatal day 3. Placentome diameter by birthweight ratio was not different between groups suggesting that the decreased placentome diameter was accompanied by decreased lamb birthweight. During the first week postnatal, lambs exposed to fluoxetine in utero had decreased blood pH and decreased total carbon dioxide, bicarbonate, and base excess and increased lactate (days 3 to 6), collectively indicative of metabolic acidemia. Additionally, ionized calcium was decreased between postnatal days 0 to 4 in lambs exposed to fluoxetine in utero. Using a non-depressed animal model clearly defines a role for SSRI on the occurrence of perinatal complications and neonatal morbidity. The decreased placentome diameter, shortened gestation, decreased birthweight, decreased calcium

levels, and neonatal acidemia suggest the occurrence of intrauterine growth restriction. The persistence of neonatal acidemia for several days postpartum suggests poor neonatal adaptation to extrauterine environment.

Introduction

Selective serotonin reuptake inhibitors (SSRI) are the primary class of antidepressants prescribed to treat depression in pregnant women and fluoxetine (Prozac®), the first SSRI introduced to the market, is still one of the most popular antidepressants worldwide (1). Approximately 15% of pregnant women suffer from depression and 8-13% are prescribed antidepressants during gestation (2, 3). Although SSRI use during pregnancy has increased in the past 20 years, numerous studies have described an increase in adverse outcomes for both mother and infant related to SSRI use (4-6). However, interpretation of these adverse pregnancy outcomes in women is confounded by the effects of underlying depression itself (5).

Circulating serotonin is primarily transported by platelets upon its uptake by serotonin transporter (SERT). Inhibition of SERT by SSRI leads to decreased platelet concentrations of serotonin (as measured in serum or whole blood samples) while plasma concentrations are increased (7, 8). Elevated plasma concentrations of serotonin during SSRI treatment potentially alter placental function. The involvement of serotonin in intrauterine growth restriction (IUGR) has been investigated (9-11). Serotonin is a potent vasoconstrictor; therefore increased serotonergic signaling decreases uterine/placental vascular perfusion (12, 13) giving rise to placental complications such as placental insufficiency, the main cause of IUGR (10, 14) Indeed, placentae from women undergoing SSRI treatment during pregnancy have several lesions of malperfusion in the maternal and fetal sides of the placenta along with decreased birthweight (15). However, the effects of SSRI on placenta size/growth, another landmark of IUGR (16, 17), are still poorly understood.

Neonatal disorders associated with intrauterine exposure to SSRI are numerous and associated with increase neonatal morbidity (4, 5, 18). Besides fluoxetine’s effects on

neurodevelopment (19, 20), treatment during pregnancy is related to increased neonatal risk for adverse respiratory and cardiovascular functions (21, 22). The potential decreased uterine/placental vascular perfusion associated with compromised neonatal respiratory and cardiovascular functions caused by maternal SSRI treatment during pregnancy may result in inadequate oxygen and carbon dioxide exchange in the peripartum period. However, little is known about the effects of intrauterine exposure to SSRI on neonatal blood gas and acid-base homeostasis.

Because of the potential adverse effects of SSRI on placental function and the neonatal cardiorespiratory system, we investigated the effects of fluoxetine exposure during the last month of gestation on placental growth, pregnancy outcomes, and neonatal health in an ovine model. We aimed to (1) explore the role of fluoxetine on the occurrence of IUGR, decreased gestation length, decreased birthweight, neonatal morbidity and (2) evaluate the effect of fluoxetine on placental growth in a non-depressed animal model. We hypothesized that fluoxetine treatment during late pregnancy (1) decreases gestation length, (2) causes IUGR and (3) affects neonatal health.

Materials and Methods

Animal management

Timed-bred multiparous Hampshire ewes (4.4 ± 0.4 years old) were obtained from the Arlington Sheep Research Unit from the University of Wisconsin-Madison. Beginning on day 112 ± 1 postbreeding, 20 pregnant ewes were housed in individual pens at the Livestock Laboratory at the University of Wisconsin-Madison and maintained at a constant temperature at 18˚C and a 14/10 hour light/dark cycle. All ewes received ad libitum access to water and were

individually fed haylage, whole-shell corn, and mineral supplement based on live weight according to National Research Council Nutrient Requirements for pregnant ewes (23). After 7 days postpartum, ewes and lambs returned to the Arlington Sheep Research Unit and were grouped housed in an open shelter under natural light.

On day 117 ± 1 postbreeding, jugular catheters were placed in all ewes for intravenous treatment and blood collection. The catheter was aseptically placed, fixated to the neck and protected by a bandage. Catheter patency was maintained during the entire period of treatment/ blood collection.

Ewes were pregnant with a range of one to three fetuses; the average number of fetuses was not different between groups ($p = 0.5$; 1.6 ± 0.2 vs 1.8 ± 0.2). Delivery was assisted as needed and lambs were fed colostrum by assisted nursing or bottle-fed fresh colostrum from their dam.

Experimental design

On day 119 ± 1 postbreeding (exp. day 0) of a 151 day expected gestation, ewes were randomly assigned to control or fluoxetine groups. Fluoxetine-treated ewes received fluoxetine hydrochloride (C845, AK Scientific, Union City, California, USA) at 10 mg/kg on exp. days 0 and 1 and at 5 mg/kg daily thereafter until parturition. Fluoxetine dosage was based on a previous experiment from our laboratory aiming to be representative of systemic fluoxetine concentrations in humans. Lyophilized fluoxetine was reconstituted daily in ethanol and diluted into 0.9% NaCl saline (07983-02, Hospira, Lake Forest, Illinois, USA) to the appropriate concentration for each ewe based on body weight. Final ethanol concentration was < 3.5%. Body weight was assessed weekly and fluoxetine dose was adjusted accordingly. Ewes in the control

group received saline + ethanol at similar ethanol concentration to fluoxetine-treated ewes. All ewes were treated at a continuous infusion rate of 200 mL for 15 minutes using an automated mini pump (Heska Vet/IV 2.2, Heska, Loveland, Colorado, USA).

Transabdominal ultrasonography

Placentome (functional unit of placenta in sheep) diameter was evaluated by transabdominal ultrasonography (MindrayZ5, Nanshan, China; 7.5 MHz transducer).(24) A baseline assessment was made prior to treatment (exp. day –1) followed by weekly assessments thereafter (E7, 14, and 21). At least three placentomes were measured per ewe in each evaluation.

Blood and milk collection

Blood samples from ewes were collected from jugular catheters immediately before each treatment during the prepartum period and for 6 days postpartum. An additional blood sample from ewes was collected within 30 minutes of parturition. Lambs had a jugular blood sample collected within 25 minutes of birth (before colostrum intake) and daily for the 6 days postpartum. Blood samples were immediately used for blood gas analysis; remaining blood was centrifuged at 2000g for 15 minutes and serum was stored at -20˚C until assayed. Colostrum was collected within 30 minutes of parturition (before lamb intake) and milk was collected on postpartum day 6.

Hormone assays and blood gas analysis

Serum serotonin was determined by EIA (IM1749, Beckman Coulter, Czech Republic). The intra- and inter-assay CV were 4.2 and 7.4%, respectively. Serum lactate was determined using Catachem reagents (C454-01, Oxford, Connecticut, USA) on ChemWell-T analyzer. The intra-assay CV was 3.1%. Colostrum and milk calcium concentrations were determined as described (25). The intra-assay CV was 8.0%.

Blood gas analysis was carried out immediately after jugular blood collection using a portable clinical analyzer (i-STAT, Abbott Laboratories, Abbot Park, Illinois, USA) with a CG8+ cartridge. Blood pH, partial pressure of carbon dioxide, bicarbonate, total carbon dioxide, base excess, and ionized calcium were analyzed. Since venous blood was collected, oxygen saturation and oxygen partial pressure data were not used.

Statistical analysis

All statistical analysis was performed using SAS (version 9.4; SAS Institute Inc., Cary, North Carolina, USA). Data were analyzed with PROC MIXED procedure using one-way ANOVA and two-way ANOVA for repeated measures. Tukey HSH was used for post hoc comparisons. Studentized residuals with deviations from assumptions of normality and/or homogeneity of variance were transformed into square root, logarithms, or ranks. Survival analysis was assessed with PROC LIFETEST using Wilcoxon test. A probability of ≤ 0.05 indicated a difference was significant and a probability between > 0.05 and ≤ 0.1 was considered a tendency for significance. Data are presented as the mean ± standard error of mean (SEM) unless otherwise indicated.

Results

A fluoxetine-treated ewe with triplets had a foot abscess, stopped eating haylage, and was treated with antibiotics for 5 days during prepartum; therefore, this ewe was not included in the analysis. The same ewe had dystocia; one of the lambs was stillborn and the other two died within 18 hours of birth. Anatomopathological findings suggested pneumonia and sepsis. Two lambs (one lamb per ewe) from saline-treated ewes with twin gestation were stillborn due to dystocia. Data from these animals were not used in the final analysis. Overall, 10 control and 9 fluoxetine-treated ewes and 14 and 15 lambs from saline and fluoxetine groups, respectively, were included in the final analysis.

Fluoxetine treatment decreased serum serotonin concentrations in ewes and lambs (Fig. 1). Serotonin concentrations were greater in lambs than in their respective dam on postnatal day 0 ($P < 0.0007$) for both control and fluoxetine groups. However, fluoxetine did not affect the neonatal/maternal ratio of serotonin. Additionally, the decrease in serum serotonin concentrations between control and fluoxetine groups was similar for ewes and lambs (82.9 and 81.6% reduction relative to control, respectively).

In the cotyledonary placenta of sheep, maternal-fetal exchange occurs exclusively at the placentome, the functional unit of placenta in ruminants (24). Placentome diameter was similar ($P > 0.1$) between groups before onset of treatment (not shown). After the onset of fluoxetine treatment placentome growth significantly decreased in fluoxetine-treated ewes while it increased in control animals (Fig. 2). Mean placentome diameter was 9.5% smaller ($P = 0.07$) in ewes treated with fluoxetine than controls, although there was no difference in placentome

diameter (exp. day 21) by lamb birthweight ratio indicating that reduced placentome growth was accompanied by reduced fetal growth.

Fluoxetine-treated ewes lost weight ($P = 0.0026$) compared to controls during the first week of treatment but had similar weight gain as controls from Day 7 until parturition (Fig. 2). Similarly, maternal feed intake was decreased ($P = 0.0005$) in the fluoxetine group only during the first week of treatment (4.1 ± 0.04 and 3.6 ± 0.1 kg/day averaged from exp. day 0 to 7 for control and fluoxetine groups, respectively; not shown). In the control group, placentome diameter by maternal weight ratio increased during the 21-day treatment period; conversely, it decreased in fluoxetine-treated ewes indicating reduced placentome growth was occurring even when ewes were gaining weight.

Fluoxetine treatment decreased mean gestation length (Fig. 3). Lamb weight was reduced in lambs born to fluoxetine-treated ewes at birth and on postnatal days 1 to 3 (Fig. 4). Lamb weight gain until day 35 was not different between groups.

Fluoxetine treatment did not significantly affect maternal pH and blood gas status on postnatal days 0 (shortly after parturition) and 6 (Table 1). Maternal lactate was increased in the fluoxetine group only immediately after parturition. However, lambs born to fluoxetine-treated ewes had overall decreased blood pH during the evaluated period (Fig. 5). Additionally, total carbon dioxide, bicarbonate and base excess were decreased in lambs from fluoxetine-treated ewes. For serum lactate, there were overall significant effects of treatment and day and an interaction indicating increased lactate in fluoxetine lambs on postnatal days 3 to 6.

Maternal concentrations of ionized calcium were not different between control and fluoxetine groups (Fig. 6). Similarly, total calcium concentration was not different between groups in the colostrum ($P > 0.1$; 3.7 ± 0.4 vs 2.5 ± 0.2 ng/dL) or milk on day 6 postpartum ($p >$

0.1; 1.8 ±. 0.2 vs 1.5 ± 0.1 ng/dL). Nevertheless, ionized calcium in the newborn lamb was decreased (P = 0.06) at birth and overall (P = 0.08) during postnatal days 0 to 6 (Fig 6). An overall analysis from postnatal days 0 to 4 found ionized calcium concentrations were decreased (P = 0.01) in lambs born to fluoxetine-treated ewes than controls.

Discussion

Understanding the effects of maternal medication on pregnancy complications and neonatal outcomes is vital to comprehensively assess the risk of perinatal exposure to psychotropic medication on maternal and newborn wellbeing during the peripartum period. Findings from the present study are especially relevant because they clearly establish a role for fluoxetine treatment during gestation on the occurrence of perinatal complications and extend previously known effects of in utero exposure to fluoxetine into the postpartum period. More specifically, findings from the present study support the hypotheses that fluoxetine treatment during late pregnancy (1) decreases gestation length, (2) causes IUGR (decreased placentome growth and decreased birthweight) and (3) affects neonatal health (neonatal acidemia, hyperlactemia, and hypocalcemia). Furthermore, the decreased capacity of the neonate to establish adequate acid-base balance in the present study extends previously reported short-term intrauterine fluoxetine-induced fetal acidemia (26) to neonatal acidemia during the first week of life in neonates exposed to fluoxetine in utero.

Increased plasma and/or placenta serotonin content are associated with placenta pathology and IUGR in humans and animal models: idiopathic IUGR in humans (10, 11, 27); serotonin or serotonin precursor treatment in rodents (28, 29); SSRI treatment in humans (4, 6, 15), mice (30-32), and sheep (present study); SERT null mouse model (33, 34). Increased

serotonin signaling caused decreased blood perfusion to the placenta (13, 35) resulting in abnormal placenta function and growth (19). Similarly, women undergoing SSRI treatment during gestation (36) and pregnant rats treated with 5HTP (serotonin precursor) (28) exhibit decreased placental weights. In the present study, the functional area of the placenta was reduced due to fluoxetine treatment – a strong indication of IUGR (37, 38). The reduced uterine blood flow (26) and decreased placenta growth/size (28, 36) in addition to altered placenta morphology (15) caused by fluoxetine treatment might be the cause of placental insufficiency and IUGR resulting in decreased newborn weight. Placental insufficiency is an important cause of preterm birth (39). Accordingly, fluoxetine-induced placental insult leading to placental insufficiency is likely associated with the increased incidence of preterm birth in women and the shorter gestation length in sheep exposed to fluoxetine.

Fluoxetine-induced decrease in uterine artery blood flow has been associated with decreased partial pressure of oxygen and oxygen saturation of hemoglobin, as well as fetal acidemia accompanied by increased partial pressure of carbon dioxide (26). Although lambs are normally born in an acidotic state (40, 41), intrauterine exposure to fluoxetine caused further reductions in neonatal blood pH and this was maintained during the first week of life. Hypoxia-induced neonatal acidosis after parturition is a clinical indicator of placental insufficiency (42). Fluoxetine-induced fetal acidemia has similarities to respiratory acidosis since there is increased partial pressure of carbon dioxide perhaps due to reduced diffusion of carbon dioxide from fetal to maternal circulation related to reduced placental blood flow. Conversely, the postnatal acidemia is quite different since there is no change on partial pressure of carbon dioxide. In addition, the decreased bicarbonate and base excess are consistent with metabolic acidosis suggesting an effect of increased plasma serotonin on kidney function as it has been shown (28).

Similarly, the increased lactate on postnatal days 3 to 6 may be related to altered renal function (28) or hypoxia due to respiratory distress commonly related to in utero exposure to SSRI (43).

The similar reduction in serum serotonin concentrations in ewes and lambs highlights the capacity of fluoxetine to inhibit fetal/neonatal SERT, thereby, increasing plasma serotonin and possibly giving rise to serotonin toxicity/syndrome in newborns exposed to serotonergic drugs as it has been reported (44-48). Other SSRI with reduced placental transfer may cause more mild symptoms (49). Additionally, although persistent pulmonary hypertension of the newborn is the most recognizable complication from in utero SSRI exposure (5, 18), we described less commonly reported SSRI-induced homeostatic imbalance – neonatal acidemia, hyperlactemia, and hypocalcemia. Neonatal providers should be aware of possible outcomes to discern between serotonin syndrome, drug withdraw, and other SSRI-related perinatal morbidity (45, 50).

Most studies in women have failed to determine whether adverse pregnancy outcomes are related to the use of SSRI or with depression itself (5). However, the observed outcomes in this ovine model clearly establishes a role for fluoxetine in perinatal complications independent of the effects of depression and highlights possible perinatal complications that arise from maternal use of fluoxetine. In a recent report, only moderate and high doses of SSRI taken during entire gestation were related to preterm birth while low doses or dose reduction/discontinuation during the first trimester did not (43). Along with the present results, it seems likely that SSRI exposure primarily during late pregnancy is the main driver of preterm birth. Of special relevance, less than 30 days of treatment during late gestation reduced pregnancy length and increased neonatal morbidity.

Although it was not investigated in the present study, SSRI use has been associated with other pregnancy complications such as increased risk of gestational hypertension, preeclampsia,

and postpartum hemorrhage. Interestingly, SSRI-induced increased serotonin may be involved in the occurrence of these conditions. As a vasoactive hormone, increased plasma serotonin affect blood pressure and may lead to hypertension as reported in patients taking SSRI (51). Additionally, SSRI increases the risk for preeclampsia likely due to altering uteroplacental blood perfusion (52). Noteworthy, gestational hypertension, preeclampsia, and SSRI use during gestation increase the risk of preterm birth (2, 5, 51, 52). On a similar note, COVID-19 infection has also been associated with preeclampsia, preterm birth, and low birthweight in infants. It has been suggested that the effects of COVID-19 infection are likely associated with uteroplacental vasoconstriction and endothelial disfunction due to SARS-CoV-2 modulation of renin-angiotensin-aldosterone system by binding to angiotensin-converting enzyme 2 (53, 54). The pathophysiology of uteroplacental blood perfusion and its impact on preterm birth under different scenarios such as SSRI use, gestational hypertension, preeclampsia, and COVID-19 needs to be addressed to improve maternal and fetal health. Lastly, the decreased platelet serotonin content caused by SSRI use may be related to the increased risk for postpartum hemorrhage because platelet serotonin plays an important role in platelet aggregation and vasoconstriction (55, 56). Accordingly, SSRI use has been associated with abnormal bleeding (55).

The main strengths of this study are related to the animal model. Although rodent models are widely used in biomedical research, similarities between humans and sheep (number of fetuses per gestation, fetal intrauterine development, and stage of fetal organ maturation at birth) as opposed to humans and mice (57) make sheep a superb animal model for studies of pregnancy that may allow more direct translation to human medicine (14, 58, 59). Because this sheep model recapitulated several findings associated with fluoxetine exposure during pregnancy in women

(3, 4, 6, 43), it should be considered for further investigation of the mechanistic effects of fluoxetine on pregnancy outcomes and neonatal heath. The ovine model has been widely used to study IUGR (14, 58, 59) but our study emphasizes the power of this model in understanding pharmaceutical effects on placental development, pregnancy, and neonatal outcomes. Specifically, our experiment further supports the use of the ovine model in translational pregnancy studies for investigating mechanistic actions of fluoxetine on the regulation of placental function and fetal development and to explore preclinical implementation of preventive therapies to overcome the adverse effects. Furthermore, the rapid onset (within seven days) of reduction of placental size in this model can be useful to identify SSRI-dependent and independent early placental changes, physiologic mechanisms, and possibly placental markers of placental insufficiency that culminate with decreased placental growth, fetal growth restriction, and preterm birth for future clinical triage of pregnancies at greater risk.

A limitation of our study is the difference in normal blood values (pH, lactate, blood gases, calcium) between human babies and lambs which challenges a direct comparison between species. Additionally, we did not investigate the underlying cause of fluoxetine-induced fetal/neonatal acidemia and hypocalcemia. Lastly, while the intravenous administration of the drug allowed consistent delivery of the desired amount of drug it does not represent the pharmacokinetics of absorption of fluoxetine in humans taking the drug orally.

In conclusion, maternal fluoxetine treatment during late gestation reduced placental growth, caused IUGR, and decreased gestation length similar to the effects of fluoxetine treatment in pregnant, depressed women. Additionally, lambs exposed to fluoxetine in utero exhibited metabolic acidemia and hypocalcemia during the first week of life, as observed in preterm and IUGR babies. However, more than recapitulating findings in women, we established

a role for fluoxetine in perinatal complications and neonatal morbidity in our non-depressed sheep model shedding light on the interpretation of the effects of SSRI on pregnancy outcomes in women previously obscured by the effects of depression itself.

Contribution to the filed statement

Selective serotonin reuptake inhibitors (SSRI) are the primary class of antidepressants prescribed to pregnant women and about 8-13% of pregnant women are exposed to antidepressants during gestation. Yet, the use of SSRI during gestation has been associated with multiple pregnancy complications such as: pregnancy loss, developmental malformations, intrauterine growth restriction (IUGR), preterm birth, and neonatal morbidity and mortality. Interpretation of studies in humans, however, are typically complicated by the adverse pregnancy outcomes caused by depression itself. In the present study we used a non-depressed sheep model to shed light on the interpretation of the effects of SSRI on pregnancy outcomes. Fluoxetine treatment during late gestation reduced placental growth, caused IUGR, and decreased gestation length. Additionally, lambs exposed to fluoxetine in utero exhibited metabolic acidemia and hypocalcemia during the first week of life extending previously reported fluoxetine-induced intrauterine fetal acidemia in sheep and as observed in preterm and IUGR babies from mothers unexposed to fluoxetine. Collectively, results from our study suggest that, regardless of underlying maternal depression, SSRI treatment primarily during late gestation affects pregnancy outcomes (reduced placental growth, IUGR, preterm birth) and fetal adaptations to extrauterine environment increasing neonatal morbidity .

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RRD, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization, Project administration; ADB, Validation, Investigation, Data curation, Writing – Review & Editing, Project administration; MKC, Validation, Investigation, Data curation, Writing – Review & Editing; MCW, Conceptualization, Methodology, Resources, Writing – Review & Editing, Visualization, Supervision, Funding acquisition; LLH, Conceptualization, Methodology, Resources, Writing – Review & Editing, Visualization, Supervision, Funding acquisition.

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Tables

Table 1. Maternal acid-base status.

Outcomes	Saline (n = 10)	Fluoxetine (n = 9)	P value
Postnatal day 0			
pH	7.508 ± 0.01	7.439 ± 0.04	0.14
Partial pressure of carbon dioxide, mmHg	33.1 ± 1.1	35.2 ± 3.7	0.6
Bicarbonate, mmol/L	23.5 ± 2.5	23.4 ± 1.6	0.9
Total carbon dioxide, mmol/L	27.2 ± 0.8	25.0 ± 1.3	0.2
Base excess, mmol/L	3.3 ± 0.9	0.0 ± 1.7	0.1
Lactate, mmol/L	3.2 ± 0.7	7.0 ± 1.6	0.02
Postnatal day 6			
pH	7.485 ± 0.01	7.478 ± 0.02	0.8
Partial pressure of carbon dioxide, mmHg	39.5 ± 1.2	39.3 ± 0.9	0.9
Bicarbonate, mmol/L	29.7 ± 0.7	29.0 ± 0.9	0.6
Total carbon dioxide, mmol/L	30.8 ± 0.8	30.1 ± 0.9	0.6
Base excess, mmol/L	6.2 ± 0.8	5.6 ± 1.2	0.9
Lactate, mmol/L	1.7 ± 0.4	2.8 ± 0.8	0.2

Figures

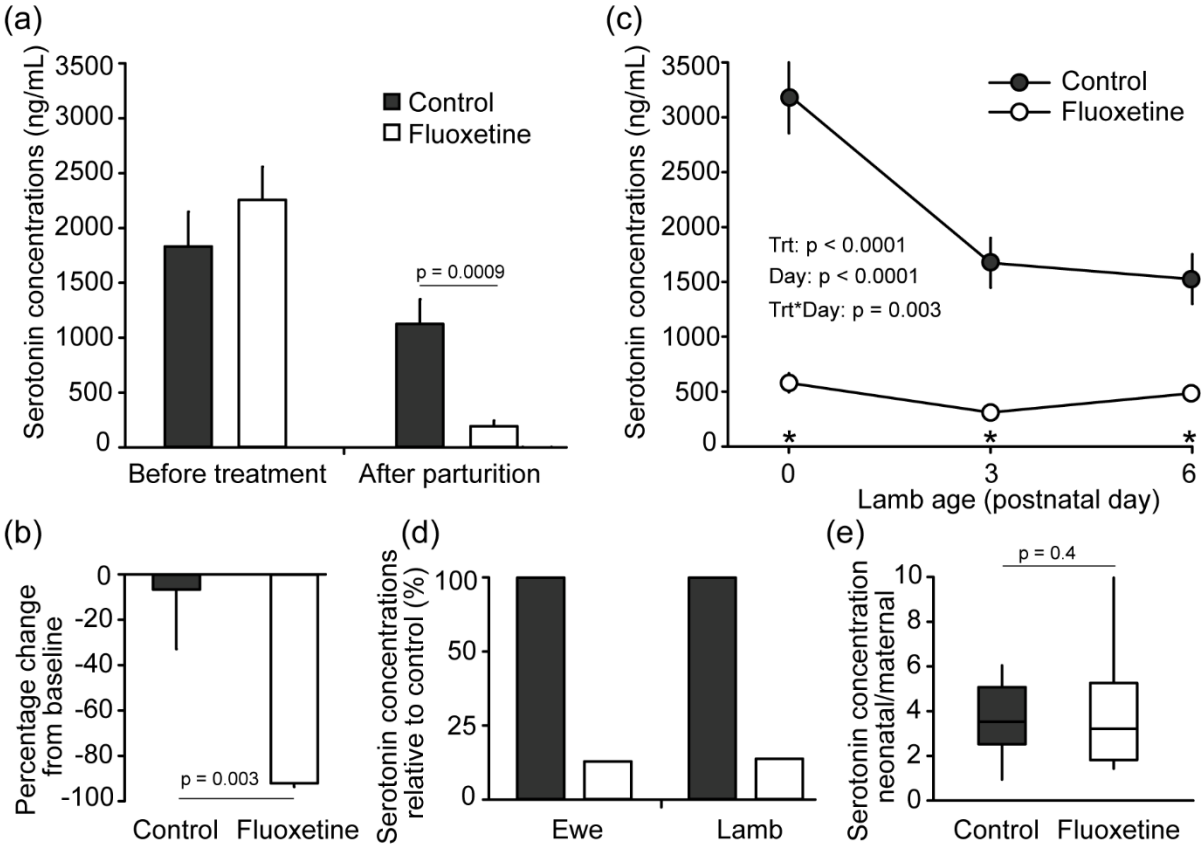

Figure 1. Serotonin concentrations. (a) Serum serotonin concentrations in ewes before treatment (baseline) and after parturition. Days from beginning of treatment until parturition varied between 26 to 34 days for the control group and 24 to 31 days for the fluoxetine group. (d) Pecentage change in serum serotonin conentrations in ewes between baseline and after parturition. (c) Serum serotonin concentrations in lambs. Blood sample on postnatal day 0 was collected before colostrum ingestion. (d) Serum serotonin concentrations in ewes and lambs relative to control group on postnatal day 0. (e) Neonatal/maternal ratio of serum serotonin concentrations on postnatal day 0; serotonin concentratations in each lamb was divided by serotonin concentrations in their respective dam.

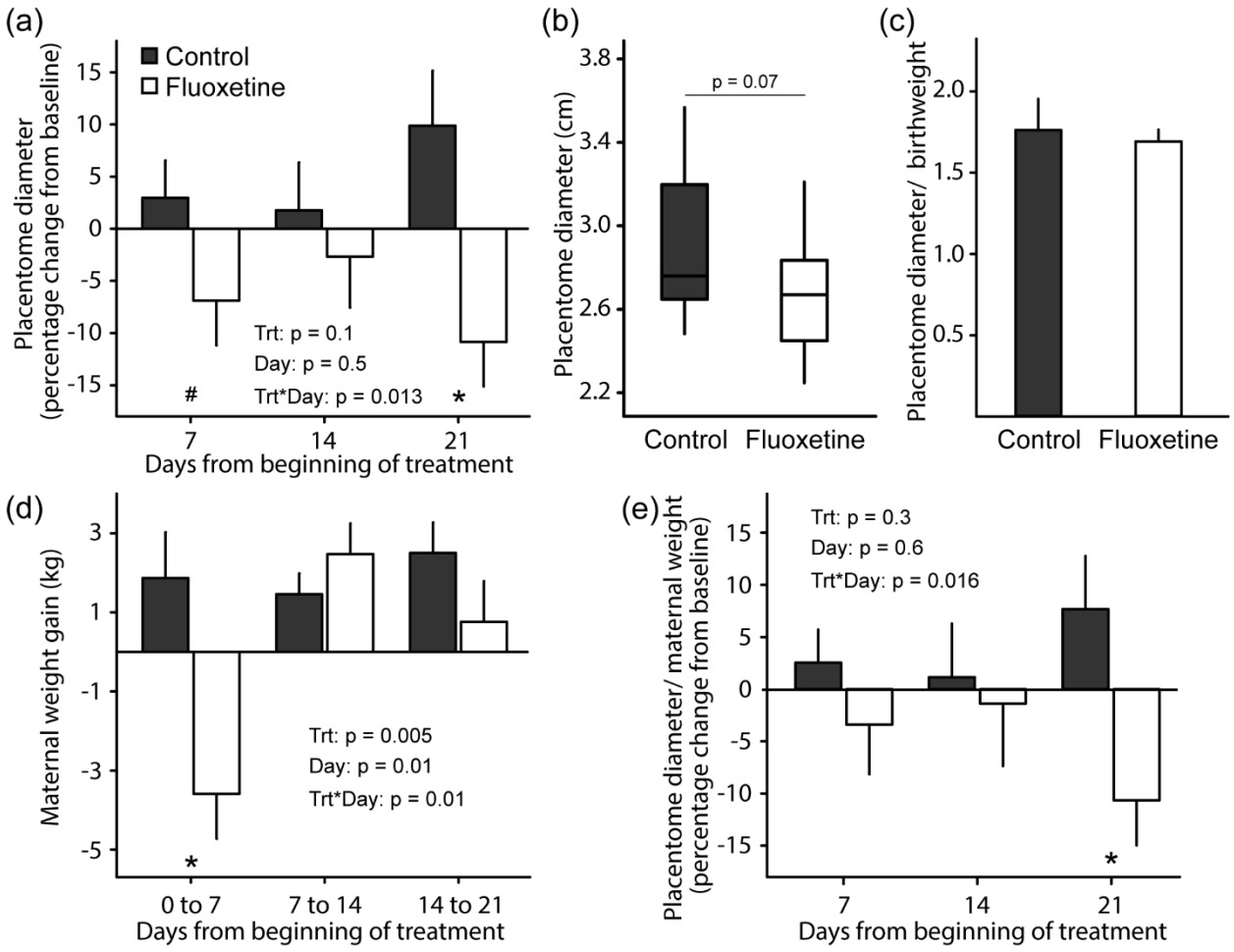

Figure 2. Placentome diameter. (a) Placentome diameter throughout experimental period relative to pretreatment. (b) Boxplot of placentome diameter on experimental day 21 (gestational day 140 ± 1). Boxplot depicts median, 10th, 25th, 75th, and 90th percentile. (c) Placentome diameter by lamb birthweight ratio. (d) Maternal weight change from baseline. (e) Placentome diameter by maternal weight ratio change from baseline. Baseline maternal weight and placentome diameter were assessed on experimental day –1 (gestational day 118 ± 1). * indicates significant difference between groups and # indicates a tendency for significance.

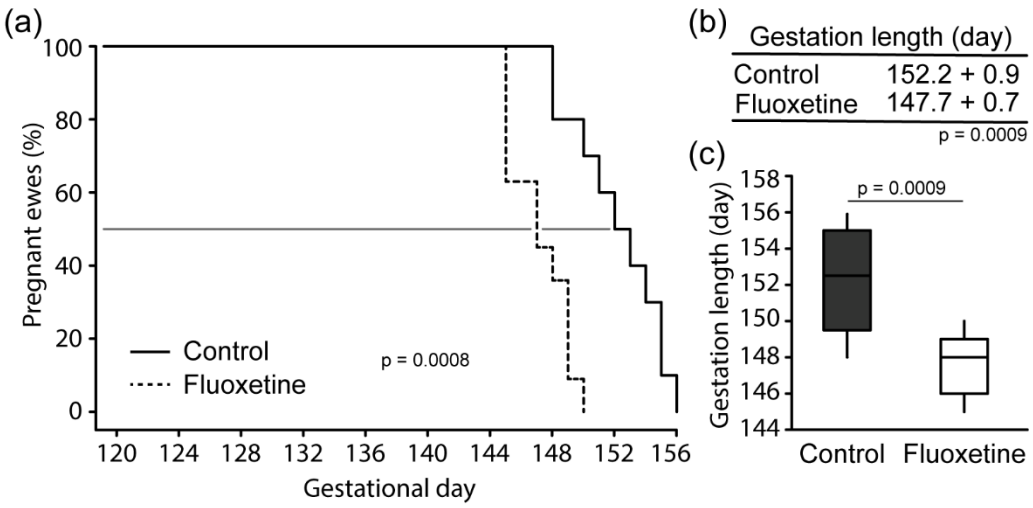

Figure 3. Pregnancy length. (a) Survival analysis depicting number of animals giving birth each day. The 50% point is shown. (b) Mean gestation length. (c) Boxplot of gestation length. Boxplot depicts median, 10th, 25th, 75th, and 90th percentile.

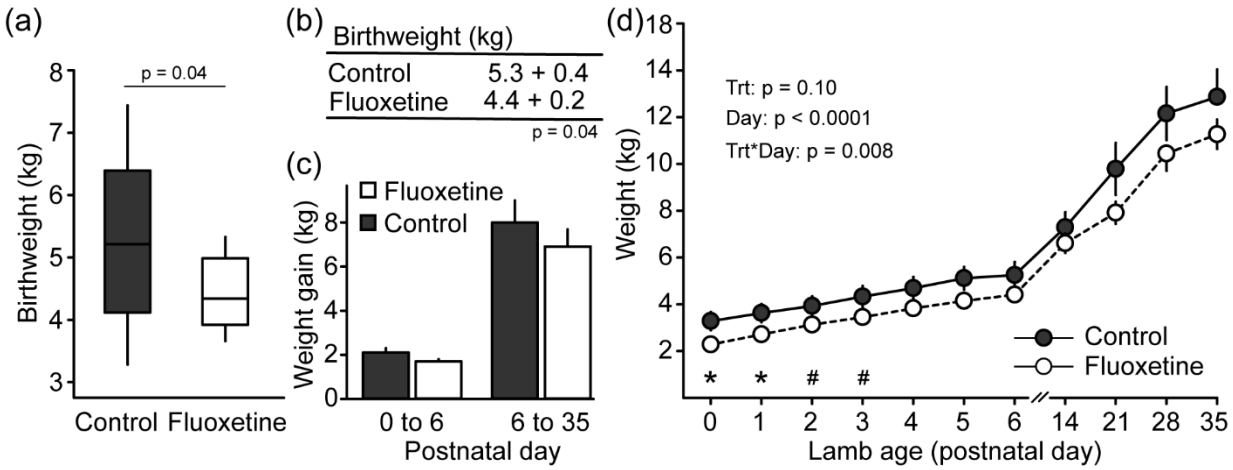

Figure 4. Lamb weight. (a) Boxplot of neonatal birthweight. Boxplot depicts median, 10th, 25th, 75th, and 90th percentile. (b) Mean lamb birthweight. (c) Lamb weight during the evaluated period. * indicates significant difference between groups and # indicates a tendency for significance.

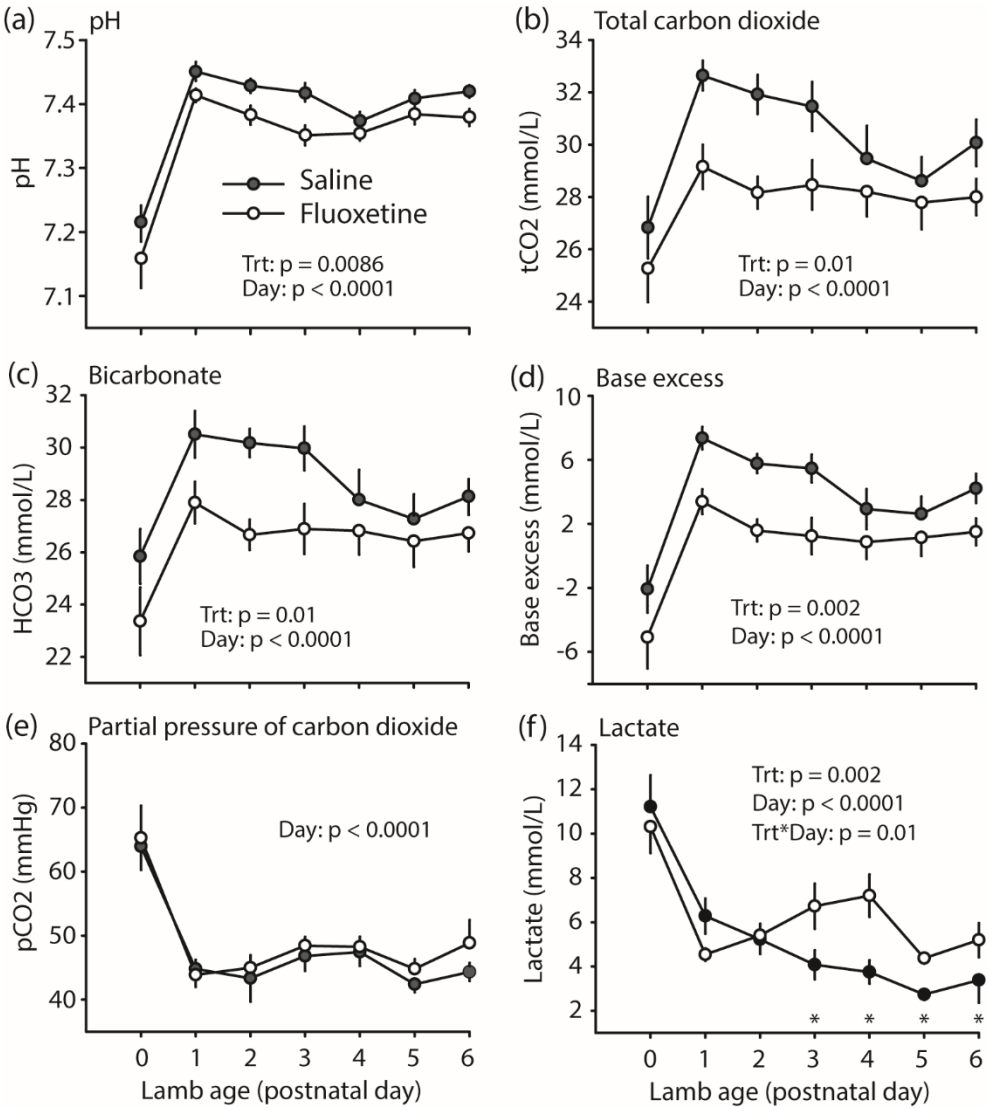

Figure 5. Lamb acid-base status. (a) pH. (b) Total carbon dioxide. (c) Bicarbonate. (d) Base excess. (e) Partial pressure of carbon dioxide. (f) Lactate. * indicates significant difference between groups.

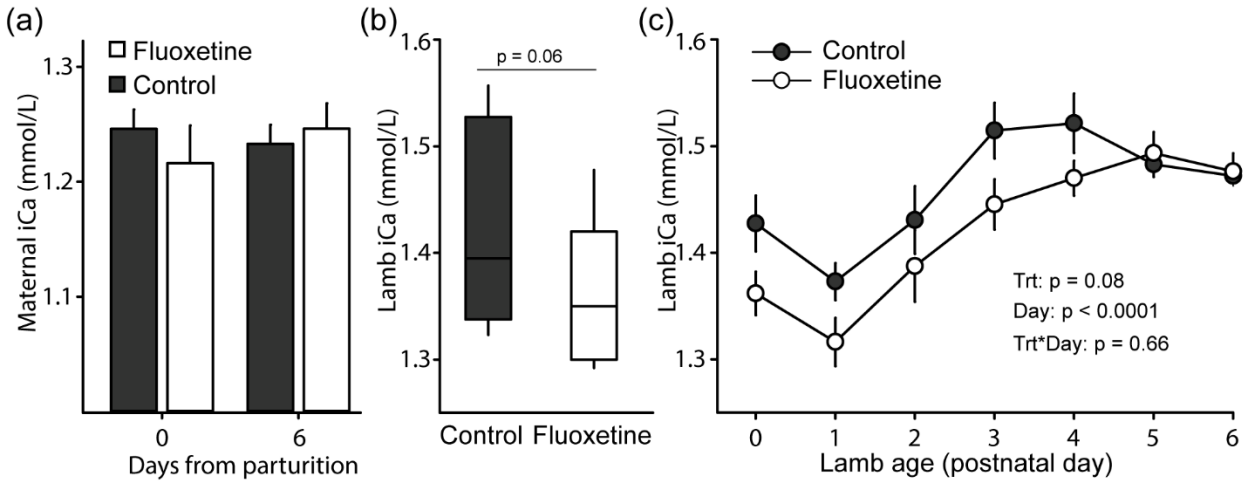

Figure 6. Ionized calcium (iCa) concentrations. (a) Maternal levels of iCa at parturition and 6 days postpartum. (b) Boxplot of neonatal concentrations of iCa after lambing. Boxplot depicts median, 10th, 25th, 75th, and 90th percentile. (c) Concentrations of iCa in lambs during days 0 to 6 of age.

Chapter 5

Adverse pregnancy outcomes caused by selective serotonin reuptake inhibitors are due to increased serotonin signaling and can be mitigated by a serotonin receptor antagonist

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Abstract

Selective serotonin reuptake inhibitors (SSRI) are the most prescribed antidepressant; however, its use during gestation is associated with pregnancy and neonatal complications. We sought to investigate the pathophysiology of SSRI-induced adverse pregnancy and neonatal outcomes and implement a therapeutic approach to mitigate them. Utilizing a mouse model genetically deficient for the serotonin transporter (Sert), the biological target for SSRI, and SSRI treatments, we demonstrated that (1) SSRI affect pregnancy and neonatal outcomes via SERT as opposed to off-target pharmacological effects and (2) inhibition/lack of maternal but not placental/embryonic SERT leads to placental transcriptomic and histopathological alterations, and consequently placental dysfunction ultimately associated with the adverse pregnancy outcomes. Lastly, a pharmacological therapy targeting the serotonin antagonist for receptors 2A and 2C mitigates SSRI induced placental changes and prevents embryonic mortality in both SSRI-treated and Sert-null dams. This is the first demonstration of a preventative treatment for SSRI-related pregnancy complications with potential benefits for women and infant wellbeing and health.

Introduction

The use of antidepressants has increased over the last several decades with greater rise in women, particularly during reproductive ages [1]. Selective serotonin reuptake inhibitors (SSRI) are the most prescribed antidepressant class. Despite the widespread use of SSRI by women during reproductive period and pregnancy, numerous studies warn of possible detrimental effects of maternal use of SSRI during pregnancy with possible detriments on maternal and fetal/neonatal health and wellbeing [2-8]. Treatment with SSRI during gestation is associated with increased risk for preterm birth, reduced birthweight/small for gestational age, persistent pulmonary hypertension of the newborn, developmental delays, among others.

Although numerous studies in humans and in animal models have outlined the outcomes related to maternal SSRI exposure during pregnancy [9-13], little is known about the biological mechanisms resulting in adverse pregnancy and neonatal outcomes. All SSRI inhibit serotonin transporter (SERT) [14, 15]; however, it is not known whether the adverse effects of SSRI on pregnancy outcomes are mediated through inhibition of SERT or through potential off-target effects independent of SERT. We recently reported several pregnancy complications in SERT-null mice (Sert-/-) that are similar to effects of pharmacological inhibition of SERT by SSRI suggesting that modulation of SERT function might be the underlying cause for adverse pregnancy outcomes [16]. Nevertheless, the role for modulation of maternal vs placental vs embryonic SERT function/expression on pregnancy complications has not yet been assessed.

Because inhibition/lack of expression of SERT leads to increased free circulating serotonin and serotonin signaling [14], we recently proposed a mechanism in which increased serotonin due to SSRI treatment promotes vasoconstriction of uterine/placental vascular beds reducing blood perfusion to these organs [2]. The inadequate blood supply leads to placental

alterations and decreased fetal development that, ultimately, are associated with the adverse pregnancy outcomes. In the present study we performed a series of experiments testing these hypotheses. Utilizing pharmacological and genetic approaches, we aimed to (1) investigate the pathophysiology of SSRI-induced adverse pregnancy outcomes and (2) implement a therapeutic approach to mitigate them.

Results

SSRI act through SERT to negatively impact pregnancy and neonatal outcomes

To access whether SSRI affect pregnancy outcomes through an off-SERT pathway and to evaluate the impact of altered SERT function on pregnancy outcomes, we treated wild-type (WT, Sert+/+) and Sert-/- pregnant mice (bred to males of their respective genotype) with saline or fluoxetine at low dose (2 mg/kg/d) and high dose (20 mg/kg/d) beginning on E10.5. Dams were euthanized on E13.75 or E18.75. Number of implantation sites, live embryos, and embryonic resorptions were not different within each group for E13.75 and E18.75 ($P > 0.1$); therefore, data were combined. The number of implantation sites was similar ($P > 0.1$) among WT and Sert-/- dams treated with saline, low and high doses of fluoxetine (Fig. 1.A). The percentage of live embryos decreased, and embryonic resorption increased in WT dams treated with fluoxetine (low and high dose) and all Sert-/- groups (saline, low and high dose fluoxetine) compared to WT saline-treated group (Fig. 1.B-C). There was no difference in either percentage of live embryos or embryonic resorption among WT treated with fluoxetine (low and high dose) and Sert-/- dams (saline, low and high fluoxetine dose). The lack of effect of fluoxetine treatment on pregnancy outcomes in Sert-/- indicated that SSRI-induced adverse pregnancy outcomes are mediated through SERT but not by an off-target mechanism. The similar pregnancy outcomes

due to pharmacological inhibition of SERT (fluoxetine treatment) or genetic ablation of SERT (Sert-/-) suggests a critical role for functional SERT on pregnancy outcomes and highlights the use of Sert-/- mice to investigate the impact of altered SERT function on pregnancy outcomes to unravel the pathophysiology of SSRI-induced pregnancy complications.

Maternal SERT modulates pregnancy outcomes

To investigate whether altered maternal, placental, or embryonic/neonatal SERT function was the main cause of adverse pregnancy outcomes, we utilized a genetic approach to breeding WT, Sert+/-, and Sert-/- mice. There was no effect ($P > 0.1$) of paternal or embryonic/neonatal genotype on any of evaluated pregnancy outcome (pregnancy loss, litter size, birthweight, neonatal mortality). We only observed effects of maternal genotype on pregnancy outcomes. Pregnancy outcomes for WT and Sert+/- dams were similar due to normal SERT function on Sert+/- mice. Therefore, data were combined for WT and Sert+/- dams and compared to Sert-/- dams, regardless of paternal or embryonic/neonate genotype (Fig. 2.A-F). The Sert-/- dams had increased pregnancy loss after E10.5 (17.6% vs 1.1%), increased pup mortality within 24 hours of birth (49.1 ± 6.8% vs 13.5 ± 2.5%) and overall during lactation period (52.3 ± 6.4% vs 22 ± 3.3%), decreased neonatal birthweight (1.294 ± 0.01 g vs1.183 ± 0.044 g), and decreased number of pups weaned per litter (5.9 ± 0.3 vs 3.6 ± 0.5). Death of entire litter within 24 of birth was greater in Sert-/- than WT + Sert+/- (36.6% vs 3.4%, respectively, $P < 0.0001$). Taken together, maternal genotype, rather than placental or embryonic/neonatal, is associated with adverse pregnancy and neonatal outcomes.

Embryonic genotype is not associated with embryonic or pup mortality

To specifically assess the impact of embryonic/neonatal genotype on embryonic and neonatal survival/mortality, Sert+/- dams were mated to Sert-/- males to produce either Sert+/- or Sert-/- offspring. Dams and offspring were euthanized on E18.75 or at the end of lactation (d21) and all live embryos and pups were individually harvested and genotyped. Based on Mendelian genetics, 50% of embryos were expected to be Sert-/- and 50% Sert+/- [17]. Overall, there were similar rates of Sert+/- and Sert-/- embryos per litter and for all live embryos (45.2% vs 54.8%) (Fig. 2.G). Similarly, frequency of pup genotype was similar for Sert+/- and Sert-/- pups per litter and for all surviving pups (50.4% vs 49.6%) (Fig. 2.H). Taken together, embryonic survival during gestation and neonatal survival during the lactational period were not associated with offspring genotype. Instead, the increased embryonic resorption and neonatal mortality observed in Sert-/- dams is due to maternal genotype confirming our previous experiments.

Adverse pregnancy outcomes in SSRI-treated and Sert-/- dams are associated with alterations in placental histopathology

We performed histopathological evaluation of the placenta. Fluoxetine treatment was associated with increased placental pathologies such as fibrosis, necrosis, infarction, mineralization, and inflammatory lesions in all placental regions (labyrinth, junctional zone, and decidua). In E18.75 placenta, fluoxetine treatment increased TUNEL-positive staining in the junctional zone and labyrinth while caspase 3- positive staining was increased only in the labyrinth suggesting different molecular pathways are associated with cell death in different regions of the mouse placenta. Additionally, Ki67-positive staining was increased in the junctional zone and labyrinth while PCNA- positive staining was increased only in the junctional

zone. Taken together, these data suggest that maternal use of SSRI results in increased placental lesions that compromise placental function.

Maternal but not fetal/placental genotype impacts the placental transcriptome

We performed RNA sequencing of E18.75 placenta from Sert+/- and SERT-/- pups generated from a Sert+/- female x Sert-/- male and from Sert-/- female x Sert+/- male breeding scheme in a 2x2 factorial design to evaluate the effect of maternal vs embryonic genotype on the placental transcriptome. There was no effect of embryonic genotype on the placental transcriptome with a false discovery rate of 5% or 10%. For maternal genotype, there were 1351 differently expressed genes (DEG) (FDR 5%). Of these genes, 965 genes were upregulated and 386 were downregulated in placenta from Sert-/- dams (Figure 4.A). Using the Ingenuity Pathway Analysis (Qiagen), we uncovered 219 and 129 altered canonical pathways with P-values of 0.05 and 0.01, respectively. The top 10% altered pathways are shown (Fig. 4. B). Using the disease and function annotation, we uncovered significant predicted upregulation of placental molecular transport, lipid metabolism, inflammatory response, immune cell trafficking, cell function and maintenance, and cell death and survival (Fig. 4.C). Other altered pathways are shown in Supplemental Figures.

Maternal circulating concentrations of serotonin are altered by SSRI treatment and genotype

Maternal serum concentrations of serotonin are affected by fluoxetine treatment and genotype (Supplemental Table 1). Fluoxetine caused dose-dependent decrease in maternal serum serotonin in WT mice. Low and high doses of fluoxetine reduce serum serotonin by 17% and

97% respectively. In Sert+/- mice, fluoxetine decreased maternal serum serotonin by about 50%. In Sert-/- mice, fluoxetine does not affect maternal serum serotonin. The serum serotonin concentrations are similar between WT and Sert+/- dams but dramatically reduced in Sert-/- (~99%).

Pup serum serotonin concentrations are affected by in utero exposure to fluoxetine and by pup genotype. In WT embryos on E18.75, fluoxetine decreased serum serotonin in a dose-dependent manner. Low and high doses of fluoxetine reduce serum serotonin by ~33% and ~88% respectively. This confirms the placental transfer of the drug and its capacity to inhibit neonatal SERT. Serum serotonin in Sert+/- was reduced by ~30% compared to untreated WT embryos, similarly to WT embryos exposed to low dose of fluoxetine in utero. In Sert-/- embryos, serum serotonin is drastically reduced (~98%). Maternal genotype (Sert+/- or Sert-/-) does not affect embryonic serum serotonin confirming previous studies suggesting that during late pregnancy circulating serotonin concentrations are regulated by the embryo.

As the majority of serotonin in the bloodstream is transported into the platelets, the type of blood sample (serum vs plasma vs platelet-poor plasma) affects measurable concentrations of the hormone [2, 14, 18]. Treatment with SSRI increases free (platelet-poor) concentrations of serotonin while decreasing serum concentrations of the hormone. Unfortunately, we were unbale to determine the free, platelet-poor concentrations of serotonin in mice given the low amount of blood collected and the volume required for assay. Nevertheless, we were able to demonstrate that SSRI inhibits SERT with decreased serum serotonin indirectly indicating increased free concentrations of serotonin as demonstrated in humans. Similarly, in Sert-/- mice, decreased serum serotonin indirectly confirms increased free serotonin as previously demonstrated. Because free circulating serotonin is readily available for binding its receptors, serotonin

signaling is increased in WT mice treated with SSRI [14, 18]. In Sert-/- mice increased circulating serotonin and serotonin signaling is independent of SSRI treatment.

Increased maternal serotonin in SSRI-treated and Sert-/- mice causes adverse pregnancy and neonatal outcomes which can be prevented by a serotonin receptor antagonist

To confirm that the adverse pregnancy and neonatal outcomes are due to increased serotonin signaling, and to explore a possible therapy to mitigate them, we treated WT mice with a serotonin antagonist for receptors 2A and 2C. Importantly, we utilized the low dose fluoxetine (2 mg/kg/d) as it results in systemic concentration similar to humans taking fluoxetine and might be more translationally relevant [19]. The serotonin antagonist for receptors 2A and 2C, ketanserin, was administered one hour prior to SSRI treatment from E10.5 to E18.5 (Fig. 5). To demonstrate that fluoxetine's actions are due to increased serotonin and subsequent signaling with the serotonin 2A and 2C receptor subtypes, a serotonin-treated group served as a positive control and a serotonin+ketanserin-treated group served to demonstrate that ketanserin mitigates the effects of increased serotonin and its interaction with the serotonin 2A and 2C receptor subtypes. Ketanserin mitigated the effects of serotonin on decreased maternal weight gain during gestation, decreased embryonic weight (E18.75) and birthweight, decreased number of pups born, and increased neonatal mortality (Supplemental Fig. 1). Fluoxetine decreased maternal weight gain from E10.5 to E18.5 (10.7 ± 0.9) compared to the saline control group (10.7 ± 0.5) which was prevented by treatment with ketanserin (12.3 ± 0.6) (Fi. 5.A). Fluoxetine treatment reduced the number of pups born (6.6 ± 0.5) compared to the saline control group (7.9 ± 0.3) and was prevented by ketanserin treatment (7.4 ± 0.4) (Fig. 5.B). Pup mortality within 24 hours of birth and overall pup mortality during lactation was increased in the fluoxetine + ketanserin (24.1

± 6.5% and 26.7 ± 6.6%) group compared to the saline control group (5.3 ± 2.2% and 9.5 ± 4.1%) (Fig. 5.D). Pup mortality was not different between fluoxetine and fluoxetine + ketanserin groups. Neonatal birthweight (Fig. 5.C) and pup weight at weaning were not different among groups.

We also treated Sert-/- dams with ketanserin daily from E10.5 to E18.5. Ketanserin treatment did not affect number of pups born (7.1 ± 0.3 treated vs 6.7 ± 0.5 untreated) and neonatal birthweight (1.250 ± 0.018 treated vs 1.231 ± 0.014 untreated) (Fig. 5.F-G). However, ketanserin decreased neonatal mortality within 24 hours of birth (23.8 ± 6.7 vs 49.0 ± 9.9) and overall during lactation period (31.7 vs 44.4% of all pups born) (Fig. 5.H-E).

In WT dams treated with fluoxetine, ketanserin mitigated fluoxetine-induced increase in TUNEL, caspase 3, Ki67, and PCNA staining suggesting amelioration of fluoxetine-induced placental damage consistent with prevention of reduced placental blood perfusion and restoration of number of pups born. Taken together, amelioration of fluoxetine-induced placental alteration and adverse pregnancy outcomes confirms that fluoxetine effects are mediated by increased serotonin signaling through receptors 2A and/or 2C.

Discussion

Understanding the pathophysiology of SSRI-induced adverse pregnancy outcomes is critical to improve maternal and fetal/neonatal wellbeing when SSRI treatment is needed by the mother during gestation. In the present study we delineated the mechanisms that result in adverse pregnancy outcomes associated with abnormal SERT function either due to SSRI treatment or genetic manipulation of SERT expression. Interestingly, both methods resulted in similar placental pathologic alterations and pregnancy outcomes highlighting the ability of the Sert-/-

mouse model to recapitulate findings in dams treated with SSRI to understand the impact of maternal SERT function on reproductive physiology.

Maternal, but not fetal/placental, SERT function/expression is a key determinant for pregnancy and neonatal outcomes. Notably, the effect of maternal but not fetal/placental genotype on placenta transcriptome highlights the placental changes caused by lack of maternal SERT. These findings in our untreated, genetic mouse model are important as they establish that the adverse pregnancy and neonatal outcomes are due to physiological alterations in the dam causing impaired fetal/neonatal development. In other words, increased murine neonatal mortality, for example, occurs due to maternal altered physiology associated with lack of SERT independently of fetal/neonatal genotype. Taking this finding into the context of SSRI exposure during gestation, it suggests that neonatal outcomes are associated with a direct impact of SSRI on the mother leading to adverse fetal development with consequent impairment of neonatal health. Nevertheless, our findings do not exclude a direct impact of SSRI on neonatal health. For example, serotonin syndrome in newborns from SSRI-treated mothers appears to be related to increased neuronal serotonin caused by a direct effect of SSRI in the neonatal brain [20-22].

The use of ketanserin, a serotonin antagonist for receptors 2A and 2C, confirms that the adverse pregnancy outcomes observed in SSRI-treated and Sert-/- dams are due to increased serotonin. In other studies [23, 24], as observed in our positive control group, ketanserin prevented serotonin-induced reduced litter size, fetal and neonatal mortality. Although it was not definitively tested in our studies, we believe serotonin affects pregnancy outcomes by decreasing blood perfusion to the uterus rather than acting directly on the placenta. Although serotonin receptor 2A is expressed in the mouse and human trophoblasts, serotonin increases cell viability, promotes cell cycle progression, and activates intracellular signaling pathways associated with

survival, differentiation, migration, and invasion in BeWo and JEG-3 cell lines of human trophoblast [25-27]. Conversely, serotonin activation of receptor 2A, expressed in human uterine [28] and chorionic [29, 30] arteries, promotes vasoconstriction with consequent reduction of uterine and placental blood perfusion as demonstrated in pregnant rodents and sheep [31-33]. Serotonin treatment of pregnant rats on the day of implantation or the day after implantation, prior to placental development, reduces uterine blood flow by 50-70% and intrauterine oxygen tension by 50% [32, 34, 35]. This resulted in embryonic reportion in about 85% of implantation sites demonstrating the impact of reduction of uterine blood perfusion on embryonic development. However, serotonin treatment does not affect pregnancy outcomes when treatment takes place prior to embryonic implantation indicating the serotonin itself is not fetotoxic [32]. Therefore, the effects of serotonin on placental pathology and pregnancy outcomes appear to be related to regulation of blood perfusion to the uterus and placenta rather than a direct effect of serotonin on the placenta. It has previously been demonstrated that ketanserin prevents serotonin-dependent fluoxetine-induced vasoconstriction of pulmonary vessels that lead to persistent pulmonary hypertension of the newborn in a preclinical sheep model [36]. We believe ketanserin acted in a similar manner in the present study preventing serotonin-dependent fluoxetine-induced vasoconstriction of uterine/placental vessels and consequent embryonic resorption in SSRI-treated and Sert-/- mice resulting in restoration of placenta structure/function and litter size.

The placental pathology in SSRI-treated and Sert-/- mice observed in the present study are consistent with reduced blood perfusion to the uterus/placenta caused by increased free circulating serotonin due to SSRI or genetic ablation of SERT [14, 37]. These placental changes in mice are comparable to the placental pathologies in women taking SSRI during gestation

(vascular lesions of maternal malperfusion, composite lesions of fetal malperfusion, inflammatory lesions) [38]. Likewise, similar placental alterations are found in women and animal models of hypertension when reduced uterine/placental blood perfusion also occurs [39]. Furthermore, the placental transcriptomic changes related to increased cell stress and apoptosis in SSRI-treated and Sert-/- dams corroborate the placental lesions, whereas the upregulation of pathways associated with inflammatory function are likely associated with modulation of substantial cellular reorganization. Placental hypoxia, as it occurs in women with chronic hypobaric hypoxia or experimentally induced in in vitro studies, leads to oxidative stress and endoplasmic reticulum stress in the placenta with activation of eukaryotic initiation factor 2 (EIF2) signaling and decreased mammalian target of rapamycin (mTOR) signaling [40]. These are associated with reduced placental and embryonic development. On the contrary, when fetal growth is increased, as in experimentally induced maternal obesity, placental EIF2 signaling is decreased while mTOR is increased [41]. In a recent report, expression of the placental EIF2 pathway was negatively correlated with birthweight [42]. The main altered pathway in the present study, EIF2, has been associated with placental inflammation, reduced cellular proliferation, intrauterine growth restriction, preeclampsia, and preterm birth [40, 43, 44]. In the present study, increased EIF2, along with decreased mTOR, insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) pathways and increased hypoxia-inducible factor alpha (HIF-α) signaling corroborate the reduced placental perfusion indicating placental dysfunction with compromised nutrient transport to the embryo consistent with the observed adverse pregnancy outcomes.

A groundbreaking aspect of the present study was the implementation of a therapeutic intervention using a serotonin antagonist, ketanserin, to mitigate the impacts of SSRI on

pregnancy outcomes. This was the first successful attempt to prevent SSRI-induced adverse pregnancy outcomes and is critical to allow women to benefit from the antidepressant effects of SSRI without compromising fetal health. Importantly, ketanserin does not interfere with the antidepressant effects of SSRI [45-47]. Therefore, concurrent SSRI and ketanserin treatment during gestation may benefit women suffering from a diverse set of psychological conditions that require treatment without the detrimental effects of SSRI on pregnancy and neonatal outcomes. Ketanserin is approved for medical use in pregnant women in some countries in the European Union to treat gestational hypertension suggesting that it safe for use in pregnant women [48].

In conclusion, we demonstrated that modulation of maternal SERT function via pharmacological inhibition (SSRI) or genetic ablation (Sert-/-) in mouse models promotes placental dysfunction and compromises pregnancy and neonatal outcomes by increasing serotonin signaling. Importantly, we mitigated the effects of increased serotonin signaling using a serotonin antagonist restoring placental function and pregnancy and neonatal outcomes.

Online Methods

Ethics statement

All experimental procedures and animal management were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin-Madison (protocol # A005789-R03).

Mice and Breeding

Wilt-type C57Bl/6J (strain 000664) and SERT-/- (strain 008355, C57Bl/6J background) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). A colony of WT, SERT+/-

, and SERT-/- mice were maintained in our animal vivarium. Mice were individually housed in a controlled environmental facility for biological research in the Animal and Dairy Sciences Department vivarium at the University of Wisconsin-Madison. The vivarium was maintained at a temperature of 25°C and a humidity of 50% to 60%, with a 12:12 hour light-dark cycle with *ad libitum* water and food (LabDiet 5015, TestDiet, Richmond, IN). For timed breeding, 1-2 sexually mature females (7-10 weeks-old) were placed overnight in a male cage. Mating was confirmed by detection of a vaginal plug the next morning (E0.5).

Blood collection

Euthanasia was performed on designated day by carbon dioxide followed by cervical dislocation or by isoflurane anesthesia followed by cervical dislocation when embryonic assessment was needed. Maternal blood was collected by cardiac puncture while embryonic (E13.75 and E18.75) and neonatal trunk blood was collected after decapitation. Blood was collected into uncoated tubes, allowed to clot overnight at 4°C, and centrifuged at 2000g for 20 minutes. Serum was stored at -80°C until assayed.

Placenta collection

Individual placenta was collected on designated day, allocated according to pup sex, and immediately sectioned in half. One half was frozen in liquid nitrogen for RNA analyses and the other half was placed in 4% paraformaldehyde for histopathological and immunohistochemistry evaluations.

RNA sequencing

Placenta RNA were extracted with Quick-RNA MiniPrep Plus (Zymo Research, CA, USA) kit according to manufacture instruction. RNA library was developed at Novogene (CA, USA). Ingenuity Pathway Analysis software (IPA, Qiagen) was used for pathway analysis.

Histopathology evaluation

Placentae (2-5 per dam) were embedded into paraffin blocks, sectioned, and stained with conventional H&E staining.

Immunofluorescence

The following primary antibody were used: SERT, Ki67 (ab15580, ABCAM), Caspase 3 (43-7800, Invitrogen), PCNA (sc-56, Santa Cruz Biotecnology. The following secondary antibody were used: AlexaFluor 594 (A11005, goat anti-mouse), 594 (A11012, goat anti-rabbit), 488 (A11078, rabbit anti-goat), 488 (A11001, goat-anti-mouse) from Life Technology. DAPI (D3571, Invitrogen) was used at 1:1500. The TUNEL staining was performed as per manufacture instruction (11684795910, Roche). Placenta were evaluated and imaged under a Zeizz AX10 macroscope coupled to a Basler acA2440-35µm camera.

Statistical analysis

Transcriptomic data analyses were performed on R. All other statistical analyses were performed on SAS (9.4). One-way and two-way ANOVA was used for comparisons among groups.

Conflict of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions: RRD conceptualization, methodology, investigation, data curation, formal analysis, project administration, original draft, reviewed and edited the manuscript; NNT investigation, data curation, formal analysis, reviewed and edited the manuscript; LMD investigation, data curation, formal analysis, reviewed and edited the manuscript; FP methodology, investigation, data curation, formal analysis, reviewed and edited the manuscript; MCW conceptualization, methodology, formal analysis, funding acquisition, supervision, reviewed and edited the manuscript; LLH conceptualization, methodology, formal analysis, funding acquisition, supervision, reviewed and edited the manuscript.

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Table

Supplemental table 1. Effect of fluoxetine treatment and genotype on circulating serum serotonin.

Maternal genotype	Embryonic genotype	Fluoxetine treatment	Serum serotonin (ng/mL)
Pregnant dams E18.75			
WT	.	-	2463 ± 212
WT	.	2 mg/kg/d	2044 ± 397
WT	.	20 mg/kg/d	64 ± 23
SERT+/-	.	-	2128 ± 178
SERT+/-	.	2 mg/kg/d	1254 ± 269
SERT-/-	.	-	6.0 ± 1.1
SERT-/-	.	2 mg/kg/d	6.7 ± 0.9
SERT-/-	.	20 mg/kg/d	8.2 ± 3.1
Embryos E18.65			
WT	WT	-	561.0 ± 60.5
WT	WT	2 mg/kg/d	374.7 ± 58.2
WT	WT	20 mg/kg/d	66.9 ± 23.0
Sert+/-	Sert+/-	-	386.2 ± 118.8
Sert+/-	Sert-/-	-	6.5 ± 1.9
Sert-/-	Sert+/-	-	396.9 ± 153.7
Sert-/-	Sert-/-	-	13.8 ± 6.1

Figures

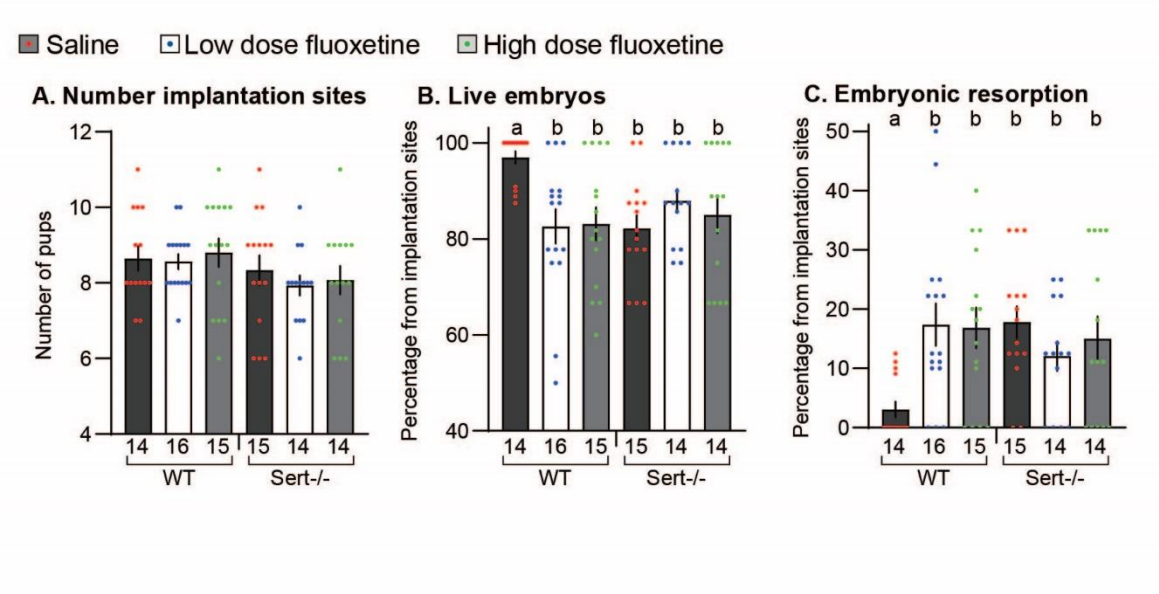


Figure 1. Effect of genotype and fluoxetine treatment on (A) number of implantation sites, (B) live embryos, and (C) embryonic resorption. Dams were mated to males of their respective genotype. [ab]Different superscript letters indicated P value < 0.05. Number of dams in each group is indicated.

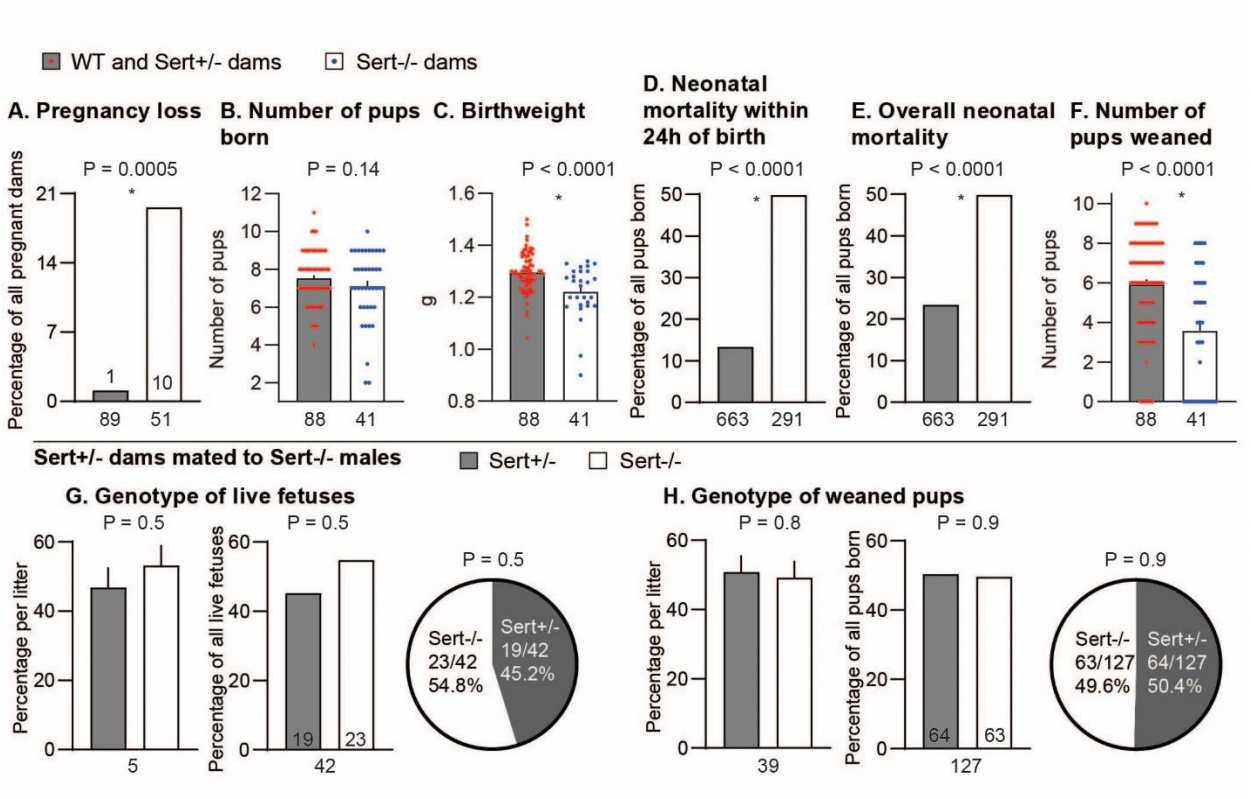


Figure 2. Effect of maternal genotype (WT and Sert+/- combined vs Sert-/-) on pregnancy outcomes: (A) pregnancy loss after E10.5, (B) number of pups born, (C) birthweight, (D) neonatal mortality within 24h of birth, (E) overall neonatal mortality during lactation (0 to 21 days postpartum), and (F) number of pups weaned. Number of dams (A, B, C, F) or total number of pups born (D,E) are indicated. Frequency of genotype for (G) live embryos and (H) weaned pups for Sert+/- dams mated to Sert-/- males. Number of dams and total number of pups born are indicated.

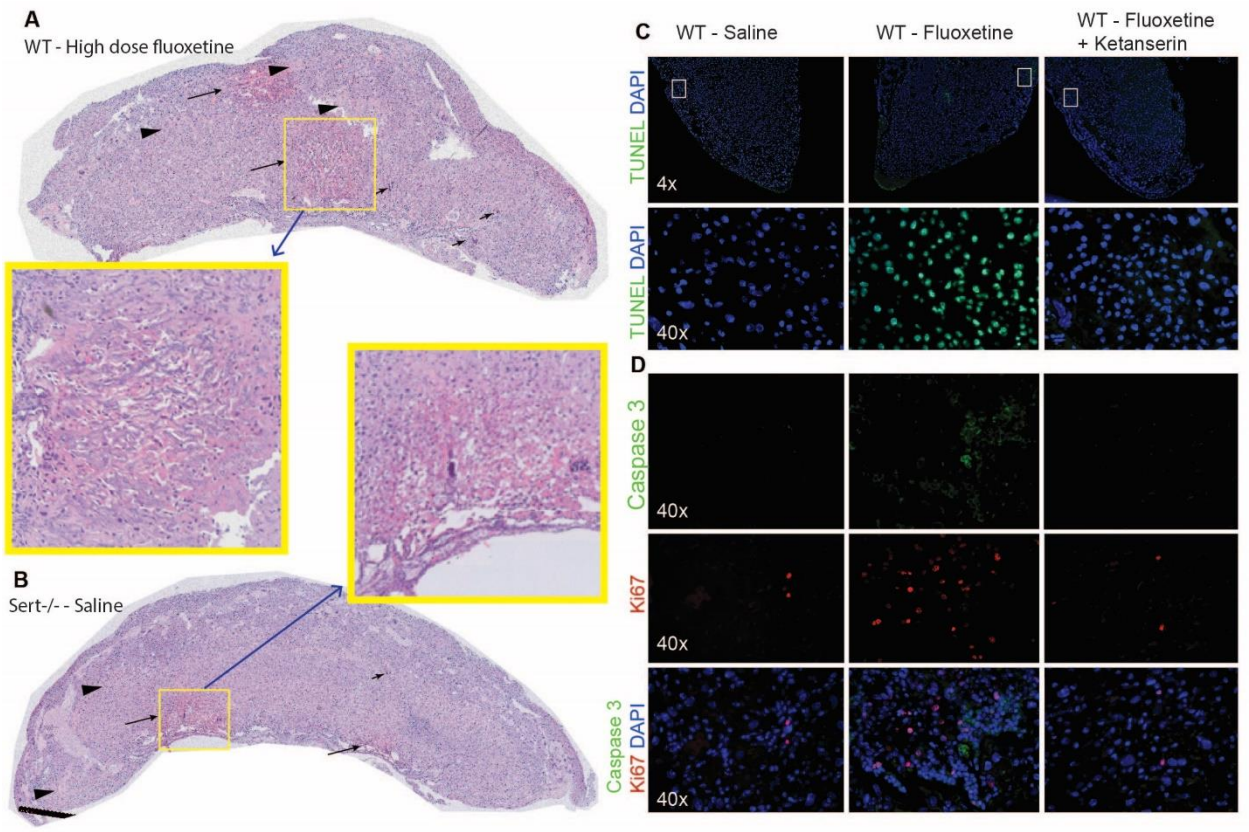


Figure 3. Placenta pathology on E18.75 in (A) WT mice treated with fluoxetine from E10.5 to E18.5 and in (B) untreated Sert-/- dam carrying Sert-/- offspring. Highlighted inserts indicate areas of infarction. Long arrows indicate areas of infarction. Short arrows indicate areas of mineralization. Arrowheads indicate areas of fibrosis and necrosis. (C) TUNEL and (D) Caspase 3 and Ki67 staining in WT mice treated with saline, fluoxetine (low dose) and fluoxetine + ketanserin from E10.5 to 18.5.

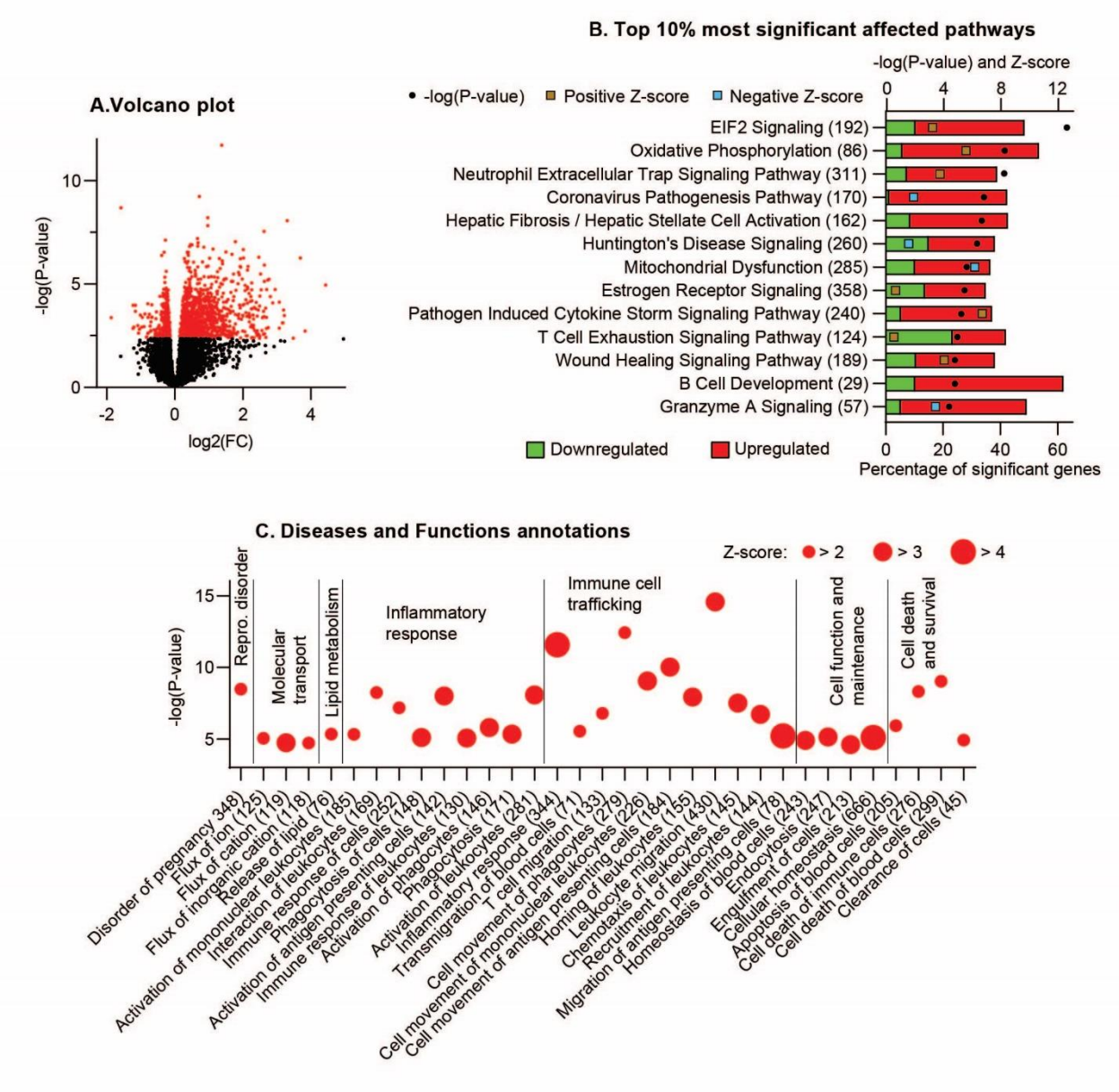


Figure 4. Effect of maternal genotype (Sert-/- vs Sert+/-) on placental transcriptome. (A) Volcano plot. (B) Top 10% (n = 13) most significant affected pathways. (C) Selected altered pathways identified with diseases and function annotation.

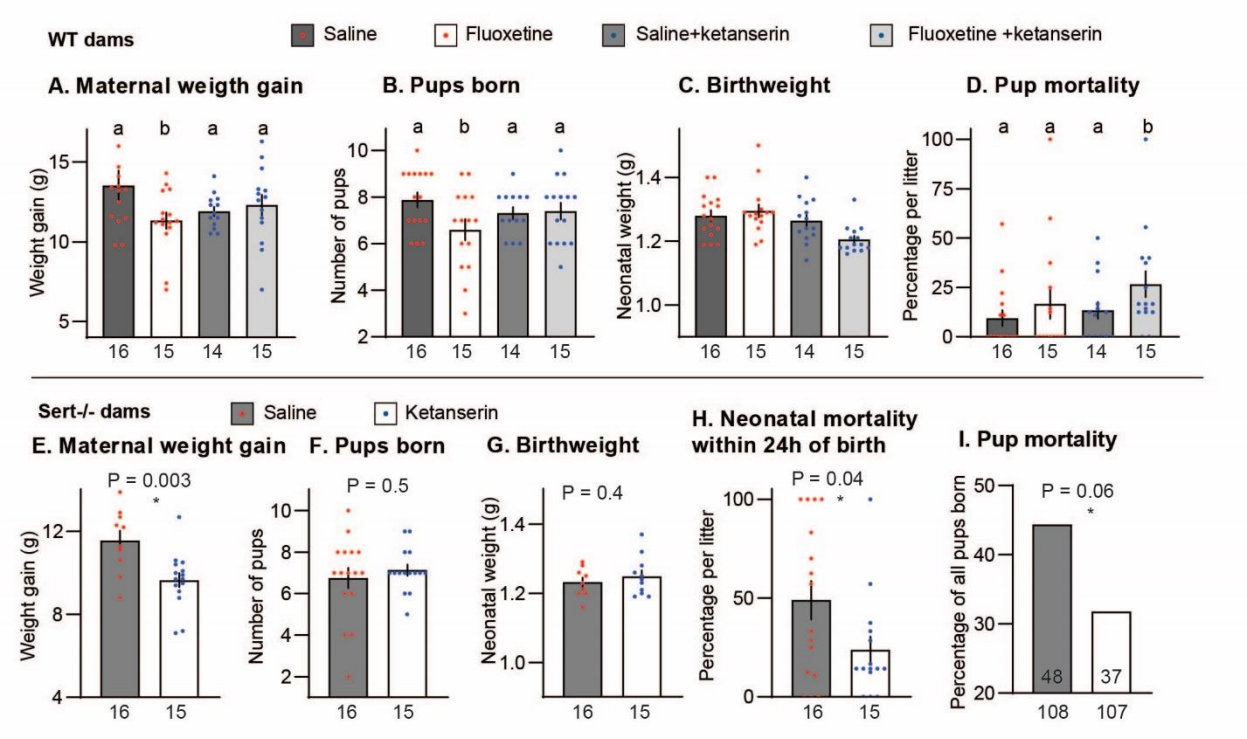
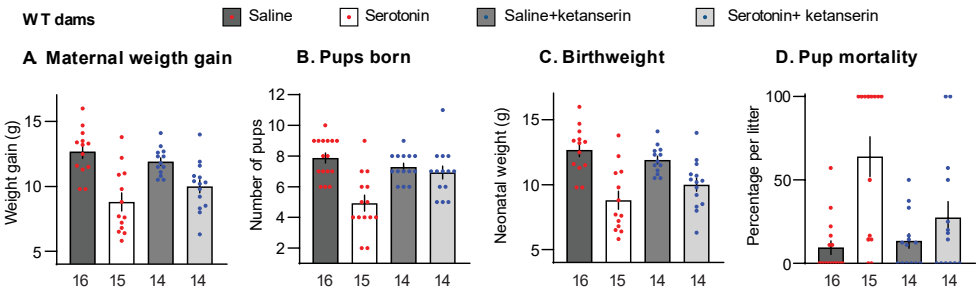


Figure 5. Effect of ketanserin treatment on pregnancy outcomes in WT fluoxetine-treated mice (A-D) or Sert-/- mice (E-I). (A,E) Maternal weight gain during treatment period (E10.5 to E18.5). (B,F) Number of pups born. (C,G) Neonatal birthweight. (D,I) Pup mortality during lactation period (21 days postpartum). (H) Neonatal mortality within 24 hours of birth. [ab]Different superscript letters indicated P value < 0.05. Number of dams (A-H) or total number of pups born (I) in each group is indicated.

Supplemental Figure



Supplemental Figure 1. Effect of ketanserin treatment on pregnancy outcomes in WT serotonin-treated mice. (A) Maternal weight gain during treatment period (E10.5 to E18.5). (B) Number of pups born. (C) Neonatal birthweight. (D) Pup mortality during lactation period (21 days postpartum). (H) Neonatal mortality within 24 hours of birth. [ab]Different superscript letters indicated P value < 0.05. Number of dams in each group is indicated.

Chapter 6

The antidepressant fluoxetine (Prozac®) modulates estrogen signaling in the uterus and alters estrous cycles in mice

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Abstract

Selective serotonin reuptake inhibitors (SSRI) are the most used antidepressants. However, up to 80% of women taking SSRI suffer from sexual dysfunction. We investigated the effects of fluoxetine (Prozac®) (low and high dose, n=6-7/group) on reproductive function and the regulation of the estrous cycle. All mice treated with high dose of fluoxetine had interruption of estrous cycles within a few days after onset of treatment. When treated for 14 days, mice in the high dose group had fewer CL, often lack of any CL, and antral follicles. Uterine expression of estrogen receptor alpha, G-protein coupled estrogen receptor, and steroidogenesis enzymes were upregulated in the high dose group. Nevertheless, decreased expression of connexin 43 and alkaline phosphatase and increased expression of insulin-like growth factor-binding protein 3 and monoamine oxidase A are consistent with decreased estrogen signaling and the decreased uterine weight. Taken together, fluoxetine modulates estrogen synthesis/signaling and dysregulates estrous cycles.

Introduction

About 6% of adolescents aged 12-19 have used psychotropic medications and 17.7% of women older than 18 years of age have used antidepressants [1]. Among these drugs, selective serotonin reuptake inhibitors (SSRI)are the most commonly prescribed. Although SSRI improve mental health, multiple studies have reported side effects of SSRI including reproductive issues [2-6]. Indeed, the effects of SSRI on pregnancy outcomes have been extensively explored in the past decade and appear to be associated with modulation of serotonin signaling [4, 6-12]. However, the effects of SSRI on reproductive function in a nonpregnant state are poorly understood [13].

The main biological target of SSRI is serotonin transporter (SERT) [14]. Accordingly, SSRI inhibition of SERT modulates serotonin availability and signaling in the brain (antidepressant effect) and in the periphery (possible side effects) [15]. Indeed, SSRI inhibition of platelet SERT leads to increased free (plasma) concentrations of serotonin [15]. However, SSRIs seem to also modulate synthesis and signaling of other hormones [16-18]. All clinically available SSRI have endocrine disrupting effects on steroid hormone synthesis in vitro [19, 20]. Fluoxetine and sertraline stimulate aromatase activity leading to increased estradiol synthesis. Additionally, SSRI use has been associated with hyperprolactinemia in women and rodents [18, 21]. However, the endocrine disruption impacts of SSRI on the regulation of reproductive cycles are poorly understood.

In humans, SSRI cause sexual dysfunction and in rodent models SSRI affect sexual behavior [10, 13, 22]. In addition to the increased rates of women suffering from depression compared to men, and consequently undergoing SSRI treatment, women display more severe SSRI-induced sexual dysfunction [9]. It has been reported that up to 80% of women taking SSRI

display some sort of sexual dysfunction. Because sexual dysfunction is often due to endocrine influence [23], delineating the mechanisms underpinning the effects of SSRI on endocrine, ovarian, and uterine function will be critical for defining the role of SSRI on sexual dysfunction and to mitigate these effects without affecting the beneficial antidepressant effects.

Due to the potential endocrine disrupting effects of SSRI on women's reproductive function, we aimed to delineate the effect of fluoxetine on reproductive function and the regulation of estrous cycles in sexually mature mice. We hypothesized that fluoxetine modulates estrogen synthesis/signaling and dysregulates estrous cycles.

Materials and Methods

Animals

All experimental procedures were approved by the Research Animal Care and Use Committee at the University of Wisconsin-Madison and were performed under protocol number A005789-A01. Mice were individually housed in a controlled environmental facility for biological research in the Animal and Dairy Sciences Department vivarium at the University of Wisconsin-Madison. Animal facility was maintained at a temperature of 25°C and a humidity of 50% to 60%, with a 12:12 hour light-dark cycle with *ad libitum* water and food (LabDiet 5015, TestDiet, Richmond, IN). Wild-type C57BL/6J mice (N = 20) were obtained from Jackson Laboratories (stock # 000664, Jackson Laboratories, Bar Harbor, ME).

Experimental design

Beginning at six-weeks of age, stage of estrous cycle of virgin female mice was determined daily for 20 days to establish normal estrous cyclicity in all animals. After

establishing normal cyclicity in all mice, daily intraperitoneal injection began (day 0) on randon days of the estrous cycle. Mice were randomly allocated to a vehicle (saline, n = 6), low dose fluoxetine (2 mg/kg/d, n = 7; fluoxetine hydrochloride, F312; Sigma-Aldrich, St. Louis, MO), and high dose fluoxetine (20 mg/kg/d, n = 7) treatment groups. Determination of estrous cycles and treatments continued daily until day 14. Mice were weighed daily throughout the experimental period.

We have previously used these doses of fluoxetine in mice [4]. The low dose (2 mg/kg/d) results in systemic concentration similar to that of humans taking fluoxetine. The high dose (20 mg/kg/d) results in greater systemic concentrations although it is commonly used in mice studies [24, 25].

Determination of phases of estrous cycle

To determine stage of estrous cycles vaginal lavage was performed daily as described [26] between 9 and 10 am by the same technician throughout the experimental period. Slides were stained with Wright-Giemsa (Hema3 Stat Pack, Fisherbrand, Pittsburgh, PA, USA) and observed using a light microscope.

Blood and tissue collection

Mice were euthanized approximately 6 hours after the last treatment (day 14) with carbon dioxide followed by cervical dislocation. Cardiac blood was collected immediately after euthanasia. Uterus was excised and weighed. One uterine horn along with the ovaries were fixed in 4% paraformaldehyde overnight and stored in 70% ethanol until histological processing. Histology samples were embedded in paraffin, sectioned into 8 μm sections, stained with

conventional hematoxylin-eosin and observed by light microscopy for image collection and analyzed qualitatively by a single technician unaware of treatment group. The other uterine horn was snap frozen in liquid nitrogen and stored at -80 °C and used for evaluation of gene expression.

Extraction of RNA, complementary DNA, and quantitative PCR

Extraction of RNA was performed with Trizol reagent (Invitrogen, CA, USA) as described by the manufacturer and quantified by spectrometry with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) as described by the manufacturer using 1 μg of total RNA. The cDNA was used directly for quantitative real-time PCR (qRT-PCR). The qRT-PCR reactions were carried out on an CFX Connect Real-Time PCR system (Bio-Rad Life Science, CA, USA) using a master mix that contained a total volume of 10.5 μL per tube consisting of 6.25 μL of SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., CA, USA), 3.25 μL of nuclease-free water, and 0.5 μL of forward and reverse primers (10 μM). Two μL of cDNA at a 1:5 dilution was added to the master mix for a total reaction volume of 12.5 μL. All samples were evaluated in duplicate. The reactions were initiated with preincubation at 95°C for 3 minutes followed by 42 cycles of denaturation (95°C for 10 seconds) and annealing and extension (60°C for 30 seconds).

The primer sequences for targeted genes (Table 1) were synthesized by Integrated DNA Technologies Inc. (CA, USA) using sequences reported in previous studies or designed by our laboratory. Efficiencies of qRT-PCR for amplification of targeted genes were determined in our laboratory and ranged from 95% to 107%. The amplification data obtained from the qRT-PCR

were the cycle threshold (Ct) for each mRNA and these was used to calculate the mRNA relative abundance of each sample by the $2^{-\Delta\Delta Ct}$ method [27] using vehicle group as baseline and the geometric mean of the housekeeping genes 36b4 and Hrpt1.

Statistical analysis

All statistical analyses were performed with SAS (version 9.4; SAS Institute Inc., Cary, North Carolina, USA). Data were analyzed with the PROC MIXED procedure using one-way ANOVA and two-way ANOVA for repeated measures. Tukey HSH was used for post hoc comparisons. Residuals with deviations from assumptions of normality and/or homogeneity of variance were transformed into square root, logarithms, or ranks. A probability of ≤ 0.05 indicated a difference was significant and a probability between > 0.05 and ≤ 0.1 indicated tendency significance. Data are presented as the mean ± standard error of mean (SEM).

Results and Discussion

Understanding the endocrine disruptive effects of psychotropic medications and their implications on reproductive function is critical so that patients and physicians can make informed decisions when developing treatment plans. Furthermore, understanding the side effects of currently prescribed drugs is essential for the development of new, improved treatments with fewer side effects.

To determine the effect of fluoxetine on estrous cyclicity, we examined daily vaginal smears of sexually mature mice. Before onset of treatment, all mice had normal estrous cycles (Figure 1). After treatment onset, vehicle and low dose groups continued to cycle regularly while estrous cycles were dramatically reduced in mice receiving the high dose of fluoxetine. All mice

receiving high doses of fluoxetine had irregular estrous cycles after treatment onset. Vaginal cytology of mice receiving high doses of fluoxetine presented with mixed cell types with predominance of leukocytes and some anucleated keratinized epithelial cells, which is characteristic of metestrus [26, 28] and typically observed in rodents chronically treated with estradiol or exposed to estrogenic endocrine disrupting compounds [29].

Fewer CL, often lack of any CL, and large antral follicles were observed in the ovaries from mice in the high dose group (Figure 2). In previous studies, fluoxetine treatment caused follicle and oocyte abnormalities, increased number of atretic follicles, and decreased number of ovulated oocytes [30-32]. Furthermore, fluoxetine increased the number of small and medium preantral follicles which might be associated with decreased follicle development beyond that stage resulting in decreased ovulation and subsequent development of CL, as observed in the present study [30]. Similarly, fluoxetine reduced ovulation in Balb/C mice [32] and rabbits [33]. The ovarian findings reported in the present and previous studies are consistent with fluoxetine-induced disruption of ovarian function and interruption of estrous cycles directing some caution for women and girls of reproductive age.

Fluoxetine and sertraline, the most commonly used SSRI [3], increase estradiol synthesis in vitro and fluoxetine has estrogenic effects in vivo [17, 19, 20]. Interestingly, in vivo short-term fluoxetine treatment seems to increase systemic concentrations of estrogen [17] whereas long-term treatment appears to reduce it [34, 35]. The fluoxetine-induced increase in concentrations of estradiol may affect hypothalamic secretion of GnRH and pituitary secretion of FSH/LH [36]. Alternatively, the fluoxetine-induced increase in neuronal serotonin signaling may directly alter GnRH and LH pulses [37-39]. Previous studies have shown that fluoxetine treatment rapidly decreases LH pulses in rats [40] and four weeks of treatment decreases estradiol and FSH. The

effects of fluoxetine on the hypothalamic-pituitary-gonadal axis [41] may lead to decreased follicle progression into preovulatory stages resulting in decreased ovulation rate as observed in the present and previous studies [30, 32, 33]. Noteworthy, chronic estrogen treatment alters estrous cycles causing decreased number of cycles, absence of CL, and ovary atrophy [36] consistent with findings in the present study. Decreased follicle development is consistent with decreased concentrations of estradiol as observed after long-term fluoxetine treatment since ovarian follicles are the main source of circulating estradiol. Although estradiol concentrations were not measured in the present study because of the confounding effect of euthanasia/blood collection on different days of the estrous cycles, the ovarian and uterine findings in the present study are consistent with decreased systemic concentrations of estradiol.

Uterine weight was reduced (P= 0.002) by 53% in the high dose group (45.7 ± 3.7 mg) compared to the vehicle (98.0 ± 9.1 mg) and low dose (98.6 ± 14.2 mg) groups (Figure 3). Furthermore, uterine weight adjusted to body weight was reduced (P = 0.0014) in the high dose group (2.6 ± 0.2) compared to the vehicle (5.5 ± 0.4) and low dose (5.3 ± 0.7) groups. The reduced uterine weight in the high dose group is consistent with decreased concentrations of estradiol and the interruption of estrous cycles. No major histological abnormalities were observed in the uterus.

The high dose of fluoxetine increased uterine expression of estradiol receptor alpha (Esr1; 2.4-fold), G-protein coupled estrogen receptor (Gper; 2.2-fold), and steroidogenic enzymes (Hsd3b [1.5-fold], Cyp11a1 [2.1-fold]) (Figure 4). Expression of aromatase in the uterus was too low to be reliably analyzed (qRT-PCR Ct > 40). Although the uterus is not commonly thought as a steroidogenic organ, it does synthesize estrogen under different stages of the estrous cycle, pregnancy, and in some disease conditions [42-44]. In the present study, the

increased uterine expression of estrogen receptors and steroidogenic enzymes are inconsistent with decreased uterine weight since estrogen signaling typically increases uterine cell proliferation and uterine weight [45]. Nevertheless, the decreased expression of connexin 43 (Cx43; ~39% reduction) and alkaline phosphatase (Alkp; ~95% reduction), major regulators/markers of uterine stromal differentiation, are consistent with decreased estrogen signaling and decreased uterine weight [42]. Additionally, classic estrogen responsive genes were not different among groups (Igf1, Gadd45g, Ramp3) [46, 47] while expression of genes typically downregulated by estrogens were increased (Igfbp3 [2-fold] and Maoa [1.9-fold]) [48-50]. Furthermore, Igfbp3 had been reported to be inversely related to uterine weight [48] as observed in the present study. Taken together, uterine gene expression along with uterine weight suggest decreased estrogen signaling. Further studies are needed to confirm the effects of fluoxetine on uterine function after short and long-term exposure and implications for reproductive health and pregnancy.

Mouse body weights were not different ($P = 0.67$) among groups before the onset of treatment (overall mean body weight was 17.3 ± 0.2 g). During the 14-day treatment regimen, body weight relative to the pretreatment period was reduced in the high dose fluoxetine group compared to vehicle and low doses groups (Figure 5). Nevertheless, daily weight change relative to day before was not different among groups when evaluated during the overall treatment period. However, after the first treatment the vehicle and low dose groups gained weight (1.1 ± 2.0 and 0.4 ± 1.0%, respectively) while the high dose fluoxetine group lost weight (-3.1 ± 0.7%; $P = 0.02$). Collectively, the high dose of fluoxetine caused only a transient weight loss after the first day of treatment. Similarly, we have previously observed that the high dose of fluoxetine caused transient weight loss in pregnant mice [4]. Furthermore, other studies also reported

reduced weight gain in mice treated with fluoxetine, particularly at higher doses [22, 51-53]. A previous study suggested that the fluoxetine-induced anovulation in Fishers rats was due to decreased food consumption and weight loss caused by fluoxetine [51]. However, in another study from the same laboratory using Sprague Dawley rats the reproductive effects of fluoxetine were only mild with longer estrous cycles in 40% of animals but only after 10 days of treatment and no anovulation [22]. Previous research in our laboratory did not observe a significant decrease in feed consumption in C57Bl/6J mice treated with fluoxetine and, similar to this study, weight loss only lasted for a day (Hernandez, unpublished). Therefore, it is unlikely that interruption of estrous cycles in the present study was due to decreased feed intake/weight loss.

Another important effect of SSRI on reproduction is its modulation of sexual behavior in both humans and rodent models. Clinical evidence of a role for SSRI on modulation of sexual behavior is its use to delay ejaculation in men with premature ejaculation and in men with erectile failure [54, 55]. In women, SSRI use has been associated with decreased sexual desire, excitement, and delayed orgasm [56]. In rodent models, medication that increase serotonin concentrations and signaling in the brain, such as SSRI, negatively affect female sexual behavior [57]. Accordingly, fluoxetine has an acute (within 30 minutes) dose-dependent reduction in lordosis response to mounting in intact and in ovariectomized, hormone primed rats suggesting a central effect of the drug on sexual behavior [58]. Taken together, the effects of SSRI on reproductive function go beyond physiological changes in reproductive organs but also affect sexual behavior which may extend its compromise on reproductive efficiency and women try to become pregnant.

Conclusions

In conclusion, we report that fluoxetine treatment results in the disruption of ovarian and uterine function with consequent interruption of estrous cycles. Treatment with the high dose of fluoxetine for 14 days caused anovulation and altered ovarian morphology. Additionally, uterine weight and gene expression were altered. Taken together, the interruption of estrous cycles along with ovarian and uterine changes suggest lack of follicle development/ovulation and decreased estrogen signaling. This is a critical finding given the number of adolescent and adult women prescribed SSRI and provides a framework for other research exploring the endocrine disrupting effects of SSRI.

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Conflict of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table

Table 1. Primer sequences for quantitative real time PCR.

Gene	Primer sequence	GenBanker ID
36b4	F: CCTATAAAAGGCACACGCGG R: ACGCGGGGTTTAAAGACGAT	NM_007475.5
Hrpt1	F: GCTTCCTCCTCAGACCGCTT R: ATCGCTAATCACGACGCTGG	NM_013556
Esr1	F: TGATGCCAGGAGAGGCCAATGC R: TGTCGCCCAGAGACTGCCTTCTT	NM_007956.4
Esr2	F: GCCAGCCCTGTTACTAGTCCAA R: CAGACGGCGCAGAAGTGA	NM_207707.1
Gper	F: CTGCACGAGCGGTACTACGA R: CAGATGAGGCCACAGCTCAG	XM_036165593.1
Pgr	F: TATGGCGTGCTTACCTGTGG R: TGCCAGCCTGACAACACTT	NM_008829.2
StAR	F: CTGCAGGACTCAGGACCTTG R: ACACAGCTTGAACGTAGCGA	NM_011485.5
Cyp11a1	F: TGCTCTGCAAAGCCGAATAC R: TGCTCTGCAAAGCCGAATAC	NM_001346787.1
Hsd3b	F: GTGCGCCCTGGGACTTACTA R: ACCAGGTATACCAGTGTTGGC	NM_133943.2
Cyp17a1	F: GGCCCAAGTCAAAGACACCT R: CGTCTGGGGAGAAACGGTAG	NM_007809.3

Hsd17b1	F: ATGTGCTTGGGACCATTCGG R: GTGGAATGGCAGTCCCATCA	NM_010475.2
Cx43	F: ACGCTTTTACGAGGTATCAGCA R: GTCTGCTGCTGTTGGGTACT	NM_010288.3
Alkp	F: CTACGCACCCTGTTCTGAGG R: GACCTCTCCCTTGAGTGTGG	XM_006538498.4F
Igf1	F: GCTCTTCAGTTCGTGTGTGGAC R: AGCCTGTGGGCTTGTTGAAGTA	NM_001111276.1
Gadd45g	F: AGTCCTGAATGTGGACCCTGACA R: GCAGAACGCCTGAATCAACGTG	NM_011817.2
Ramp3	F: GTTGCTGCTTTGTGGTGAGTGT R: AGACAGCCACCTTCTGCATCAT	NM_019511
Igfbp3	F: TGTGTGGACAAGTATGGGCAGC R: TGAGCTCCATATTATGTGGCACGG	NM_008343.2
Maoa	F: ACAGCAACACAGTGGAGTGG R: GGAACATCCTTGGACTCAGG	NM_173740.3

Figures

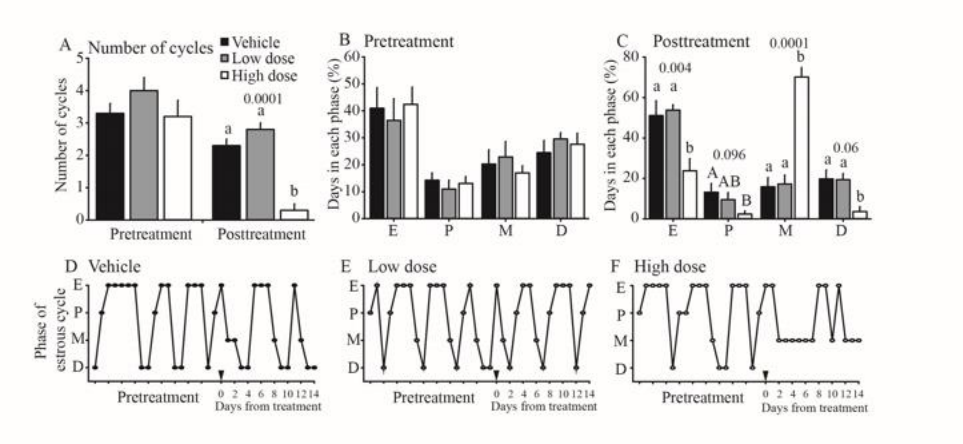

Figure 1. Estrous cycles before and after onset of treatment (day 0) for mice treated with vehicle (saline), low dose fluoxetine (2 mg/kg/d), and high dose fluoxetine (20 mg/kg/d) for 14 days. (A) Number of estrous cycles. Percentage of period in each stage of the estrous cycle during pretreatment (B) and after onset of treatment (posttreatment; C). Estrous cycle pattern in a representative mouse during experimental period for vehicle (D), low dose (E), and high dose (F). E, estrus; M, metestrus; D, diestrus; P, proestrus. abc, indicate significant difference among groups. ABC, indicate tendency for significant difference among groups.

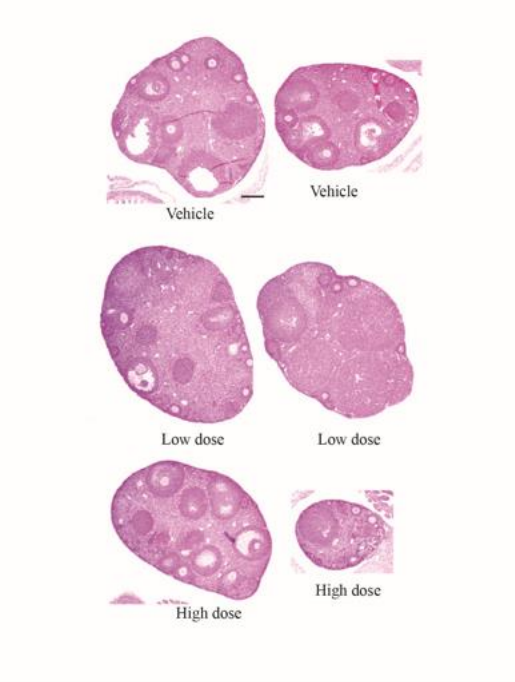

Figure 2. Ovarian histology of two representative mice treated for 14 days with vehicle (saline), low dose fluoxetine (2 mg/kg/d), and high dose fluoxetine (20 mg/kg/d). Images were taken at 4x magnification (scale bar: 2 mm)

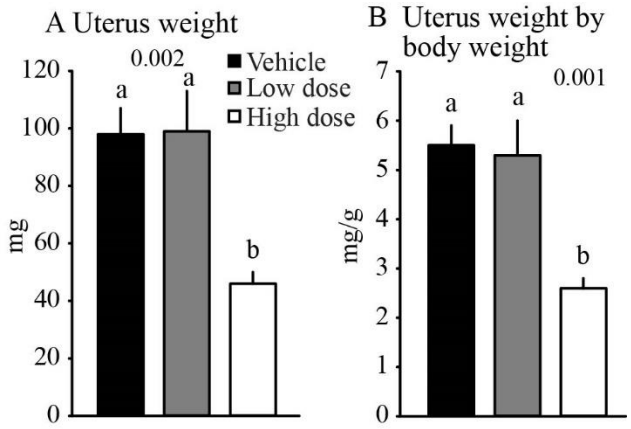

Figure 3. Uterus weight (A) and uterus weight by body weight (B) for mice treated for 14 days with: vehicle (saline), low dose fluoxetine (2 mg/kg/d), and high dose fluoxetine (20 mg/kg/d). abc, indicate significant difference among groups.

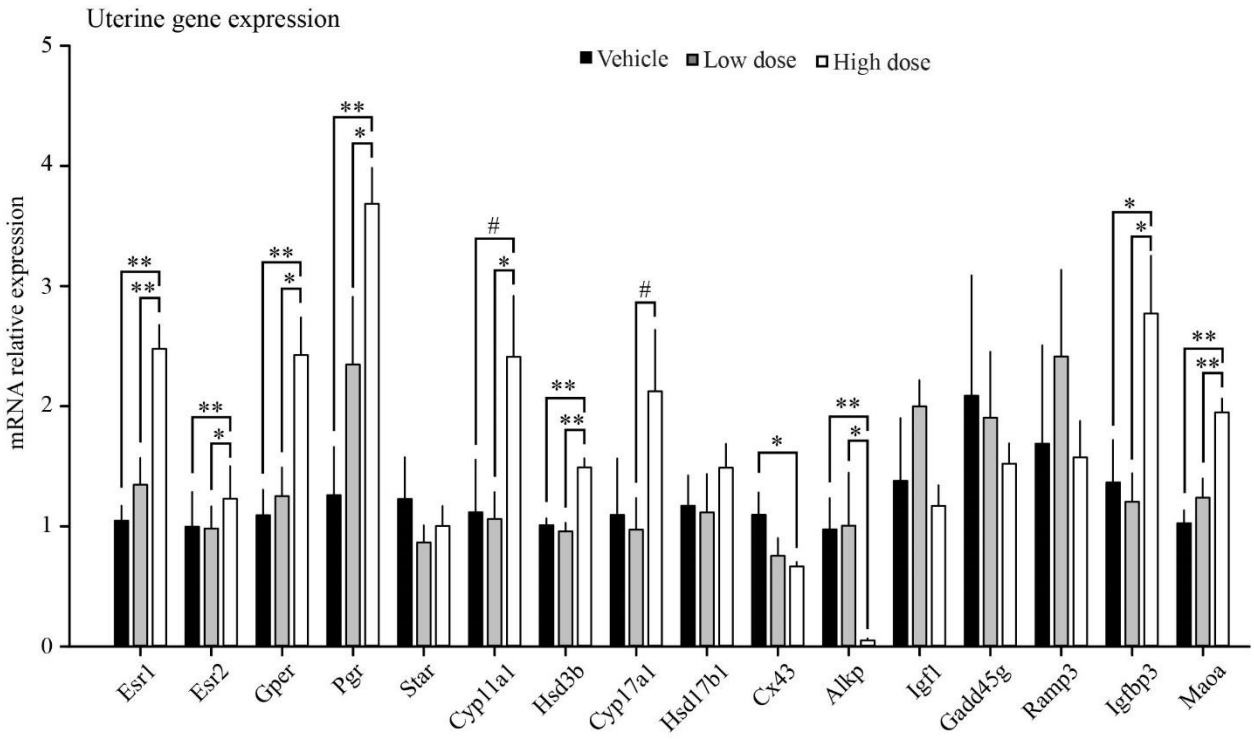

Figure 4. Uterine gene expression for mice treated for 14 days with: vehicle (saline), low dose fluoxetine (2 mg/kg/d), and high dose fluoxetine (20 mg/kg/d). ** indicate P-value < 0.01; * indicate P-value < 0.05; # indicate P-value < 0.1. ABC, indicate tendency for significant difference among groups.

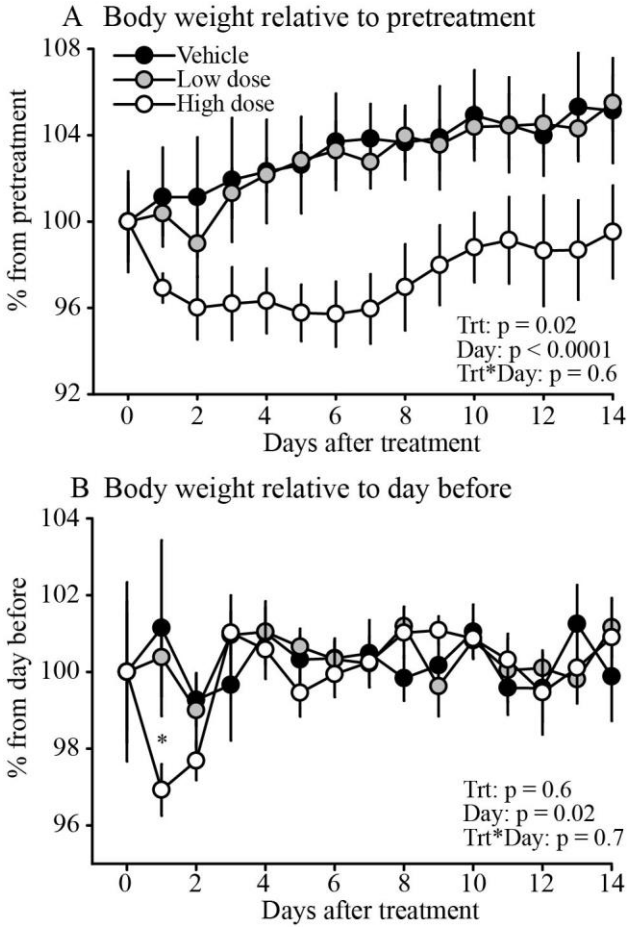

Figure 5. Body weight for mice treated for 14 days with: vehicle (saline), low dose fluoxetine (2 mg/kg/d), and high dose fluoxetine (20 mg/kg/d). Weight change relative to pretreatment (A). Weight change relative to day before (B). *, indicate significant difference among groups based on separate analysis for day 1.

Chapter 7

Serotonin-dependent serotonin transporter-mediated endocrine disrupting effect of the antidepressant fluoxetine (Prozac®)

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Abstract

Selective serotonin reuptake inhibitors (SSRI) are the most commonly used antidepressants. However, they appear to have an endocrine disrupting effect on the modulation of steroidogenesis, particularly on estrogen. We sought to investigate the in vivo effects of fluoxetine, the most commonly known SSRI, on estrogen synthesis/signaling. In prepubertal mice, the lack of increase in uterine weight in a three-day uterotropic assay indicated fluoxetine does not directly bind estrogen receptor to elicit its estrogenic effects. Treatment with a low dose of fluoxetine (2 mg/kg/d) for six days resulted in increased uterine weight in virgin, sexually mature mice or in pseudopregnant mice (43% and 100% compared to the control group, respectively). Uterine mRNA expression of alkaline phosphatase was increased 3.1-fold and insulin-like growth factor binding protein 3 was downregulated by 47.8%. Further we observed increased uterine thickness, increased epithelial height, and increased endometrial grands, and all are consistent with increased estrogen signaling. In mice with genetic ablation of the serotonin transporter (Sert-/-, biological target site for SSRI) and depleted nonneuronal synthesis of serotonin (Tph1-/-), fluoxetine treatment did not affect uterine weight. Taken together, estrogenic actions of SSRI are mediated by inhibition of SERT and are dependent on peripheral serotonin. Given that SSRI treatment increases serotonin signaling and that serotonin enhances ovarian synthesis of estrogen, it appears that the increased estrogen synthesis/signaling was elicited by SSRI-induced increase serotonin signaling.

Introduction

Selective serotonin reuptake inhibitors (SSRI) are the main class of antidepressants used to treat multiple psychological conditions including depression, anxiety, and obsessive-compulsive disorder [1-3]. The use of SSRI has increased over the last several decades with 13.2% of adults using antidepressants in the US. Noteworthy, the frequency of use of antidepressants is two-fold greater in women compared to men as 17.7% of adult women are prescribed antidepressants [4]. Nevertheless, SSRI use is associated with reproductive side-effects that include sexual dysfunction, amenorrhea, and galactorrhea in addition to adverse pregnancy outcomes [5-9]. The incidence of sexual dysfunction in women taking SSRI is up to 80% and women have more severe SSRI-induced sexual disfunction compared to men [10]. Given that sexual dysfunction is often due to endocrine causes, including owing to endocrine disruption [11], understanding the effects of SSRI on endocrine homeostasis can provide insight into the role for SSRI on sexual dysfunction.

Inhibition of the serotonin transporter (SERT) by SSRI occurs in the brain and in the periphery resulting in increased serotonin signaling throughout the body [12-14]. At the tissue level, inhibition of SERT prevents serotonin transport inside cells for degradation prolonging the presence of serotonin in the extracellular space where it binds to its cell-surface receptors [1, 15]. Additionally, SSRI increases free circulating serotonin [14]. Several studies have reported that SSRI modulates steroid synthesis in vivo and in vitro [16-22]. However, whether the effects of SSRI on steroidogenesis are mediated by inhibition of SERT or through an off-target mechanism remains to be elucidated. Additionally, the impact of SSRI-induced increased serotonin signaling on endocrine homeostasis is unknown.

In a previous experiment in our lab (unpublished), we observed increased uterine weight in female mice mated to a fertile male, as confirmed by detection of a vaginal plug, that did not become pregnant. Because increased uterine weight typically indicates increased estrogen signaling, we aimed to investigate the in vivo effects of fluoxetine as an endocrine disrupting agent modulating estrogen signaling. Additionally, we sought to assess the role of SERT and serotonin on the SSRI-induced modulation of estrogen signaling.

Materials and Methods

Animals

All experimental procedures were approved by the Research Animal Care and Use Committee at the University of Wisconsin-Madison and were performed under protocol number A005789-A01. Wild-type C57BL/6J (strain 000664) and Sert-/- (strain 008355, C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, ME) and colonies were maintained in our facility. We have also maintained a colony of Tph1-/- mice [23]. Mice were individually housed in a controlled environmental facility for biological research in the Animal and Dairy Sciences Department vivarium at the University of Wisconsin-Madison. The vivarium was maintained at a temperature of 25°C and a humidity of 50% to 60%, with a 12:12 hour light-dark cycle with *ad libitum* water and food (LabDiet 5015, TestDiet, Richmond, IN).

Fluoxetine treatments

In each experiment, mice were randomly allocated to a treatment group. Low dose fluoxetine (2 mg/kg/d) and high dose fluoxetine (20 mg/kg/d) were administered via intraperitoneal injection. Fluoxetine hydrochloride (F312; Sigma-Aldrich, St. Louis, MO) was

dissolved in saline. The control group received intraperitoneal saline alone. We have previously used these doses of fluoxetine in mice [24]. The low dose (2 mg/kg/d) of results in fluoxetine concentrations similar to that of humans taking fluoxetine. The high dose (20 mg/kg/d) results in greater systemic fluoxetine concentrations than those observed in humans although it is routinely used in mice studies [25, 26].

Blood and tissue collection

Mice were euthanized approximately 6 hours after the last treatment (day 14) with carbon dioxide followed by cervical dislocation. Cardiac blood was collected immediately after euthanasia, and the uterus was excised and weighed. One uterine horn along with the ovaries were fixed in 4% paraformaldehyde overnight and stored in 70% ethanol until histological processing. Histology samples were embedded in paraffin, sectioned into 8 μm sections, stained with conventional hematoxylin-eosin and observed by light microscopy for image collection and analyzed qualitatively by a single technician unaware of treatment group. Uterine thickness and epithelium height were measured (at least three measurements for each sample). The other uterine horn was snap frozen in liquid nitrogen and stored at -80 °C and used for evaluation of gene expression.

Extraction of RNA, complementary DNA, and quantitative PCR

Extraction of RNA was performed with Quick-RNA MiniPrep Plus kit (Zymo Research, CA, USA) as described by the manufacturer and quantified by spectrometry with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA,

USA) as described by the manufacturer using 1 µg of total RNA. The cDNA was used directly for quantitative real-time PCR (qRT-PCR). The qRT-PCR reactions were carried out on an CFX Connect Real-Time PCR system (Bio-Rad Life Science, CA, USA) using a master mix that contained a total volume of 10.5 µL per tube consisting of 6.25 µL of SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., CA, USA), 3.25 µL of nuclease-free water, and 0.5 µL of forward and reverse primers (10 µM). Two µL of cDNA at a 1:5 dilution was added to the master mix for a total reaction volume of 12.5 µL. All samples were evaluated in duplicate. The reactions were initiated with preincubation at 95°C for 3 minutes followed by 42 cycles of denaturation (95°C for 10 seconds) and annealing and extension (60°C for 30 seconds).

The primer sequences for targeted genes were synthesized by Integrated DNA Technologies Inc. (CA, USA) and have been previously reported by our laboratory [17]. Efficiencies of qRT-PCR for amplification of targeted genes were determined in our laboratory and ranged from 95% to 107%. The amplification data obtained from the qRT-PCR were the cycle threshold (Ct) for each mRNA and these was used to calculate the mRNA relative abundance of each sample by the $2^{-\Delta\Delta Ct}$ method [27] using vehicle group as baseline and the geometric mean of the housekeeping genes 36b4 and Hrpt1.

Statistical analysis

All statistical analyses were performed with SAS (version 9.4; SAS Institute Inc., Cary, North Carolina, USA). Data were analyzed with the PROC MIXED procedure using one-way ANOVA, each treatment group was compared to the control group. Residuals with deviations from assumptions of normality and/or homogeneity of variance were transformed into square root, logarithms, or ranks. A probability of ≤ 0.05 indicated a difference was significant and a

probability between > 0.05 and ≤ 0.1 indicated tendency significance. Data are presented as the mean ± standard error of mean (SEM).

Results

Experiment 1.

To assess whether fluoxetine increases estrogen signaling by binding to ER, we performed a uterotrophic assay in immature female mice. Nineteen-day old female mice were treated with saline, 17b-estradiol (positive control, 10 μg/kg), and fluoxetine (low and high dose) for three days and euthanized 24 hours after the last treatment (mice were 22-days old). Fluoxetine at either dosage did not increase uterine weight in contrast to the positive control estradiol-treated group indicating that fluoxetine does not directly bind the ER to stimulate estrogen signaling. Similarly, uterine weight corrected to body weight was increased only in the positive control group.

Experiment 2.

Sexually mature (6-7-weeks old), virgin females were treated with saline or fluoxetine (low and high dose) from experimental day 0 to 6 and euthanized six hours after the last treatment. Uterine weight was different among all groups: with the highest uterine weights observed in the low dose group, intermediate in the control, and lowest in the high dose fluoxetine group suggesting increased estrogen signaling with the low dose fluoxetine treatment. Uterine weight corrected to body weight followed a similar pattern. Expression of alkaline phosphatase (Alkp) was increased 3.1-fold and insulin-like growth factor binding protein 3 (Igfbp3) was downregulated by 47.8% in the low dose group. Compared to control and low dose

fluoxetine groups, expression of Esr2 in the high dose fluoxetine was increased 4.5- and 3.1-fold, respectively. Expression of other evaluated genes was not different among groups. Uterine histology in the low fluoxetine group was consistent with increased estrogen signaling (increased uterine thickness 35.6%, increased epithelial height 58.7%, and increased endometrial grands).

Experiment 3.

To investigate whether fluoxetine disrupts endocrine signaling during decidualization, we mated sexually mature (6-weeks old) females to vasectomized males. After detection of a vaginal plug (experimental day [d] 0), females were treated daily from d0 to d6 and euthanized six hours after the last treatment. Sample collection on d6 was chosen based on the maximal production of estrogen by the uterus of pregnant/pseudopregnant mice [28] which coincided with the timeline of observed increased uterine weight our previous experiments. Uterine weight was increased only in the low dose fluoxetine group.

Experiment 4.

To assess whether the effects of fluoxetine are dependent on SERT, we treated sexually mature (7-8 weeks old), virgin Sert-/- females with saline and low dose fluoxetine. Further, to assess whether the effects of fluoxetine are dependent on peripheral serotonin concentrations, we treated sexually mature (7-8 weeks old), virgin TPH1-/- females with saline and low dose fluoxetine. Mice deficient in TPH1 are unable to produce peripheral serotonin while brain serotonin synthesis is maintained [23, 29]. Because only the low dose fluoxetine treatment appeared to have estrogenic actions (Experiments 2 and 3), it was the only dose of fluoxetine used in the mutant mice experiments. Mice were treated daily from experimental day 0 to 6 and

euthanized six hours after the last treatment. Uterine weight was not affected by fluoxetine treatment in Sert-/- and TPH1-/- genetic models.

Discussion

Given the widespread use of SSRI, understanding its side-effects, particularly those related to reproductive function, is critical for the assessment of the effects on women's health. The present study demonstrates a role for SSRI treatment on modulation of estrogen signaling in vivo. Although the endocrine disrupting effects of SSRI are side-effects of its intended action as an antidepressant, they are mediated by interaction with its biological target site, SERT, rather than an off-target mechanism. Furthermore, these effects are dependent on SSRI modulation of peripheral, but not neuronal, serotonin signaling. Although the notion that SSRI modulates estrogen synthesis/signaling is not novel, we demonstrate for the first time that fluoxetine's in vivo effect on modulation of estrogen signaling is serotonin-dependent and through by inhibition of SERT.

Uterotrophic assays in immature, prepubertal rodents are used for in vivo assessment of the ability of a substance to elicit biological activity consistent with agonist of estrogen receptor [30-32]. As the hypothalamic-pituitary-ovarian axis is not functional in prepubertal animals, endogenous synthesis of estrogen is minimal. Therefore, if a given substance binds estrogen receptor it will stimulate biological response in target tissues such as the uterus with activation of molecular pathways resulting in cell division and growth with consequent increase in uterine weight [30, 31, 33]. The lack of increase in uterine weight in sexually immature mice treated with fluoxetine in the present study differs from the study of Muller and colleagues [16] when using a similar experimental approach and doses of fluoxetine in Wistar rats. In both studies, the

increase in uterine weight in the estradiol-treated positive control group was about 400% compared to the negative control group. In contrast, fluoxetine did not increase uterine weight at either tested dose in our study whereas doses of 1.7 and 17 mg/day, but not 0.4 mg/kg, of fluoxetine increased uterine weight in about 50% compared to the control group in the previous study. This conflicting result may be related to a differential effect of fluoxetine in mice vs rats. However, for uterotrophic assays in immature rodents, it is critical that animals have not entered puberty. In our study, all mice used for the uterotrophic assay were born in our colony and were 19-days old at onset of treatment and 22-days old at euthanasia/assessment of uterus weight. This is in contrast to the later onset of treatment and assessment of uterine weight in the previous study which may had obscured interpretation of the effects of the drug [16]. Nevertheless, it appears that fluoxetine does not bind estrogen receptor to elicit its estrogenic actions.

The increase in uterus weight in sexually mature mice at different stages of the reproductive cycle (experiments 2 and 3) demonstrates the endocrine disruption effects of fluoxetine by modulating estrogen synthesis/signaling [28, 34]. The increase in Alkp, a regulator/marker of uterine stromal differentiation, and decreased Igfbp3 support increased estrogen signaling in addition to the histological alteration in the low dose fluoxetine group [28, 33]. Furthermore, expression of Igfbp3 has been reported to be inversely related to uterine weight as in the present study [33]. Indeed, fluoxetine treatment has been shown to inhibit serotonin uptake into murine primary granulosa cell [15] and oocyte [35] and to increase systemic estradiol in rodents [36]. An important novelty of our study is that the fluoxetine-induced increased estrogen synthesis/signaling is serotonin-dependent and mediated by fluoxetine inhibition of SERT. This was evident by the lack of increase in uterine weight in mice lacking serotonin transporter (Sert-/-) and mice with depleted peripheral synthesis of serotonin

(Tph1-/-). The neuronal synthesis of serotonin is likely unaltered as Tph2 is the main tryptophan dehydrogenase in the brain whereas Tph1 is mostly expressed in the periphery [29]. Therefore, the effects of SSRI on estrogen synthesis/signaling are indirect, that is, dependent on SERT and peripheral serotonin.

Previous studies have shown that serotonin binding to its receptor 2A subtype stimulates estrogen synthesis by enhancing aromatase activity, the enzyme responsible for aromatization of androgens into estrogens [21, 37-39]. Further studies suggested that other serotonin receptors subtype may be involved in this mechanism as multiple serotonin receptors are present in granulosa cells and oocyte [15, 40]. Nevertheless, serotonin stimulates steroid synthesis and secretion by human and rodent ovarian granulosa cells[39-42]. The effects of SSRI on estrogen synthesis have also been investigated human adrenocortical carcinoma cell line (H295R) that can synthesize all steroid hormones [18, 19, 43]. Hansen and colleagues [18] demonstrated that all tested SSRI (fluoxetine, paroxetine, citalopram, escitalopram, sertraline, and fluvoxamine) appear to stimulate estrogen synthesis by enhancing aromatase activity in H295R cells. Similarly, apparent increases in aromatase activity by SSRI have been reported by others using H295R cells [19, 43]. Additionally, fluoxetine also appears to stimulate aromatase activity in human trophoblast-like BeWo cells [20, 21]. Based on the present study, it seems likely that the apparent effects of SSRI on aromatase may be indirect by increasing serotonin signaling as fluoxetine has been shown to inhibit serotonin uptake into granulosa cells [15]. Noteworthy, adrenocortical neoplastic tissues, such as the H295R cell line used in previous experiments, have an increased serotonin synthesis phenotype [44]. Therefore, it is likely that SSRI inhibition of SERT in H295R cells increased serotonin signaling which ultimately increased estrogen synthesis in this cell line. Further studies are warranted to confirm whether fluoxetine itself or the fluoxetine-

induced increase in serotonin, modulate aromatase function to upregulate estrogen synthesis giving rise to its endocrine disruption effects observed in vivo.

Findings from the present study add to our previous study [17] demonstrating that treatment with high dose of fluoxetine for 14 days promotes interruption of estrous cyclicity in mice. The effects of fluoxetine on endocrine regulation and estrous cycles, at least in rodents, appear to be dose-dependent and time-dependent [16, 17, 45-47]. In the present study, only the low dose of fluoxetine increased estrogen signaling after six days of treatment. However, based on our previous study [17], treatment with a low dose of fluoxetine does not affect estrous cyclicity and does not affect uterine weight after 14 days of treatment suggesting that the effects of fluoxetine on estrogen synthesis/signaling may be transient. On the contrary, the high dose of fluoxetine caused interruption of the estrous cycle within days after onset of treatment along with decreased estrogen signaling by day 14 of treatment as observed by decreased uterine weight and altered uterine gene expression. In the present study, the high dose fluoxetine treatment also decreased uterine weight (experiment 3) suggesting that this dose of fluoxetine causes more abrupt effects on estrogen synthesis/signaling with longer lasting effects.

In conclusion, we demonstrated the endocrine disrupting effects of fluoxetine treatment on sexually mature mice is coincident with apparent stimulation of estrogen synthesis/signaling. This effect was serotonin-dependent and SERT-mediated indicating that SSRI act through SERT to increase peripheral serotonin signaling, ultimately increasing estrogen synthesis/signaling. These findings are important given the increasing number of women at reproductive age that are exposed to SSRI medications and the potential adverse side-effects that they may suffer from, including endocrine disruption and sexual dysfunction.

Conflict of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions: RRD conceptualization, methodology, investigation, data curation, formal analysis, project administration, original draft, reviewed and edited the manuscript; JF methodology, investigation, data curation, formal analysis, reviewed and edited the manuscript; ED investigation, reviewed and edited the manuscript; MCW conceptualization, methodology, formal analysis, funding acquisition, supervision, reviewed and edited the manuscript; LLH conceptualization, methodology, formal analysis, funding acquisition, supervision, reviewed and edited the manuscript.

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Figures

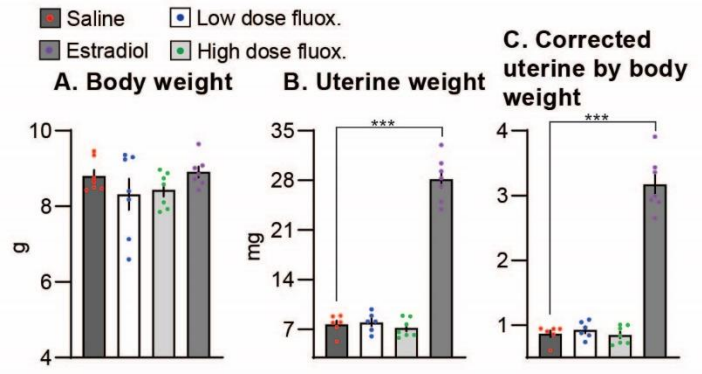

Figure 1. Uterotrophic assay in WT prepubertal mice. Effect of fluoxetine treatment on (A) body weight (B) uterine weight (C) uterine corrected to body weight. Saline n = 7, low dose fluoxetine n = 7, high dose fluoxetine n = 7, estradiol n = 7. ***indicates P value < 0.0001.

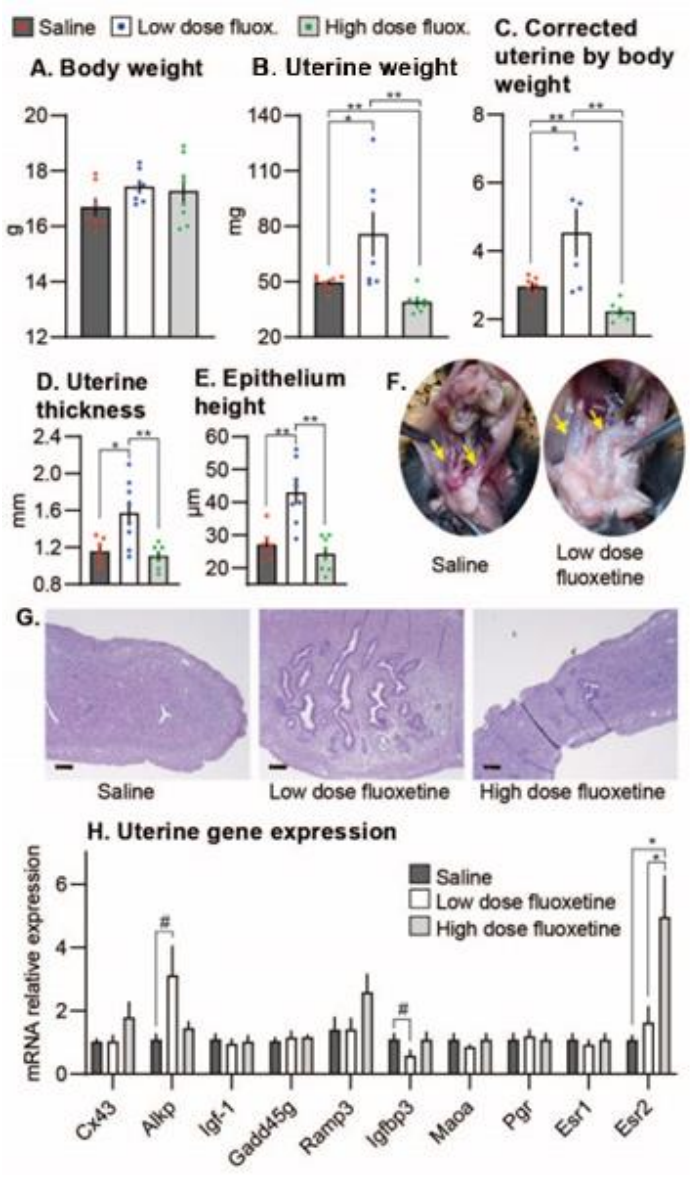

Figure 2. Effect of fluoxetine treatment on WT mice. (A) Body weight (B) Uterine weight (C) Uterine weight corrected to body weight (D) Uterine thickness (E) Epithelium height (F) Representative images of uterus collected from mice treated with saline and low dose fluoxetine. Arrows indicate uterine horns. (G) Representative histological images of the uterus collected from mice treated with saline, low dose fluoxetine, and high dose fluoxetine. Bar = 200 µm. (H) Relative gene expression in uterus from mice treated with saline, low dose fluoxetine, and high

dose fluoxetine. Saline n = 7, low dose fluoxetine n = 8, high dose fluoxetine n = 8. *indicate P < 0.05, **indicates P value < 0.01, #indicates < value < 0.1.

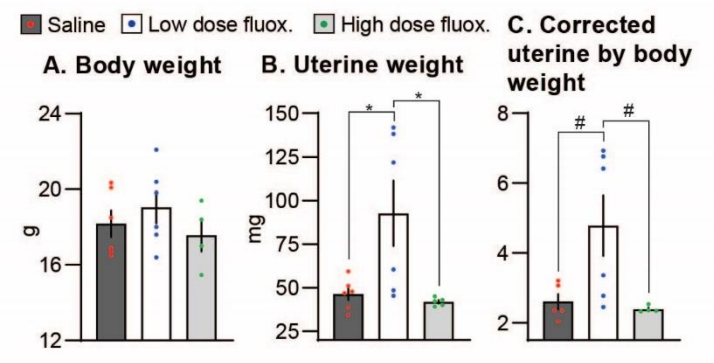

Figure 3. Effect of fluoxetine treatment on (A) body weight (B) uterine weight (C) uterine corrected to body weight in WT pseudopregnant mice. Saline n = 7, low dose fluoxetine n = 6, high dose fluoxetine n = 6. *indicates P value < 0.05, #indicates P Value < 0.1.

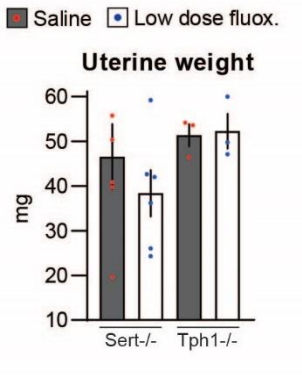

Figure 4. Effect of fluoxetine treatment on uterine weight in serotonin transporter deficient mice (Sert-/-) and tryptophan hydroxylase 1 deficient mice (TPH1-/-). Sert-/- saline n = 6, Sert-/- low dose fluoxetine n = 6, TPH1-/- saline n = 3, TPH1-/- low dose fluoxetine n = 3.

Chapter 8

Conclusions and Future Directions

Impacts of selective serotonin reuptake inhibitors on reproductive biology: maternal use of SSRI during pregnancy

Using murine and ovine animal models we showed that SSRI increases embryonic/fetal mortality, decreases gestation length, decreases birthweight, and increases neonatal morbidity and mortality (Fig. 1). Additionally, we demonstrated the disrupting effects of SSRI on placental homeostasis causing hypoxia, decreased growth, increased pathology (fibrosis, necrosis, infarction, cell death), and transcriptomic alterations. These placental alterations are consistent with our hypothesis that increased serotonin signaling due to fluoxetine treatment decreases placental perfusion, as indicated by upregulation of hypoxia-induced pathways, which, in turn, is associated with placental dysfunction and embryonic/fetal abnormal development.

A groundbreaking aspect of the present study was the implementation of a therapeutic intervention using a serotonin antagonist, ketanserin, to mitigate the impacts of SSRI on pregnancy outcomes (Fig. 1). This was the first successful attempt to prevent SSRI-induced adverse pregnancy outcomes and is critical to allow women the benefit of the antidepressant effects of SSRI without compromising fetal health. Importantly, ketanserin does not interfere with the antidepressant effects of SSRI. Therefore, concurrent SSRI and ketanserin treatment during gestation may benefit women suffering from diverse psychological conditions that require treatment without the detrimental effects of SSRI on pregnancy and neonatal outcomes.

Ketanserin is approved for medical use in pregnant women to treat gestational hypertension in several countries in Europe suggesting that it safe for use in pregnant women.

The utilization of the two animal models was one of the great assets of our studies as we took advantage of intrinsic features of each animal model. For example, we utilized genetic manipulations in mice to evaluate the effect of modulation of maternal vs fetal/placental SERT on pregnancy outcomes. Nevertheless, the short gestation length in mice precluded detailed information on the impact of SSRI on pregnancy length. Similarly, neonatal characteristics of mice precluded assessment of neonatal morbidity. On the contrary, sheep are a better, more translationally relevant model for evaluation of pregnancy length and neonatal assessments of SSRI-induced morbidity. Moreover, in sheep we evaluated placentome size during SSRI treatment period via transabdominal ultrasonography which is not easily available to accomplish in mice. Conversely, we performed detailed evaluation of placenta utilizing histology, immunofluorescence, and RNA sequencing in mice. Taken together, the implementation of murine and ovine animal models provided comprehensive assessment of the effects of SSRI exposure during gestation on pregnancy and neonatal outcomes.

Impacts of selective serotonin reuptake inhibitors on reproductive biology: endocrine disruption

In our studies we observed that treatment with a low dose of fluoxetine causes transient endocrine disruption with modulation of estrogen synthesis/signaling after short-term treatment (six days) but without interruption of estrous cycles (Fig. 2). By day 14 of treatment, endocrine disruption with modulation of estrogen signaling was not observed. Conversely, treatment with high dose of fluoxetine interrupts estrous cycles shortly after onset of treatment and reduced

uterine weight on days 6 and 14 of treatment. Taken together, the effects of fluoxetine on endocrine regulation, particularly estrogen synthesis/signaling, appear to be time- and dose-dependent. Nevertheless, given that high dose treatment of fluoxetine causes transient decrease in weight, the interruption of estrous cycle and decreased uterine weight may be related to physiological alterations associated with the weight loss.

Noteworthy, the endocrine disruptive effects of a low dose of fluoxetine treatment are serotonin-dependent and SERT-mediated. This suggests that the apparent effects of SSRI on aromatase may be indirect by increasing serotonin signaling and serotonin itself ultimately increases estrogen synthesis.

Future directions

Unsurprisingly, while the present thesis has led to the answering of many questions and a better understanding of SSRI effects, a multitude of questions have arisen. Thus, determining where we go from this thesis and other current data is imperative.

An important aspect to consider when evaluating the impacts of SSRI on reproductive physiology is the possible different effect of each SSRI compound: fluoxetine, sertraline, paroxetine, citalopram, escitalopram, and fluvoxamine. All SSRI inhibit SERT uptake of serotonin throughout the body; however, their pharmacokinetics (absorption, half-life, degradation, metabolites, placental transfer) differ quite markedly [21, 38, 49, 53]. The variability in the pharmacokinetics among SSRI, in addition to an individual's capacity to metabolize drugs [55], result in tremendous disparity in systemic concentrations of each SSRI in the maternal circulation and subsequent impact on circulating concentrations of serotonin which may partially account for the apparent differential impact of each SSRI on pregnancy outcomes.

Additionally, the distinct placental transfer of each SSRI may result in different fetal exposure of each drug which may account for differences on neonatal outcomes. These conundrums of the different SSRI add complexity to interpretation of studies that evaluate all SSRI as a single drug category as it is typically done in human studies. It would be important to evaluate the effect of each SSRI compound in animal studies to assess how each SSRI affects serotonin signaling, placental function, pregnancy and neonatal outcomes to possibly identify a drug with more mild effect.

Another important aspect to consider is the effect of exposure to SSRI at different periods of gestation. The impact of interruption of SSRI treatment at different periods of gestation remains to be elucidated. Given the short gestation of mice, the translational relevance regarding period of exposure is limited in this animal model. On the contrary, sheep with a 151-days pregnancy may be more appropriate; nevertheless, given the larger size of sheep, the costs per animal and drugs would be much greater compared to small rodents. Additionally, little is known about the effect of SSRI exposure prior to conception on fertilization rates and early embryonic development. Addressing these issues may create opportunities for more collaborative research, for example to evaluate the impact of SSRI on maternal mental health, fetal physiology and development.

Although our studies demonstrated that increased serotonin signaling is the underlying cause of SSRI-related adverse pregnancy outcomes with consequent placental hypoxia, we did not directly show the serotonin-induced vasoconstriction of uterine/placental blood vessels. Direct demonstration of the uterine and placental vascular perfusion associated with SSRI exposure would be appealing. A possible way to evaluate this is by Doppler ultrasonography of blood vessels and placenta. Aiming to demonstrate the usefulness of this technique for a grant

proposal, we had pregnant mice evaluated at the Small Animal Imaging and Radiotherapy Laboratory (Department of Medicine, University of Wisconsin-Madison). Two Sert-/- dams included in other experiments were available at the time of this procedure. One of them remained untreated while the other one received ketanserin (serotonin antagonist for receptors 2A and 2C) from E10.5 to E17.5. We were able to detect about 40% increase in placental vascular area in the mouse receiving ketanserin (Fig. 3). Although the limited number of mice prevents definitive conclusions, it suggests that ketanserin prevents serotonin-dependent vasoconstriction of uterine/placental blood vessels and decreased placental perfusion. Further studies evaluating placental vascular perfusion with Doppler ultrasound would potentially demonstrate the effects of SSRI modulation of serotonin signaling and the preventive effect of ketanserin.

In relation to the endocrine disruption effects of SSRI, further research is needed to address the interrelationship between SSRI-serotonin-estradiol as several questions remain unanswered. For example, we showed that short-term (six days) fluoxetine exposure (low dose) has estrogenic effect that is not observed after 14 days of treatment. Does a similar phenomenon occur in humans? If so, how long after onset of treatment do the fluoxetine estrogenic actions wear off? Does fluoxetine have similar effects regardless of phase of menstrual/estrous cycle at onset of treatment? These questions are important from a basic science aspect as well as from a translational standpoint. Future research should address the issues related to endocrine disrupting properties of SSRI.

An important step in the area of SSRI medication and reproductive biology is the dissemination of information regarding the risks of SSRI exposure to clinicians. There appears to be a gap between basic sciences pointing out the possible side-effects of SSRI and the increasing number of people prescribed SSRI. There must be a two-way road where basic scientists produce

more translationally relevant research publishing their findings in journals more accessible for clinicians. At the same time, clinicians, particularly psychiatrists, should become more aware of advancements in basic sciences pertaining to the risks of SSRI use. Ultimately, the combination of basic, translational, and clinical research will strength the body of evidence regarding SSRI exposure during gestation bringing basic scientists and clinicians together to improve human health and wellbeing.

Conclusion

The aim of this thesis was to understand the pathophysiology of SSRI-induced adverse pregnancy outcomes and to provide a preventive measure to ameliorate the effects of SSRI. Utilizing murine and ovine animal models and genetic manipulation of the biological target site for SSRI, SERT, we successfully accomplished our objectives. Additionally, the observational finding of the possible effect of SSRI as an endocrine disrupting agent has created a new framework of research related to other unforeseen effects of SSRI on reproductive biology. We were also able to determine the endocrine disruptive effects of SSRI on modulation of estrogen signaling advancing the understanding of SSRI-serotonin-estrogen axis. Taken together, the work in this thesis greatly advanced the knowledge of undesired SSRI effects in the body. We expect the knowledge generated in this thesis will benefit society worldwide, particularly people under diverse psychological distress in need of antidepressant medication.

Figures

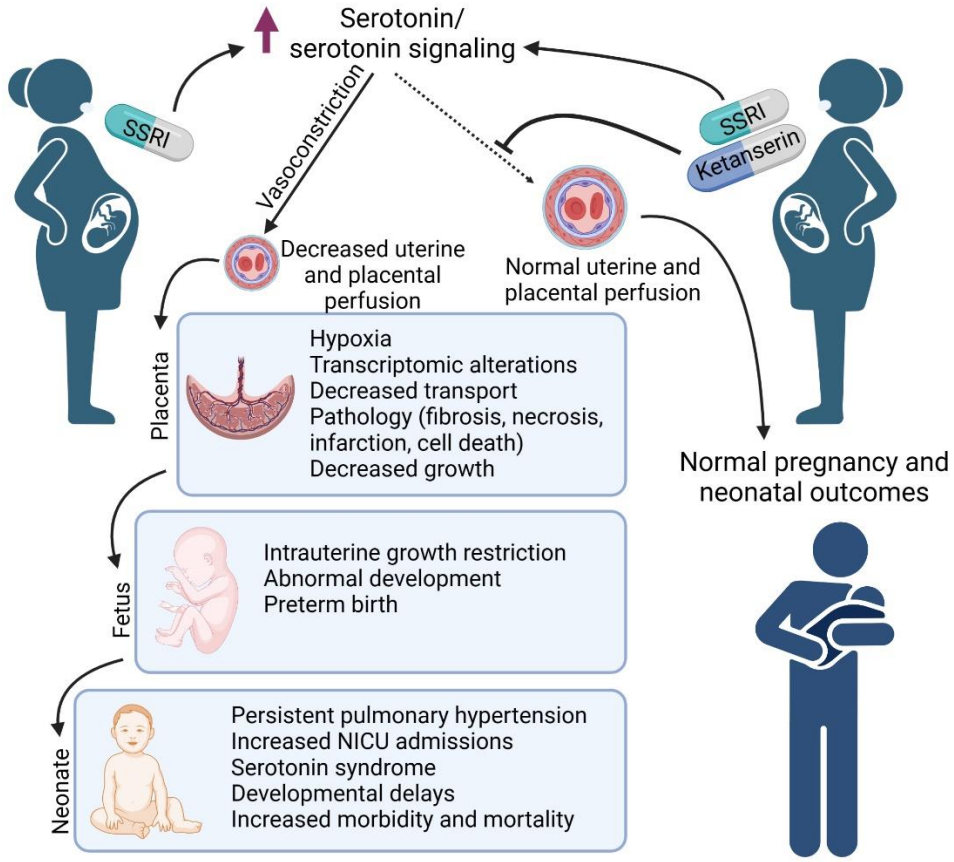

Figure 1. Model for SSRI-induced adverse pregnancy and neonatal outcomes and preventive effect of ketanserin. Treatment with SSRI leads to placental alterations that are associated with adverse embryonic/fetal development ultimately affecting neonatal health. Ketanserin mitigates the effects of SSRI on modulation of uterine/placental blood perfusion mitigating most downstream effects on the placenta, fetal, and neonate.

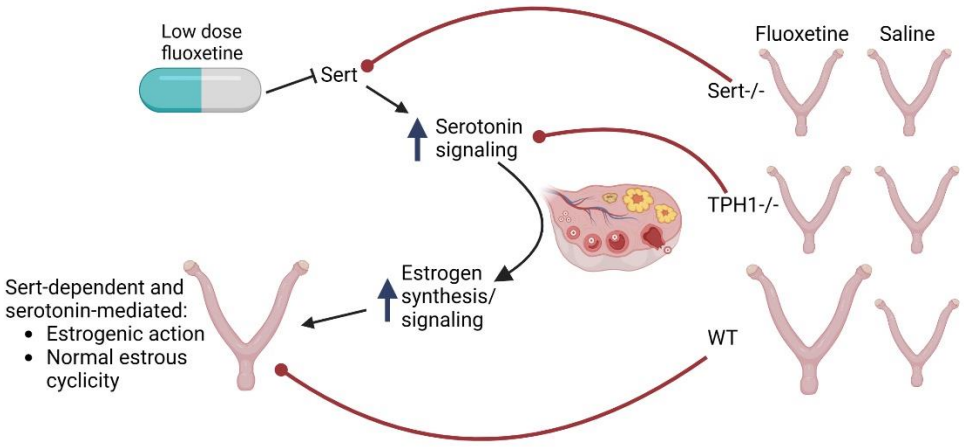

Figure 2. Model for SSRI-induced endocrine disruption. Fluoxetine treatment modulates estrogen synthesis/signaling resulting in increased uterine weight in WT mice. In Sert-/- and TPH1-/- mice, uterine weight is unaltered by fluoxetine treatment indicating fluoxetine's effect are SERT-mediated and serotonin-dependent.

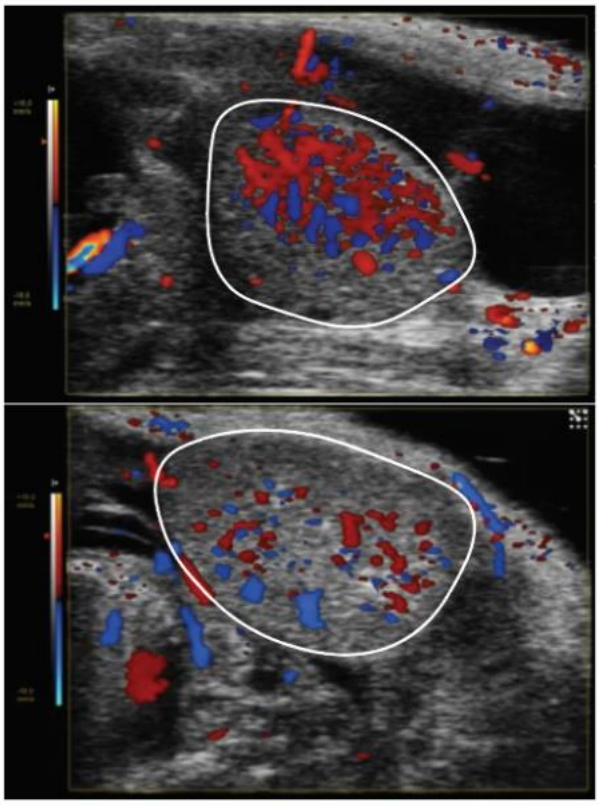

Figure 3. Doppler ultrasonography of placenta vascular area. Note the 40% increase in vascular area in the mouse receiving ketanserin (upper panel) compared to the untreated mouse (lower panel). The placenta is highlighted.