Modulation of Multidrug Resistance Phosphoglycoprotein in the Mouse Placenta and Fetal Brain by the Selective Serotonin Reuptake Inhibitor Sertraline and Maternal Bacterial Infection

by

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A thesis submitted to conformity with the requirements for the degree of Master of Science

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ABSTRACT

Thesis title: Modulation of Multidrug Resistance Phosphoglycoprotein in the Mouse Placenta and Fetal Brain by the Selective Serotonin Reuptake Inhibitor Sertraline and Maternal Bacterial Infection

Degree: MSc

Year of convocation: 2011

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Multidrug resistance phosphoglycoprotein (P-gp) is expressed in the placenta and fetal blood-brain barrier (BBB) and plays a critical role in reducing fetal accumulation of xenobiotics. In other tissues, P-gp activity is inhibited by selective serotonin reuptake inhibitors (SSRIs) and by lethal doses of LPS modeling a bacterial infection. However, nothing is known with respect to the effects of SSRIs or nonlethal infection on P-gp activity in the placenta or fetal tissues. In the studies presented in this thesis, we hypothesized that (1) the SSRI sertraline and (2) a nonlethal maternal bacterial infection would decrease P-gp activity in the placenta and fetal BBB. The first study shows that sertraline affects P-gp activity at these barrier sites in a tissue-specific manner. The second study shows that nonlethal infection does not significantly affect P-gp activity at either site. However, nonlethal infection may still influence substrate biodistribution by altering hepatic elimination of these substrates.
Acknowledgments

This thesis is dedicated to my mother, Hena, and my father, Azad.

If I began to list all of the individuals that I am indebted to for their support over the past two years, the length of that list would rival the length of this thesis. So, rather than thanking them here, I would prefer to thank most of them privately. Nevertheless, I would like to acknowledge a few individuals who have been monumental in shaping my development as a graduate student.

First and foremost, I am eternally grateful to Dr. Stephen Matthews for all of his guidance, patience, and encouragement. He is a compassionate supervisor and a truly remarkable PI. I wish him all the best in the future and am certain that he will continue to do great work in the field of reproductive biology and endocrinology.

I would also like to thank my committee members, Dr. Isabella Caniggia, Dr. Stephen Lye, and Dr. William Gibb. I am especially indebted to Dr. Gibb whose cheerful face appears in all of our weekly lab meetings and whose invaluable feedback enhances all of my work.

I must also acknowledge all of my friends in the Matthews lab and at the Department of Physiology. Only my friends from the 2010 UofT/Karolinska exchange will understand the following analogy. Being a graduate student can sometimes feel like being stuck in an elevator at SickKids. You have an idea of where you’re going but aren’t really sure when you’re going to get there. At first, you feel like you’re going to suffocate. But as time goes by and you get to know your mates better, things don’t seem so bad because ultimately you’re all in this together.
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Abbreviations

ABC: ATP-binding cassette
ABCB1: ATP-binding cassette, sub-family B
BBB: blood-brain barrier
BCRP: breast cancer resistance protein
CYP3A2: cytochrome P450 3A2
E: embryonic day
HPA: hypothalamic-pituitary adrenal
i.v.: intravenous
IL-1β: interleukin-1 beta
IL-6: interleukin-6
IL-10: interleukin-10
KO: knockout
LPS: lipopolysaccharide
mRNA: messenger ribonucleic acid
MRP1: multidrug resistance protein-1
NBD: nucleotide-binding domain
Oatp: organic anion-transporting polypeptide
Oatp2: organic anion-transporting polypeptide 2
OATP-4C1: organic anion-transporting polypeptide-4C1
OATP-8: organic anion-transporting polypeptide-8
P-gp: multidrug resistance phosphoglycoprotein
PVN: paraventricular nucleus
qPCR: quantitative polymerase chain reaction

PND: post-natal day

SEM: standard error mean

SRY: sex-determining region Y

SSRI: selective serotonin reuptake inhibitor

TM: transmembrane

TMD: transmembrane domain

TNF-α: tumor necrosis factor alpha

VEH: vehicle
Chapter 1: Introduction
1.1 MULTIDRUG RESISTANCE PHOSPHOGLYCOPROTEIN

1.1.1 Discovery, Localization, Function

In 1976, Rudolph L. Juliano and VA Ling discovered a carbohydrate-containing component of 170 kDa in drug-resistant cell membranes of the Chinese hamster ovary. This component reduced membrane permeability of a wide range of amphiphilic drugs. Through the use of selective proteolysis, they showed that this component was a cell surface glycoprotein. This glycoprotein was unique to mutant cells displaying reduced permeability. Consequently, it was named P-glycoprotein (P-gp; ‘P’ for permeability). In the mid-1980s, P-gp was discovered to be involved in conferring multidrug resistance to tumor cells. By actively effluxing chemotherapeutic drugs out of their target cells, P-gp prevented accumulation of these drugs in the cytoplasm of multidrug-resistant cells. It was soon discovered concurrently by several groups that P-gp was the product of a gene, \( ABCB1 \). This was followed by the discovery of two multidrug resistance mouse genes, \( Abcb1a \) and \( Abcb1b \), that together functionally resemble human \( ABCB1 \).

Since their discovery in cancer cells, the \( ABCB1 \) genes and P-gp have been identified in normal tissues including the heart, intestinal epithelium, liver, lungs, kidneys, as well as various barrier sites such as the blood-brain barrier, blood-placental barrier, and blood-testes barrier. In these normal tissues, P-gp is believed to reduce absorption, assist with excretion, and prevent xenobiotic accumulation. Many clinically relevant P-gp substrates have been identified since it was shown that P-gp could efflux chemotherapeutic drugs out of tumor cells. These include various analgesics (e.g., morphine), cardiac glycosides (e.g., digoxin), herbicides and pesticides (e.g., ivermectin),
HIV protease inhibitors (e.g., saquinavir), and synthetic and endogenous steroids (e.g., dexamethasone, cortisol) \(^{18-24}\). By preventing xenobiotic accumulation, P-gp plays a protective role in these normal tissues. Nevertheless, the same qualities that allow P-gp to protect tissues from xenobiotic accumulation make it a hindrance to effective cancer therapy. Many P-gp substrates are clinical drugs. By actively effluxing these drugs out of their target tissues, P-gp decreases the therapeutic benefits of these drugs. This requires them to be administered at higher doses and thus increases their deleterious side effects. For example, side effects for saquavir, digoxin, and other P-gp substrates may include diarrhea, vomiting, stomach pains, skin rash.

In humans, one gene (\(ABCB1\)) encodes P-gp in all of the tissues in which it is expressed \(^4\)\(^-\)\(^6\). Conversely, in the mouse, two genes encode P-gp: \(Abcb1a\) and \(Abcb1b\) \(^{25,26}\). Each gene encodes a unique P-gp protein isoform and shares an 84% amino acid identity with the other \(^{25}\). To date, the key distinction between these two P-gp protein isoforms is that they have been found to be differentially expressed in different tissues. While \(Abcb1a\) is predominantly expressed in the intestinal epithelium, liver, lungs, kidneys, and blood-brain barrier, \(Abcb1b\) is almost exclusively expressed in steroidogenic tissues such as the adrenal, ovary, and placenta \(^{12,27,28}\). A functional difference between the two isoforms has yet to be conclusively elucidated.

1.1.2 Structure

P-gp can be divided into two halves, each sharing a high degree of sequence similarity with the other \(^{29}\). Studies comparing the two halves suggest that P-gp formed from the
duplication of a primordial gene. Each half comprises one hydrophobic transmembrane domain (TMD) and one hydrophilic nucleotide-binding domain (NBD). Thus, the entire protein contains four domains: two hydrophobic TMDs and two hydrophilic NBDs. Each TMD contains six transmembrane segments (TM). Thus, the entire protein comprises 12 TMs. These are localized to the apical part of the plasma membrane. Each TMD is associated with one NBD. The NDB is located on the cytoplasmic side of the membrane. Each NBD possess three characteristic motifs. These motifs are called Walker A, Walker B, and the ABC signature (C). A 90-120 amino acid-long spacer region separates the Walker A and Walker B sites. These two sites extensively hydrogen bond with the attached ATP. The ABC signature motif is found upstream of the Walker B site. Substrate-binding sites have been found in five TMs: 1, 5, 6, 11, and 12.

The transport mechanism for P-gp is not completely understood. The most accepted model of P-gp substrate transport is the ‘flip-flop’ model. In this model, xenobiotics are pumped from the plasma membrane’s inner leaflet to its outer leaflet and subsequently pumped out of the cell. The name of the model derives from the notion that substrate binding leads to structural rearrangements of the P-gp molecule. This is subsequently followed by conformational changes that allow the substrate to move across the membrane.
1.1.3 Regulation

The physiological function of P-gp is a double-edged sword. While P-gp protects normal tissues by effluxing xenobiotics back into circulation, it also hinders the efficacy of cancer therapy by effluxing anticancer drugs out of tumour cells. Thus, there has been an ongoing search for effective P-gp modulators that could be used to reduce P-gp-mediated substrate efflux in tumour cells thereby allowing anticancer drugs to accumulate in these cells and subsequently destroy them. This search is into its third generation. First-generation P-gp modulators were identified among drugs already approved for other conditions. These include drugs such as cyclosporin A, verapamil, and progesterone. In cancer therapy, these drugs produced a plethora of deleterious effects and thus are only used today as comparative indices for newly-designed P-gp modulators. Second- and third-generation P-gp modulators were produced after chemical modifications of first-generation P-gp modulators. These include MS-209, PSC833, VX-710, and XR9576. Studies have also shown that phosphorylation of P-gp by protein kinase C plays an important modulatory role in the P-gp-mediated substrate efflux process. Specifically, it has been shown that basal phosphorylation of P-gp in multidrug-resistant human carcinoma cells reduced accumulation of the P-gp substrate [3H]vinblastine indicating that phosphorylation increases P-gp activity. Studies also suggest that phosphorylation of P-gp by protein kinase A and protein kinase C modulate cell swelling-activated chloride currents.

There are three in vitro criteria for determining the identity and potency of a P-gp modulator: drug accumulation, drug efflux, and cytotoxicity. ‘Drug accumulation’ and
‘drug efflux’ are mechanistic criteria. With respect to ‘drug accumulation’, the idea is that incubating P-gp-expressing tumor cells with an anticancer drug, in the presence and absence of the P-gp modulator, should result in higher intracellular drug accumulation in the latter case. With respect to ‘drug efflux’, efflux of the anticancer drug from these cells should be significantly faster in the absence of the P-gp modulator than when it is present. ‘Cytotoxicity’ is a functional criterion. The idea is that treating P-gp-expressing tumor cells with a combined regimen of the anticancer drug and the P-gp modulator should significantly increase cell demise when compared to treatment with the anticancer drug alone. If a compound meets all three criteria, it will be assessed further with respect to its usefulness in cancer therapy.

Nevertheless, the benefits of P-gp modulators must not be considered limited to their usefulness in cancer therapy. As already mentioned, P-gp is expressed in numerous normal tissues in the body and effluxes numerous therapeutic drugs. Thus, it is possible that effective modulation of P-gp at these tissue sites may influence the accumulation of these drugs. For example, analgesics may be more easily targeted to μ-opioid receptors in the brain, cardiac glycosides may be more easily targeted to the heart, and protease inhibitors may be more easily targeted to HIV protease-1. Conversely, P-gp modulators could deleteriously affect tissues by increasing unwanted intracellular accumulation of P-gp substrates. Consequently, it is important to identify P-gp modulators so that precautions are taken during drug therapy. This is because if a certain drug being given to a pregnant woman is also a P-gp modulator, it may affect placental P-gp and thereby cause unwanted fetal accumulation of circulating P-gp substrates.
1.2 P-GLYCOPROTEIN IN THE PLACENTA

1.2.1 The Placenta

The placenta was once believed to be a complete physical barrier between maternal and fetal circulation. Today, it is well-known that the placenta attaches the growing fetus to the uterine wall and permits the exchange of gas, the removal of waste, and the transport of nutrients. The placenta is also responsible for producing and releasing hormones, guarding the fetus against the maternal immune system, and sustaining a favourable uterus for the fetus. The placenta is of fetal origin but functions as a fetomaternal organ. It has two components: the chorion frondosum (fetal) and the decidua basalis (maternal).

There are three types of placentation in eutherian mammals. They are distinguished based on the relationship between the fetal trophoblast cells and the maternal blood. In the epitheliochorial placenta, the trophoblast cells of the placenta are directly apposed to the uterine epithelium. There is no trophoblast cell invasion beyond this layer. In the endotheliochorial placenta, trophoblast cells invade the uterine epithelium and find themselves directly apposed to endothelial cells of maternal uterine blood vessels. In the hemochorial placenta, trophoblast cells proceed to invade these endothelial cells. This leads to rupture of the maternal uterine blood vessels causing blood to be discharged into the intervillous space. The outer syncytiotrophoblast layer is consequently bathed in blood. The human and mouse placentas are both of this type.
Structurally, the hemochorial placentas of the human and the mouse are very similar. Each comprises three sections. The section closest to the mother comprises the decidualized maternal endometrium and myometrium. The middle section is known as the “junctional zone” in mice and the “basal plate” in humans. Maternal blood vessels pass through this section, which allows blood to flow in and out of the growing fetus. The section closest to the fetus is known as the “labyrinth” in mice and the “fetal placenta” in humans. This section possesses the interhemal barrier and is the site of exchange between maternal and fetal circulation. This is also the section that most clearly distinguishes the human placenta from the mouse placenta. While the interhemal barrier of the human placenta has a single layer of syncytial trophoblasts, that of the mouse has two syncytial trophoblast layers. Consequently, the human placenta is termed “hemomonochorial” while the mouse placenta is termed “hemotrichorial”. Despite this difference, the two syncytial trophoblast layers in the mouse placenta is considered functionally analogous to the single syncytial trophoblast layer in the human placenta. The placenta is considered to be functional once hemotrophic exchange begins. This exchange begins during the first trimester in humans and on embryonic day 10.5 (E10.5) in mice.

The placenta is the gateway to the fetus. It is the organ responsible for ensuring that the developing fetus continually receives proper nutrition and that it is protected from a plethora of potential xenobiotics. Studies suggest that between 64-95% of pregnant women in the United States take at least one prescription drug in order to treat various ailments, including depression, diabetes, HIV, and hypertension. It has also
been noted that 5-10% of pregnant women take prescription drugs that are considered teratogens. Many of these xenobiotics may alter placental transfer or be transferred across the placenta themselves. Consequently, it is imperative to understand the role that P-gp plays in the placenta and at the fetal blood-brain barrier in influencing the relative biodistribution of these drugs.

While it would be ideal to examine the role of P-gp directly in the human, several issues associated with the use of the human placenta, and of human tissues in general, make it nearly impossible to do the studies presented in this thesis in humans. For one, there are various ethical issues associated with the acquisition of preterm human placentas from terminated pregnancies. Even after overcoming these obstacles, human placental tissue is not always readily available and not always consistent in quality. To further this point, some studies, such as those presented in this thesis, require placentas and other tissues (i.e., fetal tissues, maternal brain) to be collected during very specific points in gestation in order to examine changes that occur during very narrow critical windows of fetal development. Thus, issues associated with the relative unavailability of human placentas become exponentially more problematic when considering the requirement that the placentas be from specific points in gestation. Finally, it may be impossible to collect human fetal tissues and maternal brains for the aims presented in this study. Consequently, these issues are overcome through the use of the mouse model. For one, it is significantly easier to collect maternal and fetal tissues during specific points in mouse gestation. One can also ensure that the quality of the tissues is consistent. These factors then ensure that the measurement of the dependent variables is significantly more
reliable. In addition, it is also easier to control the independent variables in the mouse. One can imagine how difficult it would be to analogize the maternal and fetal effects of sertraline to that of fluoxetine considering the varying pharmacological effects of different SSRIs as one will soon see. Confound this analysis with the varying doses and timing schedules that mothers are typically put on and one can see that the human is an impractical species for the aims of these studies. With the mouse model, one can control for the type of drug, the dose of drug, the timing of administration as well as a variety of other parameters. In addition, since the human and mouse placentas are structurally and functionally very similar, the mouse represents a good model for the aims of these studies. While there are some disadvantages to using the mouse model specifically with respect to the small size of the tissues, the relative immaturity of newborns, and the matter of shallow trophoblast invasion, these limitations are not major issues in terminal transport studies where mothers are euthanized prior to delivery and substrates accumulation is measured in whole tissue. However, one key issue still remains: how similar is P-gp expression and function in the mouse and the human?

1.2.2 Placental P-glycoprotein

In both the mouse and the human, placental P-gp is localized to the apical membrane of the syncitium (Fig. 1); levels peak in each species during mid- and early gestation, respectively, and dramatically decline near term. In the mouse, Abcb1a/1b mRNA levels are most elevated at E12.5 in the mouse. While the peak is somewhat more difficult to ascertain in the human due to the limited availability of human placentas, it has been shown that ABCB1 mRNA levels are elevated between 7-13 weeks and significantly
decline over the course of gestation. This decrease in placental P-gp expression correlates with an increase in transport of P-gp substrates across the placenta suggesting that the fetus becomes more susceptible to xenobiotics with advancing gestation.

The few studies that have examined the role of placental P-gp have shown significantly elevated transplacental transfer of various radiolabelled P-gp substrates, including [\(^3\)H]digoxin, [\(^3\)H]paclitaxel, and [\(^3\)H]saquinavir, during mid-gestation in placental Abcb1a/b KO mice. [\(^3\)H]digoxin is considered to be the pharmacological gold standard for measuring placental P-gp-mediated drug transport, in vivo. Collectively, these studies show that placental P-gp plays an important role in limiting the transfer of xenobiotics into the fetus.

Even fewer studies have examined the regulation of P-gp in the placenta. While there have been a few studies examining the role that steroids, such as progesterone and glucocorticoids, can play in modulating the expression and activity of placental P-gp, there has been a lack of studies that assess the effects of the maternal environment on P-gp-mediated drug transport in the placenta. Considering the ‘gatekeeper’ role that placental P-gp plays at the interface between mother and fetus and the wide variety of substrates that P-gp can effectively transport, it is critical to understand how placental P-gp is modulated, both for the benefits of fetal protection and fetal therapy.
1.3 P-GLYCOPROTEIN IN THE BLOOD-BRAIN BARRIER

1.3.1 The Blood-Brain Barrier (BBB)

While the placenta was once believed to be a complete physical barrier between the maternal and fetal circulations, the blood-brain barrier (BBB) was once thought to be a passive barrier. Today, it is well-known that the BBB is a complex structure that regulates the transfer of solutes between the circulatory system and the brain. At this interface, endothelial cells are bound together by tight junctions composed of transmembrane proteins. Each of these proteins is anchored to the endothelial cells by other protein complexes including zonula occludens-1 and other associated proteins. While these tight junctions prevent large proteins and hydrophobic compounds from crossing the BBB, smaller, lipid-soluble molecules can simply diffuse across the BBB. The cellular and molecular mechanisms responsible for the development and differentiation of a functional fetal BBB have yet to be clearly understood.

1.3.2 BBB P-glycoprotein

While the development of the BBB is a complex process, one of the key determinants of a functional barrier is the presence of drug efflux transporters, including P-gp. In fact, in both the mouse and the human, one of the first indicators of a developing BBB is the presence of P-gp. This is supported by the fact that tight junctions and high electrical resistance, indicating a functional BBB, are evident as early as E11 while P-gp is found in the mouse fetal BBB as early as E10.5 and in the human fetal BBB as early as 8 weeks of gestation.
In both the adult and fetal BBB, P-gp is localized to the apical surface of the capillary endothelial cells (Fig. 2) where it functions to reduce xenobiotic accumulation in the brain by pumping them back into the capillary lumen \(^{73}\). Studies have shown that \(Abcb1a\) encodes the predominant P-gp isoform in the BBB \(^{19}\) and that \(Abcb1a\) expression increases dramatically from E15.5 to E18.5 \(^{74}\). This increase in fetal BBB P-gp expression correlates to a decrease in transport of P-gp substrates across the fetal BBB suggesting that the fetal brain becomes less susceptible to xenobiotics with advancing gestation \(^{74}\). This expression profile at the fetal BBB is opposite of that at the placenta.

There is a scarcity of knowledge on the role of P-gp in the human fetus. It is very difficult to acquire human fetal brains, and even tissues acquired post-mortem may be inappropriate for studies such as those presented in this thesis because the death of the subject from whom these tissues were acquired was most likely the result of a confounding pathological condition that may have altered the expression of P-gp. Consequently, the mouse model serves as an appropriate substitute for assessing fetal BBB P-gp function because: 1) P-gp is present so early in gestation in both the mouse and the human, and 2) its expression profile is similar in these two species.

There have also been very few studies that examine regulation of fetal BBB P-gp. Our lab has recently shown that dexamethasone treatment during mid-gestation modified \(Abcb1a\) mRNA expression and P-gp function in a dose-, age-, and sex-specific manner in the fetal brain \(^{74}\). Considering that fetal BBB P-gp is present at such an early point in gestation, that its expression increases over the course of gestation, that adult BBB P-gp can prevent
a wide variety of substrates from entering the brain, and that P-gp can be regulated in other tissues, it is also critical to understand how fetal BBB P-gp is modulated.

1.4 SELECTIVE SEROTONIN REUPTAKE INHIBITORS (SSRIs)

1.4.1 Overview

Selective serotonin reuptake inhibitors (SSRIs) are a class of compounds used as antidepressants in the treatment of depression, anxiety disorders, and some personality disorders. They increase the extracellular concentration of serotonin by inhibiting its reuptake into the presynaptic neuron. This increases the amount of serotonin in the synaptic cleft available to bind to the postsynaptic receptor. SSRIs are most widely prescribed antidepressants in many countries.

1.4.2 SSRIs and Pregnancy

10-15% of pregnant women are afflicted with a mood disorder. Maternal depression has been linked to a greater risk of miscarriage, preterm delivery, and lower newborn birth weight. It is therefore recommended that pregnant women be prescribed the minimal dose of SSRIs needed to control depression during pregnancy. While SSRI use during pregnancy has been shown to be effective in controlling maternal depression, it has been linked to an increased risk of preeclampsia in pregnant women and to a variety of complications in the fetus.

Preeclampsia is a condition that is characterized by gestational hypertension and proteinuria. Studies have shown that gestational hypertension and preeclampsia have a
significantly higher incidence rate in pregnant women treated with SSRIs, particularly those who continued to receive treatment beyond the first trimester. This relationship may be mediated by activin A. Studies have shown that activin A is increased in the plasma of pregnant women given SSRIs. Maternal serum activin A has been shown to be a predictor for intrauterine growth restricted fetal and maternal preeclampsia. Consequently, maternal SSRI use may increase the risk of preeclampsia, especially when use is continued beyond the first trimester.

With respect to the fetus, it has been reported that chronic prenatal exposure to SSRIs produces a host of complications, particularly affecting behavioural development and neurodevelopment of the neonate. Studies have shown that chronic SSRI treatment during pregnancy significantly increases the rate of neonatal behavioural syndrome wherein the newborn presents signs of irritability, prolonged crying, seizures, tremors, and difficulty feeding and sleeping. In addition, there have been reported deficits in cognitive ability in neonates that are prenatally exposed to SSRI. It has been shown that children (6 to 40 months) prenatally exposed to SSRIs and other antidepressants produced lower scores on the psychomotor and behavioural development subscales of the Bayley Scales of Infant Development, a cognitive test to assess global IQ. Chronic prenatal SSRI exposure has also been shown to produce changes to the programming of the HPA axis. Newborn humans prenatally exposed to SSRIs have shown significantly reduced plasma cortisol levels at birth and at 3 months of age. Chronic fluoxetine treatment has also been shown to produce an attenuated ACTH and cortisol response in rats and humans.
One reason for these behavioural and neurodevelopmental effects could be due to the effects that these SSRIs are having on substrate transport at the BBB. Studies have shown that SSRIs inhibit P-gp function in the adult BBB. If chronic SSRI treatment causes a prolonged downregulation of fetal BBB P-gp activity, this could facilitate the continuous accumulation of teratogens in the fetal brain. The detrimental effects of these teratogens on fetal brain development may be responsible for many of these behavioural and neurodevelopmental abnormalities. For example, natural and synthetic steroids are P-gp substrates. Synthetic glucocorticoid exposure in utero has been shown to cause a lifelong reduction in basal HPA function and stress response. Consequently, many natural steroids circulating in the fetal body may accumulate in the fetal brain while the fetus is being exposed to SSRIs during critical windows of development. This may alter programming of the HPA axis. Major depression has been linked with elevated cortisol secretion. It is also likely that these behavioural and neurodevelopmental effects are due to interactions between the serotonergic system and the HPA axis during development. Serotonin is important for the mediation of the stress response and for the secretion of cortisol from the adrenal cortex. Many serotonergic projections that originate at the raphe nucleus regulate the activity of the paraventricular nucleus (PVN). Consequently, chronic prenatal SSRI exposure may modify serotonin levels at these synapses in the fetal brain, which may alter the normal development of the HPA axis. In all likelihood, these behavioural and neurodevelopmental abnormalities are the cumulative outcome of the direct effects of chronic SSRI treatment on the serotonergic...
system and HPA axis in addition to the effects that these SSRIs have on substrate accumulation in the fetal brain.

Due to the prevalence of SSRI use during pregnancy, it is important to understand how the SSRIs affect P-gp-mediated substrate efflux at the placenta and fetal BBB and how they affect fetal exposure to xenobiotics. Studies have already shown that SSRIs can affect P-gp function in other tissues.

### 1.4.3 SSRIs and P-glycoprotein

The first study to find an association between P-gp transport and antidepressants examined BBB transport of citalopram using monolayers of bovine brain microvessel endothelial cells (BMECs)\(^9^5\). It was shown that citalopram crossed the BBB using a non-stereoselective and bidirectional carrier-mediated mechanism\(^9^5\).

Uhr and colleagues published the first study to directly assess the relationship between P-gp and the SSRIs, \textit{in vivo}\(^9^6\). They found that plasma concentrations and brain:spleen ratios of the antidepressant amitriptyline and its metabolites were significantly higher in Abcb1a knockout mice. Conversely, there were no differences between groups in the tissue distribution of the SSRI fluoxetine and its metabolites among the various tissues tested. Nevertheless, this was the first paper to show that antidepressants could act as P-gp substrates and that Abcb1a activity at the level of the BBB reduces the penetration of these substances into the brain. Uhr and colleagues went further to show that the antidepressants paroxetine and venlafaxine were significantly increased in the brains of
*Abcb1a/1b* knockout mice both after a single dose administration and after treatment for 11 days\textsuperscript{89}. This latter study was the first to suggest that SSRIs could act as P-gp substrates. Considering the severe effect of SSRIs on the serotonergic system and the ubiquitous localization of P-gp in the body, over the past decade more groups began to examine any potential interactions between the SSRIs and P-gp in the plethora of tissues in which P-gp is expressed.

Weiss and colleagues were the first to demonstrate the ability of antidepressants to inhibit P-gp\textsuperscript{90}. Specifically, they determined the effects of various SSRIs on P-gp function in L-MDR1 cells and primary cultures of porcine brain capillary endothelial cells and found sertraline, desmethylsertraline and paroxetine to be the most potent P-gp inhibitors.

Peer and colleagues demonstrated that the SSRI fluoxetine meets all three *in vitro* criteria for being a P-gp inhibitor: drug efflux, drug accumulation, and cytotoxicity\textsuperscript{97}. Specifically, in the P-gp-expressing human HCT-15 cell line, fluoxetine slowed drug efflux and increased cellular accumulation (12-fold), resulting in increased cytoxocity (10- to 100-fold) of the anticancer drug, and P-gp substrate, doxorubicin\textsuperscript{97}. In mice, doxorubicin treatment combined with fluoxetine significantly decreased tumor progression compared to doxorubicin treatment alone\textsuperscript{98}. This suggested that fluoxetine reduced P-gp-mediated efflux of doxorubicin from tumor cells, thereby increasing the therapeutic effect of the anticancer drug.
Despite the discoveries made over the past decade, no study has investigated the in vivo effects of SSRIs on P-gp-mediated substrate efflux. To our knowledge, the studies presented in the thesis are the first of their kind to examine any such effects.

1.5 BACTERIAL INFECTION

1.5.1 Overview

While the vast majority of bacteria are harmless, those that are pathogenic cause a plethora of different types of infections. Bacterial infections, including pneumonia, tetanus, and typhoid are significant problems in developing nations. Tuberculosis, caused by the bacterium *Mycobacterium tuberculosis*, has one of the highest disease burdens in the world killing millions of people ever year. While some of the more prevalent bacterial infections, such as tuberculosis and pneumonia, affect pregnant and non-pregnant women alike, others have been specifically shown to produce devastating effects to mother and fetus during pregnancy.

1.5.2 Bacterial Infection and Pregnancy

Bacterial infections can affect pregnant women from implantation of the fertilized ovum through the time of delivery and peripartum period. They have also been shown to affect the fetus. Many pregnant women with these infections are asymptomatic. This highlights the key importance of clinical awareness and adequate screening.

Experimentally, bacterial infection is modeled by lipopolysaccharide (LPS). LPS is a component of the outer membrane of gram-negative bacteria. Gram-positive bacteria lack
LPS because they do not possess an outer membrane. While the prevalence of gram-positive and gram-negative bacterial infections is unclear, infections caused by the latter are a major concern in North America and Europe. It is estimated that annual treatment costs for gram-negative infections range from $5 billion to $10 billion. Gram-negative bacterial infections afflicting pregnant women include urinary tract infections, bacterial vaginosis, and various STIs such as Chlamydia and gonorrhea. Some urinary tract infections (UTIs), such as acute pyelonephritis, are more common in pregnant women. Other UTIs, such as asymptomatic bacteriuria and cystitis, are difficult to diagnose because of a lack of presentable symptoms. Chlamydia is the most common bacterial STD in the United States and is a major cause of complications in pregnancy and disease transmission in newborns. The infection is asymptomatic in 75% of women and can cause acute PID, acute urethral syndrome, cervicitis, and endometritis in all women. It can also lead to chorioamnionitis, gestational bleeding, and postpartum endometritis in pregnant women. Gonorrhea is second to Chlamydia in terms of its prevalence in the United States and is asymptomatic in approximately 50% of patients. Gonorrhea during pregnancy has been linked to an increased risk of chorioamnionitis, endocervicitis, intrauterine growth retardation, postpartum sepsis, premature rupture of fetal membranes, prematurity, and septic abortion. Bacterial vaginosis affects 16% of pregnant women in the United States and can lead to premature labor.

Since it has been shown that bacterial infections are prevalent during pregnancy, it is important to know whether and how they affect P-gp-mediated substrate efflux at the placenta and fetal BBB. Studies have already shown that bacterial infections inhibit P-gp
expression in other tissues. Thus, it is quite possible that much of the teratogenic outcomes associated with bacterial infections are due to an infection-induced inhibition of P-gp-mediated substrate efflux at these barrier sites.

1.5.3 Bacterial Infection and P-glycoprotein

The first study to identify an association between infection and P-gp function specifically examined the effects of an acute turpentine-induced infection on P-gp expression in the rat liver\textsuperscript{101}. The infection significantly reduced hepatic \textit{Abcb1a/1b} mRNA expression and P-gp protein expression by 50-70\%. It also caused a significant reduction (45-65\%) in efflux of the P-gp substrate, Rho-123.

Hartmann and colleagues were the first to use lipopolysaccharide (LPS) to assess the effects of infection on multidrug resistance\textsuperscript{102}. They also delineated the role of different pro-inflammatory cytokines in modulating P-gp expression and function. LPS (5 mg/kg) produced significant reductions in the hepatic \textit{Abcb1a/1b} mRNA expression. The pro-inflammatory cytokine IL-6 produced a 40-70\% reduction in \textit{Abcb1a/1b} mRNA levels and a 70\% reduction in P-gp protein expression. These findings indicated that IL-6 plays a critical mediating role in the downregulation of P-gp in the livers of mice during an inflammatory response. Interestingly, IL-1\(\beta\) decreased \textit{Abcb1a} mRNA expression but significantly increased \textit{Abcb1b} mRNA and protein expression. TNF-\(\alpha\) also increased \textit{Abcb1b} mRNA expression.
Kalitsky-Szirtes and colleagues were the first to investigate the effects of bacterial infection on tissues other than the liver. They found that LPS (5 mg/kg) significantly reduced Abcb1a mRNA expression in the intestines and Abcb1b mRNA expression in the jejunum. They also observed reductions in [\(^{3}\)H]digoxin transport 24h after LPS treatment to levels similar to that produced by the established P-gp inhibitor PSC-833.

Wang and colleagues compared the effects of LPS on Abcb1a/1b mRNA expression and activity in pregnant and non-pregnant rats. LPS downregulated Abcb1a/1b mRNA levels in the placenta and significantly increased fetal accumulation of \(^{99}\)mTc-sestamibi. In non-pregnant rats, LPS downregulated Abcb1a expression in the brain, heart, and liver 6h after treatment. LPS also increased accumulation of \(^{99}\)mTc-sestamibi in these tissues. Interestingly, LPS upregulated Abcb1b expression in the liver suggesting that it affects P-gp in an isoform-specific manner. In a follow-up study, Petrovic and colleagues discovered that LPS downregulated Abcb1a/1b expression 18h and 24h after LPS injection at doses of 0.5 mg/kg and 1.0 mg/kg.

While these latter two studies have shown that LPS affects placental P-gp expression and activity, there are a few issues with these studies that must be addressed. Firstly, these studies show that LPS modulates placental P-gp activity only with doses of 0.5 mg or 1.0 mg/kg. However, doses this high have been shown to cause significant intrauterine fetal deaths (IUFD). While most maternal bacterial infections produce deleterious fetal effects, they do not typically cause fetal demise. Consequently, it is important to understand how a maternal bacterial infection, that does not cause IUFD, affects P-gp-
mediated drug transport at various barrier sites during pregnancy. Secondly, these studies do not investigate the effects of LPS on P-gp-mediated drug transport at the fetal BBB. Thirdly, these studies were conducted at only one point in gestation. Studies have shown that P-gp expression and P-gp-mediated substrate efflux vary depending on the gestational day\textsuperscript{12,16}. Finally, studies have shown that LPS increases cytokine levels in the placenta in a sex-specific manner. Specifically, trophoblast cells of male placentas showed greater elevations in TNF-\(\alpha\) and IL-10 in response to LPS compared to those of female placentas\textsuperscript{107}. In addition, levels of TLR4 were greater in placentas of male fetuses. Since LPS is speculated to regulate Abcb1a/1b mRNA expression via activation of TLR4 and various proinflammatory cytokines, it is very likely that any effect of LPS on P-gp function will be sex-specific.

1.6 RATIONALE

It is clear that P-glycoprotein plays a critical role in protecting tissues from the accumulation of a wide variety of xenobiotics and endogenous compounds. At the same time, P-gp can remove various clinical drugs from their target tissues thereby reducing their therapeutic effects. Clearly, the function and regulation of P-gp must be further examined in order to deepen our understanding of drug biodistribution so that we can manipulate this distribution with the goal of improving current paradigms for treating a plethora of pathological conditions. This is particularly important during pregnancy when, not one, but two humans are symbiotically affected by the presence of various endogenous substances and xenobiotics and by the activities of P-gp to manipulate the relative distribution of these substrates.
While fetal exposure to endogenous substances present in maternal circulation is a requirement for normal development, the fetus may also be susceptible to xenobiotic accumulation as a direct result of maternal drug therapy. While the maternal liver reduces fetal exposure of many of these drugs by metabolizing them, the developing fetus is primarily protected from these potential teratogens by two barriers: the placenta and the fetal BBB. As already stated, the placenta is not a complete physical barrier and can be breached by a plethora of xenobiotics that could produce harm to the fetus. P-gp appears to represent a vital ‘gatekeeper’ at both of these barriers. It plays a significant role in preventing passage of teratogens into the fetus and fetal brain. However, P-gp function can be modulated by other factors. By better understanding how this ‘gatekeeper’ operates and is modulated at these sites, it may be possible to significantly improve current pharmacological paradigms for treating both mother and fetus for a wide variety of debilitating conditions.

While researchers have worked tirelessly over the last decade to supplement our understanding of P-gp expression and function in the placenta and at the fetal BBB, little is still known with respect to regulation of P-gp by SSRIs and bacterial infection. SSRIs have previously been shown to regulate P-gp activity in cancerous tissues. However, little is known with regard to SSRI regulation of P-gp in normal tissues. The importance of examining any interactions between SSRIs and placental and fetal BBB P-gp is highlighted by the prevalence of mood disorders during pregnancy. 10-15% of pregnant women are afflicted with a mood disorder and many of these women are prescribed
SSRIs. At the blood-placental barrier, P-gp protects the fetus by limiting the passage of xenobiotics present in maternal circulation across the syncytiotrophoblast. Therefore, it is critical to determine how SSRIs affect the placental barrier and fetal BBB, and how they could influence fetal exposure of maternal drugs. If SSRIs inhibit placental P-gp, this would increase fetal exposure of various xenobiotics. At the same time, this also introduces the possibility of a novel approach to drug treatment of the fetus. Specifically, one could treat fetal conditions by administering much lower quantities of drug thereby decreasing maternal side effects. The first aim presented in this thesis was to determine the acute effects of the SSRI sertraline on P-gp function in the placenta and at the fetal blood-brain barrier.

Another common feature of pregnancy is the presence of infection. Specifically, bacterial infections have been shown to be prevalent during pregnancy and have also been shown to regulate P-gp in a variety of different tissues, including the placenta. However, there have yet to be any studies that have looked at the effects of a nonlethal maternal infection on P-gp-mediated substrate efflux. In this study, ‘nonlethal’ was defined as the most potent bacterial infection that does not cause any maternal or fetal death. The second aim presented in this thesis was to determine the effects of a nonlethal maternal bacterial infection on P-gp function in the placenta and at the fetal blood-brain barrier during mid- (E15.5) and late (E17.5) gestation.
1.7 OBJECTIVES

The objectives of the studies undertaken in this thesis are to: 1) determine the acute effects of the SSRI sertraline on P-gp function in the placenta and at the fetal BBB during mid-gestation and 2) determine the effects of a nonlethal maternal bacterial infection on P-gp function in the placenta and at the fetal blood-brain-barrier during mid- and late gestation.

1.8 HYPOTHESES

- The SSRI sertraline will inhibit P-gp-mediated substrate efflux (1) at the placenta resulting in increased accumulation of P-gp substrate in the fetus, and (2) at the fetal BBB resulting in increased substrate accumulation in the fetal brain. (3) These effects would be time-, dose-, and sex-dependent.

- A nonlethal maternal bacterial infection will inhibit P-gp-mediated substrate efflux (1) at the placenta resulting in increased accumulation of P-gp substrate in the fetus, and (2) at the fetal blood-brain barrier resulting in increased substrate accumulation in the fetal brain. (3) These effects would be time-, age-, and sex-dependent.
Figure 1. Localization of P-glycoprotein in the placenta. P-gp is expressed in the apical membrane of the syncytiotrophoblast layer in the human placenta and in the maternal-facing membrane of syncytial layer II in the mouse placenta. Modified from a figure created by Dr. Stephen G. Matthews.
Figure 2. Localization of P-glycoprotein at the fetal BBB. P-gp is expressed in apical membrane of the brain capillary endothelium. Modified from a figure created by Dr. Stephen G. Matthews.
Chapter 2: Method
2.1 ALL STUDIES

2.1.1 Animals

Female mice (Charles River, Germantown, NY) were bred as has been described in previous studies. Briefly, the appearance of a vaginal plug marked the onset of pregnancy and was designated as E0.5 (average gestation period < 19.5 days). These studies were conducted using protocols approved by the Animal Care Committee at the University of Toronto and in accordance with the Canadian Council for Animal Care.

2.1.2 In Vivo Distribution of $[^3]$H]digoxin

At embryonic day (E) 15.5, pregnant mice were injected (i.v.) with modulator and $[^3]$H]digoxin (1µCi/dam; PerkinElmer, Boston, MA). We previously demonstrated that placental P-gp expression peaks at E12.5 and progressively declines towards term, and that this is mirrored by changes in P-gp activity. In the fetal brain, we have previously demonstrated expression of Abcb1a mRNA on E15.5 and a progressive increase with advancing gestation. We chose E15.5 because P-gp is abundant in both the placenta and fetal brain at this stage of gestation. $[^3]$H]digoxin is the gold standard pharmacological marker for assessing P-gp-mediated substrate efflux because of its biological stability. Schinkel and colleagues have shown that digoxin distribution is strongly affected by P-gp activity and that levels remain high in the plasma 4h after injection (i.v.). The dose administered has been previously used in our lab to assess transplacental transfer and transfer into the fetal brain in the mouse. Animals were anaesthetised with isofluorane and euthanized at subsequent time points after injection. Litters were collected: 1) half of the fetuses from each litter were collected as fetal units, where fetal
membranes containing amniotic fluid were left intact in order to assess total transplacental transfer and 2) brains were removed from the other half of the fetuses in order to assess [\(^3\)H]digoxin transfer from the fetal body into the brain, as has been described previously \(^{16}\). Maternal blood and maternal brains were also collected. Maternal blood (heparinized) was centrifuged and plasma was isolated. Fetal units, fetal bodies (from fetuses where brains were removed), fetal brains, and maternal brains were processed and analyzed as has been described previously \(^{16}\). Briefly, tissue was homogenized, and tissue homogenate (200 µL) and maternal plasma (100 µL) was solubilized in SOLVABLE (PerkinElmer). Hydrogen peroxide (100 µL; 30%) was added to optimize counting efficiency. Scintillation fluid (10 mL; Ultima-Gold; PerkinElmer) was added and radioactivity (DPM) determined using a Tri-Carb β-Counter (PerkinElmer). To determine net transplacental transfer (i.e., ‘drug ratio’) for each mother, the DPM value for each fetal unit was divided by the DPM value of maternal plasma. ’Drug ratios’ were averaged per litter and used for statistical analysis. In order to determine drug distribution into the brain: the fetal brain to fetal body drug ratio and the maternal brain to maternal plasma drug ratio were also calculated. Fetal BBB P-gp substrate-mediated efflux was defined as the fetal brain to fetal body drug ratio instead of the fetal brain to fetal plasma ratio as it was not possible to collect sufficient fetal plasma.

### 2.1.3 Sex Determination

DNA was extracted from fetal tails and fetal homogenate in order to determine the sex of the fetuses \(^{74}\). qPCR was performed to amplify Sry forward: 5' GAG AGC ATG GAG GGC CAT G 3' and Sry reverse: 5' ATG CCA CTC CTC TGT GAC AC 3' primers
according to manufacturer’s guidelines (Sigma REDExtract-N-AMP Tissue PCR Kit (XNAT), Sigma Chemical Co.). Amplification product was detected by 1% gel electrophoresis.

2.1.4 Statistical Analysis

For each dam, ratio values derived for individual fetal units and fetal brains were averaged to provide a litter mean and to prevent litter bias. Each ‘n’ number represents the litter mean of one mother. All data are presented as mean ± standard error of the mean (SEM). [\(^3\)H]digoxin accumulation data (ratios and individual tissue DPMs) for the sertraline study were statistically analyzed using two-way ANOVA followed by the Bonferroni method of post hoc comparison. [\(^3\)H]digoxin accumulation data (ratios and individual tissue DPMs) for the bacterial infection study and [\(^14\)C]mannitol ratio data for the sertraline study were statistically analyzed using unpaired Student t test using Prism (GraphPad Software Inc., San Diego, California, USA). Significance was set at \(P<0.05\).

2.2 SERTRALINE AND P-GP IN THE PLACENTA AND FETAL BBB

2.2.1 Passive Diffusion of [\(^14\)C]mannitol

[\(^14\)C]mannitol has been previously used to assess passive permeability at the placenta.\(^{108}\) This was carried out in order to determine whether any effect of sertraline could be attributed to alterations in passive permeability at the placenta. At E15.5, pregnant FVB mice were injected (i.v.) with sertraline (10 mg/kg; Sigma Chemical Co. St Louis, MO; \(n=3\)) or vehicle (saline; \(n=3\)). 236 min after this injection, animals were injected (i.v.)
with $[^{14}C]$mannitol (1.75 µCi/dam; PerkinElmer) and euthanized 4 min after injection, as described previously $^{109}$. Tissues were collected and drug ratios were assessed as described in Section 2.1.2.

2.3 INFECTION AND P-GP IN THE PLACENTA AND FETAL BBB

2.3.1 Dose Titration

At E13.5, a pregnant C57BL/6 were injected (i.p.) with a 20 ug/kg dose of LPS (Sigma Chemical Co.). This dose was titrated upward until a dose (termed ‘lethal dose’) was reached that either promoted preterm delivery or produced intrauterine fetal deaths (IUFD) within 48h after LPS injection (i.e., by E15.5). The LPS dose below the ‘lethal dose’ was 150 ug/kg and used for all experiments, as it was the highest dose of LPS that did not promote preterm delivery or produce IUFD.

2.3.2 Measurement of Plasma Interleukin (IL)-6 Levels

In order to determine whether the LPS causes a pro-inflammatory response in the mother, an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) was used to measure interleukin (IL)-6 levels in the maternal plasma. Briefly, a 96-well microplate was coated with 100 uL of primary antibody diluted to a working concentration of 2.0 ug/mL in PBS (0.2 uM filtered) and incubated overnight at room temperature. On the following day, the plate was washed three times with wash buffer (0.05% Tween20 in PBS). After the first wash, the plate was blocked by adding 300 uL of reagent diluent (1% BSA in PBS, 0.2 um filtered) and incubated for 1h at room temperature. Following the blocking step, the plate was washed. After the second wash,
two sets of standards were prepared in triplicate. The first set was made from diluting stock standard in reagent diluent at concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 pg/mL. The second set was made from diluting stock standard in mouse maternal plasma at the same concentrations. Plasma samples were diluted in reagent diluent and prepared in triplicate. After standard and sample preparation, 100 uL of sample or standards were added to the wells of the plate. The plate was subsequently incubated for 2h at room temperature. Following the sample incubation step, the plate was washed. After the third wash, the plate was coated with secondary antibody diluted to a working concentration of 200 ng/mL in reagent diluent and incubated for 2h at room temperature. Subsequently, the plate was washed. After the fourth wash, the plate was coated with Streptavidin-HRP and incubated for 20 min at room temperature. Subsequently, the plate was washed. After the fifth and final wash, the plate was coated with substrate solution and incubated 20 min at room temperature. Following the 20 min incubation period, 50 uL of Stop Solution was added to each well. Finally, the optical density of each well was immediately determined at 540 or 570 nm subtracted by 450 nm. The samples were examined in triplicate, and results within the standard curve range are reported.
Chapter 3: The SSRI Sertraline and P-gp in the Placenta and Fetal BBB

Sertraline Alters Multidrug Resistance Phosphoglycoprotein Activity in the Mouse Placenta and Fetal Blood-Brain Barrier (in press at Reproductive Sciences)

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*All of the experiments presented in this chapter and in the article in press at Reproductive Sciences were carried out by Manzerul Bhuiyan.
3.1 RATIONALE

Mood disorders afflict 10-15% of pregnant women and have been linked to an greater risk of miscarriage, preterm delivery, and low birth weight. Thus, the use of SSRIs has been recommended during pregnancy and has been shown to be effective in controlling depression. Nevertheless, maternal SSRI use has also been linked to a variety of fetal complications. These include various behavioural impairments and neurodevelopmental deficits. Chronic maternal SSRI treatment has been shown to increase the rate of neonatal behavioural syndrome, produce deficits in cognitive ability, affect programming of the hypothalamic-pituitary axis, and alter circadian rhythms. It is possible that these behavioural and neurodevelopmental effects are due to the effects that SSRIs are having on substrate transport at the BBB. It has shown that the antidepressants paroxetine and venlafaxine increased significantly in the brains of Abcb1a/1b knockout mice both after a single dose administration and after treatment for 11 days. This suggests that SSRIs could act as P-gp substrates, and therefore P-gp inhibitors. Studies have gone further using L-MDR1 cells and primary cultures of porcine brain capillary endothelial cells to show that sertraline, desmethylsertraline, and paroxetine are the most potent P-gp inhibitors of all SSRIs tested. These findings collectively suggest that SSRIs inhibit P-gp function in the adult BBB. If chronic SSRI treatment causes a prolonged downregulation of fetal BBB P-gp activity, this could allow for the continuous accumulation of teratogens in the fetal brain. These teratogens can then alter proper fetal brain development and subsequently cause many of these behavioural and neurodevelopmental abnormalities.
While studies have shown that SSRIs act as P-gp inhibitors at the adult BBB, no study has investigated the effects of SSRIs on P-gp function in the placenta or fetal BBB. The function of P-gp in the placenta and fetal BBB is to limit fetal exposure to various xenobiotics. Our lab has previously shown that expression of Abcb1b, the predominantly expressed P-gp gene in the mouse placenta, together with P-gp protein and activity are high at mid-gestation (E12.5) but low near term (E18.5) in the mouse.12 We’ve also shown that expression of Abcb1a, the predominantly expressed P-gp gene in the mouse brain, dramatically increases near term.74 While the function and the expression of P-gp in the placenta and at the fetal BBB have been well characterized, the current state of knowledge with respect to regulation of P-gp-mediated drug transport at these barrier sites is limited. Understanding the regulation of placental and fetal BBB P-gp during pregnancy becomes especially important when considering the prevalence of SSRI use during this time and the regulatory effects that the SSRIs have had on P-gp function in other tissues. SSRI-induced alterations in fetal BBB P-gp function may contribute to many of the neonatal complications associated with chronic prenatal SSRI exposure. The first aim presented in this thesis was to determine the acute effects of the SSRI sertraline on P-gp-mediated substrate efflux in the placenta and at the fetal BBB.

3.2 HYPOTHESES

In the present study, we hypothesized that the SSRI sertraline will inhibit P-gp-mediated substrate efflux (1) at the placenta resulting in increased accumulation of P-gp substrate in the fetus and (2) at the fetal blood-brain barrier resulting in increased substrate
accumulation in the fetal brain. (3) These effects would be time-, dose- and sex-dependent.

3.3 EXPERIMENTAL DESIGN

3.3.1 Animals

Female FVB mice (Charles River, Germantown, NY) were bred as described in Section 2.1.1. This strain was selected because the Abcb1a/1b KO mice were established in this strain.

3.3.2 In Vivo Distribution of [³H]digoxin

At embryonic day (E) 15.5, pregnant FVB mice were injected (i.v.) with: sertraline (1 mg/kg (n=17) or 10 mg/kg (n=17)) or vehicle (saline; n=16) simultaneously with [³H]digoxin (1µCi/dam). Sertraline was selected because it is the most prescribed antidepressant in the United States \(^{110}\) and has been shown to produce the most potent inhibitory effects on P-gp-mediated substrate efflux in L-MDR1 cells and endothelial cells derived from brain microvessels of all SSRIs tested \(^{90}\). The half-life of sertraline is approximately 26 hours in humans. Experiments were undertaken with doses of 10 and 1 mg/kg. The latter is similar to the dose that is used clinically to manage depression \(^{111}\). Animals were anaesthetized with isoflurane and euthanized 5 min (n=5-6/gp), 60 min (n=5-6/gp), or 240 min (n=5-6/gp) after injection. These three time points were chosen because it was at these times that our lab has previously observed SSRI-induced effects on P-gp transport at the male BBB. After euthanasia, tissues were collected, processed, and analyzed as described in Section 2.1.2.
3.3.3 Passive Diffusion of $[^{14}\text{C}]$mannitol

At E15.5, pregnant FVB mice were injected (i.v.) with sertraline (10 mg/kg; n=3) or vehicle (n=3). 236 min after this injection, animals were injected (i.v.) with $[^{14}\text{C}]$mannitol (1.75 µCi/dam) and euthanized 4 min after injection, as described previously. Tissues were collected and drug ratios were assessed as described in Section 2.1.2.

3.3.4 Sex Determination

DNA was extracted from fetal tails and fetal homogenate in order to determine the sex of the fetuses as described previously. It was analyzed as described in Section 2.2.2. There was no significant effect of sex on P-gp-mediated substrate efflux or any interaction between sex and sertraline treatment. As a result, data from male and female fetuses were combined within each litter.

3.4 RESULTS

3.4.1 In Vivo Distribution of $[^{3}\text{H}]$digoxin: Placenta

Figure 3 illustrates the fetal unit to maternal plasma drug ratio. The ‘fetal unit’ comprises the fetus, the chorionic and amniotic membranes, and the amniotic fluid. This ratio represents the net transfer of $[^{3}\text{H}]$digoxin from the maternal plasma across the placental barrier into the fetal unit. A larger ratio indicates greater net transfer of $[^{3}\text{H}]$digoxin across the placenta and higher accumulation of the substrate in the fetal unit. Higher accumulation indicates reduced placental P-gp-mediated substrate efflux. There were no effects of either dose of sertraline on the accumulation of $[^{3}\text{H}]$digoxin in the fetal unit at 5
min (Fig. 3A) and 60 min (Fig. 3B). However, at 240 min (Fig. 3C), sertraline produced a significant dose-dependent decrease in the accumulation of $[^3]$Hdigoxin in the fetal unit, indicating an increase in P-gp-mediated substrate efflux in the placenta. This difference in the fetal unit to maternal plasma drug ratio resulted from significant reductions in $[^3]$Hdigoxin levels in the fetus (Fig. 3D) with no significant change in $[^3]$Hdigoxin levels in the maternal plasma (Fig. 3E).

### 3.4.2 Blood-Brain Barrier

Figure 4 illustrates the fetal brain to fetal body drug ratio. This ratio represents the net transfer of $[^3]$Hdigoxin from the fetal body across the fetal BBB and into the fetal brain. A higher ratio indicates greater accumulation of $[^3]$Hdigoxin in the fetal brain, and reduced P-gp-mediated substrate efflux at the fetal BBB. There was no effect of either dose of sertraline on the fetal brain to fetal body ratio of $[^3]$Hdigoxin at 5 min (Fig. 4A) and 60 min (Fig. 4B). However, at 240 min (Fig. 4C), sertraline administration resulted in a significant dose-dependent increase in the fetal brain to fetal body drug ratio of $[^3]$Hdigoxin accumulation. This difference in the fetal brain to fetal body drug ratio resulted from a substantial reduction in $[^3]$Hdigoxin levels in the fetal body (Fig. 4E). There was a significant elevation in absolute fetal brain accumulation of $[^3]$Hdigoxin at 240 min with the 1 mg/kg dose (Fig. 4D).

Figure 5 illustrates the maternal brain to maternal plasma drug ratio. This ratio represents the net transfer of $[^3]$Hdigoxin from the maternal plasma across the BBB into the maternal brain. At 5 min (Fig. 5A) and 60 min (Fig. 5B), there were no differences in this
ratio between the sertraline- and vehicle-treated groups, suggesting that sertraline did not affect P-gp-mediated substrate efflux at the maternal BBB at these time points. However, at 240 min (Fig. 5C), the higher dose of sertraline (10 mg/kg) produced a significant increase in [³H]digoxin accumulation in the maternal brain, suggesting an inhibitory effect of this dose on P-gp-mediated substrate efflux at the maternal BBB. This difference in the maternal brain to maternal plasma drug ratio resulted from a significant elevation in [³H]digoxin levels in the maternal brain (Fig. 5D) with no significant change in [³H]digoxin levels in the maternal plasma (Fig. 3E).

3.4.3 Passive Diffusion of [¹⁴C]mannitol

There were no significant differences in the fetal unit to maternal plasma ratio of mannitol (VEH: 0.024 +/- 0.006; SER: 0.021 +/- 0.009), the fetal brain to fetal body ratio (VEH: 0.036 +/- 0.017; SER: 0.064 +/- 0.040), or the maternal brain to maternal plasma ratio (VEH: 0.018 +/- 0.007; SER: 0.015 +/- 0.008) between the sertraline- and vehicle-treated groups. This suggests that sertraline does not affect passive permeability at the placental barrier or at the BBB.

3.5 DISCUSSION

In the present study, we have shown that the SSRI sertraline can influence transfer of [³H]digoxin (P-gp substrate) across the placenta and across the fetal and maternal BBB. This effect is time-dependent and only occurs 240 min after sertraline administration. This is the first study to directly investigate the effects of an SSRI on transport of P-gp substrates in normal tissues, in vivo. Importantly, we have shown that the effects of
sertraline on P-gp function appear to be tissue-specific, with increased P-gp-mediated substrate efflux in the placenta and reduced P-gp-mediated substrate efflux at the fetal and maternal BBB. Further, we have shown that sertraline-mediated changes in the accumulation of [3H]digoxin in the fetus and the fetal and maternal brain, do not occur as a result of altered passive diffusion at these blood-barrier sites. Finally, there were no sex-specific differences in any of the data.

Further analysis of the absolute levels of [3H]digoxin in the various tissues measured provide some insight into the benefits of using drug ratios in transport studies. At 240 min, sertraline significantly decreased fetal accumulation (Fig. 3D) and increased maternal brain accumulation of [3H]digoxin (Fig. 5D) without affecting the source of substrate for these tissues, the maternal plasma [3H]digoxin levels (Fig. 3E). The fact that sertraline did not alter [3H]digoxin levels in the plasma is important since it suggests that sertraline does not significantly affect maternal substrate distribution/metabolism. Consequently, any differences in [3H]digoxin accumulation in the fetal unit and maternal brain can be attributed to the effects of sertraline on P-gp-mediated substrate efflux at the placenta and maternal BBB, respectively. On the other hand, sertraline did not alter fetal brain accumulation of [3H]digoxin (Fig. 4D). At 240 min, the sertraline-induced increase in the fetal brain to fetal body drug ratio was due to a significant decrease in fetal body accumulation of [3H]digoxin (Fig. 4E). Absolute levels of [3H]digoxin in the fetal brain only differed between the 1 mg/kg sertraline- and vehicle-treated groups (Fig. 4D). Nevertheless, the differences in the fetal brain to fetal body drug ratio suggest that sertraline inhibits P-gp-mediated substrate efflux at the fetal BBB. This is because the
fetal body is the source of substrate for the fetal brain. If sertraline was not influencing drug transfer into the fetal brain, there would be parallel reductions in fetal body and fetal brain $[^3]$H]digoxin levels. This was not seen as sertraline significantly decreased fetal body accumulation of substrate without producing a corresponding decrease in fetal brain accumulation. Thus, despite the lower levels of substrate in the fetal body of sertraline-treated animals, the fetal brain is still receiving a disproportionately high concentration of substrate equivalent to that of vehicle-treated animals. This observation would be missed if one were to simply look at absolute levels of substrate in various tissues. Drug ratios are much more effective in illustrating these types of findings and are a well-established way of expressing data in drug transport studies $^{104,105,112-114}$. The fetal unit to maternal plasma $[^3]$H]digoxin ratio and the maternal brain to maternal plasma $[^3]$H]digoxin ratio has been used in published studies $^{16}$. The fetal brain to fetal body $[^3]$H]digoxin ratio has been also been used $^{74}$. The fact that sertraline increases the fetal brain to fetal body drug ratio suggests that sertraline is limiting drug transfer into the fetal brain from the fetal body.

It has been demonstrated that the SSRI fluoxetine meets all three in vitro criteria for being a P-gp inhibitor: drug efflux, drug accumulation, and cytotoxicity $^{97}$. Specifically, fluoxetine slowed drug efflux (70%), increased cellular accumulation of drugs (32%), resulting in increased cytoxocity (10-fold) of the anticancer drug, and P-gp substrate, doxorubicin $^{98}$. The potency of fluoxetine as a P-gp inhibitor was comparable to the potency of other P-gp inhibitors, verapamil and cyclosporin A, in vitro $^{41}$. Oral administration of fluoxetine has been shown to produce a significant increase in doxorubicin accumulation in lung tumors, slow down tumor progression, and increase
survival by 2-3 fold, \textit{in vivo} \textsuperscript{97}. In the present study, sertraline was selected over fluoxetine because it has been shown to be a more potent P-gp inhibitor in cell systems \textsuperscript{90}. In L-MDR1 cells (which over-express the human P-gp) and porcine brain capillary endothelial cells the SSRIs sertraline, desmethylsertraline, and paroxetine inhibit P-gp activity to a degree comparable to the established P-gp inhibitor verapamil \textsuperscript{90}. The advantage of SSRIs over other known P-gp inhibitors is that they do not appear to alter drug metabolism or distribution, \textit{in vivo} \textsuperscript{115}. In the present study, sertraline did not affect levels of $[^3]H$digoxin in the maternal circulation at any time point confirming these previous observations.

While the SSRIs have been shown to inhibit P-gp function in tumor cells, the present study is the first to demonstrate that an SSRI may have tissue-specific effects on P-gp function in normal tissues, \textit{in vivo}. In the fetal and maternal BBB, the SSRI sertraline exerted an inhibitory effect on P-gp resulting in increased substrate transfer into the brain. This is consistent with the inhibitory effects that have been reported in tumor and normal cell lines, \textit{in vitro} \textsuperscript{90,97,98}. In contrast, in the placenta, sertraline reduced the net transfer of P-gp substrate from the maternal circulation to the fetal unit, suggesting that it increased P-gp-mediated substrate efflux in the placenta, enhancing the ability of the placenta to prevent xenobiotics from entering the fetus. Previous studies have shown that the same drug can have different effects on P-gp function in different tissues \textsuperscript{116,117}. For example, the antibiotic rifampin has been shown to increase intestinal accumulation of digoxin but have no effect on accumulation of digoxin in the kidneys \textsuperscript{117}. In another study, adjuvant arthritic rats were used to investigate the effect of arthritis on hepatic and intestinal P-gp
activity in rats \(^{116}\). Adjuvant arthritic rats have been used as an animal model for rheumatoid arthritis because these rats have a systemic inflammatory disease that produces changes in bone and cartilage comparable to those seen in rheumatoid arthritis. In AA rats, hepatic P-gp expression and activity were much lower than in normal rats. However, no such differences were seen with respect to intestinal P-gp. The authors concluded that different regulation systems might be involved in the liver and intestine. They based this conclusion on the notion that the upregulation of P-gp in the kidney specifically involves NF-kB, implying that specific transcription factors may play a role in the regulation of \(Abcb1a/1b\) gene expression in different tissues.

The mechanism for SSRI-induced inhibition of P-gp function is not known. Competitive inhibition occurs when a modulator outcompetes a substrate for access to the drug-binding site of a transporter. Consequently, the modulator is transported across the membrane and is thus considered a substrate of the transporter. Conversely, non-competitive inhibition occurs when a modulator binds to an allosteric site and reduces the ability of a substrate to interact with the drug-binding site. In this form of inhibition, the modulator will not be transported across the membrane. The SSRIs are believed to be competitive inhibitors of P-gp substrates. Uhr and colleagues showed that concentrations of the antidepressants doxepin and venlafaxine and the SSRI paroxetine concentrations in the cerebrum were higher in \(Abcb1a/1b\) knockout mice, suggesting that some antidepressants may act as P-gp substrates \(^{89}\). If this is the case, SSRIs may effectively inhibit P-gp-mediated substrate efflux by competing with other substrates for access to the drug-binding site.
If the SSRIs are P-gp substrates, it may seem counter-intuitive that sertraline is stimulating P-gp-mediated digoxin efflux in the placenta. However, previous studies have shown that one P-gp substrate can increase P-gp-mediated transport of another substrate. Shapiro and Ling showed this in plasma membrane vesicles of the hamster ovary. They state that this observation is most easily explained as being due to the presence of two transport-competent drug-binding sites, the H site and the R site, that interact in a positively cooperative manner. Currently, it is not clear whether the ratio of H-to-R sites can vary between different tissues. Nevertheless, studies have shown that the H and R sites bind and transport specific substrates, but can also have overlapping specificities. Consequently, it is possible that sertraline and digoxin interact with distinct drug-binding sites on placental P-gp. For instance, if sertraline binds to the H site while digoxin binds to the R site on placental P-gp, these two drugs may stimulate transport of one another in a positive cooperative manner. While this is occurring in the placenta, it is possible that sertraline competes with digoxin for access to the same drug-binding site on BBB P-gp, subsequently leading to sertraline-induced inhibition of digoxin efflux at the BBB. Further studies are required to determine the mechanisms by which SSRIs interact with P-gp drug-binding sites and consequently influence the transport of other substrates in both normal and cancer cells. For example, one group of animals could be treated with a substrate that preferentially binds to the H-site while another group is treated with a substrate that preferentially binds to the R-site. Along with the substrate, both groups would be simultaneously administered sertraline. If sertraline-induced modulation of P-gp fits into the Shapiro-Ling model, then a reduction of substrate efflux should occur in
one group of animals while an elevation of efflux occurs in the other. The major limitation to conducting this study is that there are still no unanimously accepted examples of H-site and R-site specific substrates.

Other studies have shown that some compounds can modulate P-gp by binding to sites distinct from the drug-binding site. For example, some steroid modulators bind to a high-affinity site within NBD1 domain that is close to the ATP-binding site. In addition, XR9576 has been shown to bind to a site that is distinct from the drug-binding site where established substrates vinblastine and paclitaxel bind. As another example, it has also been shown that some P-gp modulators, such as disulfiram, can bind to the drug-binding pocket of P-gp and simultaneously modify cysteine residues at the catalytic site. All of these findings suggest that P-gp modulation is not just a simple process involving competitive inhibition. Clearly, there are several potential ways in a modulator, such as sertraline, might interact with P-gp binding sites to influence transport of digoxin and other P-gp substrates. Unique tissue-specific binding mechanisms may contribute to the tissue-specific effects seen in the placenta and BBB.

Tissue-specific effects of the SSRIs on P-gp mediated drug transport may also result from unique functional properties of the predominant P-gp isoforms found in the placental syncytiotrophoblast and in the endothelial cells of the brain microvasculature. In the mouse, two genes encode P-gp: Abcb1a and Abcb1b. While the P-gp protein isoforms encoded by these genes share 84% amino acid identity, they are differentially expressed in the body. P-gp encoded by Abcb1a predominates in the intestine, liver, lung, and
blood-brain barrier \cite{19}, whereas P-gp encoded by \textit{Abcb1b} predominates in tissues associated with steroid biosynthesis and distribution, such as the adrenal, ovary and placenta \cite{28}. In addition to differences in localization, there have been reported differences in the biochemical properties of P-gp encoded by \textit{Abcb1a} and \textit{Abcb1b}. One study showed that progesterone could distinguish the transport properties of the two P-gp isoforms \cite{124}. Progesterone inhibited drug efflux with more potency in cells expressing \textit{Abcb1b}-encoded P-gp compared to cells expressing \textit{Abcb1a}-encoded P-gp. This study also discovered a single class of vinblastine binding sites on \textit{Abcb1b}-encoded P-gp and two classes of such sites on the \textit{Abcb1a}-encoded P-gp. There have also been reports that the two P-gp isoforms differ in their ability to confer cellular resistance to drugs, including colchicine and actinomycin D, \textit{in vitro} \cite{25,124-126}. However, these earlier studies used indirect tests to assess substrate specificity and their significance has been questioned by more recent studies, which have reported no difference in affinity of the two isoforms towards vinblastine \cite{123}. It was concluded in these more recent studies that if any difference in transport exists between the two P-gp isoforms, it must occur at a step subsequent to binding, such as the translocation step or linkage between drug binding and ATP hydrolysis \cite{123}. Since there is only one P-gp isoform in the human, the discovery of isoform-specific effects in the mouse would pose very significant consequences for research since it would present a novel limitation of the mouse model in P-gp transport studies. Clearly, further studies are required to investigate the novel tissue-specific nature of the effects of sertraline on P-gp function. One such study could involve investigating how sertraline affects substrate accumulation in cells transfected \textit{Abcb1a} compared to cells transfected with \textit{Abcb1b}. 

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It is also possible that various proinflammatory cytokines play a mediating role in promoting sertraline-induced changes in P-gp activity during pregnancy. Studies have shown that the SSRIs are immunodulatory. Specifically, SSRI use has been linked with reduced production of the proinflammatory cytokines TNF-α and IL-6 \(^{127,128}\). This process is thought to be mediated by cyclic-AMP, which, upon activation by the SSRIs promotes downstream signaling events that suppress the production of these proinflammatory cytokines. These cytokines have been shown to interact with P-gp. Specifically, *in vitro* TNF-α has been shown to decrease *ABCB1* mRNA expression and inhibit transport of rhodamine123 in P-gp-expressing human Caco-2 cells \(^{129}\) while *in vivo* IL-6 has been shown to reduce P-gp protein expression and mRNA levels of *Abcb1a* and *Abcb1b* in the rat liver \(^{102}\). Consequently, it is possible that suppressed production of proinflammatory cytokines by sertraline in the placenta and fetal BBB leads to alterations in the expression and function of P-gp in these tissues.

The fact that acute SSRI treatment increases P-gp substrate accumulation in the fetal brain may contribute to many of the neonatal complications associated with chronic prenatal SSRI exposure \(^{83,130}\). The prevalence of mood disorders during pregnancy is estimated to be 10-15% \(^{77}\). Many of these women are treated with SSRIs, including sertraline \(^{77}\). Chronic prenatal exposure to SSRIs has been associated with significant increases in the rate of neonatal behavioural syndrome which includes irritability, prolonged crying, seizures, tremors, and difficulty feeding and sleeping \(^{82,83}\). In addition, there have been reported deficits in cognitive ability in neonates that are prenatally
exposed to SSRI \textsuperscript{84}. Chronic maternal SSRI treatment during pregnancy has also been shown to produce an attenuated ACTH and cortisol response in rats and humans \textsuperscript{87,88}. It is possible that these neurodevelopmental effects result, in part, from the effects that SSRIs are having on substrate transport at the fetal BBB. If chronic SSRI treatment maintains the inhibition of P-gp-mediated substrate efflux at the fetal BBB, this could allow for increased accumulation of xenobiotics, as well as other endogenous substrates, that are normally excluded from the fetal brain by P-gp. While our data suggests that sertraline provides some protection to the fetus by stimulating placental P-gp, the fetal brain is still unprotected from compounds already present in fetal circulation. So while the fetus of mothers given SSRIs could be protected from P-gp substrates in maternal circulation, its organs are left unprotected from substrates in its own circulation. Finally, many of these fetal complications are associated with SSRI use during different points in gestation. The present study only allows us to make conclusions about a brief window in mid-late gestation. This window was selected because fetal deficits have been shown to be common with maternal SSRI use during mid-late gestation \textsuperscript{131}. Nevertheless, it is important to repeat this experiment during early gestation since SSRI use is common throughout pregnancy \textsuperscript{132}.

Finally, potential interactions of sertraline and/or digoxin with other transporters must be taken into consideration. With respect to the SSRIs, it has been shown that fluoxetine modulates MRP1 function albeit to a lesser extent than it modulates P-gp function \textsuperscript{41}. However, any such interaction with another transporter has yet to be discovered for sertraline. With respect to digoxin, it has been shown that the drug is a substrate of the rat
Oatp2 transporter in the liver and brain capillary endothelial cells and of human OATP-8 in the liver and OATP-4C1 at the basolateral membrane of the proximal tubule cell in the kidney. Nevertheless, the Oatp family and other transporter families of the ABC superfamily have not been shown to influence transport of digoxin in the placenta. Furthermore, while Oatp2 affects digoxin transport in adult rat brain capillary endothelial cells, it is not believed to contribute to digoxin biodistribution in the mouse fetal brain. Consequently, the influence of transporters other than P-gp in the sertraline-digoxin interaction is minimal.

In conclusion, sertraline influences transfer of P-gp substrates across the placenta and across the fetal and maternal BBB. This effect is dose- and time-dependent, only occurring at 240 min after sertraline administration. Importantly, we are the first to show that the effects of SSRIs on P-gp function are tissue-specific, with sertraline increasing P-gp-mediated substrate efflux in the placenta and reducing P-gp-mediated substrate efflux at the fetal and maternal BBB. This means that sertraline simultaneously increases the placenta’s resiliency and the fetal brain’s susceptibility to the influx of teratogens. Given the widespread use of SSRIs during human pregnancy, our present findings have important clinical implications. In particular, it will be important to exercise discretion when prescribing sertraline to pregnant woman during mid gestation. Future studies will show whether these effects are limited to mid gestation or to sertraline (i.e., can be replicated with other SSRIs).
Figure 3. Effects of Sertraline on P-gp Activity in the Placenta. Transplacental transfer of [³H]digoxin to the fetal unit at (A) 5 min, (B) 60 min, and (C) 240 min after treatment with sertraline at 1 mg/kg (grey bar), 10 mg/kg (solid bar) or vehicle control (open bar). Absolute [³H]digoxin levels in the maternal plasma (D) and fetal unit (E). Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=5-6/group. ***P<0.001, *P<0.05 versus vehicle, ±±P<0.01 versus 1 mg/kg.
Figure 4. Effects of Sertraline on P-gp Activity at the Fetal BBB. Net transfer of $[^3]$H]digoxin from the fetal body to the fetal brain at (A) 5 min, (B) 60 min, and (C) 240 min after treatment with sertraline at 1 mg/kg (grey bar), 10 mg/kg (solid bar) or vehicle control (open bar). Absolute $[^3]$H]digoxin levels in the fetal body (D) and fetal brain (E). Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=5-6/group. ***$P<0.001$, *$P<0.05$ versus vehicle. ±±$P<0.01$, ±$P<0.05$ versus 1 mg/kg.
Figure 5. Effects of Sertraline on P-gp Activity at the Maternal BBB. Net transfer of $[^3$H]digoxin from the maternal plasma to the maternal brain at (A) 5 min, (B) 60 min, and (C) 240 min after treatment with sertraline at 1 mg/kg (grey bar), 10 mg/kg (solid bar) or vehicle control (open bar). Absolute $[^3$H]digoxin levels in the maternal brain (D). Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=5-6/group. ***$P<0.001$, *$P<0.05$ versus vehicle. +++$P<0.001$ versus 1 mg/kg.
Chapter 4: Bacterial Infection and P-gp in the Placenta and Fetal BBB
4.1 RATIONALE

Bacterial infections can affect pregnant women and their fetuses throughout pregnancy. These infections can include group B Streptococcus (GBS), urinary tract infections, listeriosis, and sexually transmitted diseases (STDs) such as Chlamydia and gonorrhea. Many have been shown to cause serious harm to mother and fetus leading to conditions such as amnionitis, cystitis, and endometritis. To complicate matters, many of these infections are asymptomatic. Bacteriuria and cystitis do not present symptoms. Approximately 20-25% of pregnant women are asymptomatic carriers of GBS, and only 0.5-1% of these women develop symptoms of disease. Chlamydia is asymptomatic in 75% of women while gonorrhea shows no symptoms in 50% of women. Chlamydia and gonorrhea are the two most common bacterial STDs in the United States and are major causes of complications in pregnancy. In fact, untreated STDs can cause stillbirth or neonatal death. Thus, it is apparent that bacterial infections are common during pregnancy and can have deleterious effects on the mother and growing fetus.

Infection-induced inhibition of P-gp-mediated substrate efflux at the placenta and fetal BBB may contribute to many of the fetal complications associated with bacterial infections. Studies have already shown that bacterial infections, modeled by lipopolysaccharide (LPS), can affect P-gp expression in other tissues. LPS reduced Abcb1a/lb mRNA expression in the liver, intestines, brain and placenta in a time-dependent manner. Studies have also shown that high doses of LPS reduce P-gp activity in the intestines and placenta. Nevertheless, there is much left to be explored particularly with respect to the fetal effects of bacterial infection.
While some studies have shown that LPS affects placental P-gp expression and activity \cite{104,105}, these studies have administered doses of LPS that have been shown to cause significant IUFD \cite{106}. Dose selection is very important for pregnancy studies. Some doses may not appropriately model maternal infection. Most maternal bacterial infections do not cause fetal demise \cite{99} making it important to select a dose of LPS that does not cause IUFD. The dose that we selected for our study was the highest dose that did not cause any IUFD. Since the bacterial infection that we modeled was nonlethal to all mothers and fetuses, it will hereby be referred to as a ‘nonlethal maternal bacterial infection’. In addition, there are no studies investigating the effects of LPS on P-gp-mediated substrate efflux at the fetal BBB. Finally, studies conducted on pregnant animals are typically carried out on E17 by which point there is a significant reduction in placental P-gp expression/function and a significant elevation of fetal BBB P-gp expression/function compared to earlier gestational days \cite{12,16}. Thus, our understanding of the effects of bacterial infection on placental transport is limited to one point in gestation. Age-related changes in P-gp expression/function suggest that the effects of LPS will be age-dependent. Consequently, the second aim presented in this thesis was to address these three issues and determine the effects of a nonlethal maternal bacterial infection on P-gp-mediated substrate efflux in the placenta and at the fetal blood-brain barrier during mid (E15.5) and late (E17.5) gestation.
4.2 HYPOTHESES

In the present study, we hypothesized that a nonlethal maternal bacterial infection will inhibit P-gp-mediated substrate efflux: (1) at the placenta resulting in increased accumulation of P-gp substrate in the fetus and (2) at the fetal blood-brain barrier resulting in increased substrate accumulation in the fetal brain. (3) These effects would be time-, age-, and sex-dependent.

4.3 EXPERIMENTAL DESIGN

4.3.1 Animals

Female C57BL/6 mice (Charles River, Germantown, NY) were bred as described in Section 2.1.1. This strain was selected because the TLR4 KO mice were established in this strain (Jackson Lab) and so it could be used for future studies investigating mechanisms for LPS-induced effects on P-gp expression/function.

4.3.2 Dose Titration

The dose of LPS was titrated as described in Section 2.3.1.

4.3.3 In Vivo Distribution of [3H]digoxin

Pregnant C57BL/6 mice were injected (i.p.) with: LPS (150 ug/kg; n=10) or vehicle (saline; n=11) 4h or 24h prior to euthanasia on E15.5. The 24h time point was chosen because previous studies have shown that LPS inhibits placental Abcb1a/1b 24h after injection\(^{105}\). The 4h time point was selected because previous studies have shown LPS-induced increases in fetal accumulation of the P-gp substrate,\(^{99}\)m-Tc-sestamibi 4h after...
injection and also because IL-6 levels are significantly elevated in the plasma between 2-6h after LPS injection. An hour prior to euthanasia on E15.5, all animals were injected (i.v.) with [3H]digoxin. After euthanasia, tissues were collected and drug ratios were assessed as described in Section 2.1.2. There were only two distinctions. The first was that maternal hearts were also collected in this study and a maternal heart to maternal plasma drug ratio was calculated in order to determine drug distribution into the maternal heart. It has been shown in rats that high dose LPS significantly inhibits Abcb1a expression in the heart 6h after injection and increases substrate accumulation 1h after injection. Thus, it is interesting to consider the effects of nonlethal infection on P-gp-mediated efflux in the heart. The second distinction was that this experiment was repeated at late gestation (E17.5) to determine whether the effects differ with age.

4.3.4 Measurement of Plasma Interleukin (IL)-6 Levels

In order to determine whether the LPS causes a pro-inflammatory response in the mother, an enzyme-linked immunosorbent assay (ELISA) was used to measure interleukin (IL)-6 levels in the maternal plasma. ELISA protocols for IL-6 (R&D Systems, Minneapolis, MN) were carried out as described in Section 2.3.2. The samples were examined in triplicate, and results within the standard curve range are reported.

4.3.5 Sex Determination

DNA was extracted from fetal tails and fetal homogenate in order to determine the sex of the fetuses as described previously. It was analyzed as described in Section 2.2.2. There was no significant effect of sex on P-gp-mediated substrate efflux or any interaction.
between sex and LPS treatment. As a result, data from male and female fetuses were combined within each litter.

4.4 RESULTS

4.4.1 Dose Titration

Beginning with a dose of 20 ug/kg, pregnant mice were injected (i.p.) with increasingly higher doses of LPS until there was evidence of preterm delivery or IUFD. This occurred with a dose of 200 ug/kg (the ‘lethal dose’) with which we saw, on average, 52% dead fetuses per litter. The LPS dose below the ‘lethal dose’ was 150 ug/kg and used for all experiments, as it was the highest dose of LPS that did not promote preterm delivery or produce IUFD.

4.4.2 Measurement of Plasma Interleukin (IL)-6 Levels

Figure 6 illustrates IL-6 concentrations in the maternal plasma. At E15.5 (Fig. 6A), there is a significant increase in plasma IL-6 levels in LPS-treated animals at the 4h time point. These levels return to normal by 24h. At E17.5 (Fig. 6B), plasma IL-6 levels are also elevated in 4h LPS-treated animals. However, comparison with vehicle was not possible because plasma IL-6 levels in these animals were below the minimum detectable concentration of 22.4 pg/mL. Plasma IL-6 levels in the 24h groups were also below the concentration limit of detection of 22.4 pg/mL. Regardless, our data suggests that LPS significantly increases IL-6 concentrations in the plasma 4h after treatment and that these levels return to baseline by 24h. Combined with the dose titration data, these results
indicate that our experiment did indeed model a nonlethal maternal bacterial infection. The inter- and intra-assay coefficients of variability were 15.38 and 4.872, respectively.

4.4.3 *In Vivo Distribution of \[^3\text{H}\text{digoxin: Placenta}*

The fetal unit to maternal plasma drug ratio at E15.5 is illustrated in Figure 7A. Higher accumulation indicates reduced placental P-gp-mediated substrate efflux. Absolute DPMs for the maternal plasma (Fig. 7B) and fetal unit (Fig. 7C) are also presented. At E15.5, there are trends towards higher fetal accumulation of \[^3\text{H}\text{digoxin in the LPS-treated groups at 4h and 24h. However, this did not reach significance for either time point.}

The fetal unit to maternal plasma drug ratio at E17.5 is illustrated in Figure 8A. Again, while there are trends towards higher fetal accumulation of \[^3\text{H}\text{digoxin in the LPS-treated groups at 4h and 24h, this did not reach significance for either time point. Nevertheless, when assessing the DPMs of this ratio individually, there are significantly higher levels of \[^3\text{H}\text{digoxin in the maternal plasma (Fig. 8B) and the fetal unit (Fig. 8C) of the LPS-treated group at 4h.}

4.4.4 *Blood-Brain Barrier*

The fetal brain to fetal body drug ratio at E15.5 is illustrated in Figure 9A. A higher ratio indicates greater accumulation of \[^3\text{H}\text{digoxin in the fetal brain and reduced P-gp-mediated substrate efflux at the fetal BBB. Absolute DPMs for the fetal body (Fig. 9B) and fetal brain (Fig. 9C) are also presented. There was no effect of LPS on the fetal brain to fetal body ratio of \[^3\text{H}\text{digoxin at 4h or 24h.}
The fetal brain to fetal body drug ratio at E17.5 is illustrated in Figure 10A. There was no effect of LPS on the fetal brain to fetal body ratio of $[^3]$Hdigoxin at 4h or 24h. However, when assessing the DPMs of this ratio individually, there are significantly higher levels of $[^3]$Hdigoxin in the fetal body (Fig. 10B) and fetal brain (Fig. 10C) of the LPS-treated group at 4h.

The maternal brain to maternal plasma drug ratio at E15.5 is illustrated in Figure 11A. This ratio represents the net transfer of $[^3]$Hdigoxin from the maternal plasma across the BBB into the maternal brain. Absolute DPMs for the maternal brain are also presented (Fig. 11B). There was no effect of LPS on the maternal brain to maternal plasma ratio of $[^3]$Hdigoxin at 4h or 24h.

The maternal brain to maternal plasma drug ratio at E17.5 is illustrated in Figure 11C. There was no effect of LPS on the maternal brain to maternal plasma ratio of $[^3]$Hdigoxin at 4h or 24h. However, when assessing maternal brain DPMs individually (Fig. 11D), there is an overall effect of treatment on $[^3]$Hdigoxin accumulation in the maternal brain.

4.4.5 Heart

The maternal heart to maternal plasma drug ratio at E15.5 is illustrated in Figure 12A. This ratio represents the net transfer of $[^3]$Hdigoxin from the maternal plasma into the maternal heart. Absolute DPMs for the maternal heart are also presented (Fig. 12B).
There was no effect of LPS on the maternal brain to maternal plasma ratio of $[^3]$Hdigoxin at 4h or 24h.

The maternal heart to maternal plasma drug ratio at E17.5 is illustrated in Figure 12C. While there was no effect of LPS on the maternal brain to maternal plasma ratio of $[^3]$Hdigoxin at 24h, there was a significant increase in the maternal heart to maternal plasma drug ratio at 4h. When assessing maternal heart DPMs individually (Fig. 12D), there is also a significantly higher level of $[^3]$Hdigoxin in the maternal heart of the LPS-treated group at 4h.

4.5 DISCUSSION

This is the first study to investigate the effects of a gram-negative maternal bacterial infection, not causing preterm delivery or fetal demise, on transport of P-gp substrates in normal tissues. In the present study, we have shown that a nonlethal (to the mother and fetuses) gram-negative maternal bacterial infection, modeled by LPS, elevates IL-6 levels in the maternal plasma but does not influence transfer of $[^3]$Hdigoxin (P-gp substrate) across the placenta and across the fetal and maternal BBB. In addition, there were no sex-specific differences in any of the data. Since P-gp in the placenta and fetal BBB is unaffected by a nonlethal dose of LPS, it suggests that at these sites the transporter is resilient to the influence of infection. Conversely, LPS did produce an early increase in P-gp-mediated substrate efflux in the maternal heart at E17.5. This suggests that infection inhibits P-gp-mediated substrate efflux during late gestation. Importantly, we have also shown that LPS increases the absolute levels of $[^3]$Hdigoxin in the maternal plasma, fetal
unit, fetal body, fetal brain, maternal brain, and maternal heart during late gestation. This
suggests that while infection is not directly affecting P-gp-mediated substrate efflux at the
placental barrier or maternal and fetal BBB, it could be influencing substrate distribution
through its effects on hepatic elimination of [3H]digoxin. This will be further explored
below. For now, we must understand the possible mechanisms behind the infection-
induced inhibition of P-gp expression/function that is observed in other tissues and why
this effect is not observed in the present study.

Bacterial infection, modeled by LPS, has been shown to reduce Abcb1a/1b mRNA
expression in the liver \(^{102}\), intestines \(^{103}\), jejunum \(^{103}\), and placenta \(^{104,105}\). This reduction
correlates with a significant increase in plasma concentrations of the proinflammatory
cytokines, IL-6 and TNF-\(\alpha\) \(^{102,105}\). Thus, it is speculated that IL-6 and TNF-\(\alpha\) are
important mediators in the LPS-induced downregulation of Abcb1a/1b mRNA
expression. Importantly, the present study shows that IL-6 levels were significantly
elevated by LPS administration at 4h. With respect to P-gp-mediated substrate efflux,
studies have shown that LPS increases fetal accumulation of \(^{99}\)mTc-sestamibi \(^{104}\).
However, the studies in pregnant rodents were undertaken only during late gestation
(E17) by which point placental Abcb1b expression has decreased significantly \(^{12}\). In
addition, these studies show that LPS-induced effects occur with a minimum dose of 0.5
mg/kg. These types of doses have been shown to be lethal to the fetus, causing significant
IUFD \(^{106}\). While it is important to understand whether fetal demise in an infection
environment can be attributed to alterations in expression/function of placental and BBB
transporters, it is also crucial to determine how the relative distribution of compounds in
the mother and fetus is affected by nonlethal maternal bacterial infections. Assessing the transport effects of this type of infection will allow us to understand how vulnerable the mother and growing fetus will be to various P-gp substrates. Many P-gp substrates are environmental agents (e.g., pesticides/herbicides) and therapeutic drugs (e.g., anticancer drugs) so it is important to understand how a nonlethal maternal infection influences transport of these substrates into the fetus. The distinction between lethal infection and nonlethal infection is important because the majority of maternal bacterial infections do not result in fetal death. While past studies have shown that lethal doses of LPS influence P-gp-mediated substrate efflux at the placental barrier during late gestation, the results of the present study indicate that placental and BBB P-gp is comparatively unaffected by nonlethal doses of LPS at mid and late gestation.

Infection has been shown to affect Toll-like receptor (TLR) signaling. Specifically, LPS has been shown to activate TLR4. TLR4 recruits the adaptor protein MyD88, which, after a series of downstream signaling events, allows NF-kB to translocate into the nucleus. Within the nucleus, NF-kB regulates the expression of pro-inflammatory cytokines, such as IL-6 and TNF-α. Interestingly, LPS has also been shown to induce cytokine production by macrophages and dendritic cells in a MyD88-independent manner. While cytokine production and release is an important indicator that an infection is present, it is changes in NF-kB signaling that have been shown to be associated with changes in P-gp expression. NF-kB is a family of ubiquitous transcription factors that has been shown to play an anti-apoptotic role in cancer cells. Some studies have shown that NF-kB is involved in TNF-α-induced Abcb1b expression in rat hepatocytes, in 2-
acetylaminofluorene-induced \(ABCBl\) expression in human hepatoma cells and in \(ABCBl\) expression in MDR cells \(^{142-144}\). Another study showed that inhibition of NF-kB activity decreases \(ABCBl\) expression in HCT15 human colon cancer cells \(^{141}\). It has been suggested that an NF-kB binding site is located at bp -167 to -158 of the rat \(Abcb1b\) promoter \(^{145}\). In the present study, it is possible that LPS activates the My-D88 signaling pathway, which ultimately leads to NF-kB translocation to the nucleus. This subsequently leads to \(Abcb1a/1b\) downregulation in the liver, a decrease in P-gp function, and a reduction in hepatic elimination of P-gp substrates.

TLR function in the placenta is not well understood. TLR4 is believed to play a role in innate immunity in the placenta \(^{146}\). Activation of TLR4 in the placenta has been shown to promote NF-kB signaling and cytokine production \(^{146}\). MyD88 is believed to be a mediator in this pathway with respect to LPS-induced cytokine production \(^{147}\). We had speculated that LPS-induced activation of TLR4 would, via the MyD88-dependent pathway, promote NF-kB signaling and cytokine production as shown in previous studies \(^{141}\). This would subsequently lead to changes in P-gp expression and function. While our study suggests that LPS does not significantly alter P-gp function in the placenta, there are trends suggesting that it could inhibit placental P-gp-mediated substrate efflux at E15.5 (Fig. 7A) and E17.5 (Fig. 8A). It is possible that this would have reached significance with a slightly higher dose of LPS. However, our dose titration data suggests that a higher dose causes IUFD. Nevertheless, it is interesting to consider that the dose at the threshold of lethality (150 ug/kg) produces results that are at the borderline of significance. Significant undesirable fetal accumulation of P-gp substrates may play a
role in altering normal fetal development to the extent that it leads to IUFD. In a future study, it would be interesting to see if P-gp function is significantly inhibited in the placentas of surviving fetuses from mothers administered the minimum lethal dose (200 ug/kg in our experiment). This may highlight the important role that P-gp function plays in maintaining fetal viability.

With respect to the BBB, previous studies have shown in the adult brain that LPS inhibits *Abcb1a* expression 6h after injection and increases P-gp-mediated substrate efflux 24h after injection\(^{104}\). These effects may be mediated by TNF-\(\alpha\) as shown in more recent studies\(^ {148}\). Contrary to these previous studies, our data suggests that LPS does not affect P-gp-mediated substrate efflux at 4h or 24h after injection at either the fetal BBB (Figs. 9, 10) or the maternal BBB (Fig. 11). This may have to do with differences in the doses administered. In previous studies, effects at the adult BBB were observed with doses ranging from 0.5 to 5.0 mg/kg. In our study, these doses would have led to IUFD, thereby defeating one of the key conditions of the study in that the infection must be nonlethal to mother and fetus. As observed in the placenta, the nonlethal dose may be responsible for the lack of significant effect at the fetal BBB. However, there are no trends in the data to suggest that LPS affects P-gp-mediated efflux at the fetal BBB at all. One contributing factor to the lack of any noticeable trends at the fetal BBB could be the route of administration. It is still not clear whether sufficient levels of LPS cross the placenta. Consequently, most studies that have investigated the fetal effects of bacterial infection administered LPS through the intraamniotic route\(^ {149}\) or directly into fetal veins\(^ {150}\). However, these studies intended to model an infection that directly affected the fetus or
the fetal-maternal interface. In our study, the intraperitoneal route was selected because it would allow us to model a global maternal infection. Use of these other routes would have changed another key condition of our study in that the infection must be global and maternal. One study that investigated the effects of LPS (i.p.) on the fetal guinea pig brain identified elevations of maternal plasma IL-6 with an LPS dose of 25 ug/kg and plasma TNF-α with a dose of 100 ug/kg. This coincided with IUFD, which began occurring with a dose of 50 ug/kg. In contrast to what was observed in the maternal plasma, there were no significant elevations of IL-6 or TNF-α in the amniotic fluid even with an LPS dose of 300 ug/kg. This could suggest that insufficient LPS crosses the placenta at nonlethal doses since there were no differences in amniotic cytokine levels at nonlethal, or even lethal, doses. It was only with a minimum dose of 300 ug/kg, by which point more than 50% of the fetuses were dead, that there were effects in the fetal brain. Specifically, this super-lethal dose of LPS caused significant cell death in the corpus callosum and the cortical grey matter of the central fissure. Again, the dose of LPS plays an important role in these studies. A dose of LPS modeling a nonlethal maternal bacterial infection may not significantly affect P-gp-mediated substrate efflux at the fetal BBB because sufficient LPS is unable to cross the placenta and enter fetal circulation.

Our study suggests that nonlethal maternal infection significantly inhibits P-gp-mediated substrate efflux in the heart at late gestation, 4h after LPS injection. ABCB1 is expressed in the endothelium of heart arterioles and capillaries. Studies conducted in the adult rat heart have shown that high dose LPS significantly inhibits Abcb1a expression 6h after injection and increases substrate accumulation 1h after injection. Nevertheless, this is
the first study to show that a nonlethal infection can also affect P-gp-mediated substrate efflux in heart suggesting that the heart is one of the organs most sensitive to transport modulators. Interestingly, this effect was seen only at the 4h time point and only at E17.5. The 4h effect may be due to the significant elevation of proinflammatory cytokines observed early after LPS injection. This was seen in the present study with IL-6 (Fig. 6) and in other studies with other cytokines \textsuperscript{105,136,137}. With respect to gestational day, the LPS-treated groups at both ages trended towards increased accumulation of [\textsuperscript{3}H]digoxin. Nevertheless, the fact that it only reached significance at E17.5 suggests that the maternal heart is more susceptible to P-gp modulators at late gestation. This could have important clinical consequences for pregnant women prescribed digoxin for cardiac arrhythmias. For example, tachyarrhythmias have been shown to be common during pregnancy and digoxin is the preferred drug in many cases \textsuperscript{153}.

One of the key findings in our study was the effect that LPS had on absolute levels of substrate in various tissues. While LPS did not significantly alter placental or fetal BBB drug ratios at E15.5 or E17.5, it significantly increased absolute [\textsuperscript{3}H]digoxin levels in the various tissues at E17.5 and produced a trend toward increased [\textsuperscript{3}H]digoxin levels in these tissues at E15.5. It is possible that while nonlethal bacterial infection has little effect on placental or BBB transfer of P-gp substrates, it can influence drug distribution through its effects on P-gp-mediated substrate efflux in the liver. This was suggested in a previous study in which LPS (approximately 100 µg/kg) was injected directly into the lateral ventricles of male rats in order to model a CNS infection \textsuperscript{114}. Animals were then euthanized at various time points after LPS administration, but not before being injected
with [³H]digoxin 2h prior to euthanasia. LPS treatment was found to produce significant elevations of [³H]digoxin levels in the plasma, liver, brain, and kidney, but have no effect on the drug ratios of these tissues taken against plasma. An elevation of substrate levels in the plasma might have suggested that metabolism was being affected. However, the study showed that the percentage radioactivity recovered as unmetabolized “parent” digoxin was identical in saline- and LPS-treated animals at all time points tested suggesting that altered metabolism was not primarily responsible for the observed increase in plasma radioactivity. In fact, in female rodents, metabolism of digoxin is even less influential as a confounding variable because female rodents lack the CYP3A2 enzyme, which is one of primary enzymes responsible for breaking down digoxin. The study went further to assess P-gp function in the liver and found that in the presence of the P-gp inhibitor cyclosporin-A, the ratio of parent [³H]digoxin to metabolite detected in the bile was significantly reduced. This provided evidence that LPS inhibited P-gp-mediated transport of [³H]digoxin into the bile. The liver:plasma ratio fails to capture this phenomenon since the ratio uses whole liver substrate accumulation as opposed to substrate accumulation in the bile and hepatocytes, separately. This key finding suggested that an LPS-induced inhibition of P-gp-mediated transport of substrate into the bile could lead to a buildup of that substrate in liver hepatocytes. This could subsequently hinder substrate transfer into the liver from the plasma, which could ultimately hinder substrate efflux into the plasma from the brain and kidney. Thus, the study concluded that these elevations in [³H]digoxin levels in the liver, plasma, brain, and kidney were specifically due to an LPS-induced reduction in liver P-gp function and a perturbation of hepatic elimination of digoxin. The present study demonstrates that these findings are not
exclusive to male rats. During late gestation, we found that pregnant mice treated with LPS had significant elevations in $[^3]H$digoxin levels in the maternal plasma, fetal unit, fetal body, fetal brain, maternal brain, and maternal heart. The effects in the Goralski study were most evident at the earliest time point, 6h $^{114}$. In the present study, the effects were only significant at 4h. This suggests that they could be mediated by proinflammatory cytokines, as levels are significantly elevated at 4h as seen in the present study. Again, as observed with the heart:plasma ratio, these effects were age-dependent in that they only occurred at E17.5. Since placental $Abcb1a/1b$ expression significantly decreases by E17.5 $^{12}$, it is possible that the $Abcb1a/1b$ expression profile in various maternal tissues begins to more closely resemble that of the non-pregnant animals in the Koralski study. In a future study, it will be interesting to compare $[^3]H$digoxin in the bile and liver hepatocytes at E15.5 and E17.5 to determine if LPS does indeed significantly reduce hepatic elimination of substrate in pregnant mothers during late gestation. Another proposed future study would be to investigate whether an inhibitor of IL-6 expression/function reduces the LPS-induced elevation in tissue accumulation of P-gp substrate. If it does, it would confirm the critical role that IL-6 plays in mediating LPS-induced inhibition of P-gp function.

In conclusion, a nonlethal maternal bacterial infection does not affect transfer of P-gp substrates across the placenta and across the fetal BBB. However, our results suggest that infection could still influence the accumulation of P-gp substrates in the fetal and maternal compartments potentially through its effects on hepatic elimination of these substrates. Further studies are required specifically to assess hepatic elimination of
[³H]digoxin by measuring substrates in bile and liver hepatocytes. Nevertheless, while liver P-gp may be susceptible to nonlethal infection, the fact that P-gp-mediated substrate efflux at the placenta and fetal BBB were unaffected suggests that at these two barrier sites P-gp is relatively resilient to the influence of a nonlethal maternal bacterial infection.
Figure 6. IL-6 Concentration in the Maternal Plasma. Plasma concentrations of IL-6 in pregnant mice euthanized 4h or 24h after treatment with LPS or vehicle control on (A) E15.5 and (B) E17.5. Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=3-6/group. ***P<0.001 versus vehicle.
Figure 7. Effects of LPS on P-gp Activity in the Placenta at E15.5. (A) Transplacental transfer of $[^3]$H]digoxin to the fetal unit at 4h and 24h after treatment with LPS (solid bar) or vehicle control (open bar) on E15.5. Absolute $[^3]$H]digoxin levels in the (B) maternal plasma and (C) fetal unit. Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=5-6/group.
Figure 8. Effects of LPS on P-gp Activity in the Placenta at E17.5. (A) Transplacental transfer of $[^3]$H]digoxin to the fetal unit at 4h and 24h after treatment with LPS (solid bar) or vehicle control (open bar) on E17.5. Absolute $[^3]$H]digoxin levels in the (B) maternal plasma and (C) fetal unit. Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=3-4/group. ***$P<0.001$, *$P<0.05$ versus vehicle.
**Figure 9. Effects of LPS on P-gp Activity at the Fetal BBB at E15.5.** (A) Net transfer of $[^3]$Hdigoxin from the fetal body to the fetal brain at 4h and 24h after treatment with LPS (solid bar) or vehicle control (open bar) on E15.5. Absolute $[^3]$Hdigoxin levels in the (B) fetal body and (C) fetal brain. Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=5-6/group.
Figure 10. Effects of LPS on P-gp Activity at the Fetal BBB at E17.5. (A) Net transfer of [³H]digoxin from the fetal body to the fetal brain at 4h and 24h after treatment with LPS (solid bar) or vehicle control (open bar) on E17.5. Absolute [³H]digoxin levels in the (B) fetal body and (C) fetal brain. Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=3-4/group. ***P<0.001, **P<0.01 versus vehicle.
Figure 11. Effects of LPS on P-gp Activity at the Maternal BBB. (A) Net transfer of $[^3]$Hdigoxin from the maternal plasma to the maternal brain at 4h and 24h after treatment with LPS (solid bar) or vehicle control (open bar) on E15.5 and (C) E17.5. Absolute $[^3]$Hdigoxin levels in the maternal brain on (B) E15.5 and (D) E17.5. Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=5-6/group. *P<0.05 versus vehicle.
Figure 12. Effects of LPS on P-gp Activity at the Maternal Heart. (A) Net transfer of $[^3\text{H}]$digoxin from the maternal plasma to the maternal heart at 4h and 24h after treatment with LPS (solid bar) or vehicle control (open bar) on E15.5 and (C) E17.5. Absolute $[^3\text{H}]$digoxin levels in the maternal heart on (B) E15.5 and (D) E17.5. Values are expressed as litters averaged per dam. Bar represents mean +/- SEM drug ratio for n=5-6/group. **$P<0.01$, *$P<0.05$ versus vehicle.
Chapter 5: Conclusions and Future Directions
5.1 OVERVIEW

The studies presented in this thesis suggest that the SSRI sertraline is a modulator of placental and fetal BBB P-gp-mediated substrate efflux during mid-gestation (Fig. 13) while nonlethal maternal bacterial is not a modulator during mid- or late gestation (Figs. 14-15).

5.2 SERTRALINE AND P-GP IN THE PLACENTA AND FETAL BBB

In the first study, sertraline significantly stimulated placental P-gp-mediated substrate efflux at the same time as it inhibited P-gp-mediated substrate efflux at the BBB at mid gestation, 4h after sertraline injection. This suggests that the effects of sertraline on P-gp activity are tissue-specific. One possible explanation for these tissue-specific effects is that two distinct genes encode P-gp in the mouse. *Abcb1a* is the predominantly expressed gene at the BBB while *Abcb1b* is the predominantly expressed gene at the placental barrier. Previous studies have shown that the distinct isoforms encoded by these two genes have some functional differences. Specifically, it has been shown that progesterone inhibits drug efflux with more potency in cells expressing *Abcb1b*-encoded P-gp compared to cells expressing *Abcb1a*-encoded P-gp \(^{124}\). This study also discovered a single class of vinblastine binding sites on *Abcb1b*-encoded P-gp and two classes of such sites on the *Abcb1a*-encoded P-gp \(^{124}\). Consequently, it is also possible that sertraline differentially binds *Abcb1a* and *Abcb1b* and thus modulates BBB P-gp and placental P-gp in unique ways. A future study needs to be carried out in order to assess this possibility. This can be done using viral vectors to transfect cells with either *Abcb1a* or *Abcb1b*. If
sertraline increases substrate accumulation in Abcb1a-expressing cells while it simultaneously decreases accumulation in Abcb1b-expressing cells, this would suggest that sertraline does indeed have isoform-specific effects. The earliest research on the effects of SSRIs on P-gp were carried out in various normal and tumour cell lines and sertraline has been shown to be one of the most potent inhibitors of P-gp function\textsuperscript{90,97,98}. Compounds such as rhodamine-123 and calcein-AM would most likely be the substrates of choice since they are well-established P-gp substrates, \textit{in vitro}\textsuperscript{119,155}. If the effects are found to be isoform-specific, this may present a novel limitation of the mouse model in \textit{ABCB1} transport studies. Human P-gp is encoded by only one gene, \textit{ABCB1}\textsuperscript{17}. While the discovery of isoform-specific effects in the mouse may allow us to better understand mouse biology, its clinical relevance to humans may be questionable. Regardless, the discovery of such a limitation will certainly help us to design better experiments for transport studies in the future.

If the effects are found to not be isoform-specific, a future study must be carried out in order to confirm tissue-specificity. This could be made possible by comparing P-gp substrate accumulation in brain endothelial cells and placental cells treated with sertraline. In fact, sertraline-induced inhibition of P-gp function was first discovered in porcine brain capillary endothelial cells\textsuperscript{90} and it would be interesting to repeat that experiment alongside human cytotrophoblast cells such as BeWo cells\textsuperscript{156}. If sertraline increases substrate accumulation in porcine brain capillary endothelial cells while it simultaneously decreases accumulation in BeWo cells, this would confirm our findings.
that sertraline inhibits P-gp at the BBB while it simultaneously stimulates P-gp in the placenta.

One major finding from these seminal studies was that modulation of P-gp function was not exclusive to just one SSRI \(^90,97,98\). A future study needs to assess the potential \(\textit{in vivo}\) modulatory properties of fluoxetine, paroxetine, and even the sertraline metabolite, desmethylsertraline. Fluoxetine and paroxetine are the third and fifth most prescribed antidepressant in the United States, respectively \(^110\). Desmethylsertraline is an active metabolite of sertraline and also functions as an SSRI. Thus, the question will be asked: are these modulatory effects shared by other SSRIs or is there something unique about sertraline that allows it to be such a potent modulator of P-gp \(\textit{in vitro}\) and \(\textit{in vivo}\)? Answering this question may provide us with a better understanding of how modulators influence P-gp expression and activity.

If the effects are discovered to be shared by other SSRIs, this information may be important when prescribing antidepressants during pregnancy. As already stated, maternal SSRI use has been shown to be linked with various behavioural and neurodevelopmental abnormalities. This may be partially due to an SSRI-induced inhibition of P-gp function in the fetal BBB. While it is highly recommended that pregnant women take antidepressants to control their depression, selectivity of that antidepressant may be crucial especially if other SSRIs prove to be effective inhibitors of fetal BBB P-gp function.
A final study should assess if the effects of sertraline, and potentially other SSRIs, are age-specific. The purpose of this study was to assess the effects of sertraline on P-gp function during mid-late gestation when fetal complications associated with maternal SSRI use have been shown to be common\textsuperscript{131}. However, SSRI use is common throughout pregnancy\textsuperscript{132} so it would be important to repeat this experiment during early gestation. This will provide insight on when the gateway in the placenta and at the BBB is most vulnerable to modulation thereby offering clinicians crucial information on the time periods during pregnancy when the fetus is most susceptible to potential teratogenic effects induced by sertraline (and potentially other SSRIs).

5.3 INFECTION AND P-GP IN THE PLACENTA AND FETAL BBB

In the second study, a nonlethal maternal bacterial infection did not significantly affect P-gp-mediated substrate efflux at the placenta or the BBB during mid or late gestation, 4h or 24h after the onset of infection. This suggests that at these sites P-gp is resilient to the influence of an acute nonlethal maternal infection. Despite the lack of significant effect, trends suggest that there was greater accumulation of substrate in the fetuses of LPS-treated mice at both ages. Our data also indicates that the threshold of dose lethality is at the borderline of statistical significance. In a future study, it will be interesting to investigate whether P-gp function is significantly inhibited in the placenta of surviving fetuses from mothers injected with the minimum lethal dose (200 ug/kg in our experiment). Perhaps the data will reach significance at this dose. This would certainly highlight the vital role that placental P-gp plays in maintaining fetal viability by reducing unwanted fetal accumulation of xenobiotics.
Nevertheless, while the ratios did not reach significance, our data suggests that nonlethal infection could still influence substrate accumulation in various maternal and fetal tissues through its effect on hepatic elimination in the liver as seen in previous studies \(^{141}\). In a future study, P-gp substrate levels in the bile must be compared to levels in liver hepatocytes in order to assess this possibility. If bile accumulation of substrate is reduced in LPS-treated pregnant mice, this will suggest that hepatic elimination is indeed being affected. This would also suggest that liver P-gp plays a decisive role in the biodistribution of substrates in the pregnant body, one that overshadows the roles of placental P-gp and fetal BBB P-gp. This would not be surprising since the liver is generally the ultimate destination for all P-gp substrates. If the function of liver P-gp is indeed found to perturbed by nonlethal doses of LPS, this may have important clinical implications. Specifically, it would mean that fetal and maternal tissues are more susceptible to unwanted P-gp substrate accumulation in the event of a maternal infection. This information could be useful for clinicians when writing prescriptions for anticancer drugs, HIV inhibitors, cardiac glycosides, SSRIs, and other drugs that are P-gp substrates. It may be wise to reduce intake of these drugs during the course of a maternal infection. The value of this information may not be exclusive to pregnant women. It remains to be seen whether hepatic elimination of P-gp substrates is altered by infection. If it is, further studies might suggest that male and non-pregnant female animals are equally vulnerable.

Since P-gp in the placenta, BBB, and liver has been shown to be significantly inhibited by lethal doses of LPS in previous studies, a future study should also focus on two
potential mediators of this relationship, specifically the transcription factor NF-kB and the proinflammatory cytokine IL-6. NF-kB and IL-6 have been shown to be very important mediators in infection-induced alterations in P-gp expression. It is already known that LPS activates TLR4 which, via the MyD88-dependent pathway, leads to NF-kB signaling and cytokine production \(^{141}\). Studies have already shown that NF-kB is involved in changes in \(ABCB1\) expression in hepatocytes, tumor cells, and other \(ABCB1\)-expressing cells \(^{141-144}\). This could be mediated by IL-6, which has been shown to be elevated in LPS-treated pregnant and non-pregnant animals \(^{105,136,137}\). In fact, one study has shown that IL-6, by itself, produces a 70% reduction in protein expression and a 40-70% reduction in mRNA levels of \(Abcb1a\) and \(Abcb1b\) in the liver \(^{102}\). Thus, a possible future study could revolve around IL-6, specifically to see if IL-6 modulates P-gp function at the placenta, BBB, and liver. If IL-6 does indeed play a mediator role in this relationship, then one would expect the proinflammatory cytokine to increase placental and fetal BBB P-gp-mediated substrate efflux, or at the very least, influence substrate biodistribution through interactions with liver P-gp. To make this study more interesting, an IL-6 inhibitor could be used to see if any IL-6-induced changes in substrate biodistribution return to control levels.

The results of this study suggest that acute maternal bacterial infection does not significantly alter P-gp-mediated substrate efflux at the placenta or fetal BBB. It is possible that the short release of proinflammatory cytokines observed with a single injection of LPS \(^{105,136,137}\) is insufficient to produce any significance changes in transport.
The effects may be different when LPS is administered chronically as this would maintain the elevation in plasma proinflammatory cytokine levels.

Some infection studies have administered LPS chronically to assess various outcomes. Wang and colleagues injected (i.p.) pregnant CD-1 mice with LPS (8 ug/kg) daily from E8 to E15. They subsequently performed neurobehavioural testing on post-natal day (PND) 70, 200, 400, and 600 and discovered that spatial learning, memory ability, and sensorimotor function were impaired in an age-dependent manner in the offspring of LPS-treated mothers. While it is simple to assess age-dependent effects in post-natal animals, it may be more problematic to do so for growing fetuses. By injecting chronically over the course of a few days, it may be difficult to attribute an effect to any specific period during this treatment program. This is especially true with the mouse, which has a very short gestation. By injecting over the course of E8 to E15, one covers a developmental period spanning early to mid gestation and involves significant development of the fetal body, fetal brain, and other fetal organs. Thus, future studies must take into consideration what the model of infection represents clinically. For example, if the comparison is between the effects of a mid gestation infection, a late gestation infection, and no infection, one group of pregnant mice could receive LPS/VEH from E12 to E15 while another group receives LPS/VEH from E15 to E18. The constant must be that the infection is nonlethal to mother and fetus. This may be problematic since previous chronic infection studies conducted on pregnant mice have shown that even very low doses of LPS can cause fetal death. Zhao and colleagues injected (i.p.) CD-1 mice with LPS (10-30 ug/kg) daily from E8 to E12. They discovered significant fetal
mortality, facial and eye malformations, and skeletal deformities in litters from mothers chronically injected with 20 and 30 ug/kg. The antioxidant, phenyl-N-t-butyl nitrone, protected fetuses against these deficits. Thus, it becomes apparent that modeling a nonlethal maternal infection may be difficult to accomplish using a chronic LPS treatment paradigm.

Not many studies have administered LPS chronically simply using syringes and needles. Indeed the most popular way with which to provide continuous, timely administration of a compound has been through the use of osmotic pumps. These pumps have been implanted intraperitoneally, subcutaneously, intraamniotically, and intratracheally and used to infuse LPS in a variety of test animals including rats, mice, and fetal sheep. These pumps can deliver solutions continuously for 28 days without the need for external connections or frequency handling of animals and that they can deliver substrates into any tissues via the use of a catheter that does not alter the pumping rate of the pump. Nevertheless, they cannot be implanted intraperitoneally in mice, though one may be able to insert a cannula into the intraperitoneal cavity. In addition, while they have been used to deliver LPS in non-pregnant mice and pregnant sheep, they have not yet been used to deliver LPS in pregnant mice. Any future study to do this will be the first to use these pumps for this application.

In conclusion, the issues of age-specific treatment, fetal toxicity and mode of administration must be addressed when designing a chronic LPS treatment program. As already suggested, the first issue may be addressed by dividing pregnancy into distinct,
non-overlapping periods, such as mid and late gestation, and modeling infections specifically during each of these periods. The second issue can be addressed pragmatically through dose titration. The third issue may be overcome through either the use of an osmotic pump and a cannula inserted into the intraperitoneal cavity or daily injections (i.p.) of LPS. One suggestion to overcome at least two of these issues would be to switch the animal and perhaps use the guinea pig. This animal has a much longer gestation (~60-70 days) and can be implanted intraperitoneally with osmotic pumps.

5.4 CLOSING STATEMENTS

It is the grandest of understatements to suggest that the maternal environment plays a critical role in fetal outcome. Tragic, isolated historical events such as the Dutch famine of 1944 have given us insight into the grim, enduring consequences of an impoverished maternal environment. Since, decades of research have elucidated the intricate relationship between a mother and her growing fetus. What has become widely accepted is that the organ that connects these two individuals, the placenta, plays a pivotal role in maintaining a healthy relationship between them. The fetal BBB also plays a role by protecting the fetal brain from teratogens present in the maternal environment during critical periods of development. In these organs, the task of protection has been delegated to a series of membrane-associated transporters. P-gp is one of the most important of these gatekeepers. Thus, in order to improve our understanding of the influence of the maternal environment on fetal outcome, we must further explore how P-gp is regulated so that we can appreciate how fetal protection is maintained and how it is perturbed. It is already known that many therapeutic drugs and environmental agents present in the
The maternal environment can regulate P-gp in the placenta and fetal BBB. The studies presented in this thesis introduce two more. First, the SSRI sertraline significantly increases placental P-gp function at the same time as it decreases fetal BBB P-gp function. This suggests that protection in the placenta is maintained (and possibly augmented) at the same time as it is taken away at the fetal BBB. Second, a maternal bacterial infection, nonlethal to mother and fetus, influences accumulation of substances in the fetus and fetal brain and may do so through its effects on P-gp function in the liver. Maternal SSRI use and bacterial infections have been linked to various fetal complications. The implication of the studies in this thesis is that disturbances in the protection provided by P-gp may contribute to many of these complications, specifically those in developing brain. Consequently, it will be wise to consider this information when prescribing SSRIs to pregnant women and in the event of a maternal bacterial infection. The nine months spent in utero leave a permanent scar. If the ultimate goal is to ensure optimal fetal development, understanding and improving the maternal environment should be the primary objective.
**Figure 13. Summary of Sertraline Effects on P-gp Activity.** Levels of P-gp substrate in the various tissues of (A) vehicle-treated, (B) 1 mg/kg sertraline-treated, and 10 mg/kg sertraline-treated pregnant animals. Compared to vehicle-treated animals, sertraline at doses of 1 mg/kg and 10 mg/kg stimulates P-gp activity in the placenta at the same time as it inhibits P-gp activity in the BBB in a dose-dependent manner. This leads to a dose-dependent decrease in substrate transfer into the fetus and an increase in transfer into the fetal brain. Net accumulation in the fetal brain is similar because placental P-gp stimulation reduces the availability of substrate in fetal circulation. Modified from a figure created by Dr. Stephen G. Matthews.
Figure 14. Summary of LPS Effects on P-gp Activity at E15.5. Levels of P-gp substrate in the various tissues of (A) vehicle-treated and (B) LPS-treated pregnant animals at E15.5. LPS does not affect placental or fetal BBB P-gp activity. Consequently, the levels of P-gp in the fetus and the fetal brain are similar in vehicle-treated and LPS-treated animals. Modified from a figure created by Dr. Stephen G. Matthews.
Figure 15. Summary of LPS Effects on P-gp Activity at E17.5. Levels of P-gp substrate in the various tissues of (A) vehicle-treated and (B) LPS-treated pregnant animals at E17.5. LPS does not affect placental or fetal BBB P-gp activity. Thus, the relative amount of substrate being transferred across the two barriers is similar in both groups. Nevertheless, absolute substrate levels in tissues are different between groups possibly because of an LPS-induced reduction in hepatic elimination of substrate. Modified from a figure created by Dr. Stephen G. Matthews.
REFERENCES


