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

HARRIET LAW, B.Sc.

A thesis submitted to the School of Graduate Studies in Partial Fulfilment of the requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2014) Hamilton,
Ontario (Medical Sciences)

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

AUTHOR: Harriet Law, B.Sc. (McMaster University)

Supervisor: Dr. Alison C. Holloway

NUMBER OF PAGES: xiii,147

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 SSRI 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.

ACKNOLWEDGEMENTS

First and foremost, I would like to take this opportunity to thank my supervisor, Dr. Alison Holloway. There are no words to describe how exceptional you are. As a mentor, you clearly saw my potential. This was not confined to the walls of academia; it spilled outwards, starting with my introduction to CrossFit (have you seen the WOD?!). My journeys in both CrossFit and graduate school began in parallel, but they have become progressively integrated as my confidence grew. You were the teacher that treated me like an adult and talked to me like an equal, making me feel like my opinion mattered. I feel extremely fortunate and privileged to have joined your lab. Thank you for making not only making this experience about the science, but one that opened my eyes to life itself and taking risks. For your unyielding support, honesty and encouragement, I am truly grateful.

To my lab mates past and present and friends that I have made, Nicole De Long ("D"), Taylor VanDuzer, Nicole Barra ("B"), Caroline Moore, Chandni Pattni, Marie Gutgesell, Catherine Nicholson, Robyn Pereira (Raha lab), Kaitlyn Chan (Sloboda lab), Wajiha Gohir (Sloboda lab) and Jocelyn Wessels (Foster lab), you all accepted me for me and never failed to put a smile on my face every day. I have learned incredible things from each and every one of you. Lunch time and "coffee breaks" will never be the same. Furthermore, I would like to thank "D" for generating the animal model used in this study and "B" for helping with the editing of my thesis.

I would like to acknowledge the feedback and guidance from my committee members, Drs Katherine Morrison and Michael Surette. Beginning with Dr. Morrison, who planted the seed during my fourth year senior thesis; thank you for providing me with the opportunity to gain experience in a field that solidified my catalyst to pursue graduate school. Dr. Surette, thank you for your expertise; and for graciously opening up your lab up to me to complete the gut microbiota sequencing portion of my project. I would also like to thank all the members of the Surette lab- especially Jennifer Lau, Laura Rossi, Michelle Shah and Fiona Whelan- for welcoming me with open arms and providing me with assistance whenever I needed it. Jennifer, thank you for seeing me through what has arguably been the most challenging aspect of my project; I really appreciate all of your guidance and patience throughout these past couple of years.

To my parents, who have been my pillars of support through all of my life choices, I would not be the person I am today without you. You have taught me the definition of working smart, perseverance and discipline. Rosalind, even though we were apart you unflinchingly rode the waves with me even in the face of my occasional irrational state through this graduate experience.

TABLE OF CONTENTS

LISTS OF TABLES

LIST OF FIGURES

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 ACHEIVEMENT

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

[65, 66], 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-hydroxytrptamine; 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 liming 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-HT$_{1A/1B/2A/2C}$*, 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-HT$_{2A/2C}$* 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-HT$_{2B}$* 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-hydroxyindoleacedic 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 *SERT* [294-296]. Similar results have been found in human studies. Indeed, IBS-D and IBS-C patients show a decrease in rectal *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 *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, *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 *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].

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-HT$_{2A}$* and *5-HT$_3$* receptors results in a 5-HT-induced increase in regulators in inflammation, prostaglandin E2 (PGE2) expression [324]. In vivo, the *5-HT$_3$* receptor appears to be a predominant mediator of inflammation and immune responses [325, 326]. Administration of 5-HT$_3$ receptor antagonists has been shown to lead to the inhibition of inflammatory cytokine production in colitis rat models [327-329]. Similarly, the *5-HT$_7$* receptor has recently been shown to have pro-inflammatory effects [330]. Kim and colleagues [330] reported that inhibition of *5-HT$_7$* 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-HT$_{7-/-}$* 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-micobiota 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 *Bifidocbacterium 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 $\Delta G < -9$ 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*)

2.2 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 $\Delta G < -9$ 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 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 $\Delta G < -9$ 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 $NaPO_4$ (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 H_2O), 50 µL of mutanolysin (10 U/µl) and 10 µL of RNase A (10 mg/mL in H_2O). 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 certrifuged 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 ($MgCl_2$) (50 mM) solution, 6 µL 10 x PCR buffer, 1 uL dNTPs (10 mM each), 34.25 uL dH_2O, 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.

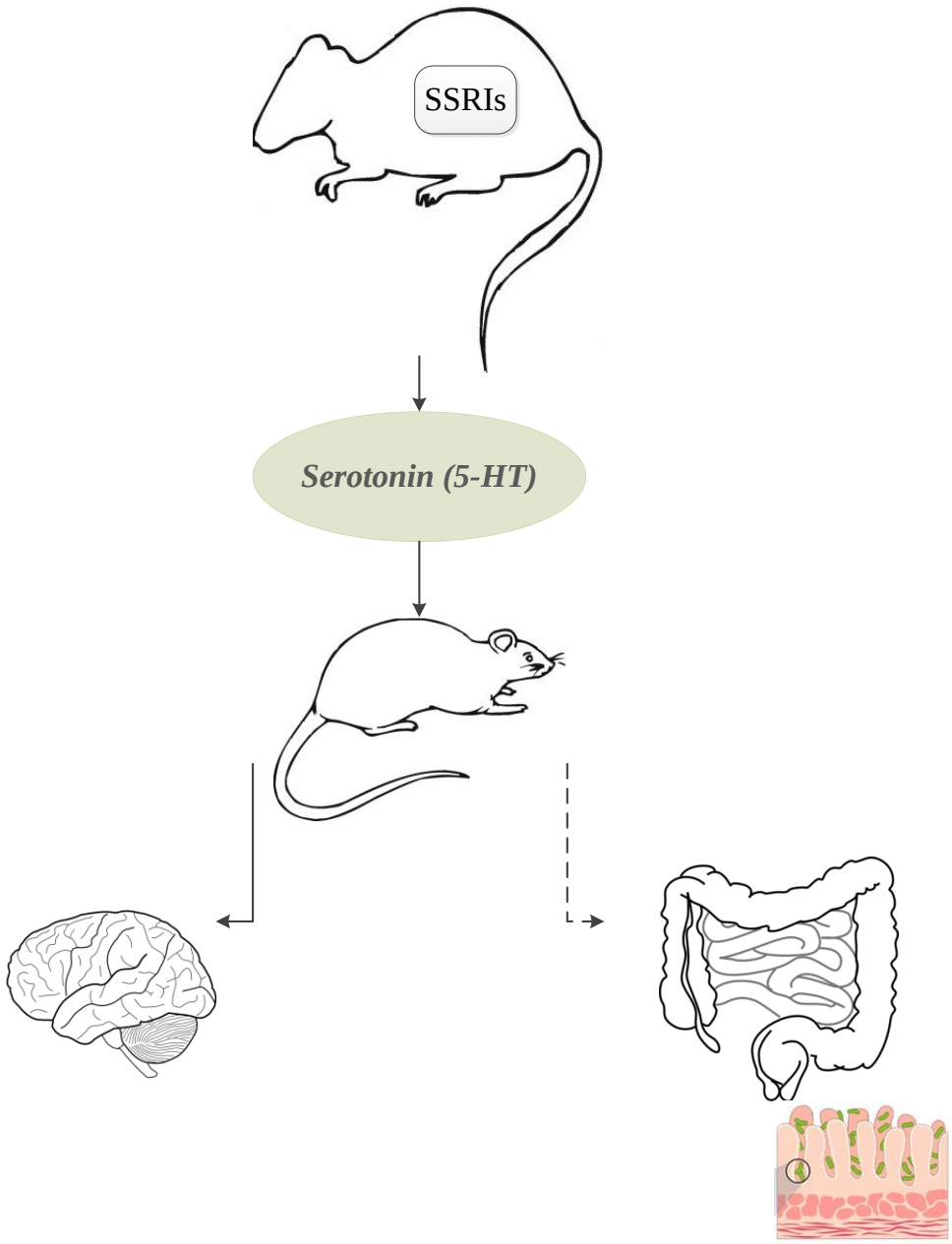

Figure 1 Study aims. Evidence from animal studies has shown that perinatal exposure to selective serotonin reuptake inhibitors (SSRIs) can disrupt the development of the central serotonergic system resulting in alterations in brain 5-HT content, peripheral 5-HT levels, 5-HT receptor density and serotonin 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.

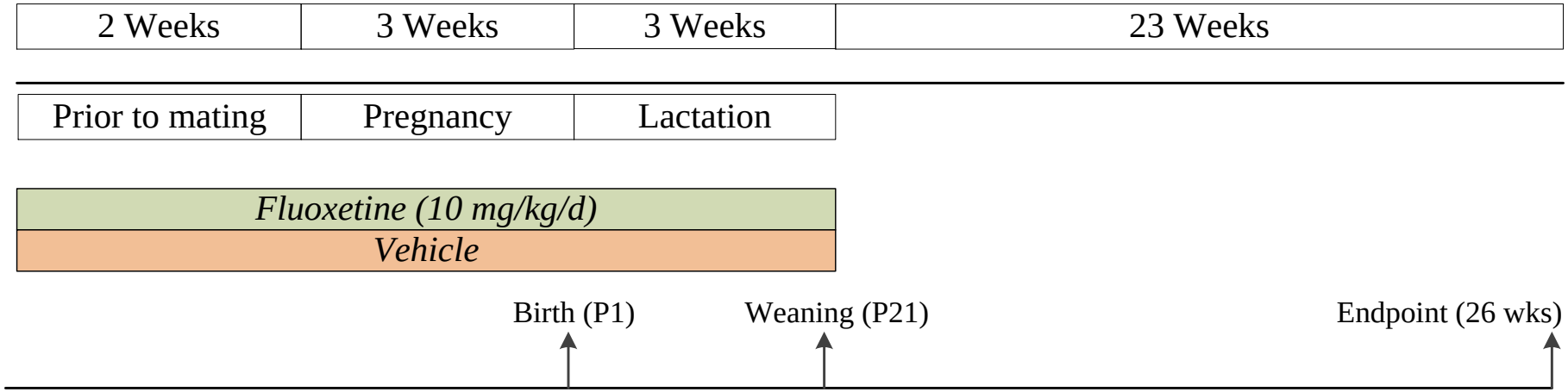

Figure 2 Production of the animal model. Nulliparous female Wistar rats were randomized to receive vehicle or fluoxetine hydrochloride (10 mg/kg/d), Toronto Research Chemicals, North York, ON) daily by oral administration 2 weeks prior to mating until weaning (postnatal day 21; PND 21). Outcome measures 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.

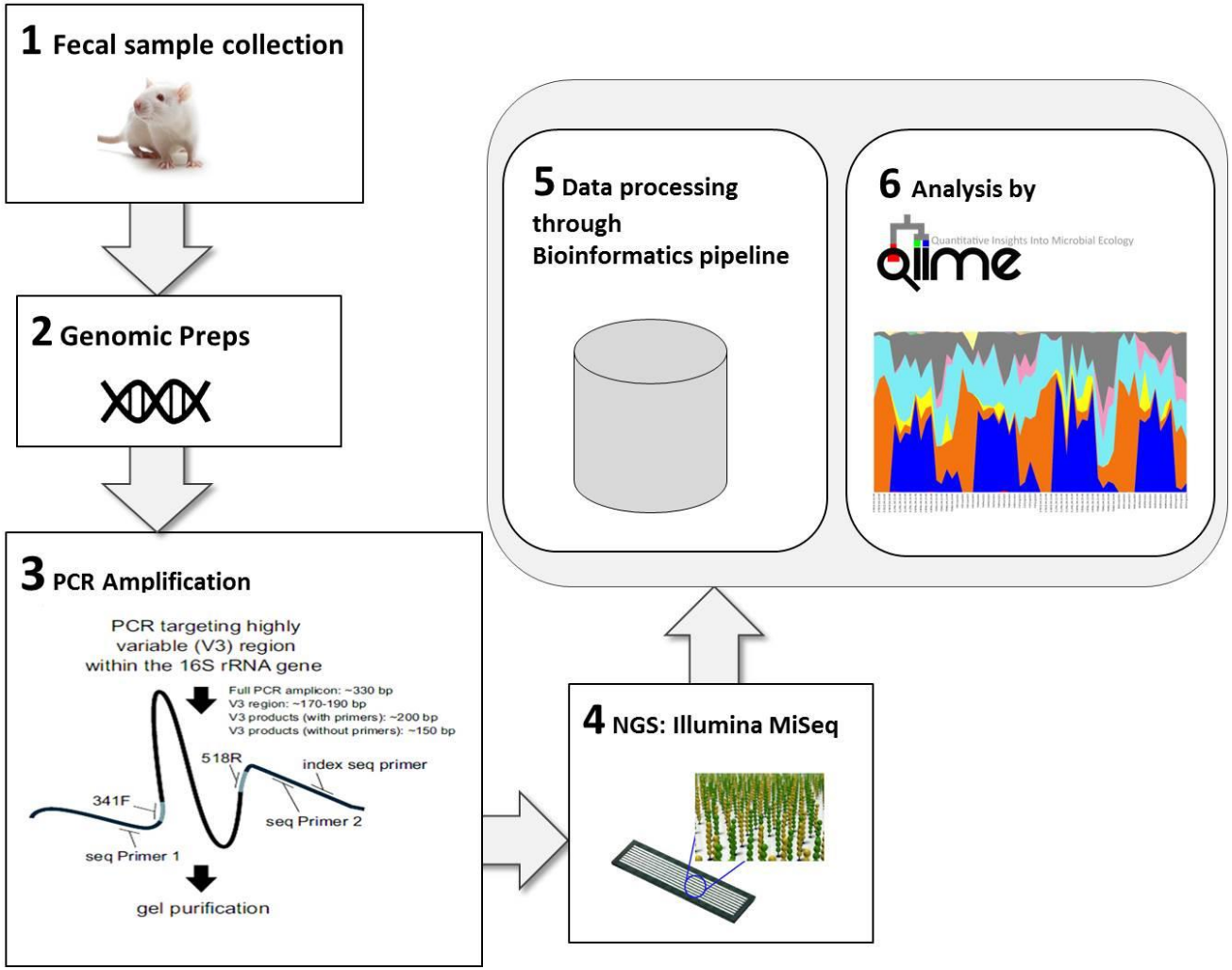

Figure 3 Overview of methods involved in phylogenetic identification and detection of microbial groups or species for Specific Aim #3. DNA was extracted from fecal samples and amplicons of the V3 hypervariable region from the16S rRNA were made by PCR for Illumina sequencing. Data analysis occurred by 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 *5HT$_{2A}$* 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-HT$_{1a}$, 5-HT$_{1b}$, 5-HT$_{1d}$, 5-HT$_{2b}$, 5-HT$_{3a}$, 5-HT$_{3b}$, 5-HT$_{4}$, and 5-HT$_{7}$*) 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-HT$_{1a}$, 5-HT$_{1b}$, 5-HT$_{1d}$, 5-HT$_{2a}$, 5-HT$_{2b}$, 5-HT$_{3a}$, 5-HT$_{3b}$, 5-HT$_{4}$, and 5-HT$_{7}$*) 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, *MCP*1 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 *Bifidocbacterium 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 *CLND1, 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 Bacteoridetes) 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.

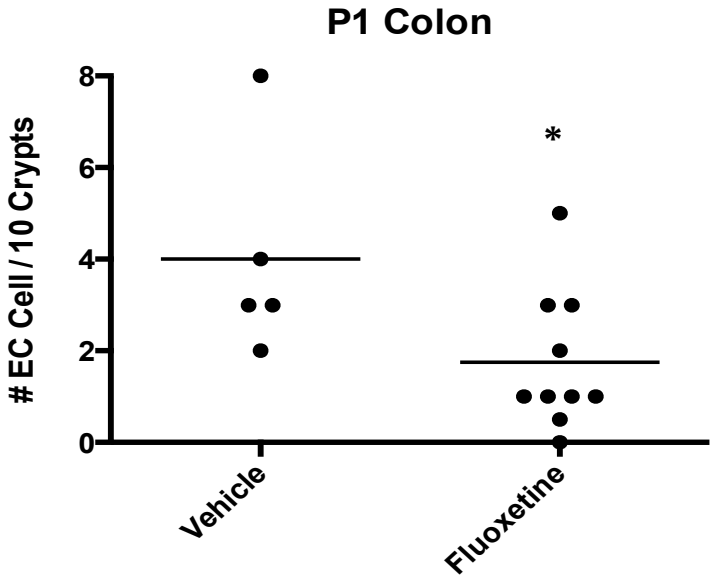

Figure 4 At postnatal day 1 (PND1), there was a significant decrease in colonic enterochromaffin (EC) cell number in the fluoxetine-exposed group compared to controls (P=0.041). Pups were sacrificeed at PND1 and colon was collected for immunohistochemistry. EC cells were labelled using polyclonal rabbit anti-rat antibody directed against 5-HT (1:5000 dilution; Immunostar); and goat anti-rabbit Alexa Fluor 488 (1:200 dilution; Molecular Probe).

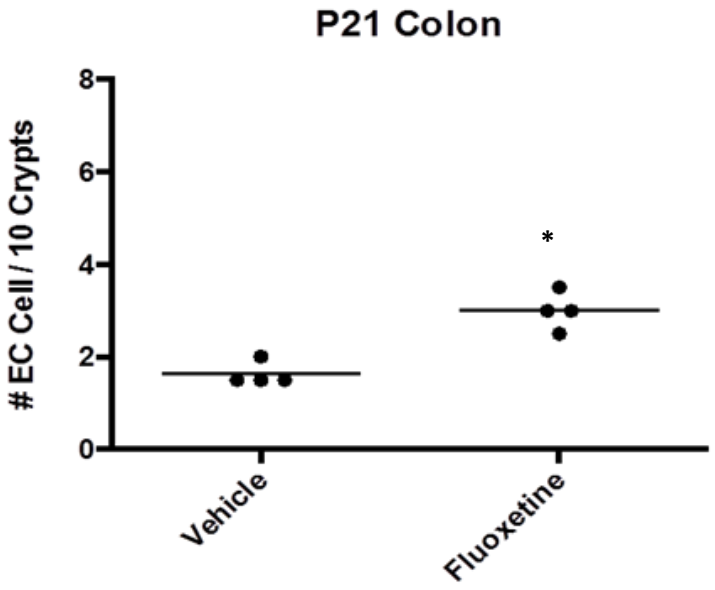

Figure 5 By weaning (Postnatal day 21; P21), fluoxetine exposure led to a significant increase in enterochromaffin (EC) cell number (P= 0.0012). Pups were sacrificed at P21 (weaning) colon was collected for immunohistochemistry. EC cells were labelled using polyclonal rabbit anti-rat antibody directed against 5-HT (1:5000 dilution; Immunostar); and goat anti-rabbit Alexa Fluor 488 (1:200 dilution; Molecular Probe).

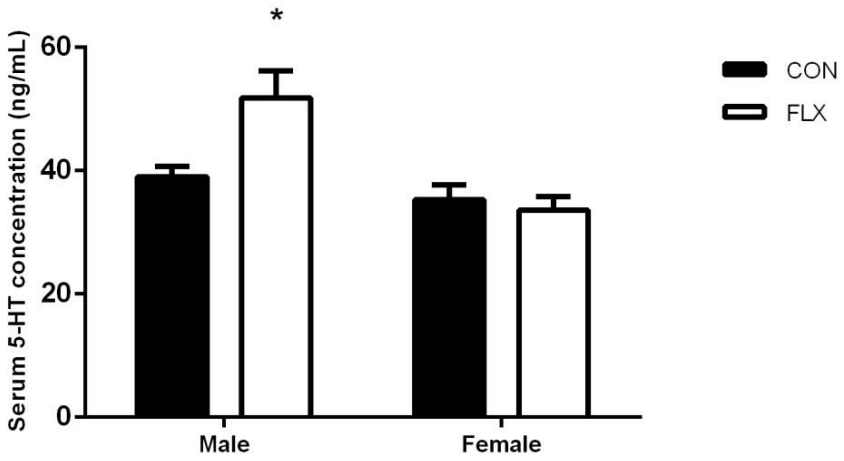

Figure 6 In adulthood (26 weeks), serum 5-HT levels were significantly elevated in treated male (mean ± SEM; 51.8 ± 4.41 vs. 39.0 ± 1.64; p= 0.005) but not female offspring (35.3 ± 2.44 vs. 33.6 ± 2.21; p= 0.616). Serum 5-HT levels were determined by blood samples collected using a commercially available rat ELISA kit.

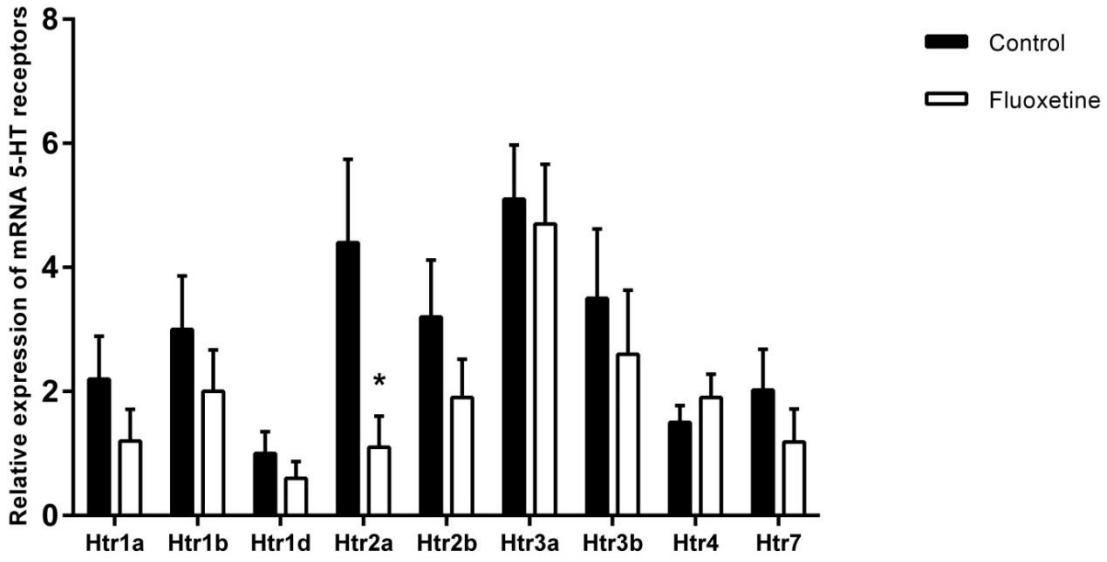

Figure 7 Evaluation of colonic serotonergic genes in male offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included serotonin receptors. Transcripts encoding 5HT2A receptor (mean ± SEM; 1.1±0.50 vs. 4.4±1.34; p-value= 0.009) expression were significantly 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.

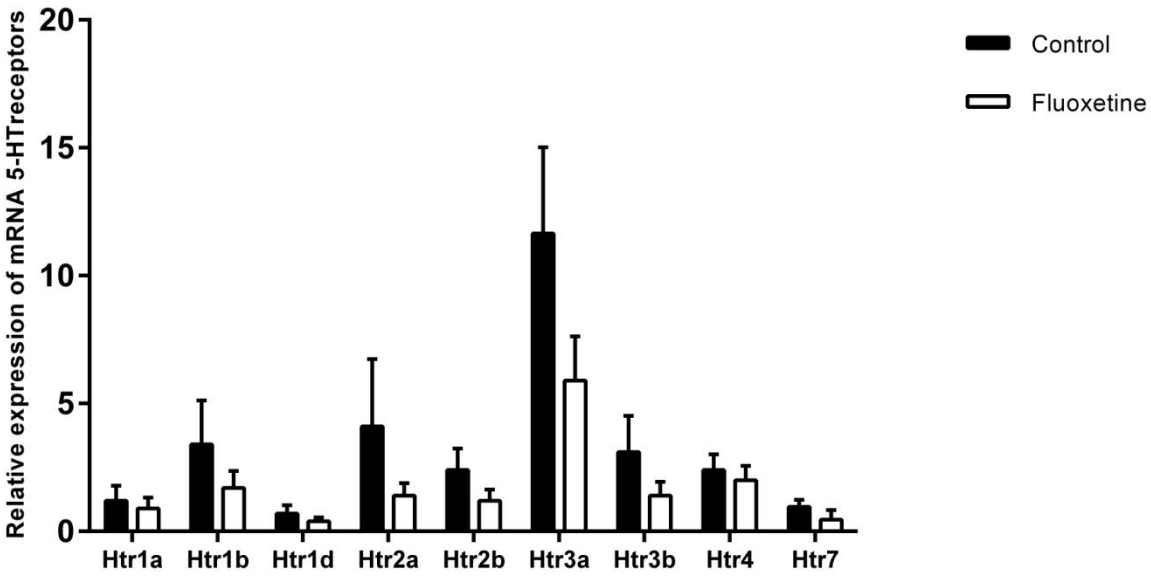

Figure 8 Evaluation of colonic serotonergic genes in female offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included serotonin receptors. Transcripts encoding receptor subtypes (*Htr1a, Htr1b, Htr1d, Htr2a, Htr2b, Htr3a, Htr3b, Htr4, and Htr7)* remained unchanged between treatment groups. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.

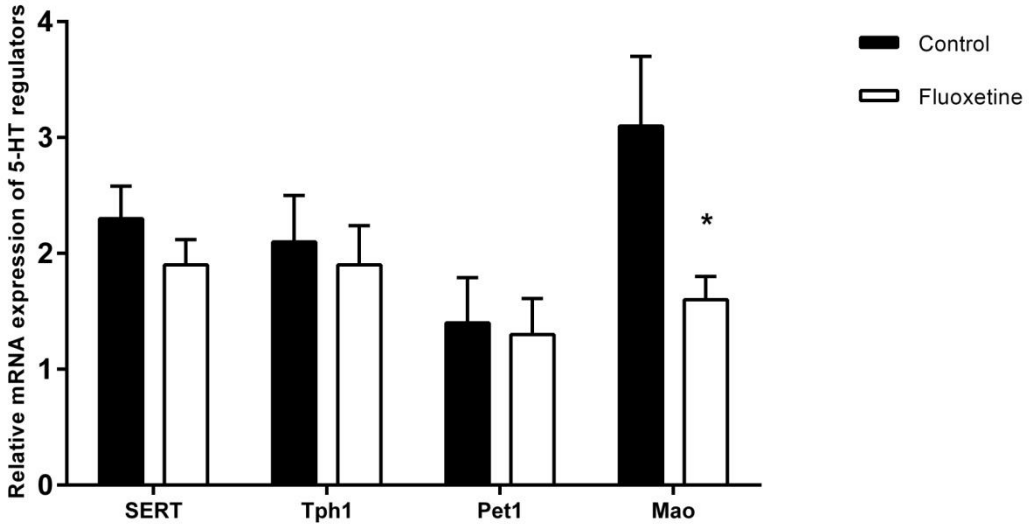

Figure 9 Evaluation of colonic serotonergic genes in male offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included the plasma membrane serotonin transporter (*SERT*); rate-limiting enzyme in 5-HT synthesis (*Tph1*); transcription factor *PET1*, and Monoamine oxidase-A (*Mao A*; enzyme involved in 5-HT degradation). Transcripts encoding *Mao* (1.6 ± .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.

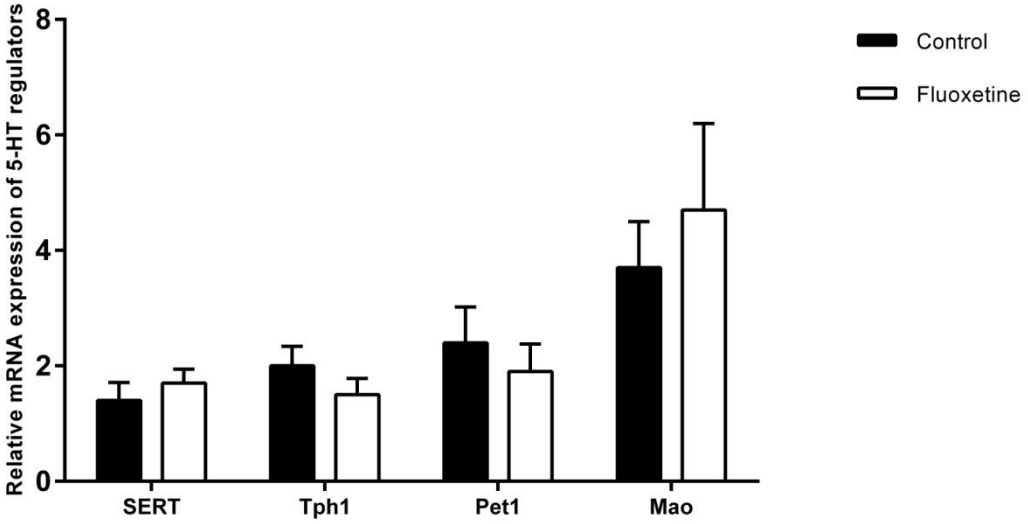

Figure 10 Evaluation of colonic serotonergic genes in female offspring at 26 weeks was done by quantitative real-time PCR (qPCR). Gene targets for this analysis included the plasma membrane serotonin transporter (*SERT*); rate-limiting enzyme in 5-HT synthesis (*Tph1*); transcription factor *PET1*, and 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 (estrus and diestrus) can be found in.

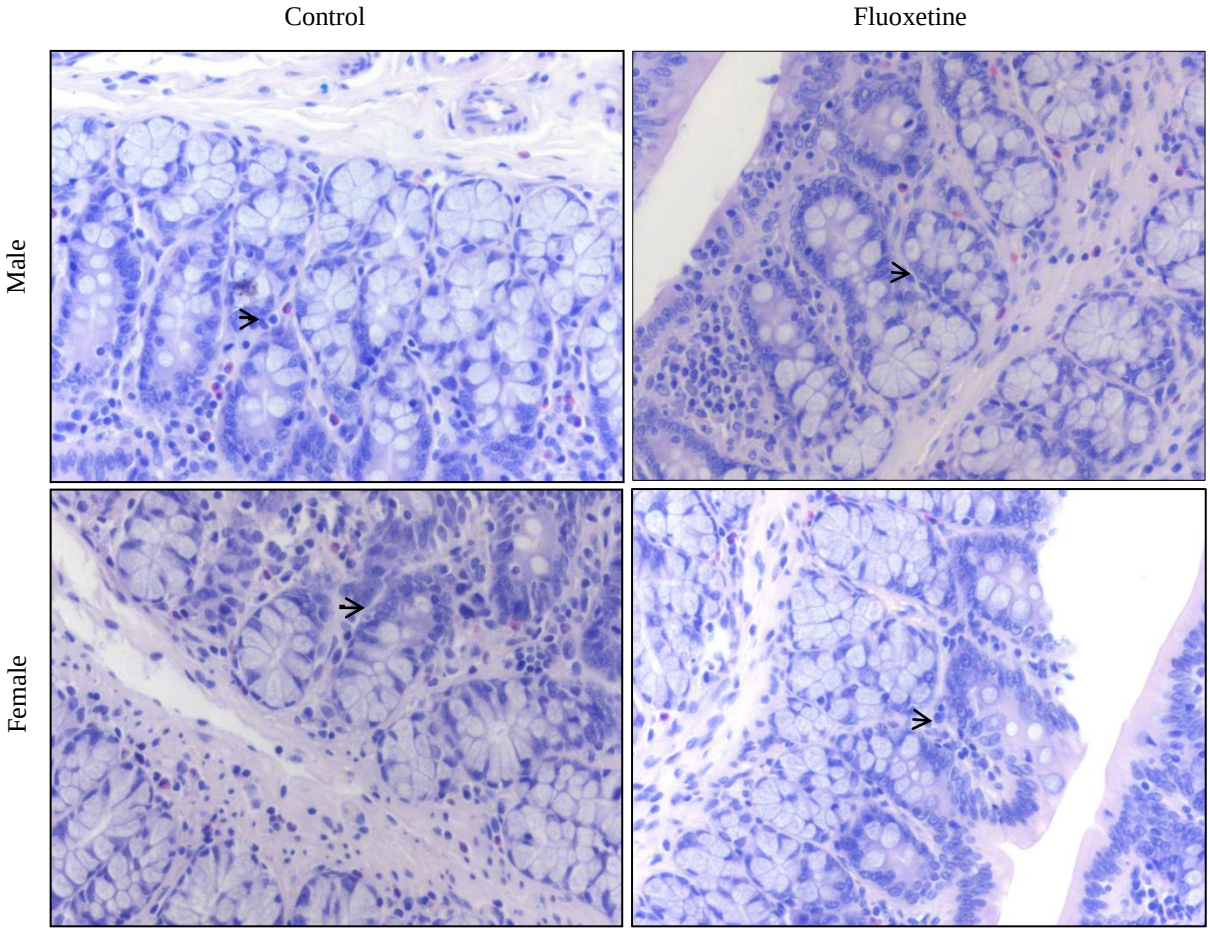

Figure 11 Hematoxylin- and eosin-stained histological sections of the colon taken from control and fluoxetine exposed offspring at 26 weeks of age. Top left, control male; Top right, Fluoxetine Male; Bottom left, control female; Bottom right, Fluoxetine female. Arrows indicate cellular infiltration.

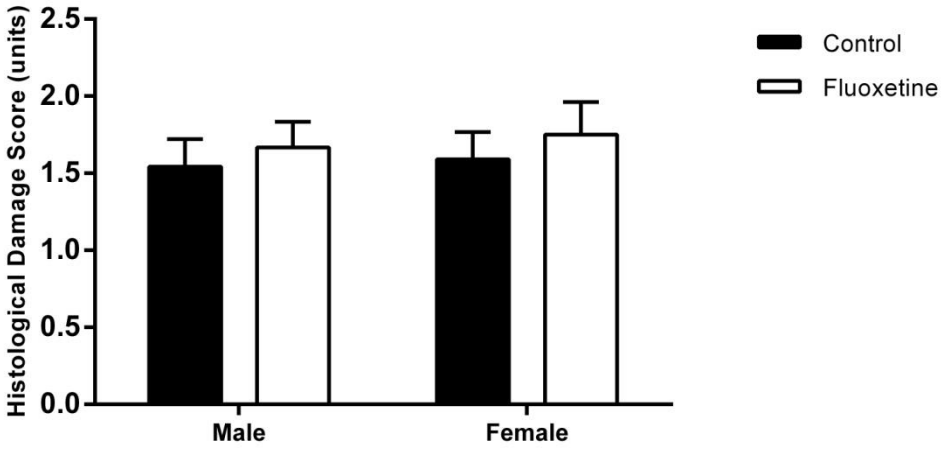

Figure 12 Fetal and neonatal exposure to fluoxetine does not increase damage in the colon postnatally. Microscopic criteria for damage and inflammation were investigated by light microscopy on hematoxylin- and eosin-stained histological sections of the colon taken from control- and SSRI-exposed offspring 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).

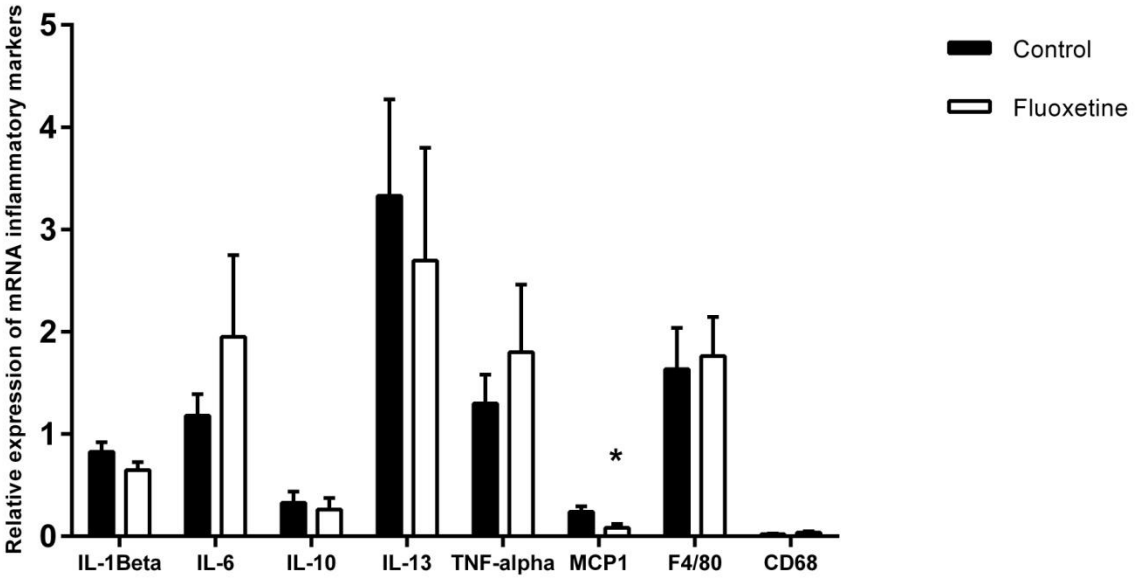

Figure 13 Evaluation of colonic inflammation (Anti- and pro- inflammatory cytokines: IL-10 (Interleukin), TNF-α (tumour necrosis factor α), IL1B, IL6, and IL13; MCP 1 (monocyte chemoattractant protein 1); cluster of differentiation 68, CD68; F4/80, (Epidermal Growth Factor) in male offspring at 26 weeks 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.

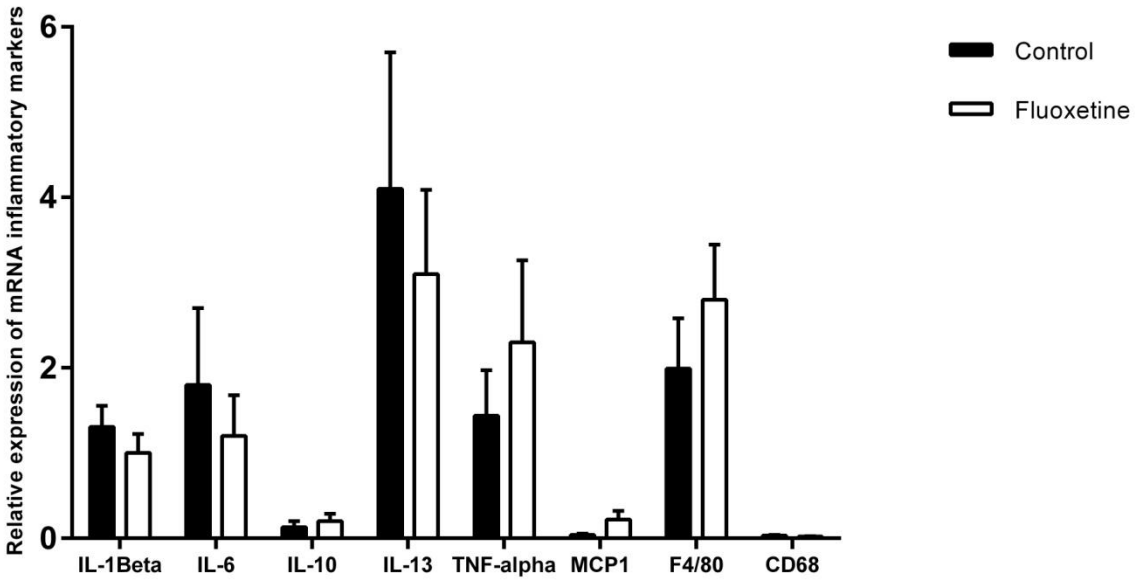

Figure 14 Evaluation of colonic inflammation in female offspring at 26 weeks by quantitative real-time PCR (qPCR). Fetal and neonatal exposure to fluoxetine does not increase colonic inflammation postnatally in adult females. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S. 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)

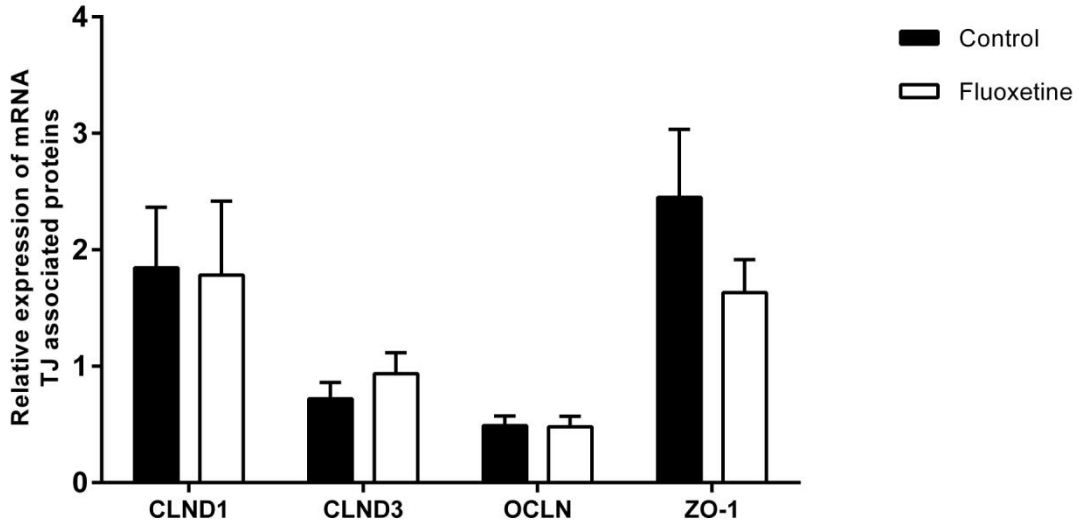

Figure 15 Evaluation of tight junction-associated proteins (claudin-1, CLND1/3; Occludin, OCLN; Zona Occludin-1, ZO-1) in male offspring at 26 weeks by quantitative real-time PCR (qPCR). Fetal and neonatal exposure to fluoxetine does not alter markers of gut permeability in adult males. Comparisons between treatment groups were done by independent samples t-test. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.

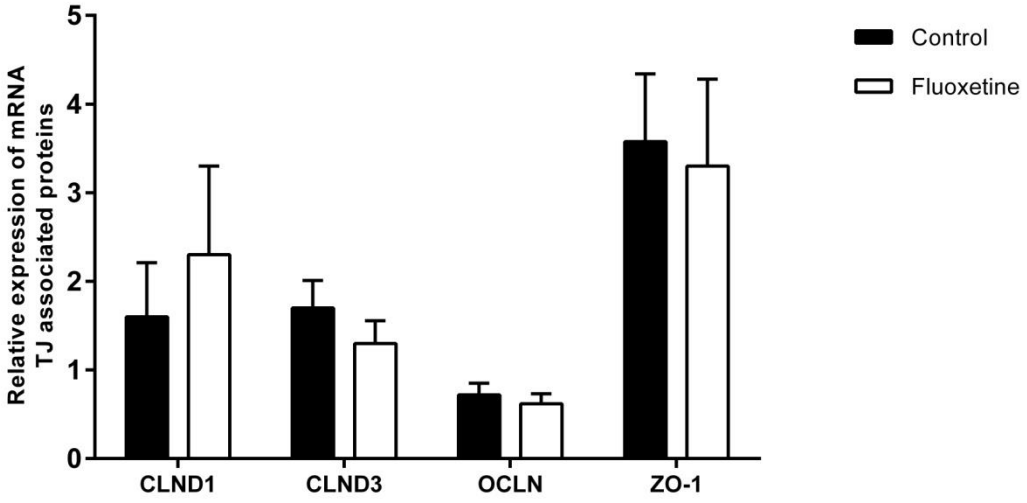

Figure 16 Evaluation of tight junction-associated proteins (claudin-1, CLND1/3; Occludin, OCLN; Zona Occludin-1, ZO-1) in female offspring at 26 weeks by quantitative real-time PCR (qPCR). Fetal and neonatal exposure to fluoxetine does not alter markers of gut permeability in adult males. Comparisons between treatment groups were done by independent samples t-test. Expression level is relative to housekeeping genes β-Actin, HPRT, and 18S.

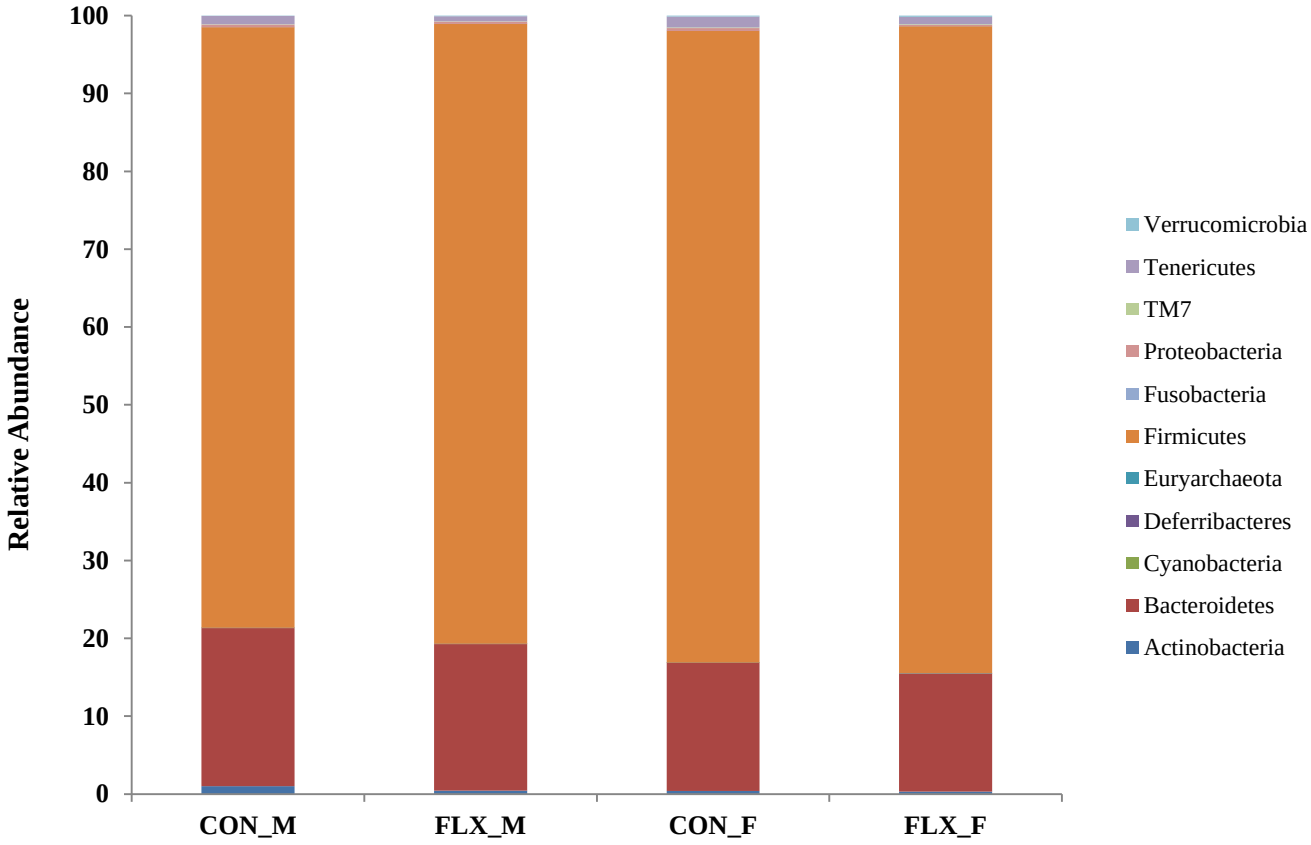

Figure 17 Percent relative abundance of bacteria at the phyla level in control and fluoxetine-exposed offspring in adulthood. This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age.

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.

Phyla	Male (mean±SEM)		P-value	Female (mean±SEM)		P-value
	CON	FLX		CON	FLX	
Actinobacteria	0.330 ± 0.067	0.442 ± 0.112	.383	0.442 ± 0.0777	0.248 ± 0.0247	.166
Bacteroidetes	20.3 ± 2.85	20.0 ± 5.33	.961	16.9 ± 2.21	14.3 ± 2.17	.439
Cyanobacteria	$1.07 \times 10^{-2} \pm 2.89 \times 10^{-3}$	$9.60 \times 10^{-3} \pm 2.71 \times 10^{-3}$	.734	$3.94 \times 10^{-3} \pm 9.71 \times 10^{-4}$	$6.85 \times 10^{-3} \pm 1.78 \times 10^{-3}$	.142
Deferribacteres	$7.67 \times 10^{-3} \pm 2.93 \times 10^{-3}$	$0.0176 \pm 5.65 \times 10^{-3}$	.112	$5.24 \times 10^{-3} \pm 1.80 \times 10^{-3}$	$2.22 \times 10^{-3} \pm 7.82 \times 10^{-4}$	.237
Firmicutes	76.8 ± 2.85	78.5 ± 5.35	.764	80.6 ± 2.37	83.9 ± 2.12	.338
Fusobacteria	$8.76 \times 10^{-4} \pm 5.81 \times 10^{-5}$	$4.31 \times 10^{-4} \pm 1.45 \times 10^{-4}$	.043	$3.91 \times 10^{-4} \pm 1.64 \times 10^{-4}$	0 ± 0	.116
Proteobacteria	0.3480 ± 0.0402	0.258 ± 0.0417	.143	0.266 ± 0.0767	0.18227 ± 0.0267	.059
TM7	$0.0383 \pm 8.74 \times 10^{-3}$	$0.0277 \pm 9.41 \times 10^{-3}$	.432	0.0717 ± 0.0185	0.0759 ± .0218	.885
Tenericutes	1.03 ± 0.158	0.675 ± 0.142	.128	1.50 ± 0.313	0.961 ± 0.149	.199
Verrucomicrobia	0.0308 ± 0.0114	0.0205 ± 0.0113	.535	0.0167 ± 0.0105	0.139 ± 0.0466	.0120

*Bonferroni correction $P<0.005$

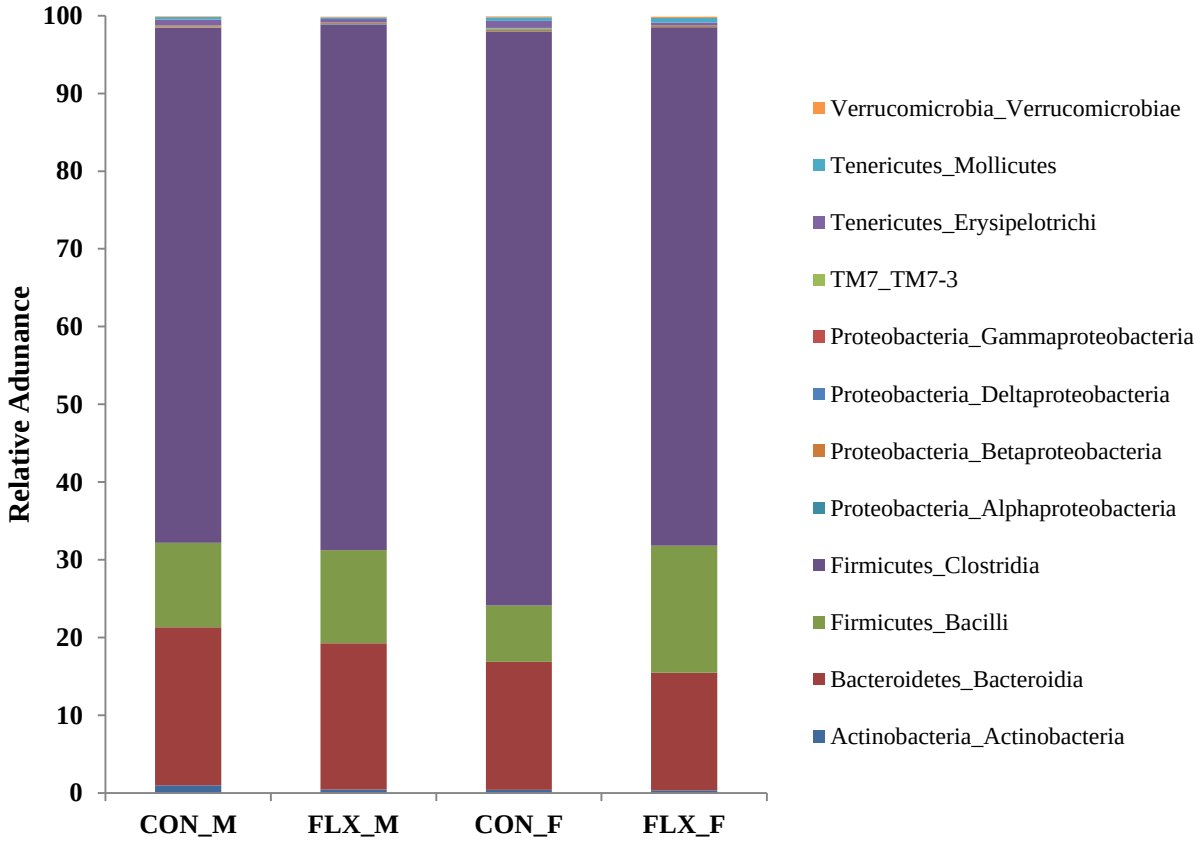

Figure 18 Percent relative abundance of bacteria at the class level in control and fluoxetine-exposed offspring in adulthood (for all representative sequences >0.01%). This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 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.

Class	Male (mean±SEM)		P-value	Female (mean±SEM)		P-value
	CON	FLX		CON	FLX	
Actinobacteria_Actinobacteria	0.330 ± 0.0671	0.442 ± 0.112	.383	0.399 ± 0.0777	0.248 ± 0.0247	.166
Bacteoridetes_						
Bacteroidia	20.2 ± 2.85	20.0 ± 5.33	.961	16.9 ± 2.21	14.3 ± 2.17	.439
Cyanobacteria_class						
4C0d-2	$4.89x10^{-3} \pm 2.25x10^{-3}$	$2.07 x10^{-3} \pm 8.17x10^{-3}$	.319	$1.78 x10^{-3} \pm 8.27 x10^{-4}$	$4.44 x10^{-3} \pm 1.97 x10^{-3}$	.185
Chloroplast	$6.79 x10^{-3} \pm 1.67 x10^{-3}$	$3.25 x10^{-3} \pm 9.15 x10^{-3}$	.124	$2.28 x10^{-3} \pm 4.40 x10^{-4}$	$2.40 x10^{-3} \pm 4.96 x10^{-4}$	.851
Deferribacteres_Deferribacteres	$7.67 x10^{-3} \pm 2.93 x10^{-3}$	0.0176 ± 0.00565	.112	$5.24 x10^{-3} \pm 1.80 x10^{-3}$	$2.23 x10^{-3} \pm 7.82 x10^{-4}$	.237
Firmicutes_class						
Bacilli	11.6 ± 2.53	12.5 ± 3.22	.825	6.62 ± 1.14	16.2 ± 2.54	.00200*
Clostridia	65.0 ± 3.85	65.8 ± 6.47	.915	72.2 ± 3.21	67.6 ± 3.31	.350
Fusobacteria	$8.76 x10^{-5} \pm 5.81 x10^{-5}$	$4.31 x10^{-4} \pm 1.45 x10^{-4}$	.0400	$3.92 x10^{-4} \pm 1.64 x10^{-4}$	0.00	.116
Proteobacteria_class						
Alpha-Proteobacteria	0.0269 ± 0.00833	0.0457 ± 0.00796	.133	.0564 ± .0139	.0302 ± .0120	.185
Beta-Proteobacteria	0.185 ± 0.0342	0.128 ± 0.0238	.215	.194 ± .0378	.112 ± .0135	.103
Delta-Proteobacteria	.0200 ± .00544	0.0161 ± .00220	.215	$.0216 \pm 5.07 x10^{-3}$	.0266 ± .00722	.564
Gamma-Proteobacteria	.0296 ± .00748	.0139 ±.00292	.0930	$.0215 \pm 6.70 x10^{-3}$	$6.68 x10^{-3} \pm 1.49 x10^{-3}$	.117
TM7_TM73	.0382 ± .00874	.0277 ± .00941	.432	.0941 ± .0280	.0759 ± .0218	.645
Tenericutes_class						
Erysipeltrichi	.703 ± .133	.466 ± .115	.212	1.01 ± .232	.366 ± .0717	.0420

Mollicutes	.295 ± .0930	0.153 ± .0219	.239	.461 ± .115	.596 ± .151	.487
Verrucomicrobia_Verrucomicrobiae	.0308 ± .0113	.0205 ± .0113	.535	$6.54 \times 10^{-3} \pm 3.06 \times 10^{-3}$	.132 ± .0530	.0170

*Bonferroni correction $P<0.003$

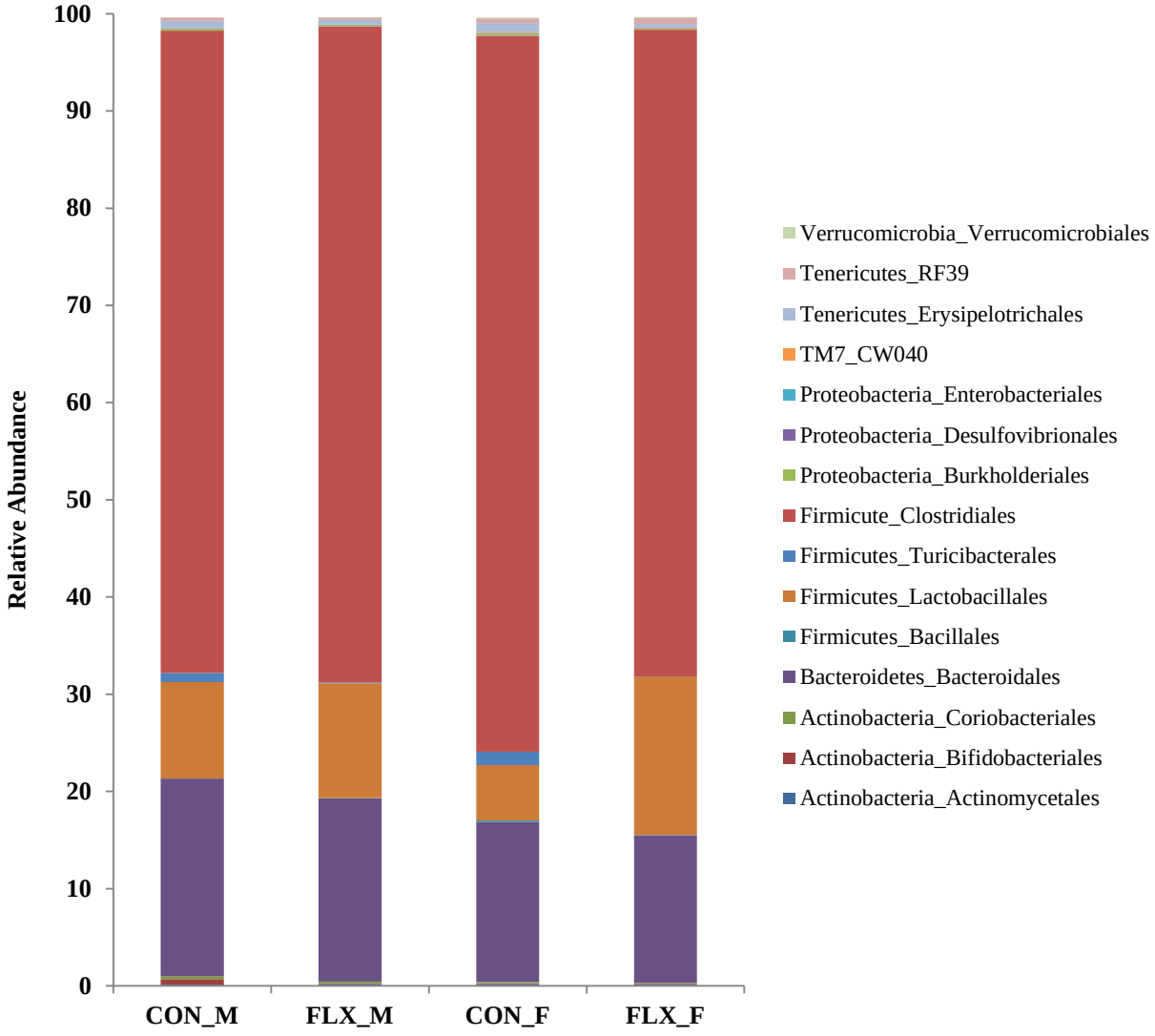

Figure 19 Percent relative abundance of bacteria at the level order in control and fluoxetine-exposed offspring in adulthood (for all representative sequences >0.01%). This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 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.

Order	Male (mean±SEM)		P-value	Female (mean±SEM)		P-value
	CON	FLX		CON	FLX	
Actinobacteria_order_						
Actinomycetales	.102 ± .0228	.191 ± .0491	.091	.167 ± .0276	.134 ± .0274	.413
Bifidobacteriales	.0475 ± .0174	.0103 ± .00614	.086	.0233 ± .0975	$2.31 \times 10^{-3} \pm 8.76 \times 10^{-4}$	.122
Coriobacteriales	.0647 ± .00821	.160 ± .0576	.121	.0943 ± .0172	.118 ± .0308	.483
Bacteroidetes_order_						
Bacteroidales	20.2 ± 2.85	20.0 ± 5.32	.961	16.9 ± 2.21	14.3 ± 2.17	.439
Cyanobacteria_YS2	$4.89 \times 10^{-3} \pm 2.25 \times 10^{-3}$	$2.07 \times 10^{-3} \pm 8.17 \times 10^{-4}$	.261	$1.78 \times 10^{-3} \pm 8.27 \times 10^{-4}$	$4.44 \times 10^{-3} \pm 1.97 \times 10^{-3}$	.185
Deferribacteres_order_						
Streptophyta	$6.79 \times 10^{-3} \pm 1.67 \times 10^{-3}$	$3.25 \times 10^{-3} \pm 9.15 \times 10^{-4}$	.081	$2.28 \times 10^{-3} \pm 4.40 \times 10^{-4}$	$2.40 \times 10^{-3} \pm 4.96 \times 10^{-4}$	.851
Deferribacterales	$7.67 \times 10^{-3} \pm 2.93 \times 10^{-3}$	.0176 ± .00565	.112	$5.24 \times 10^{-3} \pm 1.80 \times 10^{-3}$	$2.22 \times 10^{-3} \pm 7.82 \times 10^{-4}$	.237
Firmicutes_order_						
Bacillales	$7.38 \times 10^{-3} \pm 2.23 \times 10^{-3}$	.0375 ± .0162	.069	.0331 ± .0126	$7.93 \times 10^{-3} \pm 2.70 \times 10^{-3}$	.139
Lactobacillales	10.5 ± 2.54	12.3 ± 3.25	.647	5.35 ± .709	16.1 ± 2.54	<0.001
Turicibacterales	1.11 ± .331	.0180 ± .00636	.024	$.0106 \pm 3.60 \times 10^{-3}$	$7.38 \times 10^{-3} \pm 2.43 \times 10^{-3}$	.495
Clostridiales	64.9 ± 3.85	65.7 ± 6.45	.916	72.1 ± 3.21	67.5 ± 3.32	.349
Fusobacteria_fusobacteriales	$8.76 \times 10^{-5} \pm 5.81 \times 10^{-5}$	$4.31 \times 10^{-4} \pm 1.45 \times 10^{-4}$	.043	$3.91 \times 10^{-4} \pm 1.64 \times 10^{-4}$	0.00	.116
Proteobacteria_order_						
Rhizobiales	$3.14 \times 10^{-4} \pm 1.32 \times 10^{-4}$	<0.001	.080	$1.24 \times 10^{-4} \pm 6.59 \times 10^{-5}$	0.00	.191
Burkholderiales	.185 ± .0340	.128 ± .0238	.217	.194 ± .0378	.0136	.104

Neisseriales	$1.06 \times 10^{-4} \pm 5.44 \times 10^{-5}$	0.00	.102	$1.97 \times 10^{-4} \pm 9.03 \times 10^{-5}$	0.00	.149
Desulfovibrionales	$.0200 \pm 5.44 \times 10^{-3}$	$.016 \pm 2.20 \times 10^{-3}$	.543	$.0215 \pm 5.07 \times 10^{-3}$	$.0266 \pm 7.22 \times 10^{-3}$	.560
Enterobacteriales	$.0231 \pm 7.75 \times 10^{-3}$	$.0116 \pm 2.49 \times 10^{-3}$	.285	$.0184 \pm 6.43 \times 10^{-3}$	$4.44 \times 10^{-3} \pm 1.65 \times 10^{-3}$	.124
Pasteurellales	$5.31 \times 10^{-3} \pm 1.41 \times 10^{-3}$	$3.13 \times 10^{-3} \pm 1.24 \times 10^{-3}$	.285	$1.74 \times 10^{-3} \pm 3.09 \times 10^{-4}$	$1.13 \times 10^{-3} \pm 3.52 \times 10^{-4}$	.219
Pseudomonadales	$4.46 \times 10^{-4} \pm 1.53 \times 10^{-4}$	$3.03 \times 10^{-4} \pm 1.82 \times 10^{-4}$	.555	$6.83 \times 10^{-4} \pm 3.07 \times 10^{-4}$	$7.49 \times 10^{-4} \pm 1.81 \times 10^{-4}$	.870
TM7_CW040	$.0381 \pm 8.80 \times 10^{-3}$	$.0276 \pm 9.36 \times 10^{-3}$	.438	$.0941 \pm .0280$	$.0759 \pm .0218$	.645
Tenericutes _order_						
Eryspeltrichales	$.703 \pm .133$	$.466 \pm .115$	.212	$1.01 \pm .017$	$.366 \pm .0717$	.0428
Anaeroplasmatales	$2.16 \times 10^{-3} \pm 9.44 \times 10^{-4}$	$1.26 \times 10^{-3} \pm 5.15 \times 10^{-4}$	.489	$3.52 \times 10^{-3} \pm 1.06 \times 10^{-3}$	$6.16 \times 10^{-4} \pm 2.84 \times 10^{-4}$	.152
Mycoplasmatales	$3.49 \times 10^{-5} \pm 3.49 \times 10^{-4}$	$1.82 \times 10^{-4} \pm 1.22 \times 10^{-4}$	.216	$3.91 \times 10^{-5} \pm 3.91 \times 10^{-5}$	$4.46 \times 10^{-3} \pm 2.68 \times 10^{-3}$	.132
Verrucomicrobia_ Verrucomicrobiales	$.0308 \pm .0114$	$.0205 \pm .0113$	.535	$6.54 \times 10^{-3} \pm 3.06 \times 10^{-3}$	$.139 \pm .0465$	.008

*Bonferroni correction $P<0.002$

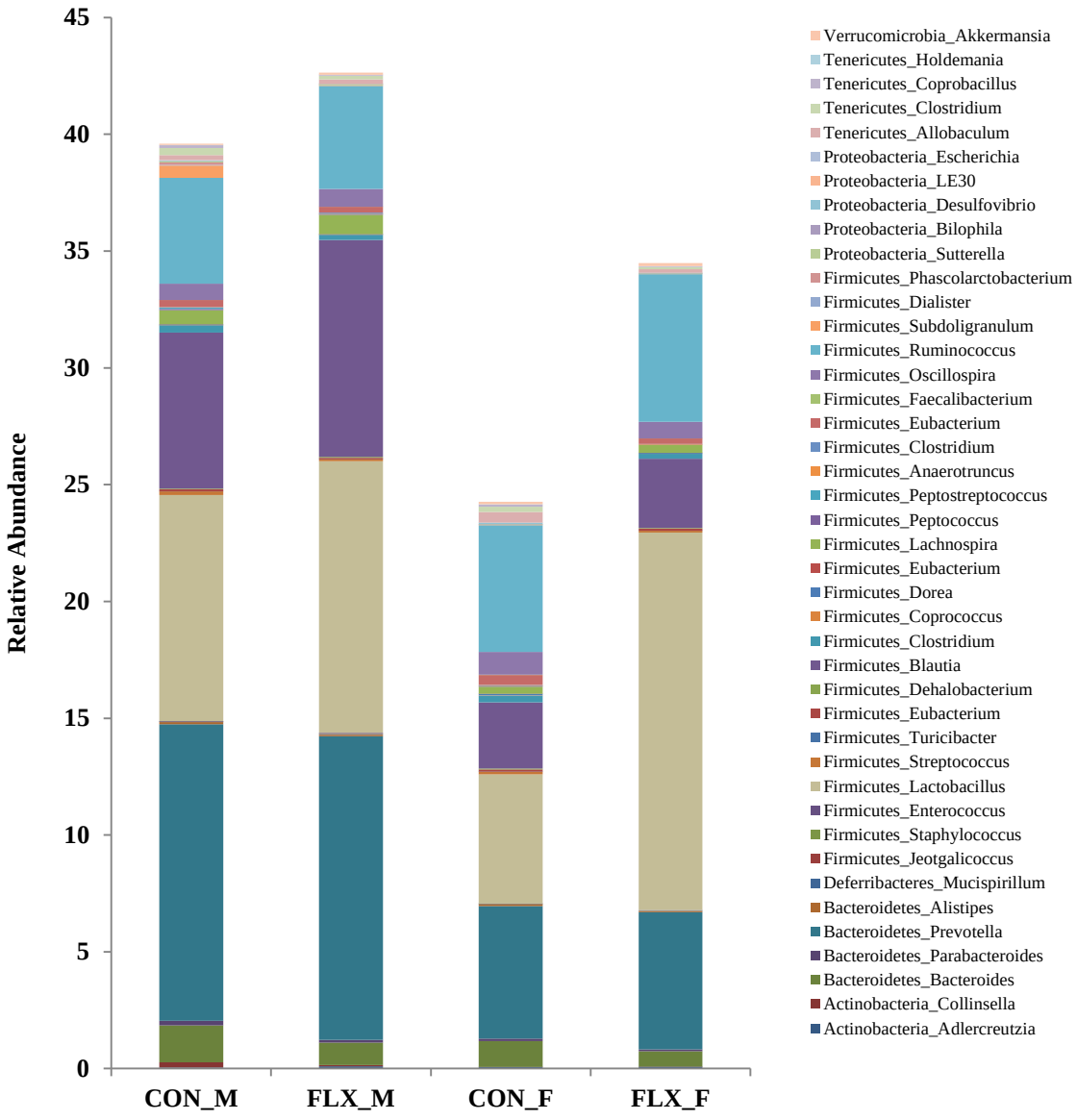

Figure 20 Percent relative abundance of bacteria at the genus level in control and fluoxetine-exposed offspring in adulthood (for all representative sequences >0.01%). This was done by bacterial profiling of 16S rRNA genes using Illumina Miseq Sequencing from fecal samples taken from offspring at 24 weeks of age. Legend is presented as Phyla_Genus.

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 e 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 10% of all samples and (3) have non-zero abundance.

Genus	Male (mean±SEM)		P-value	Female (mean±SEM)		P-value
	CON	FLX		CON	FLX	
Actinobacteria_genus_						
Corynebacterium	$2.87 \times 10^{-4} \pm 1.42 \times 10^{-4}$	$8.43 \times 10^{-4} \pm 2.64 \times 10^{-4}$	.067	$1.14 \times 10^{-3} \pm 5.50 \times 10^{-3}$	$3.27 \times 10^{-4} \pm 2.17 \times 10^{-4}$	.275
Bifidobacterium	.0475 ± .0174	.0103 ± .00614	.086	.0233 ± .0975	$2.31 \times 10^{-3} \pm 8.76 \times 10^{-3}$	.122
Adlercreutzia	.0135 ± .00242	$.0141 \pm 3.62 \times 10^{-3}$	.880	.0259 ± .00515	$.0201 \pm 6.64 \times 10^{-3}$	.501
Collinsella	$1.07 \times 10^{-4} \pm 1.07 \times 10^{-4}$	0.00	.362	$4.31 \times 10^{-4} \pm 1.41 \times 10^{-4}$	$2.31 \times 10^{-4} \pm 1.24 \times 10^{-4}$	.333
Bacteroidetes_genus_						
Bacteroides	1.14 ± .172	.979 ± .202	.541	.745 ± .0714	.545 ± .0477	.053
Parabacteroides	$.122 \pm 9.90 \times 10^{-3}$	.111 ± .0294	.743	.0959 ± .0186	.0745 ± .0155	.425
Prevotella	13.0 ± 3.22	9.72 ± 3.55	.519	6.18 ± 1.66	5.37 ± 1.15	.724
Allstipes	.0460 ± .0139	$.0419 \pm 6.92 \times 10^{-3}$	.800	.0448 ± .0106	$.0325 \pm 8.62 \times 10^{-3}$	.413
Deferribacteres_ Mucispirillum	$7.6 \times 10^{-3}7 \pm 2.93 \times 10^{-3}$	$.0176 \pm 5.65 \times 10^{-3}$	.112	$5.24 \times 10^{-3} \pm 1.80 \times 10^{-3}$	$2.23 \times 10^{-3} \pm 7.82 \times 10^{-4}$	.237
Firmicutes_genus						
Jeotgalicoccus	0.00	$1.35 \times 10^{-4} \pm 8.70 \times 10^{-5}$	.098	$1.56 \times 10^{-3} \pm 6.80 \times 10^{-4}$	0.00	.102
Staphylococcus	$2.44 \times 10^{-3} \pm 9.46 \times 10^{-4}$	$4.01 \times 10^{-3} \pm 1.17 \times 10^{-3}$	.315	$4.01 \times 10^{-3} \pm 1.02 \times 10^{-3}$	$3.53 \times 10^{-3} \pm 1.08 \times 10^{-3}$	.755
Enterococcus	$.0142 \pm 5.80 \times 10^{-3}$	$9.12 \times 10^{-3} \pm .0279$	.497	$6.48 \times 10^{-3} \pm 1.27 \times 10^{-3}$	$.0112 \pm 2.39 \times 10^{-3}$	.0750
Lactobacillus	10.2 ± 2.56	9.37 ± 1.87	.970	5.20 ± .700	16.0 ± 2.54	<0.001*
Lactococcus	$2.88 \times 10^{-4} \pm 1.46 \times 10^{-4}$	0	.107	$2.42 \times 10^{-4} \pm 1.28 \times 10^{-4}$	0.00	.158
Streptococcus	.0849 ± .0136	.0944 ±.0206	.693	.133 ±.0346	.0579 ± .0166	.130
Clostridium	$1.30 \times 10^{-4} \pm 6.52 \times 10^{-5}$	0.00	.0630	$4.34 \times 10^{-4} \pm 1.59 \times 10^{-4}$	0.00	.0560

Eubacterium	.996 ± .0224	.0642 ± 6.07 $x10^{-3}$	.200	.103 ± .0149	.0870 ± .0122	.439
Dehalobacterium	.0833 ± .0221	.0760 ± .0162	.804	.0802 ± .0992	.0533 ± .0963	.081
Blautia	5.54 ± 1.82	7.97 ± 2.81	.461	3.14 ± .677	2.71 ± .831	.687
Clostridium	.0904 ± .0240	.0954 ± .0301	.896	.0597 ± .0102	.0694 ± .0153	.586
Dorea	.0140 ± 5.40 $x10^{-3}$	.0198 ± 5.55 $x10^{-3}$	.467	.0530 ± .0180	.0104 ± .00302	.107
Lachnospira	.590 ± .196	.974 ± .472	.419	.0808 ± .0202	.162 ± .0621	.171
Moryella	.0517 ± .0206	.142 ± .0508	.093	.0952 ± .0308	.043 ± .0124	.229
Ruminococcus	2.17 $x10^{-4}$ ± 1.16 $x10^{-4}$	2.03 $x10^{-4}$ ± 1.47 $x10^{-4}$	.940	3.64 $x10^{-4}$ ± 1.38 $x10^{-4}$	3.51 $x10^{-4}$ ± 1.85 $x10^{-4}$	.955
Peptococcus	3.65 $x10^{-3}$ ± 2.67 $x10^{-3}$	.0300 ± .0124	.0540	2.65 $x10^{-4}$ ± 1.66 $x10^{-4}$	1.34 $x10^{-4}$ ± 1.34 $x10^{-4}$	.580
Peptostreptococcus	1.79 $x10^{-4}$ ± 7.86 $x10^{-5}$	2.72 $x10^{-4}$ ± 1.10 $x10^{-4}$	.489	3.77 $x10^{-4}$ ± 1.31$x10^{-4}$	0.00	.032
Anaerotruncus	9.19 $x10^{-3}$ ± 3.43 $x10^{-3}$	.0201 ± 5.76 $x10^{-3}$	.106	.0150 ± 4.41$x10^{-3}$	.0251 ± 9.28 $x10^{-3}$	.295
Clostridium	.0295 ± 5.23 $x10^{-3}$	.0604 ± .0125	.021	.0473 ± .00639	.0308 ± 3.70 $x10^{-3}$	.102
Eubacterium	.301 ± .0863	.266 ± .0533	.756	.300 ± .989	.296 ± .102	.975
Oscillopsira	.870 ± .240	.803 ± .139	.827	.938 ± .174	.894 ± .252	.883
Ruminococcus	4.44 ± 1.13	4.28 ± .564	.909	6.59 ± 1.21	6.70 ± 1.07	.948
Subdoligranulum	9.76 $x10^{-3}$ ± 2.09 $x10^{-3}$	9.00 $x10^{-3}$ ± 2.77 $x10^{-3}$	.824	.0100 ± 2.24 $x10^{-3}$	.0105 ± 4.01 $x10^{-3}$	.915
Veillonella	4.68 $x10^{-5}$ ± 4.68 $x10^{-5}$	0.00	.362	1.39 $x10^{-3}$ ± 5.96 $x10^{-4}$	5.35 $x10^{-4}$ ± 3.81 $x10^{-4}$	.292
Proteobacteria_Genus_						
Sutterella	.0550 ± .0177	.0500 ± .0177	1.00	5.74 $x10^{-4}$ ± 3.49 $x10^{-4}$	.0288 ± .0124	.028
Comamonas	0.00	2.31$x10^{-4}$ ± 1.32 $x10^{-4}$	.100	3.16 $x10^{-3}$ ± 1.76 $x10^{-4}$	0.00	.147
Neisseria	6.97 $x10^{-5}$ ± 4.68 $x10^{-4}$	0.00	.204	1.97 $x10^{-4}$ ± 9.03 $x10^{-5}$	0.00	.149
Bilophila	3.12 $x10^{-3}$ ± 1.13 $x10^{-3}$	1.15 $x10^{-3}$ ± 4.76 $x10^{-3}$	.192	5.03 $x10^{-3}$ ± 1.61 $x10^{-3}$	4.89 $x10^{-3}$ ± 2.09 $x10^{-4}$	.048

Desulfovibrio	$4.85 \times 10^{-3} \pm .0311$	0.00	.184	$2.00 \times 10^{-4} \pm 1.46 \times 10^{-4}$	$4.58 \times 10^{-4} \pm 4.58 \times 10^{-4}$	.539
LE30	$9.00 \times 10^{-3} \pm 2.64 \times 10^{-3}$	$8.15 \times 10^{-3} \pm 1.43 \times 10^{-3}$	.794	$.0117 \pm 2.46 \times 10^{-3}$	$8.09 \times 10^{-3} \pm 2.49 \times 10^{-3}$	.337
Escherichia	$.0120 \pm 3.42 \times 10^{-3}$	$3.60 \times 10^{-3} \pm 1.03 \times 10^{-3}$	.0470	$9.13 \times 10^{-3} \pm 2.42 \times 10^{-3}$	$1.37 \times 10^{-3} \pm 4.89 \times 10^{-3}$	.041
Pasteurella	$2.72 \times 10^{-3} \pm 8.67 \times 10^{-3}$	$1.77 \times 10^{-3} \pm 8.61 \times 10^{-3}$	.460	$8.28 \times 10^{-4} \pm 1.95 \times 10^{-4}$	$5.82 \times 10^{-4} \pm 2.42 \times 10^{-4}$	.437
Pseudomonas	$4.68 \times 10^{-5} \pm 4.68 \times 10^{-5}$	0.00	.396	$2.96 \times 10^{-4} \pm 1.16 \times 10^{-4}$	$4.40 \times 10^{-4} \pm 1.69 \times 10^{-4}$	.476
Tenericutes_Genus_						
Allobaculum	$.179 \pm .0428$	$.180 \pm .0685$	.987	$.498 \pm .141$	$.154 \pm .0612$	.0730
Clostridium	$.271 \pm .0890$	$.172 \pm .0605$	.404	$.238 \pm .0727$	$.0260 \pm .0129$	.0560
Coprobacillus	$.0260 \pm 7.58 \times 10^{-3}$	$.0332 \pm 8.27 \times 10^{-3}$	.532	$.0696 \pm .0198$	$.0287 \pm 9.94 \times 10^{-3}$	.118
Holdemania	$.0111 \pm 4.25 \times 10^{-3}$	$4.31 \times 10^{-3} \pm 9.76 \times 10^{-4}$	.194	$7.88 \times 10^{-3} \pm 1.95 \times 10^{-3}$	$4.84 \times 10^{-3} \pm 2.17 \times 10^{-3}$	.283
Anaeroplasma	$2.16 \times 10^{-3} \pm 9.44 \times 10^{-4}$	$1.26 \times 10^{-3} \pm 5.16 \times 10^{-4}$	.489	$2.53 \times 10^{-3} \pm 1.06 \times 10^{-3}$	$6.16 \times 10^{-3} \pm 2.84 \times 10^{-4}$	.152
Mycoplasma	$3.49 \times 10^{-5} \pm 3.49 \times 10^{-5}$	$1.82 \times 10^{-4} \pm 1.22 \times 10^{-4}$	.216	$3.91 \times 10^{-5} \pm 3.91 \times 10^{-4}$	$4.46 \times 10^{-3} \pm 2.68 \times 10^{-3}$	.132
Verrucomicrobia_ Akkermansia	$.0307 \pm .0114$	$.0203 \pm .0118$	.529	$6.50 \times 10^{-3} \pm 3.03 \times 10^{-3}$	$.139 \pm .0465$	.00800

*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 $5HT_{1A}$ 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 $5\text{-}HT_{1A}$ receptor concentrations in individuals suffering from depression. *MAO* knockout mouse models show increased extracellular 5-HT levels and decreased $5\text{-}HT_{1A}$ receptor sensitivity and concentrations [408-410]. The reduced expression of $5\text{-}HT_{1A}$ 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 $5\text{-}HT_{2A}$ and *MAO* expression and increase in serum 5-HT levels in our animal model. We found that $5\text{-}HT_{2A}$ 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 $5\text{-}HT_{2A}$ 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 (estrus 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$_{2A}$* 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$_{2A}$* 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$_{2A-/-}$* 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$_{2A}$ 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$_{2A}$* receptor signaling plays an anti- or pro-inflammatory role. For instance, rheumatoid arthritis patients show considerably lower *5-HT$_{2A}$* 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$_{2A}$* receptors involved in the pathophysiology of inflammatory conditions. Recently, however, agonism of *5-HT$_{2A}$* 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$_{2A}$* 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 aetiological 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 *Bifidocbacterium* 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 ($5\text{-}HT_{2A}$) 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

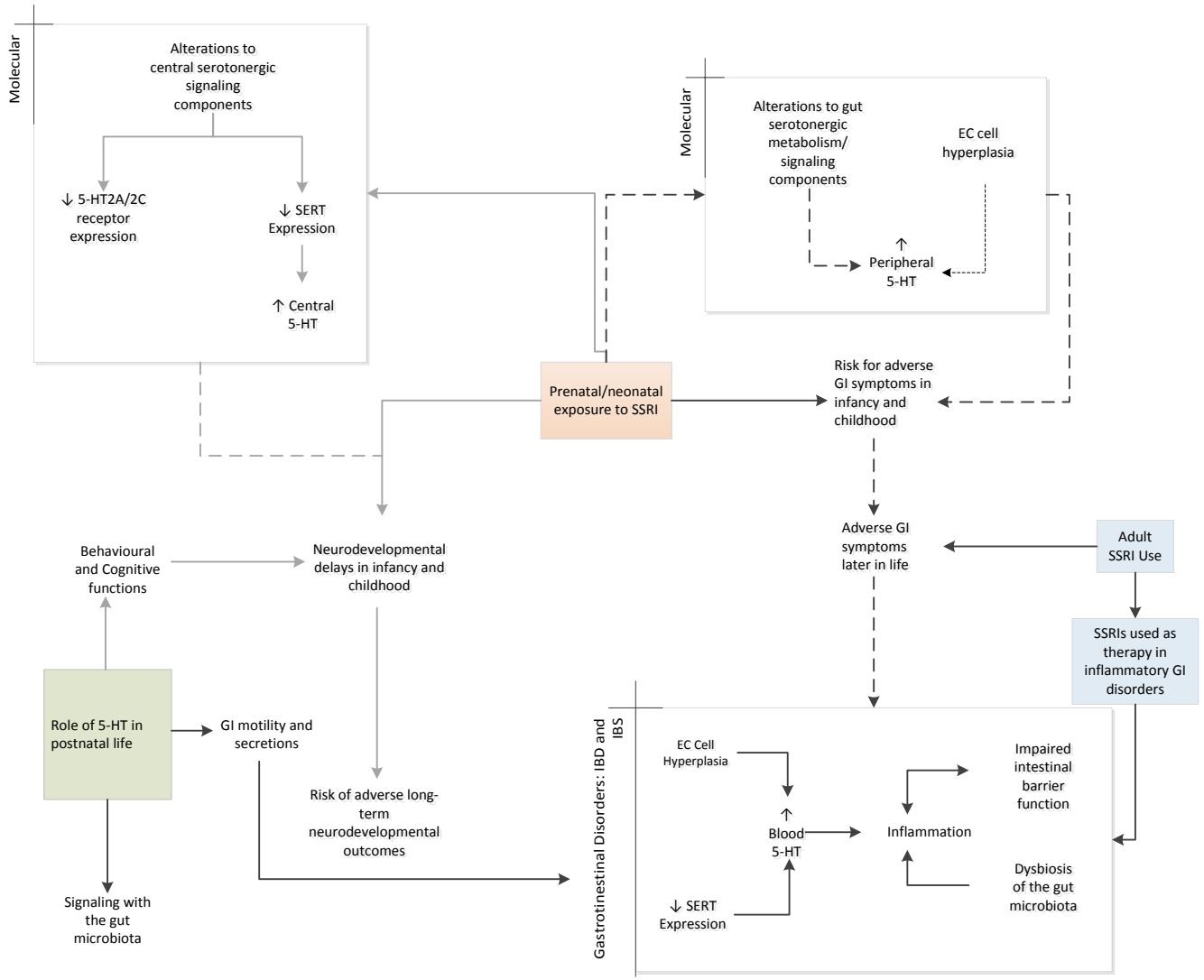

Figure 21 Summary of the (1) effects of prenatal/neonatal SSRI exposure on the central serotonergic system and known developmental consequences; (2) role of 5-HT in postnatal life; (3) effects of prenatal/neonatal SSRI exposure on the GI system; and (4) potential mechanisms in which SSRIs can affect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

APPENDIX B

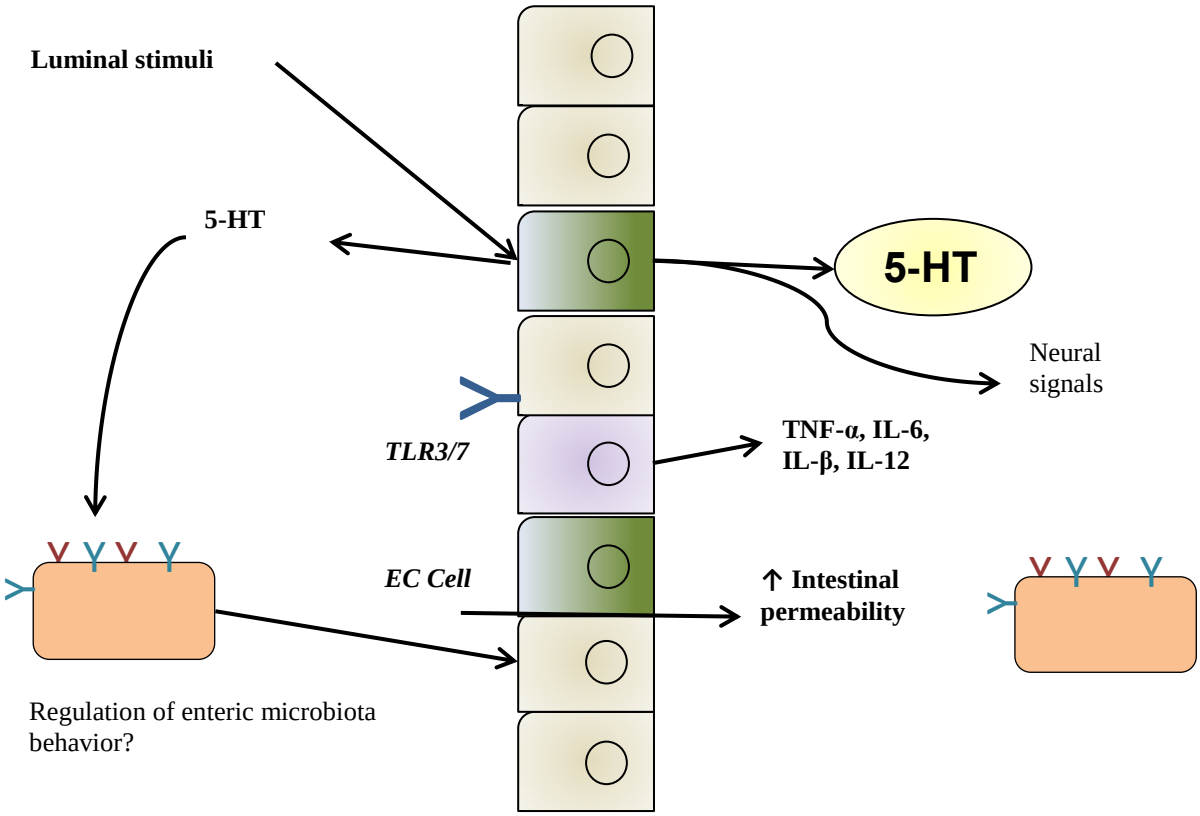

Figure 22 Schematic representation of endocrine cell-mediated signaling from enteric microbiota and host. Presence of Gram-negative bacteria and gut-derived 5-HT in the lumen might influence endocrine cells in the epithelium (enterochromaffin cells). Furthermore, the microbiota may influence intestinal 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.

Gene	Density	Exposure	First Author
SERT	Reduced	P8-14 (neonatal) 15 mg/kg	Hansen et al., 1998
		GD13-20 10 mg/kg	Cabrera et al., 1998
5-HT	Reduced in prepubescent male offspring	GD13-20 10 mg/kg	Cabrera et al., 1998
5-HT $_{1A/1B}$ receptor	Unchanged	P8-14 (neonatal) 15 mg/kg	Hansen et al., 1998
5-HT$_{2A/2C}$ receptor	Reduced		Cabrera et al., 1994

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 diestrus females. All comparisons were done by independent samples t-test.

Genes	Estrous (Mean ± SEM)		p-value	Diestrous Mean (Mean ± SEM)		p-value
	Control	Fluoxetine		Control	Fluoxetine	
Tph1	3.11 ± .640	1.32 ± .247	.032	1.28 ± .288	1.79 ± 1.50	.692
Pet-1	1.42 ± .490	1.61 ± .520	.799	1.38 ± .310	1.43 ± 1.16	.966
SERT	1.28 ± .609	1.73 ± .301	.501	1.48 ± .556	1.31 ± 1.06	.887
Mao	5.38 ± 1.64	2.90 ± 1.13	.251	3.75 ± 2.00	6.35 ± 5.60	.634
HTR 1a	2.21 ± 1.90	.325 ± .195	.195	.883 ± .763	.856 ± .850	.983
HTR 1b	6.74 ± 5.56	.659 ± .234	.249	2.42 ± 2.00	.240 ± .240	.460
HTR 1d	1.16 ± 1.01	.147 ± .052	.283	.520 ± .450	.528 ± .441	.990
HTR 2a	9.65 ± 8.687	1.22 ± .803	.302	2.57 ± 2.30	.693 ± .667	.577
HTR 2b	4.17 ± 2.47	.734 ± .181	.159	1.37 ± 1.05	.970 ± .815	.804
HTR 3a	16.0 ± 1.2	6.41 ± 2.76	.343	5.15 ± 2.16	.094 ± .805	.168
HTR 3b	5.54 ± 4.39	.741 ± .261	.250	2.52 ± 2.17	1.10 ± .555	.650
HTR 4	3.42 ± 1.30	1.29 ± .257	.118	2.35 ± 1.45	2.63 ± 2.25	.917
HTR 7	3.01 ± 2.15	1.62 ± .930	.539	1.31 ± .735	17.3 ± 17.2	.302

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 diestrus females. All comparisons were done by independent samples t-test.

Genes	Estrous (Mean ± SEM)		p-value	Diestrous (Mean ± SEM)		p-value
	Control	Fluoxetine		Control	Fluoxetine	
IL1β	.696 ± .057	.747 ± .124	.798	.944 ± .270	1.18 ± 1.00	.790
IL6	4.88 ± 3.93	.601 ± .268	.252	2.88 ± 2.66	1.17 ± .778	.656
IL10	.565 ± .423	.069 ± .040	.223	.228 ± .209	.022 ± .013	.501
TNFα	3.71 ± 2.62	.682 ± .235	.229	1.75 ± 1.48	1.15 ± .891	.785
MCP1	.592 ± .494	.074 ± .040	.268	.195 ± .188	.008 ± .003	.497
IL13	5.70 ± 4.54	1.39 ± .594	.316	3.68 ± 3.33	3.64 ± 2.87	.994
F4/80	2.52 ± 1.27	2.34 ± .921	.914	1.17 ± .579	1.41 ± .646	.811
CD68	1.18 ± .161	.916 ± .396	.615	.646 ± .329	1.36 ± 1.20	.523
OCLDN	1.09 ± .274	.617 ± .148	.160	.396 ± .193	.316 ± .280	.716
CLDN1	4.84 ± 3.79	.426 ± .201	.224	2.12 ± 1.81	2.14 ± 1.99	.995
CLDN3	1.40 ± .156	1.09 ± .302	.455	.830 ± .113	1.44 ± 1.02	.491
ZO-1	3.09 ± .594	2.42 ± .829	.570	1.69 ± .504	5.50 ± 4.78	.360
TLR4	2.64 ± .465	1.48 ± .718	.271	.346 ± .173	8.46 ± 11.8	.284

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 diestrus females. All comparisons were done by independent samples t-test.

Genes	**Estrous (Mean ± SEM)**		**p-value**	**Diestrous (Mean ± SEM)**		**p-value**
	Control	Fluoxetine		Control	Fluoxetine	
GLUT2	5.45 ± 4.54	1.05 ± .386	.303	2.47 ± 2.14	2.39 ± .944	.982
SGLT1	3.58 ± .611	1.37 ± .333	.019*	1.56 ± .327	2.20 ± 1.44	.622
Gcg	2.78 ± 1.21	3.83 ± 1.35	.604	1.52 ± .178	.675 ± .657	.218
FIAF	4.38 ± 2.52	1.27 ± .207	.203	2.35 ± 1.45	3.60 ± .033	.552
GPR41	3.02 ± 2.25	.720 ± .281	.283	1.68 ± 1.35	1.92 ± 1.70	.918
GPR43	3.77 ± 1.58	1.59 ± .290	.173	2.02 ± .60	2.36 ± 1.67	.828
FAT/CD 36	1.54 ± .518	1.14 ± .645	.670	.362 ± .125	.415 ± .106	.788

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.

OTU (#)	Base Mean (CON)	Log_2Fold Change (FLX)	P-value (adjusted)
Bacteroidetes_Bacteroidia_Bacteroidales (81)	19.9	-1.83	0.0720
Bacteroidetes_Bacteroidia_Bacteroidales (482)	4.33	-1.92	0.0403
Firmicutes_Clostridia_Clostridiales (585)	5.47	1.33	0.912
_Lachnospiraceae (88)	55.3	1.92	0.0335
_Lachnospiraceae (165)	6.54	-2.12	0.0165
_Lachnospiraceae (545)	5.70	1.34	0.917
_Lachnospiraceae (673)	3.25	-1.20	0.918
Firmicutes_Bacilli_Lactobacillales_Aerococcaceae_Aerococcus (535)	2.32	-1.29	0.787
_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcus (135)	6.79	-1.28	0.918
Tenericutes_Mollicutes_RF39 (162)	5.88	-1.72	0.145

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 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, none of the 6037 examined OTUs showed significant differences between control and treated offspring. A P-value of <0.05 was considered statistically significant.

OTU (#)	Base Mean (CON)	Log_2Fold Change (FLX)	P-value (adjusted)
Firmicutes (235)	14.504	1.932	0.2781
Firmicutes (693)	4.202	-1.722	0.2781
_Bacilli (860)	2.307	-1.367	0.808
_Clostridia_Clostridiales_Lachnospiraceae (52)	158.968	-1.110	0.826
_Lachnospiraceae (391)	14.368	-1.238	0.826
_Ruminococcaceae (353)	16.792	1.410	0.826
Proteobacteria_Alphaproteobacteria (292)	14.149	1.799	0.278
Bacteroidetes_Bacteroidia_Bacteroidales (161)	100.565	1.320	0.826
_Bacteroidaceae_Bacteroides (78)	230.893	1.416	0.324
Tenericutes_Erysipelotrichi_Erysipelotrichales_Erysipelotrichacea (248)	7.836	-1.927	0.2781

APPENDIX G

Supplemental figures

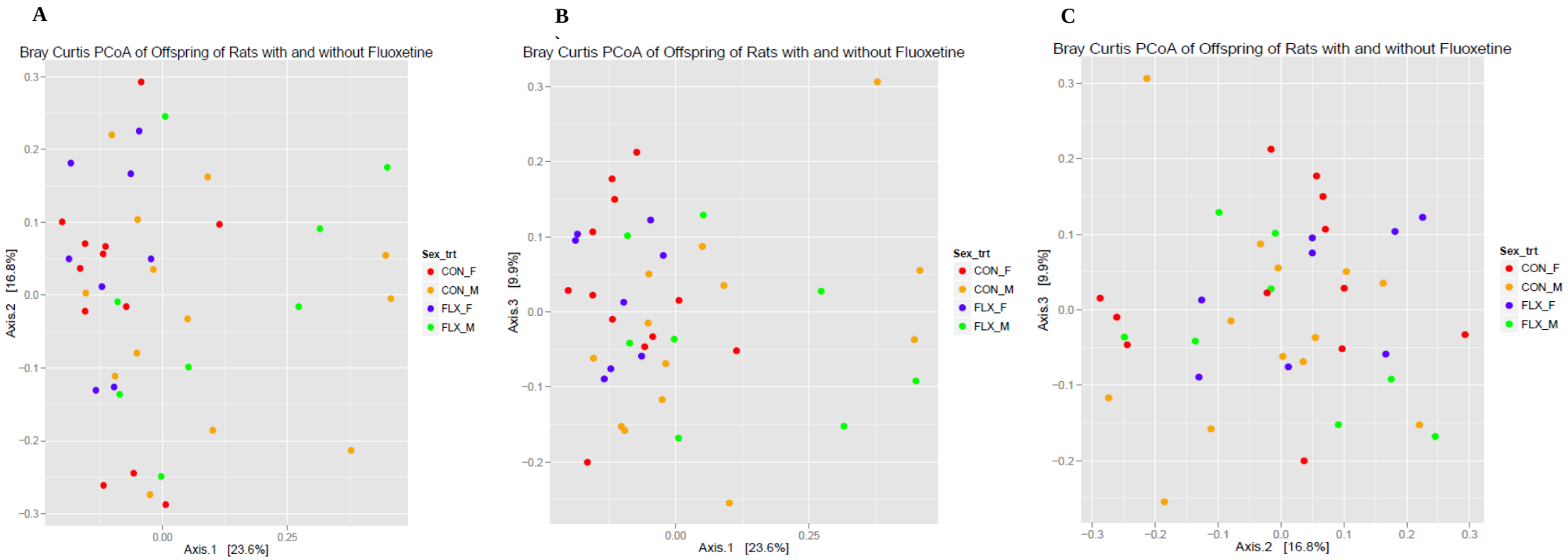

Figure 22 Two-dimensional Principal Coordinate Analysis (PCoA) plots based on the relative abundance distance matrix. Percentage of the diversity explained by each axes is indicated on the figure. There are no distinct clusters between groups, meaning that the gut microbiota of control and fluoxetine-exposed 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

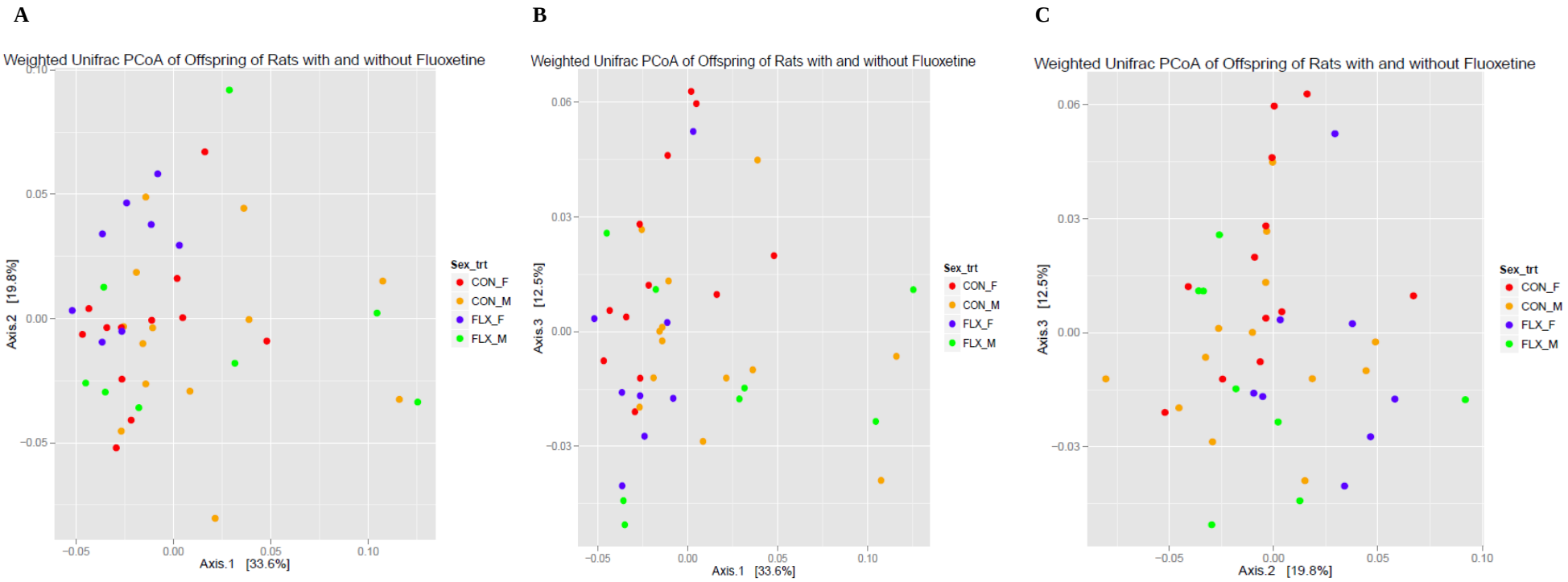

Figure 23 Two-dimensional Principal Coordinate Analysis (PCoA) plots based on the phylogenetic distance between OTUs. Percentage of the diversity explained by each axes is indicated on the figure. There are no distinct clusters between treatment groups, meaning that the gut microbiota of control and 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

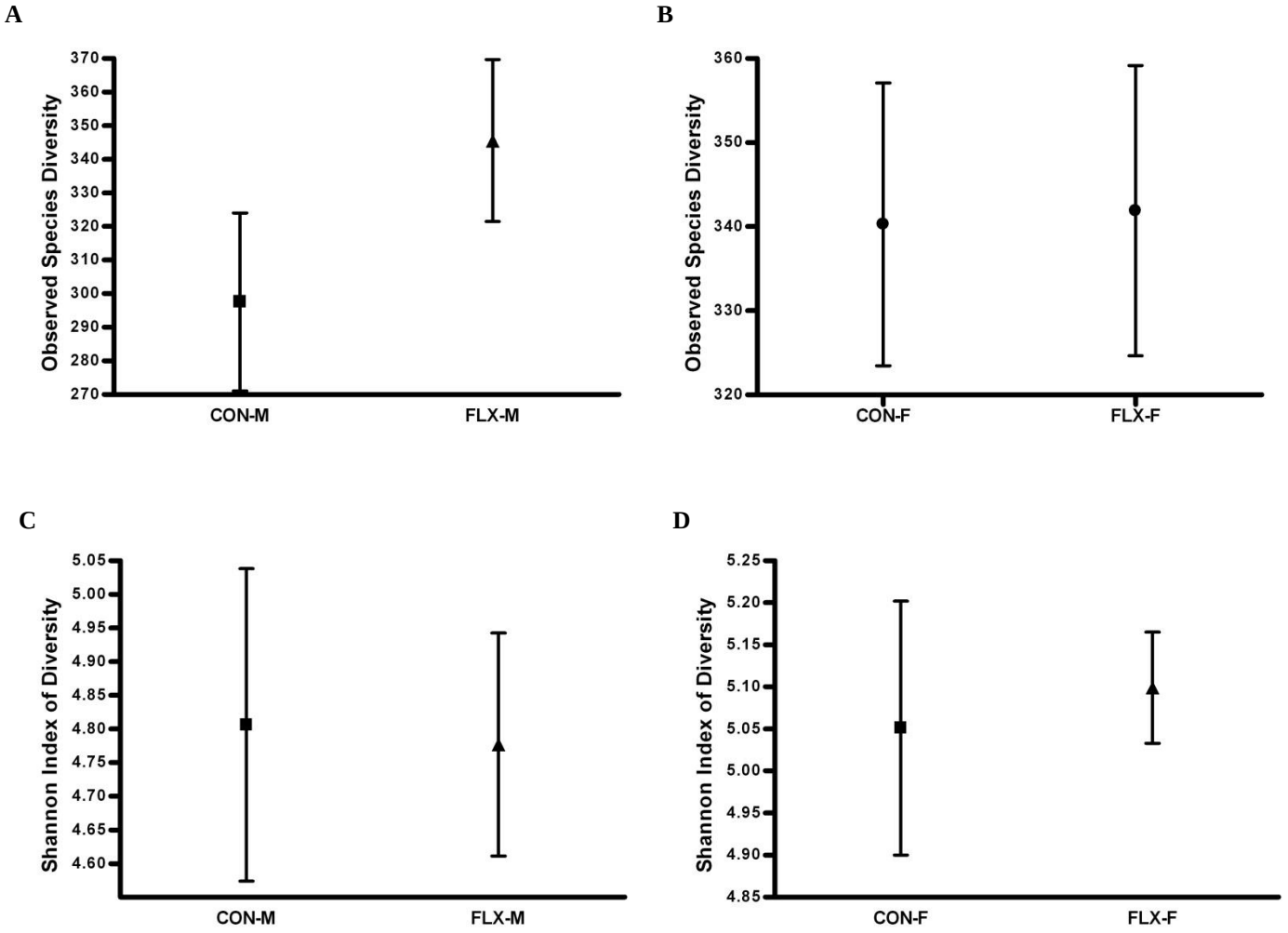

Figure 24 The number of observed species (richness) and the Shannon Diversity Index in control and treated offspring at 24 weeks of age separated by sex. A) Observed species diversity between male control (mean ± SEM; 298 ± 26.5) and fluoxetine-exposed offspring (346 ± 24.1; P= 0.247). B) Observed species 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.

Gene	Forward Sequence	Reverse Sequence
18S	5’-GCG ATG CGG CGG CGT TAT-3’	5’-AGA CTT TGG TTT CCC GGA AGC-3’
B-actin	5’-ACG AGG CCC AGA GCA AGA-3’	5’-TTG GTT ACA ATG CCG TGT TCA-3’
CLDN1	5'-CGT GAC TGC TCA GGC CAT CT-3'	5'-CGG TGC TTT GCG AAA CG-3'
CLDN3	5'-GAC CAC CCC ACC TTC CAG AT-3'	5'-CTG TCC TCT TCC AGC CTA GCA-3'
GLUT2 (SLC2A2)	5'CTG TCT GTG TCC AGC TTT GCA-3'	5'-CAA GCC ACC CAC CAA AGA AC-3'
GPR41	5'-GCT TGT GTG CCT TGG ACT CA-3'	5'-TGG CTC TTC TCC GTT CTT TAC CT-3'
GPR43	5'-TCG TGG AAG CTG CAT CCA-3'	5'-GCG CGC ACA CGA TCT TT-3'
HPRT	5’-GCA GTA CAG CCC CAA AAT GG-3’	5’-GGT CCT TTT CAC CAG CAA GCT-3’
HTR 1a	5'-CTC TGT TGC TGG GTA CTC TCA TT-3'	5'-ACT TGT TGA GCA CCT GGT ACA GA-3'
HTR 1b	5'-CTT TCT ATT TAC CCA CCC TGC TC-3'	5'-GTC TGA GAC TCG CAC TTT GAC TT-3'
HTR 1d	5'-CCC GGA GTC GAA TCC TGA A-3'	5'-TGA TAA GCT GTG CTG TGG TGA A-3'
HTR 2a	5'-AGC TCT GTG CGA TCT GGA TT-3'	5'-CCC CTC CTT AAA GAC CTT CG-3'
HTR 2b	5'-TGG CAG TTT CAT GCT CTT TG-3'	5'-TTC CCT TTG GAG AAC TGT GG-3'
HTR 3a	5'-TGG CTT CTT CTT CAG CTC ACT TG-3'	5’-CCC CCC GTT GGT TGA TG-3’
HTR 3b	5'-CCG AAC TCG GGA TCA GGT TT-3'	5'-CAG TAT AGA GCC CCA GCA CG-3'
HTR 4	5'-GAG ACC AAA GCA GCC AAG AC-3'	5'-AGG AAG GCA CGT CTG AAA GA-3'
HTR7	5’-TGC TGG CTG CCG TTT TTC-3'	5’-CTA CAG GAG GTG CCA CAG ATA AAG-3’
IL10	5'-CCC AGA AAT CAA GGA GCA TTT G-3'	5'-CAG CTG TAT CCA GAG GGT CTT CA-3'
IL13	5’-GAC AGC TGG CGG GTT CTG T-3’	5’- GGC ATT GCA ACT GGA GAT GTT-3’
IL1β	5’-GAC CTG TTC TTT GAG GCT GAC A-3’	5’-AGT CAA GGG CTT GGA AGC AA-3’
IL6	5’-CCC ACC AGG AAC GAA AGT CA-3’	5’-GCG GAG AGA AAC TTC ATA GCT GTT-3’
MAO	5'-TGG GTT GAA GAA CCC GAG TC-3'	5'-TGA TCT TGA GCA GAC CAG GC-3'
MCP1	5'-CGG TTT CTC CCT TCT ACT TCC TG-3'	5'-GCT CTG CCT CAG CCT TTT ATT G-3'
OCLN	5'-GAG AGA TGC ACG TTC GAC CAA-3'	5'-GAA TTT CGT CTT CCG GGT AAA A-3
Pet-1	5'-CCC TGC TGA TCA ACA TGT ACC-3'	5'-GCC AGC AGC TCC AGT AGA AA-3'
SERT	5'-AGC GAT GTG AAG GAG ATG CT-3'	5'-GGA CGA CAT CCC TAT GCA GT-3'

SGLT1 (SLC5A1)	5'-GGA ACT GGA AGC TGC ATG GA-3'	5'-AGT GGA CCC CGC AGA TGA T-3'
TNFα	5’-CCC AGA AAA GCA AGC AAC CA-3’	5’-GCC TCG GGC CAG TGT ATG-3’
Tph1	5'-GCC TGC TTT CTT CCA TCA GT-3'	5'-AGA CAT CCT GGA AGC TTG TGA-3'
ZO-1	5'-GCT CAC CAG GGT CAA AAT GTT T-3'	5'-AGT GTC ATT CAC ATC CTT CTT GTT CT-3'

References

1. Caryn Pearson, T.J., Jennifer Ali, *Mental and substance use disorders in Canada*. Statistics Canada, 2013.
2. Kessler, R.C., et al., *The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R).* JAMA, 2003. **289**(23): p. 3095-105.
3. Ferrari, A.J., et al., *The epidemiological modelling of major depressive disorder: application for the Global Burden of Disease Study 2010.* PLoS One, 2013. **8**(7): p. e69637.
4. Ferrari, A.J., et al., *Burden of depressive disorders by country, sex, age, and year: findings from the global burden of disease study 2010.* PLoS Med, 2013. **10**(11): p. e1001547.
5. Murray, C.L.J., Lopez, A.D. , *The Global Burden of Disease: a comprehensive assessment of mortality and disabiltiy from diseases, injuries, and risk factors in 1990 and projected to 2020.* Harvard School of Public Health on behalf of the World Health Organization & World Bank, 1996.
6. Ustun, T.B., et al., *Global burden of depressive disorders in the year 2000.* Br J Psychiatry, 2004. **184**: p. 386-92.
7. Association, A.P., *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, DSM-5.* American Psychiatric Association, 2013.
8. Organization, W.H., *International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) Version for 2010.* 2010. **Chapter V Mental and hebavioural disorders (F00-F99)**.
9. Angst, J., et al., *Gender differences in depression. Epidemiological findings from the European DEPRES I and II studies.* Eur Arch Psychiatry Clin Neurosci, 2002. **252**(5): p. 201-9.
10. Kuehner, C., *Gender differences in unipolar depression: an update of epidemiological findings and possible explanations.* Acta Psychiatr Scand, 2003. **108**(3): p. 163-74.

11. Culbertson, F.M., *Depression and gender. An international review.* Am Psychol, 1997. **52**(1): p. 25-31.

12. Dietz, P.M., et al., *Clinically identified maternal depression before, during, and after pregnancies ending in live births.* Am J Psychiatry, 2007. **164**(10): p. 1515-20.

13. Kessler, R.C., et al., *Sex and depression in the National Comorbidity Survey. II: Cohort effects.* J Affect Disord, 1994. **30**(1): p. 15-26.

14. Moses-Kolko, E.L. and E.K. Roth, *Antepartum and postpartum depression: healthy mom, healthy baby.* J Am Med Womens Assoc, 2004. **59**(3): p. 181-91.

15. Kessler, R.C., *Epidemiology of women and depression.* J Affect Disord, 2003. **74**(1): p. 5-13.

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.

17. Yonkers, K.A., et al., *Depression screening of perinatal women: an evaluation of the healthy start depression initiative.* Psychiatr Serv, 2009. **60**(3): p. 322-8.

18. Weich, S., et al., *Mental health and the built environment: cross-sectional survey of individual and contextual risk factors for depression.* Br J Psychiatry, 2002. **180**: p. 428-33.

19. Soares, C.N. and B. Zitek, *Reproductive hormone sensitivity and risk for depression across the female life cycle: a continuum of vulnerability?* J Psychiatry Neurosci, 2008. **33**(4): p. 331-43.

20. Burt, V.K. and K. Stein, *Epidemiology of depression throughout the female life cycle.* J Clin Psychiatry, 2002. **63 Suppl 7**: p. 9-15.

21. Cohen, L.S., et al., *Diagnosis and treatment of depression during pregnancy.* CNS Spectr, 2004. **9**(3): p. 209-16.

22. Cohen, L.S., et al., *Relapse of depression during pregnancy following antidepressant discontinuation: a preliminary prospective study.* Arch Womens Ment Health, 2004. **7**(4): p. 217-21.

23. Bennett, H.A., et al., *Prevalence of depression during pregnancy: systematic review.* Obstet Gynecol, 2004. **103**(4): p. 698-709.

24. Milgrom, J., et al., *Antenatal risk factors for postnatal depression: a large prospective study.* J Affect Disord, 2008. **108**(1-2): p. 147-57.

25. Robertson, E., et al., *Antenatal risk factors for postpartum depression: a synthesis of recent literature.* Gen Hosp Psychiatry, 2004. **26**(4): p. 289-95.

26. Banti, S., et al., *From the third month of pregnancy to 1 year postpartum. Prevalence, incidence, recurrence, and new onset of depression. Results from the perinatal depression-research & screening unit study.* Compr Psychiatry, 2011. **52**(4): p. 343-51.

27. Gavin, N.I., et al., *Perinatal depression: a systematic review of prevalence and incidence.* Obstet Gynecol, 2005. **106**(5 Pt 1): p. 1071-83.

28. Gaynes, B.N., et al., *Perinatal depression: prevalence, screening accuracy, and screening outcomes.* Evid Rep Technol Assess (Summ), 2005(119): p. 1-8.

29. Yonkers, K.A., et al., *The management of depression during pregnancy: a report from the American Psychiatric Association and the American College of Obstetricians and Gynecologists.* Gen Hosp Psychiatry, 2009. **31**(5): p. 403-13.

30. Field, T., M. Diego, and M. Hernandez-Reif, *Prenatal depression effects on the fetus and newborn: a review.* Infant Behav Dev, 2006. **29**(3): p. 445-55.

31. Leech, S.L., et al., *Predictors and correlates of high levels of depression and anxiety symptoms among children at age 10.* J Am Acad Child Adolesc Psychiatry, 2006. **45**(2): p. 223-30.

32. Marcus, S.M. and J.E. Heringhausen, *Depression in childbearing women: when depression complicates pregnancy.* Prim Care, 2009. **36**(1): p. 151-65, ix.

33. Marcus, S.M., *Depression during pregnancy: rates, risks and consequences--Motherisk Update 2008.* Can J Clin Pharmacol, 2009. **16**(1): p. e15-22.

34. Grote, N.K., et al., *A meta-analysis of depression during pregnancy and the risk of preterm birth, low birth weight, and intrauterine growth restriction.* Arch Gen Psychiatry, 2010. **67**(10): p. 1012-24.

35. Nulman, I., et al., *Neurodevelopment of children following prenatal exposure to venlafaxine, selective serotonin reuptake inhibitors, or untreated maternal depression.* Am J Psychiatry, 2012. **169**(11): p. 1165-74.

36. O'Connor, D.L., et al., *Growth and development of premature infants fed predominantly human milk, predominantly premature infant formula, or a combination of human milk and premature formula.* J Pediatr Gastroenterol Nutr, 2003. **37**(4): p. 437-46.

37. Crowther, N.J., *Early determinants of chronic disease in developing countries.* Best Pract Res Clin Endocrinol Metab, 2012. **26**(5): p. 655-65.

38. *Use of Psychiatric medications.* ACOG Practice Bulletin, 2008(111): p. 1001-1020.

39. Alwan, S., et al., *Patterns of antidepressant medication use among pregnant women in a United States population.* J Clin Pharmacol, 2011. **51**(2): p. 264-70.

40. Andrade, S.E., et al., *Use of antidepressant medications during pregnancy: a multisite study.* Am J Obstet Gynecol, 2008. **198**(2): p. 194 e1-5.

41. Cooper, W.O., et al., *Increasing use of antidepressants in pregnancy.* Am J Obstet Gynecol, 2007. **196**(6): p. 544 e1-5.

42. Egen-Lappe, V. and J. Hasford, *Drug prescription in pregnancy: analysis of a large statutory sickness fund population.* Eur J Clin Pharmacol, 2004. **60**(9): p. 659-66.

43. Rahola, J.G., *Antidepressants: pharmacological profile and clinical consequences.* Int J Psychiatry Clin Pract, 2001. **5**(1): p. 19-28.

44. Fitzgerald, K.T. and A.C. Bronstein, *Selective serotonin reuptake inhibitor exposure.* Top Companion Anim Med, 2013. **28**(1): p. 13-7.

45. Nonacs, R. and L.S. Cohen, *Assessment and treatment of depression during pregnancy: an update.* Psychiatr Clin North Am, 2003. **26**(3): p. 547-62.

46. Caley, C.F. and J.C. Kando, *SSRI efficacy-finding the right dose.* J Psychiatr Pract, 2002. **8**(1): p. 33-40.

47. Pirraglia, P.A., R.S. Stafford, and D.E. Singer, *Trends in Prescribing of Selective Serotonin Reuptake Inhibitors and Other Newer Antidepressant Agents in Adult Primary Care.* Prim Care Companion J Clin Psychiatry, 2003. **5**(4): p. 153-157.

48. Mitchell, A.A., et al., *Medication use during pregnancy, with particular focus on prescription drugs: 1976-2008.* Am J Obstet Gynecol, 2011. **205**(1): p. 51 e1-8.

49. Shuey, D.L., T.W. Sadler, and J.M. Lauder, *Serotonin as a regulator of craniofacial morphogenesis: site specific malformations following exposure to serotonin uptake inhibitors.* Teratology, 1992. **46**(4): p. 367-78.

50. Sloot, W.N., H.C. Bowden, and T.D. Yih, *In vitro and in vivo reproduction toxicology of 12 monoaminergic reuptake inhibitors: possible mechanisms of infrequent cardiovascular anomalies.* Reprod Toxicol, 2009. **28**(2): p. 270-82.

51. Baldwin, J.A., et al., *The reproductive toxicology of paroxetine.* Acta Psychiatr Scand Suppl, 1989. **350**: p. 37-9.

52. Nordeng, H. and O. Spigset, *Treatment with selective serotonin reuptake inhibitors in the third trimester of pregnancy: effects on the infant.* Drug Saf, 2005. **28**(7): p. 565-81.

53. Einarson, A., et al., *Incidence of major malformations in infants following antidepressant exposure in pregnancy: results of a large prospective cohort study.* Can J Psychiatry, 2009. **54**(4): p. 242-6.

54. Hayes, R.M., et al., *Maternal antidepressant use and adverse outcomes: a cohort study of 228,876 pregnancies.* Am J Obstet Gynecol, 2012. **207**(1): p. 49 e1-9.

55. Ververs, T., et al., *Prevalence and patterns of antidepressant drug use during pregnancy.* Eur J Clin Pharmacol, 2006. **62**(10): p. 863-70.

56. Hemels, M.E., G. Koren, and T.R. Einarson, *Increased use of antidepressants in Canada: 1981-2000.* Ann Pharmacother, 2002. **36**(9): p. 1375-9.

57. Bakker, M.K., et al., *Increase in use of selective serotonin reuptake inhibitors in pregnancy during the last decade, a population-based cohort study from the Netherlands.* Br J Clin Pharmacol, 2008. **65**(4): p. 600-6.

58. Tuccori, M., et al., *Safety concerns associated with the use of serotonin reuptake inhibitors and other serotonergic/noradrenergic antidepressants during pregnancy: a review.* Clin Ther, 2009. **31 Pt 1**: p. 1426-53.

59. Barker, D.J., et al., *Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease.* BMJ, 1989. **298**(6673): p. 564-7.

60. Hochberg, Z., *Developmental plasticity in child growth and maturation.* Front Endocrinol (Lausanne), 2011. **2**: p. 41.

61. Eriksson, J.G., *Early growth and coronary heart disease and type 2 diabetes: findings from the Helsinki Birth Cohort Study (HBCS).* Am J Clin Nutr, 2011. **94**(6 Suppl): p. 1799S-1802S.

62. Osmond, C., et al., *Early growth and death from cardiovascular disease in women.* BMJ, 1993. **307**(6918): p. 1519-24.

63. Hovi, P., et al., *Glucose regulation in young adults with very low birth weight.* N Engl J Med, 2007. **356**(20): p. 2053-63.

64. Sato, R., et al., *A cross-sectional study of glucose regulation in young adults with very low birth weight: impact of male gender on hyperglycaemia.* BMJ Open, 2012. **2**(1): p. e000327.

65. Laitinen, J., et al., *Predictors of abdominal obesity among 31-y-old men and women born in Northern Finland in 1966.* Eur J Clin Nutr, 2004. **58**(1): p. 180-90.

66. Leong, N.M., et al., *Early life risk factors in cancer: the relation of birth weight to adult obesity.* Int J Cancer, 2003. **103**(6): p. 789-91.

67. Fernandez-Twinn, D.S. and S.E. Ozanne, *Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome.* Physiol Behav, 2006. **88**(3): p. 234-43.

68. Motte, E., et al., *[Programming nutritional and metabolic disorders: the diabetic environment during gestation]*. Arch Pediatr, 2010. **17**(1): p. 60-70.

69. Byatt, N., K.M. Deligiannidis, and M.P. Freeman, *Antidepressant use in pregnancy: a critical review focused on risks and controversies.* Acta Psychiatr Scand, 2013. **127**(2): p. 94-114.

70. Misri, S., A. Burgmann, and D. Kostaras, *Are SSRIs safe for pregnant and breastfeeding women?* Can Fam Physician, 2000. **46**: p. 626-8, 631-3.

71. Misri, S., D. Kostaras, and X. Kostaras, *The use of selective serotonin reuptake inhibitors during pregnancy and lactation: current knowledge.* Can J Psychiatry, 2000. **45**(3): p. 285-7.

72. Hendrick, V., et al., *Placental passage of antidepressant medications.* Am J Psychiatry, 2003. **160**(5): p. 993-6.

73. Kim, J., et al., *Stereoselective disposition of fluoxetine and norfluoxetine during pregnancy and breast-feeding.* Br J Clin Pharmacol, 2006. **61**(2): p. 155-63.

74. Rampono, J., et al., *Placental transfer of SSRI and SNRI antidepressants and effects on the neonate.* Pharmacopsychiatry, 2009. **42**(3): p. 95-100.

75. Heikkine, T., U. Ekblad, and K. Laine, *Transplacental transfer of citalopram, fluoxetine and their primary demethylated metabolites in isolated perfused human placenta.* BJOG, 2002. **109**(9): p. 1003-8.

76. Hendrick, V., et al., *Fluoxetine and norfluoxetine concentrations in nursing infants and breast milk.* Biol Psychiatry, 2001. **50**(10): p. 775-82.

77. Ray, S. and Z.N. Stowe, *The use of antidepressant medication in pregnancy.* Best Pract Res Clin Obstet Gynaecol, 2014. **28**(1): p. 71-83.

78. Capello, C.F., et al., *Serotonin transporter occupancy in rats exposed to serotonin reuptake inhibitors in utero or via breast milk.* J Pharmacol Exp Ther, 2011. **339**(1): p. 275-85.

79. Wisner, K.L., J.M. Perel, and J. Blumer, *Serum sertraline and N-desmethylsertraline levels in breast-feeding mother-infant pairs.* Am J Psychiatry, 1998. **155**(5): p. 690-2.

80. Stowe, Z.N., et al., *Sertraline and desmethylsertraline in human breast milk and nursing infants.* Am J Psychiatry, 1997. **154**(9): p. 1255-60.

81. Mammen, O.K., et al., *Sertraline and norsertraline levels in three breastfed infants*. J Clin Psychiatry, 1997. **58**(3): p. 100-3.

82. Addis, A. and G. Koren, *Safety of fluoxetine during the first trimester of pregnancy: a meta-analytical review of epidemiological studies.* Psychol Med, 2000. **30**(1): p. 89-94.

83. Colvin, L., et al., *Dispensing patterns and pregnancy outcomes for women dispensed selective serotonin reuptake inhibitors in pregnancy*. Birth Defects Res A Clin Mol Teratol, 2011. **91**(3): p. 142-52.

84. Davis, R.L., et al., *Risks of congenital malformations and perinatal events among infants exposed to antidepressant medications during pregnancy*. Pharmacoepidemiol Drug Saf, 2007. **16**(10): p. 1086-94.

85. Einarson, A., et al., *Pregnancy outcome following gestational exposure to venlafaxine: a multicenter prospective controlled study*. Am J Psychiatry, 2001. **158**(10): p. 1728-30.

86. Kulin, N.A., et al., *Pregnancy outcome following maternal use of the new selective serotonin reuptake inhibitors: a prospective controlled multicenter study*. JAMA, 1998. **279**(8): p. 609-10.

87. Lund, N., L.H. Pedersen, and T.B. Henriksen, *Selective serotonin reuptake inhibitor exposure in utero and pregnancy outcomes.* Arch Pediatr Adolesc Med, 2009. **163**(10): p. 949-54.

88. Reis, M. and B. Kallen, *Delivery outcome after maternal use of antidepressant drugs in pregnancy: an update using Swedish data.* Psychol Med, 2010. **40**(10): p. 1723-33.

89. Wen, S.W., et al., *Selective serotonin reuptake inhibitors and adverse pregnancy outcomes.* Am J Obstet Gynecol, 2006. **194**(4): p. 961-6.

90. Goldstein, D.J., K.L. Sundell, and L.A. Corbin, *Birth outcomes in pregnant women taking fluoxetine.* N Engl J Med, 1997. **336**(12): p. 872-3; author reply 873.

91. Simon, G.E., M.L. Cunningham, and R.L. Davis, *Outcomes of prenatal antidepressant exposure.* Am J Psychiatry, 2002. **159**(12): p. 2055-61.

92. Grigoriadis, S., et al., *Prenatal exposure to antidepressants and persistent pulmonary hypertension of the newborn: systematic review and meta-analysis.* BMJ, 2014. **348**: p. f6932.

93. Bar-Oz, B., et al., *Paroxetine and congenital malformations: meta-Analysis and consideration of potential confounding factors.* Clin Ther, 2007. **29**(5): p. 918-26.

94. Kalra, S., et al., *The safety of antidepressant use in pregnancy.* Expert Opin Drug Saf, 2005. **4**(2): p. 273-84.

95. Grigoriadis, S., et al., *The effect of prenatal antidepressant exposure on neonatal adaptation: a systematic review and meta-analysis.* J Clin Psychiatry, 2013. **74**(4): p. e309-20.

96. Hemels, M.E., et al., *Antidepressant use during pregnancy and the rates of spontaneous abortions: a meta-analysis.* Ann Pharmacother, 2005. **39**(5): p. 803-9.

97. Nikfar, S., et al., *Increasing the risk of spontaneous abortion and major malformations in newborns following use of serotonin reuptake inhibitors during pregnancy: A systematic review and updated meta-analysis.* Daru, 2012. **20**(1): p. 75.

98. Riggin, L., et al., *The fetal safety of fluoxetine: a systematic review and meta-analysis.* J Obstet Gynaecol Can, 2013. **35**(4): p. 362-9.

99. Nikfar, S., M. Abdollahi, and P. Salari, *The efficacy and tolerability of exenatide in comparison to placebo; a systematic review and meta-analysis of randomized clinical trials.* J Pharm Pharm Sci, 2012. **15**(1): p. 1-30.

100. Ehrenthal, D.B., et al., *Independent relation of maternal prenatal factors to early childhood obesity in the offspring.* Obstet Gynecol, 2013. **121**(1): p. 115-21.

101. Goldstein, D.J., *Effects of third trimester fluoxetine exposure on the newborn.* J Clin Psychopharmacol, 1995. **15**(6): p. 417-20.

102. Einarson, A., et al., *Rates of major malformations in infants following exposure to duloxetine during pregnancy: a preliminary report.* J Clin Psychiatry, 2012. **73**(11): p. 1471.

103. Greene, M.F., *Teratogenicity of SSRIs--serious concern or much ado about little?* N Engl J Med, 2007. **356**(26): p. 2732-3.

104. Nakhai-Pour, H.R., P. Broy, and A. Berard, *Use of antidepressants during pregnancy and the risk of spontaneous abortion.* CMAJ, 2010. **182**(10): p. 1031-7.

105. Chambers, C.D., et al., *Birth outcomes in pregnant women taking fluoxetine.* N Engl J Med, 1996. **335**(14): p. 1010-5.

106. Louik, C., et al., *First-trimester use of selective serotonin-reuptake inhibitors and the risk of birth defects.* N Engl J Med, 2007. **356**(26): p. 2675-83.

107. Ericson, A., B. Kallen, and B. Wiholm, *Delivery outcome after the use of antidepressants in early pregnancy.* Eur J Clin Pharmacol, 1999. **55**(7): p. 503-8.

108. El Marroun, H., et al., *Maternal use of selective serotonin reuptake inhibitors, fetal growth, and risk of adverse birth outcomes.* Arch Gen Psychiatry, 2012. **69**(7): p. 706-14.

109. Lennestal, R. and B. Kallen, *Delivery outcome in relation to maternal use of some recently introduced antidepressants.* J Clin Psychopharmacol, 2007. **27**(6): p. 607-13.

110. Malm, H., T. Klaukka, and P.J. Neuvonen, *Risks associated with selective serotonin reuptake inhibitors in pregnancy.* Obstet Gynecol, 2005. **106**(6): p. 1289-96.

111. Costei, A.M., et al., *Perinatal outcome following third trimester exposure to paroxetine.* Arch Pediatr Adolesc Med, 2002. **156**(11): p. 1129-32.

112. Ramos, E., M. St-Andre, and A. Berard, *Association between antidepressant use during pregnancy and infants born small for gestational age.* Can J Psychiatry, 2010. **55**(10): p. 643-52.

113. Kallen, B., *Neonate characteristics after maternal use of antidepressants in late pregnancy.* Arch Pediatr Adolesc Med, 2004. **158**(4): p. 312-6.

114. Klieger-Grossmann, C., et al., *Prenatal exposure to mycophenolate mofetil: an updated estimate.* J Obstet Gynaecol Can, 2010. **32**(8): p. 794-7.

115. Zeskind, P.S. and L.E. Stephens, *Maternal selective serotonin reuptake inhibitor use during pregnancy and newborn neurobehavior.* Pediatrics, 2004. **113**(2): p. 368-75.

116. Oberlander, T.F., et al., *Neonatal outcomes after prenatal exposure to selective serotonin reuptake inhibitor antidepressants and maternal depression using population-based linked health data.* Arch Gen Psychiatry, 2006. **63**(8): p. 898-906.

117. Yonkers, K.A., et al., *Depression and serotonin reuptake inhibitor treatment as risk factors for preterm birth.* Epidemiology, 2012. **23**(5): p. 677-85.

118. Toh, S., et al., *Antidepressant use during pregnancy and the risk of preterm delivery and fetal growth restriction.* J Clin Psychopharmacol, 2009. **29**(6): p. 555-60.

119. Grzeskowiak, L.E., A.L. Gilbert, and J.L. Morrison, *Neonatal outcomes after late-gestation exposure to selective serotonin reuptake inhibitors.* J Clin Psychopharmacol, 2012. **32**(5): p. 615-21.

120. Nulman, I., et al., *Neurodevelopment of children exposed in utero to antidepressant drugs.* N Engl J Med, 1997. **336**(4): p. 258-62.

121. Cohen, L.S., et al., *Birth outcomes following prenatal exposure to fluoxetine.* Biol Psychiatry, 2000. **48**(10): p. 996-1000.

122. Pearson, K.H., et al., *Birth outcomes following prenatal exposure to antidepressants.* J Clin Psychiatry, 2007. **68**(8): p. 1284-9.

123. Wisner, K.L., et al., *Major depression and antidepressant treatment: impact on pregnancy and neonatal outcomes.* Am J Psychiatry, 2009. **166**(5): p. 557-66.

124. Huang, H., et al., *A meta-analysis of the relationship between antidepressant use in pregnancy and the risk of preterm birth and low birth weight.* Gen Hosp Psychiatry, 2014. **36**(1): p. 13-8.

125. Lattimore, K.A., et al., *Selective serotonin reuptake inhibitor (SSRI) use during pregnancy and effects on the fetus and newborn: a meta-analysis.* J Perinatol, 2005. **25**(9): p. 595-604.

126. Ross, L.E., et al., *Selected pregnancy and delivery outcomes after exposure to antidepressant medication: a systematic review and meta-analysis.* JAMA Psychiatry, 2013. **70**(4): p. 436-43.

127. Roca, A., et al., *Obstetrical and neonatal outcomes after prenatal exposure to selective serotonin reuptake inhibitors: the relevance of dose.* J Affect Disord, 2011. **135**(1-3): p. 208-15.

128. Oberlander, T.F., et al., *Hypothalamic-pituitary-adrenal (HPA) axis function in 3-month old infants with prenatal selective serotonin reuptake inhibitor (SSRI) antidepressant exposure.* Early Hum Dev, 2008. **84**(10): p. 689-97.

129. Alwan, S., et al., *Use of selective serotonin-reuptake inhibitors in pregnancy and the risk of birth defects.* N Engl J Med, 2007. **356**(26): p. 2684-92.

130. Klinger, G. and P. Merlob, *Selective serotonin reuptake inhibitor induced neonatal abstinence syndrome.* Isr J Psychiatry Relat Sci, 2008. **45**(2): p. 107-13.

131. Oberlander, T.F., et al., *Pharmacologic factors associated with transient neonatal symptoms following prenatal psychotropic medication exposure.* J Clin Psychiatry, 2004. **65**(2): p. 230-7.

132. Levinson-Castiel, R., et al., *Neonatal abstinence syndrome after in utero exposure to selective serotonin reuptake inhibitors in term infants.* Arch Pediatr Adolesc Med, 2006. **160**(2): p. 173-6.

133. Lewis, A.J., et al., *Neonatal growth outcomes at birth and one month postpartum following in utero exposure to antidepressant medication.* Aust N Z J Psychiatry, 2010. **44**(5): p. 482-7.

134. Margulis, A.V., et al., *Use of selective serotonin reuptake inhibitors in pregnancy and cardiac malformations: a propensity-score matched cohort in CPRD.* Pharmacoepidemiol Drug Saf, 2013. **22**(9): p. 942-51.

135. Leibovitch, L., et al., *Short-term neonatal outcome among term infants after in utero exposure to serotonin reuptake inhibitors.* Neonatology, 2013. **104**(1): p. 65-70.

136. Chambers, C.D., et al., *Selective serotonin-reuptake inhibitors and risk of persistent pulmonary hypertension of the newborn.* N Engl J Med, 2006. **354**(6): p. 579-87.

137. Kallen, B. and P.O. Olausson, *Maternal use of selective serotonin re-uptake inhibitors and persistent pulmonary hypertension of the newborn.* Pharmacoepidemiol Drug Saf, 2008. **17**(8): p. 801-6.

138. Kieler, H., et al., *Selective serotonin reuptake inhibitors during pregnancy and risk of persistent pulmonary hypertension in the newborn: population based cohort study from the five Nordic countries.* BMJ, 2012. **344**: p. d8012.

139. Reis, A., et al., *[Guidelines for the management of pulmonary hypertension patients]*. Rev Port Cardiol, 2010. **29**(2): p. 253-89.

140. Occhiogrosso, M., S.S. Omran, and M. Altemus, *Persistent pulmonary hypertension of the newborn and selective serotonin reuptake inhibitors: lessons from clinical and translational studies.* Am J Psychiatry, 2012. **169**(2): p. 134-40.

141. Wichman, C.L., et al., *Congenital heart disease associated with selective serotonin reuptake inhibitor use during pregnancy.* Mayo Clin Proc, 2009. **84**(1): p. 23-7.

142. Wilson, K.L., et al., *Persistent pulmonary hypertension of the newborn is associated with mode of delivery and not with maternal use of selective serotonin reuptake inhibitors.* Am J Perinatol, 2011. **28**(1): p. 19-24.

143. Boucher, N., A. Bairam, and L. Beaulac-Baillargeon, *A new look at the neonate's clinical presentation after in utero exposure to antidepressants in late pregnancy.* J Clin Psychopharmacol, 2008. **28**(3): p. 334-9.

144. Bertilsson, L., M.L. Dahl, and G. Tybring, *Pharmacogenetics of antidepressants: clinical aspects.* Acta Psychiatr Scand Suppl, 1997. **391**: p. 14-21.

145. Moses-Kolko, E.L., et al., *Neonatal signs after late in utero exposure to serotonin reuptake inhibitors: literature review and implications for clinical applications.* JAMA, 2005. **293**(19): p. 2372-83.

146. Isbister, G.K., et al., *Neonatal paroxetine withdrawal syndrome or actually serotonin syndrome?* Arch Dis Child Fetal Neonatal Ed, 2001. **85**(2): p. F147-8.

147. Mulder, E.J., et al., *Selective serotonin reuptake inhibitors affect neurobehavioral development in the human fetus.* Neuropsychopharmacology, 2011. **36**(10): p. 1961-71.

148. Popa, D., et al., *Lasting syndrome of depression produced by reduction in serotonin uptake during postnatal development: evidence from sleep, stress, and behavior.* J Neurosci, 2008. **28**(14): p. 3546-54.

149. Hansen, H.H. and J.D. Mikkelsen, *Long-term effects on serotonin transporter mRNA expression of chronic neonatal exposure to a serotonin reuptake inhibitor.* Eur J Pharmacol, 1998. **352**(2-3): p. 307-15.

150. Ansorge, M.S., et al., *Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice.* Science, 2004. **306**(5697): p. 879-81.

151. Bairy, K.L., et al., *Developmental and behavioral consequences of prenatal fluoxetine.* Pharmacology, 2007. **79**(1): p. 1-11.

152. Lisboa, S.F., et al., *Behavioral evaluation of male and female mice pups exposed to fluoxetine during pregnancy and lactation.* Pharmacology, 2007. **80**(1): p. 49-56.

153. Forcelli, P.A. and S.C. Heinrichs, *Teratogenic effects of maternal antidepressant exposure on neural substrates of drug-seeking behavior in offspring.* Addict Biol, 2008. **13**(1): p. 52-62.

154. Casper, R.C., et al., *Follow-up of children of depressed mothers exposed or not exposed to antidepressant drugs during pregnancy.* J Pediatr, 2003. **142**(4): p. 402-8.

155. Casper, R.C., et al., *Length of prenatal exposure to selective serotonin reuptake inhibitor (SSRI) antidepressants: effects on neonatal adaptation and psychomotor development.* Psychopharmacology (Berl), 2011. **217**(2): p. 211-9.

156. Oberlander, T.F., et al., *Pain reactivity in 2-month-old infants after prenatal and postnatal serotonin reuptake inhibitor medication exposure.* Pediatrics, 2005. **115**(2): p. 411-25.

157. Oberlander, T.F., et al., *Externalizing and attentional behaviors in children of depressed mothers treated with a selective serotonin reuptake inhibitor antidepressant during pregnancy.* Arch Pediatr Adolesc Med, 2007. **161**(1): p. 22-9.

158. Misri, S., et al., *Internalizing behaviors in 4-year-old children exposed in utero to psychotropic medications.* Am J Psychiatry, 2006. **163**(6): p. 1026-32.

159. Oberlander, T.F., et al., *Prenatal effects of selective serotonin reuptake inhibitor antidepressants, serotonin transporter promoter genotype (SLC6A4), and maternal mood on child behavior at 3 years of age.* Arch Pediatr Adolesc Med, 2010. **164**(5): p. 444-51.

160. Croen, L.A., et al., *Antidepressant use during pregnancy and childhood autism spectrum disorders.* Arch Gen Psychiatry, 2011. **68**(11): p. 1104-12.

161. Gentile, S. and M. Galbally, *Prenatal exposure to antidepressant medications and neurodevelopmental outcomes: a systematic review.* J Affect Disord, 2011. **128**(1-2): p. 1-9.

162. Gidaya, N.B., et al., *In Utero Exposure to Selective Serotonin Reuptake Inhibitors and Risk for Autism Spectrum Disorder.* J Autism Dev Disord, 2014.

163. Harrington, R.A., et al., *Serotonin hypothesis of autism: implications for selective serotonin reuptake inhibitor use during pregnancy.* Autism Res, 2013. **6**(3): p. 149-68.

164. Hviid, A., M. Melbye, and B. Pasternak, *Use of selective serotonin reuptake inhibitors during pregnancy and risk of autism.* N Engl J Med, 2013. **369**(25): p. 2406-15.

165. Sorensen, M.J., et al., *Antidepressant exposure in pregnancy and risk of autism spectrum disorders.* Clin Epidemiol, 2013. **5**: p. 449-59.

166. Lee, L.J. and L.J. Lee, *Neonatal fluoxetine exposure alters motor performances of adolescent rats.* Dev Neurobiol, 2012. **72**(8): p. 1122-32.

167. Bakker, M.K., et al., *Fluoxetine and infantile hypertrophic pylorus stenosis: a signal from a birth defects-drug exposure surveillance study.* Pharmacoepidemiol Drug Saf, 2010. **19**(8): p. 808-13.

168. Potts, A.L., et al., *Necrotizing enterocolitis associated with in utero and breast milk exposure to the selective serotonin reuptake inhibitor, escitalopram.* J Perinatol, 2007. **27**(2): p. 120-2.

169. Nijenhuis, C.M., ter Horst, G.J., Lolkje, T.W., de Jong-van den Berg, L.T., Wilffert, B., *Disturbed development of the enteric nervous system after in utero exposure of selective serotonin re-uptake inhibitors and tricyclic antidepressants. Part 1: Literature Review.* Br J Clin Pharmacol, 2011. **73**(1): p. 16-26.

170. Nijenhuis, C.M., ter Horst, van Rein, N., Wilffert, B., Jong-van den Berg, L.T., *Disturbed development of the enteric nervous system after in utero exposure of selective serotonin reuptake inhibitors and tricyclic antidepressants. Part 2: Testing the hypotheses.* Br J Clin Pharmacol, 2011. **73**(1): p. 126-134.

171. de Montigny, C., Y. Chaput, and P. Blier, *Modification of serotonergic neuron properties by long-term treatment with serotonin reuptake blockers.* J Clin Psychiatry, 1990. **51 Suppl B**: p. 4-8.

172. Homberg, J.R., D. Schubert, and P. Gaspar, *New perspectives on the neurodevelopmental effects of SSRIs.* Trends Pharmacol Sci, 2010. **31**(2): p. 60-5.

173. Gershon, M.D., *V. Genes, lineages, and tissue interactions in the development of the enteric nervous system.* Am J Physiol, 1998. **275**(5 Pt 1): p. G869-73.

174. Li, Z., et al., *Dependence of serotonergic and other nonadrenergic enteric neurons on norepinephrine transporter expression.* J Neurosci, 2010. **30**(49): p. 16730-40.

175. Grahame-Smith, D.G., *Serotonin function in affective disorders.* Acta Psychiatr Scand Suppl, 1989. **350**: p. 7-12.

176. Shelton, R.C., *Classification of Antidepressants and Their Clinical Implications.* Primary Care Companion J Clin Psychiatry, 2003. **5 (suppl 7)**: p. 27-32.

177. Thomas, D.R., et al., *m-Chlorophenylpiperazine (mCPP) is an antagonist at the cloned human 5-HT2B receptor.* Neuroreport, 1996. **7**(9): p. 1457-60.

178. Zhang, S., et al., *5-HT2B receptors are expressed on astrocytes from brain and in culture and are a chronic target for all five conventional 'serotonin-specific reuptake inhibitors'.* Neuron Glia Biol, 2010. **6**(2): p. 113-25.

179. Derijks, H.J., et al., *The association between antidepressant use and hypoglycaemia in diabetic patients: a nested case-control study.* Pharmacoepidemiol Drug Saf, 2008. **17**(4): p. 336-44.

180. Owens, M.J. and C.B. Nemeroff, *Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter.* Clin Chem, 1994. **40**(2): p. 288-95.

181. Sommer, C., *Serotonin in pain and analgesia: actions in the periphery.* Mol Neurobiol, 2004. **30**(2): p. 117-25.

182. Blundell, J.E. and N.A. King, *Effects of exercise on appetite control: loose coupling between energy expenditure and energy intake.* Int J Obes Relat Metab Disord, 1998. **22 Suppl 2**: p. S22-9.

183. Yadav, V.K., et al., *A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure.* Cell, 2009. **138**(5): p. 976-89.

184. Feijo Fde, M., M.C. Bertoluci, and C. Reis, *[Serotonin and hypothalamic control of hunger: a review].* Rev Assoc Med Bras, 2011. **57**(1): p. 74-7.

185. Buhot, M.C., S. Martin, and L. Segu, *Role of serotonin in memory impairment.* Ann Med, 2000. **32**(3): p. 210-21.

186. Gallager, D.W. and G.K. Aghajanian, *Effects of chlorimipramine and lysergic acid diethylamide on efflux of precursor-formed 3-H-serotonin: correlations with serotonergic impulse flow.* J Pharmacol Exp Ther, 1975. **193**(3): p. 785-95.

187. Erspamer, V. and A. Testini, *Observations on the release and turnover rate of 5-hydroxytryptamine in the gastrointestinal tract.* J Pharm Pharmacol, 1959. **11**: p. 618-23.

188. Camilleri, M., *Serotonin in the gastrointestinal tract.* Curr Opin Endocrinol Diabetes Obes, 2009. **16**(1): p. 53-9.

189. Gershon, M.D., *5-HT (serotonin) physiology and related drugs.* Curr Opin Gastroenterol, 2000. **16**(2): p. 113-20.

190. Goyal, R.K. and I. Hirano, *The enteric nervous system.* N Engl J Med, 1996. **334**(17): p. 1106-15.

191. Wade, P.R., et al., *Analysis of the role of 5-HT in the enteric nervous system using anti-idiotopic antibodies to 5-HT receptors.* Am J Physiol, 1994. **266**(3 Pt 1): p. G403-16.

192. Gershon, M.D., *Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility.* Aliment Pharmacol Ther, 2004. **20 Suppl 7**: p. 3-14.

193. Walther, D.J., et al., *Synthesis of serotonin by a second tryptophan hydroxylase isoform.* Science, 2003. **299**(5603): p. 76.

194. Kim, D.Y. and M. Camilleri, *Serotonin: a mediator of the brain-gut connection.* Am J Gastroenterol, 2000. **95**(10): p. 2698-709.

195. Berger, M., J.A. Gray, and B.L. Roth, *The expanded biology of serotonin.* Annu Rev Med, 2009. **60**: p. 355-66.

196. Gershon, M.D., *Plasticity in serotonin control mechanisms in the gut.* Curr Opin Pharmacol, 2003. **3**(6): p. 600-7.

197. Hansen, M.B. and A.B. Witte, *The role of serotonin in intestinal luminal sensing and secretion.* Acta Physiol (Oxf), 2008. **193**(4): p. 311-23.

198. Cirillo, C., P. Vanden Berghe, and J. Tack, *Role of serotonin in gastrointestinal physiology and pathology*. Minerva Endocrinol, 2011. **36**(4): p. 311-24.

199. Stasi, C., Bellini, M., Bassotti, G., Blandizzi, C., Milani, S., *Serotonin receptors and their role in the pathophysiology and therapy of irritable bowel syndrome.* Tech Coloproctol, 2013. **18**(7): p. 613-21.

200. Mawe, G.M. and J.M. Hoffman, *Serotonin signalling in the gut--functions, dysfunctions and therapeutic targets.* Nat Rev Gastroenterol Hepatol, 2013. **10**(8): p. 473-86.

201. Choi, D.S. and L. Maroteaux, *Immunohistochemical localisation of the serotonin 5-HT2B receptor in mouse gut, cardiovascular system, and brain.* FEBS Lett, 1996. **391**(1-2): p. 45-51.

202. Fuller, R.W. and D.T. Wong, *Serotonin uptake and serotonin uptake inhibition.* Ann N Y Acad Sci, 1990. **600**: p. 68-78; discussion 79-80.

203. Iversen, L., *Neurotransmitter transporters: fruitful targets for CNS drug discovery.* Mol Psychiatry, 2000. **5**(4): p. 357-62.

204. Chen, J.G., S. Liu-Chen, and G. Rudnick, *Determination of external loop topology in the serotonin transporter by site-directed chemical labeling.* J Biol Chem, 1998. **273**(20): p. 12675-81.

205. Nichols, D.E. and C.D. Nichols, *Serotonin receptors.* Chem Rev, 2008. **108**(5): p. 1614-41.

206. Chen, J.X., et al., *Guinea pig 5-HT transporter: cloning, expression, distribution, and function in intestinal sensory reception.* Am J Physiol, 1998. **275**(3 Pt 1): p. G433-48.

207. Chen, J.J., et al., *Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters.* J Neurosci, 2001. **21**(16): p. 6348-61.

208. Coates, M.D., et al., *Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome.* Gastroenterology, 2004. **126**(7): p. 1657-64.

209. Wade, P.R., et al., *Localization and function of a 5-HT transporter in crypt epithelia of the gastrointestinal tract.* J Neurosci, 1996. **16**(7): p. 2352-64.

210. Gershon, M.D. and L.L. Ross, *Location of sites of 5-hydroxytryptamine storage and metabolism by radioautography.* J Physiol, 1966. **186**(2): p. 477-92.

211. Gershon, M.D., D.L. Sherman, and J.E. Pintar, *Type-specific localization of monoamine oxidase in the enteric nervous system: relationship to 5-hydroxytryptamine, neuropeptides, and sympathetic nerves.* J Comp Neurol, 1990. **301**(2): p. 191-213.

212. Gaspar, P., O. Cases, and L. Maroteaux, *The developmental role of serotonin: news from mouse molecular genetics.* Nat Rev Neurosci, 2003. **4**(12): p. 1002-12.

213. Hansson, S.R., E. Mezey, and B.J. Hoffman, *Serotonin transporter messenger RNA expression in neural crest-derived structures and sensory pathways of the developing rat embryo.* Neuroscience, 1999. **89**(1): p. 243-65.

214. Fiorica-Howells, E., L. Maroteaux, and M.D. Gershon, *Serotonin and the 5-HT(2B) receptor in the development of enteric neurons.* J Neurosci, 2000. **20**(1): p. 294-305.

215. Tharayil, V.S., et al., *Lack of serotonin 5-HT2B receptor alters proliferation and network volume of interstitial cells of Cajal in vivo.* Neurogastroenterol Motil, 2010. **22**(4): p. 462-9, e109-10.

216. Wouters, M.M., et al., *Exogenous serotonin regulates proliferation of interstitial cells of Cajal in mouse jejunum through 5-HT2B receptors.* Gastroenterology, 2007. **133**(3): p. 897-906.

217. Daws, L.C. and G.G. Gould, *Ontogeny and regulation of the serotonin transporter: providing insights into human disorders.* Pharmacol Ther, 2011. **131**(1): p. 61-79.

218. Ansorge, M.S., R. Hen, and J.A. Gingrich, *Neurodevelopmental origins of depressive disorders.* Curr Opin Pharmacol, 2007. **7**(1): p. 8-17.

219. Haenisch, B. and H. Bonisch, *Interaction of the human plasma membrane monoamine transporter (hPMAT) with antidepressants and antipsychotics.* Naunyn Schmiedebergs Arch Pharmacol, 2010. **381**(1): p. 33-9.

220. Haenisch, B. and H. Bonisch, *Depression and antidepressants: insights from knockout of dopamine, serotonin or noradrenaline re-uptake transporters.* Pharmacol Ther, 2011. **129**(3): p. 352-68.

221. Roth, B.L., *Multiple serotonin receptors: clinical and experimental aspects.* Ann Clin Psychiatry, 1994. **6**(2): p. 67-78.

222. Roth, B.L. and Z. Xia, *Molecular and cellular mechanisms for the polarized sorting of serotonin receptors: relevance for genesis and treatment of psychosis.* Crit Rev Neurobiol, 2004. **16**(4): p. 229-36.

223. Anderson, G.M., *Peripheral and central neurochemical effects of the selective serotonin reuptake inhibitors (SSRIs) in humans and nonhuman primates: assessing bioeffect and mechanisms of action.* Int J Dev Neurosci, 2004. **22**(5-6): p. 397-404.

224. Anderson, G.M., et al., *Platelet serotonin in newborns and infants: ontogeny, heritability, and effect of in utero exposure to selective serotonin reuptake inhibitors.* Pediatr Res, 2004. **56**(3): p. 418-22.

225. Borue, X., J. Chen, and B.G. Condron, *Developmental effects of SSRIs: lessons learned from animal studies.* Int J Dev Neurosci, 2007. **25**(6): p. 341-7.

226. Cabrera-Vera, T.M., et al., *Effect of prenatal fluoxetine (Prozac) exposure on brain serotonin neurons in prepubescent and adult male rat offspring.* J Pharmacol Exp Ther, 1997. **280**(1): p. 138-45.

227. Nebigil, C.G., et al., *Developmentally regulated serotonin 5-HT2B receptors.* Int J Dev Neurosci, 2001. **19**(4): p. 365-72.

228. Gershon, M.D., *Serotonin and its implication for the management of irritable bowel syndrome.* Rev Gastroenterol Disord, 2003. **3 Suppl 2**: p. S25-34.

229. Musumeci, G., et al., *Serotonin (5HT) expression in rat pups treated with high-tryptophan diet during fetal and early postnatal development.* Acta Histochem, 2014. **116**(2): p. 335-43.

230. Kong, E.K., et al., *Up-regulation of 5-HT2B receptor density and receptor-mediated glycogenolysis in mouse astrocytes by long-term fluoxetine administration.* Neurochem Res, 2002. **27**(1-2): p. 113-20.

231. Anji, A., et al., *Regulation of 5-HT(2A) receptor mRNA levels and binding sites in rat frontal cortex by the agonist DOI and the antagonist mianserin.* Neuropharmacology, 2000. **39**(11): p. 1996-2005.

232. Kuoppamaki, M., et al., *Regulation of serotonin 5-HT2C receptors in the rat choroid plexus after acute clozapine treatment.* Eur J Pharmacol, 1994. **269**(2): p. 201-8.

233. Pranzatelli, M.R., J.N. Murthy, and P.T. Tailor, *Novel regulation of 5-HT1C receptors: down-regulation induced both by 5-HT1C/2 receptor agonists and antagonists.* Eur J Pharmacol, 1993. **244**(1): p. 1-5.

234. Roth, B.L., et al., *5-Hydroxytryptamine2-family receptors (5-hydroxytryptamine2A, 5-hydroxytryptamine2B, 5-hydroxytryptamine2C): where structure meets function.* Pharmacol Ther, 1998. **79**(3): p. 231-57.

235. Uphouse, L., *Multiple serotonin receptors: too many, not enough, or just the right number?* Neurosci Biobehav Rev, 1997. **21**(5): p. 679-98.

236. Burns, A.J. and N. Thapar, *Advances in ontogeny of the enteric nervous system.* Neurogastroenterol Motil, 2006. **18**(10): p. 876-87.

237. Spiller, R. and C. Lam, *The shifting interface between IBS and IBD.* Curr Opin Pharmacol, 2011. **11**(6): p. 586-92.

238. Loftus, E.V., Jr., *Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences.* Gastroenterology, 2004. **126**(6): p. 1504-17.

239. Burisch, J., *Crohn's disease and ulcerative colitis. Occurrence, course and prognosis during the first year of disease in a European population-based inception cohort.* Dan Med J, 2014. **61**(1): p. B4778.

240. Moris, G., *Inflammatory bowel disease: an increased risk factor for neurologic complications.* World J Gastroenterol, 2014. **20**(5): p. 1228-37.

241. Canavan, C., J. West, and T. Card, *The epidemiology of irritable bowel syndrome.* Clin Epidemiol, 2014. **6**: p. 71-80.

242. Camilleri, M. and D.A. Katzka, *Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Genetic epidemiology and pharmacogenetics in irritable bowel syndrome.* Am J Physiol Gastrointest Liver Physiol, 2012. **302**(10): p. G1075-84.

243. Drossman, D.A., et al., *Severity in irritable bowel syndrome: a Rome Foundation Working Team report.* Am J Gastroenterol, 2011. **106**(10): p. 1749-59; quiz 1760.

244. Lovell, R.M. and A.C. Ford, *Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis.* Clin Gastroenterol Hepatol, 2012. **10**(7): p. 712-721 e4.

245. Monnikes, H., *Quality of life in patients with irritable bowel syndrome.* J Clin Gastroenterol, 2011. **45 Suppl**: p. S98-101.

246. Spiller, R. and E. Campbell, *Post-infectious irritable bowel syndrome.* Curr Opin Gastroenterol, 2006. **22**(1): p. 13-7.

247. Spiller, R.C., *Overlap between irritable bowel syndrome and inflammatory bowel disease.* Dig Dis, 2009. **27 Suppl 1**: p. 48-54.

248. Spiller, R.C., *Irritable bowel syndrome: gender, infection, lifestyle or what else?* Dig Dis, 2011. **29**(2): p. 215-21.

249. Gwee, K.A., *The many faces of irritable bowel syndrome.* Singapore Med J, 1999. **40**(7): p. 441-2.

250. Morris-Yates, A., et al., *Evidence of a genetic contribution to functional bowel disorder.* Am J Gastroenterol, 1998. **93**(8): p. 1311-7.

251. Mohammed, I., et al., *Genetic influences in irritable bowel syndrome: a twin study.* Am J Gastroenterol, 2005. **100**(6): p. 1340-4.

252. Levy, R.L., et al., *Irritable bowel syndrome in twins: heredity and social learning both contribute to etiology.* Gastroenterology, 2001. **121**(4): p. 799-804.

253. van der Veek, P.P., et al., *Role of tumor necrosis factor-alpha and interleukin-10 gene polymorphisms in irritable bowel syndrome.* Am J Gastroenterol, 2005. **100**(11): p. 2510-6.

254. Zucchelli, M., et al., *Association of TNFSF15 polymorphism with irritable bowel syndrome.* Gut, 2011. **60**(12): p. 1671-7.

255. Spiller, R. and K. Garsed, *Infection, inflammation, and the irritable bowel syndrome.* Dig Liver Dis, 2009. **41**(12): p. 844-9.

256. Spence, M.J. and R. Moss-Morris, *The cognitive behavioural model of irritable bowel syndrome: a prospective investigation of patients with gastroenteritis.* Gut, 2007. **56**(8): p. 1066-71.

257. Levy, R.L., et al., *Psychosocial aspects of the functional gastrointestinal disorders.* Gastroenterology, 2006. **130**(5): p. 1447-58.

258. Gibbs-Gallagher, N., et al., *Selective recall of gastrointestinal-sensation words: evidence for a cognitive-behavioral contribution to irritable bowel syndrome.* Am J Gastroenterol, 2001. **96**(4): p. 1133-8.

259. Longstreth, G.F., et al., *Characteristics of patients with irritable bowel syndrome recruited from three sources: implications for clinical trials.* Aliment Pharmacol Ther, 2001. **15**(7): p. 959-64.

260. Chaudhary, N.A. and S.C. Truelove, *The irritable colon syndrome. A study of the clinical features, predisposing causes, and prognosis in 130 cases.* Q J Med, 1962. **31**: p. 307-22.

261. Drossman, D.A., *The functional gastrointestinal disorders and the Rome III process.* Gastroenterology, 2006. **130**(5): p. 1377-90.

262. Longstreth, G.F., et al., *Functional bowel disorders.* Gastroenterology, 2006. **130**(5): p. 1480-91.

263. Podolsky, D.K., *The current future understanding of inflammatory bowel disease.* Best Pract Res Clin Gastroenterol, 2002. **16**(6): p. 933-43.

264. Ng, S.C., et al., *Incidence and phenotype of inflammatory bowel disease based on results from the Asia-pacific Crohn's and colitis epidemiology study.* Gastroenterology, 2013. **145**(1): p. 158-165 e2.

265. Molodecky, N.A., et al., *Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review.* Gastroenterology, 2012. **142**(1): p. 46-54 e42; quiz e30.

266. Berg, A.M., A.N. Dam, and F.A. Farraye, *Environmental influences on the onset and clinical course of Crohn's disease-part 2: infections and medication use.* Gastroenterol Hepatol (N Y), 2013. **9**(12): p. 803-10.

267. Dam, A.N., A.M. Berg, and F.A. Farraye, *Environmental influences on the onset and clinical course of Crohn's disease-part 1: an overview of external risk factors.* Gastroenterol Hepatol (N Y), 2013. **9**(11): p. 711-7.

268. Kronman, M.P., et al., *Variation in Antibiotic Use for Children Hospitalized With Inflammatory Bowel Disease Exacerbation: A Multicenter Validation Study.* J Pediatric Infect Dis Soc, 2012. **1**(4): p. 306-313.

269. Virta, L.J. and K.L. Kolho, *Trends in early outpatient drug therapy in pediatric inflammatory bowel disease in Finland: a nationwide register-based study in 1999-2009.* ISRN Gastroenterol, 2012. **2012**: p. 462642.

270. Card, T., Logan, R.G., Rodrigues, L.C., Wheeler, J.G., *Antibiotic use and the development of Crohn's disease.* Gut, 2004. **53**(2): p. 246-250.

271. Shaw, S.Y., J.F. Blanchard, and C.N. Bernstein, *Association between the use of antibiotics and new diagnoses of Crohn's disease and ulcerative colitis.* Am J Gastroenterol, 2011. **106**(12): p. 2133-42.

272. Ohman, L. and M. Simren, *Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions.* Nat Rev Gastroenterol Hepatol, 2010. **7**(3): p. 163-73.

273. Zhou, Q. and G.N. Verne, *New insights into visceral hypersensitivity--clinical implications in IBS.* Nat Rev Gastroenterol Hepatol, 2011. **8**(6): p. 349-55.

274. Racke, K., et al., *Regulation of 5-HT release from enterochromaffin cells.* Behav Brain Res, 1996. **73**(1-2): p. 83-7.

275. Belai, A., et al., *Neurochemical coding in the small intestine of patients with Crohn's disease.* Gut, 1997. **40**(6): p. 767-74.

276. Camilleri, M., et al., *Efficacy and safety of alosetron in women with irritable bowel syndrome: a randomised, placebo-controlled trial.* Lancet, 2000. **355**(9209): p. 1035-40.

277. El-Salhy, M., et al., *Colonic endocrine cells in inflammatory bowel disease.* J Intern Med, 1997. **242**(5): p. 413-9.

278. Bearcroft, C.P., D. Perrett, and M.J. Farthing, *Postprandial plasma 5-hydroxytryptamine in diarrhoea predominant irritable bowel syndrome: a pilot study.* Gut, 1998. **42**(1): p. 42-6.

279. Bertrand, P.P., et al., *Analysis of real-time serotonin (5-HT) availability during experimental colitis in mouse.* Am J Physiol Gastrointest Liver Physiol, 2010. **298**(3): p. G446-55.

280. Spiller, R.C., et al., *Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome.* Gut, 2000. **47**(6): p. 804-11.

281. Oshima, S., M. Fujimura, and M. Fukimiya, *Changes in number of serotonin-containing cells and serotonin levels in the intestinal mucosa of rats with colitis induced by dextran sodium sulfate.* Histochem Cell Biol, 1999. **112**(4): p. 257-63.

282. Linden, D.R., et al., *Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis.* Am J Physiol Gastrointest Liver Physiol, 2003. **285**(1): p. G207-16.

283. Khan, W.I., et al., *Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells.* Am J Physiol Gastrointest Liver Physiol, 2006. **291**(5): p. G803-11.

284. Bischoff, S.C., et al., *Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice.* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(3): p. G685-95.

285. Haub, S., et al., *Enhancement of intestinal inflammation in mice lacking interleukin 10 by deletion of the serotonin reuptake transporter.* Neurogastroenterol Motil, 2010. **22**(7): p. 826-34, e229.

286. Ghia, J.E., et al., *Serotonin has a key role in pathogenesis of experimental colitis.* Gastroenterology, 2009. **137**(5): p. 1649-60.

287. Mawe, G.M., M.D. Coates, and P.L. Moses, *Review article: intestinal serotonin signalling in irritable bowel syndrome.* Aliment Pharmacol Ther, 2006. **23**(8): p. 1067-76.

288. Kidd, M., et al., *IL1beta- and LPS-induced serotonin secretion is increased in EC cells derived from Crohn's disease.* Neurogastroenterol Motil, 2009. **21**(4): p. 439-50.

289. Cremon, C., et al., *Intestinal serotonin release, sensory neuron activation, and abdominal pain in irritable bowel syndrome.* Am J Gastroenterol, 2011. **106**(7): p. 1290-8.

290. Ahonen, A., K. Kyosola, and O. Penttila, *Enterochromaffin cells in macrophages in ulcerative colitis and irritable colon.* Ann Clin Res, 1976. **8**(1): p. 1-7.

291. Bishop, A.E., et al., *Increased populations of endocrine cells in Crohn's ileitis.* Virchows Arch A Pathol Anat Histopathol, 1987. **410**(5): p. 391-6.

292. Spiller, R. and C. Lam, *An Update on Post-infectious Irritable Bowel Syndrome: Role of Genetics, Immune Activation, Serotonin and Altered Microbiome.* J Neurogastroenterol Motil, 2012. **18**(3): p. 258-68.

293. Pastorelli, L., et al., *Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics.* Front Immunol, 2013. **4**: p. 280.

294. Wheatcroft, J., et al., *Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction.* Neurogastroenterol Motil, 2005. **17**(6): p. 863-70.

295. Wang, H., et al., *CD4+ T cell-mediated immunological control of enterochromaffin cell hyperplasia and 5-hydroxytryptamine production in enteric infection.* Gut, 2007. **56**(7): p. 949-57.

296. Wang, S.H., et al., *Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome.* World J Gastroenterol, 2007. **13**(45): p. 6041-7.

297. Dunlop, S.P., et al., *Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS.* Gastroenterology, 2003. **125**(6): p. 1651-9.

298. Dunlop, S.P., et al., *Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome.* Clin Gastroenterol Hepatol, 2005. **3**(4): p. 349-57.

299. Singh, R.K., H.P. Pandey, and R.H. Singh, *Correlation of serotonin and monoamine oxidase levels with anxiety level in diarrhea-predominant irritable bowel syndrome.* Indian J Gastroenterol, 2003. **22**(3): p. 88-90.

300. Minderhoud, I.M., et al., *Serotonin synthesis and uptake in symptomatic patients with Crohn's disease in remission.* Clin Gastroenterol Hepatol, 2007. **5**(6): p. 714-20.

301. Isgar, B., et al., *Symptoms of irritable bowel syndrome in ulcerative colitis in remission.* Gut, 1983. **24**(3): p. 190-2.

302. O'Hara, J.R., et al., *Consequences of Citrobacter rodentium infection on enteroendocrine cells and the enteric nervous system in the mouse colon.* Cell Microbiol, 2006. **8**(4): p. 646-60.

303. Motomura, Y., et al., *Enterochromaffin cell and 5-hydroxytryptamine responses to the same infectious agent differ in Th1 and Th2 dominant environments.* Gut, 2008. **57**(4): p. 475-81.

304. Manocha, M., et al., *IL-13-mediated immunological control of enterochromaffin cell hyperplasia and serotonin production in the gut.* Mucosal Immunol, 2013. **6**(1): p. 146-55.

305. Shajib, M.S., et al., *Interleukin 13 and serotonin: linking the immune and endocrine systems in murine models of intestinal inflammation.* PLoS One, 2013. **8**(8): p. e72774.

306. Liu, M.T., et al., *Expression and function of 5-HT3 receptors in the enteric neurons of mice lacking the serotonin transporter*. Am J Physiol Gastrointest Liver Physiol, 2002. **283**(6): p. G1398-411.

307. Yang, G.B. and A.A. Lackner, *Proximity between 5-HT secreting enteroendocrine cells and lymphocytes in the gut mucosa of rhesus macaques (Macaca mulatta) is suggestive of a role for enterochromaffin cell 5-HT in mucosal immunity*. J Neuroimmunol, 2004. **146**(1-2): p. 46-9.

308. Beagley, K.W. and C.O. Elson, *Cells and cytokines in mucosal immunity and inflammation.* Gastroenterol Clin North Am, 1992. **21**(2): p. 347-66.

309. Eade, O.E., et al., *Lymphocyte subpopulations of intestinal mucosa in inflammatory bowel disease*. Gut, 1980. **21**(8): p. 675-82.

310. Schreiber, S., et al., *Increased in vitro release of soluble interleukin 2 receptor by colonic lamina propria mononuclear cells in inflammatory bowel disease.* Gut, 1992. **33**(2): p. 236-41.

311. Schreiber, S., et al., *The role of the mucosal immune system in inflammatory bowel disease.* Gastroenterol Clin North Am, 1992. **21**(2): p. 451-502.

312. Chadwick, V.S., et al., *Activation of the mucosal immune system in irritable bowel syndrome.* Gastroenterology, 2002. **122**(7): p. 1778-83.

313. Collins, S.M., *Peripheral mechanisms of symptom generation in irritable bowel syndrome*. Can J Gastroenterol, 2001. **15 Suppl B**: p. 14B-16B.

314. Liebregts, T., et al., *Immune activation in patients with irritable bowel syndrome.* Gastroenterology, 2007. **132**(3): p. 913-20.

315. Wang, L.H., X.C. Fang, and G.Z. Pan, *Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis.* Gut, 2004. **53**(8): p. 1096-101.

316. Schmulson, M. and W.D. Chey, *Abnormal immune regulation and low-grade inflammation in IBS: does one size fit all?* Am J Gastroenterol, 2012. **107**(2): p. 273-5.

317. Spiller, R.C., *Postinfectious irritable bowel syndrome.* Gastroenterology, 2003. **124**(6): p. 1662-71.

318. Akiho, H., E. Ihara, and K. Nakamura, *Low-grade inflammation plays a pivotal role in gastrointestinal dysfunction in irritable bowel syndrome.* World J Gastrointest Pathophysiol, 2010. **1**(3): p. 97-105.

319. Brydon, L., et al., *Psychological stress activates interleukin-1beta gene expression in human mononuclear cells.* Brain Behav Immun, 2005. **19**(6): p. 540-6.

320. Gwee, K.A., et al., *Increased rectal mucosal expression of interleukin 1beta in recently acquired post-infectious irritable bowel syndrome.* Gut, 2003. **52**(4): p. 523-6.

321. Dinan, T.G., et al., *Enhanced cholinergic-mediated increase in the pro-inflammatory cytokine IL-6 in irritable bowel syndrome: role of muscarinic receptors.* Am J Gastroenterol, 2008. **103**(10): p. 2570-6.

322. Li, N., et al., *Serotonin activates dendritic cell function in the context of gut inflammation.* Am J Pathol, 2011. **178**(2): p. 662-71.

323. Margolis, K.G., et al., *Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine.* Gut, 2014. **63**(6): p. 928-37.

324. Seidel, M.F., et al., *Serotonin mediates PGE2 overexpression through 5-HT2A and 5-HT3 receptor subtypes in serum-free tissue culture of macrophage-like synovial cells.* Rheumatol Int, 2008. **28**(10): p. 1017-22.

325. Fiebich, B.L., et al., *Antiinflammatory effects of 5-HT3 receptor antagonists in lipopolysaccharide-stimulated primary human monocytes.* Scand J Rheumatol Suppl, 2004. **119**: p. 28-32.

326. Idzko, M., et al., *The serotoninergic receptors of human dendritic cells: identification and coupling to cytokine release.* J Immunol, 2004. **172**(10): p. 6011-9.

327. Kato, S., *Role of serotonin 5-HT(3) receptors in intestinal inflammation.* Biol Pharm Bull, 2013. **36**(9): p. 1406-9.

328. Mousavizadeh, K., et al., *Anti-inflammatory effects of 5-HT receptor antagonist, tropisetron on experimental colitis in rats.* Eur J Clin Invest, 2009. **39**(5): p. 375-83.

329. Motavallian-Naeini, A., et al., *Validation and optimization of experimental colitis induction in rats using 2, 4, 6-trinitrobenzene sulfonic acid.* Res Pharm Sci, 2012. **7**(3): p. 159-69.

330. Kim, J.J., et al., *Targeted inhibition of serotonin type 7 (5-HT7) receptor function modulates immune responses and reduces the severity of intestinal inflammation.* J Immunol, 2013. **190**(9): p. 4795-804.

331. Bundeff, A.W. and C.B. Woodis, *Selective serotonin reuptake inhibitors for the treatment of irritable bowel syndrome.* Ann Pharmacother, 2014. **48**(6): p. 777-84.

332. Matricon, J., et al., *Review article: Associations between immune activation, intestinal permeability and the irritable bowel syndrome.* Aliment Pharmacol Ther, 2012. **36**(11-12): p. 1009-31.

333. Nobaek, S., et al., *Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome.* Am J Gastroenterol, 2000. **95**(5): p. 1231-8.

334. Gershon, M.D. and J. Tack, *The serotonin signaling system: from basic understanding to drug development for functional GI disorders.* Gastroenterology, 2007. **132**(1): p. 397-414.

335. Dunlop, S.P., et al., *Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes.* Am J Gastroenterol, 2006. **101**(6): p. 1288-94.

336. Piche, T., et al., *Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators.* Gut, 2009. **58**(2): p. 196-201.

337. Piche, T., *Tight junctions and IBS--the link between epithelial permeability, low-grade inflammation, and symptom generation?* Neurogastroenterol Motil, 2014. **26**(3): p. 296-302.

338. Spiller, R.C., *Role of infection in irritable bowel syndrome.* J Gastroenterol, 2007. **42 Suppl 17**: p. 41-7.

339. Marshall, J.K., et al., *Intestinal permeability in patients with irritable bowel syndrome after a waterborne outbreak of acute gastroenteritis in Walkerton, Ontario.* Aliment Pharmacol Ther, 2004. **20**(11-12): p. 1317-22.

340. Zhou, Q., B. Zhang, and G.N. Verne, *Intestinal membrane permeability and hypersensitivity in the irritable bowel syndrome.* Pain, 2009. **146**(1-2): p. 41-6.

341. Gorard, D.A., G.W. Libby, and M.J. Farthing, *Ambulatory small intestinal motility in 'diarrhoea' predominant irritable bowel syndrome.* Gut, 1994. **35**(2): p. 203-10.

342. Spigset, O., *Adverse reactions of selective serotonin reuptake inhibitors: reports from a spontaneous reporting system.* Drug Saf, 1999. **20**(3): p. 277-87.

343. Furuse, M., et al., *Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin.* J Cell Biol, 1998. **141**(7): p. 1539-50.

344. Furuse, M., et al., *A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts.* J Cell Biol, 1998. **143**(2): p. 391-401.

345. Nusrat, A., J.R. Turner, and J.L. Madara, *Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells.* Am J Physiol Gastrointest Liver Physiol, 2000. **279**(5): p. G851-7.

346. Tsukita, S., M. Furuse, and M. Itoh, *Structural and signalling molecules come together at tight junctions.* Curr Opin Cell Biol, 1999. **11**(5): p. 628-33.

347. Al-Sadi, R., et al., *Cellular and molecular mechanism of interleukin-1beta modulation of Caco-2 intestinal epithelial tight junction barrier.* J Cell Mol Med, 2011. **15**(4): p. 970-82.

348. Clayburgh, D.R., L. Shen, and J.R. Turner, *A porous defense: the leaky epithelial barrier in intestinal disease.* Lab Invest, 2004. **84**(3): p. 282-91.

349. Clayburgh, D.R., et al., *A differentiation-dependent splice variant of myosin light chain kinase, MLCK1, regulates epithelial tight junction permeability.* J Biol Chem, 2004. **279**(53): p. 55506-13.

350. Martinez, C., et al., *Diarrhoea-predominant irritable bowel syndrome: an organic disorder with structural abnormalities in the jejunal epithelial barrier.* Gut, 2013. **62**(8): p. 1160-8.

351. Piche, T., *[Alterations of intestinal epithelial barrier and flora in the irritable bowel syndrome]*. Gastroenterol Clin Biol, 2009. **33 Suppl 1**: p. S40-7.

352. Coeffier, M., et al., *Increased proteasome-mediated degradation of occludin in irritable bowel syndrome.* Am J Gastroenterol, 2010. **105**(5): p. 1181-8.

353. Bertiaux-Vandaele, N., et al., *The expression and the cellular distribution of the tight junction proteins are altered in irritable bowel syndrome patients with differences according to the disease subtype.* Am J Gastroenterol, 2011. **106**(12): p. 2165-73.

354. Chow, J., et al., *Host-bacterial symbiosis in health and disease.* Adv Immunol, 2010. **107**: p. 243-74.

355. Hooper, L.V. and J.I. Gordon, *Commensal host-bacterial relationships in the gut.* Science, 2001. **292**(5519): p. 1115-8.

356. Fujimura, K.E., et al., *Role of the gut microbiota in defining human health.* Expert Rev Anti Infect Ther, 2010. **8**(4): p. 435-54.

357. Kumar, P.S., et al., *Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing.* J Clin Microbiol, 2006. **44**(10): p. 3665-73.

358. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.

359. Sakamoto, M., et al., *Changes in oral microbial profiles after periodontal treatment as determined by molecular analysis of 16S rRNA genes.* J Med Microbiol, 2004. **53**(Pt 6): p. 563-71.

360. Conte, M.P., et al., *Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease.* Gut, 2006. **55**(12): p. 1760-7.

361. Ott, S.J., et al., *Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease.* Gut, 2004. **53**(5): p. 685-93.

362. Claud, E.C. and W.A. Walker, *Bacterial colonization, probiotics, and necrotizing enterocolitis.* J Clin Gastroenterol, 2008. **42 Suppl 2**: p. S46-52.

363. Malinen, E., et al., *Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR.* Am J Gastroenterol, 2005. **100**(2): p. 373-82.

364. Si, J.M., et al., *Irritable bowel syndrome consulters in Zhejiang province: the symptoms pattern, predominant bowel habit subgroups and quality of life.* World J Gastroenterol, 2004. **10**(7): p. 1059-64.

365. Lucke, K., et al., *Prevalence of Bacteroides and Prevotella spp. in ulcerative colitis.* J Med Microbiol, 2006. **55**(Pt 5): p. 617-24.

366. Kleessen, B., et al., *Mucosal and invading bacteria in patients with inflammatory bowel disease compared with controls.* Scand J Gastroenterol, 2002. **37**(9): p. 1034-41.

367. Rhee, S.H., C. Pothoulakis, and E.A. Mayer, *Principles and clinical implications of the brain-gut-enteric microbiota axis.* Nat Rev Gastroenterol Hepatol, 2009. **6**(5): p. 306-14.

368. Clarke, G., et al., *The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner.* Mol Psychiatry, 2013. **18**(6): p. 666-73.

369. Palmer, C., et al., *Development of the human infant intestinal microbiota.* PLoS Biol, 2007. **5**(7): p. e177.

370. DiBaise, J.K., et al., *Gut microbiota and its possible relationship with obesity.* Mayo Clin Proc, 2008. **83**(4): p. 460-9.

371. Stappenbeck, T.S., L.V. Hooper, and J.I. Gordon, *Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15451-5.

372. Velagapudi, V.R., et al., *The gut microbiota modulates host energy and lipid metabolism in mice.* J Lipid Res, 2010. **51**(5): p. 1101-12.

373. Rakoff-Nahoum, S., et al., *Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis.* Cell, 2004. **118**(2): p. 229-41.

374. Husebye, E., et al., *Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(3): p. G368-80.

375. Ait-Belgnaoui, A., et al., *Lactobacillus farciminis treatment suppresses stress induced visceral hypersensitivity: a possible action through interaction with epithelial cell cytoskeleton contraction.* Gut, 2006. **55**(8): p. 1090-4.

376. Fanaro, S., et al., *Intestinal microflora in early infancy: composition and development.* Acta Paediatr Suppl, 2003. **91**(441): p. 48-55.

377. Orrhage, K. and C.E. Nord, *Factors controlling the bacterial colonization of the intestine in breastfed infants.* Acta Paediatr Suppl, 1999. **88**(430): p. 47-57.

378. Mackie, R.I., A. Sghir, and H.R. Gaskins, *Developmental microbial ecology of the neonatal gastrointestinal tract.* Am J Clin Nutr, 1999. **69**(5): p. 1035S-1045S.

379. Penders, J., et al., *Factors influencing the composition of the intestinal microbiota in early infancy.* Pediatrics, 2006. **118**(2): p. 511-21.

380. Fak, F., et al., *Microbial manipulation of the rat dam changes bacterial colonization and alters properties of the gut in her offspring.* Am J Physiol Gastrointest Liver Physiol, 2008. **294**(1): p. G148-54.

381. Lamouse-Smith, E.S., A. Tzeng, and M.N. Starnbach, *The intestinal flora is required to support antibody responses to systemic immunization in infant and germ free mice.* PLoS One, 2011. **6**(11): p. e27662.

382. Goossens, D., et al., *Probiotics in gastroenterology: indications and future perspectives.* Scand J Gastroenterol Suppl, 2003(239): p. 15-23.

383. Maurice, C.F., H.J. Haiser, and P.J. Turnbaugh, *Xenobiotics shape the physiology and gene expression of the active human gut microbiome.* Cell, 2013. **152**(1-2): p. 39-50.

384. Benchimol, E.I., et al., *Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data.* Gut, 2009. **58**(11): p. 1490-7.

385. Reis, M., et al., *Serum concentrations of antidepressant drugs in a naturalistic setting: compilation based on a large therapeutic drug monitoring database.* Ther Drug Monit, 2009. **31**(1): p. 42-56.

386. Santarelli, L., et al., *Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants.* Science, 2003. **301**(5634): p. 805-9.

387. Lim, G.E., et al., *The rho guanosine 5'-triphosphatase, cell division cycle 42, is required for insulin-induced actin remodeling and glucagon-like peptide-1 secretion in the intestinal endocrine L cell.* Endocrinology, 2009. **150**(12): p. 5249-61.

388. Sangle, G.V., et al., *Novel biological action of the dipeptidylpeptidase-IV inhibitor, sitagliptin, as a glucagon-like peptide-1 secretagogue.* Endocrinology, 2012. **153**(2): p. 564-73.

389. Cooper, H.S., et al., *Clinicopathologic study of dextran sulfate sodium experimental murine colitis.* Lab Invest, 1993. **69**(2): p. 238-49.

390. Rogers, G.B., et al., *characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling.* J Clin Microbiol, 2004. **42**(11): p. 5176-83.

391. Sibley, C.D., et al., *Culture enriched molecular profiling of the cystic fibrosis airway microbiome.* PLoS One, 2011. **6**(7): p. e22702.

392. Martin, M.A., *Cutadapt removes adapter sequences from high-throughput sequencing reads*

2011.

393. Masella, A.P., et al., *PANDAseq: paired-end assembler for illumina sequences.* BMC Bioinformatics, 2012. **13**: p. 31.

394. Ye, Y., *Identification and Quantification of Abundant Species from Pyrosequences of 16S rRNA by Consensus Alignment.* Proceedings (IEEE Int Conf Bioinformatics Biomed), 2011. **2010**: p. 153-157.

395. DeSantis, T.Z., et al., *Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB.* Appl Environ Microbiol, 2006. **72**(7): p. 5069-72.

396. Wang, Q., et al., *Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy.* Appl Environ Microbiol, 2007. **73**(16): p. 5261-7.

397. Kuczynski, J., et al., *Using QIIME to analyze 16S rRNA gene sequences from microbial communities.* Curr Protoc Bioinformatics, 2011. **Chapter 10**: p. Unit 10 7.

398. Caporaso, J.G., et al., *Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample.* Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**: p. 4516-22.

399. Welch, K.M., J.S. Meyer, and S. Kwant, *Estimation of levels of serotonin and 5-hydroxyindoles in whole blood by an autoanalytical procedure: observations on the blood-brain barrier.* J Neurochem, 1972. **19**(4): p. 1079-87.

400. Da Prada, M. and G.B. Picotti, *Content and subcellular localization of catecholamines and 5-hydroxytryptamine in human and animal blood platelets: monoamine distribution between platelets and plasma.* Br J Pharmacol, 1979. **65**(4): p. 653-62.

401. Bertaccini, G., *Tissue 5-hydroxytryptamine and urinary 5-hydroxyindoleacetic acid after partial or total removal of the gastro-intestinal tract in the rat.* J Physiol, 1960. **153**(2): p. 239-49.

402. Oberlander, T.F., J.A. Gingrich, and M.S. Ansorge, *Sustained neurobehavioral effects of exposure to SSRI antidepressants during development: molecular to clinical evidence.* Clin Pharmacol Ther, 2009. **86**(6): p. 672-7.

403. Bengel, D., et al., *Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice.* Mol Pharmacol, 1998. **53**(4): p. 649-55.

404. Matthews, J.D., et al., *An open study of aripiprazole and escitalopram for psychotic major depressive disorder.* J Clin Psychopharmacol, 2009. **29**(1): p. 73-6.

405. Cases, O., et al., *Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA.* Science, 1995. **268**(5218): p. 1763-6.

406. Cabrera-Vera, T.M. and G. Battaglia, *Prenatal exposure to fluoxetine (Prozac) produces site-specific and age-dependent alterations in brain serotonin transporters in rat progeny: evidence from autoradiographic studies.* J Pharmacol Exp Ther, 1998. **286**(3): p. 1474-81.

407. Mickey, B.J., et al., *Monoamine oxidase A genotype predicts human serotonin 1A receptor availability in vivo.* J Neurosci, 2008. **28**(44): p. 11354-9.

408. Evrard, A., et al., *Altered regulation of the 5-HT system in the brain of MAO-A knock-out mice.* Eur J Neurosci, 2002. **15**(5): p. 841-51.

409. Owesson, C.A., et al., *Altered presynaptic function in monoaminergic neurons of monoamine oxidase-A knockout mice.* Eur J Neurosci, 2002. **15**(9): p. 1516-22.

410. Lanoir, J., G. Hilaire, and I. Seif, *Reduced density of functional 5-HT1A receptors in the brain, medulla and spinal cord of monoamine oxidase-A knockout mouse neonates.* J Comp Neurol, 2006. **495**(5): p. 607-23.

411. Moses-Kolko, E.L., et al., *Age, sex, and reproductive hormone effects on brain serotonin-1A and serotonin-2A receptor binding in a healthy population.* Neuropsychopharmacology, 2011. **36**(13): p. 2729-40.

412. Cabrera, T.M. and G. Battaglia, *Delayed decreases in brain 5-hydroxytryptamine2A/2C receptor density and function in male rat progeny following prenatal fluoxetine.* J Pharmacol Exp Ther, 1994. **269**(2): p. 637-45.

413. Lauder, J.M., J. Liu, and D.R. Grayson, *In utero exposure to serotonergic drugs alters neonatal expression of 5-HT(1A) receptor transcripts: a quantitative RT-PCR study.* Int J Dev Neurosci, 2000. **18**(2-3): p. 171-6.

414. Fabre, V., et al., *Altered expression and functions of serotonin 5-HT1A and 5-HT1B receptors in knock-out mice lacking the 5-HT transporter.* Eur J Neurosci, 2000. **12**(7): p. 2299-310.

415. Gobbi, G., et al., *Modifications of the serotonergic system in mice lacking serotonin transporters: an in vivo electrophysiological study.* J Pharmacol Exp Ther, 2001. **296**(3): p. 987-95.

416. Li, Q., et al., *Reduction of 5-hydroxytryptamine (5-HT)(1A)-mediated temperature and neuroendocrine responses and 5-HT(1A) binding sites in 5-HT transporter knockout mice.* J Pharmacol Exp Ther, 1999. **291**(3): p. 999-1007.

417. Li, Q., et al., *Reduction in the density and expression, but not G-protein coupling, of serotonin receptors (5-HT1A) in 5-HT transporter knock-out mice: gender and brain region differences.* J Neurosci, 2000. **20**(21): p. 7888-95.

418. Bouali, S., et al., *Sex hormone-dependent desensitization of 5-HT1A autoreceptors in knockout mice deficient in the 5-HT transporter.* Eur J Neurosci, 2003. **18**(8): p. 2203-12.

419. Rioux, A., et al., *Adaptive changes of serotonin 5-HT2A receptors in mice lacking the serotonin transporter.* Neurosci Lett, 1999. **262**(2): p. 113-6.

420. Li, Q., et al., *Brain region-specific alterations of 5-HT2A and 5-HT2C receptors in serotonin transporter knockout mice.* J Neurochem, 2003. **84**(6): p. 1256-65.

421. Foguet, M., et al., *Structure of the mouse 5-HT1C, 5-HT2 and stomach fundus serotonin receptor genes.* Neuroreport, 1992. **3**(4): p. 345-8.

422. Foguet, M., et al., *Cloning and functional characterization of the rat stomach fundus serotonin receptor.* EMBO J, 1992. **11**(9): p. 3481-7.

423. Hoyer, D., J.P. Hannon, and G.R. Martin, *Molecular, pharmacological and functional diversity of 5-HT receptors.* Pharmacol Biochem Behav, 2002. **71**(4): p. 533-54.

424. Engel, G., et al., *Identification of 5HT2-receptors on longitudinal muscle of the guinea pig ileum.* J Recept Res, 1984. **4**(1-6): p. 113-26.

425. Kuemmerle, J.F., et al., *Coexistence of contractile and relaxant 5-hydroxytryptamine receptors coupled to distinct signaling pathways in intestinal muscle cells: convergence of the pathways on Ca2+ mobilization.* Mol Pharmacol, 1992. **42**(6): p. 1090-6.

426. Prins, N.H., M.R. Briejer, and J.A. Schuurkes, *Characterization of the contraction to 5-HT in the canine colon longitudinal muscle.* Br J Pharmacol, 1997. **120**(4): p. 714-20.

427. Borman, R.A. and D.E. Burleigh, *Human colonic mucosa possesses a mixed population of 5-HT receptors.* Eur J Pharmacol, 1996. **309**(3): p. 271-4.

428. Hansen, M.B. and E. Skadhauge, *Signal transduction pathways for serotonin as an intestinal secretagogue.* Comp Biochem Physiol A Physiol, 1997. **118**(2): p. 283-90.

429. Imada-Shirakata, Y., et al., *Serotonin activates electrolyte transport via 5-HT2A receptor in rat colonic crypt cells.* Biochem Biophys Res Commun, 1997. **230**(2): p. 437-41.

430. Johnson, P.J., et al., *Characterization of 5-hydroxytryptamine receptors mediating mucosal secretion in guinea-pig ileum.* Br J Pharmacol, 1994. **111**(4): p. 1240-4.

431. Siriwardena, A.K., et al., *Identification of a 5-hydroxytryptamine (5-HT2) receptor on guinea pig small intestinal crypt cells.* Am J Physiol, 1993. **265**(2 Pt 1): p. G339-46.

432. Fiorica-Howells, E., et al., *5-HT(2A) receptors: location and functional analysis in intestines of wild-type and 5-HT(2A) knockout mice.* Am J Physiol Gastrointest Liver Physiol, 2002. **282**(5): p. G877-93.

433. Stappenbeck, T.S., et al., *Notes from some crypt watchers: regulation of renewal in the mouse intestinal epithelium.* Curr Opin Cell Biol, 1998. **10**(6): p. 702-9.

434. Nau, F., Jr., et al., *Serotonin 5-HT2A receptor activation blocks TNF-alpha mediated inflammation in vivo.* PLoS One, 2013. **8**(10): p. e75426.

435. Okamoto, K., et al., *5-HT2A receptor subtype in the peripheral branch of sensory fibers is involved in the potentiation of inflammatory pain in rats.* Pain, 2002. **99**(1-2): p. 133-43.

436. Okamoto, K., et al., *The role of peripheral 5HT2A and 5HT1A receptors on the orofacial formalin test in rats with persistent temporomandibular joint inflammation.* Neuroscience, 2005. **130**(2): p. 465-74.

437. Kling, A., et al., *Decreased density of serotonin 5-HT2A receptors in rheumatoid arthritis.* Ann Rheum Dis, 2006. **65**(6): p. 816-9.

438. Shochina, M., et al., *Neurochemical coding in the myenteric plexus of the upper gastrointestinal tract of hibernating hamsters.* Int J Dev Neurosci, 1997. **15**(3): p. 353-62.

439. O'Sullivan, M., et al., *Increased mast cells in the irritable bowel syndrome.* Neurogastroenterol Motil, 2000. **12**(5): p. 449-57.

440. Ordas, I., et al., *Ulcerative colitis.* Lancet, 2012. **380**(9853): p. 1606-19.

441. Zhang, Y.Z. and Y.Y. Li, *Inflammatory bowel disease: pathogenesis.* World J Gastroenterol, 2014. **20**(1): p. 91-9.

442. Pedersen, J., et al., *Inflammatory pathways of importance for management of inflammatory bowel disease.* World J Gastroenterol, 2014. **20**(1): p. 64-77.

443. Bian, X., et al., *High mucosal serotonin availability in neonatal guinea pig ileum is associated with low serotonin transporter expression.* Gastroenterology, 2007. **132**(7): p. 2438-47.

444. Leon-Ponte, M., G.P. Ahern, and P.J. O'Connell, *Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor.* Blood, 2007. **109**(8): p. 3139-46.

445. Uher, R., et al., *Adverse reactions to antidepressants.* Br J Psychiatry, 2009. **195**(3): p. 202-10.

446. Camilleri, M. and H. Gorman, *Intestinal permeability and irritable bowel syndrome.* Neurogastroenterol Motil, 2007. **19**(7): p. 545-52.

447. Goyal, N., et al., *Animal models of inflammatory bowel disease: a review.* Inflammopharmacology, 2014. **22**(4): p. 219-33.

448. McGuckin, M.A., et al., *Intestinal barrier dysfunction in inflammatory bowel diseases.* Inflamm Bowel Dis, 2009. **15**(1): p. 100-13.

449. Kunze, W.A. and J.B. Furness, *The enteric nervous system and regulation of intestinal motility.* Annu Rev Physiol, 1999. **61**: p. 117-42.

450. Forsythe, P., W.A. Kunze, and J. Bienenstock, *On communication between gut microbes and the brain.* Curr Opin Gastroenterol, 2012. **28**(6): p. 557-62.

451. Grider, J.R., J.F. Kuemmerle, and J.G. Jin, *5-HT released by mucosal stimuli initiates peristalsis by activating 5-HT4/5-HT1p receptors on sensory CGRP neurons.* Am J Physiol, 1996. **270**(5 Pt 1): p. G778-82.

452. Wikoff, W.R., et al., *Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites.* Proc Natl Acad Sci U S A, 2009. **106**(10): p. 3698-703.

453. Desbonnet, L., et al., *The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat.* J Psychiatr Res, 2008. **43**(2): p. 164-74.

454. Man, S.M., N.O. Kaakoush, and H.M. Mitchell, *The role of bacteria and pattern-recognition receptors in Crohn's disease.* Nat Rev Gastroenterol Hepatol, 2011. **8**(3): p. 152-68.

455. Woese, C.R., *Bacterial evolution.* Microbiol Rev, 1987. **51**(2): p. 221-71.

456. Ulevitch, R.J. and P.S. Tobias, *Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin.* Annu Rev Immunol, 1995. **13**: p. 437-57.

457. Carvalho, B.M. and M.J. Saad, *Influence of gut microbiota on subclinical inflammation and insulin resistance.* Mediators Inflamm, 2013. **2013**: p. 986734.

458. Ghoshal, S., et al., *Chylomicrons promote intestinal absorption of lipopolysaccharides.* J Lipid Res, 2009. **50**(1): p. 90-7.

459. Kim, K.A., et al., *High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway.* PLoS One, 2012. **7**(10): p. e47713.

460. Odenwald, M.A. and J.R. Turner, *Intestinal permeability defects: is it time to treat?* Clin Gastroenterol Hepatol, 2013. **11**(9): p. 1075-83.

461. Dunne, C., et al., *Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials.* Antonie Van Leeuwenhoek, 1999. **76**(1-4): p. 279-92.

462. Oksaharju, A., et al., *Effects of probiotic Lactobacillus rhamnosus GG and Propionibacterium freudenreichii ssp. shermanii JS supplementation on intestinal and systemic markers of inflammation in ApoE*3Leiden mice consuming a high-fat diet.* Br J Nutr, 2013. **110**(1): p. 77-85.

463. Miyauchi, E., et al., *Mechanism of protection of transepithelial barrier function by Lactobacillus salivarius: strain dependence and attenuation by bacteriocin production.* Am J Physiol Gastrointest Liver Physiol, 2012. **303**(9): p. G1029-41.

464. Miyauchi, E., et al., *Effect of D-alanine in teichoic acid from the Streptococcus thermophilus cell wall on the barrier-protection of intestinal epithelial cells.* Biosci Biotechnol Biochem, 2012. **76**(2): p. 283-8.

465. Menon, R., et al., *Diet complexity and estrogen receptor beta status affect the composition of the murine intestinal microbiota.* Appl Environ Microbiol, 2013. **79**(18): p. 5763-73.

466. Markle, J.G., et al., *Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity.* Science, 2013. **339**(6123): p. 1084-8.

467. Flak, M.B., J.F. Neves, and R.S. Blumberg, *Immunology. Welcome to the microgenderome.* Science, 2013. **339**(6123): p. 1044-5.

468. Jones, M.D. and I. Lucki, *Sex differences in the regulation of serotonergic transmission and behavior in 5-HT receptor knockout mice.* Neuropsychopharmacology, 2005. **30**(6): p. 1039-47.

469. Maswood, S., et al., *Estrous cycle modulation of extracellular serotonin in mediobasal hypothalamus: role of the serotonin transporter and terminal autoreceptors.* Brain Res, 1999. **831**(1-2): p. 146-54.

470. Bethea, C.L., et al., *Diverse actions of ovarian steroids in the serotonin neural system.* Front Neuroendocrinol, 2002. **23**(1): p. 41-100.

471. Imwalle, D.B., J.A. Gustafsson, and E.F. Rissman, *Lack of functional estrogen receptor beta influences anxiety behavior and serotonin content in female mice.* Physiol Behav, 2005. **84**(1): p. 157-63.

472. Spiller, R., *Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5-HT signalling and metabolism in human disease.* Neurogastroenterol Motil, 2007. **19 Suppl 2**: p. 25-31.

473. Ford, A.C., et al., *Efficacy of antidepressants and psychological therapies in irritable bowel syndrome: systematic review and meta-analysis.* Gut, 2009. **58**(3): p. 367-78.

474. Talley, N.J., A.R. Zinsmeister, and L.J. Melton, 3rd, *Irritable bowel syndrome in a community: symptom subgroups, risk factors, and health care utilization.* Am J Epidemiol, 1995. **142**(1): p. 76-83.

475. Ouyang, A. and H.F. Wrzos, *Contribution of gender to pathophysiology and clinical presentation of IBS: should management be different in women?* Am J Gastroenterol, 2006. **101**(12 Suppl): p. S602-9.

476. Hogan, A.M., et al., *Estrogen and its role in gastrointestinal health and disease.* Int J Colorectal Dis, 2009. **24**(12): p. 1367-75.

477. Mulak, A., Y. Tache, and M. Larauche, *Sex hormones in the modulation of irritable bowel syndrome.* World J Gastroenterol, 2014. **20**(10): p. 2433-48.

478. Heitkemper, M.M. and L. Chang, *Do fluctuations in ovarian hormones affect gastrointestinal symptoms in women with irritable bowel syndrome?* Gend Med, 2009. **6 Suppl 2**: p. 152-67.

479. Gustafsson, J.K. and B. Greenwood-Van Meerveld, *Amygdala activation by corticosterone alters visceral and somatic pain in cycling female rats.* Am J Physiol Gastrointest Liver Physiol, 2011. **300**(6): p. G1080-5.

480. Lee, H.R., et al., *Paraneoplastic gastrointestinal motor dysfunction: clinical and laboratory characteristics.* Am J Gastroenterol, 2001. **96**(2): p. 373-9.

481. Chang, L., et al., *Sensation of bloating and visible abdominal distension in patients with irritable bowel syndrome.* Am J Gastroenterol, 2001. **96**(12): p. 3341-7.

482. Altman, G., et al., *Increased symptoms in female IBS patients with dysmenorrhea and PMS.* Gastroenterol Nurs, 2006. **29**(1): p. 4-11.

483. Houghton, L.A., et al., *The menstrual cycle affects rectal sensitivity in patients with irritable bowel syndrome but not healthy volunteers.* Gut, 2002. **50**(4): p. 471-4.

484. Kane, S.V., K. Sable, and S.B. Hanauer, *The menstrual cycle and its effect on inflammatory bowel disease and irritable bowel syndrome: a prevalence study.* Am J Gastroenterol, 1998. **93**(10): p. 1867-72.

485. Cremon, C., et al., *Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms.* Am J Gastroenterol, 2009. **104**(2): p. 392-400.

486. Alonso, C., et al., *Acute experimental stress evokes a differential gender-determined increase in human intestinal macromolecular permeability.* Neurogastroenterol Motil, 2012. **24**(8): p. 740-6, e348-9.

487. Mayer, E.A. and G.F. Gebhart, *Basic and clinical aspects of visceral hyperalgesia.* Gastroenterology, 1994. **107**(1): p. 271-93.

488. Cervero, F. and J.M. Laird, *Visceral pain.* Lancet, 1999. **353**(9170): p. 2145-8.

489. Vitetta, L., et al., *Probiotics, prebiotics and the gastrointestinal tract in health and disease.* Inflammopharmacology, 2014. **22**(3): p. 135-54.

490. Grzeskowiak, L.E., A.L. Gilbert, and J.L. Morrison, *Long term impact of prenatal exposure to SSRIs on growth and body weight in childhood: evidence from animal and human studies.* Reprod Toxicol, 2012. **34**(1): p. 101-9.

491. Yonkers, K.A., et al., *Pregnant Women With Posttraumatic Stress Disorder and Risk of Preterm Birth.* JAMA Psychiatry, 2014.

492. Yonkers, K.A., et al., *The management of depression during pregnancy: a report from the American Psychiatric Association and the American College of Obstetricians and Gynecologists.* Obstet Gynecol, 2009. **114**(3): p. 703-13.

493. Suri, R., et al., *The impact of depression and fluoxetine treatment on obstetrical outcome.* Arch Womens Ment Health, 2004. **7**(3): p. 193-200.

494. Gershon, M.D. and E.M. Ratcliffe, *Developmental biology of the enteric nervous system: pathogenesis of Hirschsprung's disease and other congenital dysmotilities.* Semin Pediatr Surg, 2004. **13**(4): p. 224-35.

495. Gorard, D.A., G.W. Libby, and M.J. Farthing, *5-Hydroxytryptamine and human small intestinal motility: effect of inhibiting 5-hydroxytryptamine reuptake.* Gut, 1994. **35**(4): p. 496-500.

496. Gorard, D.A. and M.J. Farthing, *Intestinal motor function in irritable bowel syndrome.* Dig Dis, 1994. **12**(2): p. 72-84.