INFLUENCE OF MATERNAL SELECTIVE SEROTONIN REUPTAKE INHIBITOR EXPOSURE ON THE DEVELOPMENT OF THE GASTROINTESTINAL TRACT OF THE OFFSPRING

INFLUENCE OF MATERNAL SELECTIVE SEROTONIN REUPTAKE INHIBITOR EXPOSURE ON THE DEVELOPMENT OF THE GASTROINTESTINAL TRACT OF THE OFFSPRING

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TITLE: Influence of Maternal Selective Serotonin Reuptake Inhibitor Exposure on the Development of the Gastrointestinal Tract of the Offspring

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ABSTRACT

10-15% of women take antidepressants during pregnancy. Selective serotonin reuptake inhibitors (SSRIs) are most commonly used for perinatal depression. Perinatal exposure to SSRIs has been shown to disrupt the development of serotonergic signaling pathways in the central nervous system (CNS); however, the effects on the developing enteric nervous system (ENS) remain relatively unexplored. We hypothesized that early life exposure to SSRIs would influence the structural development of the gastrointestinal (GI) tract. We further hypothesized that these structural changes could lead to clinically relevant functional outcomes, such as modifications in susceptibility to inflammation and altered GI motility.

Female Wistar rats were given the SSRI, fluoxetine, or vehicle from 2 weeks prior to mating through gestation until weaning. At postnatal day 1 (P1), postnatal day 21 (P21; weaning) and 6 months of age (P6 months) intestines were harvested to assess for structural changes. At P6M, intestines were collected to assess motility *in vitro* and subsets of the offspring were treated with dextran sulfate sodium (DSS) to assess susceptibility to colitis.

At P1, there was a significant decrease in serotonergic neurons in the female colon. At P21, there was a significant increase in serotonergic neurons of both sexes in the colon. At P6M, there was a significant increase in the frequency and velocity of long-distance contractions in the colon when both sexes were combined and an increase in ZO-1 in male colon.

In conclusion, SSRI exposure in utero appears to have structural and functional consequences on the developing ENS in the SSRI exposed offspring. The structural consequences are seen in both sexes at P21 and although the structural changes to the ENS resolve by 6 months, motility in the colon continues to be significantly altered. There were no significant differences in chemical colitis, however, we did see difference of quantitative mRNA cytokines, chemokines and

extracellular matrix components which may suggest differences in mucosal immune response. The mechanisms by which these changes occur remain to be explored.

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ABBREVIATIONS

5-HT- 5-hydroxytryptamine or Serotonin

ACTA2 - Alpha (α)-2 Actin

ACTB - Actin Beta

AREG – Amphiregulin

ARG1 - Arginase 1

BDNF - Brain-Derived Neurotrophic Factor

CCL2 - C-C Motif Chemokine Ligand 2

Chi3I1 - Chitinase 3–like-1

CLEC7A - C-Type Lectin Domain Containing 7A

CNTF - Ciliary neurotrophic factor

COL1A1 - Collagen, type I Alpha 1 Chain

COL3A1 - Collagen Type III Alpha 1 Chain

ENS - Enteric Nervous System

EC Cell- Enterochromaffin Cell

FN1 - Fibronectin 1

GI - Gastrointestinal

H & E – Hematoxylin and Eosin

HIF1A - Hypoxia-inducible factor 1-alpha

HMGB1- High mobility group box 1

IBD- Inflammatory Bowel Disease

IL1A - Interleukin 1 Alpha

IL1B - Interleukin 1 Beta

IL1RAP - Interleukin 1 Receptor Accessory Protein

IL1RLR1 - Interleukin 1 Receptor Like 1

IL4 - Interleukin 4

IL4R - Interleukin 4 Receptor

IL6 - Interleukin 6

IL6R - Interleukin 6 Receptor

IL6ST - Interleukin 6 Signal Transducer

IL11 - Interleukin 11

IL11RA1 - Interleukin 11 Receptor subunit Alpha 1

IL13 - Interleukin 13

IL17A - Interleukin 17A

IL31 - Interleukin 31

IL31A - Interleukin 31 Receptor A

IL33 - Interleukin 33

IBS- Irritable Bowel Syndrome

KLP- Katherine Louise Prowse

LIF - Leukemia inhibitory factor

LIFR - Leukemia Inhibitory Factor Receptor Alpha

MMP7 - Matrix Metallopeptidase 7

MPO- Myeloperoxidase

MRC1- Mannose Receptor C-Type 1

MUC5B - Mucin 5B, Oligomeric Mucus/Gel-Forming

OSM – Oncostatin M

OSMR - Oncostatin M Receptor

P- Postnatal day

PGK1 - Phosphoglycerate Kinase 1

RETNLA - Resistin-like molecule alpha/FIZZ1

RETNLB - Resistin Like molecule Beta/FIZZ2

SERT- Serotonin Reuptake Transporter

STAT1 - Signal Transducer and Activator of Transcription 1

STAT3 - Signal Transducer and Activator of Transcription 3

SSRI- Selective Serotonin Reuptake Inhibitor

TGFB1- Transforming Growth Factor Beta 1

TGFBR1- Transforming Growth Factor Beta Receptor 1

TIMP1 - TIMP metallopeptidase inhibitor 1

TJ - Tight Junctions

TRKB - Tropomyosin Receptor Kinase B

TNF - Tumor Necrosis Factor

Tph1- Tryptophan hydroxylase- 1

Tph2- Tryptophan hydroxylase- 2

VEGFA - Vascular endothelial growth factor A

YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta)

DECLARATION OF ACADEMIC ACHIEVEMENT

All work has been either completed by me or under my direct supervision with the exception of the following: The majority if the animal work and tissue collection was done by Kristina Wiggers and Sergio Raez-Villanueva form Dr. Alison Holloways lab. They generated and maintained the animal cohort and helped with sample collection. Rajka Borojevic, research assistant in Dr. Elyanne Ratcliffe's lab was also involved in the majority of sample collection and tissue preparation for the animal work. Intestinal Motility was completed by Sean Parsons and Alex Vincent from Dr. Jan Huizinga's lab. Colonic myeloperoxidase was determined by Megan Wang in Dr. Elyanne Ratcliffe's lab. Staining and quantification of tight junctions was done by Anna Miroshnychenko under my direct supervision. Quantification of serotonergic neurons and crypt villi ratio was done by Filip Markovic under my direct supervision. Calculations of enterochromaffin cells per surface area were done by Eugene Mech under my direct supervision. Christina Hayes from Dr. Elena Verdu's lab provided the protocol for staining tight junctions and the analysis by quantification in ImageJ which was adapted to Volocity.

CHAPTER 1: INTRODUCTION

1.1 Maternal Depression

Untreated maternal depression can have devastating effects on the health and well-being of children and their mothers [1]. The American Psychiatric Association's Diagnostic and Statistical Manual, Fifth Edition (DSM-5) classifies maternal depression as Major Depressive Disorder, with the onset of depression that can occur during the perinatal period (antenatal) or following parturition (postnatal) [2]. Antenatal depression has been associated with risk of preterm (<37 week) delivery and low birth weight [3]. Postpartum depression has been associated with risk to the offspring including: impaired maternal fetal bonding [3-4], abnormal development [4-6], cognitive impairment [4-7], emotional and behavioural issues [3, 8-13].

Estimates of depression in women vary among different studies. The prevalence of postpartum depression was 9% in a prospective study done in the United States (n=994) which was similar to the depression rate in non-postpartum women 8% (n>13,000) [5]. In 2004, antepartum depression rates were 26% in women living in poor urban communities [14]. Other estimates of the rate of postpartum depression range from 10-16% [15] and according to the American College of Obstetrics and Gynecology, 9% of women in the antenatal period will screen positive for major depressive disorder [16]. Amongst women with postpartum depression, the

majority (58%) have onset of symptoms prior to delivery, either pre-pregnancy (20%) or antepartum (38%) [15,17-19]. The incidence of severe post-partum depression among 2.6 million women and 3.2 million births, was determined with the overall rate of 36.7/10,000 in 2010, which was a 34% increase from 2006 (27.4/10,000) [20].

Selective Serotonin Reuptake Inhibitors (SSRIs) are considered first line pharmacologic therapy and are the most commonly used antidepressants during pregnancy and lactation [21-22]**.** Approximately 7% of women in North America require an SSRI in the perinatal period [23]. SSRIs cross the placenta and are also present in detectable concentrations within the breastmilk [24]. The developing fetus and breastfed infants are, thus, exposed to SSRIs [24]. In breastfeeding women on fluoxetine (n=2), one infant had detectable serum levels of fluoxetine (approximately 15 ng/mL) [25] which is generally lower than maternal serum levels (ranging from 5-577 ng/mL during pregnancy and 21-506 ng/mL during lactation [26]).

1.2 Serotonin, Serotonin Receptors and Serotonin Metabolism

Serotonin is a neurotransmitter centrally and acts as a hormone peripherally [27]. It is found mainly in the GI tract but also in immune cells, platelets and the CNS [28]. Serotonin is synthesized from the amino acid L-tryptophan which is consumed in

our diet [28]. There are 2 rate limiting enzymes involved in the synthesis of serotonin, Tryptophan Hydroxylase 1 (TPH1) and Tryptophan Hydroxylase 2 (TPH2) [28]. Monoamine oxidase deaminates or breaks down serotonin to 5-hydroxyindoleacetaldehyde (5-HIAA) [28]. There are seven serotonin (5-HT) receptors with 14 different subtypes of 5-HT receptors [29]. Serotonin cannot easily pass intracellularly and therefore depends on a sodium dependent serotonin transporter (SERT). SERT is found in the brain, on intestinal epithelial cells, platelets, B cells, mast cells and monocytes [30]. Serotonin not taken up by the intestinal epithelial cell enters circulation via the capillary bed of the lamina propria where it enters circulation via SERT receptors on platelets [31]. Serotonin facilitates procoagulant activity [32] and therefore SSRI use has been associated with increased risk of upper GI bleeding [33].

1.3 Selective Serotonin Reuptake Inhibitors (SSRIs)

SSRIs are frequently used pharmacotherapy as first-line medication when clinically indicated for moderate to severe depression during pregnancy, postpartum and lactation because of their efficacy, tolerability, and general safety profile [34]. In 1972, fluoxetine was synthesized [34]. In 1987, fluoxetine was the first SSRI approved by the United States Food and Drug Administration (FDA) for treatment of major depression and continues to be the most commonly prescribed SSRI

during pregnancy and lactation [23, 34]. The FDA has pharmaceutical pregnancy categories for safety. Fluoxetine is considered pregnancy category C for both pregnancy and lactation [35]. Pregnancy category C is defined as "animal studies have shown risk to the fetus but there are no controlled studies in women, or studies in women and animals are not available" [36] and therefore, "risk cannot be ruled out" [35]. Given the risks associated with untreated maternal depression including: risk of suicide, prematurity, low birth weight, intrauterine growth restriction, higher impulsivity, maladaptive social interactions, and cognitive, behavioural, and emotional difficulties [37,38,39,40,41] along with the potential risks of the medication to the fetus and newborn baby, there has been controversy regarding the benefit of treatment with SSRIs during the perinatal period. Despite this controversy, many healthcare providers will initiate pharmacologic treatment with SSRIs as first line treatment for moderate to severe depression [35,42]. Literature regarding the safety of antidepressant medication during pregnancy and breastfeeding remains controversial with mixed results [43]. Both perinatal depression and SSRI exposure have been associated with fetal growth changes and shorter gestation [43]. These mixed results are due to challenges in clinical studies heterogeneity in methodology, for example, discrepancy related to small sample size, failure to control for the effects of ongoing maternal depression, and other potential confounders (smoking, substance use, socioeconomic statues, support, etc.) [43]. Healthcare providers must discuss both pharmacologic and non-

pharmacologic therapy including cognitive behavioural therapy, interpersonal psychotherapy and group therapy in order to obtain informed consent regarding the treatment of depression [43]. In 2007, nearly 13% of all pregnant women are taking antidepressant medication during pregnancy with SSRI's or Selective Norepinephrine Reuptake Inhibitors (SNRI's) being the most frequently prescribed [44,45]. The SSRIs vary considerably in their chemical structure and treat depression by increasing serotonergic activity in the brain [46].

In the central nervous system (CNS), serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter released in the brain [35,46]. The majority of our body's serotonin is found in our GI tract [47]. Serotonin is involved in mood, social behavior, appetite, digestion, sleep, memory, sexual desire and function. Serotonergic neurotransmission in the brain involves a variety of different types of serotonin receptors [35,46,48]. Serotonin receptors in the brain are G-coupled receptors and can be classified as excitatory or inhibitory [49]. The excitatory class of receptors include 5-HT_2 subclasses A-C, 5-HT_4, 5-HT_6 and 5-HT_7 [49]. The inhibitory receptors in the CNS include 5-HT_1 subclasses A-F, and 5-HT_5 subclasses A-B [49]. In the peripheral nervous system, serotonin receptors include excitatory G-coupled receptor 5-HT_2 subclasses A-C and the sodium-potassium ion channel receptor 5-HT_3. In the GI tract, excitatory G-coupled receptors 5-HT_2, 5-

HT_4, 5-HT_7 and the sodium-potassium ion channel 5-HT_3, as well as the inhibitory 5-HT_1 receptors are present [49].

In the CNS, SSRIs decrease the presynaptic serotonin reuptake by 60 to 80 percent [35,48]. As a result, there is an increase in the length of time that serotonin is available in the synapse [35, 46,48]. The increased availability of serotonin in the synapse is believed to be the mechanism by which SSRI's work to treat depression, which is associated with low CNS serotonin and norepinephrine levels [50]. SSRIs are absorbed in the gastrointestinal tract [35, 46,48]. SSRIs are metabolised and eliminated by the liver [48]. The half-life for fluoxetine ranges from 1 to 3 days [46,48,51,52]. Fluoxetine's metabolite norfluoxetine, has a half-life of 4 to 16 days [46,48,51,52]. The half-life of platelet serotonin is at least 3 days, on average approximately 5-6 days which corresponds to the half-life of the platelet [53-54].

Given that fluoxetine is the most commonly prescribed SSRI during the perinatal period and it also has the most available pharmacokinetic data during pregnancy, it was selected for this experiment [55-58].

1.4 SSRI's Influence the Outcome of the Fetus

SSRI exposure during pregnancy has been associated with an increase in congenital defects and long-term effects in language, behavior and gastrointestinal (GI) dysmotility [59]. Specifically, exposure to SSRIs in utero is associated with

congenital heart defects, omphalocele, clubfoot, anencephaly, and anal atresia [60-62]. Maternal SSRI exposure in third trimester has also been associated with neurodevelopmental abnormalities such as developmental delays, pervasive developmental problems and autism [62].

In a cohort of more than 30,000 women in the Netherlands on SSRI's during the second or third trimester, there was a 10-fold increase in the use of laxatives in their offspring [63,64]. SSRI exposure in utero has also been associated with the development of Hirschsprung's disease in the newborn period. Hirschsprung's disease occurs when a segment of the colon is lacking ganglia in the ENS and commonly presents with significant constipation [65].

1.5 Central Nervous System (CNS) Effects of SSRI's in Utero

Evidence from animal studies demonstrates that prenatal exposure to SSRI's can alter serotonergic biosynthesis and signaling pathways in the CNS of the offspring [66]. Furthermore, gestational exposure to SSRI's results in alterations to brain 5-HT content, elevation in peripheral 5-HT levels, and SERT expression, indicating decreased 5-HT function in animal studies [67-70].

Mice lacking serotonin transporter SERT, which is the target protein for SSRI medications, have demonstrated increased anxiety and depressive behaviours [71-72]. SERT knock out results in increased extracellular serotonin concentrations and

decreased tissue serotonin concentrations [73-75]. SERT KO mice have demonstrated changes in sensory patterning, compensatory increase in receptor 5-HT expression, alterations of sleep, and long-term behaviour deficits [76-77]. Mice who had pharmacologic manipulation of SERT with SSRI's have similar phenotype to SERT KO mice [77]. Both SERT KO mice and Fluoxetine exposure during the perinatal period have demonstrated similar effects on the offspring in mice including a hyperplastic ENS, increased serotonergic neurons, decreased total intestinal transit time, decreased small intestinal transit, decreased colonic transit, increased long distance contraction (LDC) frequency, increased LDC velocity, increased LDC propagation length, increased villus height and increased crypt depth [78]. Mice who received fluoxetine in the early post-natal period also demonstrated changes in SERT expression in the cortex and permanent reduction in TPH2 in the basal ganglia in the brain [77].

In human studies, prenatal SSRI exposure has been associated with decrease APGAR scores, slight delays in psychomotor and motor development [79-82], however, not all studies on SSRI exposure during the perinatal periods have poor developmental outcomes [83-89]. The conflicting literature in clinical studies has been attributed to small sample size and underlying maternal depression, not the effects of the medication [90].

1.6 Enteric Nervous System (ENS) Effects of SSRI's in utero

The majority of the neurotransmitters expressed in the CNS are represented in the ENS. Some consider the ENS is part of the PNS [91]. The ENS exhibits independence from the CNS [91]. The ENS is involved in GI motility, secretion and vascular tone [91].

Exposure to SSRI's in utero on the development of the ENS has yet to be fully explored. The vast majority of basic science/animal studies have been on mice. Our study utilizes a rat model as rats are thought to be more similar to humans [92]. SSRI exposure during the perinatal period in clinical studies has been associated with increased laxative use [63,64], Hirschsprung's disease [81] and infantile hypertrophic pyloric stenosis [93]. The mechanisms by which this occurs remains poorly understood and is thought to be related to inhibition of SERT leading to disturbed migration, differentiation and neural crest cell survival which will contribute to the formation of the enteric nervous system [91]. An alternative proposed mechanism is that 5-HT may act as a growth factor for the developing primitive ENS [91].

1.7 The Role of Serotonin in the Gastrointestinal (GI) Tract

Serotonin and its receptors appear early, embryonic day 10 in mice, during prenatal development [94, 97-99]; however, serotonin biosynthesis does not appear in the embryo nor in the extra-embryotic structures suggesting maternal serotonin plays a role during development and maturation of the developing prenatal brain [100]. As mentioned above, Tryptophan hydroxylase (TPH) is the rate-limiting enzyme for 5-HT synthesis [101]. There are 2 isoforms of the TPH enzyme regulating serotonin biosynthesis. TPH 2 is the rate limiting step for serotonin in the CNS, and for biosynthesis in the enteric nervous system. TPH 1 is the rate limiting enzyme in the synthesis of mucosal serotonin. Mice that lack TPH1 (*TPH1-/-*) are deficient in mucosal 5-HT (TPH1 is found in enterochromaffin cells (EC cells) and mast cells) [102,103], while mice lacking TPH2 (*TPH2-/-*) have a 5-HT deficiency in enteric neurons [104] and brain tissue [105]. Moreover, mice lacking TPH1 (tph1−/−) have shown that maternal serotonin is imperative for normal embryonic development, including that of the GI tract [100]. In human fetal brain, SERT antibodies revealed SERT-positive fibers in the raphe nucleus, and cortical regions of the brain at gestational week 8-13 [106]. Human fetal gut development begins around the same time at gestational week 8 with the myenteric plexus at gestational week 8-9, submucosal plexus development at gestational week 10-11 and completion of the ENS by gestational week 12-14 [94].

Serotonin signaling in the intestine is critical for intestinal function, and dysregulation of this pathway is associated with intestinal disease [107]. The GI tract contains 95% of the body's endogenous stores of 5-HT [108]. The vast majority (~90%) of intestinal 5-HT is synthesized and stored in EC cells, with the remainder being synthesized and released in the enteric nervous system (ENS) [108]. Normal 5-HT metabolism is crucial for maintenance of physiological intestinal functions such as motility and sensation [108,109]. Both EC cells and enteric neurons not only synthesize 5-HT, but also express 5-HT receptors and respond to 5-HT activation [108]. An essential part of this pathway includes SERT which inactivates 5-HT after it has stimulated its receptors and is expressed by both enterocytes and enteric neurons [108]. Serotonin is also important for mucosal homeostasis [110]. Mice lacking SERT (SERT KO) demonstrated enhancement of villus height, crypt depth and enterocyte proliferation thought to be related to the excess serotonin availability [111]. Fluoxetine exposure in utero has also demonstrated an increase in villus height and crypt depth in mice studies suggesting that disruption of serotonin homeostasis during the perinatal periods can cause structural abnormalities in the developing offspring [112].

The ENS is believed to contribute to GI disorders in adults, including Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD) however, the pathophysiology of both these entities remains unknown [31,113].

IBD is a chronic disease which is divided into two subtypes, ulcerative colitis and Crohn's disease [114]. It results in mucosal and/or transmural inflammation, bloody diarrhea, weight loss, and increases the risk for colonic adenocarcinoma [114]. 5-HT produced by the ENS activates the immune cells to produce pro-inflammatory mediators [115]. Serotonergic receptors have been found on B and T lymphocytes, monocytes, macrophage, and dendritic cell [116]. Animal studies have demonstrated an increase in 5-HT in chemically induced colitis, such as dextran sodium sulfate (DSS) [117]. In addition, TPH1 knockout mice had reduced colitis scores compared to wild type animals [118]. Alterations in EC cell number and 5-HT amount have been described in clinical studies of patients with IBD [119-122]. Moreover, villus blunting or decrease villus ratio in the small intestine has been demonstrated in humans with Crohn's disease [123] and, as mentioned above, serotonin has been implicated in intestinal epithelial cell homeostasis.

IBS is a functional GI disorder characterized by abdominal pain and alterations in bowel habits [95]. In adults, IBS is characterized as being either diarrheal predominant, constipation predominant or mixed [117]. In a subset of patients with IBS-D, clinical studies demonstrated decreased SERT mRNA in platelets [124]. The quantity of EC cells, 5-HT, mRNA levels of TPH, and the expression of SERT in mucosal biopsies have been associated with IBS in both animal and human studies [125-129].

1.8 Sex Dependent Differences in Serotonin Signaling

Reproductive endocrinology and the central serotonergic system have been linked given their relationship to mood and behaviour [130]. Observations and presumed interactions include increased levels of central serotonin and serotonin receptors with varying changes in reproductive steroids [131,132]. Effects of gonadal steroids are also regulated by serotonin and linked to behaviours such as aggression and reproduction which are modulated by both central serotonin and gonadal steroids [133-136]. Animal studies have shown that serotonin changes as a function of sex, phase in the estrus cycle and as a consequence of hormonal manipulation (ovariectomy and hormone replacement) [130].

Animal and human models suggest sex variances in 5-HT in the CNS [137]. In animal studies, central 5-HT levels, as well as cerebrospinal fluid concentrations of the 5-HT metabolite 5-hydroxyindole-3-acetic acid (5-HIAA), are significantly higher in female compared to male rats [138]. In human studies using positron-emission tomography (PET) scanning, females had significantly higher 5-HT_{1A} receptor and lower 5-HT binding potentials in a wide array of cortical and subcortical brain regions compared to males [137].

There is emerging evidence to support the concept of sex-dependent differences in the serotonergic system of the ENS, as well as the CNS. Colonic extracellular 5-HT visceral hypersensitivity and hyperexcitability of colon projecting sensory

neurons have been shown to be increased in female compared to male rats [139]. Identification of a sex specific role for the microbiota in the regulation of CNS serotonergic neurotransmission has been described, thereby linking the sex specific effects of 5-HT on the gut with the CNS [140].

CHAPTER 2: OBJECTIVES AND HYPOTHESIS

2.1 Rational and Hypothesis

Perinatal exposure to SSRI's has been shown to alter the developing CNS. Given that the majority of the body's endogenous serotonin exists in the EC cell, it is important to assess the consequences of early life exposure to SSRI's on the development of the ENS. The neurons and glia that comprise the ENS are derived from vagal and sacral regions of the neural crest cells [141]. Neurotrophic factors are polypeptides which are involved in the migration, differentiation and survival of neural crest cells [142]. Brain-derived neurotrophic factor (BDNF) bind to the tropomyosin receptor kinase (Trk) receptors for cell survival and has a role in both development of the central and peripheral nervous system [142]. BDNF has recently been shown to have an important role in abdominal pain associated with bowel obstruction [143]. The stimulatory effects of BDNF on gut motility are poorly understood with conflicting results [142], however, gut motility has been demonstrated to be regulated by the ENS but not directly by BDNF, instead serotonin mediates motility in the ENS by TrkB mediated transient calcium ion increase [144]. This mechanism might help better understand and provide insight into the pathogenesis of IBS which consists mainly of symptoms of abdominal pain and altered GI motility. Serotonin also has a regulatory role in mucosal homeostasis

and adaptation [145] and this is of clinical relevance because it may provide insight into the pathogenesis of IBD where mucosal homeostasis is altered.

We tested the hypothesis that perinatal exposure to SSRI's can influence the structure and function of the gastrointestinal tract of the offspring.

2.2 Objectives

1. To determine whether perinatal SSRI exposure can influence the structural development of the gastrointestinal tract of the offspring.

2. To determine whether perinatal SSRI exposure can influence clinically relevant functions of the gastrointestinal tract of the offspring.

CHAPTER 3: AIM 1- STRUCTURE

3.1 Background

Human studies have demonstrated that SSRI exposure in utero leads to altered brain development and maladaptive behaviors [146]. Moreover, SSRI's in utero have been shown to alter serotonergic signaling in the CNS [147, 148]. There is a growing body of literature that links SSRI exposure to alterations in the peripheral serotonergic system in the gastrointestinal tract.

3.2 Methods

3.2.1 Animal Experiments

All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous Wistar rats (Harlan, Indianapolis, IN, USA) were maintained under controlled lighting (12:12 L:D) and temperature (22 °C) with ad libitum access to food and water. Dams were randomly assigned to receive cookie dough or 10 mg/kg/day fluoxetine hydrochloride (Toronto Research Chemicals, Toronto, ON) orally in cookie dough from 14 days prior to mating and through the

weaning period (postnatal day 21; P21). Rats were housed until adulthood (postnatal 6 months; P6M). This method of drug administration has been shown to be a reliable method for drug administration to rats [149,55]. The dose of fluoxetine was chosen based on previous studies in Sprague Dawley rats [55-58] which have shown that this dose is expected to achieve serum fluoxetine levels in the rat which are representative of the median serum concentration of fluoxetine (i.e., 450 nmol/L) reported in humans [55, 150-153]. The same dose has been used and previously described in Wistar rats [154,155]. At birth (postnatal day 1; P1), pups were weighed and sexed and litters were culled to 10 pups per litter, preferentially selecting 5 male and 5 female offspring, to ensure uniformity of litter size between treated and control litters. Animals were sacrificed at P1, P21 and P6M to assess the effects of SSRI exposure in utero, at weaning (maximum amount of SSRI exposure) and adulthood to asses if effects lasted despite withdrawal of SSRI at weaning (Figure 1). Sex differences in serotonin signaling have been described in animals and humans [137-140], and therefore our analysis includes both male and female offspring.

3.2.2 Immunohistochemistry

3.2.2.1 Enteric Nervous System

Enteric neurons in the myenteric plexus were visualized in whole mount preparations of jejunum, ileum and colon at P1, P21 and P6M. Intestines were collected, fixed in 4% formaldehyde and stored in phosphate buffer saline (PBS) at 4°C. For whole mount preparations, tissues were collected and fixed immediately following sacrifice, cut open longitudinally and pinned serosal side down in individual Petri dishes. For cross-sections preparations, tissues were cryoprotected by overnight incubation in 30% sucrose solution in PBS prior to embedding in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Miles Laboratories, Elkhart, IN, USA). For immunohistochemistry of whole mount preparations, tissues were permeabilized and blocked by incubation in PBS containing 0.4% Triton X-100 and 4% normal goat serum. Primary antibodies were applied overnight by incubation in 48 well plates at room temperature. Primary antibodies included biotinylated mouse monoclonal antibodies to anti-human neuronal protein HuC/HuD (anti-HuC/D; dilution 1:50; Molecular Probes, Invitrogen Canada Inc., Burlington, ON, Canada), and polyclonal rabbit anti-5HT (dilution 1:2000; ImmunoStar, Cedarlane Burlington, ON, Canada). Sites of antibody binding were detected by incubation for 2 hours at room temperature with donkey anti-rabbit

antibodies labeled with Alexa 594 (1:200; Molecular Probes), with streptavidin labeled with Alexa 488 (1:200; Molecular Probes) or with goat anti-rabbit antibodies labeled with Alexa 594 (1:200; Molecular Probes). In negative control preparations, no immunostaining was seen when primary antibodies were omitted (Figure 6). Labeled tissue sections were mounted in Vectashield medium (Vector Laboratories Canada Inc., Burlington, ON, Canada) to minimize fading (Figure 5 & Figure 6).

3.2.2.2 Enterochromaffin cells

Cryostat sections were cut at 10 μm and thaw mounted onto Superfrost plus microscope slides (Thermo Scientific, Waltham, MA, USA) which were stored at -20°C until processed. Slides were air dried at room temperature and washed in PBS to remove OTC. Sections were circled in Gnome Ped hydrophobic marker (Frogga Bio, Toronto ON, Canada) and blocked with PBS containing 4% normal goat serum and 0.4% Triton-X-100 to prevent nonspecific binding. Polyclonal rabbit anti-5HT primary antibody was applied followed by goat anti-rabbit IgG conjugated Alexa Fluoro 488 for visualization of EC cells. Nuclei were labeled with fluorescent DNA stain bisbenzimide (Sigma-Aldrich, Oakville ON, Canada) and mounted with Vectashield mounting medium (Figure 8).

3.2.3 Image Analysis

All slides were coded prior to analysis such that the investigator was blinded to the experimental condition. Immunostained tissue was photographed with Retina QImaging digital camera mounted on Leica DMRXA2 microscope (Nussloch, Germany) operated by a Macintosh computer (Apple Computers, Markham ON, Canada). Images were viewed and analyzed using Volocity Imaging software (Improvision Inc., Montreal QC, Canada). Immunostained tissue was photographed at 40x objective in five selected fields and manually counted to assess relative density of myenteric neurons. The number of 5-HT positive cells were quantified by counting the number of 5-HT positive cells per 10 villus-crypt units (ileum) and 10 crypts (colon). The number of 5-HT positive cells were also quantified by number of 5-HT positive cells per surface area (um^2) [156]. A total of 3 samples per slide was analyzed and the average number of EC cells calculated.

3.2.4 Crypt/Villus Ratio

At P1, P21 and P6M, tissue was fixed, cryoprotected as described above and mounted on slides as cross sections. Using computer imaging software Volocity (Improvision Inc., Montreal QC, Canada), photographs were taken, and the ruler

feature was used to measure the depth of the crypts in the colons and the height of the villi in the small intestine. The small intestine contains both crypt depth and villi height, therefore a ratio of crypt depth to villi height was calculated per 10 complete villi as previously described [157]. As previously mentioned, crypt depth, villus height and the crypt ratio might provide further insight into the effects of SSRI exposure during the perinatal period and mucosal homeostasis.

3.2.5 Statistical Analysis

Data are presented as mean +/- standard deviation (SD). Statistical analysis was performed using unpaired non parametric T-test using Prism 7 (GraphPad Software, San Diego, CA) of SSRI exposed vs control for each sex. A $p<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Enteric Nervous System

To assess for the potential influence of perinatal SSRI exposure on the development of the ENS, whole mount preparations were examined at P1, P21 and P6M. At P1, a significant decrease percentage of serotonergic neurons was found in the SSRI-

exposed female offspring colon (13.6% vs 9.3%; p=0.04, n=6-10) (Figure 2). However, no significant differences were found in the total number of enteric neurons nor in serotonergic neurons between SSRI-exposed and control offspring in the jejunum, ileum and male colon. At P21, significant differences were found in the percentage of serotonergic neurons in both SSRI-exposed female (1.4% vs 7.6%; p=0.009) and male colons (1.7% vs 6.9%; p=0.002) (n=6-8) (Figure 3). No significant differences were found in the total number of enteric neurons nor serotonergic neurons in the ileum. At P6M, there was no significant difference in the percentage of total number of enteric neurons nor in serotonergic neurons between SSRI-exposed and control offspring (n=6-10) (Figure 4).

3.3.2 Enterochromaffin cells

Numbers of EC cells were assessed by immunohistochemistry with antibodies to 5-HT in cross-sections of small and large intestine at P21 and P6M. P1 was excluded given that the tissue was technically changing to cut/examine given the relatively small tissue size. The quantification of EC cells was done per 10 crypt villi and by surface area for 3 samples per animal [137]. At P21, no significant differences were found in the SSRI-exposed female offspring colon (20.5 vs 16; p= 0.6380 using the 10 crypt villi method and 2.937e-005 vs 2.148e-005; p= 0.2319 using surface area), nor the SSRI- exposed male offspring colon (17.5 vs 16.5; p=

0.3934 and 2.882e-005 vs 2.123e-005; p= 0.2767). At P21, no significant differences were found in the SSRI-exposed female offspring ileum (31.5 vs 25; p= 0.6373 and 1.674e-005 vs 1.223e-005; p= 0.8571) nor SSRI-exposed male offspring ileum (31 vs 36; p= 0.6373 and 1.559e-005 vs 1.807e-005; p= 0.1667). No significant difference was found in the number of EC cells in the SSRI-exposed offspring's colon in either female (5 vs 8; p>0.9999) or male (6.5 vs 8; p= 0.3571) at P6M. There was also no significant difference in the SSRI-exposed offspring's ileum in the female (8 vs 11; p= 0.4603) and male (15 vs 10; p= 0.1032) at P6M. At P21 (n=10-14 colon and n=3-6 ileum) and adult P6M, ECC number was not statistically significant using either method (Figure 7, Figure 9, and Figure 10).

3.3.3 Crypt/Villus Ratio

Crypt depth in the colon and villi height/crypt depth in the small intestine were measured using Volocity (Improvision Inc., Montreal QC, Canada). A villus to crypt ratio was also calculated in the small intestine in order to assess structural consequence related to serotonin signaling. At P1 and P6M there was no significant difference in crypt depth of the colon of SSRI-exposed vs control offspring (Figure 11 & Figure 13). At P21, the crypt depth was significantly reduced in the SSRI-exposed female offspring when compared to control (p= 0.03, n=3-7) (Figure 12).

At P1, P21 and P6M there was no significant different in the height of the villi, nor in the ratio of the villus to crypt in the small intestine of the SSRI- exposed vs control male offspring (n= 3-7).

CHAPTER 4: AIM 2- FUNCTION

4.1 Background

The results from Aim 1 of this study suggested that the structure of the ENS was altered, however, it remained to be determined whether there would be clinically relevant functional consequences related to intestinal motility. As described above, intestinal serotonin modulates the ENS development, motility, secretion and inflammation [158]. Another clinically relevant target for patients with delayed motility is the use of serotonin agonist and antagonist as pharmacologic therapy [158].

Moreover, mucosal serotonin increases in intestinal inflammation (i.e. colitis) [118] and our results suggested altered numbers of serotonergic neurons in the ENS which could alter the mucosal immune response in colitis. Our results also showed changes in the crypt villus ratio in female SSRI-exposed offspring for which serotonin plays an important role in mucosal homeostasis [145].

4.1.1 Serotonin and Inflammation

5-HT influences activity of the effector cells of the adaptive and innate immune response [159]. For instance, T cells and macrophages have been shown to produce

5-HT, while murine and rat mast cells synthesize, store, secrete and uptake serotonin [160]. 5-HT has also been shown to affect chemotaxis, proliferation, leukocyte activation, anergy, cytokine secretion and apoptosis in immune cells [160,161]. 5-HT participates in cell-mediated immunity by facilitating lymphocyte trafficking through post-capillary venules, as is it vasoactive [162]. Serotonin also accumulates in sympathetic nerve terminals and after traveling through the axon and entering the synaptic vesicles and is co-released with norepinephrine [163]. Therefore, sympathetic innervation in gut-associated lymphoid tissue could be another source of 5-HT that regulates intestinal immunity.

Recent animal studies of SSRI exposed offspring have demonstrated increased liver inflammation with sex variances [154]. SSRI-exposed male rat offspring had significantly higher mRNA expression of TNFα, IL6 and monocyte chemoattractant protein 1 [154]. The female SSRI-exposed offspring had higher levels of mRNA expression of TNFα and increased macrophage infiltration [125].

4.2 Methods

4.2.1 Animal Experiments

The same rats as described above in Aim 1were used to assess motility and response to experimental colitis using DSS.

4.2.2 Motility

18 Wistar rats aged 6 months were used to measure motility patterns, as previously described [164]. 9 (5M, 4F) rats were offspring of mothers treated with SSRI as described above and 9 (4M, 5F) rat controls. Rats were euthanized by CO_2 asphyxiation. The small intestine and colon were dissected and placed in chilled Krebs solution (composed of (in mM) 120.35 NaCl, 5.9 KCl, 15.5 $NaHCO_3$, 1.2 NaH_2PO_4, 0.1 Citric acid $.H_2O$, 0.1 Aspartic acid, 2.5 $CaCl_2$ $.2H_2O$, 1.2$MgCl_2$ $.6H_2O$, 6 glucose) for preparation. The entire mesentery was cut away and luminal contents were gently flushed with saline solution (137 NaCl and 5.9 KCl). The small intestine was cut to 25-30 cm in length and the entire colon was used. The oral and anal ends of the tissues were cannulated with 3mm polyethylene tubing and secured with cotton string. The small intestine and colon were placed in individual organ baths containing 1L of oxygenated (95% oxygen, 5% CO_2), warmed Krebs solution (pH 7.35, 36°C) which was continuously pumped to avoid precipitation of $NaHCO_3$. The oral cannulas of the tissues were connected to a peristaltic pump (World Precision Instruments, Inc.) which pumped either Krebs solution into the small intestine or PBS ((in mmol/L) 117 NaCl, 10 Na_2HPO_4, 3.9 KCl, 2 KH_2PO_4) into the colon. PBS was used in the colon as glucose has been shown to induce 5-HT release from enterochromaffin cells [165]. The anal cannulas

were placed on top of an adjustable stand, which was used to maintain an intraluminal pressure of 3cm H_2O, from which the fluid freely dripped into a reservoir. After a 30-minute baseline period, the tissues were video recorded (1/3' SONY Super HAD CCD, 700 TVL, and SONY Effio-E DSP) for 15 minutes twice under slow (0.14 mL/min), then fast (1.4 mL/min) perfusion speeds. Creation of spatiotemporal maps and all other analysis were carried out with custom plugins written for ImageJ (National Institutes of Health, Bethesda, MD), as described previously [163]. Propulsive contractions in the colon were expressed at Long Distance Contractions (LDCs) in the colon. This is synonymous with complex migrating motor complexes (CMMC) or colonic motor complexes (CMC) which are also frequently used as a description for colonic motility [166]. Pharmacology challenge with ondansetron (5-HT3 receptor antagonist) x 10 minutes and lidocaine (blockade of neural action potentials) x 10 minutes was done with the small intestine (Figure 15).

4.2.3 Experimental colitis

At P6M, a subset of the offspring was treated with dextran sulfate sodium (DSS) to assess susceptibility to colitis (n=5/sex/group for each DSS and control conditions). DSS was added to drinking water for a final concentration of 4% (w/v) for a total of 5 days, as previously described [124], using 20g of DSS (Sigma-Aldrich, Cat.

no 42867, molecular weight Mr 40,000, Oakville ON, Canada) added to 500 mL of autoclaved water. In the biosafety hood, 50 mL of 4% DSS was poured into Falcon tubes (Thermo Scientific, Waltham, MA, USA) one per cage. Drinking water was replaced with 4% DSS solution. Rats did not have any access to any other drinking water. Control rats received autoclaved drinking water alone. Rats weights and amount of 4% DSS solution consumed were recorded. Rats were monitored for dehydration and disease activity in accordance with Animal Utilization Protocol (AUP). At the end of the 5th day, colons were collected from DSS-treated and control offspring and scored for histological damage and MPO using the below validated protocols.

Severity of experimental models of colitis can be made at the tissue level, through analysis of MPO [124] and cytokine production [124]. At the tissue level, previously published and validated protocols use histology into a standardized scoring system assessing the histopathological state of the intestine using hematoxylin & eosin stained colonic tissue sections. Colonic tissue fragments can be used to determine MPO activity. MPO is an enzyme released by granulocytes such as neutrophils and is used as a surrogate marker of inflammation.

4.2.4 Histologic Inflammation Score

Colons were collected from male and female SSRI-exposed and control offspring at P21 and P6M receiving regular drinking water. Colons were also collected from the P6M subset of SSRI-exposed and control rats that received DSS. Sections were mounted in OTC (Tissue-Tek, Miles Laboratories, Elkhart, IN, USA) and frozen and stored in at -20°C. Cryostat sections were cut at 5 μm and thaw mounted onto Superfrost plus microscope slides (Thermo Scientific, Waltham, MA, USA) which were stored at -20°C until processed. Slides were air dried at room temperature and washed in PBS to remove OTC. They were subsequently stained with hematoxylin and eosin (H&E) (Figure 16) and scored using a previously established validated histology score to assess damage and inflammation (Table 1) [167]. Slides were placed in hematoxylin for 1 minute, then rinsed under running tap water for 5 minutes. Slides were then placed in eosin for 30 seconds, then rinsed under running tap water for 5 minutes. Slides were subsequently dehydrated with one change of 5 minutes in 95% alcohol, followed by 2 changes in 100% alcohol for 5 minutes. Slides were left in the fume hood to dry. Once dry, coverslips were mounted. The histological score considers changes in crypt architecture, cellular infiltration, goblet cell depletion, and crypt abscesses. Using a light microscope in the Dr. Waliul Khan Lab (Olympus CX31, Markham ON, Canada), sections were score

blinded to the various treatment groups. For consistency, all scoring was done by one person (KLP).

4.2.5 Colonic Myeloperoxidase

Colons collected from the above P6M subset of offspring treated with either 4% DSS or drinking water were collected for colonic myeloperoxidase assay (MPO). MPO activity was measured following a published protocol [168] in colonic tissue samples from SSRI-treated and control offspring. Weight of each sample after removing any feces and/or fat, using a pair of bent forceps/tweezers, were place into a 2 mL Eppendorf microcentrifuge tube with one homogenizer bead. Hexadecyltrimethylammonium bromide (HTAB) (Sigma-Aldrich, Cat. no. H5882-100G, Oakville ON, Canada) buffer was added based on the weight of the tissue sample. Tissue was then homogenized with tissue homogenizer (Retsch® Mixer Mill MM 301, Retsch USA Verder Scientific, Inc., Newtown, PA) for 4 min at 30 Hz. The homogenizer bead was removed using forceps. Once tissue had been homogenized, samples were centrifuged (6 min, 13400 x g, 4°C). The supernatant was collected, and the resulting pellet discarded. Supernatant samples were stored at -80°C until use. Subsequently at the time of analysis, O-dianisidine solution was prepared fresh for every assay. Tissue homogenates in triplicate was added into a 96-well plate. Absorbance at 450 nm was measured using a spectrophotometer.

Three readings at 30 second intervals were recorded. MPO activity was calculated as follows: MPO activity was measured in units (U) of MPO/mg tissue, where one unit of MPO is defined as the amount needed to degrade 1 μmoL of H_2O_2 per minute at room temperature. Considering that one unit (U) of MPO = 1 μmoL of H_2O_2 split and that 1 μmoL of H_2O_2 gives a change of absorbance of 1.13×10^{-2} nm/min, units of MPO in each sample was determined as change in absorbance $[\Delta A(t2\text{-}t1)]/\Delta min \times (1.13 \times 10^{-2})$. MPO activity is expressed in units per milligram of wet tissue, where 1 U is the quantity of enzyme able to convert 1 mmol hydrogen peroxide to water in 1 min at room temperature.

4.2.6 Assessment of Tight Junction Proteins by Immunohistochemistry

Immunofluorescence was performed in accordance with the established protocol as previously described. When quantifying apical tight junction proteins, the primary antibodies used were rabbit anti-occludin polyclonal antibody (Life Technologies Inc, Cat. no PA520755, Burlington ON, Canada) and rabbit anti-zonula occludens-1 (ZO-1) polyclonal antibody (Life Technologies Inc, cat. no 617300, Burlington ON, Canada). The secondary antibody was goat anti-rabbit Alexa Flour 488 (Thermo Scientific, Cat. no A-11008 Waltham, MA, USA).

Sections of the SSRI-exposed and control offspring at P6M were placed in Optimal Tissue Compound (OTC) (Tissue Tek, VWR International, Cat no. 4583,

Mississauga, ON Canada) and frozen at 20°C. Cryostat sections were cut at 5 μm and thaw mounted onto Superfrost plus microscope slides (Thermo Scientific, Waltham, MA, USA) which were stored at -20°C until processed. Slides were air dried at room temperature and washed in PBS to remove OTC (Tissue Tek, VWR International, Cat no. 4583, Mississauga, ON Canada). Once dried, circles where drawn around the tissue samples using Hydrophobic pen (Cederlane, Cat. no. Vector H-4000, Burlington ON, Canada). A blocking/diluent buffer was prepared using normal goat serum (Life Technologies Inc, cat. no. 16210064, Burlington ON, Canada), 0.4% Triton x-100 (Sigma Aldrich, cat. no 11332481001, Oakville ON, Canada) and PBS. Once hydrophobic pen (Cederlane, Cat. no. Vector H-4000, Burlington ON, Canada) had dried, blocking buffer was applied to the tissue samples for 1 hour at room temperature. Blocking buffer was then removed prior to placement of primary antibody (either zonulin-1 or occludins) in a 1:2000 dilution with the blocking buffer and was incubated overnight (12-18 hours) at room temperature. Primary antibodies where then removed in 3 washes of 5 minutes of PBS in Coplin jars (Thermo Scientific, Cat. no 22-038-489 Waltham, MA) on an orbital shaker (Barnstead Lab-Line 2309 Multi-Purpose Rotator, USA). Secondary antibody goat anti-rabbit Alexa Fluoro 488 (Thermo Scientific, Cat. no A-11008, Waltham, MA) was applied in a 1:200 dilution with the blocking buffer and incubated for 2-3 hours at room temperature. Secondary antibodies where then removed in 3 washes of 5 minutes of PBS in Coplin jars (Thermo Scientific, Cat.

no 22-038-489 Waltham, MA) on an orbital shaker (Barnstead Lab-Line 2309 Multi-Purpose Rotator, USA). The intestinal epithelial nuclei of the mucosa were labelled with bisbenzimide (Sigma Aldrich, cat. no. H33258, Oakville ON, Canada) as a counterstain for visualizing architecture. 1ug/ml Bisbenzimide (Sigma Aldrich, cat. no H33258, Oakville ON, Canada) was incubated on the slide for 4 min at room temperature and then washed for 5 minutes in water followed by 2 exchanges in PBS for 5 minutes. Slides were drained and mounted with a coverslip using mounting medium Vectashield (MJS BioLynx, Vector H-1000, Brockville ON, Canada) (Figure 18 & Figure 19).

4.2.7 Image Analysis

The difference in the quantity of tight junctions at P6M was visualized with immunofluorescence staining to occludin and ZO-1 antibodies and measured as fluorescence intensity using Volocity (Improvision Inc., Montreal QC, Canada). Colonic samples were measured in duplicate; 3 images per sample were taken at 20X magnification for a total of 6 images per animal. A protocol provided by the Verdu lab using ImageJ for measuring intensity was adapted to Volocity (Improvision Inc., Montreal QC, Canada) with the help of technical support from Volocity. This protocol included measuring standard deviation intensity per specified region (surface area) of interest. The region of interest in each image was

identified based on the following criteria: apical portion (1/3) of the intestinal crypt of approximately 100,000 micrometers in surface area. Once the area of interest was identified and selected, a series of objects with a unique intensity was computed by Volocity. The fluorescence intensity for each image was measured as weighted average in each object within the selected region of interest (ROI). The intensity was reported as average of intensities per object. The fluorescence intensity for each slide was calculated as the average of 6 images in SSRI-exposed and control groups as demonstrated below.

Sample Calculations

Fluorescence Intensity Average Per Slide (3 Samples) =

$$\frac{Mean\ Intensity\ of\ Sample\ 1\ +\ Mean\ Intesnity\ of\ Sample\ 2 + \cdots}{N\ of\ Sample}$$

Fluorescence Intensity Per Sample =

$$\frac{Intensity\ of\ Image\ 1\ +\ Intensity\ of\ Image\ 2 + \cdots}{N\ of\ Images}$$

4.2.8 Serotonin and Inflammation: Quantitative Gene Expression by Nanostring

After reviewing immunology of DSS colitis in rats [169-172], we developed an 83 gene code set with Dr. Carl Richards Lab for Nanostring genomic analysis (RatE code set).

mRNA was extracted from colonic tissue of DSS treated and water treated rats using commercially available Qiagen RNA Easy kits (Qiagen, Cat no. 74104, Toronto ON, Canada). 20-30 mg of colonic tissue was placed directly in a DNAse/RNAse free 1.5 mL microcentrifuge tube (Fisher Scientific Company, Cat. no 05-408-130, Ottawa ON, Canada) containing a metal bead with RLT buffer containing 2-Mercaptoethanol (2-ME) (Life Technologies Inc., Cat no. 21985023, Burlington ON, Canada). The tissue was homogenized using Retsch® Mixer Mill MM 301 (Retsch USA Verder Scientific, Inc., Newtown, PA) in Dr. Waliul Khan's Lab for 2 min at 30 Hz in pre-cooled -20^{0}C tube holder. The homogenizer bead was removed using forceps. Once tissue had been homogenized, samples were centrifuged (3 min, 13400 x g, 4°C). The supernatant was collected, and the resulting pellet discarded. 70% ethanol was added to the cleared supernatant and mix immediately by pipetting. Up to 700 µl of the sample was transferred to a RNeasy spin column placed in a 2 ml collection tube and centrifuge for 15 s at 10,000 rpm. The flow-through was discarded. 700 µl Buffer RW1 was then added to the RNeasy spin column and centrifuge for 15 s at 10,000 rpm. The flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuge for 15 s at 10,000 rpm. The flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuge 2 min at 10,000 rpm. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuge at full speed for 1 min. The RNeasy spin column in a new 1.5 ml collection tube,

45 µl of RNase-free water was added directly to the middle of spin column membrane and centrifuge for 1 min at 10,000 rpm to elute the RNA. The mRNA concentration and quality were assessed using Nanodrop 2000 (Thermo Scientific, Cat. no ND 2000, Waltham, MA) in Dr. Deborah Sloboda Lab and normalized to a concentration of 100 ng/µL. Samples were stored in -80°C freezer until processing in the Farncombe Metagenomics Facility by Nanostring (Figure 21). Prior to samples being run, they were analyzed once more by the Farncombe Metagenomics Facility Bioanalyzer to ensure adequate sample integrity and consistent results with Nanodrop 2000. Bioanalyzer results confirmed concentration and sample quality prior to analysis. Results were normalized in computer program nSolver 4.0 Analysis Software (NanoString Technologies, Inc. Seattle, Washington USA) prior to statistical analysis using housekeeping genes Beta Actin, YWHAZ and PGK1.

4.3 Results

4.3.1 Motility

Small intestinal motility did not appear altered while colonic motility was significantly altered at P6M. The nomenclature for propulsive contractions is diverse including complex migrating motor patterns (CMMC), colonic motor complexes and long-distance contractions (LDCs) [173]. Our results are expressed

using LDC's as the description for propulsive contractions in the colon. There was an increased frequency of long-distance contractions (LDCs) in the SSRI-exposed vs control offspring ($p = 0.04$; n=9) colon at P6M which suggest that colonic contents move significantly faster through the colon of our SSRI exposed offspring. The velocity of LDCs was also increased in the SSRI-exposed offspring ($p = 0.03$; n=9) in the colon at P6M. There was no significant difference between the SSRI exposed and control offspring in terms of propagation distance of LDCs ($p = 0.39$; n=9). It was noted that using lower pressure vs higher pressure did not result in statistically significant difference in LCDs between SSRI-exposed and control offspring ($p = 0.06$; n=9).

The pattern of small bowel motility did not show statistically significant changes in magnitude nor frequency of contractions on SSRI-exposed offspring at P6M. There was also no significant different in frequency of contractions at P6M in the small intestine with pharmacologic challenge of Ondansetron or Lidocaine (Figure 14).

4.3.2 Histologic Inflammation Score

Fetal and neonatal exposure to SSRI did not significantly affect intestinal microscopic disease at P21 or P6M. Moreover, there was no difference in severity of DSS colitis between control and SSRI-exposed offspring at P6M, although the

female offspring born to SSRI-treated mothers tended to have a higher microscopic colitis score ($p = 0.056$; $n = 9$-11) (Figure 17).

4.3.3 Colonic Myeloperoxidase

At P6M, MPO increased significantly in the DSS treated vs water treated animals (female water vs female DSS, $p= 0.0079$; male water vs male DSS, $p= 0.0079$; $n =5$ per group). MPO was also significantly increased in the SSRI exposed-offspring at P6M (female SSRI-exposed water vs female SSRI-exposed DSS, $p=0.02$; male SSRI-exposed water vs male SSRI-exposed DSS, $p=0.004$; $n=5$-6 per group). Although MPO activity was increased as expected in the DSS treated animals, there was no significant effect of SSRI exposure in utero (Figure 17).

4.3.4 Assessment of Tight Junction Proteins by Immunohistochemistry

An unpaired t-test was performed to analyze whether there was a significant difference in TJ quantity at P6M, calculated as difference in fluorescence intensity, between SSRI-exposed ($n = 3$-5) and control offspring in the colon. The difference in fluorescence intensity, measured as weighted average of object intensities, between the SSRI-exposed ($n = 3$-5) and control ($n = 3$-5) in colon cross sections stained for occludin was not statistically significant (female SSRI-exposed

offspring vs female control, p= 0.86; male SSRI-exposed offspring vs male control, p= 0.35). The difference in fluorescence intensity, measured as weighted average of object intensities, between the SSRI-exposed and control in colon cross sections stained for ZO-1 was statistically significantly increased in SSRI-exposed male offspring (male SSRI exposed vs male control, p = 0.04; n = 3-5), but not in females (female SSRI-exposed offspring vs female control, p= 0.40; n = 3-5) (Figure 20).

4.3.5 Serotonin and Inflammation: Gene Expression by Nanostring

Colonic mRNA gene expression by Nanostring was significantly different depending on sex and SSRI-exposure in chemically induced DSS colitis. Although some of our top gene targets, consistent with existing DSS colitis literature in rat [169-172] were statistically significantly altered which included IL-1β, CCL2, IL-6 and IL-4; TNF-α was not significantly different. We were also interested in the additional pathways currently being explored as therapeutic targets superficially for IBD [174] such as the JAK-STAT pathways which we did not come across in our literature review. Moreover, despite our model of DSS being an acute model we wondered if some of the markers for wound healing and fibrosis might be involved as early as the initial acute inflammatory insult, which might suggest a more aggressive inflammatory response in the SSRI exposed compare to control offspring. Finally, IL-31 has been described as a mediator of pro-inflammatory

subepithelial myofibroblasts in the human colon and therefore was also of interest [175].

The statistical analysis was done for significant p-value using unpaired t-tests with the two SSRI-exposed offspring groups (male and female separately) and the control group (male and female separately).

The following housekeeping genes were selected beta (β)-actin (ACTB), Phosphoglycerate kinase 1(PGK1) and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (YWHAZ) which are required for basic cell function and were not statistically significantly different between groups.

At P6M, in the SSRI-exposed offspring treated with DSS compared to the SSRI-exposed given normal drinking water, mRNA levels in the colon were significantly elevated for the following targets (n = 6 SSRI-exposed control males, n = 5 SSRI-exposed DSS male): gene encoding cytokines; Interleukin 1 alpha (IL1A; p = 0.009), Interleukin 31 Receptor A (IL31RA; p = 0.02), and Resistin-like molecule beta/FIZZ2 (Retnlb; p = 0.02), enzymes; Matrix Metallopeptidase 7 (MMP7; p = 0.03), and gene encoding proteins; Mannose Receptor C- Type 1 (MRC1; p = 0.03) and TIMP1 (p = 0.004) (Figure 28).

Colonic levels of tissue mRNA were significantly elevated in SSRI-exposed DSS female compared to SSRI-exposed female water controls in the following targets (n = 4 SSRI-exposed control female, n = 5 SSRI-exposed DSS female): gene encoding cytokines; Interleukin 11 receptor, alpha chain 1 (IL11RA1; p = 0.02), Interleukin

31 Receptor A (IL31RA; p = 0.03), and Interleukin 6 Signal Transducer (IL6ST; p = 0.03), gene encoding proteins; Chemokine Ligand 2 (CCL-2 p = 0.02), Ciliary neurotrophic factor (CNTF; p = 0.03), High mobility group box 1 (HMGB1; p = 0.02), MRC1 (p = 0.03), Secreted Frizzled Related Protein 1 (SFRP1; p = 0.02), and STAT3 (p = 0.03) (Figure 28).

In the DSS control offspring, colonic levels of tissue mRNA were significantly elevated in DSS male control offspring compared to male water control offspring in the following gene targets (n = 5 male control, male DSS, female control, and female DSS): gene encoding cytokines: Interleukin 1 beta (IL-1B; p = 0.008), interleukin 4 receptor (IL-4R; p = 0.008), gene encoding proteins; C-C Motif Chemokine Ligand 2 (CCL2; p = 0.03), Mannose Receptor C- Type 1 (MRC1; p = 0.02), Oncostatin M Receptor (OSMR; p = 0.02), Transforming Growth Factor Beta 1 (TGFB1; p = 0.03), Signal transducer and activator of transcription 3 (STAT3; p = 0.02), and tissue inhibitor of metalloproteinases (TIMP1; p = 0.03) (Figure 22). Colonic levels of tissue mRNA were statistically significantly elevated in DSS female compared to water controls in the Collagen Type III Alpha 1 Chain (COL3A1; p = 0.008) gene encoding protein. Colonic levels of tissue mRNA were statistically significantly decreased in DSS male and female compared to water control offspring for Interleukin-17A (IL17; p = 0.008) gene encoding cytokine (Figure 23). Figure 32 represents Venn diagram of overlapping significant quantitative mRNA in each group.

CHAPTER 5: DISCUSSION

Increasing evidence suggests that early life events may have an impact on adult health and disease [176]. The incidence and prevalence of antepartum depression treated with pharmacologic therapy is increasing [43]. There is also increasing prevalence of gastrointestinal disease such as IBS and IBD [177]. Previous studies have demonstrated an effect of SSRI exposure in utero on the developing CNS but the ENS and response to inflammation has been less well characterized. Given, that a large and increasing number of women are prescribed SSRI's during the perinatal period it is important to assess any possible effect on the developing offspring's gastrointestinal tract.

Our findings suggest that SSRI-exposure affects the development of the ENS but that these changes do not persist over time. There was no significant difference on the EC cell numbers. However, despite the fact that the ENS appeared structurally normal at P6M, colonic motility was affected. The crypt depth was decreased at P21 in the SSRI-exposed female offspring which correlated with maximal exposure to SSRI, but architecture was otherwise preserved, and these changes also did not persist into adulthood. The severity of chemically induced colitis was not significantly different nor was the amount of colonic myeloperoxidase in the SSRI-exposed offspring. Given that we are seeing an increase in inflammatory bowel

disease in the under 10-year-old age group in Canada [178], perhaps repeating the DSS experiment at an earlier timepoint would yield different results. P6M was selected as the timepoint for DSS colitis based on preliminary data of microbiota profiling which demonstrated a decrease in Bifidobacterium in SSRI-treated offspring. Human studies have demonstrated the clinical benefits of Bifidobacterium in the management of IBD and animal studies have demonstrated that Bifidobacterium species can have direct effects on the intestinal mucosa [179,180] and the ENS including inhibition after-hyperpolarizing neuronal excitability and vagal nerve stimulation [181, 182]. There were significant differences in the quantity of colonic mRNA tissue expression. Moreover, we found sex related differences in each variable assessed.

5.1 Influence on Intestinal Microanatomy

Assessment of mucosal epithelial maintenance and integrity can be quantified by analysis of the relative height of the villus to the depth of the crypt in the small intestine and by the depth of the crypt in the colon [157]. Literature has demonstrated altered crypt villus ratio in an SSRI exposed mouse model [183]. We found a statistically significant decrease in colon crypt depth at P21 in SSRI-exposed females which correlates to maximal exposure to SSRI. We did not demonstrate changes in crypt/villus ratio in the small intestine. This suggests that

the mucosal microanatomy is preserved in SSRI-exposed rat offspring with the exception of the female colon at P21. It is possible that the mucosal anatomic disturbance resolves once exposure to the SSRI is discontinued given that the mucosal epithelial cells are constantly being regenerated. It is also plausible that the sex related differences may be due to having differences in serotonin signaling or sensitivity to serotonin perturbations in the female offspring similar to previous animal and human studies mentioned above [137,138]. Finally, there may be hormonal differences, however, this would require further investigation.

The decrease in colonic crypt depth may help explain why we did not see a difference in the total number of EC cells as we had expected. Since the absolute number of EC cells was unchanged but the surface area was less, this may suggest that there are more EC cells in a smaller surface area. However, when we quantified the number of EC cells by surface area (SA) there was still no significant difference.

5.2 Influence of SSRI's on the Developing Enteric Nervous System and Serotonergic System

The percentage of serotonergic neurons were significantly reduced in the female colon at P1. At P21, there was a significant increase in the percentage of serotonergic neurons. One theory is that the pregnant dam may have higher circulating levels of SSRI's which increase extracellular serotonin and may down-

regulate the expression of serotonergic neurons in utero. After delivery, the offspring may have a rebound increase in enteric neurons given that the SSRI appears to be less bioavailable in breastmilk than maternal circulating blood. At P6M, there were no statistically significant differences in the percentage of serotonergic neurons between SSRI exposed and control offspring which may be secondary to the plasticity of the enteric nervous system once SSRI exposure is no longer present.

We did not find structural differences in the number of EC cells in the colon at P6M, however our colonic motility data had a significant increase in the LDC frequency and velocity. LDC's are migrating propulsive contractions that travel long distances from the proximal end toward the anal end of the colon [184]. These contractions are usually larger and longer, originating in the small intestine. Increased frequency and velocity of these LDC's would suggest that they travel faster through the colon which we might expect to present clinically with diarrhea. It is plausible that this increase in the frequency and velocity of LDC's is associated with abnormal serotonin signaling which may be lasting consequences of structurally abnormal ENS and warrants further investigation. This may also be a consequence of abnormal 5-HT_3 or 5-HT_4 receptors which play a role in motility [104]. Serotonin is involved in motility; increased serotonin increases motility [104]. We observed an increase in serotonergic neurons at P21 in the colon which correlates to the point of maximal exposure to SSRI, suggesting the effect of SSRI

exposure may have lasting functional effects despite resolution of the structural changes. It is also possible that the effects on motility are related to the release of serotonin from the EC cells or serotonergic neurons, or quantitative amounts of serotonin in tissue which may be altered in our animal model. This also warrants further investigation.

5.3 Functional Consequences of SSRI Treatment on Motility

We observed the frequency and velocity of LDCs in the colon of SSRI-exposed offspring were significantly increased at P6M which may suggest that despite the structurally normal colon, the effects of SSRI exposure may have long term functional consequences.

This is clinically relevant as it may be similar to patients with IBS in which the colon is structurally normal but clinically, they have increased motility, decreased motility or a mix of alternating increased and decreased motility manifested as diarrhea or constipation. The pathophysiology of IBS remains unclear but treatments for this gastrointestinal entity often include medications that work on 5-HT_3 and 5-HT_4 which are involved in GI motility. Clinically studies have also demonstrated alterations in 5-HT in intestinal mucosa of IBS patients in comparison to healthy controls [185]. Other symptoms include bloating, abdominal pain or

visceral hypersensitivity which were not assessed in this model and remain an area for future exploration.

5.4 Increased Susceptibility to Chemically Induced Colitis

There was no increased susceptibility to colitis in the SSRI-exposed vs control offspring at P6M. P6M was chosen as the timepoint to assess the effects of DSS colitis in order to test the hypothesis about developmental origins of disease [186]. In other words, assessing whether early exposure to SSRI could influence susceptibility to colitis in adults. DSS is toxic to the mucosal epithelial due to its highly negative charged sulfate group which results in erosions and increased intestinal permeability [169-172]. This inciting factor contributes to the burden of reactive oxygen species (ROS) which are also by products of normal cellular metabolism and can be beneficial at low levels (helps with host defense against invading pathogens, wound healing, tissue repair, etc.) [187]. When ROS are disproportionately high homeostasis is disrupted and results in oxidative tissue damage. It appears that immune response in DSS colitis is maintained in this rat model which suggests that the mechanism of oxidative stress (ROS) on mucosal epithelial cells is not influenced by SSRI-exposure. DSS has been used in both acute and chronic models of colitis [124]. Our group chose an acute model of colitis to assess for increased susceptibility. Given that recent literature assessing

pathways in IBD involved some of the cytokines involved in fibrosis (like OSM) [174] and wound healing (COL3A1) we assessed markers of fibrosis in our acute DSS model which is novel. Typically, these genes would be assessed in a chronic colitis model. Interestingly, we did find changes in COL3A1 and OSMR as described above.

Although colonic MPO was significantly increased in the DSS induced colitis rats, there was no difference between the SSRI-exposed and control offspring which supports that chemical induced colitis at P6M does not appear to be influenced by early life exposure to SSRIs.

We know that in the mammalian bowel, neuroendocrine signaling is important in aiding innate and adaptive immune response [177], specifically macrophages and lymphocytes [188]. Mice lacking SERT (SERT KO) had worsening response to another chemically induced colitis 2,4,6-trinitrobenzene sulfonic acid (TNBS), which suggests that 5-HT has a pro-inflammatory effect [177]. Given our animals did not have a significant difference in the structural component of the ENS nor EC cells which comprise the serotonergic signaling at the P6M, this could explain why we did not find an increased inflammatory response. Perhaps had we done the DSS experiment at P21, where there were structural changes in the ENS, we might have seen this effect.

5.5 Increased Intestinal Permeability and Inflammatory Bowel Disease

Increased intestinal permeability is seen in IBD but also in healthy first-degree relatives [186] and although the pathophysiology of IBD remains unknown, increased intestinal permeability is thought to be an important factor [188]. We also know that the prevalence of IBD is increasing, and in the pediatric population, the less than 10 age group has the most rapidly increasing rate, which could suggest early life factors such as an in-utero exposure may contribute to the burden of intestinal inflammatory bowel disease [189]. Literature has demonstrated SERT KO mice and SSRI-exposed mice offspring have increased intestinal permeability [183]. We had therefore expected our SSRI-exposed rat offspring to show increased intestinal permeability and potentially increased susceptibility to chemically induced colitis. We observed a significant increase in ZO-1 in the SSRI exposed male at P6M. It is unclear at this time whether this is a compensatory mechanism or whether at a different timepoint (i.e. P21, point of maximal exposure) we might have observed a different phenotype more consistent with the published literature, in light of the ongoing exposure to SSRI at P21.

5.6 Altered Immune Response in Perturbed Serotonin Signaling

The release of norepinephrine from vagal control has been shown to target a specific memory T cell population producing acetylcholine (ChAT) [190]. ChAT positive fibers originating mainly in the enteric nervous system (and peyer patches, however no neuronal contact) are in close proximity to intestinal macrophages in the muscularis [191]. Vasoactive intestinal peptide (VIP) is another neuropeptide in the ENS which has been shown to modulate dendritic cell (DC) activity [192] and is thought to potentially interfere with both innate and adaptive immunity [193]. DCs in the intestine express the 5-HT_7 receptor, however controversy remains whether the effects are pro or anti-inflammatory [177] which remains to be explored. 5-HT has also been shown to increase cAMP in DCs [194]. These findings demonstrate an association between our ENS and inflammatory pathways which are less well understood.

The immune system target genes via quantitative mRNA between the male DSS control and male SSRI-exposed DSS offspring are almost entirely different with the exception of MRC1 and TIMP (Figure 32) . The female control DSS group (not exposed to SSRI) had many more significantly elevated mRNA for selected gene targets in comparison to the SSRI-exposed DSS female counterparts. Given that pregnancy is a proinflammatory state, it is possible that there are hormonal differences that make the female rat more resistant to the number or quantity of

mRNA gene targets of interest in DSS induced colitis, although clinical phenotype is similar, the biochemical reactions may be different with SSRI-exposure which warrants further investigation.

Finally, in terms of target gene grouping and associations, IL6ST has shared signal transduction and receptor system with CNTF and OSMR [195]. OSMR associated with IL31 and IL31RA to activate STAT3 [196]. IL31 has been associated with stimulation of colonic myofibroblast in humans [197]. IL11RA1 is similar in structure to CNTF [198]. IL6ST and IL31RA identified at loci for CD [199]. CNTF identified as gene loci for IBD in genome-wide association study (GWAS) and immunochip studies [199]. Increased TGFB1 leads to decreased IL17A [200] which was consistent in our findings suggesting neutrophils attraction occurs by another mechanism. HMGB1 is thought to contribute to pathogenesis of IBD [201]. HMGB1 antagonist reduce inflammatory reaction and ameliorate colitis in rodent models [201].

5.7 Sex Dependent Differences in Serotonin Signaling

Our results suggest sex dependent difference exist as we observed altered number of serotonergic neurons in the SSRI exposed offspring at P1 and P21 in the female colon, decreased crypt length at P21 in the SSRI exposed offspring female colon, increased ZO-1 in the SSRI exposed male colon, and a number of quantitative mRNA genes that were significantly elevated in the SSRI exposed male offspring.

Animal studies have demonstrated central 5-HT levels, as well as cerebrospinal fluid concentrations of the 5-HT metabolite 5-hydroxyindole-3-acetic acid (5-HIAA), are significantly higher in female compared to male rats [138].

There is also emerging evidence to support the concept of sex-dependent differences in the serotonergic system of the ENS, as well as the CNS. Colonic extracellular 5-HT visceral hypersensitivity and hyperexcitability of colon projecting sensory neurons have been shown to be increased in female compared to male rats [139]. Identification of a sex specific role for the microbiota in the regulation of CNS serotonergic neurotransmission has been described, thereby linking the sex specific effects of 5-HT on the gut with the CNS [140].

In rats, 5-HT has been shown to reduce the concentration of testosterone in testis and SSRI which increases circulating 5-HT in synapses has been shown to decrease circulating estrogen [202, 203, 204]. In human studies, 5-HT has been shown to increase secretion of aldosterone in the adrenal cortex [205].

Clinically, IBS has female predominance and there is a body of literature looking at the effects of estrogen on the serotonergic pathways [206]. 5-HT_3 is involved in motility and visceral pain which has been exploited in the treatment of IBS [207]. 5-HT plasma concentration has been demonstrated to be influenced by sex and IBS subtype in human studies. IBS-D female patients had higher postprandial plasma 5-HT concentrations than health controls. IBS-C female patients had lower postprandial [208, 209]. Human studies have also demonstrated that men with IBS

have higher platelet SERT levels than healthy controls [210]. These studies and our results suggest that there are sex related differences and in our study these mechanisms warrant further exploration.

5.8 Limitations

One of the limitations of this study with respect to the animal experiments were the challenges in controlling for stress from handling the rats. We ensured minimal and similar handling of the rats to the best of our ability. Another experimental study limitation was not assessing the functional consequences at each time point and most specially at P21-26 which correlates to the point of maximal exposure to the SSRI. Experiments with DSS colitis would not have been feasible at the P1 timepoint as they require 5 days to complete and the rats are consuming breastmilk at this time.

An alternate mechanism to assess intestinal permeability would have been useful, not only assessing ZO-1 and occludins but also assessing permeability using an Ussing chamber. The method of quantification used to determine ZO-1 and occludins using Volocity, although very similar to established protocols using ImageJ, needs to be validated.

One of the challenges with the Nanostring data is that the Farncombe Metagenomics Facility changed their reagents between the two experiments

(experiment 1; control DSS vs control water, experiment 2; SSRI-exposed DSS vs SSRI-exposed water). Although we re-ran some samples and normalized the data in nSolver (NanoString Technologies, Inc. Seattle, Washington USA) to ensure no significant differences, it is possible that this is an area of potential error and for that reason the analysis does not compare directly SSRI exposed vs control offspring. In addition, we should confirm our Nanostring results by qPCR prior to determining protein level with ELISA. We also did not check MUC2 which is a gene involved in intestinal mucous secretion as an insoluble mucous barrier that protects the gut lumen which would be another important factor to assess.

Finally, an opportunity and limitation to this study, is the lack of information on the hormonal fluctuations which might have helped explain mechanisms underlying the sex difference. Despite the fact that the differences were seen prior to gonadal steroid production, it would have been beneficial to look at sex hormones fluctuations prior to sexual maturity, as well as cortisol, as these hormones have previously been shown to influence serotonin concentration. In addition to hormonal fluctuations, it would have been useful to look at 5-HT and SERT concentration in plasma and tissue to see if this might have helped explain some of the sex differences as previously demonstrated in human studies of IBS.

5.9 Future Directions

The observed sex differences prompt further exploration addressing the influence of sex and stress hormones. Physiologic fluctuations of estrogen during our lifespan, ovarian cycles and menopause have been shown to have predictable effects on serotonin by increasing TPH and inhibiting the expression of the gene for SERT [211] resulting in increased extracellular serotonin concentrations. Changes in estrogen occur in conjunction with progesterone making it another important hormone to look at when assessing changes in estrogen in relationship to serotonin levels, serotonin metabolites and receptors.

Another clinically meaningful and important hormone is cortisol which increases during times of stress and is referred to as a stress hormone. Cortisol is known to reduce serotonin concentrations [212] and therefore would be another important hormone to consider measuring.

The Nanostring results are interesting but they leave an opportunity for a future direction which would include confirmation of Nanostring results with quantitative PCR and targeted cytokine measurements at the protein level using enzyme-linked immunosorbent assay (ELISA). Moreover, given the interaction between the microbiota and the mucosal immune system, it would be important to assess for any significant differences between the SSRI-exposed and control offspring in our study. SERT KO rats in combination with early life stress (maternal separation)

have demonstrated changes in microbial composition including increased abundance of *Desulfovibrio, Mucispirillum*, and *Fusobacterium*, all of which are reported to be associated with intestinal inflammation [213]. In assessing the microbiota, in addition to assess relative abundance of species between samples, looking at metabolic by-products such as short chain fatty acids could also help understand any differences in the mucosal immune response which one might anticipated based on differences observed in quantitative mRNA of selected target genes. Stool samples would be collected at each timepoint including a maternal stool sample and sent for sequencing for 16 sRNA. Once analyzed, utilizing a computer software program such as R or QIIME, we could determine the relative abundance of various organisms and assess for any significant differences.

Additional limitations of this study design include only studying functional consequences in the adult rat at P6M. Perhaps repeating the DSS experiment at P21, which corresponds to the point of maximum exposure to SSRI and where we observed the most significant effects on the ENS would have yielded different results. For this same reason, assessing permeability at P21 would also be of interest given the increasing body of literature around neuroinflammatory pathways. In addition, assessing motility at P21, where we see the most significant difference in the ENS and is the point of maximal exposure to SSRI would be of interest. It would also be of interest to compare the findings at P21 to the altered motility at P6M. 5-HT_3 receptor antagonist and 5-HT_4 receptor agonist are currently being used as

pharmacologic therapies to treat decreased intestinal motility. Therefore, in addition to assess motility at P21 and P6M, we should also assess 5-HT_3 and 5-HT_4 receptors by qualitative PCR as both have demonstrated to be important receptors in intestinal motility.

Finally, given the controversy in human studies with respect to the effects of depression on the offspring, rather than the effect of the antidepressant medication, if one were to re-design this study, it would be ideal to have a group of rats with depression not on pharmacotherapy, as well as rats with depression being treated with pharmacotherapy.

CHAPTER 6: CONCLUSION

In conclusion, SSRI exposure does alter the development of the gastrointestinal tract and there are differences between male and female offspring. The female rat reaches sexual maturity around P32-P34, whereas the male rat reaches sexual maternity around P45-48 [214] which is shortly after the timepoint we selected. The female colon at P21 appeared to be the most sensitive to SSRI exposure which correlates to the point of maximal exposure. Structural consequences appear to resolve by P6M, but functional consequences related to motility persist. It is unclear based on this data whether SSRI exposure in utero and during breastfeeding results in increased burden of gastrointestinal diseases. SSRI-exposure in utero and breastfeeding does not appear to increase intestinal permeability and may have a protective role for intestinal mucosal epithelial barrier in the male SSRI-exposed offspring. The SSRI exposure during the perinatal and postnatal periods does not increase susceptibility to DSS colitis. However, SSRI exposure does appear to alter colonic motility at P6M which might suggest a possible role in the pathogenesis of Irritable Bowel Syndrome although this warrants further dedicated investigation.

CHAPTER 7: APPENDICES

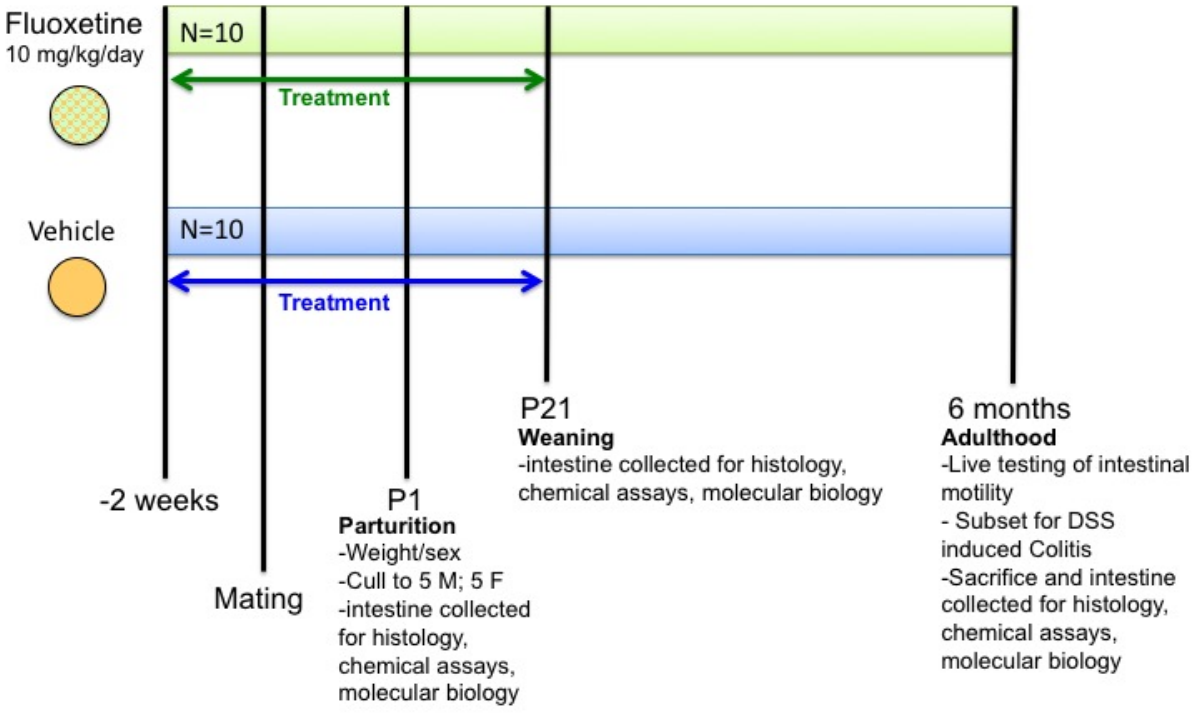

Figure 1. Summary of the Experimental Plan: Dams were randomly assigned to receive vehicle or 10 mg/kg/day fluoxetine hydrochloride orally from 14 days prior to mating and through the weaning period (P21). Rats were housed until adulthood (P6M). At birth (P1), pups were weighed and sexed and litters were culled to 10 pups per litter, preferentially selecting 5 male and 5 female offspring. Animals were sacrifices at P1, P21 and P6M to assess the effects of SSRI exposure in utero, at weaning and if effects lasted into adulthood.

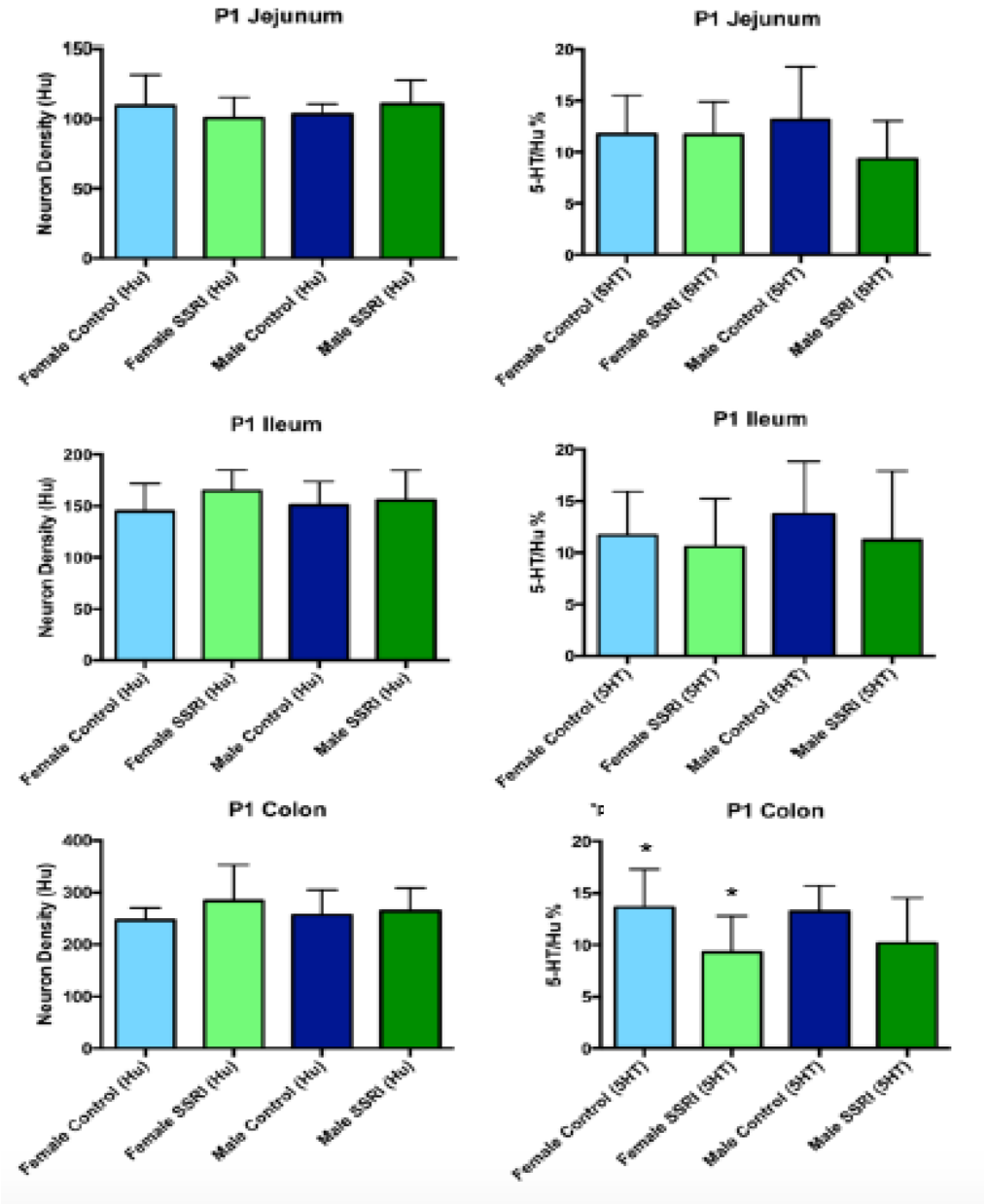

Figure 2. Whole mount preparations of small intestine and colon from the P1 offspring of fluoxetine-exposed and control rats were immunostained with antibodies to HuC/D (to visualize enteric neurons) and to 5-HT (to detect serotonergic neurons). A significant decrease in the total number of enteric neurons and serotonergic neurons were found in the fluoxetine-exposed female offspring colon (mean ± SD, 13.6% vs 9.3%; p=0.04).

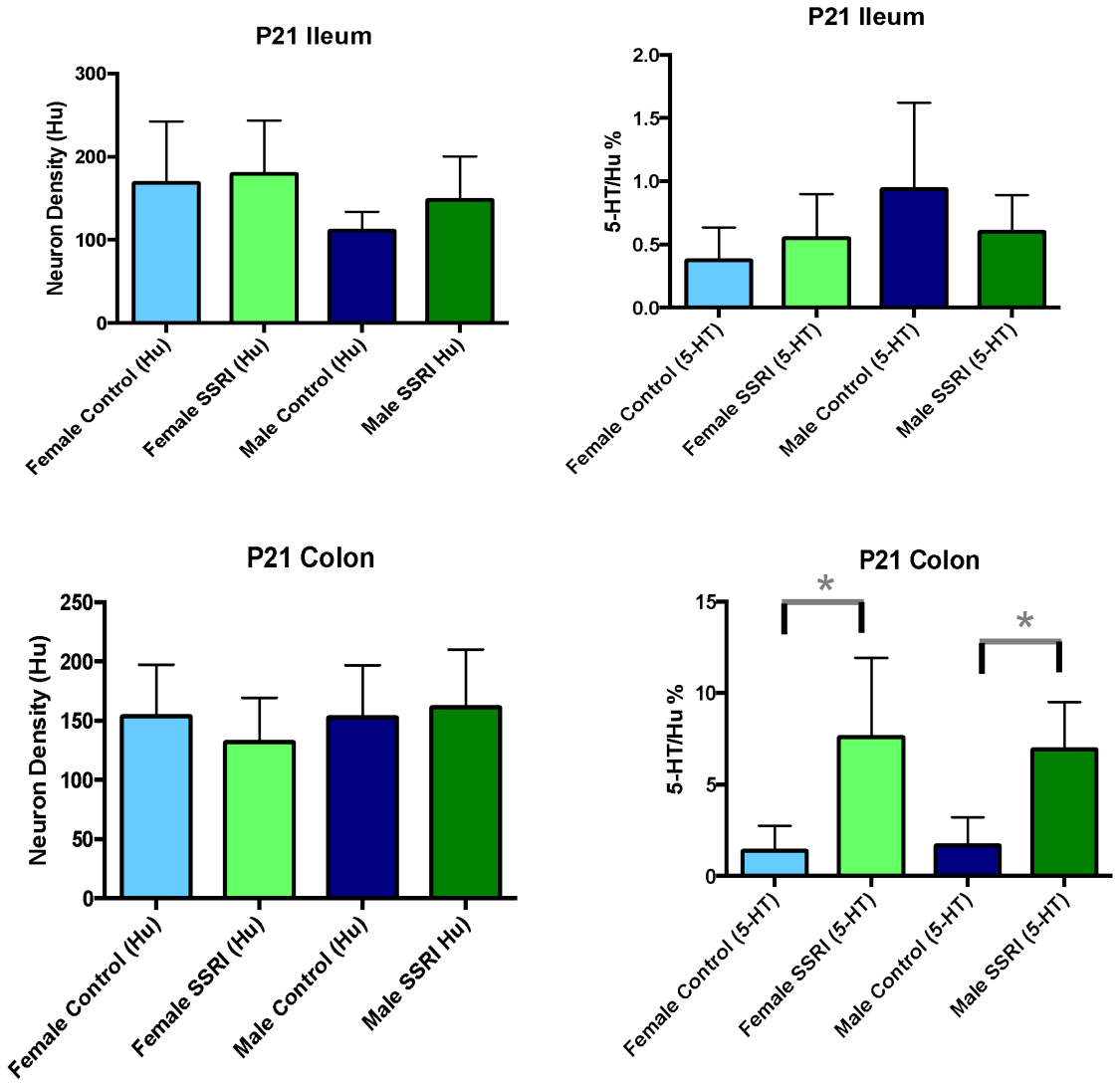

Figure 3. Whole mount preparations of small intestine and colon from the P21 offspring of fluoxetine-exposed and control rats were immunostained with antibodies to HuC/D (to visualize enteric neurons) and to 5-HT (to detect serotonergic neurons). Significant differences (mean ± SD) were found in the percentage of serotonergic neurons in both fluoxetine-exposed female (1.4% vs 7.6%; p=0.009) and male colons (1.7% vs 6.9%; p=0.002).

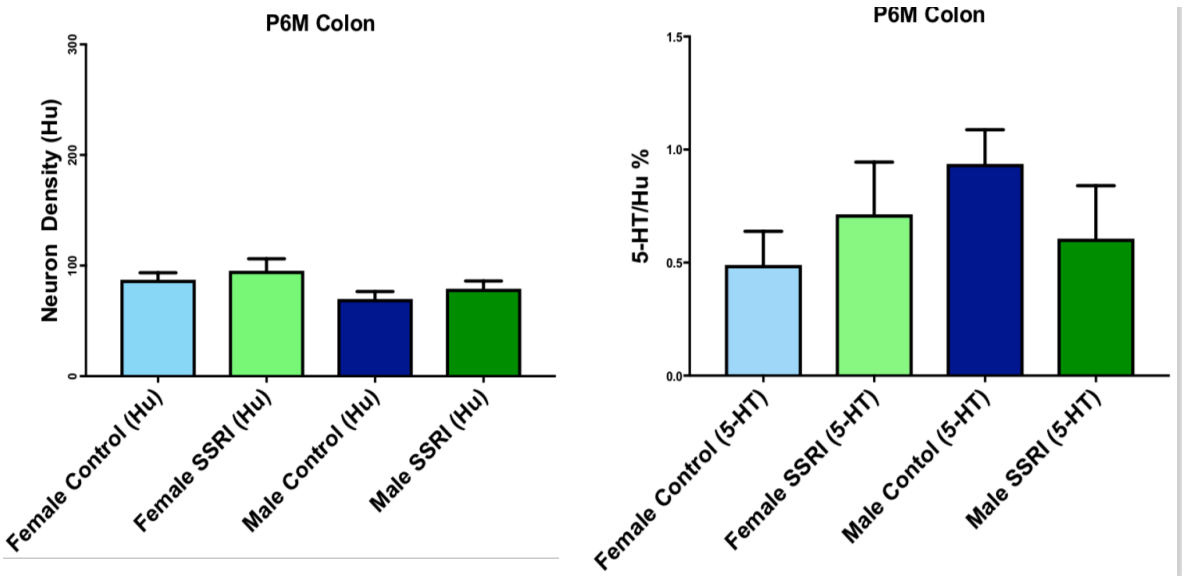

Figure 4. Whole mount preparations of small intestine and colon from the P6M offspring of fluoxetine-exposed and control rats were immunostained with antibodies to HuC/D (to visualize enteric neurons) and to 5-HT (to detect serotonergic neurons). At 6 months, no significant difference (mean ± SD) in the percentage of total number of enteric neurons was found.

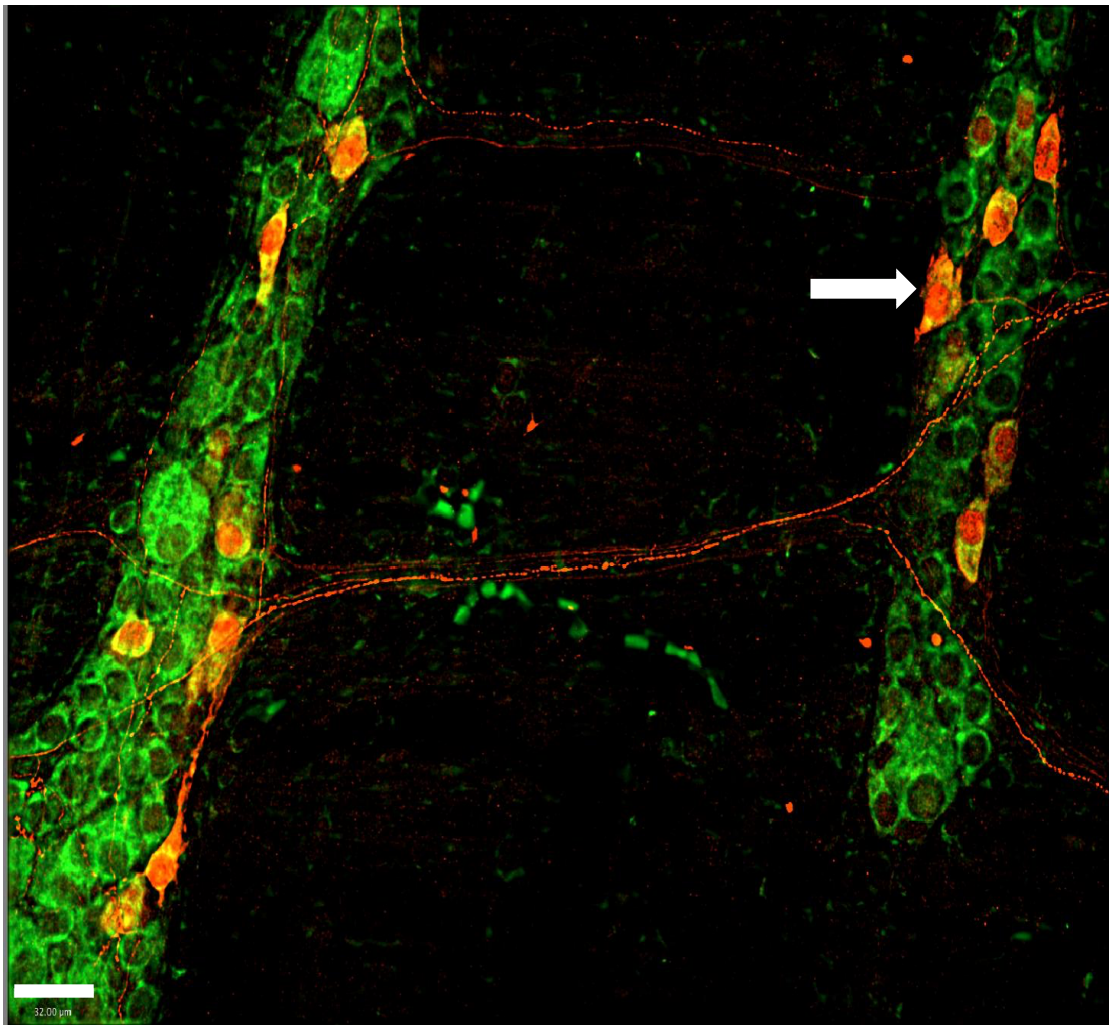

Figure 5: Immunofluorescent staining of enteric and serotonergic neuron. Neurons within the myenteric plexus of the ileum at P6M immunolabeled with antibodies to HuC/HuD (green), and serotonergic neurons visualized with antibodies to 5-HT (red), note the 5-HT myenteric neuron (arrow), 20x magnification. Scale bar =30 µm.

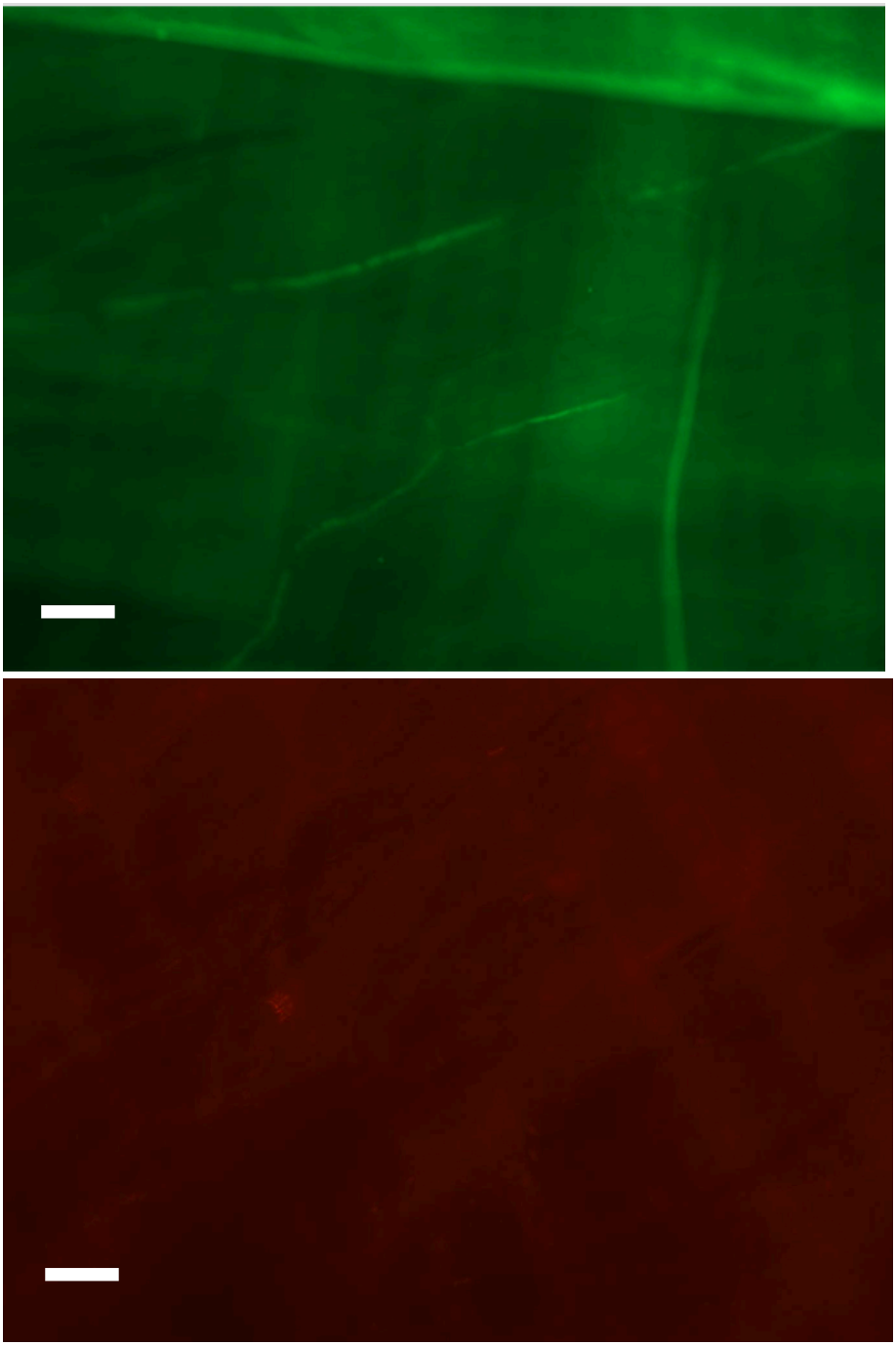

Figure 6: Expression of Hu C/D (green) and 5-HT (red) at P6M in colon visualized by immunofluorescence; negative control with primary antibody omitted. No staining visualized. Scale Bar = 60 µm

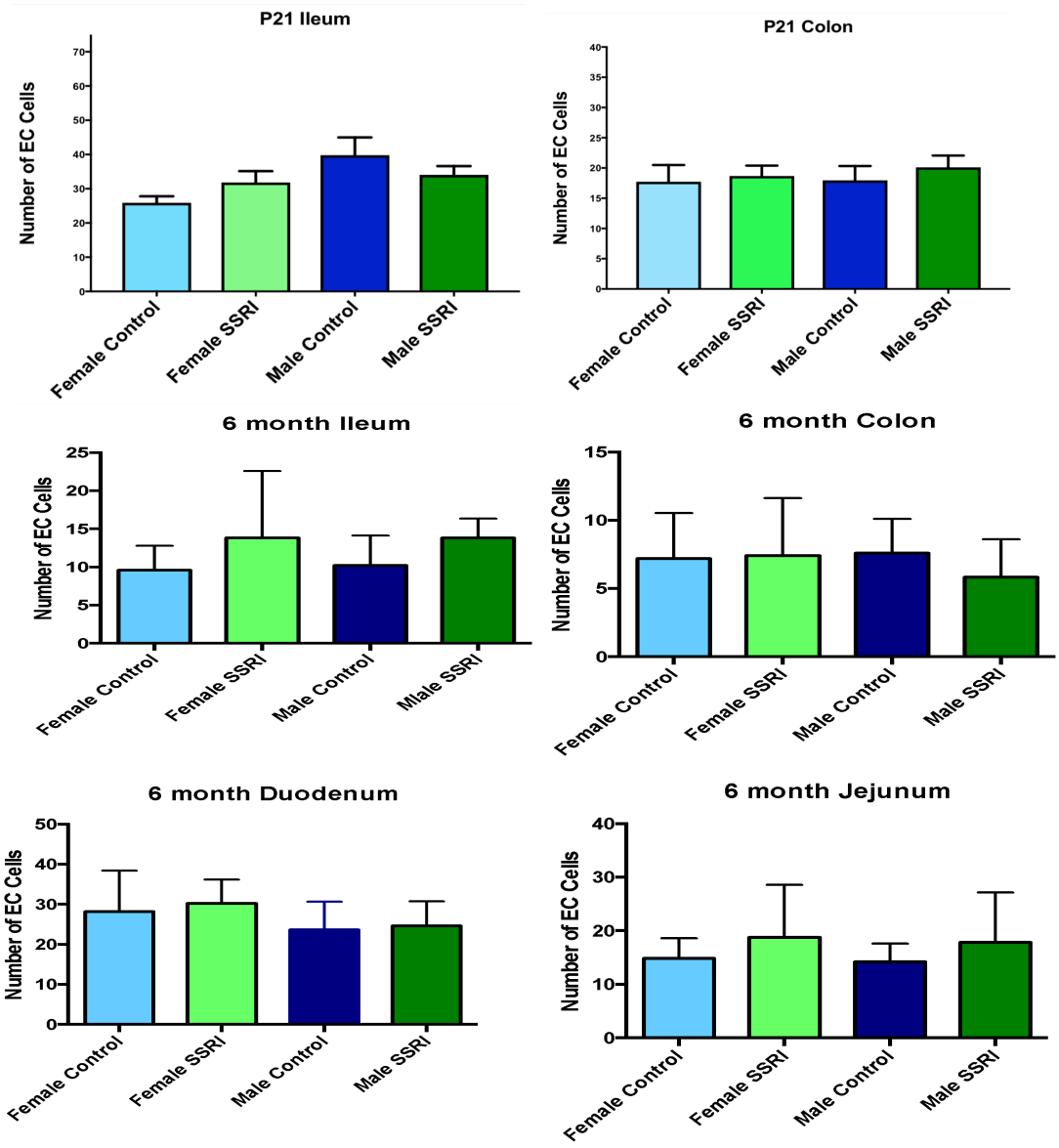

Figure 7. Cross sections were prepared of small intestine and colon from the P21 and P6M offspring of fluoxetine-exposed and control rats. Enterochromaffin cells were visualized with antibodies to 5-HT, and intestinal epithelial cell nuclei stained with bisbenzimide. Expressed as total number of enterochromaffin cells per 10 villi in the small intestine and 10 crypts in the colon (mean ± SD).

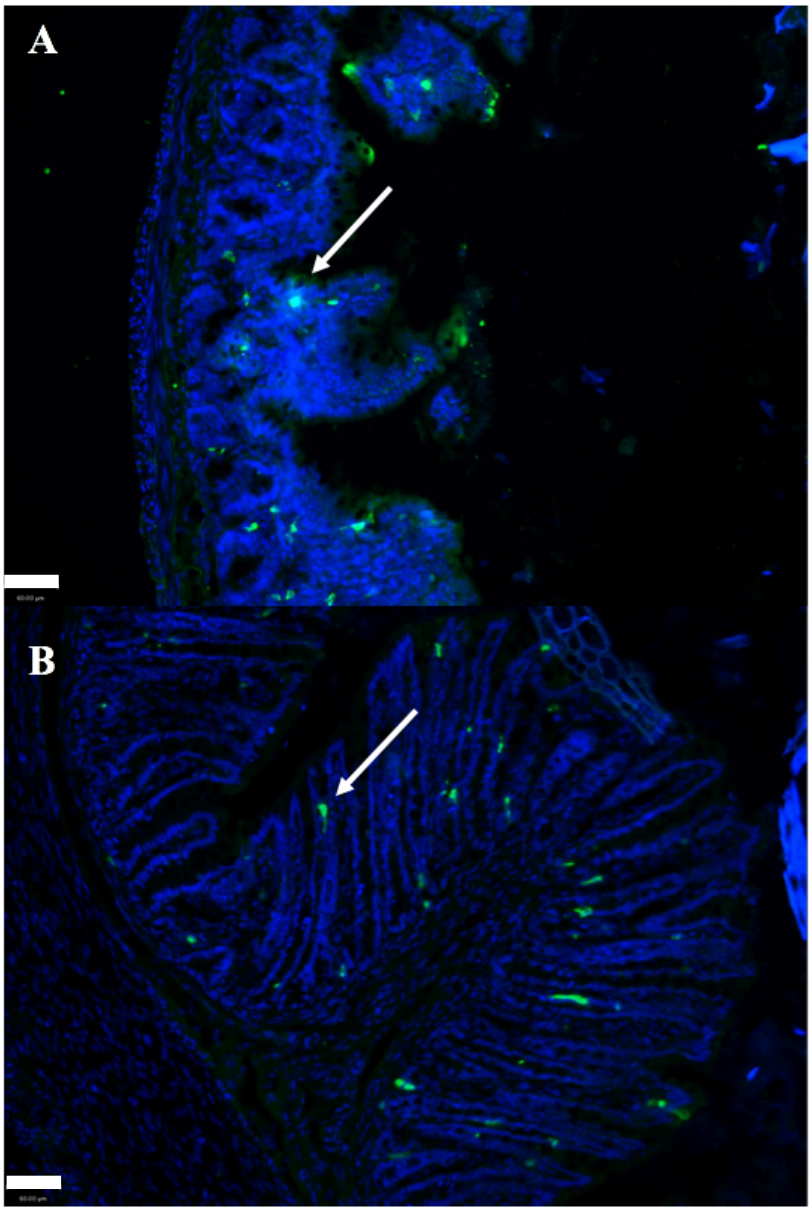

Figure 8. Expression of epithelial enterochromaffin cells in ileum **(A)** and colon **(B)** visualized by immunofluorescence to 5-HT (Alexa 488-labeled 5-HT antibodies appear green; bisbenzimide-stained nuclei appear blue; 10X magnification). The arrow in images **(A)** and **(B)** is directed at a single EC cell. Scale Bar= 60 µm.

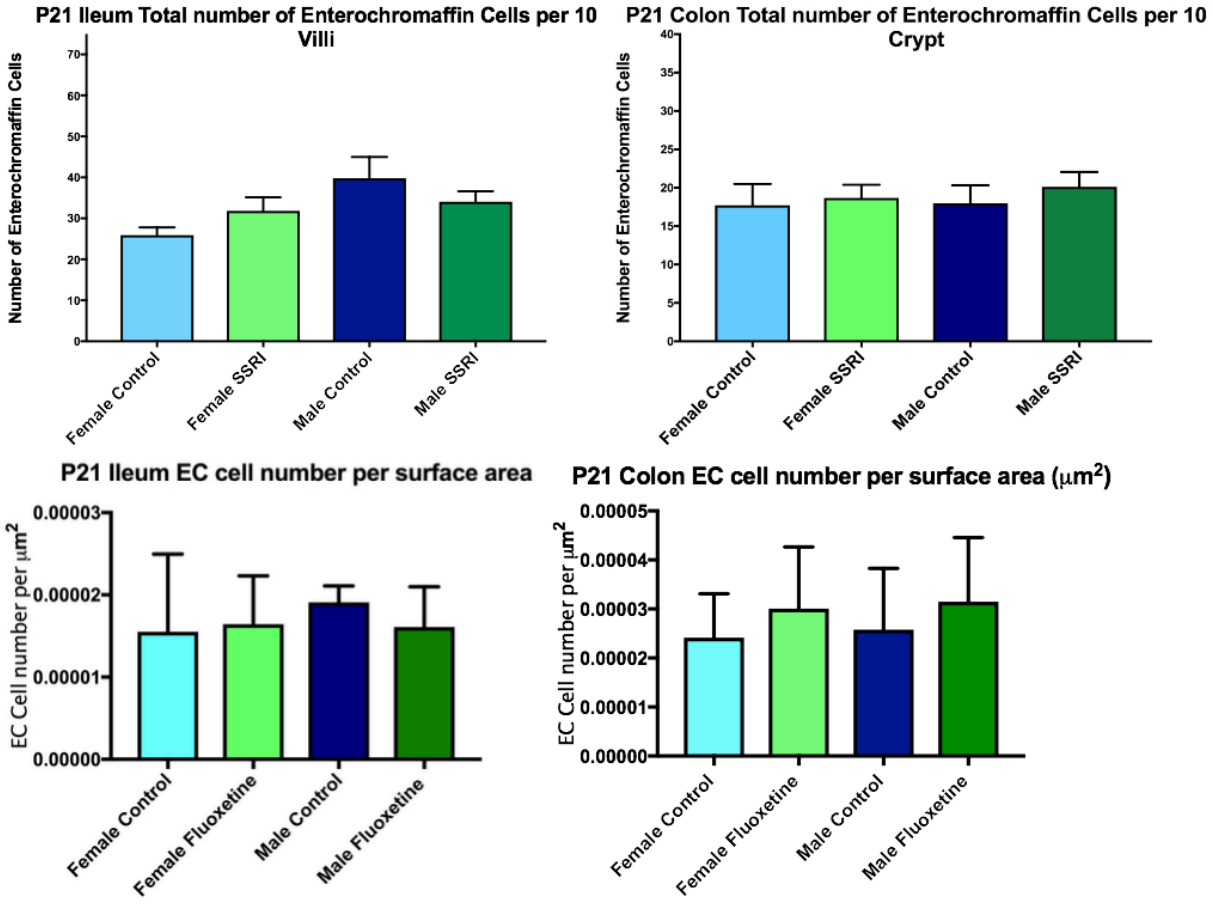

Figure 9. P21 total number of enterochromaffin cells in ileum **(A)** per 10 villi and **(B)** colon per surface area was not significant (mean ± SD) by either method.

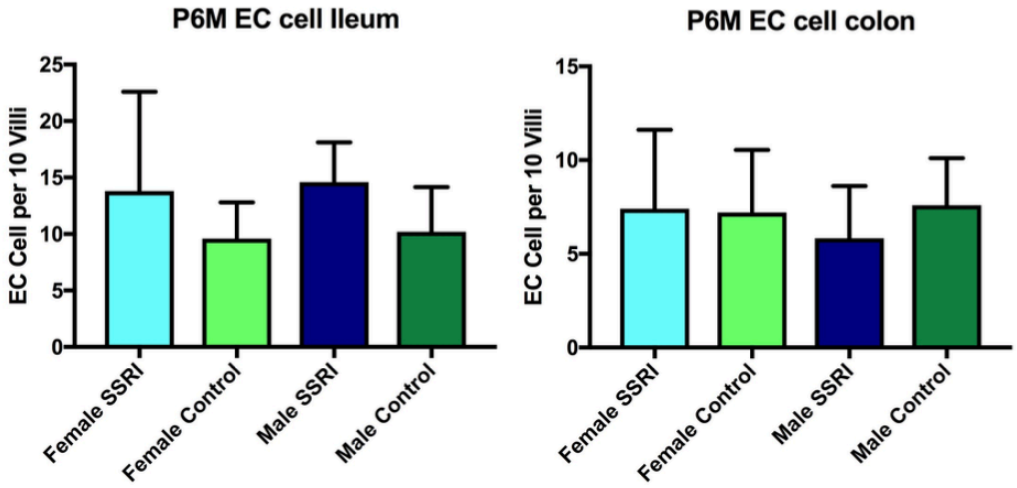

Figure 10. P6M total number of enterochromaffin cells in ileum **(A)** and **(B)** colon per 10 villi was not significant by either method (mean ± SD).

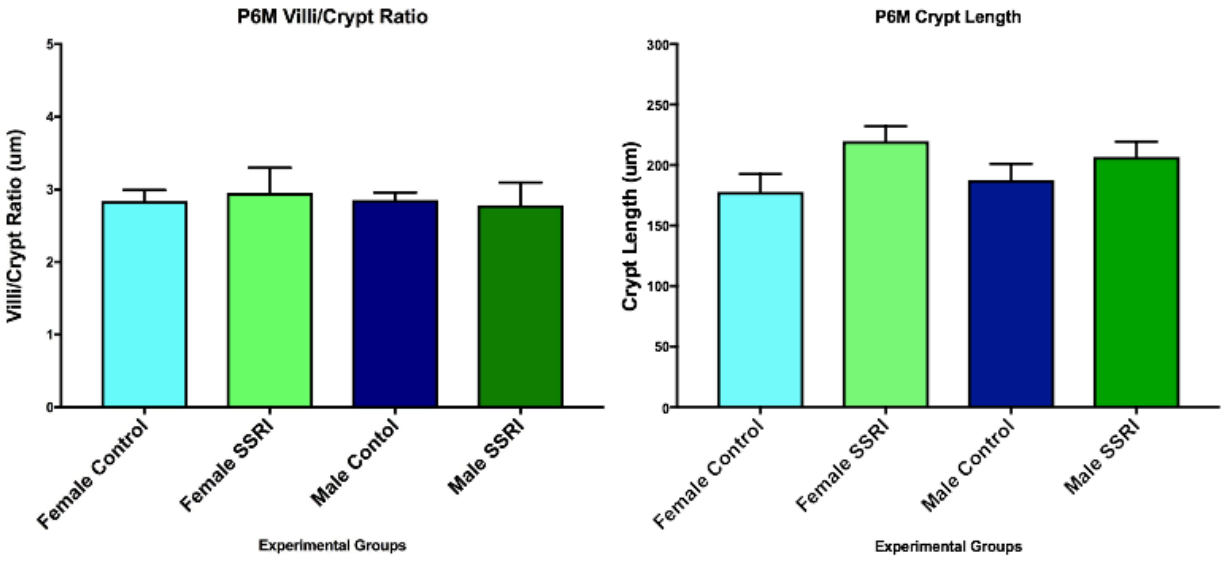

Figure 11. P6M villi to crypt ratio of the ileum and crypt length in the colon with no significant difference (mean ± SD) between SSRI exposed vs control male and female offspring. n = 5 per group.

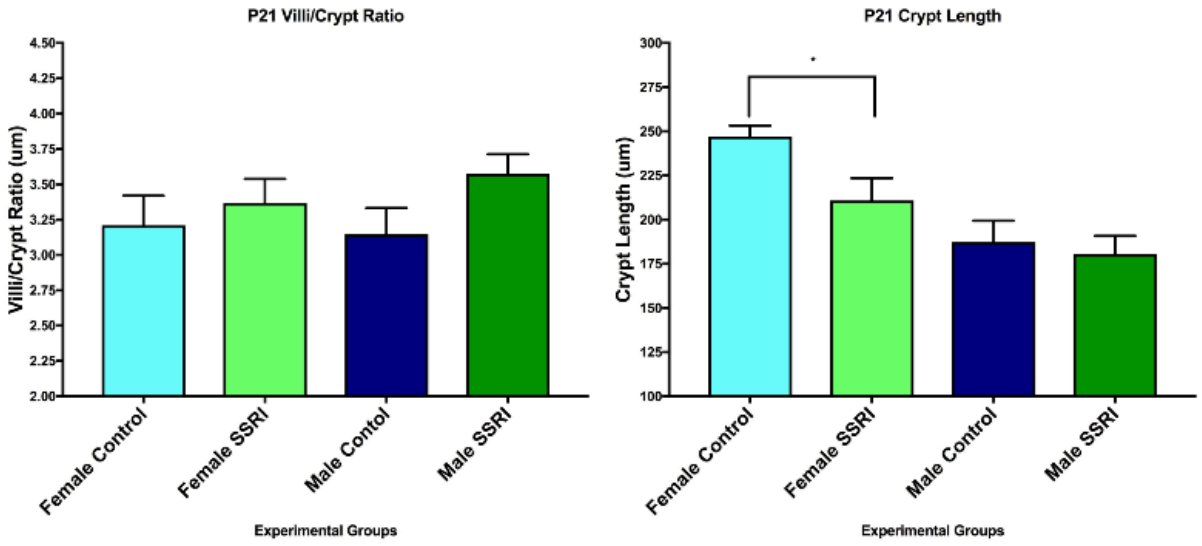

Figure 12: P21 villi to crypt ratio of the ileum with no significant difference (mean ± SD) between SSRI exposed vs control male and female offspring (n=7-9). P21 colon crypt length significantly reduced in the SSRI exposed female offspring (p= 0.0311) (n=7-9 per group).

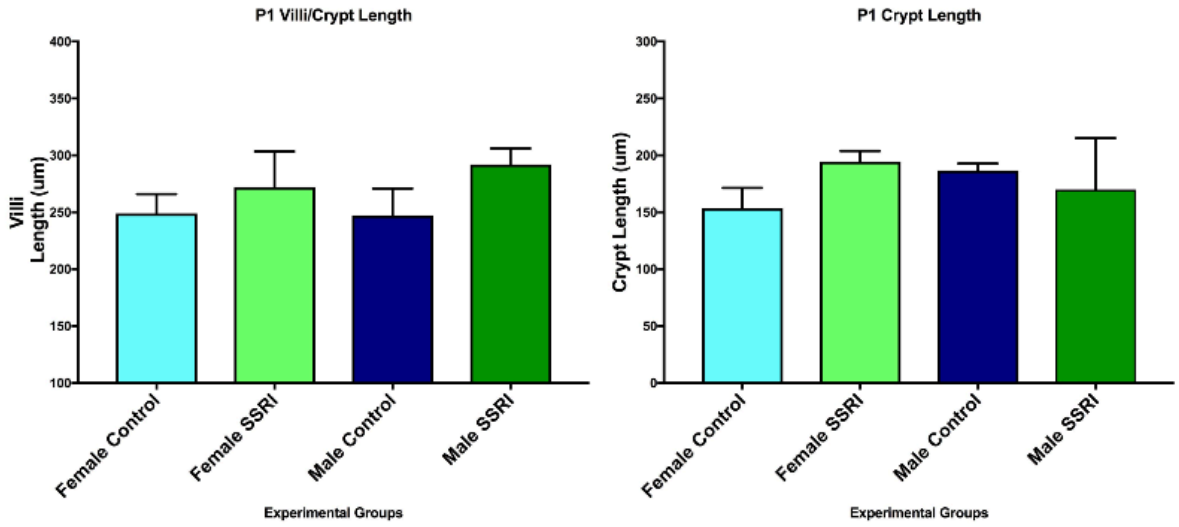

Figure 13. P1 villi length in the ileum and crypt length in the colon with no significant difference (mean ± SD) between SSRI exposed vs control male and female offspring. n = 3-4 per group.

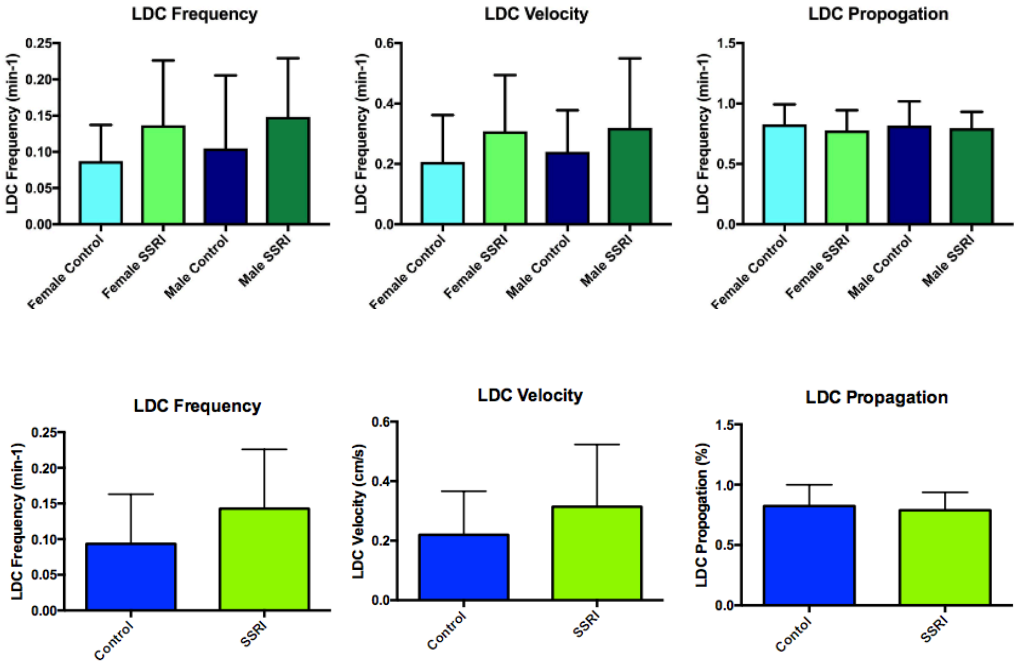

Figure 14. Long distance contractions (LDCs) measured by spatiotemporal map was not significant when grouped by sex (mean ± SD). When combined to obtain a mean LDC frequency for the SSRI and the control rats ($p = 0.046$). Velocity of LDCs was calculated on ImageJ by measuring the slope of the contraction ($p = 0.0418$). LDC percentage propagation was calculated by dividing the distance the LDC travelled by the total length of the colon. $n = 9$ per group.

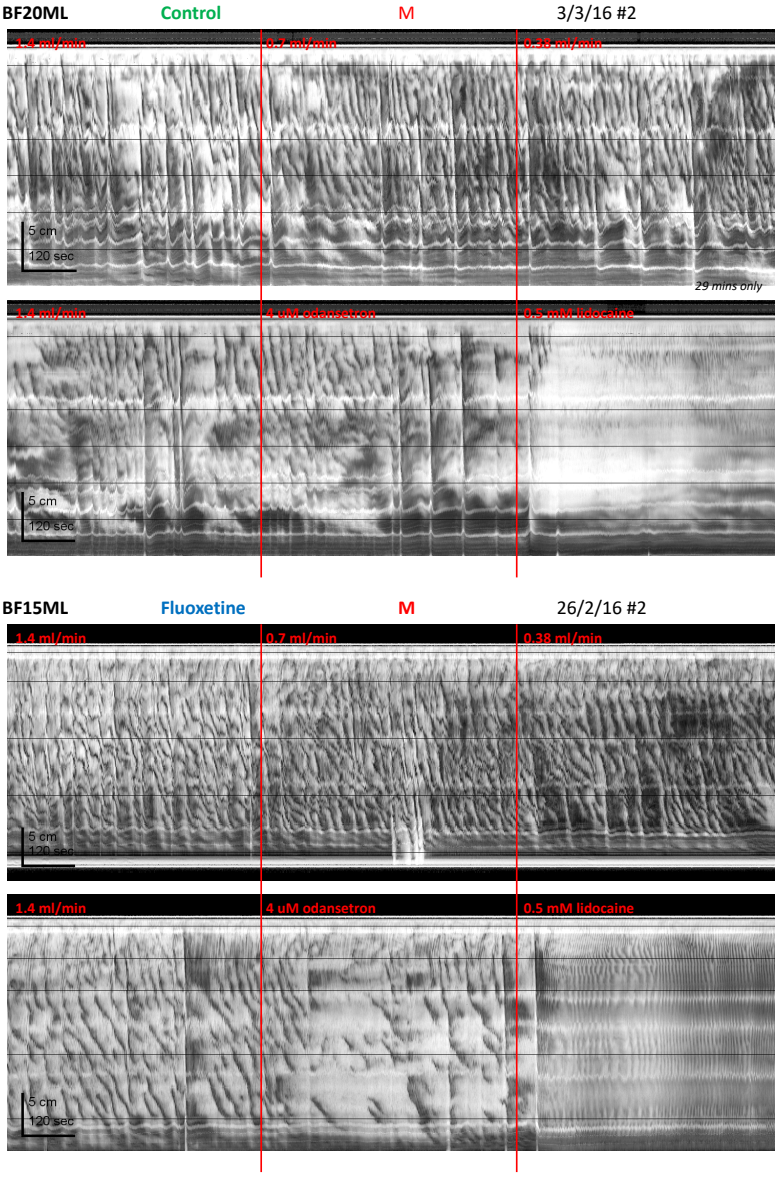

Figure 15. Photos from video recorded spatiotemporal mapping of SSRI exposed and control offspring at P6M with 5-HT_3 antagonist (Ondansetron) to increase contractility and lidocaine for decreased contractility.

Figure 16. H&E stained cross section of DSS exposed rat colon with increased inflammatory cells, loss of crypts and goblet cell depletion.

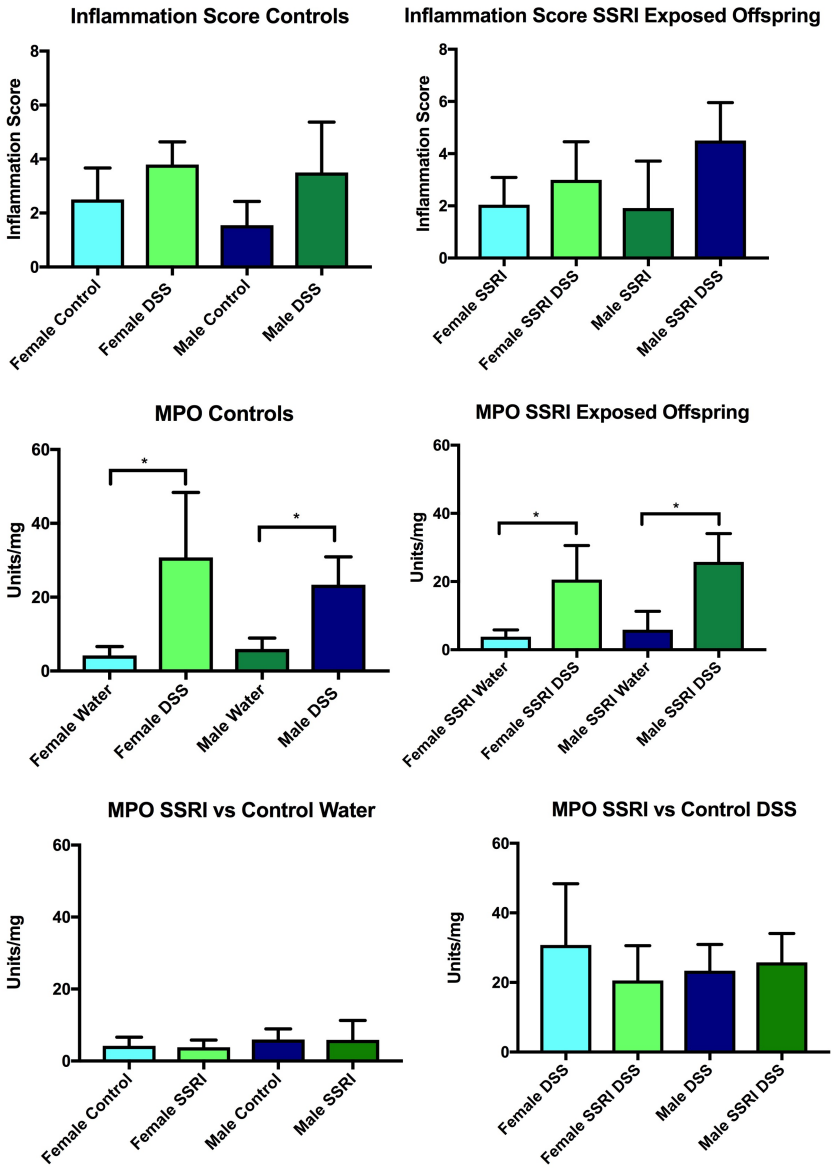

Figure 17A. Validated Histology Score and Colonic Myeloperoxidase (MPO) at P6M for water vs DSS in SSRI exposed and control offspring. Colonic MPO showed statistically significant differences (mean ± SD) between water and DSS (female water vs female DSS, p= 0.008; male water vs males DSS, p= 0.008; n=5 per group) (female SSRI-exposed water vs female SSRI-exposed DSS, p=0.02; male SSRI exposed water vs male SSRI-exposed DSS, p=0.004; n=5-6 per group).

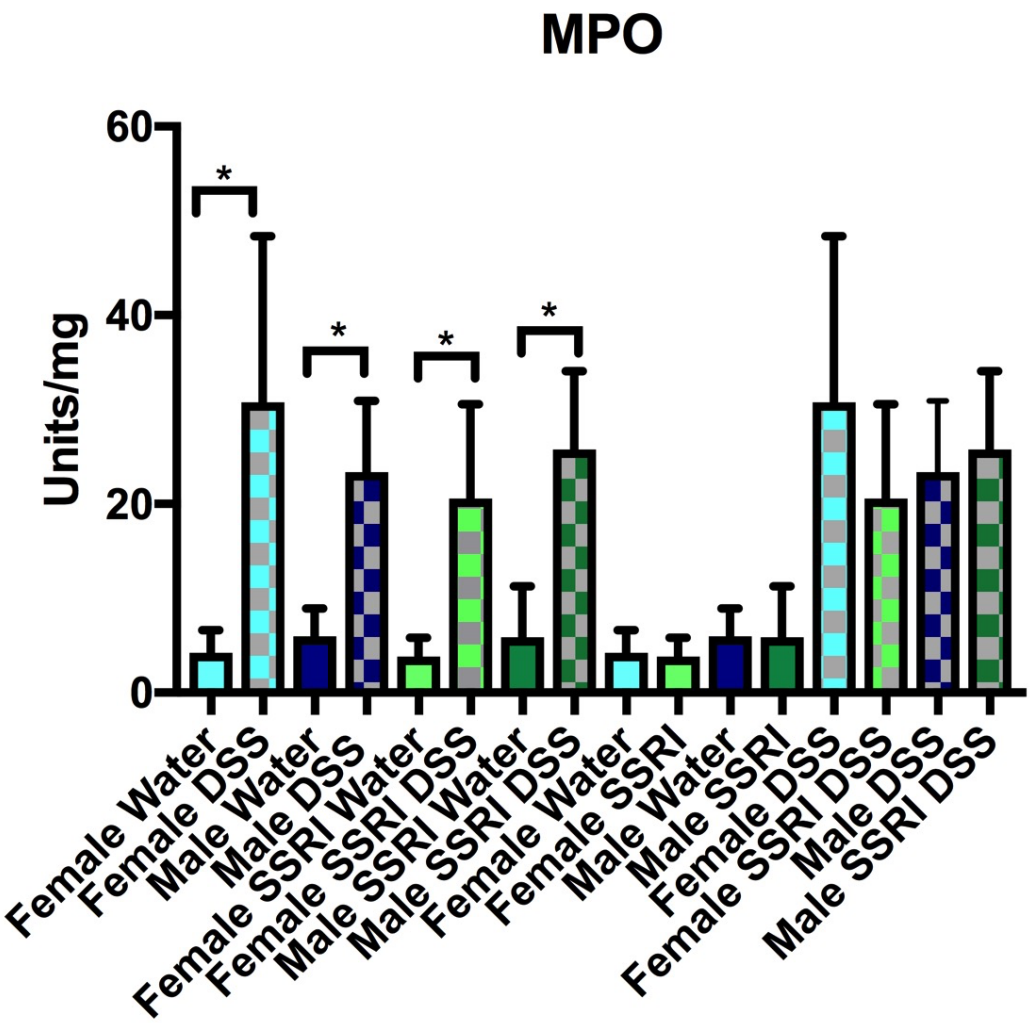

Figure 17B. Summary of Colonic Myeloperoxidase (MPO) at P6M for water vs DSS in SSRI exposed and control offspring. Colonic MPO showed statistically significant differences (mean ± SD) between water and DSS (female water vs female DSS, p= 0.008; male water vs males DSS, p= 0.008; n=5 per group) (female SSRI-exposed water vs female SSRI-exposed DSS, p=0.02; male SSRI exposed water vs male SSRI-exposed DSS, p=0.004; n=5-6 per group). No significant difference in SSRI vs Control male and female water nor DSS.

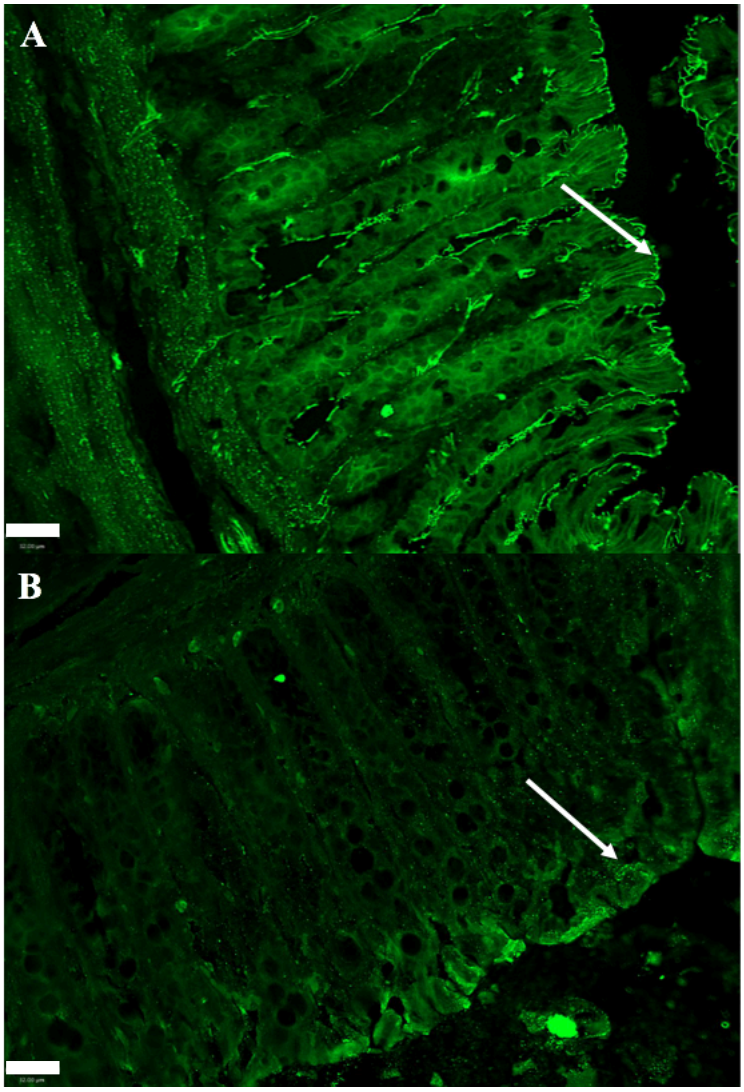

Figure 18. Expression of tight junctions at P6M in colon visualized by immunofluorescence to occludin **(A)** and ZO-1 **(B)** labeled with Alexa 488 fluorescent antibody; 10X magnification. The arrow in images **(A)** and **(B)** is directed at the location of tight junctions. Scale Bar = 10 µm

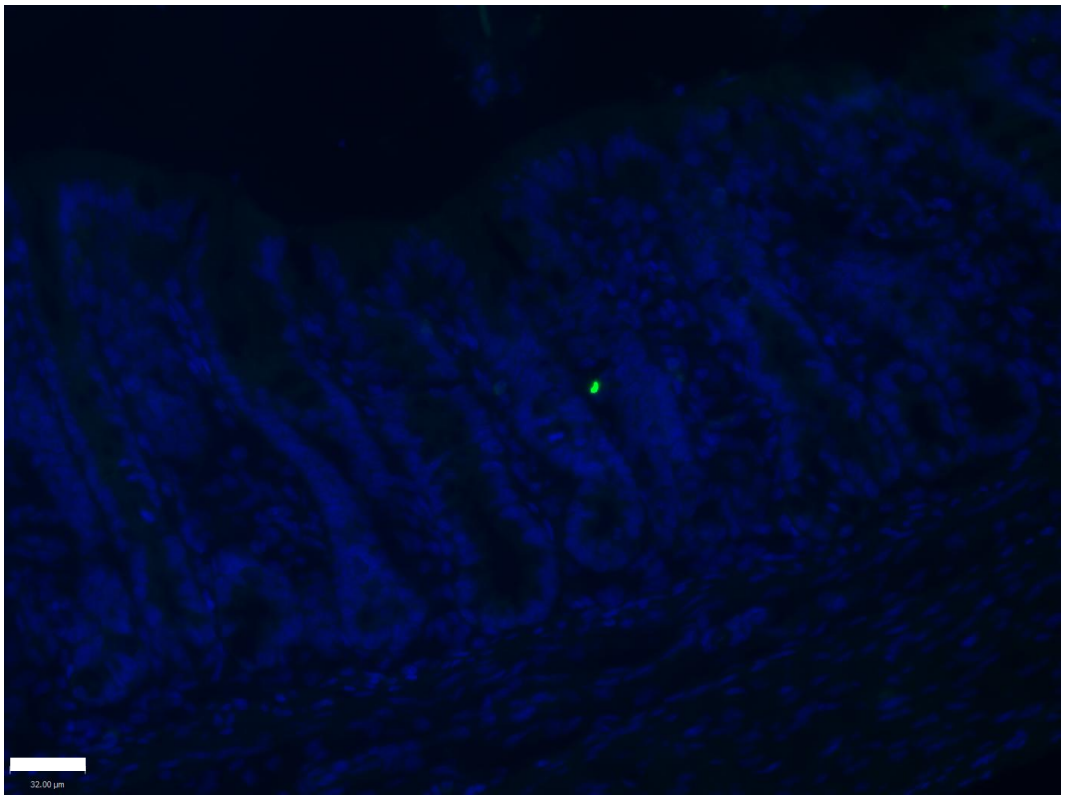

Figure 19. Expression of tight junctions at P6M in colon visualized by immunofluorescence to occluding; negative control with primary antibody omitted. No staining visualized. Scale Bar= 30 µm

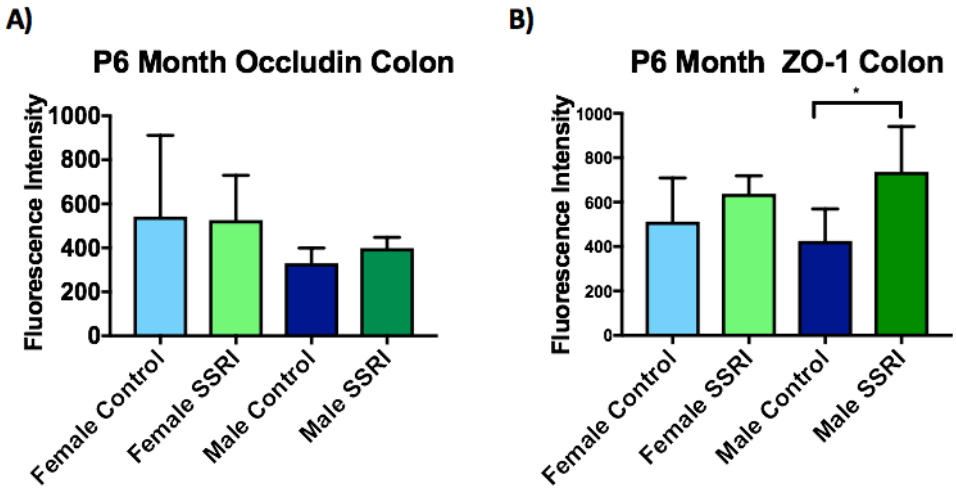

Figure 20. Fluorescence intensity measures at P6M, using weighted average, in colon visualized by immunofluorescence to occludin **(A)** and (ZO-1) **(B)**; was significantly increased (mean ± SD) in the male SSRI offspring for ZO-1.

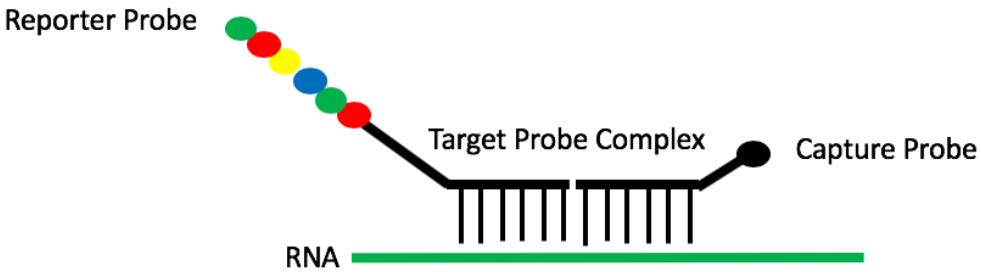

Figure 21. Nanostring technology schematic showing molecular barcodes are tagged with capture probes and reporter probes placed that are specific to the target gene of interest, creating a unique target probe complex. After hybridization, excess probes are removed, leaving only purified target probe complexes. Complexes are immobilized and aligned on the imaging surface. The sample is then scanned using an automated fluorescent microscope. Labelled barcodes are directly counted, and the data analyzed through an intuitive analysis software.

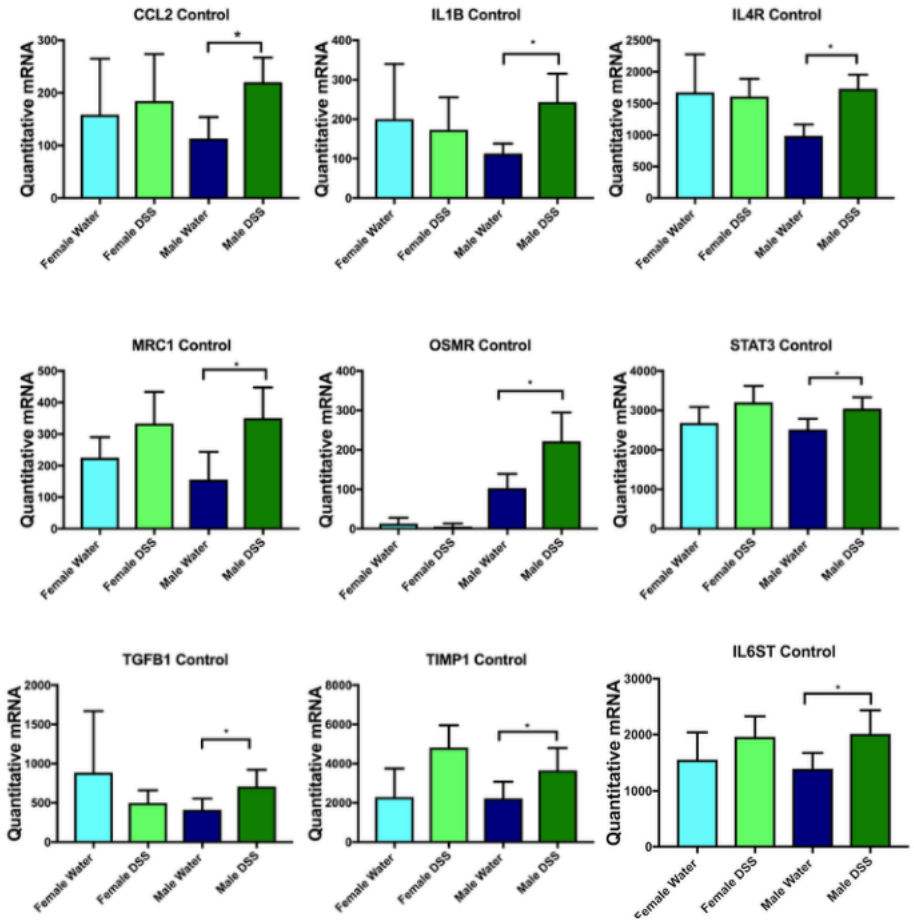

Figure 22. Quantitative amounts of mRNA in the colon of male DSS colitis vs male water control P6M Wistar rats demonstrated significant increases (mean ± SD) in CCL2 ($p = 0.0317$), IL1B ($p = 0.0079$), MRC1 ($p = 0.0159$), IL-4R ($p = 0.0079$), OSMR ($p = 0.0159$), TGFB ($p = 0.0317$), STAT3 ($p = 0.0159$), TIMP1 ($p = 0.0317$) and IL6ST ($p=0.03$). n = 5 per group.

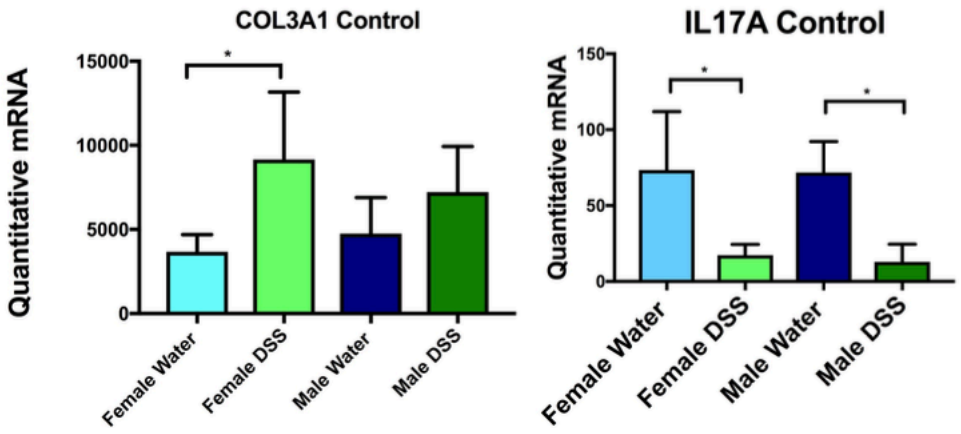

Figure 23. Quantitative amounts of mRNA in the colon of female DSS colitis vs female water control at P6M Wistar rats demonstrated a significant increase (mean ± SD) in COL3A1($p = 0.0079$), and both male and female DSS colitis vs water control in IL17A ($p = 0.0079$). n=5 per group.

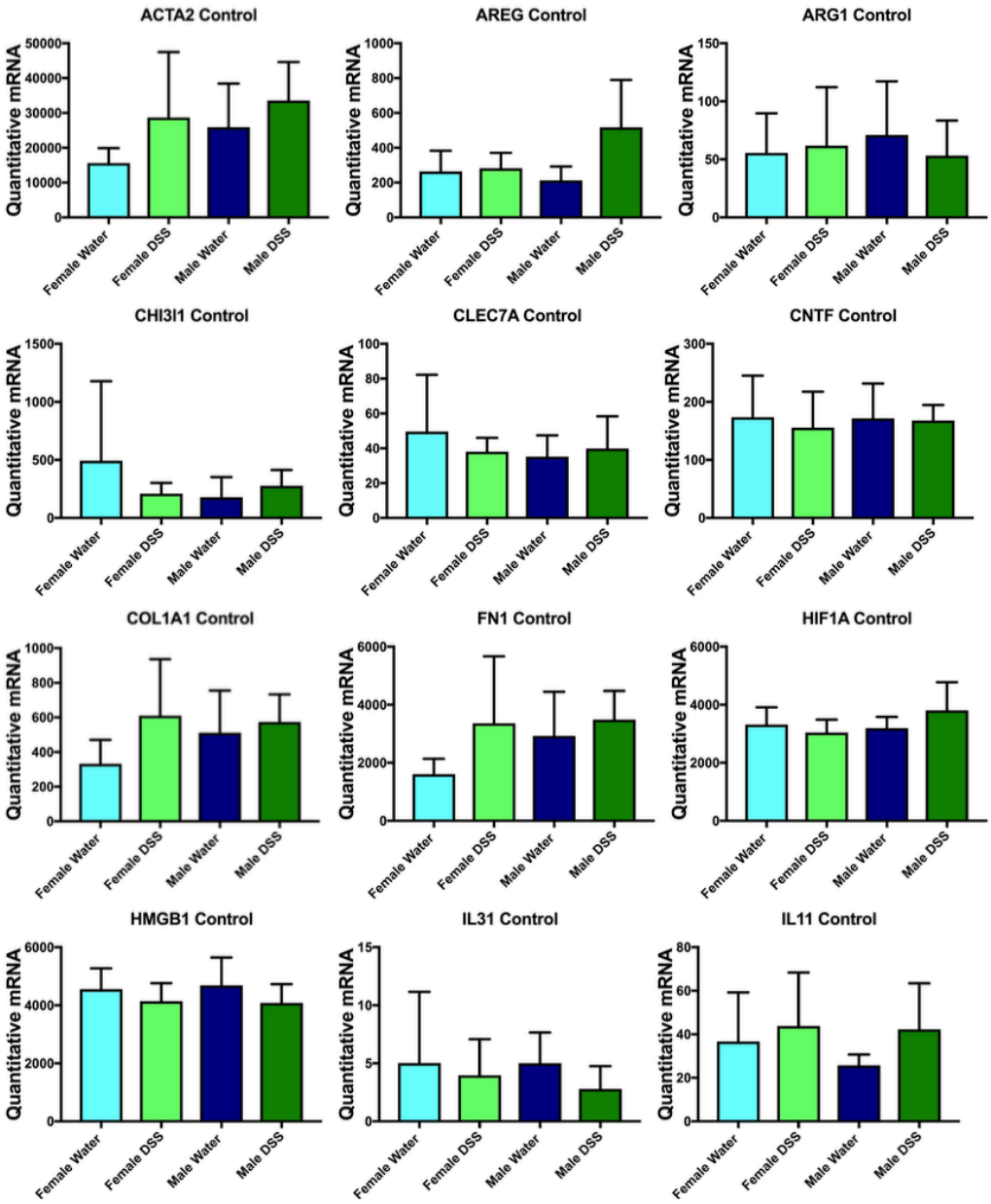

Figure 24. Quantitative amounts of mRNA in the colon of DSS colitis vs water control at P6M Wistar rats demonstrated no significant differences (mean ± SD) in ACTA2, AREG, ARG1, CHI3I1, CLEC7A, CNTF, COL1A1, FN1, HIF1A, HMGB1, IL31, and IL11. n=5 per group.

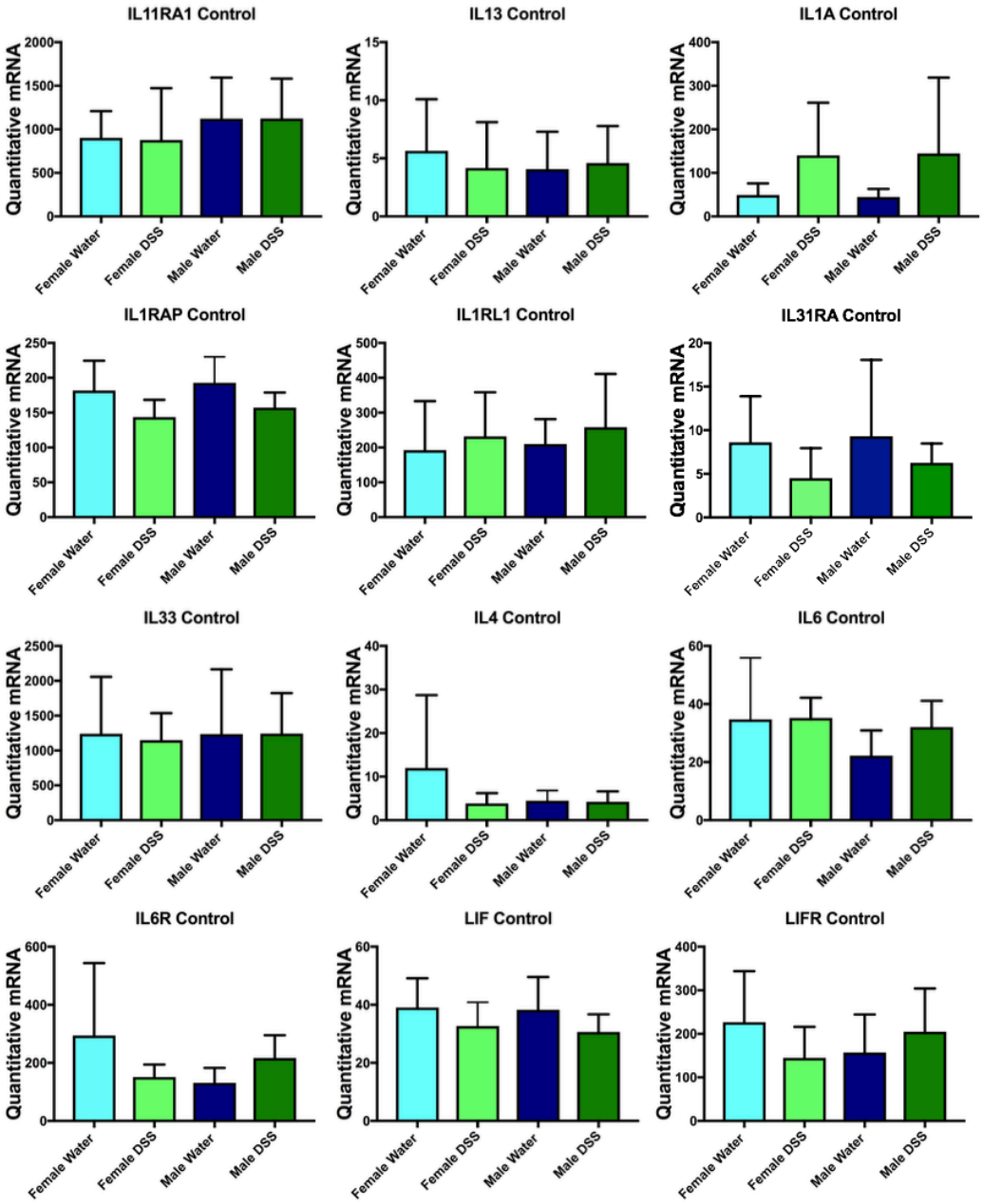

Figure 25. Quantitative amounts of mRNA in the colon of DSS colitis vs water control at P6M Wistar rats demonstrated no significant differences (mean ± SD) in IL11RA1, IL13, IL1A, IL1RAP, IL1RL1, IL31RA, IL33, IL4, IL6, IL6R, LIF, and LIFR. n=5 per group.

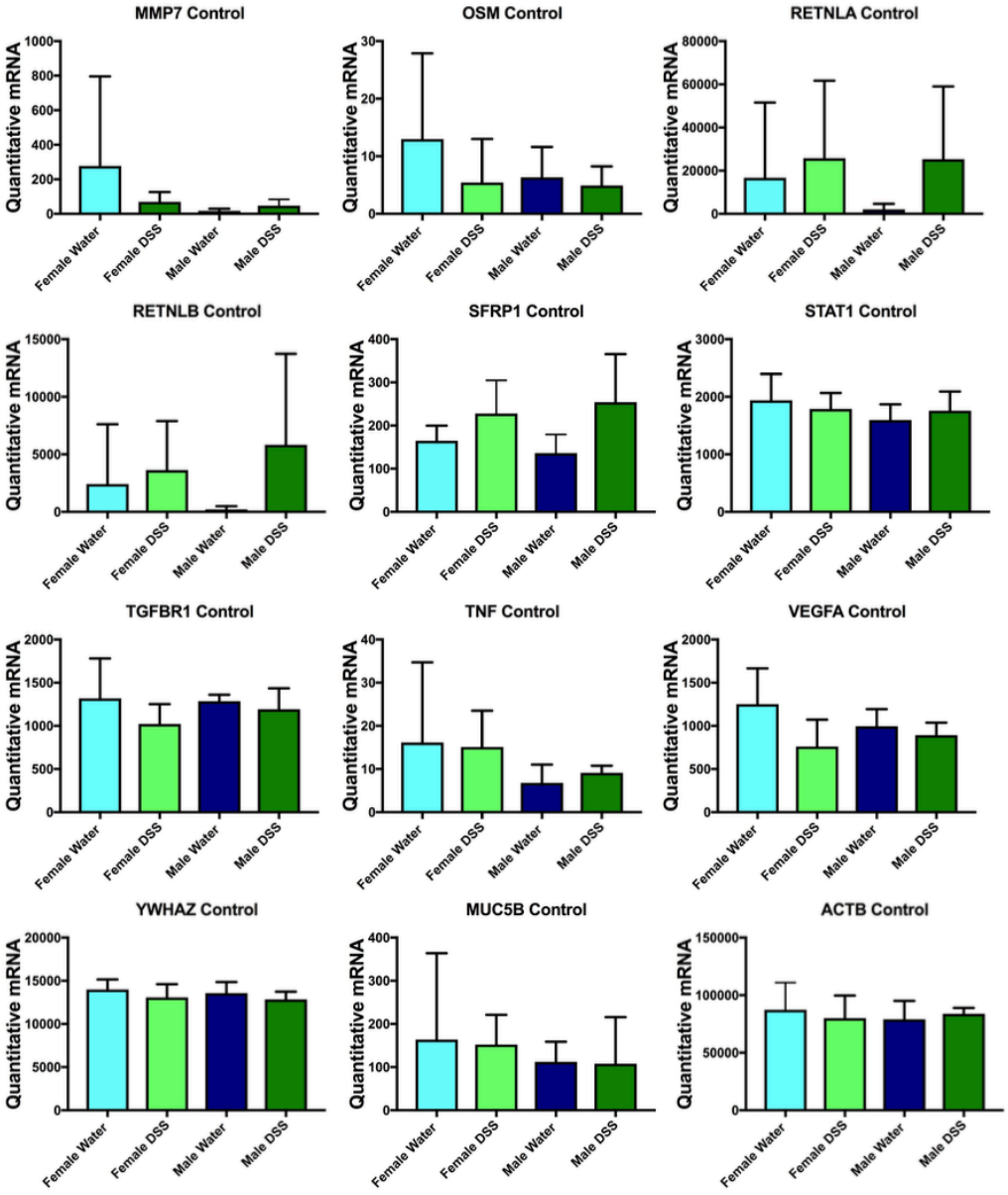

Figure 26. Quantitative amounts of mRNA in the colon of DSS colitis vs water control at P6M Wistar rats demonstrated no significant differences (mean ± SD) in MMP7, OSM, RETNLA, RETNLB, TGFBR1, TNF, VEGFA, YWHAZ, MUC5B, and ACTB. n=5 per group.

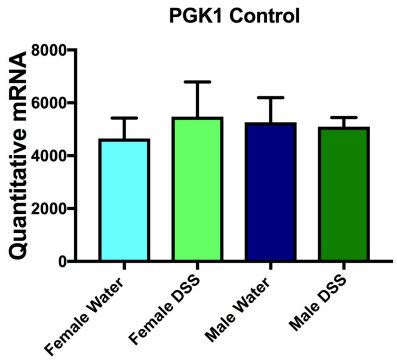

Figure 27. Quantitative amounts of mRNA in the colon of DSS colitis vs water control at P6M Wistar rats demonstrated no significant difference (mean ± SD) in PGK1. n=5 per group.

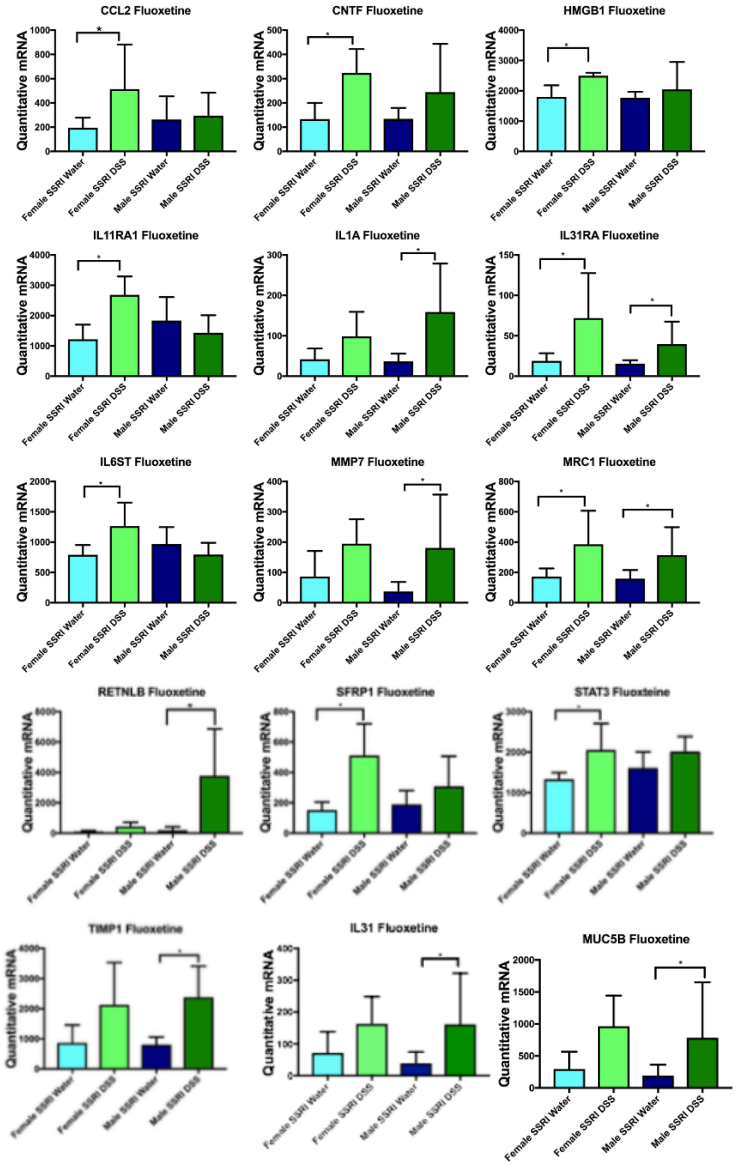

Figure 28. Quantitative amounts of mRNA in the colon of fluoxetine exposed DSS colitis vs fluoxetine exposed male water control P6M Wistar rats demonstrated a significant increase (mean ± SD) in CCL2 (p = 0.02), CNTF (p = 0.03), HMGB1 (p= 0.02), IL11RA1 (p = 0.02), IL1A (p=0.01), IL31RA (p = 0.02), IL6ST (p = 0.03), MMP7 (p = 0.0303), MRC1 (p = 0.03), RETNLB (p = 0.02), SFRP1 (p = 0.02), STAT3 (p = 0.03), TIMP (p = 0.004), IL31 (p= 0.05)and MUC5B (p= 0.05). n = 4 in female SSRI water group, n = 5 for female and male SSRI DSS group. n =6 in the male SSRI water group.

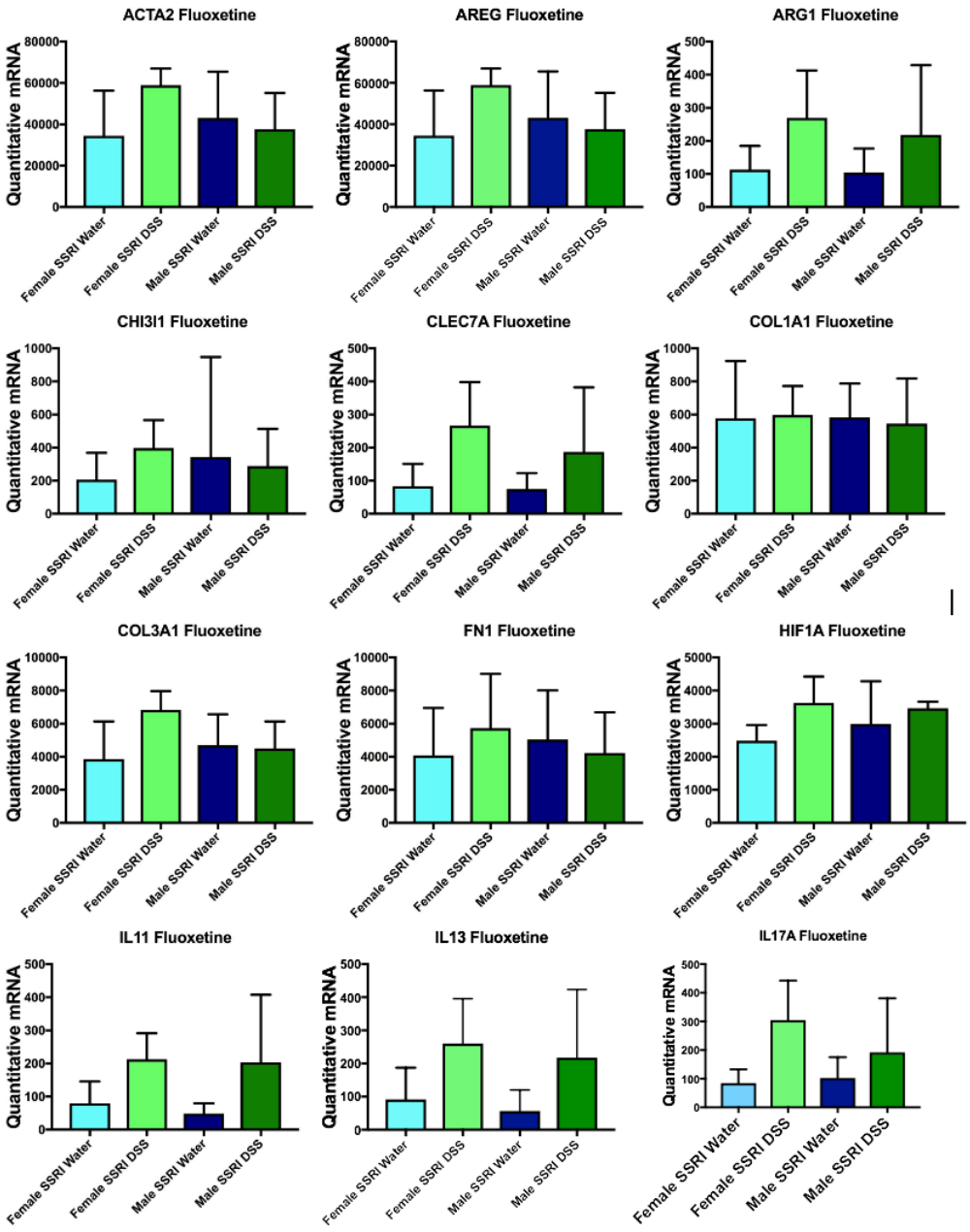

Figure 29. Quantitative amounts of mRNA in the colon of fluoxetine exposed DSS colitis vs fluoxetine exposed water control at P6M Wistar rats demonstrated no significant difference (mean ± SD) in ACTA2, AREG, ARG1, CHI3I1, CLEC7A, COL1A1, FN1, HIFA, IL11, IL31, IL17A

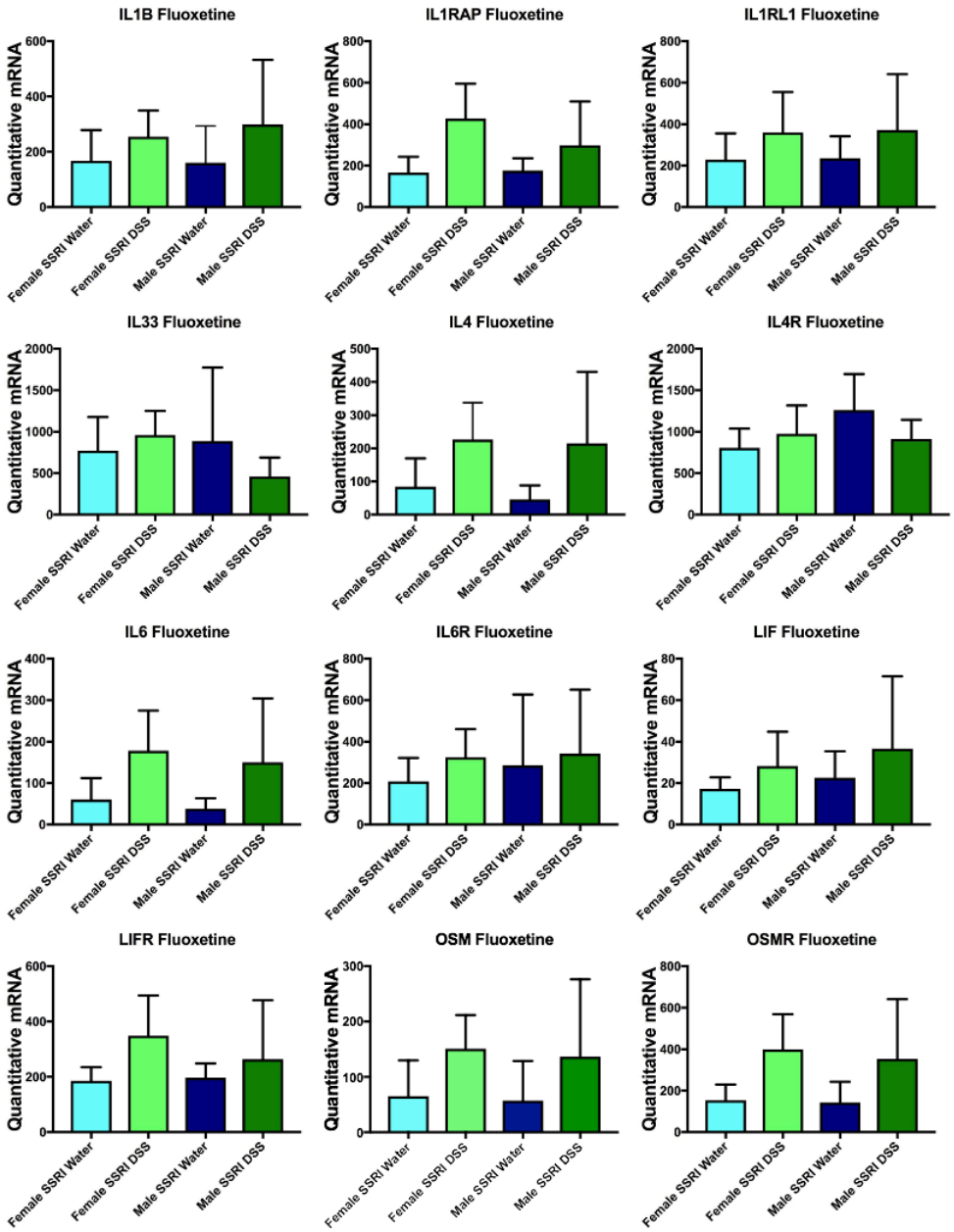

Figure 30. Quantitative amounts of mRNA in the colon of fluoxetine exposed DSS colitis vs fluoxetine exposed water control at P6M Wistar rats demonstrated no significant difference (mean ± SD) in IL1B, IL1RAP, IL1RL1, IL33, IL4, IL4R, IL6, IL6R, LIF, LIFR, OSM, and OSMR.

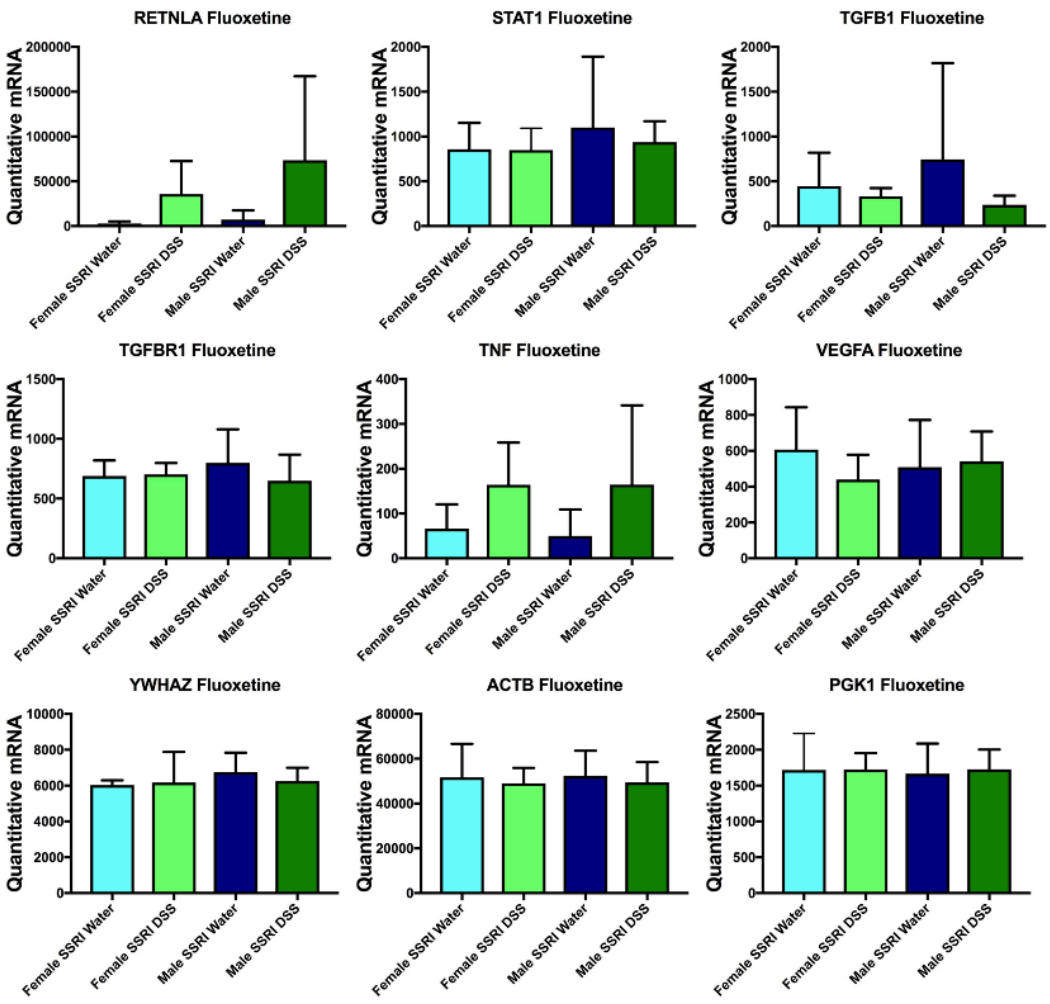

Figure 31. Quantitative amounts of mRNA in the colon of fluoxetine exposed DSS colitis vs fluoxetine exposed water control at P6M Wistar rats demonstrated no significant difference (mean ± SD) in RETNLA, STAT1, TGFB1, TGFBR1, TNF, VEGFA, YWHAZ, ACTB, and PGK1.

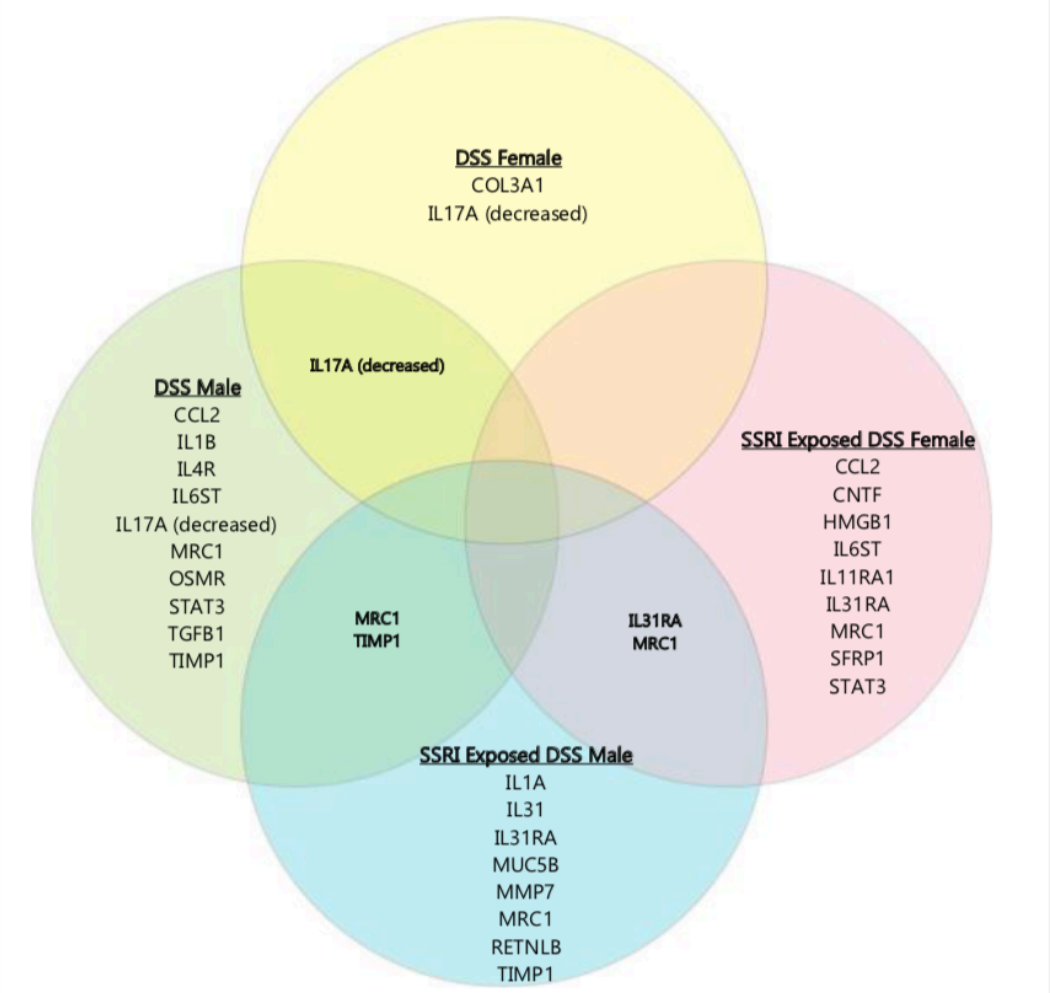

Figure 32. Venn diagram of significantly increased quantitative mRNA expression excepted where decreased shown in brackets of SSRI exposed DSS male/female and DSS male/female controls from colonic tissue samples demonstrating IL-17 does not appear to be involved in the migration of neutrophils in DSS colitis ,SSRI exposure does not appear to alter the increase in gene encoding proteins for MRC1 nor TIMP1 for males irrespective of DSS induced acute colitis, and that SSRI exposure does not alter the increase in IL31RA nor MRC1 in DSS induced acute colitis in the male and female offspring.

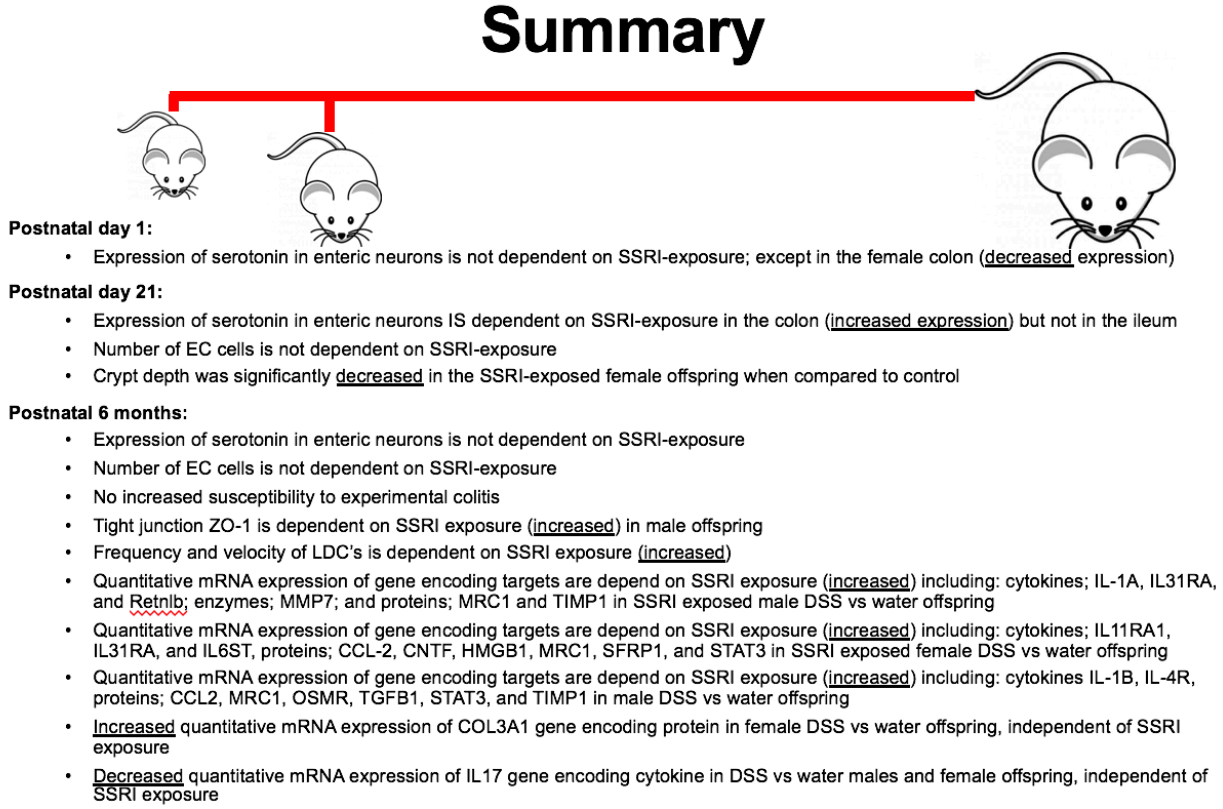

Figure 33. Summary of Findings

TABLES

Criteria	Grade	Description
Crypt Architecture	0	Normal
	1	Shortening of crypt
	2	Focal thinning of epithelium
	3	Severe crypt distortion with loss of entire crypts
Degree of Inflammatory Cell Infiltrate	0	Normal
	1	Mild inflammatory infiltrate
	2	Prominent inflammatory infiltrate
	3	Dense inflammatory infiltrate(extends into muscularis)
Muscle Thickening	0	Base of crypt sits on muscularis mucosa
	1	Loss of basal 1/3 of crypt
	2	Loss of basal 2/3 of crypt
	3	Marked muscle thickening
Goblet Cell Depletion	0	Absent
	1	Present
Crypt Abscess	0	Absent
	1	Present

Table 1. Validated Inflammation Histology Score. (Adapted from Cooper Lab Invest 1993, Kim J Immunol 2013)

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