ANTIDEPRESSANT USE DURING PREGNANCY: DETERMINING THE IMPACT ON THE GUT SEROTONERGIC SYSTEM IN THE OFFSPRING
ANTIDEPRESSANT USE DURING PREGNANCY: DETERMINING THE IMPACT ON THE GUT SEROTONERGIC SYSTEM IN THE OFFSPRING

By

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TITLE: Antidepressant use during pregnancy: Determining the impact on the gut serotonergic system in the offspring

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ABSTRACT

Approximately 10% of pregnant women take antidepressants. Prenatal exposure to selective serotonin reuptake inhibitors (SSRIs), a class of antidepressants, has been shown to alter serotonergic signaling in the brain. However, the effects of SSRIs on peripheral serotonin (5HT) synthesis and/or signaling have largely been ignored. Serotonin in the gut is critical for intestinal function and dysregulation of this pathway is associated with intestinal disease. Therefore, the goal of this study was to determine the effects of perinatal exposure to the SSRI fluoxetine (Prozac®) on intestinal health in the offspring.

Dams were given vehicle or fluoxetine hydrochloride (FLX 10 mg/kg/d; N=15) for 2 weeks prior to mating until weaning. We assessed markers of serotonergic signaling, inflammation, and composition of the gut microbiota in the offspring.

Male offspring of fluoxetine-treated dams had significantly elevated serum levels of 5-HT and decreased expression of the $5HT_{2A}$ receptor and $MAO$. In female offspring there was no effect of SSRRI exposure to alter any components of serotonergic signaling. Although we did not find any evidence of increased inflammation following fluoxetine exposure, there were significant alterations in the composition of the gut microbiota in the exposed offspring.

Male offspring of SSRIs-exposed mothers had changes in key components of the gut serotonergic system in association with elevated levels of serum 5-HT and alterations in the gut microbiota in adulthood. The impact of these changes on intestinal health and the reasons for the sex specific effects remain to be determined.
I dedicate this work to my pillars of support: Mom, Dad and Rosalind. To all present and future students, researchers, scientists and inquisitive-minds, may this thesis add a drop to your fountain of knowledge.
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ABBREVIATIONS

5-HT - Serotonin
CON - Control
EC Cell - Enterochromaffin cell
E - Embryonic Day
FLX - Fluoxetine
GDS - Gut derived serotonin
H & E - Hematoxylin and Eosin
IBD - Inflammatory Bowel Disorder
IBS - Irritable Bowel Syndrome
IHC - Immunohistochemistry
MAO - Monoamine Oxidase
MDD - Major Depressive Disorder
OTU - Operational Taxonomic Unit
P - Postnatal Day
PCoA Plot - Principal Component of Analysis Plot
SERT - Serotonin Reuptake Transporter
SSRI - Selective Serotonin Reuptake Inhibitor
TJ - Tight Junction
TLR - Toll-like receptor
Tph - Tryptophan hydroxylase-1/2
DECLARATION OF ACADEMIC ACHIEVEMENT

All work in this thesis was completed by me except the following: Serum 5-HT levels were determined by Nicole De Long from Dr. Alison Holloway’s lab. Colonic enterochromaffin cell immunohistochemistry (IHC) and counts were done by Rajka Borjevic and Katelynn Tang from Dr. Elyanne Ratcliffe’s lab. Processing of Illumina sequencing data was done by Dr. Michael Surette. Statistical analysis of gut microbiota using R and PCoA plots were constructed by Jake Szamosi.
CHAPTER 1: INTRODUCTION

1.1 The occurrence of depression

According to the Canadian Community Health Survey, the 12-month prevalence rate of depression is approximately 4.5% among all Canadians older than 12 years of age [1]. In another population-based survey, approximately 7% of adults reported depression in the preceding year [2]. Subsequently in 2010, the Global Burden of Disease (GBD) study identified depressive disorders as the leading cause of disability worldwide [3, 4]. Depressive disorders have increased significantly over the last 20 years; similar studies conducted in 1990 and 2000 ranked depressive disorders as the fourth and third leading cause of disability, respectively [5, 6]. Clearly depression is a global public health concern. Within depressive disorders, major depressive disorder (MDD or major depression) was the main contributor to disease burden, accounting for 85% of years lived with disability (YLDs) and disability adjusted life years (DALYs) [4]. The DSM-IV-TR [7] describes MDD as an episodic disorder with a chronic outcome and an elevated risk of mortality, equivalent to the World Health Organization’s (WHO) International Classification of Disease (ICD) -10’s description of recurrent depressive disorders [8]. MDD involves the presence of at least one major depressive episode, which is characterized by discrete occurrences of persistent depressed mood accompanied by a loss of interest or pleasure in all activities for at least 2 weeks duration. Among the 298 million cases of major depression reported in the most recent GBD study, the highest proportion occurred in individuals between 25 and 34 years of age [4]. Furthermore, consistent with previous reports of
sex differences [9-11], women were twice as likely as men to experience an episode of major depression (5.5% (95% uncertainty: 5.0-6.0%) vs. 3.2% (3.0-3.6%)) [4].

1.1.2 Depression in women of childbearing age

The risk of major depression among women ranges from 10% to 25%, with peak prevalence during childbearing years (18-44 years) [12-15]. When compared to other non-communicable diseases, such as hypertension and diabetes, the occurrence of depression in women of reproductive age remains significantly higher (14.7% vs. 6.9% and 3.1%) [16]. Reasons for the increased risk of depression in women are unclear but likely involve biological, psychological and sociocultural factors [12, 17, 18]. Importantly, the increased lifetime risk of depression in women has been largely attributed to the hormonal changes associated with the reproductive cycle [19]. Since hormonal changes are exacerbated during pregnancy, this period represents a vulnerable window for the onset, recurrence, or exacerbation of depression [20-22]. The prevalence rates of depression during pregnancy are 7.4%, 12.8% and 12% for the first, second and third trimesters, respectively [23]. Furthermore, antenatal depression increases the risk of developing postpartum depression; an outcome which affects approximately 10-15% of women [24, 25]. Taken together, estimates show that 10-16% of all pregnancies fulfill the diagnostic criteria for depression [23, 26-28]

1.1.3 Untreated maternal depression is associated with poor obstetrical, fetal and neonatal outcomes

Pregnancies complicated by maternal depression constitute a complex medical situation, as both the health of the mother (including self-neglect, risk of self-harm or suicide, reduced compliance with prenatal and postnatal care and increased risk taking activities such as drug and alcohol use) [29] and her unborn child (including reduced fetal growth, impaired mother-child bonding and
impaired cognitive, behavioral and emotional development in childhood) may be compromised [30-33]. A meta-analysis has demonstrated that untreated antepartum depression is a strong predictor of adverse pregnancy outcomes including neonatal intensive care unit admission, cesarean or preterm delivery (<37 weeks gestation) and low birth weight (<2,500 g) [34]. The latter two obstetrical complications are leading causes of neonatal, infant and childhood morbidity and mortality worldwide [35-37]. Therefore, drug therapy is recommended during the perinatal period for moderate to severe depression [21, 38]. As rates of perinatal depression have increased [23, 26, 32, 33], so too has the use of antidepressants during pregnancy. It has been reported that antidepressant use during pregnancy increased 300% from 1998 to 2005 [39]. This increase was mostly accounted for by increases in selective serotonin reuptake inhibitor use [40-42].

1.2 SSRI use during pregnancy
A wide variety of medications are available to treat perinatal depression including; first generation tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), serotonin norepinephrine reuptake inhibitors (SNIRs), selective norepinephrine reuptake inhibitors (NRIs), and norepinephrine and dopamine reuptake inhibitors (NDRIs) [43]. However, as a result of their proven specificity, efficacy and safety in adults relative to first generation antidepressants [44-46], the Selective Serotonin Reuptake Inhibitors (SSRIs) including fluoxetine (Prozac®), sertraline (Zoloft®), paroxetine (Paxil®), fluvoxamine (Luvox®) and citalopram (Celexa®) are recommended as first-line therapy in pregnant and postpartum women [14, 47]. Epidemiological data show that over 50% of all women will take a prescription medication in pregnancy, and the most frequently used class of agents is the SSRIs [48]. The SSRIs —which ease depression by inhibiting the reabsorption of the neurotransmitter serotonin
(5-HT) in the brain—remain the pharmacotherapy of choice for up to 15% of expectant women [40]. This is despite evidence from animal studies which link SSRI exposure with an increased risk of birth defects [49-51]. Currently SSRIs are designated a pregnancy Class C (i.e., to be used only if “the potential benefit outweighs the potential risk”) (excludes paroxetine [Paxil], which carries a grade D) by the U.S. Food and Drug Administration [52, 53]. Of the 8.7% of women prescribed antidepressants during pregnancy in the United States, 6.2% had exposure to an SSRI [41]. Indeed the SSRI antidepressants sertraline and fluoxetine were among the top 20 prescription medications taken in the first trimester of pregnancy during the years 1997 to 2003 [48]. The pattern of antidepressant use across pregnancy is variable; with peak prevalence occurring in the first trimester, decreasing in the second trimester and increasing around the time of delivery [40, 54]. Studies suggest that 25% of depressed women continue their antidepressant use throughout pregnancy whereas 0.5% of women start using antidepressants once pregnant [55]. As the use of SSRIs during pregnancy has been steadily on the rise [40-42, 56, 57], so too has the concern regarding the potential for adverse effects on fetal and postnatal development [58].

1.3 Defining Fetal Programming

Sir David Barker was the first to report that babies born with lower birth weight had an increased risk of coronary heart disease mortality in adulthood [59]. This observation was the foundation for the developmental origins hypothesis of adult disease (DOHaD) [60], otherwise known as “fetal programming”. The DOHaD hypothesis proposes that an adverse intrauterine environment can exert profound impacts on fetal development and postnatal health potential. Indeed, there is now considerable evidence that demonstrates an association between poor fetal growth and an increased incidence of cardiovascular disease [61, 62], altered glucose tolerance [63, 64], obesity
type 2 diabetes (T2DM) [67, 68] and hypertension [61] in adult life. The definition of an adverse intrauterine environment encompasses maternal exposures such as undernutrition, smoking, stress, prenatal glucocorticoid exposure, environmental toxicants, and exposure to xenobiotics including environmental toxicants and medications [67].

1.4 Perinatal exposure to SSRIs affects the offspring
Concerns about the use of antidepressants, including SSRIs, in pregnancy and the treatment of mental illness has been the focus of considerable scrutiny [69-71]. SSRIs and their metabolites (e.g. norfluoxetine) have been shown to cross from the placenta into the fetus [72-74] and can be identified in amniotic fluid, umbilical circulation, and fetal serum [75, 76]. Paired maternal and umbilical cord blood collected at delivery shows that SSRIs achieve cord blood concentrations over 50% of those seen in maternal circulation and, in some cases, cord blood concentrations are equal to maternal blood concentrations [77]. Despite the significant heterogeneity among individual SSRIs, animal exposure studies have demonstrated that placental transfer of SSRIs can achieve concentrations in the fetus which are sufficient to block over 90% of the transporter sites in the developing rodent brain [78]. In addition, SSRIs and their metabolites are also present in breast milk [77], and are commonly prescribed for postpartum depression therefore neonates are also equally exposed to these antidepressants [27, 79-81]. As a consequence there have been numerous studies on the safety of SSRI use during pregnancy [77].

1.4.1 Adverse birth outcomes, neonatal complications and neurobehavioural outcomes
There have been many studies looking at the safety of SSRI use during pregnancy; for the most part these studies have focused on the risk of congenital abnormalities [82]. Large studies using national databases confirm that the use of SSRIs during pregnancy is not associated with an increased risk of either major or minor congenital malformations to offspring when used in their
recommended doses [40, 83-90]. Similarly, smaller studies that primarily used cohorts drawn from teratogen information services do not show an effect of SSRI treatment on the overall major malformation rate [85, 86, 91]. Indeed, with the exception of an increased risk of cardiovascular malformations associated with maternal paroxetine use [92], meta-analyses do not find a significant association or pattern of malformations related to perinatal antidepressant exposure [93-100]. These results held across exposures in all trimesters of pregnancy [86, 101]. Diav-Citrin and Ornoy [102] calculated that the overall rate of major congenital malformations and of cardiovascular anomalies in published prospective studies after prenatal exposure to SSRIs were both within their baseline risk (3.8% [189/4920] and 0.9% [53/6094]) in the general population. Therefore, it is generally accepted that SSRIs are not major teratogens [103]. However, a number of epidemiological and population studies outline the relationship between SSRI use and risk of miscarriage, spontaneous abortion [53, 104], stillbirth, and rare birth defects [87, 105, 106].

The potential association between SSRI use and low birth weight has become an important consideration in the evidence supporting “fetal programming” as a model of risk for later adult illnesses. While several studies have shown an association of prenatal SSRI use with low birth weight or small size for gestational age [89, 107-118], others do not [52, 54, 83, 85-87, 105, 119-123]. Despite these conflicting results, meta-analyses associate SSRI use with a significantly increased risk for low birth weight, preterm birth, reduced APGAR scores and increased neonatal hospitalizations [124-126]. These studies and meta-analyses varied widely in design, populations, control groups and methods, and few control for the mental health status of the mother and other potential confounding variables including the drug dose [127], the timing of exposure [54, 118, 128] and the specific SSRI [129].
Short-term adverse outcomes occur in up to 30% of infants exposed in utero to SSRIs during the neonatal period [130-132]. These outcomes include persistent pulmonary hypertension for the newborn (PPHN) [83, 116, 119, 123, 133] and neonatal behavioural syndrome [130, 132, 134, 135]. There is evidence to suggest that late SSRI use in pregnancy (>20 weeks) may increase the risk for PPHN [136-139] however other studies have failed to show a similar effect [40, 140-142]. Similar to all psychotropic medications, SSRIs cross the blood-brain barrier resulting in fetal central nervous system (CNS) exposure. Therefore, maternal SSRI used has been associated with adverse events in the infant including; irritability, trouble feeding, tremor, agitation, hypertonia, respiratory distress, seizures and excessive crying [143-145]. Within the neonatal period, these SSRI-related symptoms have been attributed to both withdrawal syndrome [132] and direct drug effects [146], that result in the disruption of the aforementioned neurobehavioural outcomes [115]. Neonatal neurological symptoms include central nervous system excitation (increased motor activity, restlessness, tremors, seizures, increased arousals) and autonomic symptoms (decreased heart rate variability, temperature instability), fewer changes in behavioral state, increased motor activity and abnormal sleep patterns [115, 132, 147, 148].

Despite the widespread use of SSRIs during pregnancy there is relatively limited information regarding the long-term outcomes related to fetal and/or neonatal exposure to SSRIs. However there is data from animal models which has shown that perinatal exposure to SSRIs can cause subtle changes in brain circuitry and promote maladaptive behaviors (increased anxiety, aggression, depression) that are maintained in adulthood. Indeed, Hansen et al. [149] reported that in rats SSRI exposure during postnatal days (P) P8–P21 resulted in depression-like symptoms during adulthood. Furthermore, Ansorge et al. [150] showed that chronic SSRI treatment during postnatal days P4–21 resulted in reduced exploratory behaviour and increased
anxiety-related phenotype. Other animal studies focusing on the effects of prenatal SSRI exposure revealed that exposed offspring showed a range of behavioural abnormalities including: delayed motor development, improved spatial learning [151], reduced impulsivity, increased immobility in the forced swim test [152] and increased sensitivity to the reinforcing effects of cocaine and reduced extinction of drug-seeking behaviour [153]. There is also data from human clinical studies which suggests that exposure to SSRIs during pregnancy can permanently alter brain development leading to long-term neurodevelopmental abnormalities and behavioral changes [115] including lowered psychomotor development [111, 154, 155] and blunted response to pain [156]. In addition, children exposed to SSRIs in utero have impaired cognitive and language development [120, 154, 157], in addition to an increase in internalizing [158] and externalizing behaviors [157, 159]. More recently there has been considerable interest in the association between prenatal exposure to prenatal SSRIs and an increased risk for autism spectrum disorder (ASD) [110, 160-165]. To date, most of the evidence for altered neurodevelopmental and behavioural outcomes is in young children; whether or not these persist into adulthood is not clear. However, evidence from rodent studies demonstrates that these anxiety- and depression-like behaviors caused by the perinatal and/or neonatal administration of SSRIs may persist into adolescence [166] and adulthood [150]. Taken together these data suggest that prenatal exposure to SSRIs may have profound and persistent effects on neurodevelopment; effects which are likely mediated via alterations in central serotonergic pathways.

1.4.2 Perinatal SSRI exposure and effects on the gastrointestinal tract

In neonates whose mothers were taking SSRIs, there have been reports of adverse gastrointestinal (GI) symptoms including diarrhea, poor feeding, vomiting, necrotizing enterocolitis and infantile hypertrophic pyloric stenosis (IHPS) [106, 146, 167, 168]. However,
there is limited epidemiological evidence to suggest an association between maternal SSRI use and abnormal function of the GI tract, most of the evidence comes from small cohort studies or case reports. For example, one study reported a 10-fold increase in laxative and anti-diarrheal medication use in children exposed in utero to SSRIs (mainly fluoxetine and paroxetine) [169, 170]. These findings suggest the possibility of an association between the use of exposure to specific SSRIs during pregnancy and abnormal function of the GI tract in children. Due to the inconsistencies between this association and a paucity of data with regards to GI effects following perinatal SSRI exposure, further studies are warranted.

Although there are no studies which conclusively link prenatal SSRI exposure with abnormal GI development, it is biologically plausible SSRIs could influence both structure and function of the GI system [169]. SSRIs have significant placental transport [72-74] and fetal exposure to SSRIs has been shown to alter components of the serotonergic signaling pathway in the CNS [171, 172]. Importantly, serotonin (5-hydroxytryptamine; 5-HT) is critical for the motility of the GI tract and the development of the enteric nervous system [173, 174]. Therefore, if SSRI exposure can also perturb peripheral serotonin signaling it may have profound effects on the intestinal health in the offspring.

1.5 SSRIs and serotonin

Deficiencies in CNS levels of serotonin (5-hydroxytryptamine [5-HT]) have been proposed to be the underlying cause of MDD [175]. SSRIs act to increase serotonin availability in the brain by blocking the plasma membrane serotonin transporter (SERT; 5-HTT) preventing the reuptake of serotonin. Thus, treatment with an SSRI results in enhanced and prolonged serotonergic neurotransmission as there is an increase in the magnitude and duration of the activity of 5-HT on pre- and postsynaptic 5-HT receptors [176]. All SSRIs share a similar mechanism of action
despite having different chemical structures and affinity for SERT [177-179]. However, since SERT is expressed not only in the CNS but in a number of peripheral tissues, including gastrointestinal tract, the use of SSRIs may also alter 5-HT signaling in the periphery.

1.6 Serotonin synthesis and signaling

Serotonin or 5-hydroxytryptomine (5-HT) is well-known for its role in the CNS with well-defined roles in depression [180], arousal and pain pathways [181], appetite [182-184] and other cognitive and behavioral functions [185]. In the CNS serotonin is synthesized in the serotonergic neurons of the raphe nucleus in the brain stem [186]. However, the major source of bioavailable 5-HT in the human body is located in the gut, primarily in enterochromaffin (EC) cells, a subset of enteroendocrine cells scattered throughout the enteric epithelium from the stomach to the colon [187]. Indeed, approximately 95% of 5-HT is synthesized in the gut; 90% of which is localized in EC cells and the remainder synthesized and released in the neurons of the enteric nervous system (ENS) [188-191]. Because of its resemblance to the brain, the ENS is often cited as a “simple nervous system” [192].

The 5-HT signaling components of the gut mucosa are the same as those found in the CNS. EC cells express the enzymatic machinery, including the rate limiting enzyme tryptophan hydroxylase (TPH) to synthesize 5-HT which is then stored in secretory granules until stimulated by luminal stimuli where it is secreted as a first messenger [193]. There are two TPH isoforms, TPH1 and TPH2, the former found primarily in EC and mast cells, while the latter is localized to the brain and enteric neurons. 5-HT may be released through a variety of stimuli which include mechanical, chemical, neural factors, infection and inflammation in the GI tract [194]. Once released, 5-HT acts on receptors located on the processes of sensory neurons that pass into the lamina propria (mucosa in intestine). 5-HT released from EC cells into the blood mediates a
variety of physiological functions, including gastrointestinal motility and secretory reflexes [194-196]. Both EC cells and enteric neurons not only synthesize 5-HT but also express 5-HT receptors and respond to 5-HT activation [197]. 5-HT responsive sensory nerves in the lamina propria confer specificity on the responses because of the 5-HT receptors they express. Indeed, the action of 5-HT is mediated through 7 receptor groups (14 subtypes), $5-HT_1$ to $5-HT_7$. Most of the receptors are expressed in the GI tract, and their stimulation plays different roles (either inhibitory or excitatory) in the control of intestinal motility and secretion [188, 198-200]. Responses to 5-HT activation of pre- ($5-HT_{1A/1P/4}$) and postsynaptic ($5-HT_{1A/2A/2B/2C/3/7}$) 5-HT receptors [192, 201] are terminated by its reuptake [202, 203]. Similar to the brain, 5-HT is transported into mucosal enterocytes by the serotonin transporter (SERT; $5-HTT$), which mediates 5-HT uptake into nerve fibers [192, 204-206]. There is only one SERT gene, with an identical protein encoded in both the CNS and the gut [204, 207-209]. The mucosal epithelial cells are well equipped to catabolize the 5-HT they take up by means of enzyme monoamine oxidase (MAO) [210, 211]. These are functions of 5-HT in the adult, but there is evidence that suggests that 5-HT modulates cell migration, differentiation and survival through certain 5-HT receptors [173, 189, 212-216].

1.7 Serotonin during development: central and peripheral implications

Serotonin is critical for normal development. The serotonergic neurons are among the earliest neurons to appear in the developing embryo [217] where 5-HT is released by growing axons before conventional synapses are established [212]. Importantly, manipulation of 5-HT levels in animal models has been shown to result in neuroanatomical and functional deficits that are dependent on the timing (critical period) and direction (increase or decrease) of the perturbation [218]. For example, SERT knockout mice exhibit altered 5-HT homeostasis in the brain, as
evidenced by increased extracellular 5-HT levels and decreased expression of 5-HT receptors 5-HT1A/1B/2A/2C, resulting in an anxiety-like phenotype [219, 220]. Similarly, changes to 5-HT homeostasis by genetic alterations to components of the serotonergic system have been correlated with changes to adult behavior [212]. Indeed, dysregulation of the central 5-HT system has been implicated in the pathogenesis of many psychiatric and neurological disorders [221, 222]. There is now considerable evidence from animal studies to show that prenatal exposure to SSRIs can alter serotonergic biosynthesis and signaling pathways in the CNS of the offspring [119]. SSRIs increase synaptic 5-HT levels by inhibiting the reuptake of 5-HT via the 5-HT transporter. The subsequent elevation in synaptic 5-HT levels following prenatal exposure to SSRIs can alter the expression of many components of the central serotonergic pathway in offspring [172, 223-225]. Evidence from animal studies has shown that gestational exposure to SSRIs can result in alterations to brain 5-HT content, elevation in peripheral 5-HT levels, and 5-HT2A/2C receptor density and SERT expression, indicating decreased 5-HT function [148-150, 226]. Taken together these studies clearly show that prenatal SSRI exposure can alter central serotonergic signaling, however, whether or not similar effects occur in the peripheral serotonergic system is less well studied.

1.7.1 The effects of prenatal SSRI exposure on the peripheral serotonergic system

If like the brain, the fetal/neonatal gut serotonergic system is also sensitive to SSRI-induced perturbations; it may have long-term implications for intestinal health in the offspring. A recent review suggested that there may be an association between in utero exposure to SSRIs and enteric nervous system (ENS) function [169]. Previously conducted studies [169, 170] show that SSRIs could influence the development of the ENS in two ways: (i) through inhibition of SERT and (ii) through binding of some SSRIs to the 5-HT2B receptor. Nijenhuis and colleagues [169]
proposed that the mechanism underlying these changes may involve alterations in the peripheral serotonergic system, namely changes in the expression of 5-HT receptors (i.e. \(5-HT_{2B}\)), \(SERT\) and/or enzymes responsible for 5-HT synthesis. This is plausible since \(SERT\) plays a role in the development of the ENS by regulating 5-HT concentrations. Therefore, blockage of these transporters during fetal development could influence migration, differentiation and survival of cells. The addition of enteric neurons to the developing bowel happens gradually in the developing embryo. Enteric neurons can be detected in the mouse foregut as early as E12, however, new neurons continue to be added at least through the first 3 weeks of postnatal life in rodents [173]. Since all enteric serotonergic neurons develop early, for this reason, 5-HT’s role as a growth factor that affects the development of late-enteric neurons has long been suspected [173, 227]. Since the peripheral nerves that innervate the gut store the majority of the body’s 5-HT [228], alterations to the development of the ENS may have profound implications for the production of gut-derived serotonin. It has previously been shown that pups born to mother’s given a high tryptophan diet have significantly increased 5-HT protein expression in their gastric tissues [229]. Similar to the CNS, the development of the enteric serotonergic neurons by 5-HT is thought to occur primarily through the \(5-HT_{2B}\) receptors [189, 214-216, 230]. \(5-HT_{2B}\) expression can first be detected in the fetal mouse at embryonic day (E) 14, peaks at E15 and declines to adult levels by E18 [173]. The discovery that 5-HT is a growth factor has potential implications in that the early experience-related activity of the ENS can sculpt its subsequent development. This is significant because chronic use of the SSRI fluoxetine has been shown to initially down regulate (desensitize) and then up regulate the \(5-HT_2\) receptor family in the astrocytes of the CNS [231-235]. The peripheral \(5-HT_{2B}\) receptor located in the fundus of the stomach has been shown to be homologous to the central \(5-HT_{2C}\) receptor [169]. Therefore it is
probable that the receptors in the ENS will respond similarly to the $5-HT_{2B}$ receptors found in the fundus. SSRIs that pass through the placenta may bind to the $5-HT_{2B}$ receptor and disrupt the development of the ENS by changing the concentration of 5-HT. As a result of this disruption, the development of the fetal myenteric (Auerbach’s) plexus - which provides motor innervation to the GI tract to control peristalsis [236] may also be affected by increasing the activity of endogenously released 5-HT. Although it is biologically plausible that fetal exposure to SSRIs may disrupt gut serotonergic signaling, it has not been demonstrated in either animal models or human studies. However, if intestinal serotonergic signaling is altered by perinatal exposure to SSRIs it may have profound implications for intestinal health in the offspring.

1.8 Disorders of the gut

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) represent two common but distinct gastrointestinal (GI) disorders. IBD is a chronic and relapsing inflammatory disease of the intestines that manifests as Crohn’s disease (CD) or ulcerative colitis (UC). While CD can manifest at any part of the GI tract, UC only affects the colon. In both cases, the intestinal mucosa is infiltrated by activated cells from the innate and adaptive immune systems that lead to destructive inflammation [237]. Combined, CD and UC affect nearly 2 million people in North America [238]. Although IBD does not often lead to mortality, it gives rise to substantial morbidity and decreased quality of life [239, 240].

IBS is not inconsequential. It is classified as a chronic functional GI disorder that affects up to 11% of the population globally and approximately 20% of adults in North America [241]. Consequently, its prevalence is accompanied with high societal cost and negative impact on quality of life [242-245]. Clinical presentation of constipation, diarrhea, or a combination, constitutes the different subtype of IBS: IBS with constipation (IBS-C), IBS with diarrhea (IBS-
D), mixed IBS (IBS-M) or post-infectious IBS (PI-IBS) which is similar to D-IBS [246]. The underlying pathophysiology of each is poorly understood and has not been fully elucidated [247, 248]. However, it is considered to be multifactorial [248, 249]. Known risk factors include familial tendency [248, 250-254], physical and psychosocial factors [250, 255-258], and bacterial factors derived from acute infection such as gastroenteritis [259, 260]. Unlike IBD, IBS does not produce destructive inflammation despite abdominal pain; discomfort; increased visceral sensitivity; changes in bowel habit; impaired GI motility; imbalanced autonomic nervous system function; disrupted intestinal flora and altered intestinal secretions [261, 262].

IBD is of unknown etiology; however its development seems to involve a complex interplay between genetic predisposition and the environment. The role of genetics has been well documented as contributing to the pathogenesis of IBD; however twin studies demonstrate the significance of the environment [263]. This is further reinforced by several epidemiologic studies that highlight a rising incidence of IBD and geographic variation that has occurred over the past several decades [264, 265].

Early onset IBD is becoming increasingly common. The development or exacerbation of IBD can be brought on by childhood influences, medications, immunizations, mental health, air pollution, lifestyle choices (e.g. breast feeding, smoking, diet, exercise), and seasonal variation [266, 267]. The possibility that early life factors, such as maternal and/or childhood medication use might influence the pathogenesis of IBD has been illustrated in children where antibiotic exposure has been associated with the development of childhood CD [164, 268, 269], and where their use between 2 to 5 years of age has preceded a 1.3-fold increased risk of adult-onset IBD [270, 271]. Future prospective studies are needed to better understand and identify the early determinants on the onset and disease course of IBD. Similar to IBS [255, 272, 273], these
environmental disturbances may create a predisposition to IBD by altering the mucosal immune system and serotonergic system, influencing intestinal permeability, and disrupting the intestinal microbiota [238]. Importantly, for this thesis, disruptions in 5-HT signaling have been implicated in the pathophysiology of both IBD and IBS.

1.9 Disorders of the gut and serotonin

5-HT released from EC cells is an important signaling molecule involved in the maintenance of intestinal homeostasis [274]. Abnormalities in intestinal 5-HT signaling, which is critical for normal gut function and sensation, have been demonstrated in a range of intestinal pathologies [198, 275-277]. Elements of 5-HT signaling include: EC cell number, 5-HT content, TPH1 message levels, 5-hydroxyindoleacetic acid levels, platelet free serum 5-HT levels and SERT expression. Indeed, EC cell hyperplasia, in addition to elevated tissue and plasma 5-HT levels [196, 228, 249, 278] and increased 5-HT content have been observed in models of experimental colitis [279-286], murine models of IBS [287] and in patients with IBD and IBS as compared with control subjects [208, 275, 277, 288-291]. These alterations to 5-HT signaling may underlie the disruptions in gut motility, secretion and visceral sensation that characterize these patients [292, 293]. A common feature of these studies conducted to date is that they report changes in one or more aspects of 5-HT signaling. As reviewed by Mawe and colleagues [287], combinations of changes in EC cell populations and 5-HT content vary with IBS type. Interestingly, under basal or stimulated conditions, Coates et al. [208] reported no changes in 5-HT release in IBS-D or IBS-C release. If this finding reflects the physiological nature of 5-HT release in these individuals, it would indicate that the same amount of 5-HT is being released regardless of possible changes in EC cell numbers or 5-HT content. Therefore, changes in 5-HT
signaling upstream of 5-HT release may be irrelevant. However, inconsistencies also exist with regards to signaling downstream of 5-HT release.

Murine models of IBS exhibit EC cell hyperplasia and reduced mRNA encoding \textit{SERT} [294-296]. Similar results have been found in human studies. Indeed, IBS-D and IBS-C patients show a decrease in rectal \textit{SERT} expression [208] which is consistently accompanied by an increase in serum 5-HT levels [297, 298]. Similarly, Singh and colleagues [299] found that concentrations of 5-HT were significantly higher in individuals with D-IBS as compared to healthy volunteers. In contrast, a decrease in serum 5-HT and \textit{SERT} expression were reported in patients with IBS-C. Despite these inconsistencies in the literature, taken together these data suggest that 5-HT may be involved in the pathogenesis of IBS.

Although IBS-like conditions often co-exist with IBD, there are fewer published data on the role of 5-HT in the context of IBD [300, 301]. EC cell hyperplasia and increased 5-HT content in inflamed colon also underscore those with IBD [282]. In terms of downstream effectors of 5-HT, patients with IBD have been reported to have similar changes seen in other animal models and in mice with 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced colitis. For instance, \textit{SERT} transcription was reduced in animal models of postinfectious bowel dysfunction [294] and enteric infection [302]. These findings translate to human populations of UC and IBS, where \textit{SERT} transcription has been observed to be decreased [208]. Taken together, alterations to 5-HT signaling components and its downstream effectors may be accompanied by inflammation [282].

\subsection{1.9.1 Disorders of the gut, serotonin and inflammation}

EC cell hyperplasia and changes to 5-HT signaling may underlie the development of enteric infection and inflammation [283, 288, 295, 303]. Inflammation of the intestinal mucosa (e.g.
elevated colonic IL-13 levels) has been associated with a profound decrease in the expression of SERT [282] and up-regulation of EC cell numbers and colonic 5-HT content [304, 305] in animal models of induced colitis. Clinically, biopsies of human colonic mucosa from individuals affected by UC or IBS have also shown similar results [208, 306]. The effects predicted to be exerted by changes in serotonergic expression components underlie the abnormalities of gastrointestinal function and sensation [287].

EC cells are located in very close proximity to or in contact with key immune cells [307]. Considering the strategic location of EC cells in the GI mucosa, it is probable that 5-HT plays an important role in immune activation and generation of gut inflammation in GI disorders. Indeed, the intestinal mucosa of patients with IBD is characterized by ulcerative lesions accompanied by a prominent infiltrate of activated cells from both innate and adaptive immune systems [308-311]. This pathology is accompanied by an enhanced immune response (and increase in CD3-positive T cells) and/or low-grade systemic inflammation [246, 280, 295, 312-315]. It is also becoming increasingly clear that low grade inflammation and immune activation may accompany a subset of IBS cases [312, 316-318]. Particularly, increases in pro-inflammatory cytokines IL-1β [315, 319, 320], IL-6 [321], TNF-α [314] have been reported in PI-IBS and D-IBS patients.

Several independent lines of evidence from SERT and TPH1 knockout models support a proinflammatory role of 5-HT in the pathogenesis of mucosal inflammation in both chronic and functional GI disorders [174, 285, 286, 322]. In one approach, TNBS-induced model of colitis was further exacerbated in mice that lacked SERT (SLC6A4) as seen by changes in histological assessment of the colonic mucosa and an increase in myeloperoxidase (MPO) activity caused by the increased potentiating of serotonergic signaling. An increase in colitis severity was also seen
in the IL-10 mutant mouse model [285]. There is also relevance of TPH1 in intestinal epithelial 5-HT production and modulation of intestinal inflammation. Ghia and colleagues (2009)[286] demonstrated that TPH1-deficient (TPH1-/-) mice, which have significantly lower amounts of 5-HT in the gut, were almost completely protected in two different chemical models of colitis. Also in this model, Li et al. [322] demonstrated that the absence of TPH1 is accompanied by reduced colitis severity and down regulation of IL-17 and IFN-ϒ levels in colonic tissue. Similarly, Margolis et al. [323] found that depletion of 5-HT by oral administration of peripheral TPH inhibitors led to a reduced severity of TNBS-induced colitis; and at least a four-fold reduction in expression of 17% of 84 genes encoding inflammation-related cytokines and chemokines.

5-HT-induced inflammation appears to be potentiated by several receptor subtypes. In vitro models of osteoarthritis demonstrate that stimulation of 5-HT2A and 5-HT3 receptors results in a 5-HT-induced increase in regulators in inflammation, prostaglandin E2 (PGE2) expression [324]. In vivo, the 5-HT3 receptor appears to be a predominant mediator of inflammation and immune responses [325, 326]. Administration of 5-HT3 receptor antagonists has been shown to lead to the inhibition of inflammatory cytokine production in colitis rat models [327-329]. Similarly, the 5-HT7 receptor has recently been shown to have pro-inflammatory effects [330]. Kim and colleagues [330] reported that inhibition of 5-HT7 receptor signaling ameliorated both acute and chronic colitis induced by DSS and lowered histological damage and proinflammatory cytokine levels. Colitis severity was significantly lower in 5-HT7-/- mice, thus highlighting the role of this receptor in intestinal inflammatory disorders such as IBD. 5-HT receptor antagonists have therefore been suggested as therapeutic targets in the treatment of IBS [199, 331]. Disruption of
5-HT may also cause GI deficits due to altered barrier function and/or dysbiosis of the gut microbiota [332, 333].

1.10 Serotonin disruption and altered gut barrier function

Animals with genetic ablation of SERT, which leads to an increase in bioavailable 5-HT, are well characterized as having abnormal gastrointestinal motility [334] and impaired intestinal barrier function [285]. These models also frequently exhibit diarrhea associated with watery stools interspersed with periods of constipation [207]. The use of SSRIs in adulthood has been frequently associated with increased incidence of diarrhea [341, 342]. Similarly in humans, the loss of epithelial barrier integrity, triggered by multiple factors coming from the lumen or the mucosa, may contribute to the generation or perpetuation of C- and/or D- IBS symptoms [208, 335-340]. This increase in barrier dysfunction may arise as the result of a combined process of low-grade mucosal inflammation and immune activation caused by altered gastrointestinal 5-HT homeostasis [237].

Throughout the body, epithelial cells are connected by junctional complexes that form boundaries between compartments of the body and the external environment. The intestinal epithelium forms the largest barrier that separates the intestinal lumen and its bacterial population and products from surrounding peritoneal tissues. Its paracellular permeability is maintained by the expression of tight junctions (TJ). The tight junction associated proteins include the zonula occludens-1(ZO-1), claudin-1 (CLND) and occludin (OCLN) comprises the TJ multi-protein complex [343-346]. In the digestive tract, these transmembrane proteins regulate intestinal permeability to macromolecules while acting as a barrier against pro-inflammatory cytokines [347]. Their expression is dynamic and may be regulated by intracellular
processes and extracellular stimuli. Modification to the tight-junction barrier function is closely associated with health and susceptibility to both intestinal and systemic diseases [348-350].

The accompanying symptoms that characterize functional and chronic GI disorders are accompanied by structural changes to these TJ complexes. Indeed, Piche et al. [351] have shown that IBS patients with a decrease in ZO-1 mRNA level are associated with an increase in colonic paracellular permeability. In this study, OCLN mRNA expression remained unchanged between groups, while in another reported a decreased expression in the colonic mucosa [352]. This decrease was the result of a higher degradation of OCLN by the proteasome system, whereas its mRNA level remained unaffected. Bertiaux-Vandaële et al. [353] demonstrated that while colonic mRNA levels remained unaffected, the protein expression and the cellular distribution of TJ proteins, ZO-1 and claudin-1, but not OCLN, were significantly lower in D-IBS patients.

At present, there exists no evidence between fetal and neonatal exposure to SSRIs and altered intestinal barrier function in the offspring. However, since animal models with dysregulated 5-HT homeostasis exhibit changes and SSRI use in adulthood are both associated with alterations in intestinal permeability it is plausible that prenatal SSRI exposure will have similar effects in the offspring.

1.11 Dysbiosis is associated with intestinal pathology

The role of the gut microbiota in health and disease is becoming increasingly apparent. A shift from a normal (commensal) host-microbiota relationship to a pathogenic relationship (termed dysbiosis) increases the risk of adverse health outcomes [354, 355]. Many of the diseases and disorders associated with adult gut microbiota dysbiosis exhibit an overall reduction of bacterial diversity [356]. When compared to healthy subjects who exhibit distinct, diverse and temporally
stable microbiota, those displaying disease symptoms have dramatically altered bacterial community composition [357-359]. Dysbiosis of the gut microbiota has been implicated in the pathogenesis of chronic intestinal disorders such as irritable bowel syndrome (IBS) (Malinen et al., 2005), inflammatory bowel disease (IBD) [360, 361] and necrotizing enterocolitis [362].

1.11.1 Gut microbiota, disorders of the gut and serotonin

Inflammatory gastrointestinal disorders including IBS and IBD are characterized by instability of the enteric microbiota and aberrant serotonergic functioning [292]. Earlier studies have shown reductions of *Lactobacillus* spp. and *Bifidobacterium* spp. and increased number of Enterobacteriaceae in the gut flora of IBS patients when compared to healthy volunteers [363, 364]. Furthermore, patients with increased colonic colonization to Bacteroides/Prevotella cluster have an increased susceptibility to UC [365, 366]. Importantly, there also appears to be a link between alterations in the microbiota and 5-HT signaling.

In adults, interactions between the microbiota and 5-HT signaling in the GI tract have been demonstrated [367]. Moreover, Clarke et al. [368] reported that animals lacking gut microbiota (i.e. germ free [GF] animals) have perturbations in hippocampal 5-HT production versus conventionally colonized control animals. Furthermore, concentrations of tryptophan, the precursor of 5-HT were increased in the plasma of male GF animals [368]. Interestingly, the absence of the gut microbiota did not affect expression of *Tph2*, *SERT* or the range of serotonergic receptor (*5-HT_{1A}, 5-HT_{6} and 5-HT_{2C}* gene expression evaluated in either the male or female GF animals compared with their respective control counterparts. Nevertheless, results from this study strongly suggest a link between the gut microbiota and regulation of serotonergic signaling pathways. However, whether or not the gut microbiota in the offspring can be altered following maternal SSRI use remains to be determined.
1.11.2 The gut microbiota is susceptible to environmental perturbations

The adult human gut contains an immense number of microorganisms, collectively known as the microbiota [369]. When healthy, the gut microbiota has a symbiotic relationship with its host, serving a multitude of functions which include maintenance of the immune system (Hooper et al., 2012), fat storage [370], stimulation of intestinal angiogenesis [371], regulation of host energy metabolism [372] and epithelial barrier function [373]. The enteric microbiota can also directly influence gut homeostasis by the regulation of bowel motility and modulation of intestinal pain, immune responses and nutrient processing [367, 374, 375].

The gut microbiota is established during infant life; the infant GI tract progresses from being sterile to being colonized by a dense mixture of microbiota resembling that found in the adult GI tract [376-378]. Multiple factors have been found to influence the composition of the intestinal microbiota in early life, including gestational age, mode of delivery, maternal contact, and type of infant feeding and administration of antibiotics [376, 378-382]. More recently, it has been suggested that medication use may also affect the establishment of the enteric microbiota [383], suggesting that prenatal exposure to maternal medication, including SSRIs, may alter the gut microbiota in the offspring.

1.12 Overall aims of the study

Serotonin (5-HT) is critical for normal gut function and sensation. Abnormalities in intestinal 5-HT signaling have been demonstrated in a range of intestinal pathologies including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Evidence from animal studies has shown that perinatal exposure to selective serotonin reuptake inhibitors (SSRIs) can disrupt the development of the central serotonergic system resulting in alterations in brain 5-HT content,
peripheral 5-HT levels, 5-HT receptor density and serotonin transporter expression [171, 172]. If like the brain, the fetal/neonatal gut serotonergic system is also sensitive to SSRI-induced perturbations; it may have long-term implications for intestinal health in the offspring. Taking cues from the pathophysiology of these gastrointestinal disorders and given that early onset IBD is becoming increasingly common [384], I hypothesized that prenatal and neonatal exposure to the SSRI Fluoxetine (Prozac®) will lead to changes to the signaling and biosynthesis components of the gut serotonergic pathway, and that such changes will also be associated with alterations to 5-HT levels and serotonergic signaling components, leading to increased intestinal inflammation, impaired barrier permeability and/or changes in the gut microbiota. Therefore my aims were (Figure 1):

1) To determine whether fetal and neonatal exposure to an SSRI will lead to altered 5-HT levels and changes to the components of the gut serotonergic system.

2) To determine whether changes to the gut serotonin signaling system and 5-HT levels are accompanied by an increase in colonic damage and inflammation.

3) To determine whether disruption to the serotonergic signaling pathway will be accompanied by dysbiosis of the gut microbiota and altered gene expression of key proteins involved in intestinal barrier function.
CHAPTER 2: MATERIALS AND METHODS

2.1 Aim 1- Determining EC cell number, 5-HT levels and expression of the components of the gut serotonergic system.

2.1.2 Production of animal model
Nulliparous female Wistar rats (N=15 per group) were randomized to receive vehicle (flavoured gelatin base) or fluoxetine hydrochloride (10 mg/kg/d, Toronto Research Chemicals, North York, ON) daily 2 weeks prior to mating until weaning (postnatal day 21; PND21) (Figure 2). Based on prior studies in pregnant rats, this dose of fluoxetine is predicted to yield serum concentrations in the rat which are comparable to serum levels in humans (ranging from 5-577 ng/mL during pregnancy and 21-506 ng/mL during lactation) determined from a large therapeutic drug monitoring database [385]. Previous studies have demonstrated that in rodents, five days of oral fluoxetine administration is sufficient to yield steady state serum levels [386]. All dams were allowed to deliver normally. After parturition animals were sacrificed and colon tissue was collected at birth (P1), weaning (P21) and adulthood (26 weeks of age) as previously described [387, 388]. Briefly, the colon was washed in PBS and then either fixed in 10% neutral buffered formalin for 24 hours or snap frozen in liquid nitrogen. Fixed samples were dehydrated in 70% ethanol and embedded in paraffin and sectioned for histological analysis. Frozen samples were stored at -80°C until needed for molecular analysis.
2.1.3 Determining EC cell number by immunohistochemistry

SSRI treated and control offspring were sacrificed during the early postnatal period (P1 and P21). The colon was collected and processed for frozen sections. EC cells were immunolabeled in cryosections of colon by overnight incubation with polyclonal rabbit anti-rat antibody directed against 5-HT (1:5000 dilution; Immunostar). Sites of antibody binding were detected by incubation for 3 hours with goat anti-rabbit Alexa Fluor 488 (1:200 dilution; Molecular Probe) and nuclei were identified by staining DNA with Bisbenzimide (1μg/mL dilution in PBS; Sigma). The slides were cover slipped with Vectashield mounting medium and the tissues photographed. EC cell numbers were quantified by counting the number of 5-HT positive cells per 10 crypts (for colon) as previously published [303].

2.1.4 Measuring serum 5-HT levels

Fasting blood samples were collected from offspring of rats sacrificed in adulthood (age 26 weeks). After solid-phase extraction, the serum levels of 5-HT were analyzed using a commercially available rat ELISA kit (MyBioSource; San Diego, California, USA).

2.1.5 Determining gene expression of the gut 5-HT pathway

2.1.5.1 Tissue homogenization and RNA Isolation

For the duration of the process, colon samples were kept on crushed dry ice and all collection tubes pre-cooled. Frozen tissue was placed in the mortar and ground to a fine powder and kept chilled by liquid nitrogen. The resulting powder was stored at -80°C. Following disruption of tissue, purification and isolation of total colonic RNA was done following protocol from the Qiagen Allprep mini kit and homogenized using a needle and syringe. A spectrophotometer (ND-1000, Nanodrop Technologies Inc, Wilmington, DE) measured RNA yield and purity.
Single-stranded cDNA was synthesized using 1 μg of RNA and a High Capacity reverse transcription kit (Applied Biosciences®). A starting material of 30 mg was used. Sample lysates were washed in 70% ethanol by running them through an RNeasy spin column and were further purified through subsequent buffer washes. Resultant RNA was eluted from column in 30 μL of RNase-free water and immediately stored at -80°C until use for extraction of total RNA for quantitative real-time PCR analysis.

2.1.5.2 Evaluation of gene expression

Expression of selected genes was evaluated by quantitative real-time PCR (qPCR). qPCR was performed using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences), a Light-cycler 480 real-time PCR detection system (Roche Applied Sciences) using specific primers coding for components of the 5-HT signaling and biosynthesis pathway including, serotonin receptors (Htr1a, Htr1b, Htr1d, Htr2a, Htr2b, Htr3a, Htr3b, Htr4, and Htr7); the plasma membrane serotonin transporter (SERT); rate-limiting enzyme in 5-HT synthesis (Tph1) and aromatic L-amino acid decarboxylase (enzyme involved in serotonin synthesis); transcription factor PET1 (Fev), and Monamine oxidase-A (Mao A; enzyme involved in 5-HT degradation). Primers were designed for each target using Pubmed’s nucleotide database. The FASTA sequence was entered in Primer Express® Software (Version 3.0; Life Technologies) under the sequence tab section. Appropriate sequences were selected based on a ΔG < -9 and with minimal hairpins using Oligo Analyzer (Integrated DNA Technologies®). Primers were synthesized by MOBIX, McMaster University’s DNA sequencing and oligo synthesis facility. Before use, they were validated by assessing standard and melting point curves. Expression data were normalized to β actin, 18S, and HPRT messenger RNA (mRNA) expression and presented as a relative message level. (Primer sequences can be found in Appendix J, Table 11)
2.2 Aim 2- Determining macroscopic disease score and targets of inflammation

2.2.1 Production of animal model

Nulliparous female Wistar rats (N=15 per group) were randomized to receive vehicle (flavoured gelatin base) or fluoxetine hydrochloride (10 mg/kg/d, Toronto Research Chemicals, North York, ON) daily 2 weeks prior to mating until weaning (postnatal day 21; PND21) (Figure 2). Based on prior studies in pregnant rats, this dose of fluoxetine is predicted to yield serum concentrations in the rat which are comparable to serum levels in humans (ranging from 5-577 ng/mL during pregnancy and 21-506 ng/mL during lactation) determined from a large therapeutic drug monitoring database [385]. Previous studies have demonstrated that in rodents, five days of oral fluoxetine administration is sufficient to yield steady state serum levels [386]. All dams were allowed to deliver normally. After parturition animals were sacrificed and colon tissue was collected at birth (P1), weaning (P21) and adulthood (26 weeks of age) as previously described [387, 388]. Briefly, the colon was washed in PBS and then either fixed in 10% neutral buffered formalin for 24 hours or snapped frozen in liquid nitrogen. Fixed samples were dehydrated in 70% ethanol and embedded in paraffin and sectioned for histological analysis.

2.2.2 Evaluation of colon morphology and histology

Formalin-fixed colon segments were paraffin embedded and stained with hematoxylin and eosin (H&E) to assess colon damage. The sections were examined with a light microscope and photographed. Images were scored by a single investigator who was blinded to the experimental group using a previous scoring system that considers changes in crypt architecture, cellular infiltration, goblet cell depletion and crypt abscess [389].
2.2.3 Tissue homogenization and RNA Isolation

For the duration of the process, colon samples were kept on crushed dry ice and all collection tubes pre-cooled. Frozen tissue was placed in the mortar and ground to a fine powder and kept chilled by liquid nitrogen. The resulting powder was stored at -80°C. Following disruption of tissue, purification and isolation of total colonic RNA was done following protocol from the Qiagen Allprep mini kit and homogenized using a needle and syringe. A spectrophotometer (ND-1000, Nanodrop Technologies Inc, Wilmington, DE) measured RNA yield and purity. Single-stranded cDNA was synthesized using 1 µg of RNA and a High Capacity reverse transcription kit (Applied Biosciences®). A starting material of 30 mg was used. Sample lysates were washed in 70% ethanol by running them through an RNeasy spin column and were further purified through subsequent buffer washes. Resultant RNA was eluted from column in 30 µL of RNase-free water and immediately stored at -80°C until use for extraction of total RNA for quantitative real-time PCR analysis.

2.2.4 Evaluation of inflammatory gene expression

Expression of selected genes was evaluated by quantitative real-time PCR (qPCR). qPCR was performed using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences), a Light-cycler 480 real-time PCR detection system (Roche Applied Sciences) using specific primers for inflammatory markers including: IL (Interleukins) -1β, 6, 10, and 13, MCP1 (monocyte chemoattractant protein 1), TNFα (tumour necrosis factor α), F4/80 (Macrophage marker) and CD68 (cluster of differentiation 68). Primers were designed for each target using Pubmed’s nucleotide database. The FASTA sequence was entered in Primer Express® Software (Version 3.0; Life Technologies) under the sequence tab section. Appropriate sequences were selected based on a ΔG < -9 and with minimal hairpins using Oligo Analyzer (Integrated DNA
Technologies®). Primers were synthesized by MOBIX, McMaster University’s DNA sequencing and oligo synthesis facility. Before use, they were validated by assessing standard and melting point curves. Expression data were normalized to β actin, 18S, and HPRT messenger RNA (mRNA) expression and presented as a relative message level. (Primer sequences can be found in Appendix J, Table 11)

2.3 Aim 3- Determining the composition of the gut microbiota and tight-junction associated protein expression

2.3.1 Production of animal model
Nulliparous female Wistar rats (N=15 per group) were randomized to receive vehicle (flavoured gelatin base) or fluoxetine hydrochloride (10 mg/kg/d, Toronto Research Chemicals, North York, ON) daily 2 weeks prior to mating until weaning (postnatal day 21; PND21) (Figure 2). Based on prior studies in pregnant rats, this dose of fluoxetine is predicted to yield serum concentrations in the rat which are comparable to serum levels in humans (ranging from 5-577 ng/mL during pregnancy and 21-506 ng/mL during lactation) determined from a large therapeutic drug monitoring database [385]. Previous studies have demonstrated that in rodents, five days of oral fluoxetine administration is sufficient to yield steady state serum levels [386]. All dams were allowed to deliver normally. After parturition animals were sacrificed and colon tissue was collected at birth (P1), weaning (P21) and adulthood (26 weeks of age) as previously described [387, 388].

2.3.2 Evaluation of tight-junction associated protein gene expression
Expression of selected genes was evaluated by quantitative real-time PCR (qPCR). qPCR was performed using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences), a Light-cycler 480
real-time PCR detection system (Roche Applied Sciences) using specific primers for the evaluation of gut permeability by examining expression of tight-junction associated proteins: *CLDN* (claudin) 1, 3, *OCLN* (occludin), and *ZO-1* (zonula occludin). Primers were designed for each target using Pubmed’s nucleotide database. The FASTA sequence was entered in Primer Express® Software (Version 3.0; Life Technologies) under the sequence tab section. Appropriate sequences were selected based on a ∆G < -9 and with minimal hairpins using Oligo Analyzer (Integrated DNA Technologies®). Primers were synthesized by MOBIX, McMaster University’s DNA sequencing and oligo synthesis facility. Before use, they were validated by assessing standard and melting point curves. Expression data were normalized to β actin, 18S, and HPRT messenger RNA (mRNA) expression and presented as a relative message level. (Primer sequences can be found in Appendix J, Table 11)

2.3.3 Fecal Sample Collection

Stool was collected from offspring at 24 weeks of age for gut microbiota profiling. Samples were individually stored at -80°C immediately after collection (*Figure 3*).

2.3.3.1 Extraction of DNA from fecal samples

DNA was extracted from a single fecal sample taken from each rat using a standard extraction/purification method for mixed clinical samples as previously described [390, 391]. This approach involved the basic steps of mechanical lysis, chemical lysis, and DNA purification in a series of 10 steps. Approximately 300 µL of feces was placed in a 2mL plastic screw top tube containing 0.2 g of 2.0 mm diameter ceramic beads, and suspended in 800 µL of 200 mM NaPO₄ (pH 8) and 100 µL of GES. The tube was homogenized at 3000 r.p.m for 3 minutes in a bead-beater instrument two times. Approximately 0.2 grams of 0.1 mm diameter ceramic beads were added and then homogenized at 1500 r.p.m for an additional 3 minutes. Samples were then
subjected to a two-step enzymatic lysis. The first comprised of an incubation at 37°C water bath for 1-1.5 hours in a 110 µL solution of 50 µL of lysozyme (100 mg/mL in H₂O), 50 µL of mutanolysin (10 U/µl) and 10 µL of RNase A (10 mg/mL in H₂O). In the second stage, samples were incubated for 0.5-1.5 hours in 125 µL solution of 25 µL 25% SDS, 25 µL Proteinase K, and 75 µL 5 M NaCl. Screwcap tubes were then centrifuged at max speed for 5 minutes and then 900 µL of supernatant was removed and transferred to a 2 mL tube containing 900 µL (equal volume) of 25:24:1 phenol-chloroform-isoamyl alcohol. The solution was vortexed and then centrifuged at max speed (15000 rpm; Eppendorf 5424) for 10 minutes, and the top layer transferred to a sterile 1.5 mL tube. Purification and final elution of DNA was done using a Zymo DNA clean and concentrator 250 kit. DNA was eluted in 50 µL of sterile DNase/RNase free water pre-heated at 65°C. DNA concentration and quality in the extracts was determined with a Nanodrop 1000 spectrophotometer Thermo Scientific. Extracted DNA was stored at -80°C until needed for PCR amplification.

2.3.3.2 Bacterial profiling of 16S rRNA genes using Illumina MiSeq Sequencing

Variable region 3 (V3) of bacterial 16S ribosomal RNA genes present in each fecal community was amplified by PCR, and the resulting amplicons were sequenced on an Illumina MiSeq 2000 instrument. Samples were amplified in triplicate using a Veriti® 96-Well Fast Thermal Cycler, model 9902. The PCR reaction mixture in a volume of 60 µL contained 6 µL (10 pmol/µL) each of V3 forward and barcoded reverse primers, 1.5 µL magnesium chloride (MgCl₂) (50 mM) solution, 6 µL 10 x PCR buffer, 1 uL dNTPs (10 mM each), 34.25 uL dH₂O, 0.25 uL Taq Polymerase, and 5 uL Template DNA (30 ng total) with the following cycling conditions: 30 cycles (94°C, 30 s, 50°C, 30 s; 72°C, 30 s) after an initial denaturation of 2 min at 94°C.
Amplicons from the triplicate reactions were pooled together, and separated electrophoretically on a 2% agarose gel.

2.3.3.3 Microbial Sequencing and analysis

Analysis was performed using an in-house bioinformatics pipeline that generates clusters of operational taxonomic units (OTUs), taxonomic assignment and various measures of alpha and beta-diversity. PCR products were sequenced using the Illumina Miseq with paired-end reads. Custom Perl scripts were developed in-house to process the sequences. First, Cutadapt [392] was used to trim these sequences to the V3 region, ridding of any sequences surpassing this region. Next, sequences were aligned with their pair using PANDAseq [393]; during this alignment, any mismatches or ambiguous bases were culled. Operational taxonomic units (OTUs) were picked using AbundantOTU and as described previously [394] with a clustering cutoff of 97%. Taxonomy of the resultant OTUs was assigned via comparison of a representative sequence of the unit to the Greengenes reference database [395] using the Ribosomal Database Project (RDP) classifier [396].

Comparative 16S rRNA gene sequence analysis was used to determine differences in the bacterial composition between groups were summarized with the QIIME (Quantitative insights Into Microbial Ecology) software package [397, 398]. Comparisons were made between control and treated offspring within each sex by Student’s t-test with Bonferroni correction. Taxonomic units were excluded from analysis if 1) they were undefined at the level of analysis or 2) less than 10% of all samples had detectable levels of the OTU. Pricincipal Component of Analysis (PCoA) plots were made using R 3-1-0.
2.4 Statistical Analysis

Analysis was performed using SPSS software (SPSS release 20.0, IBM, Chicago, IL, USA) and plotted using GraphPad Prism version 6.00 for Windows, (GraphPad Software Inc., San Diego, CA, USA). The results are expressed as means ± S.E.M. Data were tested for normality with the Kolmogorov-Smirnov test and Grubbs test method to identify outliers. Comparisons between two means were tested with the Student’s t test. All tests were two-sided and significance level was set at 0.05.
Figure 1 Study aims. Evidence from animal studies has shown that perinatal exposure to selective serotonin reuptake inhibitors (SSRIs) can disrupt the development of the central serotonergic system resulting in alterations in brain 5-HT content, peripheral 5-HT levels, 5-HT receptor density and serotonin transporter expression. If like the brain, the fetal/neonatal gut serotonergic system is also sensitive to SSRI-induced perturbations; it may have long-term implications for intestinal health in the offspring. Therefore I hypothesized that prenatal and neonatal exposure to SSRI fluoxetine will lead to changes to the components of the gut serotonergic pathway. This will also be accompanied by alterations to peripheral serotonin levels. Disruptions to serotonin levels caused by changes in expression of key serotonergic pathway components may independently or mediate changes to the gut microbiota exacerbate intestinal inflammation. Solid lines denote determined associations according to previous literature; broken line depicts possible associations.
Figure 2 Production of the animal model. Nulliparous female Wistar rats were randomized to receive vehicle or fluoxetine hydrochloride (10 mg/kg/d), Toronto Research Chemicals, North York, ON) daily by oral administration 2 weeks prior to mating until weaning (postnatal day 21; PND 21). Outcome measures related to obesity, including body weight and visceral fat were determined. Colon was collected at P1, P21 and week 24. Fecal samples were collected from offspring postnatally at 24 weeks of age.
Figure 3 Overview of methods involved in phylogenetic identification and detection of microbial groups or species for Specific Aim #3. DNA was extracted from fecal samples and amplicons of the V3 hypervariable region from the 16S rRNA were made by PCR for Illumina sequencing. Data analysis occurred by QIIME.
CHAPTER 3: RESULTS

3.1 Determine whether prenatal and neonatal fluoxetine exposure will lead to altered peripheral 5-HT levels and changes to the components of the gut serotonergic system

Prenatal exposure to SSRIs, a class of antidepressants, has been shown to alter serotonergic signaling in the brain. However, the effects of SSRIs on peripheral serotonin (5HT) synthesis and/or signaling have largely been ignored. A recent review suggested that there may be an association between in utero exposure to SSRIs and enteric nervous system (ENS) function [169]. 5-HT in the gut is critical for intestinal function and elevated peripheral 5-HT levels and dysregulation of the serotonergic pathway is associated with intestinal diseases including IBS and IBD.

Therefore, the goal of this first aim was to determine the effects of perinatal exposure to the SSRI fluoxetine (Prozac®) on EC cell number- the primary source of 5-HT in the body; serum 5-HT levels and components of the 5-HT signaling pathway. A comparison of the number of EC cells between control and fluoxetine-exposed offspring was determined by sacrificing, collecting and processing colon for frozen sections at P1 and P21. Colonic EC cells were immunolabeled in cryosections with polyclonal rabbit antibody directed against 5-HT. Sites of antibody binding were detected with goat anti-rabbit Alexa Fluor 488 and nuclei identified by staining DNA with Bisbenzimide. EC cell numbers were quantified by counting 5-HT positive cells per 10 crypts (for colon) as previously published [303]. Serum 5-HT levels were determined by ELISA from fasting blood samples collected from sacrificed offspring in adulthood. Gene expression of the
gut serotonergic pathway was determined by isolating RNA from snap frozen colon sections collected from animals sacrificed in adulthood and evaluating selected genes by qPCR. The purpose behind the aforementioned experiments was to establish whether prenatal and neonatal SSRI exposure has similar effects in the periphery as it does in the central compartment.

3.1.2 Perinatal exposure to fluoxetine alters EC cell number and increases blood 5-HT concentrations

At postnatal day 1 (P1), there was a significant decrease in colonic EC cell number in the fluoxetine exposed group compared to controls (Figure 4). By weaning (Postnatal day 21; P21), EC cell number was significantly increased in the treatment group (Figure 5). At 26 weeks (adulthood), serum 5-HT levels were significantly elevated in treated male (51.8 ± 13.2 vs. 39.0 ± 5.90; p= 0.005) but not female offspring (33.6 ± 2.21 vs. 35.3 ± 2.44) (Figure 6).

3.1.3 Sex-dependent changes in serotonergic synthesis, catabolism and signaling

In male adult offspring, there were no differences in the expression of genes involved in 5-HT synthesis between treatment groups (Tph1 2.1 ± 0.40 vs. 1.9 ± 0.34; p= 0.696) and PET1 (1.4 ± 0.39 vs. 1.3 ± 0.31; p=1.00). However, transcripts encoding the enzyme MAO (1.6 ± 0.2 vs. 3.1 ± 0.60; p value= 0.043) (Figure 9) and 5HT2a receptor (1.1 ± 0.50 vs. 4.4±1.34; p-value= 0.009) (Figure 7) were significantly decreased in fluoxetine-exposed offspring. Other receptor subtypes (5-HT1a, 5-HT1b, 5-HT1d, 5-HT2b, 5-HT3a, 5-HT3b, 5-HT4, and 5-HT7) and the serotonin transporter (SERT; 5-HTT), levels remained unchanged between treatment groups (Figure 7). In female offspring, fluoxetine exposure did not significantly alter the expression of any genes involved in 5-HT synthesis (TPH1), transport (SERT), signaling (5-HT1a, 5-HT1b, 5-HT1d, 5-HT2a, 5-HT2b, 5-HT3a, 5-HT3b, 5-HT4, and 5-HT7) or degradation (MAO) ((Figures 8 and 10).
3.2 Determine macroscopic disease score and expression of inflammatory targets

In animal models, 5-HT has been considered to have a proinflammatory role, particularly in the pathogenesis of intestinal inflammation. Patients with colonic inflammation and inflammatory bowel disease (IBD) demonstrate an increase in the number of 5-HT-producing enterochromaffin (EC) cells and subsequently elevated 5-HT levels. EC cells respond to inflammatory responses such as cytokines and bacterial infection by increasing their release of 5-HT. Such pathologies are accompanied by increased in pro-inflammatory markers IL-1β and 6 in the colon. Indeed, mouse models of DSS induced colitis have elevated colonic IL-13 levels accompanied by up-regulation of EC cell numbers and colonic 5-HT content. Many components of the serotonergic system itself have been shown to directly mediate this response. Therefore, my second aim was to determine whether changes to the gut serotonergic system and 5-HT levels are accompanied by an increase in colonic damage and inflammation.

Colon morphology and histology were determined using colon sections taken in adulthood and stained with hematoxylin and eosin (H & E) to assess damage using a previous scoring system that considers changes in crypt architecture, cellular infiltration, goblet cell depletion and crypt abscess [389]. At the molecular level, inflammation was evaluated by gene expression of pro- and anti-inflammatory targets by qPCR. The purpose behind these experiments was to evaluate whether alterations to colonic 5-HT signaling and peripheral 5-HT levels in adulthood was associated with inflammation.

3.2.1 Perinatal exposure to fluoxetine does not increase colonic damage or inflammation

Macroscopic disease and inflammation scores were similar between SSRI-treated offspring and controls in both males and females (Figures 11 and 12). In support of this finding, there were no
significant changes in the mRNA expression of the proinflammatory (*IL-1β, IL-6, and IL-13; TNF-α, and CD68*) or anti-inflammatory (*IL-10*) cytokines (*Figures 13 and 14*). However, *MCP1* was significantly lower in fluoxetine-exposed male offspring (0.238 ± 0.0538 vs. 0.0845 ± 0.0360; *p* = 0.0489) (*Figure 13*).

### 3.3 Determine the effects prenatal and neonatal exposure to fluoxetine on TJ structural components and the composition of the gut microbiota

Animals with genetic ablation of *SERT*, which leads to an increase in bioavailable 5-HT, are well characterized as having impaired intestinal barrier function [285]. Similarly in humans, the loss of epithelial barrier integrity, triggered by multiple factors coming from the lumen or the mucosa, may contribute to the generation or perpetuation of C- and/or D- IBS symptoms [208, 335-340]. This increase in barrier dysfunction may arise as the result of a combined process of low-grade mucosal inflammation and immune activation caused by altered gastrointestinal 5-HT homeostasis [237]. The use of SSRIs in adulthood has been frequently associated with increased incidence of diarrhea [341, 342]. Since animal models with dysregulated 5-HT homeostasis exhibit changes and SSRI use in adulthood are both associated with alterations in intestinal permeability it is plausible that prenatal SSRI exposure will have similar effects in the offspring.

In adults, interactions between the microbiota and 5-HT signaling in the GI tract have been demonstrated [367]. Moreover, Clarke et al. [368] reported that animals lacking gut microbiota (i.e. germ free [GF] animals) have perturbations in hippocampal 5-HT production versus conventionally colonized control animals. Results from these studies strongly suggest a link between the gut microbiota and regulation of serotonergic signaling pathways.
Inflammatory gastrointestinal disorders including IBS and IBD are characterized by instability of the enteric microbiota and aberrant serotonergic functioning [292]. Earlier studies have shown reductions of *Lactobacillus spp.* and *Bifidobacterium spp.* and increased number of Enterobacteriaceae in the gut flora of IBS patients when compared to healthy volunteers [363, 364]. Furthermore, patients with increased colonic colonization to Bacteroides/Prevotella cluster have an increased susceptibility to UC [365, 366]. The gut microbiota is established during infant life. More recently, it has been suggested that medication use may also affect the establishment of the enteric microbiota [383], suggesting that prenatal exposure to maternal medication, including SSRIs, may alter the gut microbiota in the offspring. Therefore, our final aim was to determine whether disruption to the serotonergic signaling pathway via prenatal exposure to fluoxetine will also be accompanied by dysbiosis of the gut microbiota and altered expression of tight-junction associated proteins in adulthood.

The evaluation of colonic mRNA expression of TJ associated proteins was done by qPCR. Comparative 16S rRNA gene sequence analysis was used to determine differences in the bacterial composition between groups were summarized with the QIIME (Quantitative insights Into Microbial Ecology) software package [397, 398].

### 3.3.1 Perinatal fluoxetine exposure does not alter expression of tight-junction associated proteins

Fetal and neonatal exposure to fluoxetine did not significantly alter the expression of the gut permeability markers *CLDN1, CLDN3, OCLN* or *ZO-1* between treatment groups in both sexes (*Figures 15 and 16*).
3.3.2 Perinatal fluoxetine exposure is associated with alterations to the adult gut microbiota

At 24 weeks, there were no significant differences in the major mammalian phyla Bacteroidetes and Firmicutes between treatment and control groups in either sex (Table 1). Class Bacilli (P=0.0020) (Phylum Firmicutes) was significantly higher in treated female offspring compared to controls (Table 2). At the Order level (Table 3) there were no significant differences between treatment groups in male offspring. However, Lactobacillales (Phylum Firmicutes) was significantly less abundant in female offspring exposed to fluoxetine. Similarly, at the Genus level (Table 4); there were no differences between treatment groups in male offspring. For instance,Prevotella (Phylum Bacteroidetes) was similar between treatment groups in both male (P=0.519) and female (P=0.72) offspring. However, *Lactobacillus* (Phylum Firmicutes; female only, P<0.001) were significantly higher in offspring exposed to fluoxetine.

When looking at overall β diversity (Appendix G and H; Figures 22 and 23), it appears that despite the aforementioned significant differences in certain bacterial groups between treatment and control offspring, the gut microbiota overall remain similar in both sexes. Thus, the lack of discrete clusters reflects the subtle differences in the bacteria populations observed in adulthood.
Figure 4 At postnatal day 1 (PND1), there was a significant decrease in colonic enterochromaffin (EC) cell number in the fluoxetine-exposed group compared to controls (P=0.041). Pups were sacrificed at PND1 and colon was collected for immunohistochemistry. EC cells were labelled using polyclonal rabbit anti-rat antibody directed against 5-HT (1:5000 dilution; Immunostar); and goat anti-rabbit Alexa Fluor 488 (1:200 dilution; Molecular Probe).
Figure 5 By weaning (Postnatal day 21; P21), fluoxetine exposure led to a significant increase in enterochromaffin (EC) cell number (P= 0.0012). Pups were sacrificed at P21 (weaning) colon was collected for immunohistochemistry. EC cells were labelled using polyclonal rabbit anti-rat antibody directed against 5-HT (1:5000 dilution; Immunostar); and goat anti-rabbit Alexa Fluor 488 (1:200 dilution; Molecular Probe).
Figure 6 In adulthood (26 weeks), serum 5-HT levels were significantly elevated in treated male (mean ± SEM; 51.8 ± 4.41 vs. 39.0 ± 1.64; p= 0.005) but not female offspring (35.3 ± 2.44 vs. 33.6 ± 2.21; p= 0.616). Serum 5-HT levels were determined by blood samples collected using a commercially available rat ELISA kit.
Figure 7 Evaluation of colonic serotonergic genes in male offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included serotonin receptors. Transcripts encoding 5HT2A receptor (mean ± SEM; 1.1±0.50 vs. 4.4±1.34; p-value= 0.009) expression were significantly decreased in fluoxetine exposed males. Other receptor subtypes (Htr1a, Htr1b, Htr1d, Htr2b, Htr3a, Htr3b, Htr4, and Htr7) levels remained unchanged between treatment groups. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.
Figure 8 Evaluation of colonic serotonergic genes in female offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included serotonin receptors. Transcripts encoding receptor subtypes (Htr1a, Htr1b, Htr1d, Htr2a, Htr2b, Htr3a, Htr3b, Htr4, and Htr7) remained unchanged between treatment groups. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.
Figure 9 Evaluation of colonic serotonergic genes in male offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included the plasma membrane serotonin transporter (SERT); rate-limiting enzyme in 5-HT synthesis (Tph1); transcription factor PET1; and Monoamine oxidase-A (Mao A; enzyme involved in 5-HT degradation). Transcripts encoding Mao (1.6 ± 0.20 vs. 3.1 ± 0.60; p value= 0.043) expression were significantly decreased in fluoxetine exposed males. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.
Figure 10 Evaluation of colonic serotonergic genes in female offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included the plasma membrane serotonin transporter (SERT), rate-limiting enzyme in 5-HT synthesis (Tph1); transcription factor PET1, and Monoamine oxidase-A (Mao A; enzyme involved in 5-HT degradation). No significant difference in transcript expression was seen for any gene targets between treatment groups. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S. Analysis separated by stage of cycle (estrous and diestrus) can be found in.
Figure 11 Hematoxylin- and eosin-stained histological sections of the colon taken from control and fluoxetine exposed offspring at 26 weeks of age. Top left, control male; Top right, Fluoxetine Male; Bottom left, control female; Bottom right, Fluoxetine female. Arrows indicate cellular infiltration.
Figure 12 Fetal and neonatal exposure to fluoxetine does not increase damage in the colon postnatally. Microscopic criteria for damage and inflammation were investigated by light microscopy on hematoxylin- and eosin-stained histological sections of the colon taken from control- and SSRI-exposed offspring at 26 weeks of age. The histological criteria were based on the following: degree of mucosal architectural changes, cellular infiltration, goblet cell depletion, and presence of crypt abscesses (Khan et al., 2002. Infect Immun 70:5931-7).
Figure 13 Evaluation of colonic inflammation (Anti- and pro-inflammatory cytokines: IL-10 (Interleukin), TNF-α (tumour necrosis factor α), IL1B, IL6, and IL13; MCP 1 (monocyte chemoattractant protein 1); cluster of differentiation 68, CD68; F4/80, (Epidermal Growth Factor) in male offspring at 26 weeks by quantitative real-time PCR (qPCR). Fetal and neonatal exposure to fluoxetine did not increase colonic inflammation postnatally in adult males. However, MCP1 was significantly decreased in treated offspring (0.238 ± 0.0538 vs. 0.0845 ± 0.0360; p= 0.0489). Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.
Figure 14 Evaluation of colonic inflammation in female offspring at 26 weeks by quantitative real-time PCR (qPCR). Fetal and neonatal exposure to fluoxetine does not increase colonic inflammation postnatally in adult females. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S. Analysis separated by stage of cycle (estrus and diestrus) can be found in Section 5.3 Table 2. (Anti- and pro-inflammatory cytokines: IL-10 (Interleukin), TNF-α (tumour necrosis factor α), IL1B, IL6, and IL13; MCP 1 (monocyte chemoattractant protein 1; cluster of differentiation 68, CD68; F4/80, Epidermal Growth Factor)
Figure 15 Evaluation of tight junction-associated proteins (claudin-1, CLND1/3; Occludin, OCLN; Zona Occludin-1, ZO-1) in male offspring at 26 weeks by quantitative real-time PCR (qPCR). Fetal and neonatal exposure to fluoxetine does not alter markers of gut permeability in adult males. Comparisons between treatment groups were done by independent samples t-test. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.
Figure 16 Evaluation of tight junction-associated proteins (claudin-1, CLND1/3; Occludin, OCLN; Zona Occludin-1, ZO-1) in female offspring at 26 weeks by quantitative real-time PCR (qPCR). Fetal and neonatal exposure to fluoxetine does not alter markers of gut permeability in adult males. Comparisons between treatment groups were done by independent samples t-test. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.
Figure 17 Percent relative abundance of bacteria at the phyla level in control and fluoxetine-exposed offspring in adulthood. This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age.
Table 1 Bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Relative abundance (%) of phyla separated by sex. Statistical significance between control and treated offspring within each sex was determined by Student’s t-test. Rules for inclusion of gut microbiota in analysis: (1) Had to be defined; (2) Had to have at least 10% of all samples (CON and FLX) and have non-zero abundance.

<table>
<thead>
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<th>Phyla</th>
<th>Male (mean±SEM)</th>
<th>P-value</th>
<th>Female (mean±SEM)</th>
<th>P-value</th>
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<td>CON (0.442 ± 0.0777)</td>
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<tr>
<td></td>
<td>FLX (0.442 ± 0.112)</td>
<td>.383</td>
<td>FLX (0.248 ± 0.0247)</td>
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</tr>
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<td>Deferrribacteres</td>
<td>7.67 x10⁻³ ± 2.93 x10⁻³</td>
<td>.112</td>
<td>5.24 x10⁻³ ± 1.80 x10⁻³</td>
<td>.237</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>76.8 ± 2.85</td>
<td>.764</td>
<td>80.6 ± 2.37</td>
<td>.338</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>8.76 x10⁻⁴ ± 5.81 x10⁻⁵</td>
<td>.043</td>
<td>3.91 x10⁻⁴ ± 1.64 x10⁻⁴</td>
<td>.116</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.3480 ± 0.0402</td>
<td>.143</td>
<td>0.266 ± 0.0767</td>
<td>.059</td>
</tr>
<tr>
<td>TM7</td>
<td>0.0383 ± 8.74 x10⁻³</td>
<td>.432</td>
<td>0.0717 ± 0.0185</td>
<td>.885</td>
</tr>
<tr>
<td>Tenericutes</td>
<td>1.03 ± 0.158</td>
<td>.128</td>
<td>1.50 ± 0.313</td>
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</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.0308 ± 0.0114</td>
<td>.535</td>
<td>0.0167 ± 0.0105</td>
<td>.0120</td>
</tr>
</tbody>
</table>

*Bonferroni correction P<0.005
Figure 18 Percent relative abundance of bacteria at the class level in control and fluoxetine-exposed offspring in adulthood (for all representative sequences >0.01%). This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Legend is presented as Phyla_Class.
### Table 2

Bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Relative abundance (%) of taxonomy class separated by sex. Statistical significance between control and treated offspring within each sex was determined by Student’s t-test and expressed as mean ± SEM. Rules for inclusion of gut microbiota in analysis: (1) Had to be defined; (2) had to have at least 10% of all samples and (3) have a non-zero abundance.

<table>
<thead>
<tr>
<th>Class</th>
<th>Male (mean±SEM)</th>
<th>Female (mean±SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>FLX</td>
<td></td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td>0.330 ± 0.0671</td>
<td>0.442 ± 0.112</td>
<td>.383</td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td>0.399 ± 0.0777</td>
<td>0.248 ± 0.0247</td>
<td>.166</td>
</tr>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td>20.2 ± 2.85</td>
<td>20.0 ± 5.33</td>
<td>.961</td>
</tr>
<tr>
<td></td>
<td>16.9 ± 2.21</td>
<td>14.3 ± 2.17</td>
<td>.439</td>
</tr>
<tr>
<td><strong>Cyanobacteria</strong></td>
<td>6.79 ± 1.67</td>
<td>3.25 ± 9.15 x10^{-3}</td>
<td>.124</td>
</tr>
<tr>
<td></td>
<td>2.28 ± 4.40 x10^{-4}</td>
<td>2.40 ± 4.96 x10^{-4}</td>
<td>.851</td>
</tr>
<tr>
<td><strong>Deferribacteres</strong></td>
<td>7.67 ± 2.93 x10^{-3}</td>
<td>0.0176 ± 0.00565</td>
<td>.112</td>
</tr>
<tr>
<td></td>
<td>5.24 ± 1.80 x10^{-3}</td>
<td>2.23 ± 7.82 x10^{-4}</td>
<td>.237</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacilli</strong></td>
<td>11.6 ± 2.53</td>
<td>12.5 ± 3.22</td>
<td>.825</td>
</tr>
<tr>
<td></td>
<td>6.62 ± 1.14</td>
<td>16.2 ± 2.54</td>
<td>.00200*</td>
</tr>
<tr>
<td><strong>Clostridia</strong></td>
<td>65.0 ± 3.85</td>
<td>65.8 ± 6.47</td>
<td>.915</td>
</tr>
<tr>
<td></td>
<td>72.2 ± 3.21</td>
<td>67.6 ± 3.31</td>
<td>.350</td>
</tr>
<tr>
<td><strong>Fusobacteria</strong></td>
<td>8.76 x10^{-5} ± 5.81 x10^{-5}</td>
<td>4.31 x10^{-4} ± 1.45 x10^{-4}</td>
<td>.0400</td>
</tr>
<tr>
<td></td>
<td>3.92 x10^{-4} ± 1.64 x10^{-4}</td>
<td>0.00</td>
<td>.116</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Alpha-Proteobacteria</strong></td>
<td>0.0269 ± 0.00833</td>
<td>0.0457 ± 0.00796</td>
<td>.133</td>
</tr>
<tr>
<td></td>
<td>0.0564 ± .0139</td>
<td>0.0302 ± .0120</td>
<td>.185</td>
</tr>
<tr>
<td><strong>Beta-Proteobacteria</strong></td>
<td>0.185 ± 0.0342</td>
<td>0.128 ± 0.0238</td>
<td>.215</td>
</tr>
<tr>
<td></td>
<td>0.194 ± .0378</td>
<td>0.112 ± .0135</td>
<td>.103</td>
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<tr>
<td><strong>Delta-Proteobacteria</strong></td>
<td>.0200 ± .00544</td>
<td>.0161 ± .00220</td>
<td>.215</td>
</tr>
<tr>
<td></td>
<td>.0216 ± 5.07 x10^{-3}</td>
<td>.0266 ± .00722</td>
<td>.564</td>
</tr>
<tr>
<td><strong>Gamma-Proteobacteria</strong></td>
<td>.0296 ± .00748</td>
<td>.0139 ± .00292</td>
<td>.0930</td>
</tr>
<tr>
<td></td>
<td>.0215 ± 6.70 x10^{-3}</td>
<td>6.68 x10^{-3} ± 1.49 x10^{-3}</td>
<td>.117</td>
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<tr>
<td><strong>TM7</strong></td>
<td>0.0382 ± .00874</td>
<td>.0277 ± .00941</td>
<td>.432</td>
</tr>
<tr>
<td></td>
<td>0.0941 ± .0280</td>
<td>0.0759 ± .0218</td>
<td>.645</td>
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<tr>
<td><strong>Tenericutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Erysipeltrichi</strong></td>
<td>.703 ± .133</td>
<td>.466 ± .115</td>
<td>.212</td>
</tr>
<tr>
<td></td>
<td>1.01 ± .232</td>
<td>.366 ± .0717</td>
<td>.0420</td>
</tr>
<tr>
<td>Mollicutes</td>
<td>0.295 ± 0.0930</td>
<td>0.153 ± 0.0219</td>
<td>0.239</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>-------</td>
</tr>
<tr>
<td><em>Verrucomicrobia</em> Verrucomicrobcae_</td>
<td>0.0308 ± 0.0113</td>
<td>0.0205 ± 0.0113</td>
<td>0.535</td>
</tr>
</tbody>
</table>

*Bonferroni correction P<0.003*
Figure 19 Percent relative abundance of bacteria at the level order in control and fluoxetine-exposed offspring in adulthood (for all representative sequences >0.01%). This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Legend is presented as Phyla_Order.
Table 3 Bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Relative abundance (%) of taxonomy order separated by sex. Statistical significance between control and treated offspring within each sex was determined by Student's t-test and expressed as mean ± SEM. Rules for inclusion of gut microbiota in analysis: (1) Had to be defined; (2) had to have at least 10% of all samples and (3) have a non-zero abundance.

<table>
<thead>
<tr>
<th>Order</th>
<th>Male (mean±SEM)</th>
<th>Female (mean±SEM)</th>
<th>P-value</th>
<th>P-value</th>
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</thead>
<tbody>
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<td></td>
<td>CON</td>
<td>FLX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinobacteria_order_</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycetales</td>
<td>.102 ± .0228</td>
<td>.191 ± .0491</td>
<td>.091</td>
<td>.167 ± .0276</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.134 ± .0274</td>
</tr>
<tr>
<td>Bifidobacteriales</td>
<td>.0475 ± .0174</td>
<td>.0103 ± .00614</td>
<td>.086</td>
<td>.0233 ± .0975</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.31 x10⁻³ ± 8.76 x10⁻⁴</td>
</tr>
<tr>
<td>Coriobacteriales</td>
<td>.0647 ± .00821</td>
<td>.160 ± .0576</td>
<td>.121</td>
<td>.0943 ± .0172</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>.118 ± .0308</td>
</tr>
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<td>Bacteroidetes_order_</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroidales</td>
<td>20.2 ± 2.85</td>
<td>20.0 ± 5.32</td>
<td>.961</td>
<td>16.9 ± 2.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.3 ± 2.17</td>
</tr>
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<td>Cyanobacteria_YS2</td>
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<td>1.78 x10⁻³ ± 8.27 x10⁻⁴</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>4.44 x10⁻³ ± 1.97 x10⁻³</td>
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<td>Deferrribacteres_order_</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>Streptophyta</td>
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<td>3.25 x10⁻³ ± 9.15 x10⁻⁴</td>
<td>.081</td>
<td>2.28 x10⁻³ ± 4.40 x10⁻⁴</td>
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<td>2.40 x10⁻³ ± 4.96 x10⁻⁴</td>
</tr>
<tr>
<td>Deferrribacterales</td>
<td>7.67 x10⁻³ ± 2.93 x10⁻³</td>
<td>.0176 ± .00565</td>
<td>.112</td>
<td>5.24 x10⁻³ ± 1.80 x10⁻³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.22 x10⁻³ ± 7.82 x10⁻⁴</td>
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<td>Firmicutes_order_</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillales</td>
<td>7.38 x10⁻³ ± 2.23 x10⁻³</td>
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<td>.069</td>
<td>.0331 ± .0126</td>
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<td></td>
<td>7.93 x10⁻³ ± 2.70 x10⁻³</td>
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<tr>
<td>Lactobacillales</td>
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<td>5.35 ± .709</td>
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<tr>
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<td></td>
<td>16.1 ± 2.54</td>
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<td>1.11 ± .331</td>
<td>.0180 ± .00636</td>
<td>.024</td>
<td>.0106 ± 3.60 x10⁻³</td>
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<td></td>
<td></td>
<td></td>
<td>7.38 x10⁻³ ± 2.43 x10⁻³</td>
</tr>
<tr>
<td>Clostridiales</td>
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<td>65.7 ± 6.45</td>
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<td>72.1 ± 3.21</td>
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<td></td>
<td>67.5 ± 3.32</td>
</tr>
<tr>
<td>Fusobacteria_fusobacteriales</td>
<td>8.76 x10⁻⁵ ± 5.81 x10⁻⁵</td>
<td>4.31 x10⁻⁴ ± 1.45 x10⁻⁴</td>
<td>.043</td>
<td>3.91 x10⁻⁴ ± 1.64 x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Proteobacteria_order_</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizobiales</td>
<td>3.14 x10⁻⁴ ± 1.32 x10⁻⁴</td>
<td>&lt;0.001</td>
<td>.080</td>
<td>1.24 x10⁻⁴ ± 6.59 x10⁻⁵</td>
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</tr>
<tr>
<td>Burkholderiales</td>
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<td>.128 ± .0238</td>
<td>.217</td>
<td>.194 ± .0378</td>
</tr>
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<td>Order</td>
<td>Genus</td>
<td>Relative Abundance</td>
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<td>----------------------</td>
<td>---------------------</td>
<td>--------------------</td>
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<td>Neisseriales</td>
<td>1.06 x 10^-5 ± 5.44 x 10^-5</td>
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<td>1.97 x 10^-4 ± 9.03 x 10^-5</td>
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<td>Desulfovibrionales</td>
<td>.0200 ± 5.44 x 10^-3</td>
<td>.016 ± 2.20 x 10^-3</td>
<td>.0215 ± 5.07 x 10^-3</td>
<td>.0266 ± 7.22 x 10^-3</td>
</tr>
<tr>
<td>Enterobacteriales</td>
<td>.0231 ± 7.75 x 10^-3</td>
<td>.0116 ± 2.49 x 10^-3</td>
<td>.0184 ± 6.43 x 10^-3</td>
<td>4.44 x 10^-3 ± 1.65 x 10^-3</td>
</tr>
<tr>
<td>Pasteurellales</td>
<td>5.31 x 10^-3 ± 1.41 x 10^-3</td>
<td>3.13 x 10^-3 ± 1.24 x 10^-3</td>
<td>1.74 x 10^-3 ± 3.09 x 10^-4</td>
<td>1.13 x 10^-3 ± 3.52 x 10^-4</td>
</tr>
<tr>
<td>Pseudomonadales</td>
<td>4.46 x 10^-4 ± 1.53 x 10^-4</td>
<td>3.03 x 10^-4 ± 1.82 x 10^-4</td>
<td>6.83 x 10^-4 ± 3.07 x 10^-4</td>
<td>7.49 x 10^-4 ± 1.81 x 10^-4</td>
</tr>
<tr>
<td>TM7_CW040</td>
<td>.0381 ± 8.80 x 10^-3</td>
<td>.0276 ± 9.36 x 10^-3</td>
<td>.0941 ± .0280</td>
<td>.0759 ± .0218</td>
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<tr>
<td>Tenericutes <em>order</em></td>
<td>Erysipeltrichales</td>
<td>.703 ± .133</td>
<td>.466 ± .115</td>
<td>.212</td>
</tr>
<tr>
<td>Anaeroplasmatales</td>
<td>2.16 x 10^-3 ± 9.44 x 10^-4</td>
<td>1.26 x 10^-3 ± 5.15 x 10^-4</td>
<td>3.52 x 10^-3 ± 1.06 x 10^-3</td>
<td>6.16 x 10^-4 ± 2.84 x 10^-4</td>
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<tr>
<td>Mycoplasmatales</td>
<td>3.49 x 10^-5 ± 3.49 x 10^-4</td>
<td>1.82 x 10^-4 ± 1.22 x 10^-4</td>
<td>3.91 x 10^-5 ± 3.91 x 10^-5</td>
<td>4.46 x 10^-3 ± 2.68 x 10^-3</td>
</tr>
<tr>
<td>Verrucomicrobia_</td>
<td>Verrucomicrobiales</td>
<td>.0308 ± .0114</td>
<td>.0205 ± .0113</td>
<td>.535</td>
</tr>
</tbody>
</table>

* Bonferroni correction P<0.002
Figure 20 Percent relative abundance of bacteria at the genus level in control and fluoxetine-exposed offspring in adulthood (for all representative sequences >0.01%). This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Legend is presented as Phyla_Genus.
M.Sc. Thesis- H. Law; McMaster University- Medical Sciences

Table 4: Bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Relative abundance (%) of taxonomy genus separated by sex. Statistical significance between control and treated offspring within each sex was determined by a non-zero abundance. Student’s t-test and expressed as mean ± SEM. Rules for inclusion of gut microbiota in analysis: (1) Had to be defined; (2) had to have at least zero abundance. Student’s t-test and expressed as mean ± SEM. Rules for inclusion of gut microbiota in analysis: (1) Had to be defined; (2) had to have at least 10% of all samples and (3) have non-zero abundance.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Male (mean±SEM)</th>
<th>P-value</th>
<th>Female (mean±SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>FLX</td>
<td></td>
<td>CON</td>
</tr>
<tr>
<td>Actinobacteria_genus_</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>2.87 x10^4 ± 1.42 x10^4</td>
<td>8.43 x10^4 ± 2.64 x10^4</td>
<td>.067</td>
<td>1.14 x10^3 ± 5.50 x10^3</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>.0475 ± .0174</td>
<td>.0103 ± .00614</td>
<td>.086</td>
<td>.0233 ± .0975</td>
</tr>
<tr>
<td>Adlercreutzia</td>
<td>.0135 ± .00242</td>
<td>.0141 ± 3.62 x10^3</td>
<td>.880</td>
<td>.0259 ± .00515</td>
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<tr>
<td>Collinsella</td>
<td>1.07 x10^4 ± 1.07 x10^4</td>
<td>0.00</td>
<td>.362</td>
<td>4.31 x10^4 ± 1.41 x10^4</td>
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<tr>
<td>Bacteroidetes_genus_</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Parabacteroides</td>
<td>.122 ± 9.90 x10^3</td>
<td>.111 ± .0294</td>
<td>.743</td>
<td>.0959 ± .0186</td>
</tr>
<tr>
<td>Prevotella</td>
<td>13.0 ± 3.22</td>
<td>9.72 ± 3.55</td>
<td>.519</td>
<td>6.18 ± 1.66</td>
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<tr>
<td>Allstipes</td>
<td>.0460 ± .0139</td>
<td>.0419 ± 6.92 x10^3</td>
<td>.800</td>
<td>.0448 ± .0106</td>
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<tr>
<td>Deferrribacteres_ Mucispirillum</td>
<td>7.6 x10^3 ± 2.93 x10^3</td>
<td>0.0176 ± 5.65 x10^3</td>
<td>.112</td>
<td>5.24 x10^3 ± 1.80 x10^3</td>
</tr>
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<td>Firmicutes_genus</td>
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<td></td>
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<tr>
<td>Jeotgalicoccus</td>
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<td>1.35 x10^3 ± 8.70 x10^5</td>
<td>.098</td>
<td>1.56 x10^3 ± 6.80 x10^4</td>
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<td>Staphylococcus</td>
<td>2.44 x10^3 ± 9.46 x10^4</td>
<td>4.01 x10^3 ± 1.17 x10^3</td>
<td>.315</td>
<td>4.01 x10^3 ± 1.02 x10^3</td>
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<td>Enterococcus</td>
<td>.0142 ± 5.80 x10^3</td>
<td>9.12 x10^3 ± .0279</td>
<td>.497</td>
<td>6.48 x10^3 ± 1.27 x10^3</td>
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<td>Lactobacillus</td>
<td>10.2 ± 2.56</td>
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66
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<th>M. Sc. Thesis H. Law: McMaster University - Medical Sciences</th>
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### Table 1: Bacterial Composition

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*Bonferroni correction P < 0.001
CHAPTER 4: DISCUSSION

In this study, I have shown that fetal and neonatal exposure to the SSRI fluoxetine results in altered expression of key components of 5-HT signaling and biosynthetic pathways in male, but not female, offspring. These changes in the gut serotonergic signaling pathway were not associated with increased intestinal inflammation. Interestingly, both male and female offspring of fluoxetine-exposed dams had significant changes in the composition of the gut microbiota in adulthood. Taken together these data suggest that early life exposure to SSRIs has the potential to alter gut development and perturb the normal gut microbiota. Importantly, changes in serotonergic signaling and/or composition of the gut microbiota following developmental exposure to SSRIs may be relevant in the pathophysiology of inflammatory gut disorders such as inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) later in life.

4.1 Prenatal and neonatal fluoxetine exposure alters the number of colonic 5-HT containing EC cells

Early developmental exposure to the SSRI fluoxetine may play a role in altered 5-HT production in adulthood. Although we observed changes in the number of EC cells at P1 and P21, we are not sure if these differences persist in adulthood, and how this may result in the increase in serum 5-HT in adult males born to dams exposed to fluoxetine. This increase in circulating 5-HT can only be derived from the gut itself since 5-HT produced by the CNS is unable to cross the blood brain barrier [399]. Furthermore, postprandial 5-HT enters the bloodstream where it is rapidly taken up by platelets that also express the transporter [400]. Therefore, 5-HT found in the blood arises
primarily from 5-HT released from EC cells [187, 401]. This increase in serum 5-HT observed can be accounted for either by (1) an increase in EC cell number; (2) the EC cell’s increase capacity to endogenously synthesize 5-HT and/or (3) alterations to the serotonergic degradation pathway. It is possible that increased levels of 5-HT during gestation as a result of SSRI exposure resulted in disruption in an initial acute increase in serotonergic tone (steady state levels), leading to an increase in negative feedback which inhibits the development of the serotonergic system, resulting in disruptions to 5-HT signaling tone later in life [402]. The fact that a similarly significant increase in serum 5-HT concentration was not evident in the female animals reinforces the importance of investigating sex differences. Some limitations to this part of the analysis include not examining postnatal EC cell counts, or measuring fetal 5-HT exposure in this model.

Both prenatal/neonatal SSRI exposure and SERT knockout models are known to disrupt the normal development of both serotonergic and non-serotonergic neurons in the CNS (Homberg et al., 2009). This disruption results in neurochemical and physiological changes including an increase in 5-HT synthesis and a decreased capacity to store 5-HT that results in markedly elevated extracellular 5-HT levels [220, 403, 404]. Besides the 5-HT producing neurons of the raphe nucleus, a diverse range of cell types briefly harbor SERT during neurodevelopment, thus explaining the widespread behavioural consequences that are associated with a disruption to 5-HT homeostasis. Since 5-HT is required for the development of the CNS, understanding how this affects the development of the 5-HT-containing EC cells of the gut is required to determine how alterations in the levels of 5-HT or 5-HT receptors during specific developmental times modify the formation of the peripheral serotonergic system and influence intestinal health in the offspring later in life.
4.2 Sex-dependent changes in serotonergic pathways following developmental fluoxetine exposure

Peripheral 5-HT levels depend on synthesis by Tph1, reuptake by SERT and subsequent degradation by MAO. In our model there does not appear to be a change in the former, but rather a perturbation in a key component of the degradation pathway. The increase in serum 5-HT in males coincides with a decrease in the metabolism of 5-HT as seen with the decrease in key regulator of 5-HT activity, intracellular enzyme MAO. The corresponding elevated serum 5-HT levels may indicate that potentially more 5-HT is being released as a result of higher 5-HT content due to a decrease in intracellular catabolism. We may not see these SSRI-related changes in our female offspring because the expression of MAO may be dependent on the stage of the estrus cycle (e.g. 30% reduction in expression between estrus and diestrus; Appendix D, Table 7). Furthermore, the sample size for different stages of the estrus cycle was small because although we documented the estrus cycle stage, we did not control for it. Although we did not measure 5-HT levels at an earlier time point, knockout models of the main enzyme responsible for 5-HT degradation, MAO causes a nine-fold increase in the level of 5-HT in the brain during the first postnatal week of life [405]. During this period, 5-HT accumulation was associated with marked effects in the somatosensory and visual systems regions of the brain. Thus, it is possible that a similar disturbance in the gut may result in apparent functional differences, such as gastrointestinal motility in the gut. Indeed, we have preliminary that demonstrates the myenteric plexus in the colon is hyperplastic in SSRI-treated offspring at P21.

Our finding that colonic SERT messenger expression in adulthood was not disrupted in our model means that fluoxetine has a minimal effect on the innate reuptake mechanisms in the periphery during maturity. Similar to our results, prenatal fluoxetine exposure has been
demonstrated to result in an overall increase in SERT expression in various brain regions at PND28, but not in adult life in the CNS [226, 406].

Models of neuropsychiatric disorders demonstrate a sex-specific interaction between 5HT1A receptor availability and the MAO gene of the human serotonergic system, which suggests a neurobiological basis for sexual dimorphism in serotonin-modulated phenotypes (Mickey et al., 2008). Mickey et al. [407] found that low-activity MAO genotype was associated with lower 5-HT1A receptor concentrations in individuals suffering from depression. MAO knockout mouse models show increased extracellular 5-HT levels and decreased 5-HT1A receptor sensitivity and concentrations [408-410]. The reduced expression of 5-HT1A receptors has been explained as compensatory down-regulation resulting from excess extracellular 5-HT levels in animals that lack functional MAO enzyme [408-410]. This may provide a basis for the decrease of both 5-HT2A and MAO expression and increase in serum 5-HT levels in our animal model. We found that 5-HT2A receptor expression significantly correlated with MAO transcript expression (r=0.421; p=0.015) (data not shown).

In adulthood, male offspring exposed both in the prenatal and neonatal periods to the SSRI fluoxetine had significantly lower colonic 5-HT2A messenger expression compared to their non-treated counterparts. In females, a similar trend was observed; however did not reach statistical significance (4.1 ± 2.64 vs. 1.4 ± 0.49; P= 0.689), even when separated by stage of cycle (estrous versus diestrus). The expression of this receptor in the brain is independent of sex hormones [411], confirming the similar downward pattern of expression in both sexes seen in the fluoxetine-treated group.
When compared to previous studies of prenatal exposure to fluoxetine, similar reductions in hypothalamic $5-HT_{2A/2C}$ receptors have been observed in male rats at 70 days postnatal age [412]. Interestingly, these effects were not observed at P28, suggesting a delayed decrease in receptor density and function. As demonstrated by Lauder et al. [413], both under and over-stimulation of $5-HT_{1A}$ receptors by serotonergic drugs during prenatal development has also been shown to produce a significant reduction in the expression of its transcripts in the brain; whether these effects persist into adulthood is not known. Evidence from these studies suggests that appropriate levels of serotonergic stimulation of 5-HT receptors may be required for normal developmental regulation in the CNS, which may occur in the gut.

As a consequence of altered 5-HT homeostasis in SERT knockout models, density in 5-HT receptor expression has been shown to vary (Haenisch et al., 2010). In contrast to chronic SSRI treatment which causes desensitization and thereby reduction of $5-HT_{1A/1B}$ receptor function, these receptors are additionally down-regulated at the mRNA and protein level in SERTKO mice [414, 415] in several brain areas [416-418]. $5-HT_{2A}$ receptors are similarly decreased in a variety of brain regions including the hypothalamus and cortex [419, 420]. In contrast, the $5-HT_{2C}$ receptors show up-regulation in its brain regions [420].

The $5-HT_{2A}$ receptor is part of a closely related subgroup of $5-HT_2$ G-protein coupled receptors expressed predominantly in peripheral tissues, such as the stomach, intestine, heart and kidney [421-423]. In the mammalian gut, $5-HT_{2A}$ promotes the contraction of gastric smooth muscle cells [424-426] and regulates secretions from epithelial cells [427-431]. This serotonergic receptor appears as early as embryonic day 14 (E14) on ganglia in the CNS [214], therefore it is possible that any perturbations made to its expression during this period may be accompanied by functional and/or expression abnormalities in the gut. A study by Forica-Howells et al [432]
however shows that genetic ablation of 5-HT\textsubscript{2A} receptor, did not alter GI transit time or colonic motility. Although we did not test for measures of enteric function, anecdotally we did not observe the presence of diarrhea in the fluoxetine-exposed offspring. Other studies have suggested that 5-HT\textsubscript{2A} may be involved in the continuous maintenance and development of epithelial cells in intestinal crypts and muscularis externa that persists throughout life [433]. Hence, in the colon of 5-HT\textsubscript{2A}−/− mice, enterocytes were smaller, muscle layers thinner and fewer Paneth cells were present [432]. These measures were not examined in our present study. The preservation of gross measures of motility despite the changes in the musculature suggests in the absence of a pathological insult these structural changes do not affect function and therefore, we do not expect functional changes in response to possible structural alterations in our animals.

5-HT\textsubscript{2A} receptor activation has been implicated in inflammatory responses [434], and thus has been associated with inflammatory diseases in animal models [435, 436]. However, a discrepancy exists as to whether 5-HT\textsubscript{2A} receptor signaling plays an anti- or pro-inflammatory role. For instance, rheumatoid arthritis patients show considerably lower 5-HT\textsubscript{2A} receptor density than controls, thus establishing an inverse correlation between disease severity and receptor expression [437]. The authors suggested that the down regulation of receptor expression can be a compensatory mechanism directed against stimulation of 5-HT\textsubscript{2A} receptors involved in the pathophysiology of inflammatory conditions. Recently, however, agonism of 5-HT\textsubscript{2A} has been shown to exert potent anti-inflammatory effects. When stimulated, this receptor has been shown to decrease key inflammatory markers TNF-α, and IL-6 in the rat small intestine [434]. Therefore we hypothesized that the decrease in 5-HT\textsubscript{2A} receptor messenger expression seen in fluoxetine-exposed adult male offspring might be associated with increased inflammation in the
gut. However, we found no significant differences in the inflammatory gene profile in our fluoxetine-exposed adult male offspring.

4.3 Alterations to the serotonergic pathway by prenatal/neonatal exposure to fluoxetine is not associated with colonic inflammation in adulthood

Despite the elevated levels of serum 5-HT measured and decreased expression of the 5-HT$_{2A}$ receptor and MAO enzyme, these changes in serotonergic signaling and regulation components were not accompanied by an increase in inflammation at the macroscopic level as seen by measuring cellular infiltration. This was confirmed molecularly through evaluation of messenger expression of cytokine levels and macrophage markers. This negative result is in direct contrast to the increase in intestinal inflammation that accompanies elevations in 5-HT and changes to the peripheral serotonergic signaling seen in GI disorders [208, 277, 288-291, 438]. The activation of the mucosal immune system by an excess of 5-HT accompanies the histologic and morphologic changes involving EC cells, lymphocytes, mast cells and enteric nerves and is believed to contribute to pathophysiology of IBS [280, 312, 439]. When compared to their healthy counterparts, patients with IBS have elevated levels of pro-inflammatory cytokines, including IL-β, IL-6 and TNF-α [249, 314, 315, 321]. Intestinal inflammation, as seen by increases in additional mediators including IL-4, IL-5, IL-10, IL-12, IL-13 and IL-17 has also been shown to accompany the potentiation of serotonergic signaling in both models [282, 285] and human patients with IBD [440, 441]. The concentration of these mediators is highly elevated in blood, stool and intestinal mucosa. The release of these pro-inflammatory mediators is regulated by different pathways involved in inflammation including NF-κB and MAPK pathway and JAK/STAT pathway, which results in the progression of disease [442]. It is possible that the changes in serotonergic components we observed were not large enough to overburden the
organism’s innate compensatory mechanisms as seen with SERT or TPH1 knockout (KO) models which either respond by having an increase in colitis severity or become resistant to experimentally induced colitis, respectively [284-286]. A previous study has suggested that the elevated 5-HT that occurs as a result of these models may actually be partially protective against gut inflammation [207, 306]. Bischoff et al. [284] confirms that this is so in the absence of inflammation. They anticipated that the stress of inflammation would release 5-HT and overwhelm the protective compensations in SERT KO mice, such as decreased sensitivity and rapid desensitization of 5-HT receptors as well as a low-affinity, nonspecific uptake of 5-HT by backup transporters [207, 306]. Furthermore, if enteric 5-HT is involved in inflammation, intestinal inflammatory responses would be expected to be significantly exacerbated by the potentiation of serotonergic signaling that occurs when SERT is inactive [443]. On the other hand, due to the significantly elevated 5-HT levels in our male offspring, it is likely that there is excess activation of the 5-HT$_{2A}$ receptor, which has been shown to have anti-inflammatory effects [434]. These effects, however, may too be diminished since we observed a reduced density of 5-HT$_{2A}$ receptor expression. Therefore, 5-HT levels raised through reductions in MAO expression may confer a protective mechanism. In addition to its role as a neurotransmitter, 5-HT also has immunomodulatory effects which are mediated through 5-HT receptors expressed on lymphocytes, monocytes, macrophages and dendritic cells [444]. The finding that EC cell-derived 5-HT acts as a pro-inflammatory mediator in the gut has generated interest in the potential of 5-HT antagonists for treatment of gut disorders involving inflammation [323].
4.4 Prenatal SSRI exposure does not alter the expression of tight-junction associated proteins

Although we did not observe any significant differences in the expression of tight-junction associated proteins between treatment groups, we did not measure whether similar results would translate at the protein level or lead to impairments in barrier integrity. However, these results are consistent with the finding that we did not see evidence of colonic inflammation either macroscopically or at the molecular level. At present, we are the first to demonstrate that prenatal and neonatal exposure to an SSRI does not significantly alter the structure components of TJs at the mRNA level in adult offspring. There is also a paucity of data with regards to both gut barrier structural and functional effects in adults who take SSRIs. It is well-established that GI symptoms, including diarrhea is commonly associated with adult SSRI use [445]. An increase in enteroendocrine mediators including 5-HT released from EC cells has been implicated at the origin of altered epithelial barrier functions and ENS signalling [337]. However, how specific TJ components are linked to diarrhea still remains unclear. The TJ proteins constitute a critical platform that regulates epithelial barrier integrity and maintains homeostasis of mucosal immune activation [337]. In pathological disease states, the disruption of the intestinal barrier results in dysregulated epithelial permeability, which can induce an increase in paracellular permeability and an overactive mucosal immune response leading to chronic intestinal inflammation [293]. Several lines of evidence suggest that compromised intestinal barrier function is associated with low-grade inflammation in the gut mucosa of IBS and IBD patients [297, 446-448]. Put into clinical context, the increase in 5-HT as a result of EC cell hyperplasia has similarly been suggested as the underlying cause between specific patterns of alterations in TJ and diarrhea in these inflammatory conditions of the gut [337].
4.5 Developmental SSRI exposure is associated with sex-dependent alterations to the gut microbiota

Determining the composition of the intestinal microbiota in adulthood showed small differences in the relative abundance of bacteria only at certain taxonomic levels between both treated and control offspring in female offspring. Many of the diseases and disorders associated with gut microbiota dysbiosis exhibit an overall reduction in bacterial diversity [356]. However, the sex-dependent differences seen were representatives of smaller bacterial groups, such as class Bacilli (Phylum Firmicutes; female only) which account for less than 10% of the total gut bacteria sequenced. A similar trend was observed at the genus level, whereby the only significant difference was observed was a significant increase in Lactobacillus (Phylum Firmicutes) was observed in female offspring exposed to fluoxetine. As illustrated by our (PCoA) Plots (Appendix G, Figure 22 [unifrac]; Appendix H, Figure 23 [Bray-Curtis]) the microbiota found between all treatment groups (CON-M vs FLX-M; CON-F vs FLX-F) did not form discrete clusters, which suggests that regardless of treatment, there are similar bacterial communities and there are more biological differences between sexes. We are the first to characterize the composition of the gut microbiota in adult offspring born to dams given fluoxetine during the prenatal and neonatal periods. However, others have suggested that medication use may also affect the establishment of the enteric microbiota in adults [383]. Not only does short-term exposure to xenobiotics alters bacterial physiology, but it also significantly alters the structure of the overall microbial community as seen by 16S rRNA gene sequencing [383]. This suggests that prenatal exposure to maternal medication, including SSRIs, may alter the gut microbiota in the offspring. In light of the absence in gross alterations to the gut microbiota, it is unknown whether specific bacteria have unique effects on long-term alterations in gut physiology or whether
different pathogens converse to cause common alterations resulting in similar phenotype. Thus changes to gut function do not necessarily need to occur via larger dominant bacterial groups.

The past decade has witnessed an appreciation for the importance of the symbiotic relationship regarding the vast microbial community that resides within the intestine and of their host. The topic has generated great expectations in terms of gaining a better understanding of disorders ranging from IBD to metabolic disorders and obesity. Moreover, understanding the influence of maternal medication use on the establishment of the gut microbiota in the offspring remains a relatively unexplored area of investigation.

4.5.1 Gastrointestinal Disease, serotonin and the gut microbiota

The gut microbiota is subject to influences from a diverse range of factors including diet, antibiotic usage, infection and stress. However, whether or not maternal SSRI use is one of these factors remains to be determined. 5-HT is an important mediator in the bi-directional interaction between the gut microbiota and the CNS that allows afferent signaling to the brain to modulate gut motility [194, 449, 450]. Taking cues from 5-HT’s role in the brain-gut axis, there is indirect evidence suggesting an interaction between the gut microbiota and gut-derived serotonin (GDS) itself. Indeed, in addition to other signaling peptides, enterochromaffin (EC) cells secrete 5-HT in response to physiological and pathological luminal stimuli that may be either microbial or bacterial in nature [451]. EC cells express a wide variety of receptors, and the possibility of adrenergic receptors being expressed on the brush border of epithelial cells has been proposed to serve as way in which bacteria could have a wide variety of target to influence gut 5-HT release [367]. When compared to controls, germ-free mice presented with a nearly 3-fold increase in plasma 5-HT levels [452]. Additionally, an elevation in plasma tryptophan, the amino acid precursor to 5-HT, has been observed following administration of probiotic bacteria to rats [453].
Altered composition of the gut microbiota has been considered as a potential aetiologic factor in at least a subset of patients with IBS and is becoming increasingly apparent in those with IBD. Earlier studies have shown reductions of *Lactobacillus* spp. and *Bifidobacterium* spp. and increased number of Enterobacteriaceae in the gut flora of IBS patients when compared to healthy controls [363, 364]. Patients with CD have been observed to have a reduction in microbes of the phylum Firmicutes (Gram-positive bacteria, including *Clostridium* and *Bacillus* species) and a concomitant increase in Proteobacteria (Gram-negative rods, including *Escherichia* spp.) [454]. Microbes belonging to the genus *Prevotella* (Gram-negative bacteria) are also enriched in the stool of these patients which was in contrast to our finding of a 3-fold increase in *Lactobacillus* in female offspring born to dams exposed to fluoxetine. *Lactobacillus* is a Gram positive bacterium, and therefore lacks the bacterial endotoxin lipopolysaccharide (LPS) on its cell wall [455]. It is when there is an excessive presence of LPS from groups belonging to the Gram negative Bacteoridetes that a strong inflammatory response may be elicited by the organism to protect it from infection [456] through a toll-like receptor 4 (TLR4) – dependent mechanism [457, 458]. Structural imbalances of the gut microbiota, particularly reductions in the abundance of gut-barrier-protecting bacteria such as *Bifidobacterium* spp. and increases in the abundance of Gram-negative endotoxin producing bacteria such as *Desulfovibrio* spp. and *Prevotella* spp. may lead to increases in intestinal permeability and circulating gut-originated antigens [459]. A higher level of circulating LPS has been suggested to be caused by disruptions to the intestinal mucosal TJ structure and function, thus causing increased intestinal permeability [242, 446]. Correlative data suggests that compromised intestinal barrier function is associated with intestinal immune activation that may contribute to disease progression [460]. Since we did not observe an increase in either any of these Gram-negative bacteria at the genus
level in offspring exposed perinatally to fluoxetine, this is consistent with the absence of inflammation and a decrease in TJ associated proteins observed in our model. Similar to *Bifidobacterium spp*, *Lactobacillus spp.* is considered to be a “protective” bacterium as it has proven to be efficacious as a probiotic in both animal models of disease and human clinical trials [461]. As a probiotic, *Lactobacillus spp.* has been shown to down-regulate both intestinal and systemic pro-inflammatory changes induced by a high-fat diet in a mouse model [462]. One of the mechanisms in which *Lactobacillus* confers protection is in its capacity to underpin the activation of intracellular signaling pathways involved in the relocalization of tight junction proteins through extracellular signal-regulated kinase (ERK) [463, 464]. This results in the protection of enhanced intestinal barrier function. Since this increase in *Lactobacillus* was only seen in female offspring born to dams exposed to fluoxetine, this highlights the importance of sex-dependent effects. Despite that our differences in components of the serotonergic signaling pathway and EC cell hyperplasia observed are consistent with those found in IBS and IBD disorders, our model does not, however, share the gross changes in the gut microbiota and inflammation seen in these pathologies. This potentially demonstrates more favorable long-term intestinal health outcomes in offspring exposed to an SSRI in utero.

### 4.5.2 Sex-dependent regulation of the gut serotonergic system and the gut microbiota

The sex differences observed in the gut microbiota in adulthood in this study may be explained by the fact that there is a bidirectional communication between the gut bacteria and the brain which are modulated by estrogens. In early life, the gut-microbiota brain axis regulates the hippocampal serotonergic system in a sex-dependent manner [368]. Clarke and colleagues [368] showed that male germ free mice, unlike females, display a significant elevation in hippocampal 5-HT concentration and its metabolite compared with conventionally colonized control animals.
A recent study demonstrated that steroid nuclear receptor expression including ER-β may be a determinant of the intestinal microbiota composition [465]. Furthermore, results published recently by Markle et al., [466], indicate that sex differences in the gut microbiota drive hormone-dependent regulation of autoimmunity. In this study, using a non-obese diabetic mouse model of type 1 diabetes, male puberty in mice led to changes in the gut microbiota that increase testosterone production, which is protective against the development of T and B cell functions linked to autoimmune disease [466]. The male microbiota is associated with testosterone-mediated protection from autoimmune disease which can be transferred to younger female recipients. The observations that early-life microbial exposures determine sex hormone levels and modify sex-mediated immune regulation may have crucial implications for the pathophysiology of IBS. A new concept termed “microgenderome” is emerging based on the recent observations that the sex bias present in numerous diseases is not entirely a host-intrinsic factor, but may be exercised and/or reinforced by the commensal microbiota of the host [467].

4.5.3 Sex-dependent regulation of the serotonergic system and inflammatory GI disorders

The regulation of the serotonergic system is influenced by sex [468, 469]. It is noteworthy that many of the colonic alterations (we found occurred in a sex-specific manner. We also observed potential differences in inflammatory markers when our female offspring were separated by stage of cycle (estrus and diestrus). For example, at a glance, IL-6 expression appears to be a fold-change higher in female offspring in the estrus cycle as compared to diestrus (Appendix D; Table 7). Since there is a paucity of data in this area, future studies will need to account for stage of cycle to confirm that there is no inflammation in this model. The mechanism surrounding these sex differences are not well understood but may relate to the well-known but complex influence of the oestrous cycle hormones on the CNS serotonergic system [470]. For instance,
estrogen and its receptor (ERβ) are known to modulate hippocampal 5-HT concentrations [471]. Therefore it is important to investigate the influence of SSRIs in rats of both sexes.

Various observations point to a possible role for sex hormones in the pathophysiology of IBS. For instance, in Western countries the female-to-male ratio among non-patient population of IBS sufferers is 2:1 [472], thus making female gender a significant independent risk factor for new-onset IBS (odds ratio [OR] = 2.14; 95% CI, 1.56-2.94) [473], and IBS-C subtype [474]. Hormonal differences (eg, ovarian vs. testicular) between men and women may contribute, at least in part, to the gender gap in abdominal pain and IBS symptom reporting [475]. Furthermore, sex hormones such as estrogens play a significant role in the physiological regulation of motor and sensory function in the gastrointestinal tract [476]. Therefore, their role in the pathophysiology of inflammatory gastrointestinal disorders is becoming increasingly apparent [477]. This female predominance underlies the correlation between IBS symptoms and hormonal status during menstrual cycle phases, pregnancy or menopause [478]. Dynamic changes in ovarian hormones during menstrual cycle can modulate GI contractility, transit, secretion, visceral sensitivity, and immune function in both the periphery and the brain [262]. In animal models, it has been shown that both visceral and somatic sensitivity vary over the rat estrous cycle and that high levels of ovarian hormones (proestrous/estrus stages) are associated with enhanced sensitivity [479]. Clinical studies indicate that period of low ovarian hormone levels in women, such as during menses, may contribute to the occurrence or exacerbation of GI symptoms that varies across the menstrual phase [478, 480-484]. Human data confirms that IBS symptomatology is exacerbated at menses and in contrast with healthy women, rectal sensitivity changes with the menstrual cycle [483]. This indicates that IBS patients may respond differently to fluctuations in sex hormones than healthy subjects. Variation in GI symptoms during the
menstrual cycle can be related to change in colonic motility and alterations to colonic epithelial barrier and mucosal immunity [289, 475, 485]. Estrogen-dependent intestinal barrier function is another component in sex-related differences in IBS. In humans, acute experimental stress evokes a differential sex-dependent increase in intestinal macromolecular permeability [486], thus suggesting a mechanism that may contribute to female over susceptibility to IBS.
CHAPTER 5: CONCLUSION

I have found evidence for structural changes in the intestine of pups exposed to mothers who were administered SSRI fluoxetine compared with control animals. This was illustrated by an increase in colonic EC cell number. Along with the increase in EC cells, I have demonstrated an increase in serum levels of 5-HT in the male SSRI-treated offspring in adulthood. This was accompanied by altered expression of enzymes (MAO) and receptors (5-HT₂A) involved in 5-HT function the colon of male SSRI-treated adult offspring. There was no evidence of inflammation and/or damage and in the expression of tight junction associated proteins in the colonic mucosa. However, I observed subtle sex-dependent differences in the profile of intestinal microbiota in offspring exposed to fluoxetine. These results demonstrate that similar to the CNS, the adult serotonergic system of the gut and the microbiota are susceptible to prenatal and neonatal exposure to the SSRI fluoxetine. Despite these alterations, it is not apparent that they potentiate intestinal inflammation or alter structures related to intestinal permeability.

Increased levels of 5-HT have been associated with increased susceptibility to colitis [286]. Since there was no evidence of an increase in colonic inflammation or impairments to intestinal barrier function that accompanied the structural changes, this may highlight the fact that prenatal and neonatal SSRI exposure has minimal impact on the long-term intestinal health of the offspring. It is yet to be determined whether these subtle changes in 5-HT content and signaling can lead to increased susceptibility to IBD given the right environmental triggers. However, our
results may be of greater relevance to functional GI disorders (FGIDs). FGIDs, such as IBS, are characterized by symptoms including abdominal pain, diarrhea, constipation, anxiety, or depression [262]. Unlike in IBD, these symptoms are often accompanied by altered gut motility or visceral sensitivity [487, 488] in the absence of identifiable structural or biochemical abnormalities [261].

In light of the alterations to the gut microbiota in offspring exposed to prenatal and neonatal SSRIs, this represents a possible window of opportunity for prebiotic/probiotic interventions to both mother and baby. Probiotics have been demonstrated to have an overall reduction in risk over the majority of GI inflammatory conditions [489]. Treating clinical depression in pregnant and breastfeeding mothers requires assessing the risk of using a psychotropic medication, which might affect the developing fetus, against the benefits of preventing a mother from becoming incapacitated with depressive symptoms. Despite progress to date, definitive conclusions on the use of antidepressants in pregnancy and the treatment of mental illness are limited by the methodological issues inherent in clinical research involving illness versus treatment effects in pregnancy. The risk for adverse fetal and neonatal outcomes may be an independent product of both maternal depression and prenatal SSRI use. It has been suggested that there is potential for confounding to occur and thus it is important to differentiate the effects of exposure to the drug itself from that of the underlying maternal psychiatric illness [119, 490, 491]. In addition, maternal health habits such as smoking, illicit drug and alcohol use, poor prenatal care and obesity can confound birth outcomes [492] but are not always reported accurately or are underreported in cohort studies. When compared to women who are not on antidepressant treatment, women who are on antidepressants are more likely to have poor health habits (e.g. smoking), to be older and to have elevated body mass index [54, 88]. They are also more likely
to have complications such as diabetes and hypertension [88]. However, few studies examining neonatal outcomes of prenatal SSRI exposure have attempted to account for the effect of underlying maternal depression [87, 123, 128, 493]. Animal models allow us to discern the effects of only SSRI exposure on the offspring without the potential confounding influence of maternal depression itself.
CHAPTER 6: FUTURE DIRECTIONS

Results from this study suggest that maternal use of SSRIs causes alterations in the gut serotonergic signaling pathways similar to what is seen in the CNS. However, in this model, the changes were restricted to only male offspring. There was no evidence of intestinal inflammation but there were subtle changes in the gut microbiota of offspring. Analysis of the current literature and the results from this thesis indicates several interesting directions that require further investigation.

For instance, it remains to be determined whether prenatal/neonatal exposure to SSRIs can lead to persistent structural changes in the intestine (increase in EC cells and enteric neuron density) as these are factors that promote an inflammatory state. Accordingly, my results should form the basis to characterize the temporal structural changes in EC cells, intestinal mucosa and enteric nervous system (ENS) in control and SSRI-exposed offspring. Animal models with reduced function of SERT, which leads to an increase in bioavailable 5-HT, have abnormal GI motility [494]. Furthermore, loss of SERT function has been shown to lead to impairment of intestinal barrier function [285]. Although I did not observe a difference in the expression of tight-junction associated proteins, it is possible that changes may appear at the protein level. To determine whether the observed increase in EC cells and enteric neurons are associated with functional abnormalities in the intestine of SSRI-treated offspring, it would be interesting to conduct investigations of motility and permeability. Since both EC cells, through the release of 5-HT, and the ENS are inextricably linked with the motility of the GI tract, these functional measurements
would be useful [495, 496]. Furthermore, whether SSRIs alter the microbiota in the mother remains to be determined. Since the mother is the first to colonize her offspring, characterizing the gut microbiota at earlier time points such as at birth and P21 would be beneficial to determine whether there are changes that precede the ones we observed in adulthood.

Overall, a direct link between the morphological and structural correlates of developmental SSRI exposure in rodents still needs to be established. There are few case reports or studies to date found reporting disturbed bowel function when exposed to antidepressants in utero. Second, more research is required to draw conclusions about human SSRI use from rodent studies. Third, concerns about the potential adverse effects of prenatal or neonatal SSRI exposure should be balance by observations that untreated depression could also harm the unborn or newborn child. A current limitation of the animal models is that SSRIs are applied to healthy pregnant dams, which do not fully represent the human situation. More research is needed to understand the role of 5-HT in enteric development, the long-term consequences of SSRI use during pregnancy, and the postpartum period will allow doctors and patients to make better informed decisions on its use during pregnancy.
APPENDIX A

Figure 21 Summary of the (1) effects of prenatal/neonatal SSRI exposure on the central serotonergic system and known developmental consequences; (2) role of 5-HT in postnatal life; (3) effects of prenatal/neonatal SSRI exposure on the GI system; and (4) potential mechanisms in which SSRIs can affect intestinal health. Solid lines denote what is known, dotted lines denote potential associations.
APPENDIX B

Figure 22 Schematic representation of endocrine cell-mediated signaling from enteric microbiota and host. Presence of Gram-negative bacteria and gut-derived 5-HT in the lumen might influence endocrine cells in the epithelium (enterochromaffin cells). Furthermore, the microbiota may influence intestinal permeability via a Toll-like receptor dependent mechanism, thereby increasing inflammation. Adapted from Rhee et al., 2009.
### APPENDIX C

**Table 5** Summary of the effects of prenatal and/or neonatal exposure to SSRI on the central serotonergic system in offspring.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Density</th>
<th>Exposure</th>
<th>First Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT</td>
<td>Reduced</td>
<td>P8-14 (neonatal)</td>
<td>Hansen et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 mg/kg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GD13-20</td>
<td>Cabrera et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mg/kg</td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>Reduced in</td>
<td>GD13-20</td>
<td>Cabrera et al., 1998</td>
</tr>
<tr>
<td></td>
<td>prepubescent male</td>
<td>10 mg/kg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>offspring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;1A/1B&lt;/sub&gt; receptor</td>
<td>Unchanged</td>
<td>P8-14 (neonatal)</td>
<td>Hansen et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 mg/kg</td>
<td></td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2A/2C&lt;/sub&gt; receptor</td>
<td>Reduced</td>
<td></td>
<td>Cabrera et al., 1994</td>
</tr>
</tbody>
</table>
**APPENDIX D**

**Supplemental Tables**

Table 6 qPCR data of genes involved in the gut serotonergic system of the colon separated by stage of cycle in female offspring at 26 weeks of age. No differences were observed in treatment groups of estrus and diestrous females. All comparisons were done by independent samples t-test.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Estrous (Mean ± SEM)</th>
<th>p-value</th>
<th>Diestrous Mean (Mean ± SEM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fluoxetine</td>
<td>Control</td>
<td>Fluoxetine</td>
</tr>
<tr>
<td>Tph1</td>
<td>3.11 ± .640</td>
<td>1.32 ± .247</td>
<td>.032</td>
<td>1.28 ± .288</td>
</tr>
<tr>
<td>Pet-1</td>
<td>1.42 ± .490</td>
<td>1.61 ± .520</td>
<td>.799</td>
<td>1.38 ± .310</td>
</tr>
<tr>
<td>SERT</td>
<td>1.28 ± .609</td>
<td>1.73 ± .301</td>
<td>.501</td>
<td>1.48 ± .556</td>
</tr>
<tr>
<td>Mao</td>
<td>5.38 ± 1.64</td>
<td>2.90 ± 1.13</td>
<td>.251</td>
<td>3.75 ± 2.00</td>
</tr>
<tr>
<td>HTR 1a</td>
<td>2.21 ± 1.90</td>
<td>.325 ± .195</td>
<td>.195</td>
<td>.883 ± .763</td>
</tr>
<tr>
<td>HTR 1b</td>
<td>6.74 ± 5.56</td>
<td>.659 ± .234</td>
<td>.249</td>
<td>2.42 ± 2.00</td>
</tr>
<tr>
<td>HTR 1d</td>
<td>1.16 ± 1.01</td>
<td>.147 ± .052</td>
<td>.283</td>
<td>.520 ± .450</td>
</tr>
<tr>
<td>HTR 2a</td>
<td>9.65 ± 8.687</td>
<td>1.22 ± .803</td>
<td>.302</td>
<td>2.57 ± 2.30</td>
</tr>
<tr>
<td>HTR 2b</td>
<td>4.17 ± 2.47</td>
<td>.734 ± .181</td>
<td>.159</td>
<td>1.37 ± 1.05</td>
</tr>
<tr>
<td>HTR 3a</td>
<td>16.0 ± 1.2</td>
<td>6.41 ± 2.76</td>
<td>.343</td>
<td>5.15 ± 2.16</td>
</tr>
<tr>
<td>HTR 3b</td>
<td>5.54 ± 4.39</td>
<td>.741 ± .261</td>
<td>.250</td>
<td>2.52 ± 2.17</td>
</tr>
<tr>
<td>HTR 4</td>
<td>3.42 ± 1.30</td>
<td>1.29 ± .257</td>
<td>.118</td>
<td>2.35 ± 1.45</td>
</tr>
<tr>
<td>HTR 7</td>
<td>3.01 ± 2.15</td>
<td>1.62 ± .930</td>
<td>.539</td>
<td>1.31 ± .735</td>
</tr>
</tbody>
</table>

Tph1 (Tryptophan hydroxylase-1); Pet-1 (transcription factor); SERT (serotonin transporter); Mao (Monoamine Oxidase); 5-Htr (serotonin receptor).
Table 7 qPCR data of genes involved in inflammation and gut permeability in the colon separated by stage of cycle in female offspring at 26 weeks of age. No differences were observed in treatment groups of estrus and diestrous females. All comparisons were done by independent samples t-test.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Estrous (Mean ± SEM)</th>
<th>p-value</th>
<th>Diestrous (Mean ± SEM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fluoxetine</td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>IL1β</td>
<td>0.696 ± 0.057</td>
<td>0.747 ± 0.124</td>
<td>0.798</td>
<td>0.944 ± 0.270</td>
</tr>
<tr>
<td>IL6</td>
<td>4.88 ± 3.93</td>
<td>0.601 ± 0.268</td>
<td>0.252</td>
<td>2.88 ± 2.66</td>
</tr>
<tr>
<td>IL10</td>
<td>0.565 ± 0.423</td>
<td>0.069 ± 0.040</td>
<td>0.223</td>
<td>0.228 ± 0.209</td>
</tr>
<tr>
<td>TNFα</td>
<td>3.71 ± 2.62</td>
<td>0.682 ± 0.235</td>
<td>0.229</td>
<td>1.75 ± 1.48</td>
</tr>
<tr>
<td>MCP1</td>
<td>0.592 ± 0.494</td>
<td>0.074 ± 0.040</td>
<td>0.268</td>
<td>0.195 ± 0.188</td>
</tr>
<tr>
<td>IL13</td>
<td>5.70 ± 4.54</td>
<td>1.39 ± 0.594</td>
<td>0.316</td>
<td>3.68 ± 3.33</td>
</tr>
<tr>
<td>F4/80</td>
<td>2.52 ± 1.27</td>
<td>2.34 ± 0.921</td>
<td>0.914</td>
<td>1.17 ± 0.579</td>
</tr>
<tr>
<td>CD68</td>
<td>1.18 ± 0.161</td>
<td>0.916 ± 0.396</td>
<td>0.615</td>
<td>0.646 ± 0.329</td>
</tr>
<tr>
<td>OCLDN</td>
<td>1.09 ± 0.274</td>
<td>0.617 ± 0.148</td>
<td>0.160</td>
<td>0.396 ± 0.193</td>
</tr>
<tr>
<td>CLDN1</td>
<td>4.84 ± 3.79</td>
<td>0.426 ± 0.201</td>
<td>0.224</td>
<td>2.12 ± 1.81</td>
</tr>
<tr>
<td>CLDN3</td>
<td>1.40 ± 0.156</td>
<td>1.09 ± 0.302</td>
<td>0.455</td>
<td>0.830 ± 0.113</td>
</tr>
<tr>
<td>ZO-1</td>
<td>3.09 ± 0.594</td>
<td>2.42 ± 0.829</td>
<td>0.570</td>
<td>1.69 ± 0.504</td>
</tr>
<tr>
<td>TLR4</td>
<td>2.64 ± 0.465</td>
<td>1.48 ± 0.718</td>
<td>0.271</td>
<td>0.346 ± 0.173</td>
</tr>
</tbody>
</table>

Anti- and pro-inflammatory cytokines: I-10, TNF-a, IL-1β, IL-6, and I-13; MCP 1 (monocyte chemoattractant protein 1); CD68 (Cluster of differentiation 68); F4/80 (EGF-like module-containing mucin-like hormone receptor-like 1) Tight junction proteins involved in gut permeability: OCLN (Occludin); CLDN 1 (claudin 1); CLDN 3 (claudin 3); ZO-1 (zona occludin 1).
Table 8 qPCR data of genes involved in glucose/fatty acid metabolism in the colon separated by stage of cycle in female offspring at 26 weeks of age. No differences were observed in treatment groups of estrus or diestrous females. All comparisons were done by independent samples t-test.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Estrous (Mean ± SEM)</th>
<th>p-value</th>
<th>Diestrous (Mean ± SEM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fluoxetine</td>
<td>Control</td>
<td>Fluoxetine</td>
</tr>
<tr>
<td>GLUT2</td>
<td>5.45 ± 4.54</td>
<td>1.05 ± .386</td>
<td>.303</td>
<td>2.47 ± 2.14</td>
</tr>
<tr>
<td>SGLT1</td>
<td>3.58 ± .611</td>
<td>1.37 ± .333</td>
<td>.019*</td>
<td>1.56 ± .327</td>
</tr>
<tr>
<td>Gcg</td>
<td>2.78 ± 1.21</td>
<td>3.83 ± 1.35</td>
<td>.604</td>
<td>1.52 ± .178</td>
</tr>
<tr>
<td>FIAF</td>
<td>4.38 ± 2.52</td>
<td>1.27 ± .207</td>
<td>.203</td>
<td>2.35 ± 1.45</td>
</tr>
<tr>
<td>GPR41</td>
<td>3.02 ± 2.25</td>
<td>.720 ± .281</td>
<td>.283</td>
<td>1.68 ± 1.35</td>
</tr>
<tr>
<td>GPR43</td>
<td>3.77 ± 1.58</td>
<td>1.59 ± .290</td>
<td>.173</td>
<td>2.02 ± .60</td>
</tr>
</tbody>
</table>

Glucose/fatty acid metabolism: SLC2A2 (glucose transporter 2; GLUT2); SLC5A1 (sodium/glucose co-transporter 1; SGLT1); Gcg (glucagon); Fiaf (fasting induced adipose factor); GPR41/43 (free fatty acid receptor 3; G protein coupled receptor); CD36 (cluster differentiation 36; fatty acid translocase)
### APPENDIX E

**Table 9** Significantly different Operational Taxonomic Units (OTUs) among groups from fecal samples obtained from adult male offspring at 24 weeks of age. Base mean represents the average number of counts (reads). Comparisons are made to base mean (CON) to fluoxetine expressed as a Log2fold. Positive and negative denote increase and negative denotes decrease relative to base mean. Identical OTUs but with different OTU #s represent at least two different species within the taxonomic level, but cannot be identified to a greater taxonomic resolution. OTUs presented as phyla_class_order_family_genus (OTU #). According to the statistical analysis, 4 out of the 6037 examined OTUs showed significant differences between control and treated offspring. A P-value of <0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>OTU (#)</th>
<th>Base Mean (CON)</th>
<th>Log2Fold Change (FLX)</th>
<th>P-value (adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes_Bacteroidia_Bacteroidales (81)</td>
<td>19.9</td>
<td>-1.83</td>
<td>0.0720</td>
</tr>
<tr>
<td>Bacteroidetes_Bacteroidia_Bacteroidales (482)</td>
<td>4.33</td>
<td>-1.92</td>
<td>0.0403</td>
</tr>
<tr>
<td>Firmicutes_Clostridia_Clostridiales (585)</td>
<td>5.47</td>
<td>1.33</td>
<td>0.912</td>
</tr>
<tr>
<td>_Lachnospiraceae (88)</td>
<td>55.3</td>
<td>1.92</td>
<td>0.0335</td>
</tr>
<tr>
<td>_Lachnospiraceae (165)</td>
<td>6.54</td>
<td>-2.12</td>
<td>0.0165</td>
</tr>
<tr>
<td>_Lachnospiraceae (545)</td>
<td>5.70</td>
<td>1.34</td>
<td>0.917</td>
</tr>
<tr>
<td>_Lachnospiraceae (673)</td>
<td>3.25</td>
<td>-1.20</td>
<td>0.918</td>
</tr>
<tr>
<td>Firmicutes_Bacilli_Lactobacillales_Aerococcaceae_Aerococcus (535)</td>
<td>2.32</td>
<td>-1.29</td>
<td>0.787</td>
</tr>
<tr>
<td>_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcus (135)</td>
<td>6.79</td>
<td>-1.28</td>
<td>0.918</td>
</tr>
<tr>
<td>Tenericutes_Mollicutes_RF39 (162)</td>
<td>5.88</td>
<td>-1.72</td>
<td>0.145</td>
</tr>
</tbody>
</table>
APPENDIX F

Table 10 Significantly different Operational Taxonomic Units (OTUs) among groups from fecal samples obtained from adult female offspring at 24 weeks of age. Base mean represents the average number of counts (reads). The number of reads used was 21,444 sequences/sample. Comparisons are made to base mean (CON) to fluoxetine expressed as a Log2 fold. Positive and negative denote increase and negative denotes decrease relative to base mean. Identical OTUs but with different OTU #s represent at least two different species within the taxonomic level, but cannot be identified to a greater taxonomic resolution. OTUs presented as phyla_class_order_family_genus (OTU #). According to the statistical analysis, none of the 6037 examined OTUs showed significant differences between control and treated offspring. A P-value of <0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>OTU (#)</th>
<th>Base Mean (CON)</th>
<th>Log2 Fold Change (FLX)</th>
<th>P-value (adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes (235)</td>
<td>14.504</td>
<td>1.932</td>
<td>0.2781</td>
</tr>
<tr>
<td>Firmicutes (693)</td>
<td>4.202</td>
<td>-1.722</td>
<td>0.2781</td>
</tr>
<tr>
<td>_Bacilli (860)</td>
<td>2.307</td>
<td>-1.367</td>
<td>0.808</td>
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<tr>
<td>_Clostridia_Clostridiales_Lachnospiraceae (52)</td>
<td>158.968</td>
<td>-1.110</td>
<td>0.826</td>
</tr>
<tr>
<td>_Lachnospiraceae (391)</td>
<td>14.368</td>
<td>-1.238</td>
<td>0.826</td>
</tr>
<tr>
<td>_Ruminococcaceae (353)</td>
<td>16.792</td>
<td>1.410</td>
<td>0.826</td>
</tr>
<tr>
<td>Proteobacteria_Alphaproteobacteria (292)</td>
<td>14.149</td>
<td>1.799</td>
<td>0.278</td>
</tr>
<tr>
<td>Bacteroidetes_Bacteroidia_Bacteroidales (161)</td>
<td>100.565</td>
<td>1.320</td>
<td>0.826</td>
</tr>
<tr>
<td>_Bacteroidaceae_Bacteroides (78)</td>
<td>230.893</td>
<td>1.416</td>
<td>0.324</td>
</tr>
<tr>
<td>Tenericutes_Erysipelotrichi_Erysipelotrichales_Erysipelotrichacea (248)</td>
<td>7.836</td>
<td>-1.927</td>
<td>0.2781</td>
</tr>
</tbody>
</table>
APPENDIX G

Supplemental figures

Figure 22 Two-dimensional Principal Coordinate Analysis (PCoA) plots based on the relative abundance distance matrix. Percentage of the diversity explained by each axes is indicated on the figure. There are no distinct clusters between groups, meaning that the gut microbiota of control and fluoxetine-exposed offspring are similar. The two (axes) explain the variance, respectively. Samples associated with control females are labeled as red; control male- yellow; fluoxetine female- purple; fluoxetine male- green are shown as single points.
APPENDIX H
Supplemental figures

Figure 23 Two-dimensional Principal Coordinate Analysis (PCoA) plots based on the phylogenetic distance between OTUs. Percentage of the diversity explained by each axes is indicated on the figure. There are no distinct clusters between treatment groups, meaning that the gut microbiota of control and fluoxetine-exposed offspring are similar. The two (axes) explain the variance, respectively. Samples associated with control females are labeled as red; control male- yellow; fluoxetine female- purple; fluoxetine male- green are shown as single points.
APPENDIX I

Figure 24 The number of observed species (richness) and the Shannon Diversity Index in control and treated offspring at 24 weeks of age separated by sex. A) Observed species diversity between male control (mean ± SEM; 298 ± 26.5) and fluoxetine-exposed offspring (346 ± 24.1; P= 0.247). B) Observed species diversity between female control (340 ± 16.8) and fluoxetine-exposed offspring (342 ± 17.2; P= 0.792). C) Shannon Index in male control (4.81 ± 0.232) and fluoxetine-exposed offspring (4.78 ± 0.166; P= 0.754). D) Shannon Index in female control (5.05 ± 0.151) and fluoxetine-exposed offspring (5.10 ± 0.0661; P= >0.999). The number of reads used was 21 444 sequences/sample.
## APPENDIX J

**Table 11** Full forward and reverse primer sequences for all genes evaluated via qRT-PCR.

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<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tr>
<td>18S</td>
<td>5'-GCG ATG CGG CGG CGT TAT-3'</td>
<td>5'-AGA CTT TGG TTT CCC GGA AGC-3'</td>
</tr>
<tr>
<td>B-actin</td>
<td>5'-ACG AGG CCC AGA GCA AGA-3'</td>
<td>5'-TTG GTT ACA ATG CCG TGT TCA-3'</td>
</tr>
<tr>
<td>CLDN1</td>
<td>5'-CGT GAC TGC TCA GGC CAT CT-3'</td>
<td>5'-CGG TGC TTT GCG AAA CG-3'</td>
</tr>
<tr>
<td>CLDN3</td>
<td>5'-GAC CAC CCC ACC 'TTC CAG AT-3'</td>
<td>5'-CTG TCC TCT TCC AGC CTA GCA-3'</td>
</tr>
<tr>
<td>GLUT2</td>
<td>5'CTG TCT GTG TCC AGC TTT GCA-3'</td>
<td>5'-CA A GCC ACC CAC CAA AGA AC-3'</td>
</tr>
<tr>
<td>GPR41</td>
<td>5'-GCT TGT GTG CCT TGG ACT CA-3'</td>
<td>5'-TGG TTC TCC TCC GTT CTT TAC CT-3'</td>
</tr>
<tr>
<td>GPR43</td>
<td>5'-TCG TGG AAG CTG CAT CCA-3'</td>
<td>5'-GCG CGC ACA CTA GCT TT-3'</td>
</tr>
<tr>
<td>HPRT</td>
<td>5'-GCA GTA CAG CCC CAA AAT GG-3'</td>
<td>5'-GGT CCT TTT CAC CAG CAA GCT-3'</td>
</tr>
<tr>
<td>HTR 1a</td>
<td>5'-CTC TGT TGC TGG GTA CTC TCA TT-3'</td>
<td>5'-ACT TGT TGA GCA CCT GGT ACA GA-3'</td>
</tr>
<tr>
<td>HTR 1b</td>
<td>5'-CTT TCT ATT TAC CCA CCC TGC TC-3'</td>
<td>5'-GTC TGA GAC TCG CAC TTT GAC TT-3'</td>
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<tr>
<td>HTR 1d</td>
<td>5'-CCC GGA GTC GAA TCC TGA A-3'</td>
<td>5'-TGA TAA GCT GTG CTG TGG TGA A-3'</td>
</tr>
<tr>
<td>HTR 2a</td>
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<td>5'-CCC CTC CTT AAA GAC CTT CG-3'</td>
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<tr>
<td>HTR 2b</td>
<td>5'-TGG CAG TTT CAT GCT CTT TG-3'</td>
<td>5'-TTC CCT TTG GAG AAC TGT GG-3'</td>
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<tr>
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<td>5'-CCC CCC GTT GGT TGA TG-3'</td>
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<td>5'-GAG ACC AAA GCA GCC AAG AC-3'</td>
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<td>5'-CTA CAG GAG GTG CCA CAG ATA AAG-3'</td>
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<td>IL10</td>
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<tr>
<td>IL6</td>
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<tr>
<td>MAO</td>
<td>5'-TGG GTT GAA GAA CCC GAG TC-3'</td>
<td>5'-TGA TCT TGA GCA GAC CAG GC-3'</td>
</tr>
<tr>
<td>MCP1</td>
<td>5'-CGG TTT CTC CCT TCT ACT TCC TG-3'</td>
<td>5'-GCT CTG CCT CAG CCT TTT ATT G-3'</td>
</tr>
<tr>
<td>OCLN</td>
<td>5'-GAG AGA TGC ACG TTC GAC CAA-3'</td>
<td>5'-GAA TTT CGT CTT CCG GGT AAA A-3'</td>
</tr>
<tr>
<td>Pet-1</td>
<td>5'-CCC TGC TGA TCA ACA TGT ACC-3'</td>
<td>5'-GCC AGC AGC TCC AGT AGA AA-3'</td>
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<tr>
<td>SERT</td>
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<td>5'-GGA CGA CAT CCC TAT GCA GT-3'</td>
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<td>---------------</td>
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<tr>
<td>SGLT1</td>
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<td>5'-AGT GGA CCC CGC AGA TGA T-3'</td>
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<td>(SLC5A1)</td>
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<td>TNFα</td>
<td>5'-CCC AGA AAA GCA AGC AAC CA-3'</td>
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<td>Tph1</td>
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<td>5'-AGA CAT CCT GGA AGC TTG TGA-3'</td>
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<td>ZO-1</td>
<td>5'-GCT CAC CAG GGT CAA AAT GTT T-3'</td>
<td>5'-AGT GTC ATT CAC ATC CTT CT TGT CT-3'</td>
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</tbody>
</table>
References


16. CDC, *Preventing and Managing Chronic Disease to Improve the Health of Women and Infants*. 2011, Division of Reproductive Health, National Center for Chronic Disease Prevention and Health Promotion.


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