

NICOLE E. DE LONG – PH.D. THESIS – SSRIs and the risk of T2DM

SELECTIVE SEROTONIN REUPTAKE INHIBITORS AND THE RISK OF TYPE 2
DIABETES MELLITUS

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Lay Abstract

Pregnancy is a window of vulnerability for depression with prevalence rates estimated to be approximately 10%. Guidelines recommend that antidepressant medication should be considered for pregnant women with moderate to severe depression of which selective serotonin reuptake inhibitors (SSRI) are the most common. The aim of this project was to look at SSRI exposure during pregnancy and to determine whether this exposure can predispose the offspring to obesity and/or type 2 diabetes (T2DM). We have found that SSRI use during pregnancy may increase the risk of T2DM and fatty liver in the adult offspring. These findings raise new concerns about the metabolic health of children born to women who take SSRI antidepressants during pregnancy. While these findings suggests a significant outcome in rodents, further investigations are critical to understanding the complexities within this field before suggesting similar outcomes in humans.

Abstract

Major Depressive Disorder (MDD) is a wide spread psychiatric disorder which affects more than 350 million people worldwide. Of the current available treatments, Selective Serotonin Reuptake Inhibitors (SSRIs) are the most commonly prescribed. Long-term SSRI use has been associated with the onset of Type 2 Diabetes (T2DM) in adults. T2DM is driven by beta cell dysfunction and hence we wanted to investigate the mechanism(s) by which SSRIs were causing beta cell demise. We have found that fluoxetine, more commonly known as Prozac®, does in fact disturb beta cell function by inducing mitochondrial dysfunction and thereby oxidative damage.

Interestingly, women are twice as likely to experience MDD, and this risk peaks during childbearing years. As a result, up to 1 in 10 pregnancies are complicated with SSRIs. This is an astonishing number considering the long-term effects of these drugs on the children are unknown. Within this thesis I report for the first time, the long-term metabolic outcomes of fetal and neonatal exposure to SSRIs in a rodent model. We have found that antenatal exposure to fluoxetine results in altered glucose homeostasis and impaired insulin sensitivity in the adult offspring. Furthermore we have shown that epigenetic modifications may be implicated in the increased incidence of fatty liver in these offspring. These findings demonstrate a predisposition to the development of obesity and T2DM into adulthood. These findings are novel and have clinical importance with respect to mothers taking these drugs during pregnancy. Within this thesis we are not advising mothers with depression to discontinue medication. We are merely exploring the

risks in a rodent model while acknowledging that a significant amount of research is required before applying these results to the human population.

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PREFACE

This thesis is prepared in the “sandwich” format as outlined in the “Guide for the preparation of Master’s and Doctoral Theses” available through the School of Graduate Studies at McMaster University. Chapter 1 is a general introduction regarding the content of subsequent chapters. Portions of this chapter are published. The body of this thesis consists of 4 chapters (Chapter 2-5), each one an independent study, three of which are published and chapter 5 submitted for publication at the time of thesis submission. The author of this thesis, who is also the first author on all included works, wrote all submitted and published manuscripts included in this thesis. There is a small introduction to each chapter describing the contributions of the other authors. Finally Chapter 6 includes the discussion of this thesis aimed to summarize the conclusions of the thesis and discuss possible future directions along with reviewing the clinical significance of the work.

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List of Abbreviations and Symbols

ANOVA	Analysis of Variance
bp	Base Pair
BCA	Bicinchoninic Acid Assay
BSA	Bovine Serum Albumin
C	Celsius
CD68	Cluster of Differentiation 68
cDNA	Complementary DNA
CO ₂	Carbon Dioxide
COX	Complex
CYP 7A1	Cholesterol 7 alpha-hydroxylase
D	Day
DAB	3,3'-diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic Acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorder, 5 th edition
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ETC	Electron Transport Chain
FLX	Fluoxetine
GSIS	Glucose Stimulated Inulin Secretion
GTT	Glucose Tolerance Test
H ₂ O ₂	Hydrogen Peroxide
ITT	Insulin Tolerance Test
kDA	Kilodalton
L	Liter
µg	Micrograms
µL	Microliters
µm	Microns
µM	Micromolar
MDD	Major Depressive Disorder
mRNA	Messenger RNA
MCP1	Monocyte Chemotatic Protein
mL	Milliliters
min	Minutes
N, n	Sample Size
NASH	Nonalcoholic Steatohepatitis
ng	Nanograms
p	Level of Significance/probability value
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PND	Postnatal Day
PVDF	Polyvinylidene Difluoride

RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SERT	Serotonin Transporter
SGA	Small for Gestational Age
SSRI	Selective Serotonin Reuptake Inhibitor
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with 0.1% Tween 20
TNF α	Tumor Necrosis Factor alpha
U/mL	Units/Milliliter
VEGF	Vascular Endothelial Growth Factor

Declaration of Academic Achievement

Chapter 2

Publication:

De Long NE, Hyslop JR, Raha S, Hardy DB, and Holloway AC (2014). Fluoxetine-induced pancreatic beta cell dysfunction: New insight into the benefits of folic acid in the treatment of depression. *J Affect Disord* 166; 6-13. doi: 10.1016/j.jad.2014.04.063

Contribution

This study was conceived and designed by NED and ACH. All laboratory experiments including RNA and protein extraction, Western blots, radioimmunoassays, mitochondrial ROS production and mitochondrial enzyme activities were completed by NED and JRH with the assistance from RS and DBH. Data was analyzed by NED and ACH. NED drafted the manuscript while all authors contributed to the final versions of the manuscript.

Chapter 3

Publication:

De Long NE, Gutgesell MK, Petrik JJ and Holloway AC (2015). Fetal Exposure to Sertraline Hydrochloride Impairs Pancreatic β -Cell Development. *Endocrinology* 156(6):1952-7. doi: 10.1210/en.2014-1779

Contribution:

This study was conceived and designed by NED and ACH. All laboratory experiments including animal handling, sample collection, immunoassays, immunohistochemistry, RNA extraction and real time PCR were completed by NED and MKG, with the exception of the immunohistochemical staining for VEGF and CD31 in the pancreas which was performed by JJP. NED drafted the manuscript while all authors contributed to the data analysis and final revisions of the manuscript.

Chapter 4

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De Long NE, Barry EJ, Pinelli C, Wood GA, Hardy DB, Morrison KM, Taylor VH, Gerstein HC, Holloway AC (2015). Antenatal exposure to the selective serotonin reuptake inhibitor fluoxetine leads to postnatal metabolic and endocrine changes associated with type 2 diabetes in Wistar rats. *Toxicol Appl Pharmacol.* 285(1):32-40. doi: 10.1016/j.taap.2015.03.006

Contribution:

This study was conceived by NED, KMM, VHT, HCG and ACH. All laboratory experiments including animal handling, sample collection, immunoassays, immunohistochemistry, RNA extraction and real time PCR were completed by NED and EJB. CP and GAW scored for liver pathology while the lipid analysis was analyzed at the Metabolic Phenotype Laboratory at Robart's Research Institute in London Ontario. Data was analyzed by NED and ACH. NED drafted the manuscript while all authors contributed to the final version of the manuscript.

Chapter 5

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This study was conceived by NED, DBH and ACH. All laboratory experiments including animal handling, sample collection, RNA extraction, real time PCR, acid histone extraction and Western blotting were completed by NED. Chromatin immunoprecipitation was completed by NM. Data was analyzed by NED, DBH and ACH. NED drafted the manuscript while all authors contributed to the final version of the manuscript.

Chapter 1: Introduction

1.1 Major Depressive Disorder

1.1.1 Impact

Major depressive disorder (MDD) is a highly prevalent condition which is currently estimated to affect as many as 840 million people worldwide, including more than 16 million people aged 18 or older in the United States (Demyttenaere et al., 2004; World Health Organization, 2008). It is predicted that by 2020, MDD will be the second leading cause of disease burden for developed countries (Murray and Lopez, 1996). In addition to reduced quality of life (Joffe et al., 2012), depression has a significant economic burden. The economic cost of depression is not only the direct costs associated with treatment but also indirect costs associated with decreased work productivity (Sobocki et al., 2007), and depression-related disability (Greenberg and Birnbaum, 2005). For example, in the United States of America, the cost of depression-related disability in 1990 was upwards to \$51 billion (Greenberg et al., 2003). More recent estimates from 2000 put that number as high as \$83 billion; a 150% increase over 10 years (Greenberg and Birnbaum, 2005). These trends are comparable across the world (Greenberg et al., 2015; Sobocki et al., 2006).

1.1.2 Pathology of Depression & Etiology: A Focus on the Serotonin System

MDD is diagnosed utilizing the Diagnostic and Statistical Manual of Mental Disorder, 5th edition (DSM-V). MDD is characterized by having a depressed mood (including 5 out of these 9 symptoms; irritable, loss of interest or pleasure in daily activities, significant weight change, changes in sleeping patterns, activity levels, energy levels, feelings of worthlessness, inability to concentrate along with thoughts of self-harm) nearly every day for at least 2 weeks. There is a large heterogeneity in the neuropathology of depression. Human brain imaging and postmortem studies have found that there are many areas of the brain involved in the emotional processing and stress-regulation affected in depression (Koolschijn et al., 2009). Early onset (<25 years of age) and recurrent cases have found areas of gray matter volume loss in a number of regions including the anterior cingulate cortex, prefrontal cortex, ventromedial striatum, amygdala and the hippocampus (reviewed in Drevets et al., 2008). Overall there appears to be a decrease in the volume and glial cell density along with reduced concentrations of pro-survival signals (i.e., serotonin, brain derived neurotropic factor etc.) for neurons which may be contributing to this mood disorder [reviewed in (Martinowich and Lu, 2008; Russo and Nestler, 2013)]. Nevertheless, the literature is inconsistent in regards to the structural changes in the brain associated with depression. These discrepancies may be explained through the subtypes of depression, the timing of tragic life events, the onset of neurodevelopmental diseases, and/or the interaction of these environmental factors with genetic predisposition (Bijanki et al., 2014; Drevets et al., 2008; Sheline et al.,

1999). As a result there are no established criteria to perform brain imaging for diagnosing MDD.

Given that MDD has been associated with both environmental and genetic factors, researchers have attempted to identify the genetic determinants of depression and/or genes implicated in the ability to respond to stressors that could act as diagnostic susceptibility measures for the onset of MDD (Sullivan et al., 2000). One of the promising putative targets is the serotonin transporter (SERT) gene, (*SLC6A4*). While SERT is a major site of action for the therapeutic actions of the majority of the antidepressants on the market, there is a new area of research regarding its allelic expression. The promoter region of the serotonin transporter gene (*5HTTLPR*) comprises a short (S) and a long (L) allelic variant. The S allele is linked with decreased transcription of SERT (Bradley et al., 2005). However there is conflicting evidence suggesting an associations between both alleles and MDD. For instance, a systematic review and meta-analysis found that the L allele was more predictive of suicidal behaviour (de Medeiros Alves et al., 2015) while another Japanese study reported that there may be an association between the L allele frequency and age of onset of MDD (s/s: 41.1 ± 13.6 ; s/l: 5.0 ± 15.2 ; l/l: 48.6 ± 15.7 ; $p=0.027$) (S.-Y. Watanabe et al., 2015). Conversely, in Caucasians individuals carrying two copies of the S allele have an overall increased incidence of MDD (Kiyohara and Yoshimasu, 2010) along with a heightened hypothalamic-pituitary-adrenal (HPA) axis reactivity in response to particular adverse life

events (Vinberg et al., 2014). Further studies are needed to definitively assess the contribution of 5HTTLPR polymorphisms and the onset of MDD.

The expression of SERT may also be influenced by epigenetic modifications as a result of gene-environment interactions. It has been shown that stress (either during early-life or into adulthood) results in increased methylation (transcriptional silencing) of the SERT gene (Beach et al., 2011; Duman and Canli, 2015; Kang et al., 2013; Ouellet-Morin et al., 2013; Vijayendran et al., 2012). Alexander et al., were the first to establish that altered gene methylation can compensate for allelic susceptibility to stress reactivity (Alexander et al., 2014). Furthermore Ouellet-Morin et al., demonstrated that early life stressors such as bullying resulted in methylation of SERT and decreased its expression, thereby increasing serotonergic activity (Ouellet-Morin et al., 2013). Although the genetic contribution to MDD is not entirely clear, work in this area highlights the importance of the serotonergic system in the etiology of MDD.

Serotonin is synthesized from the amino acid L-tryptophan and is regulated by the rate limiting enzyme tryptophan hydroxylase 2 (Tph) in the brain, and Tph1 in the peripheral tissues (Reviewed in [Fidalgo et al., 2013]). Serotonin is stored in synaptic vesicles at the nerve terminals and exocytosis of these vesicles releases serotonin into the synaptic cleft where it can bind to a family of serotonergic receptors (5-HT₁₋₇). Serotonin action is eventually terminated by serotonin reuptake into the pre-synaptic cell via the serotonin transporter (SERT) and its catabolism to 5-hydroxyindoleacetic acid by the action of monoamine oxidase.

It has long been hypothesized that the serotonergic system is altered in patients suffering from depressive symptoms (Artigas, 2013; Nestler et al., 2002; Nestler and Hyman, 2010) and now there is clear evidence to support this. Patients who had unsuccessfully attempted suicide had reduced serotonin levels in cerebral spinal fluid (Mann et al., 1996) along with decreased serotonin transporter (SERT) binding potentials in the midbrain and brain stem (Nye et al., 2013). Furthermore two recent meta-analysis studies reported a decreased SERT availability within the limbic system of individuals with MDD (Gryglewski et al., 2014; Kambeitz and Howes, 2015). Antidepressants that modulate key components of the serotonin signaling pathway aim to restore this imbalance (Nestler and Hyman, 2010). The importance of serotonin in depression which led to the use of SSRIs for the treatment of depression was developed based on the monoamine hypothesis of mood disorders (Hirschfeld, 2000). The monoamine hypothesis proposes that patients with depression have decreased availability of monoamines (i.e., serotonin, dopamine, norepinephrine) resulting in decreased central neural transmission (Hirschfeld, 2000) and that this suppression of monoamine effect is associated with the observed depressive symptoms. As a result most of the psychiatric medications used to treat depression attempt to restore this imbalance, and increase the signaling by monoamines, including serotonin, in the central nervous system.

1.2 Selective Serotonin Reuptake Inhibitors

1.2.1 Prevalence of Antidepressant use

Antidepressants are a first-line option for the management of moderate to severe MDD and estimates suggest that 27,000,000 persons in the USA alone are taking antidepressants (Olfson and Marcus, 2009); representing a 400% increase in the last 20 years (National Center for Health Statistics US, 2011). Similar increases in the prevalence of antidepressant use have been reported worldwide (Chien et al., 2007; Ilyas and Moncrieff, 2012; Smith et al., 2008). However, recent data from 2012 suggest that this increase in antidepressant use is leveling off at approximately 5.5% of the Canadian population (Patten et al., 2014). Although there are a wide variety of medications available for the treatment of MDD (Block and Nemeroff, 2014), drugs that target serotonin signaling (including selective-serotonin reuptake inhibitors [SSRIs] serotonin-norepinephrine reuptake inhibitors [(SNRIs], serotonin receptor antagonist and reuptake inhibitors [(SARIs], and noradrenergic and specific serotonergic antidepressants NaSSAs) are the most commonly prescribed for adults, children and adolescents (Hoffmann et al., 2014; Mojtabai and Olfson, 2008; Trifirò et al., 2013; Wemakor et al., 2014).

1.2.2 SSRI: Mechanism of Action

Over 90% of antidepressants (i.e., SSRIs, SNRIs, SARIs, and NaSSAs) on the market target SERT (Artigas, 2013). Indeed, in most countries SSRIs represent the most

commonly prescribed class of antidepressants (Smith et al., 2008; Trifirò et al., 2013; Wemakor et al., 2014). SERT plays a critical role regulating central serotonin levels by mediating the reuptake of serotonin from the synaptic cleft into the presynaptic neuron. As a result, SSRIs act to inhibit the presynaptic SERT transporter, increasing the serotonin levels in the synapse thereby restoring the balance of the monoamine levels in the brain (Fidalgo et al., 2013). However, since SSRIs require 2-6 weeks to have clinical efficacy, it suggests that there is also an adaptive response to these drugs. This delay in therapeutic action is believed to be due to the time required to desensitize the serotonin receptor 1A (5-HT_{1A}) in the brain. 5-HT_{1A} are found on the somatodendritic compartment of the serotonergic neurons located in the raphe nucleus. Activation of these receptors decreases the synthesis and packaging of serotonin at the projection sites. Following chronic exposure to SSRIs, 5-HT_{1A} become desensitized, increasing the serotonergic neurotransmission via internalization or endocytosis of these receptors (Le Poul et al., 2000; 1995). Drugs within the SSRI class include citalopram, fluoxetine, paroxetine, sertraline, escitalopram, and fluvoxamine. In addition to their actions on SERT, fluoxetine and paroxetine are also antagonists at the 5-HT_{2C} receptor (Pälvimäki et al., 1996). Similarly, SNRIs (e.g., venlafaxine, desvenlafaxine and duloxetine) inhibit both SERT and norepinephrine transporters (NET) (Celikyurt et al., 2012). NaSSAs (e.g., mirtazapine) are antagonists at both adrenergic receptors (α -2) and select serotonin receptors (i.e., 5-HT_{1C}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT₃) (Croom et al., 2009; Kent, 2000; Nash and Nutt, 2007; Pinder, 1997). Finally, SARI antidepressants (e.g., trazodone)

antagonize serotonin receptors including 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} (Cusack et al., 1994; Nash and Nutt, 2007; Odagaki et al., 2005; Spina et al., 2008).

1.3 Depression and its comorbidities

In addition to being one of the leading causes of disability worldwide, MDD is also associated with a high risk of developing metabolic disorders, including an increased risk of obesity and type 2 diabetes (T2DM) (Holt et al., 2004; Newcomer, 2007; Vancampfort et al., 2013). Established risk factors for the increased incidence of diabetes in patients with MDD include: age, sex, familiarity and genetic predisposition, socioeconomic status, and maladaptive behaviors during a depressive episode such as comfort eating and physical inactivity (Chien et al., 2012; Katon, 2008). Psychotropic medication use has also been associated with this increased risk but the exact etiology underlying this association has not been elucidated. [reviewed in (Newcomer, 2007)]

1.3.1 Bidirectional Relationship Between MDD and T2DM

There is a bidirectional relationship between MDD and T2DM, although the direction of this relationship is still unclear (Hasan et al., 2014; Kan et al., 2013; Mezuk et al., 2008; Pan et al., 2010; 2012b; Renn et al., 2011; Rotella and Mannucci, 2013). Normal glucose tolerance is tightly regulated by the availability of insulin and the ability of the peripheral tissues to utilize insulin to uptake glucose as fuel. T2DM is defined by a fasting glucose level ≥ 7.0 mmol/L or ≥ 11.1 mmol/L 2 hour post oral glucose (75g) load

and occurs in circumstances of beta cell dysfunction in conjunction with insulin resistance. However, insulin resistance, in the absence of beta cell dysfunction does not lead to the development of T2DM and therefore, beta cell demise is central in the progression of T2DM (Ahren, 2005; Chiasson and Rabasa-Lhoret, 2004; Del Prato and Marchetti, 2004; Kahn et al., 2008; Leahy, 2005). Hepatic insulin resistance is a major contributor to metabolic comorbidities of T2DM such as fatty liver, as insulin not only controls the uptake of glucose into the hepatocyte, but also suppresses the amount of glucose and lipid production in the liver. In response to hyperglycemia as a result of insulin resistance, beta cells secrete higher amounts of insulin to compensate for the peripheral insulin resistance. However, this burden over time results in beta cell damage and ultimately beta cell death. Patients with severe forms of the disease may be treated with insulin to control their blood glucose levels. Diabetes affected more than 382 million people worldwide in 2013 and this is predicted to increase by 55% to 592 million by 2035, particularly in the developing countries (King et al., 1998; Zimmet et al., 2001). These rates are steadily increasing along with the incidence of MDD and there is now new insight to suggest that these two illnesses may share a common pathophysiology.

A meta-analysis of 42 studies reported that MDD is approximately 2 times more likely in patients with diabetes compared with the general population (OR=2.0; 95% CI 1.8-2.2) (Anderson et al., 2001) with the reported global prevalence of MDD in patients with T2DM ranging from 2% (Brazil) to 84% (India) (Anderson et al., 2001; Mendenhall et al., 2014). This connection is clinically relevant given that depression in patients with T2DM is associated with increased rates of diabetic complications, poorer glycemic

control and decreased quality of life (de Groot et al., 2001; Holt et al., 2014). It has been suggested that the increased risk of MDD among diabetic patients may be attributed to the burden of the disease and its treatment, and/or to the related underlying physiological and biochemical changes associated with the pathophysiology of diabetes (Knol et al., 2007; Renn et al., 2011; Siddiqui, 2014; Talbot and Nouwen, 2000). Conversely, there is also evidence demonstrating that individuals suffering from MDD are at an increased risk of new-onset T2DM (Campayo et al., 2010; 2011; Engum, 2007; Himmerich et al., 2008; Hirschfeld, 2001; Knol et al., 2006). Most strikingly, Knol et al. report within a meta-analysis of 9 studies that there was a 37% increased risk ($RR=1.37$, 95% $CI=1.14-1.63$) (Knol et al., 2006) of developing T2DM in depressed adults while Mezuk et al. report in a subsequent meta-analysis two years later encompassing of more than 6,000 cases that people with MDD have a 60% ($RR=1.60$, 95% $CI=1.37-1.88$) (Mezuk et al., 2008) increased risk of developing T2DM. Indeed, it has been reported that the degree of insulin resistance and whole body glucose disposal rates are different depending on the clinical phenotype of MDD (Cizza et al., 2012; Schweiger et al., 2008). However, the psychiatric medications used to treat MDD may also pose an independent risk factor for the development of T2DM, thus adding a layer of complexity to the MDD–diabetes paradigm.

1.3.2 Are Antidepressants the Link?

Ultimately, all serotonin-modulating antidepressants inhibit the reuptake of MDD-associated neurotransmitters centrally. However, the development of T2DM may be an

unintended consequence associated with the use of antidepressants that target serotonin signaling (Deuschle, 2013; Pan et al., 2012a; Rubin et al., 2008). There are several studies that report an increased risk of T2DM in patients taking antidepressants that target serotonin signaling. Yoon et al. reported in a recent meta-analysis of 8 studies that there was an increased risk of T2DM among SSRI users (RR: 1.35; 95% CI: 1.15-1.58) (Yoon et al., 2013). Antidepressants such as sertraline, fluvoxamine, paroxetine, venlafaxine, fluoxetine, citalopram, and mirtazapine were associated with dysregulation of glucose homeostasis manifesting as hyperglycemia, hypoglycemia, or both, particularly when treatments were continued for more than one year (Derijks et al., 2008; Khoza and Barner, 2011). Furthermore, case reports of patients taking fluvoxamine and mirtazapine (Khoza and Barner, 2011), along with a retrospective cross-sectional health survey (OR 2.36; 95% CI: 1.00-5.60; $p=0.051$) (Raeder et al., 2006) and a long-term nested case-control study (OR: 1.20; 95% CI: 1.05-1.37) (Wu et al., 2014), have reported an elevated risk of hyperglycemia and new-onset diabetes in adults. Similarly a retrospective cohort study identified an increased odds ratio of developing T2DM in children and adolescents taking SSRIs (OR =1.37; 95% CI 1.10-1.71) (Jerrell, 2010). Furthermore, long-term use (greater than 2 years) of SSRIs is associated with an incidence rate ratio of 2.06 (95% CI=1.20-3.52) (Andersohn et al., 2009) for risk of diabetes; duration of medication use and mean daily dosage were significant contributing factors. Moreover, the risk of diabetes appeared to be drug-dependent with reported risk estimates of 9.05 (95% CI=1.08-75.58) for fluvoxamine, 1.75 (95% CI=1.13-2.72) for paroxetine, and 3.01 (95% CI=1.01-9.02) for venlafaxine (Andersohn et al., 2009). In a study reporting on cohorts

across the United States, the average absolute risk difference for new-onset diabetes between individuals taking any antidepressant medication (including participants using fluoxetine, sertraline, paroxetine, and citalopram) and those without antidepressant treatment was 2.87 per 1,000 person-years (Pan et al., 2012b). While it is still unclear if the risk of new-onset diabetes changes with respect to MDD severity it is worth noting that the increased risk of diabetes in patients taking SSRIs persisted even after controlling for a number of known risk factors for the development of T2DM (Wu et al., 2014; Yoon et al., 2013). Taken together, these studies suggest that manipulation of serotonergic signaling and transmission affects biological processes important in the pathophysiology of T2DM although the specific mechanisms underlying this relationship remain unclear (Dhavale et al., 2013; Gehlawat et al., 2013).

1.4 Mechanisms Underlying T2DM and Antidepressant Use

1.4.1 Weight Gain

It has been suggested that the increased risk of diabetes in patients taking antidepressants may be secondary to increased weight gain (Bet et al., 2013) as obesity is a well-established risk factor for T2DM (Sattar and Gill, 2014). Serotonin is an important central regulator of feeding behaviour and energy expenditure; key physiological processes through which body weight homeostasis is maintained (Berger et al., 2009; Donovan and Tecott, 2013; Lam et al., 2010; Voigt and Fink, 2015). In many studies, the long-term use of antidepressants which affect serotonergic pathways has been associated with an increased risk of abdominal obesity and weight gain (Aronne and Segal, 2003;

Blaine, 2008; Deuschle, 2013; Himmerich et al., 2004; Raeder et al., 2006; Serretti and Mandelli, 2010). Given the known effects of serotonin on feeding behaviors, these changes in body weight homeostasis might be secondary to altered food intake. However, there is also evidence that suggests that weight gain with antidepressant use may be related to direct effects of these drugs on lipid metabolism. Clinical studies have demonstrated that antidepressant use is associated with dyslipidemia, including clinically significant increases in triglyceride, low-density lipoprotein and cholesterol levels (Y.-C. Chen et al., 2010; McIntyre et al., 2010; Serretti and Mandelli, 2010). Furthermore, metabolomic profiling of the early biochemical changes associated with the use of the SSRI sertraline identified drug effects on pathways important for the regulation of lipid metabolism (Kaddurah-Daouk et al., 2013). In vitro studies have identified that SSRIs can directly alter hepatic lipid production by increasing lipogenesis and decreasing lipolysis (Feng et al., 2012; Xiong et al., 2014). Furthermore, alterations of serotonin metabolism in murine fibroblasts and human adipocytes have been shown to increase lipogenesis (Grès et al., 2012) by the activation of the transcription factor peroxisome proliferator-activated receptor gamma (PPAR γ), a key regulator of adipogenesis (Ali et al., 2013). It is also possible that increased weight gain with antidepressant use is related to their mechanism of actions at SERT and/or the serotonin receptors.

Studies in rodents have shown that administration of SSRIs can not only decrease the effectiveness of serotonin uptake by SERT but can also cause reduction in SERT expression (Descarries and Riad, 2012; Gomez et al., 2014; Shishkina et al., 2012). Importantly, clinical studies and animal experiments provide evidence to suggest that

both decreased expression and function of the serotonin transporter is associated with obesity (Bah et al., 2010). Results from a monozygotic twin study, for example, found that increased BMI, weight gain and waist circumference was associated with epigenetic silencing of SERT transcription (histone methylation) (Zhao et al., 2013). Obesity has also been directly associated with decreased platelet SERT expression (Giannaccini et al., 2013) which, given that animals with genetic ablation of SERT (i.e., SERT knockout mice) result in elevated body weight as a result of an increase in percent body fat and a reduction in percent lean body mass without any increases in food consumption (Chen et al., 2012), may explain some of the observed weight changes. Indeed, in human populations diminished SERT function as a result of an allelic (“s” allele) variation in the promoter region of SERT (HTTLPR) is commonly found in many cases of T2DM (Iordanidou et al., 2010). Therefore, it is biologically plausible that antidepressants that target the serotonin transporter may affect body weight homeostasis and the subsequent risk of T2DM through inhibition of peripheral SERT function and/or expression.

Modulation of serotonin signaling via agonism/antagonism at serotonin receptors may also be a mechanism by which serotonin-targeting antidepressants may influence body weight homeostasis. Polymorphisms of the 5-HT_{2C} receptor gene or knockout of the 5-HT_{2C} receptor itself are related to dysregulation of appetite and feeding behavior, and weight gain (Halder et al., 2007; Iordanidou et al., 2008; H. Watanabe et al., 2011; Yuan et al., 2000). Therefore, antagonism of this receptor, as is seen with many of the antidepressants which target serotonin signaling (Pälvimäki et al., 1996; Stahl, 2009), may contribute to the increased weight gain seen with antidepressant use. However,

although weight gain and dyslipidemia often leads to decreased peripheral insulin sensitivity, altered glucose homeostasis and ultimately the development of T2DM, Pan et al. (2012a) reported that patients with MDD who were taking antidepressants were 25% more likely to develop T2DM irrespective of body mass index and physical activity (Pan et al., 2012b). Moreover, not all of the drugs that target serotonergic signaling pathways are associated with weight gain (Serretti and Mandelli, 2010), suggesting that the increased risk of T2DM with antidepressant use cannot be attributed solely to the effects of increased body weight.

1.4.2 Insulin Resistance & Fatty Liver

Another mechanism through which antidepressants may lead to T2DM is by increasing insulin resistance. In normal insulin signaling, insulin binds to insulin receptors and promotes glucose uptake into cells via glucose transporters (Cartailler, Beta Cell Biology Consortium). In conditions of insulin resistance, cells are unable to appropriately take up and use glucose for metabolic or storage purposes, leading to hyperglycemia (Sinaiko and Caprio, 2012; Goldstein, 2002; Schinner et al., 2005; Ye, 2013). Serotonin-related antidepressants have been proven to disrupt insulin signaling through the involvement of insulin receptor substrate (IRS)-1 and IRS-2. The SSRIs paroxetine and sertraline have been shown to inhibit IRS kinase stimulation in vitro, which leads to serotonin-induced phosphorylation of IRS-1 and IRS-2 at selected serine residues (Isaac et al., 2013; Levkovitz et al., 2007; Li et al., 2013). Serine phosphorylation inhibits Tyr phosphorylation by up to 50-70%, thereby leading to IRS

dissociation from the insulin receptor complex and/or receptor degradation. Furthermore, sertraline exposure in liver cells induced endoplasmic reticulum stress which can also contribute to a decrease in the overall insulin responsiveness (Chen et al., 2014).

There is a strong relationship between hepatic fat accumulation and insulin resistance. Hepatic fat accumulation occurs due to an elevation in dietary fat intake, serum lipid levels, de novo hepatic lipogenesis and/or decreased hepatic lipid oxidation (Bugianesi et al., 2005). Lipid deposition in the liver is strongly correlated with metabolic disorders and T2DM patients in particular have an increased risk of nonalcoholic steatohepatitis (NASH) (Dietrich and Hellerbrand, 2014; Portillo Sanchez et al., 2015). Importantly, the use of antidepressant medications that target serotonin signaling has been shown to be associated with hypercholesterolemia and hypertriglyceridemia (Beyazyüz et al., 2013; Raeder et al., 2006). Furthermore, in vitro studies have demonstrated that SSRIs have a direct effect on hepatic lipogenesis; treatment of primary mouse hepatocytes with fluoxetine resulted in an increase in lipogenic enzyme expression and lipid deposition (Feng et al., 2012; Xiong et al., 2014). Although short term (4-8 week) treatment with SSRIs or SNRIs has not been shown to affect insulin resistance (i.e., HOMA-IR) (Chang et al., 2013), Pyykkonen et al. (2011) reported that the use of antidepressants was associated with a higher odds for belonging to the top quartile of insulin resistance (Pyykkönen et al., 2011). Therefore, despite the fact that there is mechanistic evidence demonstrating that SSRIs can lead to insulin resistance by diminishing insulin receptor sensitivity, function, and signal transduction (Boura-Halfon

et al., 2010; Isaac et al., 2013; Levkovitz et al., 2007; Q. Li et al., 2013) and increasing hepatic fat accumulation, the clinical evidence for this relationship is less clear.

1.4.3 Pancreatic Beta Cell Function

Antidepressants targeting serotonergic pathways may also contribute to T2DM by adversely affecting pancreatic beta cell function and survival, hallmarks of T2DM. The presence of serotonin in pancreatic beta cells was first reported in the late 1960s (Jaim-Etcheverry and Zieher, 1968) and since then it has been clearly demonstrated that all of the key components of the serotonergic pathway including the plasma membrane serotonin transporter, serotonin receptors, tryptophan hydroxylase (Tph1 and Tph2), aromatic L-amino acid decarboxylase and monoamine oxidase, are expressed in pancreatic islets (Huang et al., 2005; Kim et al., 2010; Kutlu et al., 2009; Paulmann et al., 2009; Richmond et al., 1996; Schraenen et al., 2010). Serotonin is synthesized in pancreatic beta cells, stored along with insulin-granules, and is released upon glucose stimulation (Paulmann et al., 2009; Richmond et al., 1996). Serotonin reuptake into the pancreas by SERT increases intracellular serotonin levels enabling serotonylation of Rab3a and Rab27a, which are key contributors in the signaling sequence involved in insulin exocytosis. Mouse models without the necessary substrates for this intracellular serotonylation and/or serotonin synthesis (tryptophan hydroxylase knockout mice; i.e., Tph1-/-), are associated with dysglycemia and a beta cell secretory defect (i.e., impaired beta cell function) without any reduction in beta cell mass (Paulmann et al., 2009). Furthermore, inhibition of serotonin uptake by blocking SERT (i.e., as seen with SSRI

and SNRI treatment) has been shown to decrease intracellular serotonin levels in the pancreatic beta cells which, resulted in inhibition of glucose-stimulated insulin secretion (Li et al., 2014). Additionally, stimulation of the 5-HT_{1A} receptor by extracellular serotonin was shown to have inhibitory effects on insulin-granule exocytosis and, consequently, on insulin signaling pathways (Luo et al., 2012; Paulmann et al., 2009). Thus, SSRIs that prevent serotonin reuptake increase extracellular levels of serotonin such that stimulation of the 5-HT_{1A} receptor increases, and insulin secretion is further inhibited. There is also evidence from animal and human studies that supports the hypothesis that SSRIs can negatively affect insulin secretion. Treatment of rats and humans with an SSRI (paroxetine and fluoxetine respectively) for 4 weeks resulted in significant reductions in the insulin response to an oral glucose tolerance test (Chen et al., 2007; Li et al., 2014). The ability of the pancreas to secrete sufficient insulin to maintain glucose homeostasis is also affected by beta cell mass, which may also be impacted by serotonin targeting antidepressant medications. In vivo models have demonstrated that perturbations of the serotonergic system, either by disruption of serotonin signaling, or by manipulation of serotonin levels (Tph1: synthesis; MAO: degradation) have adverse effects on the regulation of beta cell mass (Edvell and Lindström, 1999; Kim et al., 2010). Furthermore, there is evidence from in vitro studies that SSRIs can have direct effects on beta cell survival; treatment of isolated beta cells with SSRIs has been shown to increase apoptosis (De Long et al., 2014; Isaac et al., 2013). The mechanisms by which SSRIs can impact beta cell function (i.e., insulin secretion) and survival are not entirely clear but

direct effects on IRS signaling, mitochondrial dysfunction, oxidative stress and ER stress in pancreatic beta cells have been reported (Isaac et al., 2013).

Antidepressants that target serotonergic systems have been shown to alter IRS signaling, an important contributor to insulin secretion (Hennige et al., 2003). The SSRI sertraline was shown to inhibit glucose-stimulated insulin secretion by inhibiting tyrosine phosphorylation of IRS-2 by 40-60%, thereby inducing inhibitory downstream effects on insulin expression and secretion; paroxetine and fluoxetine, showed similar effects (Isaac et al., 2013). SSRIs have also been shown to inhibit protein kinase B (PKB/Akt) activation, a signaling molecule downstream of the insulin receptor (Isaac et al., 2013). Inactivation of Akt is central to the onset of T2DM as it is a central regulator of beta cell survival/proliferation and is crucial in the expansion of beta cell mass (Elghazi et al., 2007). Furthermore, De Long et al. (2014) reported that fluoxetine treatment caused mitochondrial dysfunction and oxidative stress in INS-1E cells, a rat insulinoma cell line, resulting in the ablation of glucose stimulated insulin secretion (De Long et al., 2014). Similarly treatment of Min6 cells, a mouse beta cell line with sertraline, also resulted in impaired glucose-stimulated insulin secretion as well as endoplasmic reticulum stress, the unfolded protein response and increased apoptosis (Isaac et al., 2013). Taken together, these data suggest that the association between the use of antidepressants that target serotonin signaling pathways and new-onset T2DM may be attributed, at least in part, to an iatrogenic decrease in beta cell function.

While the relationship between depression and diabetes is complex, evidence from in vitro, animal and clinical studies suggests that the use of antidepressant medications which target serotonin signaling may play a role in the increased incidence of T2DM seen in adults with depression. However, there still remain considerable gaps in the literature regarding the effects of antidepressant use during pregnancy on metabolic outcomes in the offspring; an important question given that approximately 6-9% of pregnant women are taking selective serotonin reuptake inhibitor (SSRIs) antidepressants during pregnancy and lactation .

1.5 Antidepressant use During Pregnancy

1.5.1 SSRI Use During Pregnancy: Epidemiologic Evidence

Women are twice as likely to suffer from a depressive episode than men (Steel et al., 2014) and current estimates conclude that approximately 10% of pregnant women suffer from depression and/or anxiety during the perinatal period (Bennett et al., 2004; Cooper et al., 2007; Melville et al., 2010). Untreated depression during pregnancy poses risks for both the mother and the child (Gavin et al., 2005; Malm et al., 2015; Wemakor et al., 2015; Wisner et al., 2009). For the mother, risks include poor nutrition, pre-eclampsia, increased vulnerability to substance abuse, impaired bonding, and increased weight gain while the fetus is at risk of preterm birth and low birth weight (LBW) (Grote et al., 2010; Lokuge et al., 2011; S. M. Marcus, 2009). Therefore, practice guidelines recommend that antidepressant medication should be considered for pregnant women with moderate to severe major depression, while acknowledging that the medications themselves may pose

a risk to the fetus (Cooper et al., 2007; Melville et al., 2010; Oberlander et al., 2006; Sie et al., 2012). Of the antidepressants on the market, SSRIs are commonly prescribed for perinatal depression (Alwan et al., 2011).

Approximately 7% of pregnant women in the US are being prescribed SSRIs during pregnancy for the treatment of depression and it appears that this prevalence is increasing (Andrade et al., 2008; Cooper et al., 2007; Huybrechts et al., 2013). Global rates of SSRI use during pregnancy has been reported between 3%-13.3% (Andrade et al., 2008; Cooper et al., 2007; Jimenez-Solem et al., 2013; Lancaster et al., 2010). There has been an increase in antidepressant use over the past ten years likely due to the increased administration for a number of off label uses (Czaja and Valuck, 2012). A Danish study found that once women were aware of their conception, 43.3% of women discontinued their antidepressant therapies, of those who continued treatment (11.3%), the majority (42%) switched to fluoxetine (Jimenez-Solem et al., 2013). When examining the reasoning behind the decisions made regarding treatment options for antenatal depression, only 33% of women thought that antidepressant use during pregnancy was acceptable and believed individual psychotherapy was the preferred treatment option (Goodman, 2009). Additionally, it is probable that SSRI use may be underrepresented and/or depression is being under treated/low adherence in this population, likely due to the perceived barriers of time commitment and the associated stigma surrounding pharmaceutical use during pregnancy (Goodman, 2009; Lupattelli et al., 2015).

There have been numerous studies on the use and safety of antidepressants during pregnancy (Ellfolk and Malm, 2010; Grote et al., 2010; Källén, 2007; Udechuku et al., 2010; Way, 2007). They have for the most part focused on teratogenicity (i.e., major and minor fetal malformations) (Gentile, 2015), fetal toxicity (assessed by stillbirth), obstetrical outcomes (e.g. preterm delivery, spontaneous abortion) and neonatal outcomes (e.g. APGAR score, neonatal intensive care admissions and birth weight) (Ellfolk and Malm, 2010; Udechuku et al., 2010). Although it is uncertain whether it is the depression itself or the drugs used to treat depression, which cause these adverse outcomes, it is known that SSRIs cross the placenta directly exposing the fetus *in utero* (Hendrick et al., 2003; Loughhead et al., 2006; Rampono et al., 2004; 2009). A recent systematic meta-analysis found that pregnant women exposed to SSRI medications during at least the first trimester had an increased odds of offspring with a major malformation (OR 1.10, 95% CI 1.03–1.16, $z=3.07$, $p=0.002$) while the subgroup analysis identified paroxetine and fluoxetine as the agents of greatest concern (Myles et al., 2013). Moreover this study re-established the link between paroxetine (Paxil®) use during the first trimester and the increased risk of cardiac malformations (Myles et al., 2013). Fluoxetine (Prozac®) has been implicated in jitteriness, hypotonia and may increase the risk of persistent pulmonary hypertension of the newborns (Grigoriadis et al., 2014; Huybrechts et al., 2015). Most concerning is that SSRI use during pregnancy has consistently been found to increase the risk of low birth weight (LBW) infants (RR: 1.44 95% CI: 1.21-1.70) (H. Huang et al., 2013).

1.5.2 SSRI Use During Pregnancy: Human Studies

While there have been various follow-up studies on the neurobehavioural effects of antenatal SSRI exposure (Casper et al., 2003; Nulman et al., 1997; Oberlander et al., 2006), there have been limited studies on the postnatal metabolic effects in these children. The longest study to follow children of mothers who took SSRIs during pregnancy is Grzeskowiak et al., who demonstrated that SSRI use during pregnancy resulted in an increased risk of being overweight in male children (i.e., BMI at or exceeding the age- and sex specific 85th percentile in exposed children) by 7 years of age (Grzeskowiak et al., 2013). Although this study only had a 7-year follow-up, it provides strong evidence that antenatal SSRI exposure may in fact affect the predisposition for fat accumulation in these children.

1.5.3 SSRI Use During Pregnancy: Evidence from Animal Models

It has been suggested that the increased risk of low birth weight (LBW) in women taking antidepressants may be due to the underlying maternal depression and not a direct effect of the medication(s). Indeed, maternal depression is independently associated with an increased risk of LBW (Grote et al., 2010). However, direct effects of antidepressants on embryonic/fetal growth have also been demonstrated in vitro and in animal models (i.e., drug exposure with no underlying depression). In the rat whole embryo culture model, all of the SSRIs tested (fluoxetine, paroxetine and citalopram) caused dose-related reductions in embryonic growth (Sloot et al., 2009). Similarly, maternal exposure to the SSRIs fluoxetine and paroxetine significantly reduced birth weight in rats (Van den Hove

et al., 2008; Vorhees et al., 1994). Taken together these data provide strong evidence that maternal use of both SSRI antidepressants may have direct effects on fetal growth that are independent from the effects of maternal depression. This is significant as delivering a LBW baby is significantly associated with metabolic dysfunction, such as the development of T2DM, in adulthood (Harder et al., 2007; Whincup et al., 2008).

1.6 Rationale

Depression is a wide spread psychiatric disorder which affects more than 840 million people worldwide (World Health Organization, 2008). According to the World Health Organization, depression is predicted to be the second leading cause of disease burden worldwide by the year 2030 (World Health Organization, 2008). Of the current available treatments, SSRIs are the most commonly prescribed pharmaceuticals. Long term SSRI use has been associated with the onset of T2DM in adults (Wu et al., 2014). T2DM is characterized by hyperglycemia and peripheral insulin resistance. This is the combination of the pancreatic beta cells to secrete sufficient amounts of insulin and the inability of the peripheral tissues to utilize the insulin efficiently in response to the hyperglycemia. These two processes are central to the onset and progression of T2DM.

Interestingly, women are twice as likely to suffer from a depressive episode than men. Furthermore, the onset or the worsening of disease state aligns with the shifts in hormonal status as the risk of depression peaks during childbearing years (Lokuge et al., 2011; S. M. Marcus and Heringhausen, 2009). Current estimates conclude that

approximately 10% of pregnant women suffer from depression and/or anxiety during the perinatal period (Bennett et al., 2004; Cooper et al., 2007; Melville et al., 2010). Consequently approximately 7% of pregnant women in Canada are being prescribed SSRIs during pregnancy for the treatment of depression and it appears that this prevalence is increasing (Whincup et al., 2008). Despite their widespread use, there have been no studies to our knowledge investigating the use of SSRIs during pregnancy and throughout lactation in regards to the long-term metabolic health of these children. Therefore, we will examine in an animal model the role of fetal and neonatal SSRI exposure on long-term metabolic health, specifically the risk of developing endocrine and metabolic changes associated with T2DM.

1.7 Hypothesis

The overall hypothesis of this thesis is that fetal and neonatal exposure to SSRIs increases the risk of T2DM in the offspring.

1.8 Objectives

1.8.1 Objective 1

The first objective of this Ph.D. thesis was to determine whether a model SSRI could directly affect pancreatic beta cell function *in vitro*. At the time of undertaking this project, there were no studies to our knowledge addressing the effects of antidepressants on beta cell function and/or survival. We hypothesized that since beta cell dysfunction is

central to the development of T2DM (Del Prato and Marchetti, 2004) and the use of SSRI antidepressants increased the risk of new onset T2DM in adults and children, that SSRIs could directly affect beta cell function and/or survival.

1.8.2 Objective 2

In my first study I identified that SSRI exposure resulted in dysregulation of beta cell function, in part due to increased oxidative stress. Since the developing pancreas (i.e., fetal pancreas) is known to be particularly sensitive to oxidative insults (Sakuraba et al., 2002), I hypothesized that fetal exposure to an SSRI antidepressant in a rat model would result in aberrant pancreatic development.

1.8.3 Objective 3

Results from Chapter 4 demonstrated that fetal exposure to an SSRI antidepressant adversely affected pancreatic development and resulted in fetal growth restriction in a rat model. Since epidemiological studies and animal experiments have demonstrated that low birth weight and impaired pancreatic development are risk factors for the development of metabolic abnormalities (e.g., T2DM, obesity and fatty liver) in postnatal life (Johansson et al., 2008; Nobili et al., 2008; Simmons et al., 2001), Chapter 4 tested the hypothesis that fetal and neonatal exposure to fluoxetine, one of the most commonly prescribed SSRIs for perinatal depression, would result in obesity, dysglycemia and/or fatty liver in the rodent postnatal life. For these studies we chose to use fluoxetine as our

model SSRI drug because as the first SSRI introduced into the market, clinical studies of fluoxetine use during pregnancy were more abundant relative to studies on the use of other, newer SSRIs. Furthermore, information on serum fluoxetine levels in pregnant women was readily available which would allow us to compare serum values in the rats with those reported in humans.

1.8.4 Objective 4

Chapter 5 demonstrated that fetal and neonatal exposure to fluoxetine hydrochloride resulted in abnormal glucose homeostasis, decreased insulin sensitivity, increased hepatic triglycerides, increased abdominal adiposity, and a greater proportion of offspring with mild to moderate NASH (i.e., fatty liver) in our rat model. The final objective of this PhD thesis was to examine the molecular pathways underpinning the increase in fatty liver in fluoxetine-exposed animals. Given that elevated intrahepatic triglyceride content is strongly correlated with obesity and insulin resistance along with metabolic inflexibility, I hypothesized that fetal and neonatal fluoxetine exposure increased *de novo* lipogenesis in these offspring.

Chapter 2

Fluoxetine-induced pancreatic beta cell dysfunction: New insight into the benefits of folic acid in the treatment of depression

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Chapter 2.1 Introduction

All serotonin-modulating antidepressants inhibit the reuptake of MDD-associated neurotransmitters centrally. However, the development of type 2 diabetes may be an unintended consequence associated with the use of antidepressants that target serotonin signaling on peripheral tissues (Lam et al., 2007). T2DM does not develop in the absence of peripheral insulin resistance, and beta cell dysfunction. Therefore, SSRI antidepressants may influence the onset of T2DM as a direct insult on pancreatic beta cells. Notably, pancreatic beta cells are particularly susceptible to oxidative damage (Grankvist et al., 1981; Lenzen et al., 1996). Studies have shown that when intracellular ROS levels exceed the antioxidant capacity of the cell, oxidative damage and impaired

beta cell function will ensue (Pi et al., 2010). Notably SSRI exposure in vitro has been shown to increase oxidative stress via the production of reactive oxygen species (ROS) (Mun et al., 2013). Furthermore, SSRIs have also shown to result in beta dysfunction and beta cell loss. Therefore, we wanted to determine whether SSRIs directly affected beta cells and whether increasing the oxidative capacity of the cell would reverse the effects on beta cell function.

Fluoxetine-induced pancreatic beta cell dysfunction; new insight into the benefits of folic acid in the treatment of depression

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Abstract

Major depressive disorder is a common psychiatric illness with reported prevalence rates of 12 to 16% in persons aged 12 and over. Depression is also associated with a high risk of new onset type 2 diabetes (T2D). This relationship between depression and diabetes may be related to depression itself and/or drugs prescribed. Importantly, the use of selective serotonin reuptake inhibitors (SSRIs), the most commonly prescribed class of antidepressants, increases the risk of developing T2D. However, the mechanism(s) underlying this association remains elusive. Here we examine the effects of the SSRI fluoxetine (Prozac®) on beta cell function utilizing INS-1E cells, a rat beta cell line, to elucidate the underlying molecular mechanisms. Fluoxetine treatment significantly reduced glucose stimulated insulin secretion (GSIS). This decreased beta cell function was concomitant with an increased production of reactive oxygen species and oxidative damage which may contribute to decreased mitochondrial electron transport chain enzyme (ETC) activity. Importantly the fluoxetine-induced deficits in beta cell function were prevented by the addition of the antioxidant folic acid. These findings suggest that use of SSRI antidepressants may increase the risk of new-onset T2D by causing oxidative stress in pancreatic beta cells. However, folic acid supplementation in patients taking SSRIs may reduce the risk of new onset diabetes via protection of normal beta cell function.

Keywords: beta cell, antidepressant, selective serotonin reuptake inhibitor, mitochondrial dysfunction, reactive oxygen species, folic acid, mitochondrial dynamics

Introduction

Patients with major mental illnesses have an increased risk of metabolic disorders including type 2 diabetes (T2D) (Pan et al., 2012; Renn et al., 2011). Established risk factors for the increased incidence of T2D in patients with depression include: familiarity and genetic predisposition; socioeconomic status; maladaptive behaviors during a depressive episode such as comfort eating and physical inactivity and psychotropic medication use (McIntyre et al., 2010; Rotella and Mannucci, 2013). Antidepressants are a first-line option for the management of moderate to severe depression, and estimates suggest that in the USA alone, 27,000,000 persons are taking antidepressants (Keller et al., 2005; Olfson and Marcus, 2009). Antidepressant use in Canada is equally astounding; in the Canadian Community Health Survey (Cycle 1.2; 2002) 5.8% of Canadians reported taking antidepressants (Beck et al., 2005). There is now considerable evidence from animal experiments and clinical studies that antidepressant use constitutes a major risk factor for impaired glucose homeostasis and T2D (Bhattacharjee et al., 2013; Rotella and Mannucci, 2013). Although there are a wide variety of medications available for the treatment of depression (Boonstra et al., 2011; Lam et al., 2009; Philip et al., 2008), selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed therapy for adults, children and adolescents (Birmaher et al., 1998; Gelenberg, 2010; Lam et al., 2009). In humans, the use of SSRIs appears to increase the risk of developing T2D. Indeed, a retrospective cohort study identified an increased odds ratio of developing T2D in children and adolescents taking SSRIs (OR =1.37; 95% CI 1.10-1.71) (Jerrell et al., 2012). Similarly, a recent large case-control study reported that long-term use of SSRIs

(i.e., greater than 24 months) in adults, was associated with an increased risk of T2D (incidence rate ratio=2.06; 95% CI=1.20-3.52) (Andersohn et al., 2009). Although the mechanisms underlying the increased risk of T2D in patients with SSRI use have not been fully explored, a recent study has reported that SSRIs can directly impact pancreatic beta cell function (Isaac et al., 2013).

Isaac et al. (2013) demonstrated that high doses of (30 μ M) of sertraline inhibited glucose-stimulated insulin secretion (GSIS) from pancreatic islets (i.e., impaired beta cell function) and led to increased beta cell death (Isaac et al., 2013). The mitochondria play a central role in regulating beta cell function and survival (Supale et al., 2012); notably SSRI exposure has been shown to cause mitochondrial dysfunction in a variety of cell types (Abdel-Razaq et al., 2011; Agostinho et al., 2011; Han and C. S. Lee, 2009; C. S. Lee et al., 2010) although the effects of SSRIs on beta cell mitochondrial function have not been explored. Moreover, SSRIs have also demonstrated to increase the production of reactive oxygen species (ROS) (Mun et al., 2013) resulting in damage to mitochondrial as well as cytoplasmic proteins, lipids and nucleic acids (Ježek et al., 2012; Supale et al., 2012; Wallace, 2005). Collectively, oxidative stress, an imbalance between the production of ROS and the cellular antioxidant defense system, plays an essential role in the development of T2D (Drews et al., 2010). Indeed, increased ROS production may have profound effects in the endocrine pancreas because pancreatic beta cells have low levels of anti-oxidant enzymes and are therefore particularly susceptible to oxidative stress (Lenzen et al., 1996; Tiedge et al., 1997). Since SSRI exposure has been shown to

increase oxidative stress in a number of cell types, it is plausible to suggest that an antioxidant therapy might ameliorate SSRI-induced beta cell deficits. One such therapy is folic acid.

Results from epidemiological studies suggest that low folate may be a risk factor for depression (Gilbody et al., 2007). As a result there has been considerable interest in the use of folic acid as a treatment for depression, alone or as an adjunct to antidepressant use (Taylor et al., 2004). Interestingly, folate deficiency increases ROS production in RINm5F pancreatic beta cells (Hsu et al., 2013) and folic acid administration reduced oxidative stress in patients with type 2 diabetes (Lazalde-Ramos et al., 2012). Taken together, these data suggest that folic acid supplementation might prevent or ameliorate SSRI-induced deficits in pancreatic beta cell function.

Methods

Cell culture maintenance and treatment

INS-1E cells were generously provided by Dr. Claes Wollheim (University of Geneva, Geneva, Switzerland). Cells between passages 60-90 were cultured at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂ in RPMI-1640 (RPMI; Sigma Aldrich, Oakville, ON), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 1mM sodium pyruvate, 50µM β-mercaptoethanol, 1mM glutamine, 10mM HEPES, 1U/ml penicillin, and 1µg/ml streptomycin (Sigma Aldrich). Unless otherwise

noted, experimental protocols were carried out in RPMI media supplemented as described above.

Cell viability

INS-1E cells were seeded in 96-well plates at a density of 1×10^4 cells/well. Cells were allowed to attach for 24 hours. After 24 hours, media was removed and cells were treated with vehicle (control) or increasing $\frac{1}{2}$ log doses of fluoxetine hydrochloride ranging from 1×10^{-10} M to 1×10^{-2} M (Toronto Research Chemicals, North York, ON). After 48 hours, cell viability was determined using the MTS assay (Promega, Madison, WI) according to the manufacturer's instructions.

ROS production and oxidative damage

ROS have been shown to act as important signalling molecules in the pancreatic beta cell (Pi et al., 2010). However, if the level of ROS exceeds the antioxidant capacity of the cell, oxidative damage and impaired beta cell function will ensue (Pi et al., 2010). Notably, pancreatic beta cells are particularly susceptible to oxidative damage (Bhattacharjee et al., 2013; Keller et al., 2005; Lenzen et al., 1996; Olfson and Marcus, 2009; Rotella and Mannucci, 2013; Tiedge et al., 1997). To examine if fluoxetine exposure led to increased oxidative damage to pancreatic beta cells, we determined the effect of fluoxetine on ROS production by measuring hydrogen peroxide levels (H_2O_2); H_2O_2 is the most common ROS produced by the mitochondria (Pomytkin, 2012). INS-1E cells were seeded in 96-well plates at a density of 20,000 cells/well. Cells were allowed to

attach for 24 hours. After 24 hours, media was removed and cells were treated with vehicle (control) or 1 μ M fluoxetine hydrochloride (Toronto Research Chemicals). This concentration approximates the 90th percentile of human serum fluoxetine concentrations (i.e., 1.2 μ M) (Keller et al., 2005; Reis et al., 2009). After 48 hours, hydrogen peroxide production in the cell culture supernatant was determined using a commercially available kit (OxiSelect™ Hydrogen Peroxide Assay Kit, Cell Biolabs, Inc. San Diego, CA) according to the manufacturer's instructions.

We further assessed oxidative damage by determining the presence of 4-hydroxy-2-nonenal [4-HNE], a marker of oxidative damage of lipids, by Western blotting. To determine the balance between ROS production and antioxidant capacity of the cell, protein expression of resident antioxidant enzymes Cu-Zn superoxide dismutase (SOD1), Mn superoxide dismutase (SOD2), catalase and glutathione peroxidase was determined by Western blotting.

Protein expression of oxidative stress markers

INS-1E cells were seeded in 100 mm dishes until they reached 80% confluence. Cells were then washed with PBS and then incubated with 0 (control) or 1 μ M fluoxetine hydrochloride (Toronto Research Chemicals) for 48 hours (N=5 independent experiments). Following the 48 hour treatment, cells were pelleted by centrifugation (2000 rpm for 5 minutes), re-suspended in RIPA lysis buffer (15mM Tris-HCL, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 167 nMNaCl, 0.5% (w/v) sodium deoxycholate acid),

with Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche Applied Science). The cellular suspension was sonicated (Microsonix 200) at 7Hz for 15 sec. Protein content in the supernatants was determined using a BCA protein assay kit (Thermo Scientific), and samples were stored at -80°C. 20 µg of total protein was subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) blotting membrane (BioRad Laboratories, Hercules, CA). Membranes were blocked for 2 hours in 5% (wt/vol) skim milk in TBST (Tris-buffered saline [TBS], 0.1% [vol/vol] Tween 20) at room temperature on a rocking platform and then incubated with primary antibody at 4°C overnight (4-HNE mouse polyclonal, 1:5000, Abcam, Toronto, ON; SOD1, rabbit polyclonal, 1:1000, Santa Cruz, Santa Cruz, CA; SOD2, rabbit polyclonal, 1:1000, Santa Cruz; Catalase, 1:500, Abcam; glutathione peroxidase, 1:1000, Abcam). Following washing with TBST, blots were incubated with peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody (1:5000; GE Healthcare, QC, CA) for 1 hour at room temperature on a rocking platform. Blots were developed using enhanced chemiluminescence (ECL) (Millipore, Billerica, MA) and quantification was carried out using ImageLab 4.1 software (ImageLab Version 4.1 build 16; Bio-Rad, Hercules, CA). Immunoblots were subsequently incubated with stripping buffer (Thermo Scientific) and re-probed with beta-actin (1:2,000, Abcam) to control for protein loading. Blots were quantified as previously described (Bruin et al., 2008).

Mitochondria as a target of ROS

Electron Transport Chain Activity: There is now considerable evidence to suggest that altered ROS production may have profound changes on mitochondrial function (N. Li et al., 2009; M Victor et al., 2011; Newsholme et al., 2007). To determine if fluoxetine-induced oxidative beta cell damage was associated with mitochondrial dysfunction, INS-1E cells were seeded in 100 mm dishes until they reached 80% confluence. Cells were then washed with DPBS and then incubated with 0 (control) or 1 μ M fluoxetine for 48 hours. Mitochondria were isolated by differential centrifugation as previously described (Graham, 2001). Briefly, cells were harvested in 2.5mL of homogenization buffer (5 mM HEPES, pH 7.4, 100 mM KCl, 70 mM sucrose, 220 mM mannitol, 1 mM EGTA, 2 mg/mL fatty acid free BSA) plus Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Laval, QC, Canada) using a Dounce homogenizer (Wheaton, Millville, NJ). Homogenates were then centrifuged (Avanti J-301, Beckman Coulter, Fullerton, CA) at 3000 x g for 10 minutes at 4°C. The supernatant was centrifuged at 12,000 x g for 10 minutes at 4°C. The pellet was then washed in 1 mL of homogenization buffer without BSA twice, and the final pellet was re-suspended in 1 mL of homogenization buffer without BSA, flash frozen in liquid nitrogen and then stored at -80°C until enzyme activity analysis.

Both citrate synthase and complex IV (cytochrome *c* oxidase) activity assays were performed using UV-spectrophotometry (Varian Inc., Palo Alto, CA) as previously described (Parise et al., 2005). Citrate synthase activity, an indicator of total

mitochondrial mass (Figueiredo et al., 2008), was measured using the thiol reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Sigma Aldrich). Complex IV activity was assessed by measuring the rate of cytochrome *c* (Sigma Aldrich) oxidation. Data is expressed as the mean complex IV activity relative to the activity of citrate synthase.

Mitochondrial Protein Content: To determine whether there was altered expression of mitochondrial membrane proteins, COXIV and CS protein expression was determined by Western blotting as described above (COXIV; 1:10,000, MitoScience, Eugene, OR; citrate synthase (generously donated by Dr. B. Robinson, Hospital for Sick Children, Toronto, ON).

Mitochondrial Dynamics: Alterations in mitochondrial dynamics (i.e., fusion and fission events) have been shown to alter beta cell function and survival, likely as a result of changes in mitochondrial function (Stiles and Shirihai, 2012). Furthermore, increased cellular levels of ROS have been shown to affect mitochondrial dynamics at the transcriptional level (Bolisetty and Jaimes, 2013). To determine whether or not deficits in mitochondrial function in fluoxetine-treated cells are related to alterations in mitochondrial dynamics, we used real time PCR to assess the effects of fluoxetine on the transcriptional control of mitochondrial fission (Dynamin-1-like protein, Drp1; Mitochondrial fission 1 protein, Fis1) and fusion (Mitofusin-1, Mfn1; Mitofusin-2, Mfn2; Optic Atrophy1, Opa1; See Supplementary Table 1). Total RNA was extracted from approximately 1.5×10^6 cells following 48 hour treatment with 0 (control) or 1 μ M

fluoxetine (N=5 independent experiments per treatment group) using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturers instructions. First-strand cDNA synthesis was performed using random primers with 2 µg of RNA, MultiScribe™ Reverse Transcriptase (Invitrogen, Carlsbad CA) in a 20µL reaction volume. Primer sequences for Mfn1, Mfn2, Fis1, Opa1, and Drp1 have been previously published (Ding et al., 2010; J. Li et al., 2010; Won-Kyu Ju, 2008). The relative abundance of each transcript was determined by real-time quantitative PCR using SYBR green supermix and the Bio-Rad CFX384 Real Time System (Bio-Rad, ON, CA). The cycling conditions included polymerase activation (95°C for 10min), followed by 40 cycles of denaturing (95°C for 15s) and annealing/elongation (60°C for 1min). Samples were assayed in triplicate and normalized to β-actin. The cycle threshold was set where the exponential increase in amplification was equivalent between all samples. Relative fold changes were calculated using the comparative cycle times (Ct) method with β-actin as the reference gene. ΔCt values for each probe set were standardized to the experimental samples with the lowest transcript abundance (highest Ct value). The relative abundance of each primer set compared with calibrator (β-actin) was determined by the formula, $2^{\Delta\Delta Ct}$, where ΔΔCt was the standardized Ct value.

Beta cell function

To determine the effects of fluoxetine on beta cell function, we measured glucose-stimulated insulin secretion. INS-1E cells were seeded in 6-well plates at a density of 3×10^5 cells/well for 24 hours. Cells were washed twice with DPBS (Dulbecco's modified

phosphate buffered saline; HyClone) and then incubated with 0 (control), or 1 μ M fluoxetine for 48 hours (N=5-9 independent experiments per treatment condition). Following the 48 hour incubation, the cells were washed twice with 1mL of DPBS (HyClone) and then incubated for 2hours in glucose free RPMI 1640 media (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 1mM sodium pyruvate, 50 μ M β -mercaptoethanol, 1mM glutamine, 10mM HEPES, 1U/ml penicillin, and 1 μ g/ml streptomycin (Sigma Aldrich). Cells were then washed twice with Krebs Ringer bicarbonate HEPES (KRBH) buffer (135mM NaCl, 3.6mM KCl, 5mM NaHCO₃, 0.5mM H₂PO₄, 0.5mM MgCl₂, 1.5mM CaCl₂, 10mM HEPES, 2.8mM glucose, pH 7.4) supplemented with 0.1% bovine serum albumin (all reagents from Sigma Aldrich) and incubated for 1 hour. Cells were then washed twice with DPBS and incubated for 1 hour in KRBH buffer containing either 3.3 mM or 16.7 mM glucose. Following the final incubation period, media was collected, centrifuged and frozen at -20°C until analysis. Insulin content in the media was determined using a commercially available rat insulin radioimmunoassay (EMD Millipore Corporation, Billerica, MA) according to the manufacturer's instructions. Cells were lysed with 0.4M NaOH, and protein content was determined using the BCA protein assay (Thermo Scientific, Rockford, IL). Insulin release was normalized to cellular protein.

Can folic acid preserve beta cell function?

To determine if supplementation with the antioxidant folic acid could preserve beta cell

function, INS-1E cells were treated with vehicle (control) or fluoxetine (1 μ M) $\pm$ folic acid supplementation (50 nM) for 48 hours. This dose of folic acid supplementation was chosen to mimic the increase in serum folate levels in humans given a 1.0 mg/d folic acid supplement for 3 months (Wald et al., 2001). Glucose-stimulated insulin secretion was determined as described above.

Statistical analysis

All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL, USA). The results are expressed as mean $\pm$ SEM. Data were tested for outliers, normality and equal variance, and when normality or variance tests failed, data were analyzed using appropriate non-parametric tests. Glucose-stimulated insulin release was determined by comparing the amount of insulin released at 16.7 mM glucose relative to 3.3 mM glucose using Student's t-test ($\alpha=0.05$). All other outcome measures were compared between control and treatment groups by either one-way ANOVA followed by appropriate *post-hoc* tests when significance was indicated or by Student's t-test ($p<0.05$).

Results

Cell viability

Within the range of reported human serum concentrations (i.e., 68 nM to 2.0 μ M) (Keller et al., 2005; Reis et al., 2009), there was no significant effect of fluoxetine to alter cell viability (data not shown).

ROS production and oxidative damage

Fluoxetine treatment significantly increased cellular ROS (i.e., H_2O_2) production relative to control treated cells ($p < 0.05$; Figure 1). Although there was an increase in the expression of SOD1 (Figure 2a) indicative of an antioxidant response in fluoxetine-treated cells, this was insufficient to prevent oxidative stress as there was significantly more oxidative damage following fluoxetine exposure (Figure 2c-d).

Mitochondrial function

It is well accepted that increased ROS and oxidative damage can adversely affect mitochondrial ETC activity (Supale et al., 2012). Treatment of INS-1E cells with fluoxetine (1 μM) for 48 hours significantly decreased mitochondrial complex IV activity (1 μM : $p = 0.03$; Figure 3a); this reduction in enzyme activity occurred in the absence of significant change in the protein expression of either complex IV or citrate synthase (Figure 3b-d). Moreover, fluoxetine treatment did not significantly alter the steady-state levels of Drp1, Fis1, Mfn1, Mfn2, or Opa1 mRNA, all markers of mitochondrial dynamics (all $p > 0.05$; Figure 4).

Beta cell function

INS-1E cells demonstrate a robust insulin response to a glucose stimulus (GSIS); incubation in 16.7 mM glucose resulted in a significant ($p < 0.05$) increase in insulin secretion relative to basal insulin secretion (i.e., at 3.3 mM glucose). However, cells treated for 48 hours with fluoxetine did not exhibit normal GSIS; there was no significant

difference between the amount of insulin secreted under basal (i.e., 3.3mM) and high (i.e., 16.7mM) glucose concentrations ($p=0.13$; Figure 5).

Folic Acid Supplementation restores Beta Cell Function

Both vehicle-treated (control) and folic acid treated cells exhibited robust GSIS. Pre-treatment of INS-1E cells for 48 hours with 1 μ M fluoxetine resulted in the ablation of GSIS; an effect which was rescued by co-administration of folic acid ($p<0.05$; Figure 6).

Discussion

Major depressive disorder (MDD) is one of the most common psychiatric illnesses and is currently estimated to affect as many as 840 million people (Demyttenaere et al., 2004; Wang et al., 2010). Pharmacotherapy is a first-line option for the management of depression (Czaja and Valuck, 2012; E. Lee and Teschemaker, 2012), and it has been reported that approximately 11% of Americans aged 12 or older take antidepressants (Pratt et al., 2011). Selective serotonin reuptake inhibitors (SSRIs) are commonly used for the treatment of depression and, in fact, account for the majority of all antidepressant use (Beck et al., 2005). Although long-term SSRI use increases the risk of developing type 2 diabetes (T2D) (Andersohn et al., 2009; Wu et al., 2014), the mechanisms underlying this association are poorly defined.

It has been suggested that the increased risk of diabetes in patients taking SSRIs is related to drug-induced weight gain (Serretti and Mandelli, 2010) given that obesity is a

significant risk factor for the development of T2D. Although weight gain is reported as a common side effect of SSRI treatment in drug naïve patients, the changes in body weight are very drug-specific (Beyazyüz et al., 2013). For example, fluoxetine leads to weight loss, paroxetine leads to weight gain, while other SSRIs (sertraline, escitalopram and citalopram) do not alter weight gain at all (Beyazyüz et al., 2013). Collectively, these results suggest that weight gain as a result of SSRI use may not fully explain the increased risk of new onset T2D in patients taking SSRIs.

Recent evidence suggests that SSRIs may increase the risk of T2D via direct effects on the pancreatic beta cell. Beta cell dysfunction and peripheral insulin resistance are central to the onset of T2D (Kahn et al., 2008; Leahy, 2005). However, insulin resistance alone, in the absence of a beta cell dysfunction, does not lead to the development of this disease (Ahren, 2005; Chiasson and Rabasa-Lhoret, 2004; Del Prato and Marchetti, 2004; Kahn et al., 2008; Leahy, 2005). Recent work by Isaac et al. has demonstrated that acute treatment of beta cells with the SSRI sertraline caused both impaired beta cell function and increased beta cell death (Isaac et al., 2013). However, the concentration of sertraline used by Isaac et al. to induce beta cell dysfunction and apoptosis (i.e., 30 μ M) was significantly higher than the mean or median human serum sertraline levels (i.e, 99 nM and 67 nM respectively) reported in naturalistic settings (Reis et al., 2009; Unterecker et al., 2012). In the current study we have demonstrated that chronic exposure to fluoxetine at a concentration which is representative of human serum levels (Reis et al., 2009), results in impaired beta cell function (i.e., blunted GSIS) in the absence of overt

cytotoxicity. Notably, this compromised beta cell function occurs in concert with altered mitochondrial function.

In pancreatic beta cells the mitochondria play a key role in maintaining function through the coupling of a glucose stimulus to insulin release (Maechler et al., 2010). ATP production by the mitochondrial electron transport chain (ETC) increases the ATP/ADP ratio in the beta cell, which in turn causes the closure of ATP-sensitive K^+ channels ultimately resulting in insulin exocytosis (Maechler, 2013). Therefore, any damage to the ETC complexes results in inefficient ATP synthesis and uncoupling of glucose-stimulated insulin release which will ultimately cause impaired beta cell function (Maechler et al., 2010). Several studies have demonstrated that the mitochondria appear to be a target for SSRIs. Early pharmacokinetic studies in rats identified that fluoxetine and its metabolite (norfluoxetine) accumulate in brain mitochondria and synaptosomes after *in vivo* administration (Caccia et al., 1990). Subsequently Souza *et al.* reported that fluoxetine treatment was able to impair the function of isolated hepatic mitochondria *in vitro* (Souza et al., 1994). More recently Abdel-Razaq et al. have reported that in a Chinese hamster ovary cell line treated with norfluoxetine, complex I, II/II and IV activity was reduced (Abdel-Razaq et al., 2011). Notably, norfluoxetine had the greatest impact on complex IV activity (Abdel-Razaq et al., 2011). Interestingly, Li *et al.* found that sertraline, another SSRI antidepressant, resulted in a significant inhibition of the activity of complexes I and V, but not complex II, III or IV of the electron transport chain (Y. Li et al., 2012). However, to date, adverse effects of SSRI exposure on beta cell mitochondrial function

have not been reported. We have demonstrated that in a pancreatic beta cell line, fluoxetine exposure causes a significant reduction in complex IV activity; an outcome that is not due to altered enzyme expression or mitochondrial dynamics. It is noteworthy that inhibition of complex IV activity has been also demonstrated to inhibit glucose-stimulated insulin secretion from pancreatic islets (MacDonald and Fahien, 1990). Similarly in our study, the reduction in complex IV activity occurred in association with impaired GSIS. Importantly, we have also shown an increase in ROS production and oxidative damage following fluoxetine exposure.

Fluoxetine has been shown to increase markers of oxidative stress and/or damage in a variety of cell types both in vivo and in vitro (Han and C. S. Lee, 2009; Inkielewicz-Stępnik, 2011; C. S. Lee et al., 2010; Mun et al., 2013), however, there are no studies which have focused on these outcomes in pancreatic beta cells. In this study we demonstrated that fluoxetine increased cellular ROS production. This increase in ROS was associated with an increase in Cu-Zn superoxide dismutase (SOD1; an antioxidant enzyme) however this compensatory mechanism was insufficient to prevent oxidative damage. Furthermore, since ROS has been shown to impair both mitochondrial function and insulin secretion (Maechler et al., 1999), we hypothesized that co-administration of an antioxidant would preserve beta cell function. We chose to investigate the effects of folic acid since there has been considerable interest in folic acid as an adjunctive treatment for depression (reviewed in Coppen, 2005; Gilbody et al., 2007) including a recently completed randomized controlled trial (NCT00514410) to assess folate

augmentation for depression (Roberts et al., 2007). Moreover, folic acid administration has been shown to reduce arsenic-induced ROS production in pancreatic islets (Majumdar et al., 2009), and to reduce oxidative stress in patients with type 2 diabetes (Lazalde-Ramos et al., 2012).

There is considerable evidence to suggest that there is a relationship between low folate levels and an elevated risk for major depressive disorder (Gilbody et al., 2007) and/or a poorer response to antidepressant therapy (Fava and Mischoulon, 2008). As a result there have been a number of clinical trials evaluating the use folic acid or folic acid metabolites (e.g., L-methylfolate) as adjunctive therapy for major depression. A 2003 Cochrane Review of folate for depressive disorders concluded that folate augmentation may have a potential role in the treatment of depression (Taylor et al., 2003). More recently a report from the American Psychiatric Association's Task Force on Complementary and Alternative Medicine concluded that although there is not enough evidence for the efficacy of folate and methylfolate monotherapy, folate augmentation of antidepressant treatment in patients with major depressive disorder is a reasonable part of a treatment plan (Freeman et al., 2010). Importantly, results from the current study suggest that the benefits of folic acid supplementation may extend beyond treatment of depression itself. In this study fluoxetine exposure resulted in oxidative damage and impaired beta cell function. We assessed whether supplementation with folic acid, an antioxidant, was sufficient to restore the ultimate function of pancreatic beta cells, glucose stimulated insulin secretion following SSRI exposure. In fact, co-administration of a physiologically

relevant concentration of folic acid restored normal beta cell function in fluoxetine-treated cells. The identification that folic acid can protect beta cells against the adverse effects of fluoxetine treatment *in vitro* raises the tantalizing possibility that folic acid supplementation may be beneficial to prevent and/or reduce the associated risk of glucometabolic outcomes in patients taking SSRI antidepressants (Renn et al., 2011). Therefore the inclusion of endocrine and metabolic outcomes in clinical trials evaluating the use of folate in major depressive disorder warrants consideration.

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Declaration of interest

The authors declare no conflicts of interest in the work reported in the present article.

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Figure Legend

Figure 1: Effect of 1 μ M fluoxetine treatment on hydrogen peroxide (H_2O_2 ; a reactive oxygen species) production by INS-1E cells. Fluoxetine treatment (white bar) significantly increased ROS production relative to control treated cells after 48 hours ($p<0.05$). Values are expressed as the mean $\pm$ SEM; N=3 independent experiments. Values with asterisks are significantly different from control ($p<0.05$).

Figure 2: Quantification of protein expression for A. SOD1 B. SOD2 C. 4-HNE in INS-1E cells treated with Control (black bar) and 1 μ M FLU (white bar). D. Representative western blot for each protein and the loading control, β -actin. Protein expression was quantified relative to β -actin and results are expressed as the mean $\pm$ SEM; N=5 independent experiments. Values with asterisks are significantly different from control ($p<0.05$).

Figure 3: Mitochondrial activity and expression A. Cytochrome c oxidase activity (nmol/min/mg protein) relative to citrate synthase activity (marker of mitochondrial mass; nmol/min/mg protein) in INS-1E cells following a 48-hour exposure to 1 μ M fluoxetine (white bar). Data are presented as mean $\pm$ SEM (N=5 independent experiments). Values with an asterisk are significantly different from control (black bar; $p<0.05$). Quantification of protein expression for B. Cytochrome c oxidase (COX IV) and C. Citrate synthase (CS) in INS-1E cells treated with CON (black bar) and 1 μ M FLU (white bar; N=6 per group) for 48 hours. D) Representative western blot for each protein. Protein

expression was quantified relative to β -actin and results are expressed as the mean $\pm$ SEM; N=5 independent experiments. Values with asterisks are significantly different from control ($p<0.05$).

Figure 4: Relative gene expression analysis of INS-1E cells treated with CON (black bar) and 1 μ M FLU (white bar; N=6 per group) for 48 hours for key genes regulating mitochondrial fission (Dynamin-1 like protein, Drp1; Mitochondrial fission 1 protein, Fis1) and fusion (Mitofusin-1, Mfn1; Mitofusin-2, Mfn2; Optic Atrophy1, Opa1) mRNA levels were determined by quantitative RT-PCR. The relative levels of each gene were normalized to that of the levels of β -actin. Results are expressed as the mean $\pm$ SEM; N=5 independent experiments.

Figure 5: Glucose-stimulated insulin secretion in INS-1E cells following a 48-hour exposure to control (CON), 1 μ M fluoxetine (FLU). Data are presented as mean $\pm$ SEM (N=5 independent experiments). Results presented as percent control on insulin released at 3.3 mM glucose. Values with an asterisk are significantly different from basal (i.e., at 3.3 mM glucose) insulin release ($p<0.05$).

Figure 6: Glucose-stimulated insulin secretion in INS-1E cells following a 48-hour exposure to control (CON), 1 μ M fluoxetine (FLU), 50nM folic acid (FA), and 1 μ M FLU + 50nM FA. Data are presented as mean $\pm$ SEM (N=5-9 independent experiments). Results presented as percent control on insulin released at 3.3 mM glucose. Values with

an asterisk are significantly different from basal (i.e., at 3.3 mM glucose) insulin release ($p < 0.05$).

Figure 1

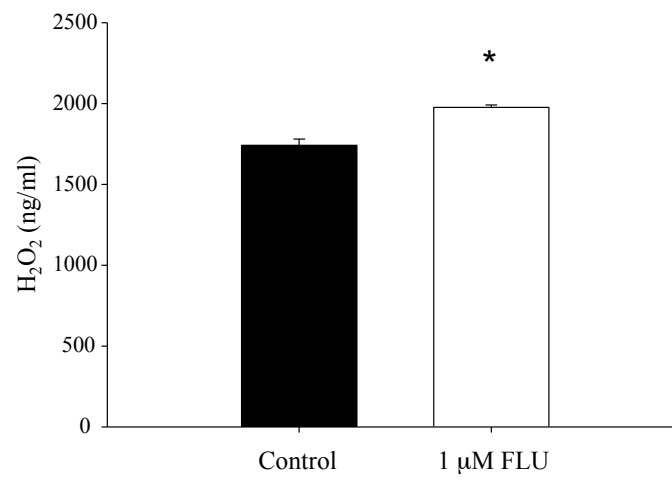


Figure 2

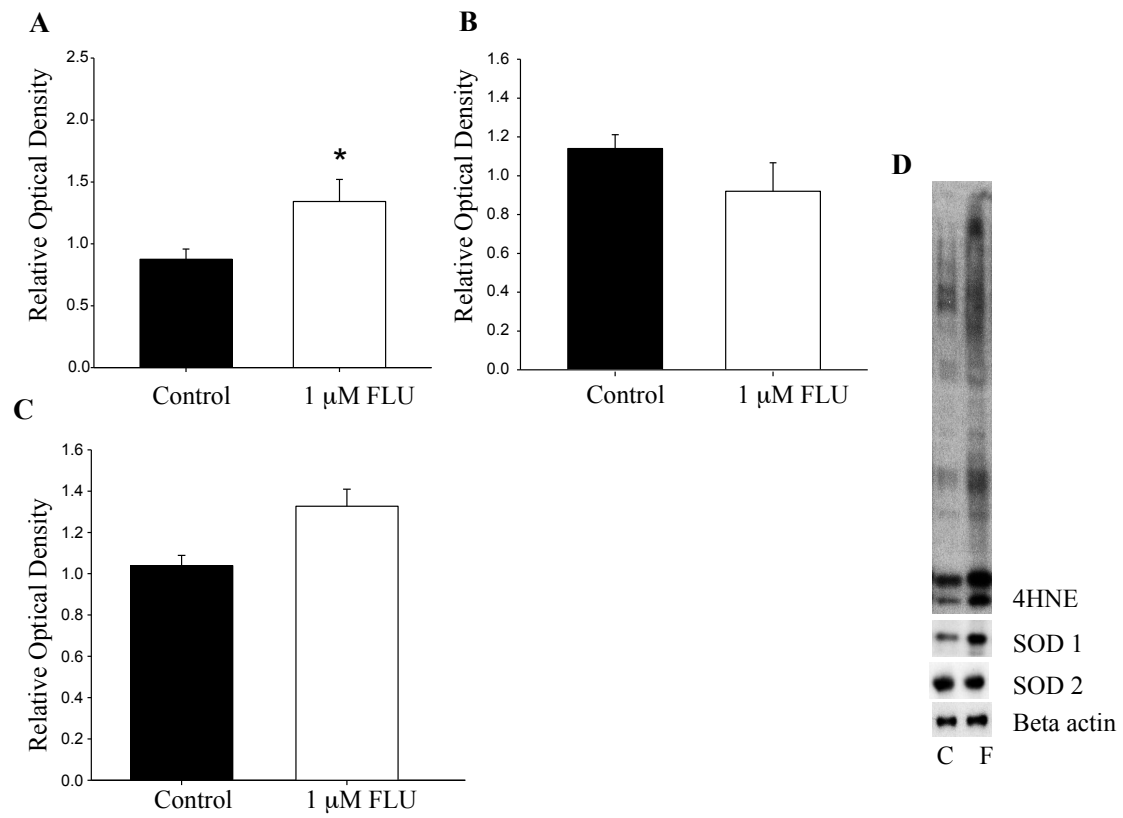


Figure 3

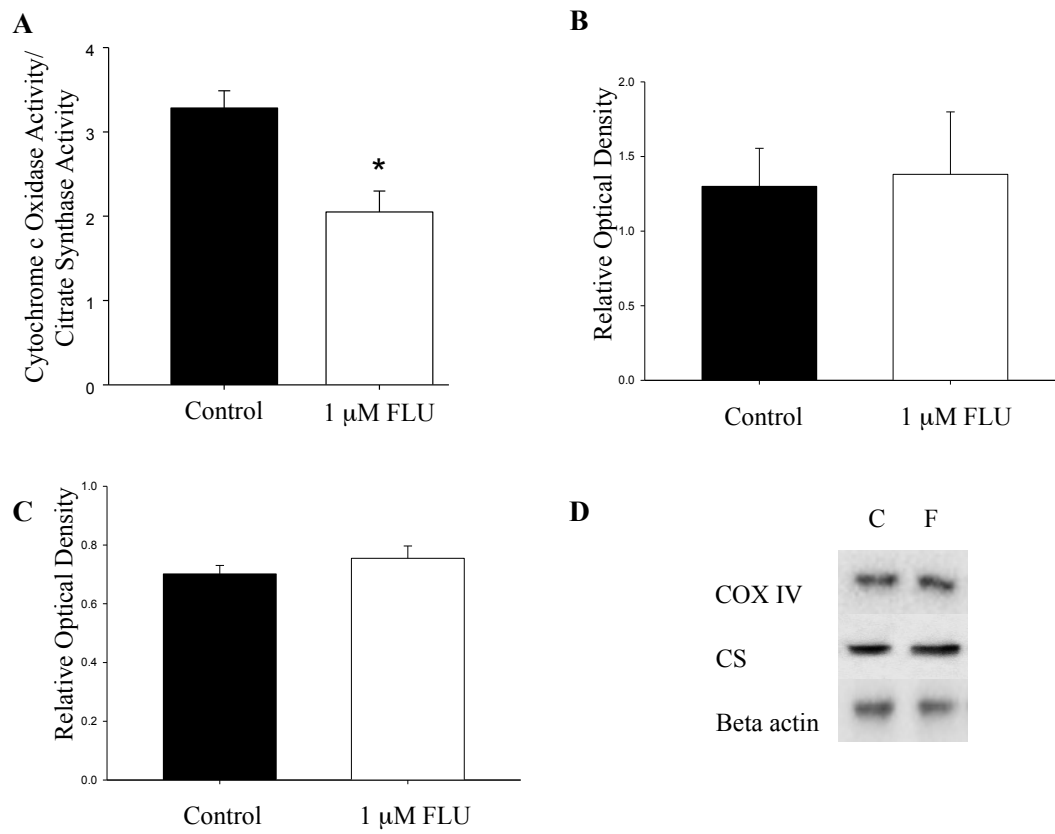


Figure 4

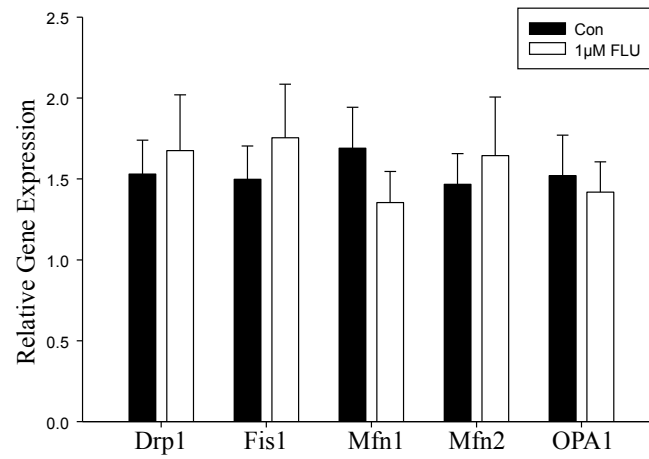


Figure 5

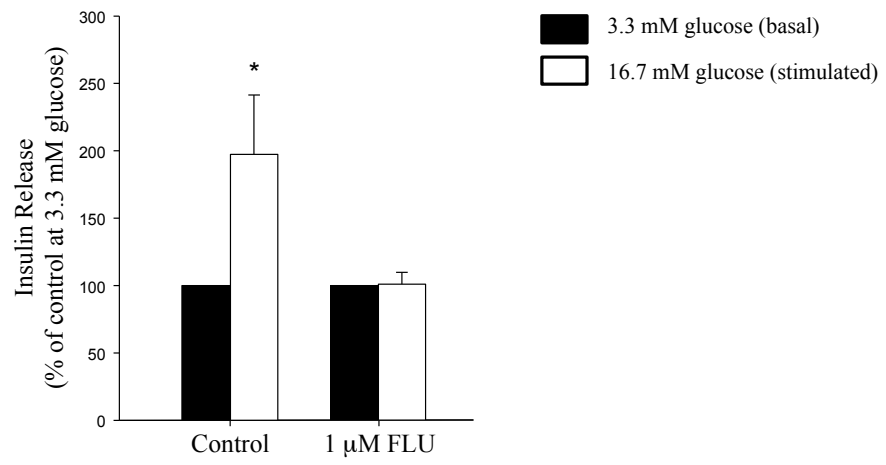
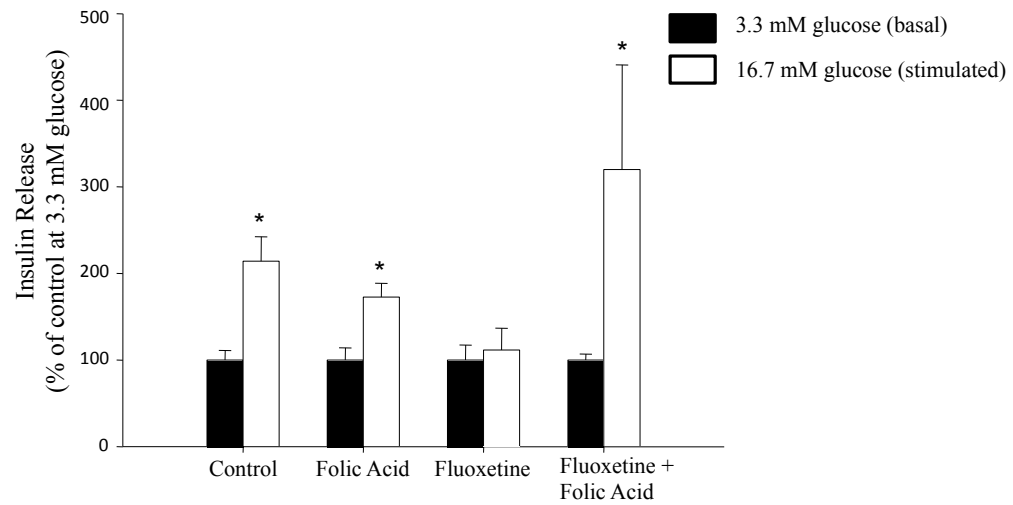


Figure 6



Supplemental Table 1

Table 1.

Primer sequences from quantitative real-time PCR.

Genes	Sense (5'-3')	Antisense (5'-3')
Mfn1	CCT TGT ACA TCG ATT CCT GGG TTC	CCT GGG CTG CAT TAT CTG GTG
Mfn2	GAT GTC ACC ACG GAG CTG GA	AGA GAC GCT CAC TCA CTT TG
Opa1	CAG CTG GCA GAA GAT CTC AAG	CAT GAG CAG GAT TTT GAC ACC
Fis1	GCA CGC AGT TTG AAT ACG CC	CTG CTC CTC TTT GCT ACC TTT GG
Drp1	AGC TGC AAG ACG TCT TCA AC	CAT TCT TCT GCT TCA ACT CC
β -actin	ACG AGG CCC AGA GCA AGA	TTG GTT ACA ATG CCG TGT TCA

Chapter 3

Fetal Exposure to Sertraline Hydrochloride Impairs Pancreatic β -Cell Development.

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Chapter 3.1 Introduction

SSRIs and their metabolites have been identified in umbilical cord blood and amniotic fluid which argues for significant placental transfer and direct in utero exposure to antidepressants during fetal development (Rampono et al., 2009; Hendrick et al., 2001). Direct effects of antidepressants on embryonic/fetal growth have also been demonstrated in vitro and in animal models (i.e., drug exposure with no underlying depression). In the rat whole embryo culture model, all of the SSRIs tested (fluoxetine, paroxetine and citalopram) caused dose-related reductions in embryonic growth. Similarly, for women taking SSRIs during pregnancy, there is an increased risk of delivering a LBW baby (RR: 95% CI: 1.44, 1.21-1.70). Taken together these data provide strong evidence that maternal use of SSRI antidepressants may have direct effects on fetal growth. LBW has been associated with an increased risk of T2DM in animal and human studies, an

association which is thought to be due in part, to altered pancreatic development (Grote et al., 2010; Chambers et al., 1996). Therefore, we sought to identify whether fetal exposure to an SSRI would cause abnormal pancreatic development.

Fetal exposure to sertraline hydrochloride impairs pancreatic beta cell development

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Abbreviated Title: *SSRI impairs rat beta cell development at birth*

Key Terms: Beta cell mass; Islet vascularization; Vascular endothelial growth factor; Antidepressants; Serotonergic E-twenty-six (ETS) transcription factor *Pet1*

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Abstract

10-15% of women take selective serotonin reuptake inhibitor (SSRI) antidepressants during pregnancy. Offspring exposed to SSRIs are more likely to be low birth weight; this is associated with an increased risk of developing diabetes in adulthood in part due to altered pancreatic development. The effects of perinatal exposure to SSRIs on pancreatic development are unknown. Therefore the objective of this study was to determine the effect of fetal exposure to sertraline hydrochloride on pregnancy outcomes and pancreatic development. Wistar rats were given vehicle (N=5) or sertraline hydrochloride (10 mg/kg/d; N=8) via daily subcutaneous injection from the confirmation of mating until parturition. Results from this animal model demonstrated that offspring born to sertraline-exposed dams have no changes in birth weight, but had a reduction in pancreatic beta cell area. The altered pancreatic islet development was a result of altered gene expression regulating islet development and survival. Therefore, fetal exposure to sertraline reduces beta cell capacity at birth, raising concerns regarding the long-term metabolic sequelae of such exposures.

Keywords: beta cell mass; islet vascularization; vascular endothelial growth factor; antidepressants; serotonergic E-twenty-six (ETS) transcription factor *Pet1*

Introduction

Depression is a wide spread psychiatric disorder which is more prevalent in women, especially during the perinatal period [1]. With reported rates as high as 20% [2], antenatal depression poses a significant risk to the health and wellbeing of both the mother and the child [3]. Consequently, approximately 6-9% of pregnant women are taking selective serotonin reuptake inhibitor (SSRIs) antidepressants during pregnancy and lactation [4, 5]. A growing body of literature suggests that antidepressant use during pregnancy is associated with an increased risk of adverse pregnancy outcomes including spontaneous abortion, preterm delivery and low birth weight [5-7].

There is now substantial epidemiological evidence to support the hypothesis that there is an association between low birth weight and the development of metabolic disorders including obesity, cardiovascular disease and type 2 diabetes in adulthood [8, 9]. In humans it has been suggested that the increased risk of type 2 diabetes in low birth weight individuals is due, in part, to abnormal pancreatic development [9]. Indeed, animal studies have demonstrated that *in utero* insults resulting in low birth weight are often associated with a significant reduction in beta cell mass and permanent deficits in pancreatic function postnatally [10-12]. However, despite the fact that in human studies there is an increased risk of low birth weight with maternal SSRI use [2, 6], to our knowledge there have been no human or animal studies that have examined the effects of maternal SSRI use on pancreatic development in the offspring. Therefore, the goal of this

study was to determine whether or not fetal exposure to an SSRI antidepressant would impact pancreatic development.

Methods

Maintenance and Treatment of Animals

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 200-250g female Wistar rats (Harlan, Indianapolis, IN) were housed under controlled lighting (12:12 light:dark) and temperature (22°C) conditions with *ad libitum* access to food and water. Dams were housed with a male until mating was confirmed by detection of sperm in a vaginal flush. Once mating was confirmed, dams were randomly assigned to receive vehicle or 10mg/kg/day sertraline hydrochloride (N=5-8 dams/treatment; Toronto Research Chemicals, Toronto, ON) via subcutaneous injection until parturition. This dose of sertraline, one of the most commonly prescribed SSRIs for perinatal depression [5], was selected to mimic the 85th percentile serum drug concentrations in pregnant women [13]. Dams were allowed to deliver normally. For each dam pregnancy outcomes were determined as previously described [14]. At postnatal day 1 (PND1), offspring were weighed and then sacrificed by decapitation. Trunk blood was collected for the determination of serum glucose concentrations using the glucose oxidase method (Pointe Scientific Inc., Canton, MI), and insulin levels using an ultra-sensitive rat insulin ELISA (Crystal Chem Inc., Downers Grove, IL). Pancreas tissue was removed and either frozen in liquid nitrogen (for gene expression analysis) or

fixed immersion in 10% (v/v) neutral buffered formalin (EMD Chemical, Gibbstown, NJ) for immunohistochemistry.

Beta Cell Area

To determine whether fetal exposure to sertraline altered beta cell development, we measured beta cell area as previously described [15]. Briefly, immunohistochemical detection of insulin was performed on 5 μ m serial sections, separated by an average of 40 μ m and immunopositive cells were identified using Image Pro Plus v 5.1 software (Media Cybernetics Inc., Silver Spring, MD, USA). The percent beta cell area was calculated as a ratio of the beta cell area (insulin positive staining only) to the total pancreas area X 100.

Markers of Pancreatic Development

In order to assess the effects of fetal exposure to sertraline on the development of the endocrine pancreas, we determined the expression of a number of transcription factors important for pancreatic formation, endocrine cell differentiation and beta cell differentiation/maturation [16]. These include; pancreatic and duodenal homeobox-1 (*Pdx1*; plays a crucial role in pancreas formation, beta cell differentiation and maintenance of mature beta cell function); neurogenin 3 (*Ngn3*) and neurogenin differentiation factor 1 (*Neurod/Beta2*) (required for pancreatic endocrine cell differentiation) and musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*; a beta cell maturation factor) [12, 17-20]. Furthermore, we determined the effect of sertraline exposure on the expression of the serotonergic E-twenty-six (ETS) transcription factor

Pet1 (gene name *Fev*) as this transcription factor has been shown to be exclusively expressed in the endocrine cells of the pancreas [21] and is also integral to the serotonin signaling pathway which is often altered with SSRI exposure [22]. Total RNA was isolated from PND1 pancreas tissue using RNeasy® mini kit (Qiagen, Hilden, Germany) and cDNA synthesized from 2µg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per manufacturer's instructions while using PerfeCta® SYBR® green FastMix® (Quanta Biosciences, Gaithersburg, MD) on the LightCycler480 (Roche, Laval, QC). The relative amount of each gene was quantitated using standard curves and normalized to the geometric mean of housekeeping genes cyclophilin A and β actin. Primer details are provided in Supplemental Table 1. To confirm whether or not changes in mRNA were also reflected in protein expression, we quantified Pdx1 and Ngn3 expression by immunohistochemistry (N=4-7 animals/group, 5 islets/animal).

Determinants of beta cell survival

To assess apoptosis and proliferation in the PND1 pancreas, we performed TUNEL (Roche Applied Science, Laval, PQ, Canada) and Ki67 immunostaining with insulin co-localization (N=5-6 animals per group, maximum 1 animal per litter, 15-20 islets/animal) as previously described [23-24]. We also determined the expression of growth factors important for beta cell proliferation and survival [vascular endothelial growth factor (*Vegf*), insulin-like growth factor1/2 (*Igf1*, *Igf2*)], and an early marker of apoptosis [i.e.,

Annexin5]. Gene expression of *Vegf*, *Igf1*, *Igf2* and *Annexin5* were determined as described above

Islet vasculature

As islets are very metabolically active tissues, extensive vascularization is necessary for proper beta cell function and expansion of beta cell mass [25]. To determine whether the decline in beta cell area was associated with impaired vascularization, blood vessels in pancreas were stained with CD31, a marker for vessel endothelial cell density, and VEGF, a regulator of islet vascularization as previously described [26]. Sections were imaged using an Olympus BX-61 fluorescent microscope utilizing Metamorph (Universal Imaging Corp., CA) image analysis software. Islet vessel area (CD31-positive vasculature divided by total islet area x 100) and the percentage of VEGF immunopositive cells per islet (VEGF-immunopositive cells divided by total islet area x 100) were determined (4 fields/section, 5 sections/animal, 5 animals/group).

Statistical Analysis

All statistical analyses were performed by Student t-test (SigmaStat, v.2.03; SPSS, Chicago, Ill) comparing the treatment group to the controls using the litter as the experimental unit. Categorical variables were analyzed using the Fisher's exact test. Data were tested for normality as well as equal variance, and non-parametric tests were used where appropriate. Values are presented as mean $\pm$ SEM. A p-value ≤ 0.05 was deemed significant.

Results

Pregnancy Outcomes

Sertraline exposure during pregnancy resulted in a significant increase in litter size without any alterations in gestation length, litter weight, number of stillbirths, pregnancy success rate, or live birth index. Initial evaluation of birth weight showed a slight, but non-significant (CON: 6.14 ± 0.2 ; SERT: 5.73 ± 0.2 ; $p=0.07$) decline in birth weight among the sertraline-exposed offspring. However, when we further characterized the proportion of pups in each treatment group that were small for gestational age (i.e., a birth weight of less than 2 standard deviations below the mean birth weight of the control pups), sertraline exposure during pregnancy resulted in a higher proportion of small for gestational age pups (CON: 0/62; SERT: 16/113; $p=0.001$).

Serum Measures and Beta Cell Mass

There was no effect of treatment on serum glucose levels (Figure 1A) however there was a significant decrease (Figure 1B; $p=0.049$) in the circulating serum insulin levels in the sertraline-exposed offspring compared to control offspring. Furthermore, fetal exposure to sertraline resulted in a significant decrease ($p<0.01$) in beta cell area compared to controls (Figure 1C-E).

Markers of Pancreatic Development

Fetal exposure to sertraline resulted in a significant decrease in the expression of *Ngn3* ($p=0.03$; Figure 2A) and its downstream target *Neurod* ($p=0.05$; Figure 2C); key

transcription factors important for the development of the endocrine pancreas. There was also a significant decrease ($p<0.01$; Figure 2E) in the expression of *Fev/Pet1* which is solely expressed in endocrine cells of the pancreas [21]. There was no significant difference in the expression of *Pdx1* or *Mafa* in the pancreas of sertraline-exposed offspring relative to controls, however Pdx1 immunostaining was significantly reduced in sertraline-exposed animals (Figure 1F). Furthermore, although it did not reach statistical significance, there was a decrease in Ngn3 immunostaining in the sertraline-exposed pups ($p=0.056$; Figure 1G).

Determinants of Beta Cell Survival

There was no effect of sertraline exposure to alter either beta cell apoptosis (i.e., *AnnexinV* gene expression or TUNEL staining) or proliferation (i.e., Ki67) (Figure 3). There was a reduction ($p=0.03$) in the expression of the islet survival factor *Vegfa* with no change in either of the insulin-like growth factors [27].

Islet Vasculature

Fetal exposure to sertraline caused a significant decrease ($p<0.05$) in pancreatic islet vascularization and islet specific expression of VEGF ($p<0.001$; Figure 4).

Discussion

Human and animal models have demonstrated that low birth weight increases the risk of developing a number of chronic diseases such as type 2 diabetes (T2D) in adulthood [8]

which may be due, in part, to abnormal pancreatic development [28]. In this study we found that fetal exposure to sertraline resulted in decreased pancreatic beta cell fraction in association with a reduction in serum insulin levels suggesting that sertraline-exposed animals have a beta cell deficit at birth.

Pancreatic organogenesis and differentiation of the endocrine pancreas requires the tightly regulated control of a number of genes. *Pdx1* is crucial for the early development of the pancreatic lineage. Genetic ablation of *Pdx1* results in the absence of pancreatic development, but in the mature pancreas its expression is restricted to differentiated beta cells [19]. *Pdx1* expression was decreased by 30% in sertraline-exposed animals, which although not statistically significant ($p=0.09$), is consistent with the reduction in the percentage of *Pdx1*⁺ islet cells and beta cell fraction (i.e., 39% reduction in insulin positive staining in sertraline exposed animals relative to controls). Furthermore, Neurogenin 3 (*Ngn3*) has been identified as the most important transcription factor specific for endocrine (i.e., pancreatic islet) differentiation and gives rise to insulin producing cells [20, 29]; pancreatic *Ngn3* gene expression was significantly lower in sertraline-exposed offspring at birth and this correlated with the reduction in *Ngn3*⁺ islet cells by immunohistochemistry. The reduction in *Ngn3*, *Fev* and *Neurod* in sertraline-exposed offspring is consistent with the fact that both *Fev* and *Neurod* have been shown to be *Ngn3* targets [17, 21]. Importantly, our observed reduction in beta cells in sertraline exposed animals is consistent with *Neurod*-knockout mice as they have a significant reduction in beta cell number at birth [30] and *Fev*^{-/-} mice which have reduced pancreatic

insulin content [21]. Taken together, our data suggest that fetal exposure to sertraline causes a reduction in the gene expression of transcription factors important for endocrine cell differentiation (*Ngn3*, *Neurod*, *Fev*). The mechanism(s) by which sertraline impacts pancreatic development are not clear, however, SSRIs have been shown to increase oxidative stress in a number of cell types including pancreatic beta cells [31,32]. Importantly, oxidative stress has been associated with a loss of Pdx1 expression and a reduction in beta cell mass [33]. However whether or not maternal exposure to SSRIs induces oxidative stress in the fetus resulting in altered pancreatic development remains to be determined. Regardless, our findings of altered expression of the transcription factors important for beta cell development following fetal sertraline exposure are consistent with our observed changes in beta cell function (i.e., decreased serum insulin) and protein expression (i.e., significant reduction in the fraction of insulin positive beta cells).

Beta cell number in the neonate depends not only on the coordinated expression of transcription factors important for pancreatic development, but also on the postnatal regulation of beta cell proliferation and apoptosis. In the neonatal pancreas insulin like growth factors (IGFs) and vascular endothelial growth factor (VEGF) are important beta cell survival factors; in the neonate IGFs inhibit beta cell apoptosis [34] and VEGF plays a major role in beta cell proliferation [35]. Moreover, IGF and VEGF expression have been shown to be altered by exposure to SSRI antidepressants [36, 37]. In our study we did not observe an increase in pancreatic apoptosis, beta cell specific apoptosis or a

reduction in the expression of the anti-apoptotic growth factors IGF1 and IGF2. However, sertraline exposure did result in a significant reduction in *Vegf* mRNA expression. VEGF is the principle regulator of islet angiogenesis and vascularization; the loss of VEGF results in abnormal vasculature and impaired insulin secretion into systemic circulation [38]. Similarly, we have demonstrated that the loss of *Vegf* expression in the neonatal pancreas of sertraline-exposed animals is associated with deficits in islet vascularization and reduced circulating insulin levels. The mechanism(s) by which fetal exposure to an SSRI antidepressant can affect VEGF expression and islet vascularization are as yet unknown, but it may represent a generalized dysregulation of VEGF in response to fetal exposure to SSRIs.

In conclusion, our study is the first to report that fetal exposure to an SSRI antidepressant, sertraline, may result in aberrant pancreatic development; effects which appear to be mediated via a reduction in transcription factors important for endocrine cell differentiation and decreased expression of the growth factor VEGF. However, whether or not these deficits in pancreatic development persist beyond birth is not currently known. Regardless, results from this study clearly suggest that further studies examining the effects of perinatal exposure to SSRIs on pancreatic development and postnatal pancreatic function are warranted.

Declaration of Interest

NED, MKG, JJP, ACH: There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

Authors Contributions

NED, ACH designed the study. NED, MKG, JJP and ACH performed the experiments.

NED, JJP and ACH performed the statistical analysis. NED wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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Figure Legend

Figure 1. Serum concentrations of A. Glucose (mg/dL) and B. Insulin (ng/mL) at postnatal day 1 in control (N=5) and sertraline-exposed (N=8) offspring. Pancreatic beta cell fraction at birth. Representative immunohistochemical images of C) Control and D) Sertraline exposed animals. E) Quantification of 5 sections per animal; 5-7 animals per group randomly selected to include only 1 animal per litter. Pancreatic and duodenal homeobox 1 (PDX-1) immunostaining at birth. Values are expressed as the mean $\pm$ SEM. Values with asterisks are significantly different from control ($p \leq 0.05$).

Figure 2. Relative gene expression analysis of key genes regulating pancreatic formation, endocrine cell differentiation and beta cell differentiation/maturation [pancreatic and duodenal homeobox-1 (PDX-1), neurogenin 3 (Ngn3), neurogenin differentiation factor 1 (NeuroD1/BETA2) and musculoaponeurotic fibrosarcoma oncogene homolog A (MafA)] in control (N=5) and sertraline-exposed (N=8) PND1 pancreas tissue. F) Quantification of the percent Pdx1⁺ islet cells was calculated as a ratio of the immunopositive (Pdx1⁺ staining only) to the total islet area X 100 (N= 4-7 animals per group randomly selected to include only 1 animal per litter). Immunopositive cells were identified using Image Pro Plus v 5.1 software (Media Cybernetics Inc.). G) Quantification of the percent Ngn3⁺ islet cells was calculated as a ratio of the immunopositive (Ngn3⁺ staining only) to the total islet area X 100 (N= 4-7 animals per group randomly selected to include only 1 animal per litter). Values are expressed as the mean $\pm$ SEM. Values with asterisks are significantly different from control ($p \leq 0.05$).

Figure 3. Pancreatic beta cell A. Apoptosis; 15-20 islets/animal were imaged on a Nikon Eclipse 90i microscope (Nikon, Melville, NY) and quantified using NIS software (Nikon) for apoptosis and reported as the percentage of TUNEL+ beta-cells and B. Proliferation; N=5-7 animals per group, maximum 1 animal per litter). Images were captured (Nikon) and analyzed with NIS software; 15-20 islets per section were quantified and reported as the percentage of Ki67⁺ beta cells. Relative gene expression of C) Annexin V; D) IGF-1, E) IGF2 and F) VEGF in pancreas tissue from control (N=5) and sertraline-exposed (N=8) offspring at birth. Values are expressed as the mean $\pm$ SEM). Values with asterisks are significantly different from control ($p \leq 0.05$).

Figure 4. A. Quantification of pancreatic islet vessel area (the total area occupied by microvessels per optical field). Representative immunohistochemical (CD31+) images of B. Control and C. Sertraline-exposed offspring. D. Percent vascular endothelial growth factor (VEGF) islet staining (% VEGF immunopositive cells per islet in six islets per section) compared to control (black bar; N=5). Representative immunohistochemical (VEGF+) images of E. Control and F. Sertraline-exposed offspring. Values are expressed as the mean $\pm$ SEM). Values with asterisks are significantly different from control ($p \leq 0.05$).

Supplemental Table 1: RT-PCR primers

Figure 1

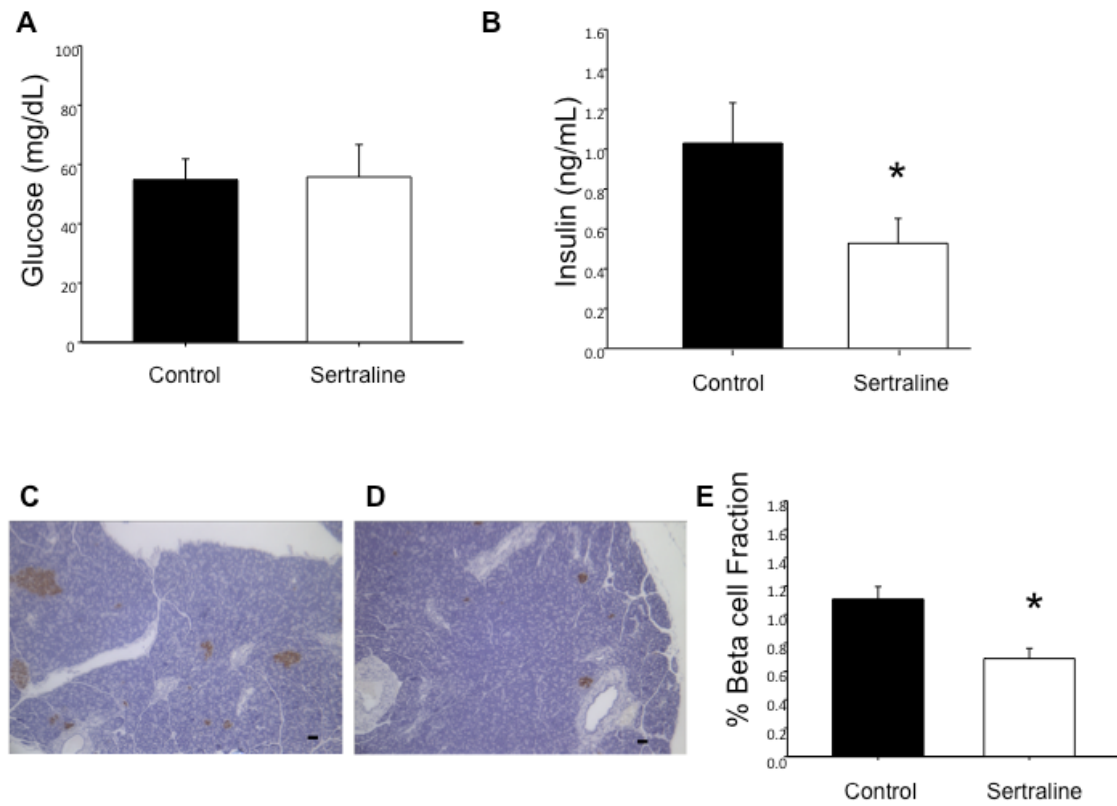


Figure 2

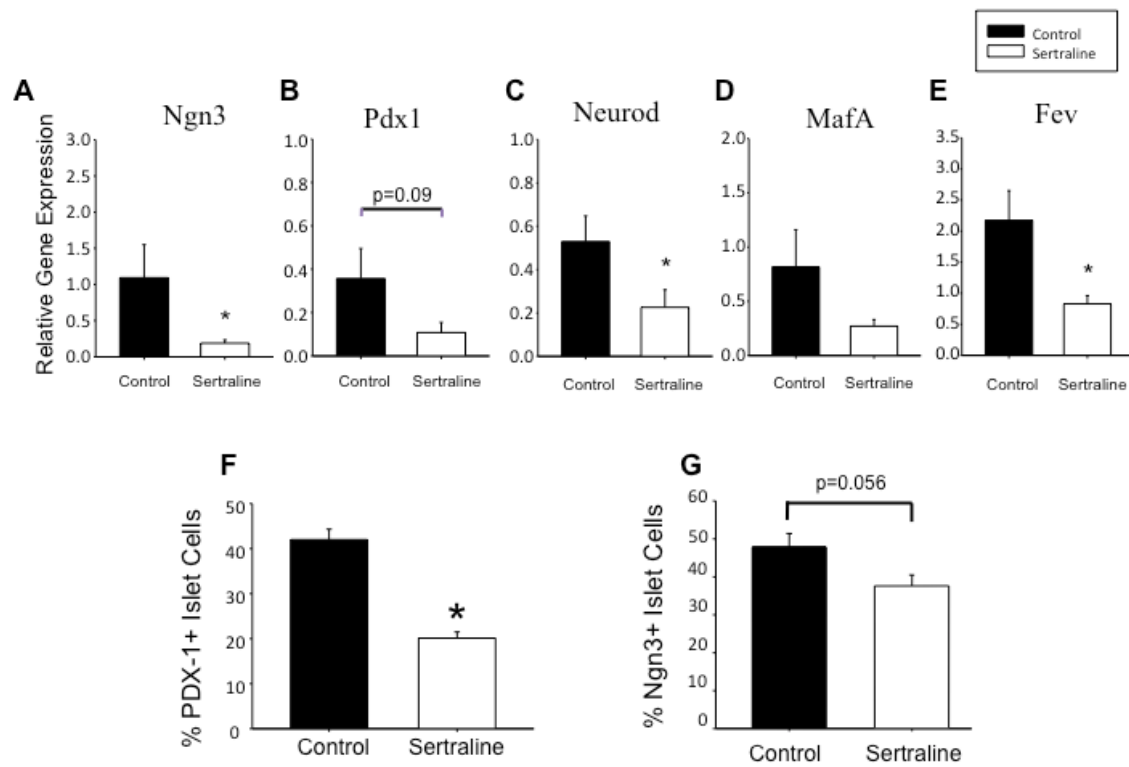


Figure 3

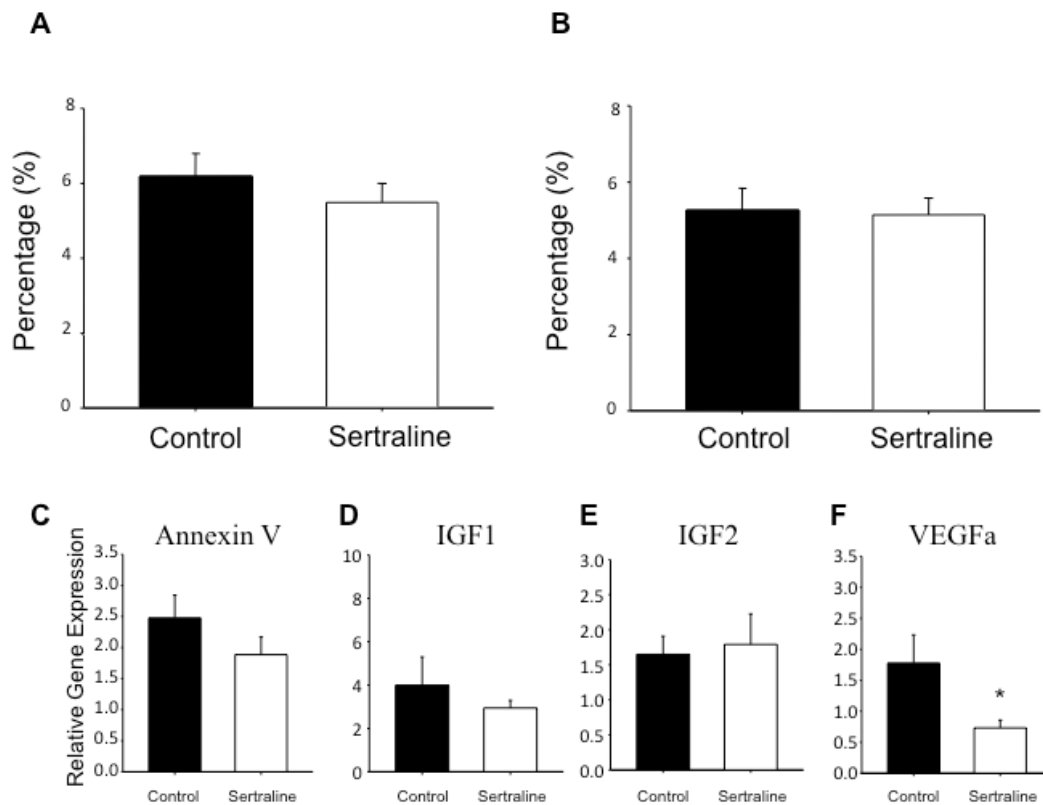
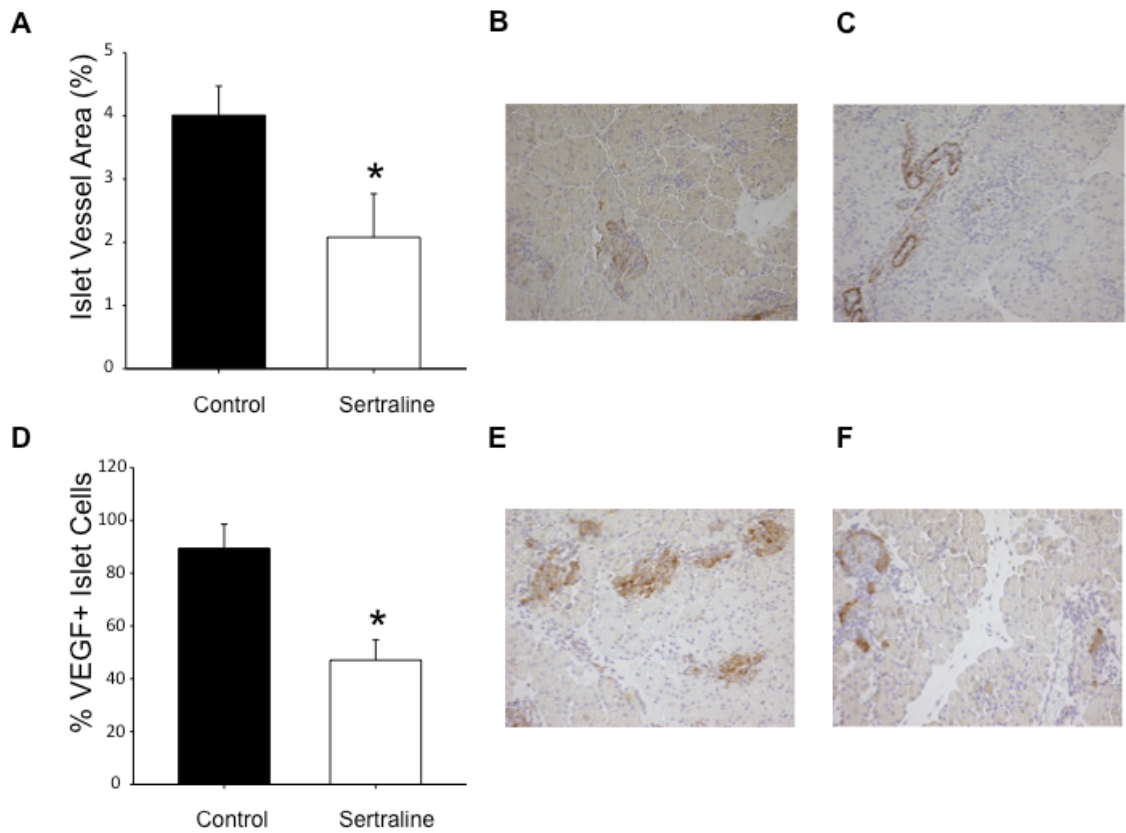


Figure 4



Supplemental Table 1

Supplemental Table 1. RT-qPCR Primer sequences

Gene	Forward	Reverse
Ngn3	5'-CATAGCGGACCACAGCTTCT	5'-CAGGCTACCAGCTTGGGAAA
PDX1	5'-GCTTGTAGCTCCCTACCCC	5'-GCGTAGTACTGCTCCTCACT
NeuroD	5'-GACAGCTCCCAITGTGTTCCA	5'-TGGTGCAATCAGTTAGGGGG
MafA	5'-AGCTGGTGTCATGTCAGTG	5'-CGTATTTCCTTGTACAGG
Pet-1	5'-CCCTGCTGATCAACATGTACC	5'-GCCAGCAGCTCCAGTAGAAA
AnnexinV	5'-CAGGGACCTTGTGAATGACATG	5'-TCAAAGCCACAATTAACCTTCTCAAA
IGF1	5'-TTGGCCTCCTCGAAAGCAAT	5'-AGGGGCGTTAAACTTGGGT
IGF2	5'-CCAGGTGACAGGACTGGCAC	5'-CCTGAAAACACCCATCCCAC
VEGFα	5'-TCTCCAGATCGGTGACAGT	5'-AAGGAATGTGTGGTGGGGAC

Chapter 4

Antenatal exposure to the selective serotonin reuptake inhibitor fluoxetine leads to postnatal metabolic and endocrine changes associated with type 2 diabetes in Wistar rats.

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Chapter 4.1 Introduction

We have demonstrated that fetal exposure to an SSRI antidepressant, sertraline, resulted in aberrant pancreatic development; effects which appear to be mediated via a reduction in transcription factors important for endocrine cell differentiation and decreased expression of the growth factor vascular endothelial growth factor (VEGF). This study was the first to show that exposure to SSRI antidepressants during pregnancy alone alters pancreatic development in the offspring. Importantly early loss of beta cell mass in animal models has been shown to be associated with the development of T2DM into adulthood (Cox et al., 2010). However, the long-term metabolic consequences of fetal and

neonatal exposure to SSRIs, as occurs in many pregnancies, are unknown. The goal of this study was to determine the effects of in utero and lactational exposure to an SSRI, on postnatal metabolic outcomes in the offspring.

Antenatal Exposure to the Selective Serotonin Reuptake Inhibitor Fluoxetine leads to Postnatal Metabolic and Endocrine Changes Associated with Type 2 Diabetes in Wistar Rats.

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Abstract

Hypothesis: 10-15% of women take antidepressant medications during pregnancy. A recent clinical study reported that the use of selective serotonin reuptake inhibitor antidepressants during pregnancy is linked with an increased risk of postnatal obesity. While obesity is often associated with fatty liver, dyslipidemia and inflammation, to date, the effects of perinatal exposure to SSRIs on these outcomes are unknown.

Methods: Female nulliparous Wistar rats were given vehicle (N=15) or fluoxetine hydrochloride (FLX 10 mg/kg/d; N=15) orally for 2 weeks prior to mating until weaning. We assessed glucometabolic changes and hepatic pathophysiology in the offspring.

Results: Fluoxetine exposed offspring demonstrated altered glucose homeostasis without any alterations to beta cell mass. FLX-exposed offspring had a significant increase in the number of offspring with mild to moderate NASH and dyslipidemia. There was also increased inflammation of the liver in FLX-exposed offspring; males had significant elevations in TNF α , IL6 and monocyte chemoattractant protein 1 (MCP1), while female offspring had higher expression of TNF α , and increased macrophage infiltration (MCP1).

Limitations: This is an animal study. Further research examining the metabolic outcomes of children exposed to antidepressants in utero are required, given the increase in childhood obesity and psychiatric medication use during pregnancy

Conclusion: These data demonstrate that fetal and neonatal exposure to FLX results in evidence of increased adiposity, fatty liver and abnormal glycemic control. Since these are all hallmarks of the metabolic syndrome, this raises concerns regarding the long term metabolic sequelae of fetal exposure to SSRIs in human populations.

Key Words: beta cell; inflammation; depression; adiposity; nonalcoholic steatohepatitis; dyslipidemia

Introduction

Women are twice as likely as men to suffer from depression, with a peak in vulnerability during childbearing years (Bebbington et al., 2003; Burt and Stein, 2002; Lokuge et al., 2011). This occurs because pregnancy appears to be a high risk period both for the exacerbation of existing depression and/or the onset of new maternal depressive symptoms (Marcus, 2009). The prevalence of antenatal and postnatal depression in North American women has been reported to be 10-15% (Hobfoll et al., 1995; Koleva et al., 2010).¹ Given the well documented maternal and fetal risks associated with untreated

¹ **Abbreviations:**

Carbon Dioxide	CO ₂
Cholesterol 7 alpha-hydroxylase	CYP 7A1
Cluster of Differentiation 68	CD68
Complementary DNA	cDNA
Day	D
Fluoxetine	FLX
Light	L
Monocyte Chemotactic Protein	MCP1
Nonalcoholic Steatohepatitis	NASH
Postnatal Day	PND
Selective Serotonin Reuptake Inhibitor	SSRI
Serotonin Transporter	SERT

depression during pregnancy for both the mother and the child (Association, 2006; Bebbington et al., 2003; Burt and Stein, 2002; Cohen et al., 2010; Leung, 2009), drug therapy is often recommended for moderate to severe depression (Association, 2006; Cohen et al., 2010). However, to date, the long-term effects of fetal exposure to these drugs are largely unknown.

Although a wide variety of medications are used to treat perinatal depression, selective serotonin reuptake inhibitors (SSRIs) are usually recommended as first-line therapy during pregnancy and lactation (Lam et al., 2009; National Collaborating Centre for Mental Health (UK), 2007). Recent estimates suggest that approximately 6% of pregnant women in the United States are taking SSRIs (Andrade et al., 2008; Cooper et al., 2007). This is relevant given that SSRIs and their metabolites have been identified in umbilical cord blood and amniotic fluid, indicating exposure of the fetus to these drugs during development [reviewed in (Ray and Stowe, 2014)]. As a consequence, the effects of SSRI use on the risk of congenital malformations, adverse neonatal outcomes (e.g., NICU admissions, respiratory distress and pulmonary hypertension), postnatal neurobehavioral development and adverse pregnancy outcomes (e.g., low birth weight, miscarriage and preterm birth) are well established [reviewed in (Andersen et al., 2014; Ray and Stowe, 2014)]. However, there is very limited information regarding metabolic outcomes in

Small for Gestational Age

SGA

Type 2 Diabetes

T2D

SSRI-exposed offspring despite considerable evidence showing an association between SSRI use in adults and metabolic disturbances.

The use of SSRIs has also been reported to increase the risk of developing type 2 diabetes (T2D) (incidence rate ratio=2.06; 95% CI=1.20-3.52) (Andersohn et al., 2009) and its comorbidities (i.e., obesity and metabolic dyslipidemia). SSRI use is also associated with an increased risk of abdominal obesity (OR=1.40, 95% CI=1.08-1.81) (Raeder et al., 2006) hypercholesterolemia and hypertriglyceridemia (Beyazyüz et al., 2013; Raeder et al., 2006). Despite these well documented associations, however, there is very little information available regarding the long-term metabolic consequences of fetal exposure to SSRI antidepressants. Therefore, the goal of this study was to determine, using a rat model, the effects of fetal and neonatal exposure to fluoxetine (FLX), one of the most commonly prescribed SSRIs for perinatal depression (Andrade et al., 2008; Morrison et al., 2005), on postnatal endocrine and metabolic changes associated with T2D.

Materials and Methods

Maintenance and treatment of animals

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 200-250g female 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 vehicle (N=15) or 10mg/kg/day fluoxetine hydrochloride (FLX; N=15; Toronto Research Chemicals, Toronto, ON) orally in flavored gelatin cubes from 14 days prior to mating until weaning (postnatal day 21; PND21). This method of drug administration has been shown to be a reliable method for drug administration to rats (Corbett et al., 2012; Flecknell et al., 1999). The dose of fluoxetine was chosen based on previous studies in Sprague Dawley rats (Capello et al., 2011; Y. Hui et al., 2007; Pawluski et al., 2014) 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 (Capello et al., 2011; Hendrick et al., 2001; Olivier et al., 2011; Reis et al., 2009; Sit et al., 2011). Maternal body weight and food consumption were monitored daily for the duration of the study. Two weeks after the initiation of treatment, dams were housed (1:1) with an age-matched male and monitored daily for confirmation of breeding (i.e., presence of sperm in the vaginal swab). The day that a positive sign of copulation was observed was designated gestational day 0 (GD0). Dams were allowed to deliver normally. At birth (postnatal day 1; PND1), pups were weighed and sexed and litters were culled to 8, preferentially selecting 4 male and 4 female offspring, to ensure uniformity of litter size between treated and control litters. For each dam, time to mating, gestation length, pregnancy weight gain, maternal food consumption, litter size, sex, birth weight and the number of stillbirths were recorded. From these data the mating index ($[\# \text{ of females mated} / \# \text{ of females cohabited}] * 100$), pregnancy success rate ($[\# \text{ of dams delivering a litter} / \# \text{ dams with a confirmed}$

mating]*100), dams with still births (*# of dams with a stillborn/# of dams delivered*), the live birth index (*[# of live offspring/# of offspring delivered]*100*), the sex ratio (*# of male offspring/# of female offspring*), and proportion of pups which were small for gestational age (SGA; (i.e., a birth weight of less than 2 standard deviations below the mean birth weight of the control pups) were calculated. At weaning (PND21), two male and two female pups per litter were euthanized by CO₂ asphyxiation. Pancreas, liver and fat pads (mesenteric, perirenal, and gonadal) were excised and weighed, and pancreas and liver tissue was either snap frozen in liquid nitrogen or fixed in 10% neutral buffered formalin (EMD Chemicals Inc., Gibbstown, NJ) for subsequent analysis. After weaning, the remaining offspring were caged in same-sex sibling pairs.

Postnatal weight gain, adiposity and serum lipid levels

Pups were weighed weekly from PND1 until 26 weeks of age to assess postnatal growth. At 26 weeks of age, one male and one female offspring from each litter were euthanized by CO₂ asphyxiation for blood and tissue collection. Fat pads (perirenal, mesenteric and gonadal) were excised and weighed. The percentage of visceral fat (mesenteric fat pad weight/body weight x 100) was calculated for each animal. Serum triglyceride and cholesterol levels were measured using commercially available kits (Pointe Scientific, Canton, MI).

Glycemic control

To determine whether fetal and neonatal exposure to fluoxetine altered postnatal glycemic control, animals were given a glucose tolerance test at 8, 16 and 24 weeks of age. Briefly, glucose was measured by glucometer (NovaMax, Nova Diabetes Care Inc., Billerica, MA) in saphenous vein samples (N=8-9 per group) following an intra-peritoneal injection of 2 g/kg glucose (Sigma-Aldrich, St. Louis, MO) as described previously (De Long et al., 2013). To control for litter effects, only one animal per sex, per litter was used for these experiments. At 24 weeks of age, serum was collected throughout the ipGTT for the determination of the insulin response to the GTT. Serum insulin levels were measured using an ultra-sensitive rat insulin ELISA (Crystal Chem Inc., Downers Grove, IL). The total glucose and insulin response to the ipGTT (area under the curve from 0-120 minutes; AUC) was calculated using the trapezoidal rule.

Beta cell mass

It is widely recognized that beta cell deficits, including a reduction in the number of beta cells, can play a key role in the pathogenesis of impaired glycemic control [reviewed in (Meier and Bonadonna, 2013)]. Therefore, we examined the effect of fetal and neonatal exposure to fluoxetine on postnatal beta cell mass at PND21 (the time at which maximal exposure to the drug has occurred) and 6 months of age. At necropsy, the pancreas was excised, weighed, fixed in 10% neutral buffered formalin (EM Science, Gibbstown, NJ, USA) and embedded in paraffin. Immunohistochemical detection of insulin was performed on 5 μ m serial sections, separated by an average of 30 μ m, for all groups (5

sections per animal; 5 animals per sex per group; maximum of one animal per litter) as previously described by our laboratory (De Long et al., 2013). Briefly, tissue sections were deparaffinized, rehydrated and endogenous peroxidase activity was quenched in methanol. Antigen retrieval in 10mmol/l citrate buffer (pH 3.0) heated to 37°C for 30 minutes followed by blocking with 10% normal goat serum and 1% BSA prior to incubation with the primary antibody, a polyclonal, guinea pig anti-swine insulin antibody (1:150 dilution) (DakoCytomation, Carpinteria, CA, USA) overnight at 4°C. Sections were then washed and immunostaining was identified using the Vectastain kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzadine as the chromogen. Tissue sections were counterstained, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). All measurements were performed at 10x magnification and the whole pancreas was analyzed by a single investigator blinded to treatment. Immunopositive cells were identified and the beta-cell area was calculated as a percentage of the total area of the section using integrated morphometry software (Image-Pro Plus, Media Cybernetics, Rockville, MD). Beta cell mass was determined by quantifying the beta cell area as a percentage of the total pancreatic area and then multiplying the total pancreatic weight by this percentage. The percent beta cell area was calculated as a ratio of the beta cell area (immunopositive staining only) to the total pancreas area (immunopositive staining and pancreas counterstaining) x 100. The beta cell mass was calculated as the product of the percent beta cell area and the corresponding total pancreas weight in milligrams.

Liver pathology, hepatic lipids and inflammation

Increased visceral adipose tissue mass in humans is strongly correlated with increased lipid deposition in liver (Kelley et al., 2003), which in turns results in abnormal glycemic control. Therefore to determine whether fluoxetine-exposed offspring had increased hepatic lipid accumulation, we assessed liver histology, hepatic lipid levels and the hepatic inflammatory profile.

Liver pathology: At 26 weeks of age livers were excised, weighed, fixed in 10% neutral buffered formalin (EM Science) and embedded in paraffin. Sections (5µm) were stained with hematoxylin and eosin using standard protocols (N=9-12 animals per sex per group, maximum of one animal per litter) and blindly scored by a single investigator (C.P.) for the presence and degree of histological criteria that contributed to a NASH grade according to Brunt et al. (Brunt et al., 1999). These criteria were comprised of macrovesicular lipid, portal inflammation, lobular inflammation, hepatocellular ballooning, mallory's hyaline, acidophil bodies, glycogenated nuclei, lipogranulomas, and fibrosis, and a grade of mild, moderate or severe NASH was derived.

Hepatic lipids: At 26 weeks of age, livers were excised and snap frozen in liquid nitrogen and then stored at -80°C until analysis. Samples were sent to the Metabolic Phenotype Laboratory at Robart's Research Institute (London, Ontario) for analysis of hepatic total cholesterol and triglyceride content. This was assessed by a Cobas® Mira S Analyzer (Roche Diagnostics, Laval, Quebec).

Inflammation: To quantify hepatic inflammation, we examined the steady-state mRNA expression of *TNF α* , *IL-6* (markers of a proinflammatory state), monocyte chemoattractant protein (*MCP-1*) and cluster of differentiation 68 (*CD68*) (markers of macrophage infiltration) by qPCR. Total RNA was isolated from 26 week liver tissue using TRIzol® (Life Technologies, Grand Island, NY) extraction combined with the RNeasy® mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from 2µg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions using PerfeCta® SYBR® green FastMix® (Quanta Biosciences, Gaithersburg, MD) on the LightCycler480 (Roche). The relative amount of each gene was quantified using standard curves and normalized to the geometric mean of four housekeeping genes hypoxanthine-guanine phosphoribosyltransferase (HPRT), Cyclophilin A (CYC), beta 2 microglobulin (B2M), and 18S ribosomal RNA (18S). Primer sequences are supplied in Supplemental Table 1.

Statistical analysis

All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL). The results are expressed as mean $\pm$ SEM. Categorical variables (mating index, pregnancy success rate, stillbirths, and number of SGA pups) were compared using Fisher's exact test. For all outcomes at birth (litter size, live birth index, sex ratio, and birth weight) and prior to weaning (i.e., survival to PND4 and survival to weaning) the statistical unit was

the litter. The proportion of animals exhibiting any evidence of NASH (mild, moderate or severe) was compared between control and fluoxetine exposed offspring using Fisher's exact test. All postnatal outcome measures results for male and female offspring were analyzed separately; outcome measures between control and fluoxetine-treated offspring were analyzed using Student's t-test ($\alpha = 0.05$). Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using the Mann-Whitney rank sum test.

Results

Pregnancy and birth outcomes:

SSRI administration in rodents has been associated with altered food consumption (Lauzurica et al., 2013), however, in this study there was no change in maternal food consumption throughout pregnancy and lactation (all $p > 0.05$; data not shown). There was no effect of fluoxetine exposure on time to mating, mating index, pregnancy success rate, gestation length, pregnancy weight gain, litter size, live birth index, sex ratio, small for gestational age pups, birth weight, survival to PND4 or survival to weaning (Supplemental Table 2).

Postnatal weight gain and adiposity at 26 weeks of age

Fetal and neonatal exposure to fluoxetine did not affect postnatal weight gain in male offspring, however, female offspring born to exposed dams were significantly heavier than controls by 26 weeks of age ($p < 0.05$; See Figure 1). Both male and female

fluoxetine-exposed offspring animals had a significant increase in visceral adiposity; this increase in adiposity occurred without any alterations in postnatal food consumption (data not shown). In addition, fluoxetine exposure did not significantly alter serum cholesterol or triglyceride levels in either male or female offspring (all $P > 0.05$; data not shown).

Glycemic control

At 8 and 16 weeks of age, there was no effect of fetal and neonatal exposure to fluoxetine on the total response to a glucose challenge (i.e., AUC_{glucose}) in either sex. Similarly, there were no significant differences in fasting glucose, peak glucose (i.e., glucose at 15 minutes post-administration), the ability to clear the glucose load (i.e., glucose levels at 120 minutes) (Supplemental Figure 3). At 24 weeks of age, male offspring born to fluoxetine-exposed dams did not exhibit significant alterations in fasting glucose (CON: 6.68 ± 0.23 mmol/L, FLX 6.78 ± 0.28 mmol/L, $P > 0.05$), fasting insulin (CON: 403.89 ± 47.64 pmol/L, FLX: 405.25 ± 32.92 , $P > 0.05$) or the total response to the ipGTT (Figure 2A). However, although fluoxetine-exposed male offspring exhibited a normal response to the glucose challenge, they required a significantly higher total insulin response (i.e., AUC_{insulin}) to normalize blood glucose levels ($p < 0.05$; Figure 2B). Conversely in female offspring, fetal and neonatal exposure to fluoxetine resulted in an abnormal response to the glucose challenge at 24 weeks of age (Figure 2C); they had a higher total glucose response (area under the curve; AUC_{glucose} ; Figure 2C) to the glucose load relative to the vehicle controls ($p < 0.05$). Moreover, female offspring born to fluoxetine-exposed dams had a higher total insulin response (AUC_{insulin}) to the glucose challenge relative to the

control offspring ($p < 0.05$; Figure 2D). There were no significant differences in either fasting glucose (CON: 6.05 ± 0.15 mmol/L, FLX 5.75 ± 0.18 mmol/L, $P > 0.05$) or fasting insulin (CON: 116.01 ± 18.59 pmol/L, FLX: 100.60 ± 16.40 , $P > 0.05$) levels in female offspring that were exposed to fluoxetine.

Beta cell mass

There was no significant effect of fluoxetine exposure on beta cell mass in either male or female offspring at any age examined (Figure 3).

Liver pathology, hepatic lipids and inflammation

Fetal and neonatal exposure to fluoxetine resulted in a significant increase in the proportion of offspring with steatohepatitis compared to vehicle-exposed offspring (Figure 4). Moreover, the proportion of offspring exhibiting microvesicular lipid accumulation was significantly higher in fluoxetine-exposed offspring when compared to controls (Figure 4E, 50% vs 17%, $p = 0.04$). Because there was evidence by histology of lipid accumulation in hepatocytes, we determined hepatic cholesterol and triglyceride levels.

Fluoxetine exposure significantly increased hepatic triglycerides in both male and female offspring relative to controls (Figure 5). Hepatic cholesterol levels were significantly increased in female offspring ($p = 0.014$) but not in male offspring ($p = 0.057$). This increase in liver damage and hepatic lipid accumulation occurred in association with elevations in the expression of pro-inflammatory cytokines *TNF α* ($p < 0.05$) and *IL-6*

(Male: $p < 0.05$; Female: $p = 0.08$; Figure 6). Furthermore, male exposed-offspring had significantly increased expression of chemoattractant protein (*MCPI*; Male: $p < 0.05$; Female $p = 0.09$) while females had increased macrophage infiltration (*CD68*; $p < 0.05$).

Discussion

Results from this study demonstrate that fetal and neonatal exposure to fluoxetine results in endocrine and metabolic changes that are consistent with type 2 diabetes (T2D) and its comorbidities, namely obesity and dyslipidemia.

Emerging evidence suggests that SSRI medications may be an important risk factor for T2D, weight gain and dyslipidemia (Beyazyüz et al., 2013; Deuschle, 2013; Raeder et al., 2006). However, despite the fact that SSRIs cross the placenta and are present in breast milk, resulting in considerable transfer to the fetus (Hendrick et al., 2001; Loughhead et al., 2006; Rampono et al., 2004; 2009), to date, the risk of adverse metabolic outcomes in the offspring following developmental exposure to SSRIs are largely unknown. Indeed most studies of SSRI use during pregnancy have focused on teratogenicity (Myles et al., 2013; Riggan et al., 2013) or the risk of adverse pregnancy outcomes. Prenatal SSRI exposure has been shown to alter postnatal components of central serotonergic signaling (Grzeskowiak et al., 2012a), a pathway that plays a critical role in regulating mammalian energy homeostasis (reviewed in Donovan and Tecott, 2013). Moreover, SSRI use during pregnancy has been reported to increase the risk of low birth weight (Huang et al., 2013), an outcome which in turn has been associated with an increased risk of T2D and obesity

in adulthood (Johansson et al., 2008). Therefore, there is increasing interest in postnatal metabolic outcomes in children who were exposed to SSRIs during the perinatal period (Grzeskowiak et al., 2012b).

To date, the results in human studies have been inconsistent. Indeed, SSRI use during pregnancy has been reported to decrease (Grzeskowiak et al., 2012b) and increase (Grzeskowiak et al., 2013) the risk of being overweight (i.e., BMI at or exceeding the age- and sex-specific 85th percentile in exposed children). In the present study we have shown that fetal and neonatal exposure to fluoxetine resulted in a significant increase in body weight in the female but not male offspring; these changes occurred in the absence of altered postnatal food consumption despite the fact that fluoxetine has been shown to affect food consumption in adult rodents (Lauzurica et al., 2013). Moreover, although other experiments in rats have reported a reduction in birth weight following fluoxetine exposure during pregnancy (de Oliveira et al., 2013; Müller et al., 2013) we did not observe any effect of fluoxetine in altering birth weight in our study, suggesting that the metabolic deficits in this model are independent of impaired fetal growth. Although the mechanism(s) underlying the sex-specific differences in body composition are unknown, sex-specific differences in the long-term effects of prenatal exposure to SSRIs and childhood overweight have also been reported in human studies (Grzeskowiak et al., 2013). It is likely that these differences are related to estrogen as there is considerable evidence 1) that estrogen plays an important role in the regulation of body weight homeostasis and 2) that there is considerable cross-talk between estrogen and

serotonergic signaling pathways as estrogen has been shown to up regulate serotonin transporter (SERT) expression, enhancing serotonergic signaling pathways (Brown and Clegg, 2010; Rivera et al., 2009).

Interestingly, while fluoxetine differentially affected body weight in males and females, both sexes had increased visceral adiposity. In humans fatty liver often occurs in concert with increased abdominal obesity [Reviewed in (E. Hui et al., 2013)]. Similarly, in our study we observed a significant increase in the proportion of fluoxetine-exposed animals with evidence of steatohepatitis and hepatic microvesicular lipid accumulation compared to controls. Moreover, fetal and neonatal exposure to fluoxetine resulted in increased levels of hepatic triglycerides in both male and female offspring ($p < 0.01$) whereas hepatic cholesterol levels were significantly elevated in female ($p = 0.014$) but not male ($p = 0.057$) offspring. It is noteworthy that hepatic fat accumulation has been strongly associated with aberrant glycemic control (Berlanga et al., 2014; Milić et al., 2014) which we also observed in the fluoxetine-exposed offspring.

Interestingly, animals born to fluoxetine-exposed dams were not hyperglycemic or hyperinsulinemic at 6 months of age. However, the total insulin response to an ipGTT (i.e., AUC insulin) was significantly increased in fluoxetine-exposed offspring; in males this enhanced insulin response was sufficient to maintain normoglycemia whereas in females it was insufficient to normalize the total glucose response (i.e., AUC_{glucose}) to the glucose challenge. Despite the fact that SSRI exposure has been shown to reduce beta cell survival and function (De Long et al., 2014; Isaac et al., 2013), the changes in

glycemic control in our model do not appear to be a result of deficits in beta cell mass but are more likely related to hepatic insulin resistance.

In humans and animal models there is a strong relationship between obesity, hepatic lipid accumulation, hepatic inflammation and insulin resistance (Gruben et al., 2014). Indeed, obesity and fatty liver have been shown to be associated with increased production of inflammatory mediators including *TNF- α* , *IL-6* and *IL-1 β* (Ma et al., 2008). There have been reports that SSRI antidepressants can increase the production of these cytokines in vitro (Munzer et al., 2013), but the effects of exposure to these drugs during fetal development on inflammatory markers in postnatal life has not been determined. Fetal and neonatal exposure to fluoxetine resulted in a significant increase in the expression of *TNF α* (male and female) at 26 weeks of age. This is of great interest considering that chronic exposure to *TNF α* has been shown to attenuate insulin signaling leading to insulin resistance (Gupta et al., 2007; Kanety et al., 1995). Furthermore, male offspring exposed to fetal and neonatal fluoxetine had a significant increase in *MCP-1* with no significant effects on *CD68* suggesting that there is increased recruitment of monocytes/macrophages from the peripheral circulation to the liver. Conversely, female offspring had significant increases in *CD68* while there was no change in *MCP-1* ($p=0.09$). These results suggest that the increase inflammation in the female offspring may be mediated due to an increase in Kupffer cells, resident macrophages, and may in fact predispose the female offspring to a more profound metabolic phenotype (demonstrated by elevated AUC_{glucose} and decreased insulin sensitivity). The mechanisms underlying this increase in adiposity, fatty

liver, inflammation and insulin resistance following fluoxetine exposure are unclear but may be related to changes in the expression of the serotonin transporter (SERT) in peripheral tissues.

There is considerable evidence demonstrating that fetal exposure to SSRIs can alter expression and activity of key components of the serotonin signaling pathways, including SERT in the brain (Anderson, 2004; Borue et al., 2007; Homberg et al., 2010). Moreover, *TNF α* has been shown to decrease the expression and activity of SERT (Foley et al., 2007; Malynn et al., 2013). The genetic ablation of SERT has been shown to result in glucose intolerance, insulin resistance, obesity and hepatic steatosis, which is similar to the phenotype we report in this study (Chen et al., 2012). Whether or not disruptions to serotonergic signaling pathways in the liver underlie the metabolic deficits in this model remains to be determined.

In summary, pregnancy is a window of vulnerability for depression with prevalence rates reported to range from 6.5 to 20% of all pregnant women (Gavin et al., 2005; Marcus, 2009; Melville et al., 2010). As a consequence, many women take antidepressants while pregnant and/or breastfeeding ultimately leading to fetal and neonatal exposure to these medications. To date, how these drugs impact the postnatal metabolic health of the offspring remained largely unexplored, despite the fact that we are seeing a considerable increase in childhood obesity and early onset T2D (Washington, 2008). Results from this study indicate that fetal and neonatal exposure to an SSRI antidepressant leads to

metabolic abnormalities in the offspring. However, these findings are limited by the fact this study was conducted in a rodent model of antenatal SSRI exposure. Regardless, the results of this study raise the possibility that similar adverse metabolic outcomes may occur in children who have been exposed to SSRIs *in utero*. Therefore, long-term endocrine and metabolic follow up studies should be considered in these children.

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Declaration of interest

The authors declare no conflicts of interest in the work reported in the present article.

Contribution

All authors contributed substantially to the conception and design of the study, collection and analysis of the data, along with the drafting and final editing of the manuscript.

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Figure Legend

Figure 1: Effect of fetal and neonatal fluoxetine exposure (10mg/kg/day; white bar; N=9) on A. Body weight B. total adiposity in rats at 26 weeks of age compared to control (black bar; N=12). Weights measured weekly and % visceral adiposity was measured as mesenteric fat as a percentage of body weight. Data presented as Mean $\pm$ SEM. Values with asterisks are significantly different from control ($p \leq 0.05$).

Figure 2: Effects of fetal and neonatal fluoxetine exposure (10mg/kg/day; white bar; N=9) on A. male and C. female serum glucose (mmol/L) following an intraperitoneal glucose load (time=0 minutes) and area under the curve (AUC) at 24 weeks of age compared to control (black bar; N=12). B. male and D. female serum insulin levels (pmol/L) in response to an intraperitoneal glucose load (time=0 min) and area under the curve (AUC) compared to control (black bar; N=12). Data presented as Mean $\pm$ SEM. Values with asterisks are significantly different from control ($p \leq 0.05$).

Figure 3: Effects of fetal and neonatal exposure to fluoxetine (10mg/kg/day; white bar; N=9) on pancreatic beta cell mass (mg) of rats at A. 3 weeks of age and B. at 26 weeks of age compared to control (black bar; N=12). Quantification of 5 sections per animal; 5 animals per group randomly selected to include only 1 animal per litter. Values are expressed as the mean $\pm$ SEM.

Figure 4: Effects of fetal and neonatal exposure to fluoxetine (10mg/kg/day; white bar; N=9) on liver histology stained with hematoxylin and eosin of 26 week liver samples of A. male control B. female control C. male fluoxetine D. female fluoxetine liver compared to control (black bar; N=12). Arrows pointing to macrovesicular fat vacuoles. E. Non-alcoholic hepatocellular steatosis (NASH) activity grade was determined based on macrovesicular steatosis, ballooning and disarray, and degree of inflammation in acinar and portal regions. Proportion of offspring scored for mild to moderate NASH shown here. Values with asterisks are significantly different from control ($p \leq 0.05$; black bar; N=12).

Figure 5: The effect of fetal and neonatal fluoxetine exposure (10mg/kg/day; white bar; N=9) on total hepatic A. cholesterol and B. triglyceride concentrations (mg of lipid/g of tissue) measurements in male and female offspring at 26 weeks of age compared to control (black bar; N=12). Values are expressed as the mean $\pm$ SEM. Values with asterisks are significantly different from control ($p \leq 0.05$).

Figure 6: Relative gene expression analysis of fetal and neonatal exposure to fluoxetine (10mg/kg/day; white bar; N=9) and control (black bar; N=12) for markers of inflammation (TNF- α , IL-6, MCP-1) and presence of macrophage (CD68) in livers of A. Male and B. Female offspring at 26 weeks of age. Values are expressed as the mean $\pm$ SEM. Values with asterisks are significantly different from control ($p \leq 0.05$).

Figure 1

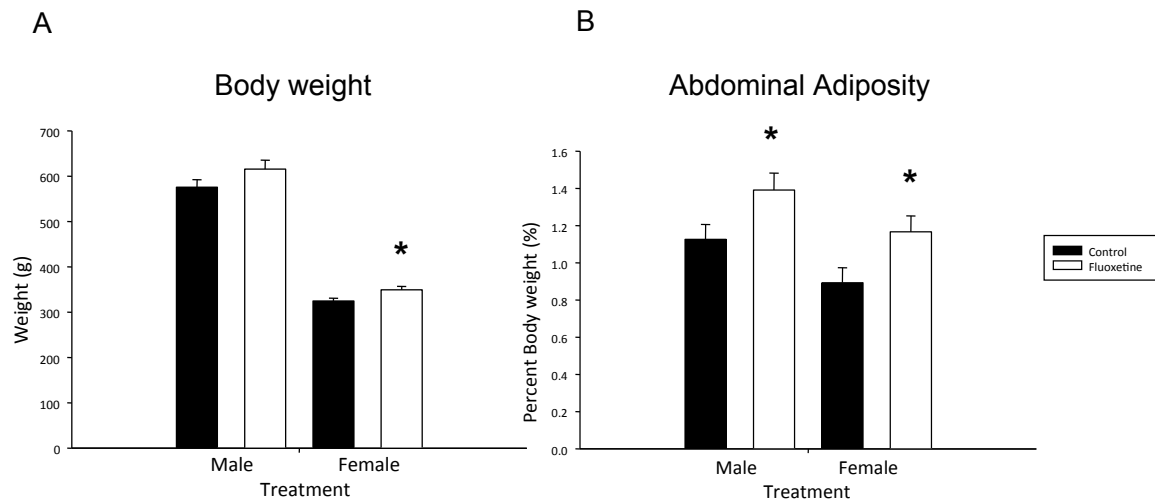


Figure 2

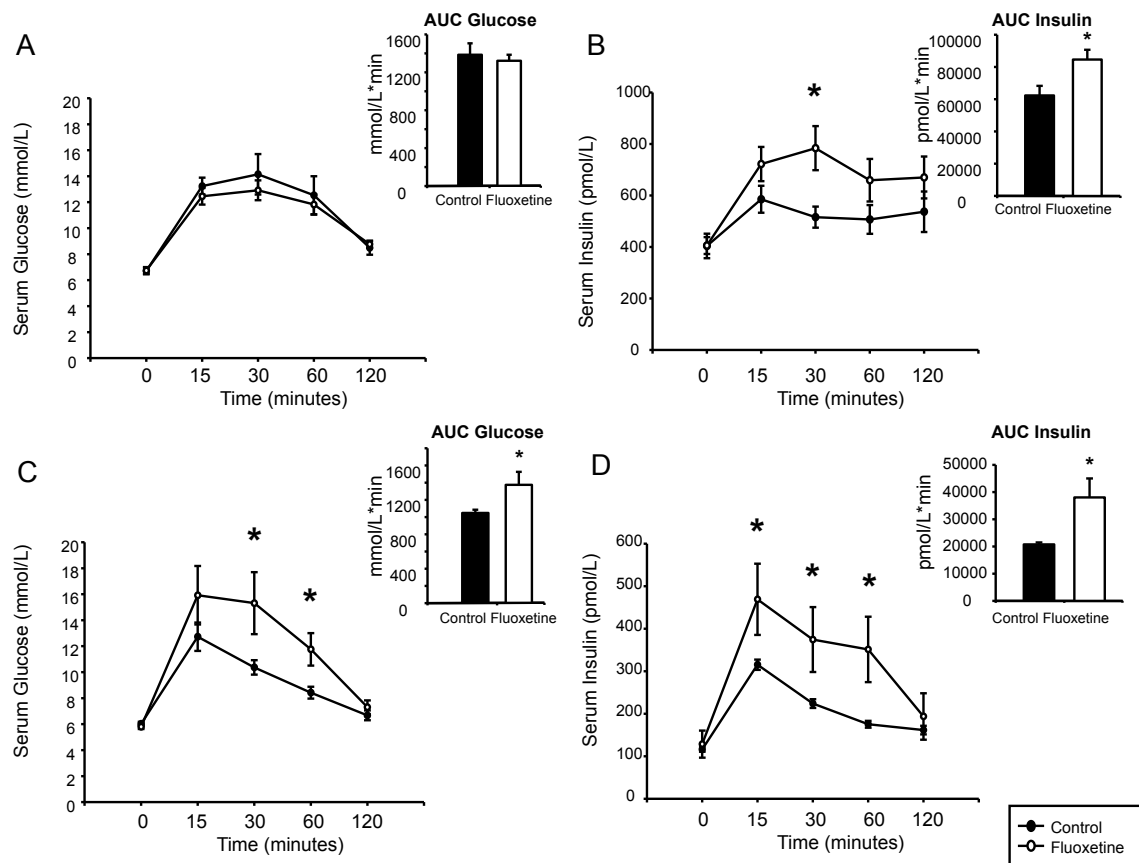


Figure 3

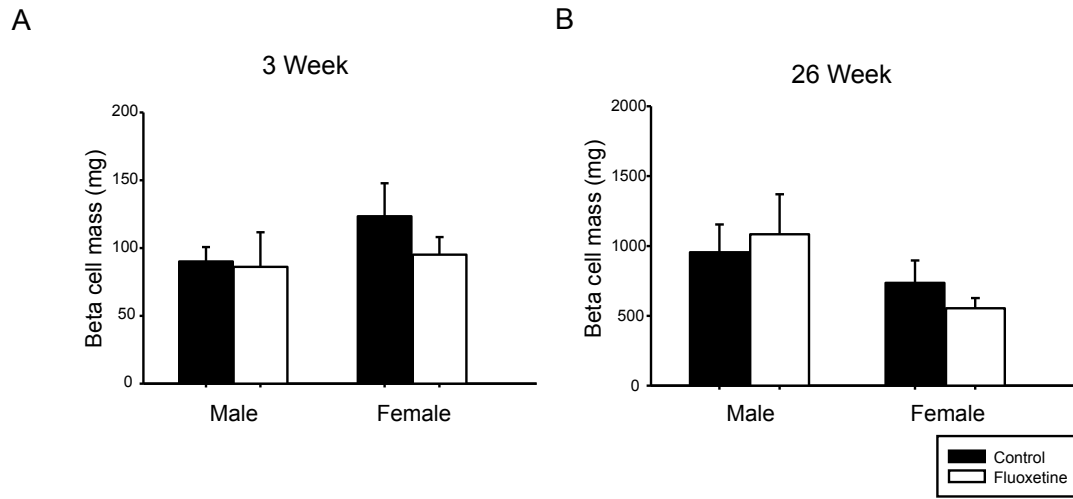


Figure 4

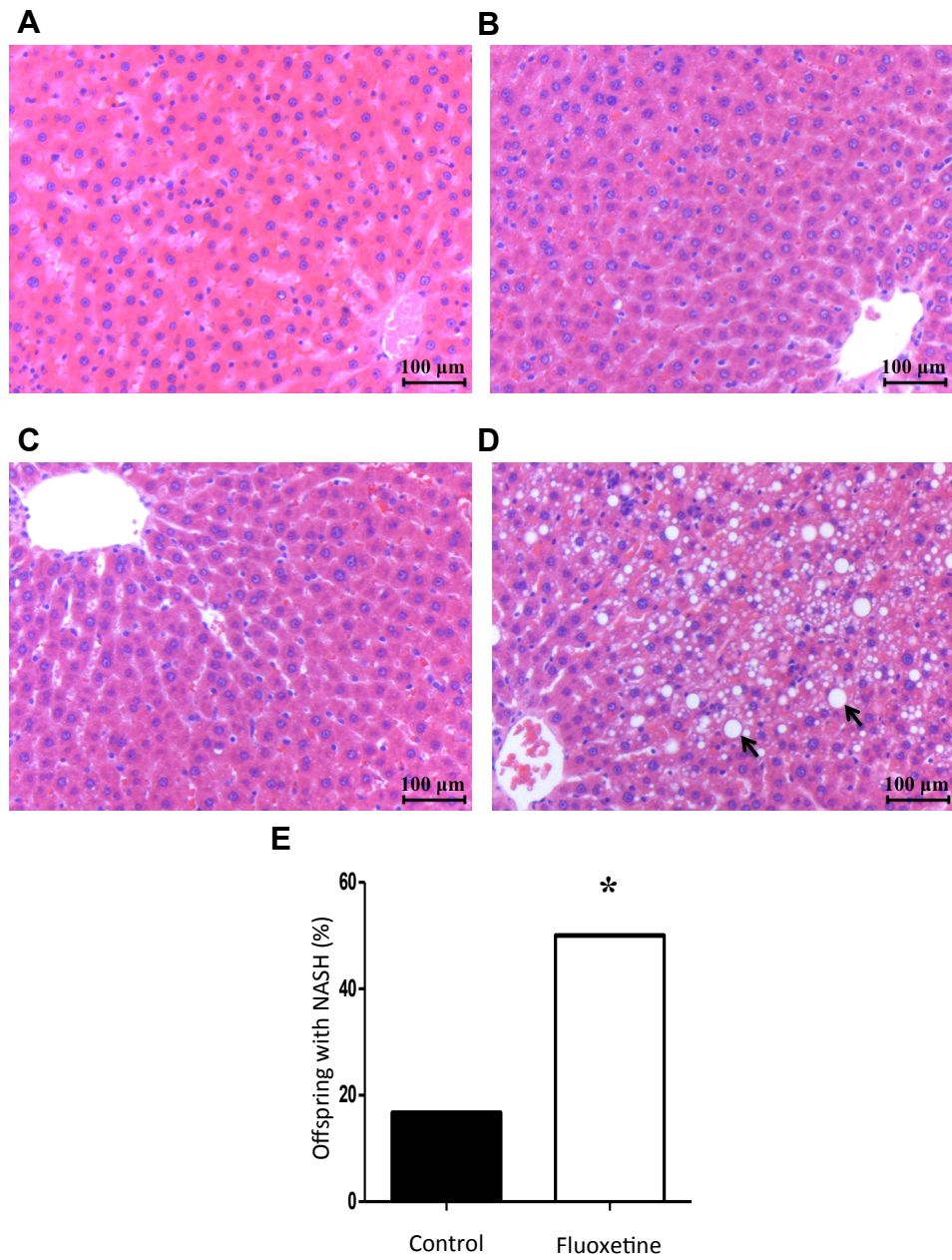


Figure 5

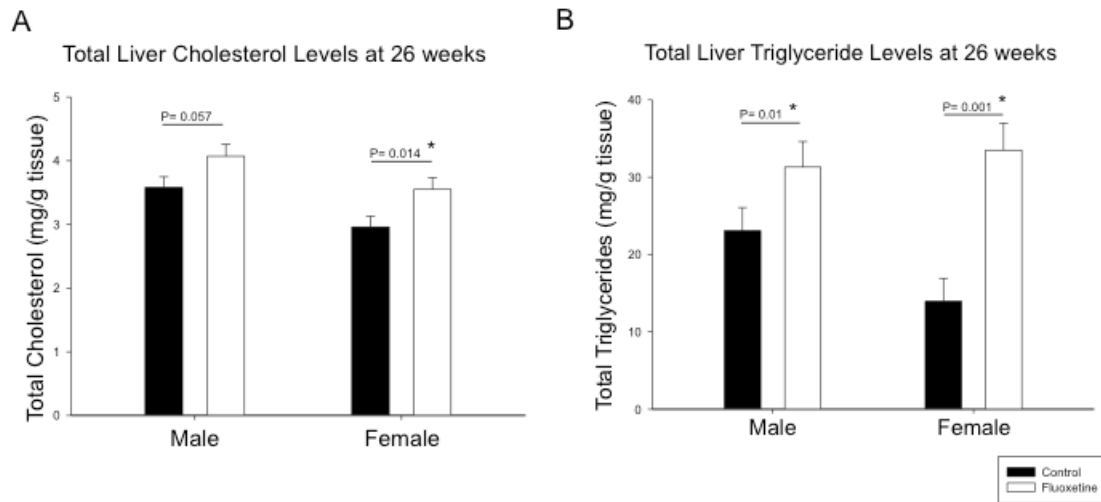
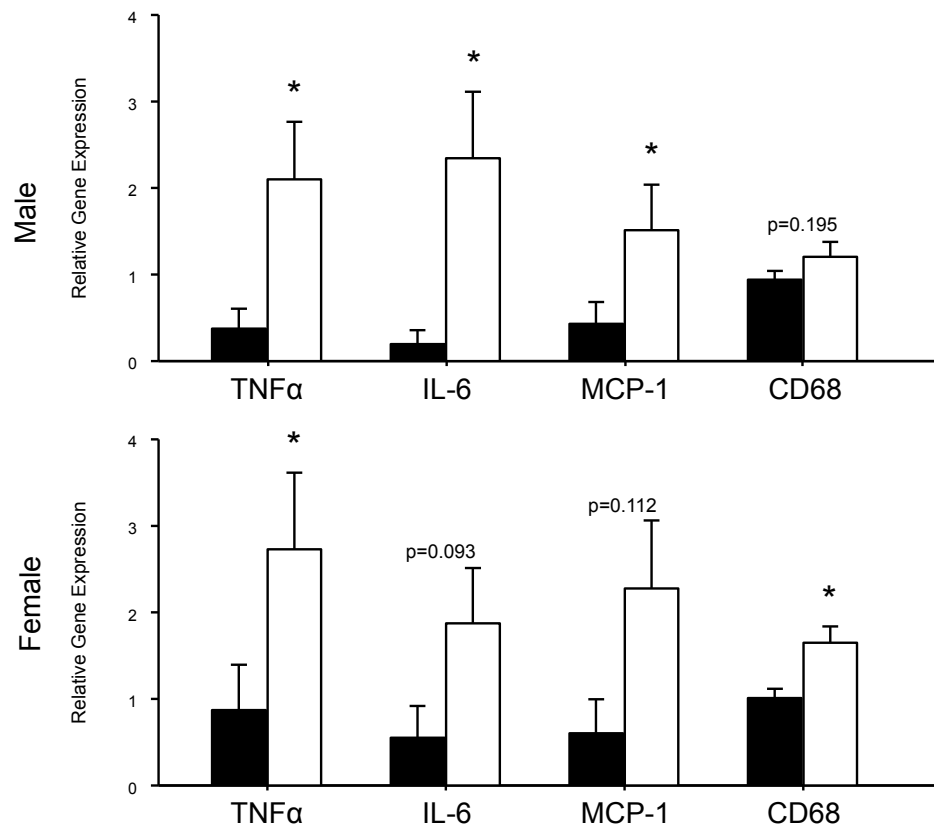


Figure 6



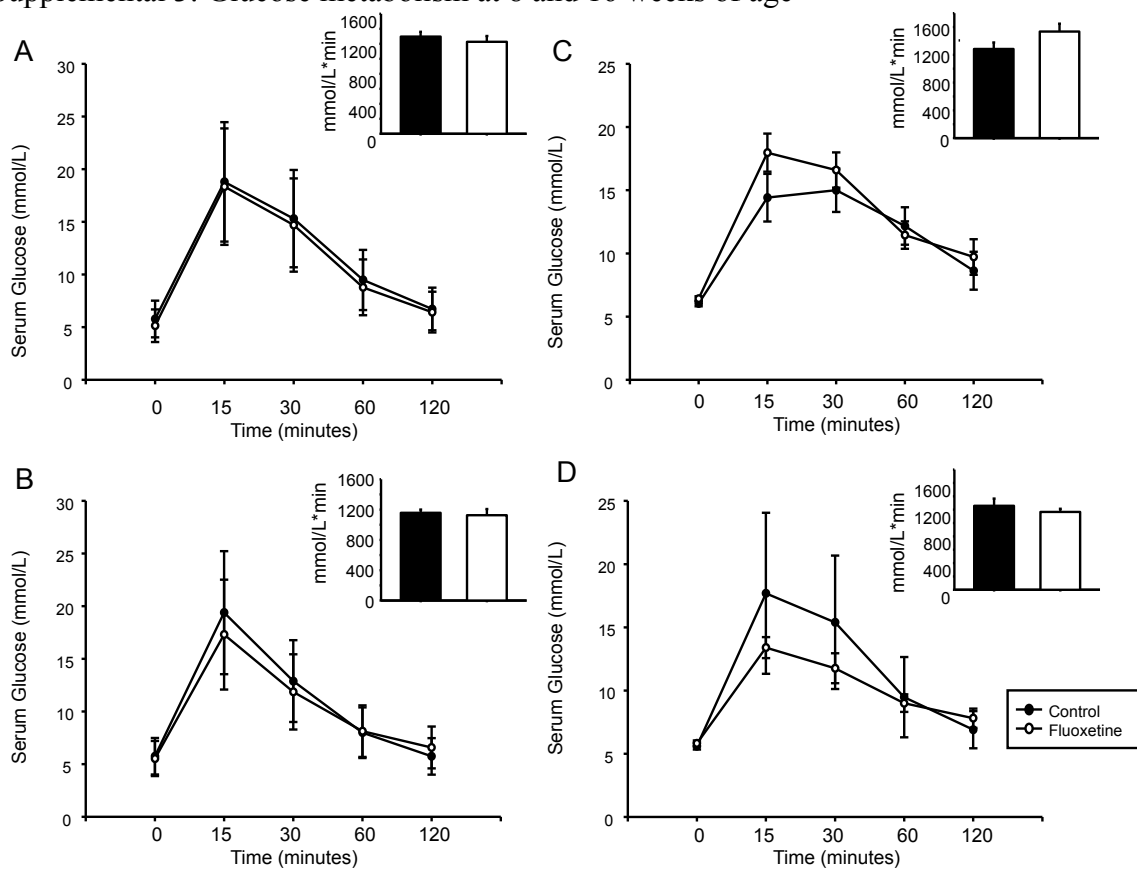
Supplemental 1: RT-PCR Primer Sequences

Genes of Interest	Forward (5'- 3')	Reverse (5'- 3')
HPRT	GCA GTA CAG CCC CAA AAT GG	GGT CCT TTT CAC CAG CAA GCT
CYC	CCG CTG TCT CTT TTC GCC	GCT GTC TTT GGA ACT TTG TCT GC
B2M	AAT TCA CAC CCA CCG AGA CC	GCT CCT TCA GAG TGA CGT GT
18S	GCG ATG CGG CGG CGT TAT	AGA CTT TGG TTT CCC GGA AGC
TNFalpha	CCG GGC AGG TCT ACT TTG CA	AGG CCA CTA CTT CAG CGT CTC G
IL-6	CTT CCA GCC AGT TGC CTT CTT G	TGG TCT GTT GTG GGT GGT ATC C
MCP1	CGG TTT CTC CCT TCT ACT TCC TG	GCT CTG CCT CAG CCT TTT ATT G
CD68	ACT GGG GCT CTT GGA AAC TAC AC	CCT TGG TTT TGT TCG GGT TCA

Supplemental 2: Birth phenotype and fertility measures: 200-250 gram females

	Control (N=13)	Fluoxetine (N=11)	p-value
Time to Mating (days)	2.33 ± 0.50	3.2 ± 0.71	0.34
Mating index (%)	87.5	81.25	1
Preg. Success Rate (%)	64.29	76.92	0.67
Gestational Length (days)	22.11 ± 0.48	22.1 ± 0.31	0.7
Preg. Weight gain (g)	115.67 ± 13.00	139.3 ± 11.36	0.15
Litter size (<i>n</i>)	9.67 ± 1.51	12.4 ± 1.88	0.28
Dam with still borns (%)	33.33	50	0.65
Live Birth Index (%)	93.41	94.89	0.89
Sex ratio (M/F)	1.54 ± 0.57	0.83 ± 0.15	0.15
# of SGA pups	4/119	12/162	0.2
Pup weight (g)	6.78 ± 0.27	6.57 ± 0.32	0.63
Survival to PND4 (%)	87.5	95.37	0.95
Survival to weaning (%)	100	100	1

Supplemental 3: Glucose metabolism at 8 and 16 weeks of age



Supplemental Figure 3: Effects of fetal and neonatal fluoxetine exposure (10mg/kg/day; white bar; N=9) on serum glucose (mmol/L) following an intraperitoneal glucose load (time=0 minutes) and area under the curve (AUC) in A. Male offspring and B. Female offspring at 8 weeks of age and C. Male offspring and D. Female offspring at 16 weeks of age compared to control (black bar; N=12). Data presented as Mean $\pm$ SEM.

Chapter 5

Antenatal Exposure to the Selective Serotonin Reuptake Inhibitor Fluoxetine leads to Increased Hepatic Triglyceride Synthesis in Adult Male Rat Offspring via Transcriptional and Epigenetic Mechanisms.

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Chapter 5.1 Introduction

We have demonstrated that fetal and neonatal exposure to fluoxetine resulted in decreased insulin sensitivity and an increased prevalence for nonalcoholic fatty liver disease (NAFLD). However, the mechanism(s) by which fluoxetine exposure promotes hepatic fat accumulation are still unknown. It is now known that intrahepatic triglyceride content is positively correlated insulin resistance in metabolically abnormal obese patients (Fabbrini et al., 2015). Therefore the study described in this next chapter aims to identify the mechanism(s) underlying the fluoxetine-induced increase in intrahepatic triglyceride content.

Antenatal Exposure to the Selective Serotonin Reuptake Inhibitor Fluoxetine leads to Increased Hepatic Triglyceride Synthesis in Adult Male Rat Offspring via Transcriptional and Epigenetic Mechanisms.

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Abstract

Introduction: Up to 10% of women take selective serotonin reuptake inhibitors (SSRI) during pregnancy. Children exposed to SSRIs in utero have an increased risk of being overweight suggesting that fetal exposure to SSRIs can cause permanent metabolic changes. The aim of this study was to identify the mechanism(s) underlying the fluoxetine-induced increase in intrahepatic triglyceride content.

Methods: Female nulliparous Wistar rats were given vehicle (N=15) or fluoxetine (FLX, 10 mg/kg/d; N=15) orally for 2 weeks prior to mating until weaning. At 6 months of age, we assessed whether SSRI exposure altered components of the hepatic triglyceride biosynthetic pathway in the offspring and determined whether epigenetic changes may have been implicated in these changes.

Results: SSRI-exposed offspring had a significant increase in the steady-state mRNA levels of *elovl6* and *dgat1*. This increased expression of *elovl6* may be associated with both increased acetylation of histone H3 [K9,14] (a hallmark of chromatin activation) and increased acetylation surrounding the proximal promoter region of *Elov16* although this did not reach significance ($p=0.09$).

Conclusion: We have previously demonstrated that antenatal exposure to SSRIs can lead to fatty liver in the offspring raising concerns regarding the long term metabolic sequelae of fetal SSRI exposure. These findings suggest that the elevated hepatic triglyceride levels observed in the SSRI-exposed offspring are due, in part, to long-term posttranslational histone modifications favouring activation of transcription within the promoter region of *Elov16*.

Introduction

Depression affects as many as 840 million people worldwide (World Health Organization, 2008). Women are especially vulnerable given they are twice as likely to be diagnosed with depression at some period in their lifetime; the highest rates occurring during reproductive age (Lokuge et al., 2011). Depression during pregnancy is associated with a number of adverse consequences for both the mother and the child (Grote et al., 2010). As a result, up to one in 10 pregnant women use antidepressants (Andrade et al., 2008; Cooper et al., 2007; Huybrechts et al., 2013). The most common antidepressants used during pregnancy are the selective serotonin reuptake inhibitors (SSRI) since they are well tolerated (Smith et al., 2008; Trifirò et al., 2013; Wemakor et al., 2014). Of the available SSRIs, fluoxetine is one of the most commonly prescribed (Jimenez-Solem et al., 2013). Given that the fetus and neonate can be exposed to SSRIs via placental and lactational transfer, it is pertinent to determine the short and long-term postnatal effects of antenatal SSRI exposure on postnatal health outcomes for the offspring (Rampono et al., 2009).

Long-term use of SSRIs in adults is associated with increased weight gain and new-onset type 2 diabetes, but little is known if these adverse outcomes are mirrored in children of mothers who used SSRIs in pregnancy (Yoon et al., 2013). Interestingly, Grzeskowiak et al., found that males were at an increased risk of being overweight by 7 years of age if prenatally exposed to an SSRI (Grzeskowiak et al., 2013), however the underlying mechanisms remain to be determined. Our lab has recently demonstrated in a rodent

model that antenatal fluoxetine exposure leads to abnormal glucose homeostasis, decreased insulin sensitivity, increased hepatic triglycerides, increased abdominal adiposity, and a greater proportion of offspring with mild to moderate nonalcoholic fatty liver disease (NAFLD) (De Long et al., 2015a). NAFLD is scored based on intrahepatic triglyceride content, degree of inflammation, and the incidence of fibrosis (Brunt et al., 1999). It is noteworthy that intrahepatic triglyceride accumulation is driven by an increase in fatty acid uptake [i.e., lipogenic diet, insulin resistance and/or an increase in *de novo* lipogenesis DNL] and/or a decrease in fatty acid output [i.e., decreased very low density lipoprotein (VLDL) secretion and beta oxidation] (Jensen-Urstad and Semenkovich, 2012). Complex radiolabeling studies in humans have identified that an increase in DNL plays a significant role in intrahepatic triglyceride accumulation (Diraison et al., 2003; Donnelly et al., 2005). Briefly, DNL involves a number of enzymes including acetyl-coA carboxylase (α) that catalyzes malonyl-CoA from acetyl-CoA. Malonyl-CoA is then used as a substrate for the fatty acid synthase (FAS) complex which synthesizes monosaturated long chain fatty acids (palmitic acid; C16:0). From this point, palmitic acid can either be elongated by elongation of very long chain fatty acids (ELOVL) and/or desaturated through stearoyl-CoA-1 (SCD-1) (Rui, 2014). Additionally, diglyceride acyltransferases (DGAT) perform the final and committed step of triglyceride formation from diacylglycerol and acyl-CoA.

Given our previous findings that fetal and neonatal fluoxetine exposure resulted in increased hepatic triglyceride content in postnatal life (De Long et al., 2015a), and that

augmented triglycerides contributes to NAFLD and obesity, this study was designed to test the hypothesis that perinatal exposure to SSRIs leads to augmented intrahepatic triglyceride synthesis. In addition, we sought to uncover the underlying transcriptional and epigenetic mechanisms involved.

Materials and Methods

Maintenance and treatment of animals

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 200-250g female 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. 14 days prior to mating until weaning (postnatal day 21; PND21), dams were randomly assigned to receive vehicle (N=15) or 10mg/kg/day fluoxetine hydrochloride (FLX; N=15; Toronto Research Chemicals, Toronto, ON) orally in flavored gelatin cubes as previously described (De Long et al., 2015a). The dose of fluoxetine was chosen based on previous studies (Capello et al., 2011; Hui et al., 2007) 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 (Olivier et al., 2011; Sit et al., 2008). Dams were allowed to deliver normally. At birth (postnatal day 1; PND1), pups were weighed and sexed and litters were culled to 8, preferentially selecting 4 male and 4 female offspring to ensure uniformity of litter size between treated and control litters. Offspring were maintained on a chow diet until 26 weeks of age. At PND21 and

26 weeks, one male offspring per litter was fasted overnight and euthanized by CO₂ asphyxiation. Liver and adipose tissue were excised and snap frozen in liquid nitrogen for subsequent analysis. Blood was collected, allowed to clot, centrifuged and serum was stored at –80 °C for analysis.

Hepatic triglyceride synthesis:

To quantify the components of the triglyceride synthetic pathway involved in intrahepatic triglyceride content, we examined the steady-state mRNA expression of enzymes responsible for the synthesis of triglycerides [acetyl-CoA carboxylase (*acc1*), fatty acid synthase (*fasn*), steroyl CoA desaturase 1 (*scd1*), elongation of very long fatty acid (*elovl6*), and diacylglycerol o-acyltransferase (*dgat1/2*)], along with key regulatory lipid-sensing nuclear receptors involved in DNL [sterol regulatory element-binding protein 1c (*srebp1c*), liver x receptor α (*lxra*)], by qPCR. Total RNA was isolated from 3 week and 26 week liver tissue using TRIzol® (Life Technologies, Grand Island, NY) extraction combined with the RNeasy® mini kit (Qiagen, Hilden, Germany) (N=9-10 animals per treatment group). Complementary DNA (cDNA) was synthesized from 2µg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions using PerfeCta® SYBR® green FastMix® (Quanta Biosciences, Gaithersburg, MD) on the LightCycler480 (Roche, Mississauga, Ontario, CA). The cycling conditions included polymerase activation (95°C for 10min), followed by 40 cycles of denaturing (95°C for 15s) and annealing/elongation (60°C for 1min). Delta delta Ct values were obtained according to Livak et al., by

normalizing gene expression to two housekeeping genes [hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Cyclophilin A (CYC)] (Livak and Schmittgen, 2001). Primer sequences are supplied in Table 1.

Acid histone extraction and Western Blotting:

Liver collected from male offspring at 3 and 26 weeks of age were ground by mortar and pestle and resuspended in lysis buffer [10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and protease cocktail inhibitor (Roche)]. Hydrochloric acid was added to a final concentration of 0.2 M. The resultant solution was then sonicated at 30% amplitude for 5 sec total, 1 sec per pulse, and incubated on ice for 30 min with periodic vortexing. It was then centrifuged at 11,000 g for 10 min at 4 °C. The supernatant was collected and was dialyzed using Pierce Slide-A-Lyzer MINI Dialysis Units (3500 MWCO) (Thermo Scientific). The order of dialysis occurred in the following manner: 2 x 200 mL of 0.1 M acetic acid for 1 hour, 200 mL of deionized water for 1 hour, 200 mL of deionized water for 3 hours, and 200 mL of water overnight. Samples were then stored at -80 °C until Western blotting was performed. 10 µg of total protein was loaded and subjected to 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) blotting membrane (BioRad Laboratories, Hercules, CA). Membranes were blocked for 3 hours in 5% (wt/vol) skim milk in TTBS (Tris-buffered saline [TBS], 0.1% [vol/vol] Tween 20) at room temperature on a rocking platform and then incubated with Histone H3 (cat #05-499, EMD Millipore) and acetylated histone H3 [K9,14] (cat #sc-06-599, EMD Millipore) primary antibody at 4°C overnight. After washing with TTBS, blots were

incubated with peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody (1:5000; GE Healthcare, QC, CA) for 1 hour at room temperature on a rocking platform. Blots were developed using enhanced chemiluminescence (ECL) (Millipore, Billerica, MA) and quantification was carried out using ImageLab 4.1 software (ImageLab Version 4.1 build 16; Bio-Rad, Hercules, CA). Immunoblots were subsequently incubated with stripping buffer (Thermo Scientific) and re-probed with beta-actin (1:4,000, Abcam) to control for protein loading. Histone H3 and acetylated histone H3 [K9,14] was normalized to β -actin. Data presented are the ratio of acetylated histone H3 [K9,14]:Total histone H3.

Chromatin immunoprecipitation (ChIP):

Chromatin was extracted from liver tissues excised at 26 weeks of age from male offspring as previously described (Sohi et al., 2011). In brief, a small piece of snap frozen liver was homogenized and incubated in 0.5 mL of 1% formaldehyde for 10 min at room temperature to cross-link proteins and DNA. Glycine (0.125 M, final concentration) was added to all samples to terminate cross-linking. Samples were micro centrifuged at 950 g at room temperature for 5 min and the supernatant was subsequently removed. The liver tissue was then washed once with cold PBS before being placed in 500 μ L of DS lysis buffer (Millipore, Etobicoke, Ontario, Canada) with protease inhibitor cocktail (Roche). Each sample was sonicated to produce sheared, soluble chromatin. The lysates were diluted ten times with the addition of ChIP dilution buffer (Millipore) and aliquoted to 300 μ L amounts. Each of the aliquots was precleared with protein A/G Plus agarose beads

(40 μ L, Millipore) and rotated for 2 h at 4 °C. In order to pellet the beads, samples were micro centrifuged at 20,000 g, and the supernatant containing the sheared chromatin was placed in new tubes. The aliquots were incubated with 3 μ g of acetylated histone H3 antibody (lysine 9,14, cat #05-399, Millipore) and rotated overnight at 4 °C. Two aliquots were reserved as ‘controls’ — one incubated without anti- body (‘input’) and another with non-immune IgG (Millipore). Protein A/G Plus agarose beads (60 μ L) were added to each tube, the mixtures rocked for 1 h at 4 °C and the immune complexes collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5 min in wash buffer I (20 mM Tris–HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), wash buffer II (same as I, except containing 500 mM NaCl), wash buffer III (10 mM Tris–HCl, pH 8.1, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.25 M LiCl), and in 2 $\times$ TE buffer. The beads were eluted with 250 μ L elution buffer (1% SDS, 0.1 mM NaHCO₃ + 20 μ g salmon sperm DNA (Sigma-Aldrich, Oakville, Ontario, Canada) at room temperature. The elution step was repeated once and eluates were combined. Crosslinking of the immunoprecipitated chromatin complexes and ‘input controls’ (10% of the total soluble chromatin) was reversed by heating the samples at 65 °C for 4 h. Proteinase K (15 μ g; Invitrogen, Carlsbad, CA, USA) was added to each sample in buffer (50 mM Tris–HCl, pH 8.5, 1% SDS, 10 mM EDTA) and incubated for 1 h at 45 °C. The DNA was purified by phenol- chloroform extraction and precipitated in ethanol overnight at –20 °C. The supernatant was removed and pellets were dried. Both samples and ‘input’ controls’ were diluted in 10–100 μ L TE buffer prior to PCR analysis. Real-time PCR was employed using forward (5’ -

CCCACATGAGGGAAAGAGAG -3') and reverse (5' - CCAGCCCCACAGGTTTT -3') primers that amplify the - 300 bp to - 200 bp region of the rat *Elvol6* promoter (NM_134383.2) and forward (5' - TGTGACTGCAGTTCCCAGAG -3') and reverse (5' - TCACCGTCGCAGAAGTAGTG -3') primers that amplify the -350 bp to -200 bp region of the rat *Dgat1* (NM_053437.1) promoter. Using serial dilutions of rat liver chromosomal DNA, the primers were demonstrated to have good linear correlation (slope ~ 3.4), strongly suggesting equal priming efficiency in priming to their target sequence. ChIP signal was calculated by comparing the relative abundance of the immunoprecipitated chromatin (*e.g.* acetylated Histone H3 [K9,14]) compared with input chromatin between experimental groups. The relative fold changes were calculated using the comparative cycle times ($2\Delta\Delta Ct$) method, as previously described (Ma et al., 2014).

Statistical analysis:

All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL). The results are expressed as mean $\pm$ SEM. The results from quantitative RT-PCR, immunoblot and ChIP analysis are expressed as the mean of arbitrary values $\pm$ SEM. Outcome measures between male control and fluoxetine-treated offspring were analyzed using Student's t-test ($\alpha = 0.05$). Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using the Mann-Whitney rank sum test.

Results

Fetal and neonatal exposure to fluoxetine leads to altered hepatic triglyceride synthesis

Previously, we demonstrated that perinatal SSRI exposure led to augmented hepatic triglyceride levels, while serum triglycerides were not altered in 26 week old offspring (De Long et al., 2015b). In this study, quantitative real-time PCR revealed that the steady-state mRNA levels of hepatic *elovl6* ($p=0.01$) and *dgat1* ($p=0.03$), key enzymes in the triglyceride biosynthetic pathway, were significantly increased in 26 week offspring of dams exposed to fluoxetine (Figure 1D and 1E). In contrast, the expressions of the triglyceride synthesizing enzymes (*acc1*, *fasn*, *scd-1*, or *dgat2*, Figure 1) were unaltered. Moreover, there was no significant increase in the steady-state mRNA expression of the key lipogenic regulatory transcription factors (*lxra*, *srebp1c*) (Figure 2). This increased gene expression of *elovl6* and *dgat1* appears to be liver-specific as they were unaltered in adipose tissue (Supplemental 1).

Fetal and neonatal exposure to fluoxetine increased global hepatic histone H3 acetylation in isolated hepatic histones along with promoter-specific histone H3 acetylation surrounding the proximal site of the Elovl-6 promoter in 26 week rat offspring

Epigenetic posttranslational histone modifications such as increased histone H3 acetylation [K9,14] favouring chromatin opening, lead to transcriptional activation (Lee et al., 1993). Therefore, we sought to investigate whether the genes that were up-regulated were associated with posttranslational histone modifications promoting

chromatin activation in their proximal promoter regions (Lee et al., 1993). To explore this further, we first investigated whether there were any global changes in total hepatic acetylated histone in the offspring of SSRI-treated dams. Immunoblotting revealed there was a significant increase in the global acetylation of H3 lysine 9 [K9,14] in the isolated histones from the livers of fluoxetine-exposed offspring at 26 weeks ($p=0.02$; Figure 3A). We next sought to determine whether this increased acetylation of histone H3 was associated, in a promoter-specific manner, with the increased transcription of *elovl-6* and/or *dgat1*. Therefore, primers were designed to target the proximal promoter region of the rat *elovl-6* and *dgat 1* genes. While there was a trend for SSRI-exposed to exhibit increased acetylation of histone H3 [K9,14] at the *elolv6* promoter, this was not significant ($p=0.09$; Figure 3B). Furthermore, there was no significant difference in the acetylation of H3 lysine 9 [K9,14] at the *dgat1* promoter region (Figure 3C; $p=0.54$).

Increased hepatic lipid accumulation in SSRI-exposed offspring at 26 weeks of age appears to be an indirect effect.

To assess if fetal and neonatal fluoxetine exposure was directly affecting the long-term changes observed in the hepatic fatty acid synthesis pathway, we sought to determine the effect of SSRIs on hepatic triglyceride synthesizing enzymes from an earlier postnatal time point. Therefore, we examined the steady state mRNA levels of *lxra*, *srebp1c*, *acc1*, *fasn*, *scd-1*, *elovl6*, *dgat1* or *dgat2* at PND21 at weaning (time point representing the end of SSRI exposure; Table 2). There were no significant changes in any of the target genes investigated. Moreover at this age there were no global changes in total hepatic acetylated

histone H3 lysine 9 [K9,14] in the offspring of SSRI-treated dams (Figure 4), suggesting that the SSRI-induced effects on histone acetylation and increased triglyceride synthesizing enzymes are indirect.

Discussion

Nonalcoholic fatty liver disease (NAFLD) is a condition marked by hepatic lipid accumulation, inflammation and fibrosis. Current estimates suggest that the current incidence of NAFLD in the United States is approximately 30% of all adults (Lazo and Clark, 2008). Our lab has previously demonstrated that fetal and neonatal fluoxetine exposure led to increased hepatic triglyceride accumulation by 6 months of age (De Long et al., 2015a); these levels depend on 1) hepatic lipid influx, 2) *de novo* lipogenesis and 3) hepatic lipid efflux. Our data demonstrates that the observed increase in hepatic lipid accumulation following fetal and neonatal fluoxetine exposure is due, in part, to epigenetic modifications favouring *de novo* lipogenesis mediated via an increased expression of *elovl6* and *dgat1* mRNA. Furthermore, this increase in *elovl6* expression occurred in association with enhanced global histone H3 acetylation [K9,14] and promoter-specific increases at the proximal promoter region of *elovl6*, although this did not reach significance. Given the limited literature surrounding the metabolic consequences of fetal and neonatal fluoxetine exposure, this study provides further insight into potential long-term effects on postnatal lipid homeostasis and metabolic disease risk.

In our model of fetal and neonatal exposure to fluoxetine, male offspring had elevated levels of hepatic triglyceride levels (De Long et al., 2015a) without a concomitant increase in circulating serum triglyceride levels. As mentioned previously, intrahepatic triglyceride accumulation occurs as a result of increased dietary intake, increased lipid production by adipocytes and/or *de novo* hepatic lipogenesis. Given that both groups of animals were maintained on the same chow diet with no differences in food consumption and that serum triglyceride levels were not different between treatment groups (De Long et al., 2015a), it is unlikely that the increased accumulation of triglycerides in the liver of fluoxetine-exposed offspring was due to increased dietary intake. Furthermore, there were no significant differences in gene expression of lipogenesis genes in adipose tissue suggesting that the changes in hepatic lipids are specific to *de novo* lipogenesis in hepatocytes (Supplemental 1).

De novo lipogenesis is the synthesis of fatty acid chains from carbohydrate precursors and is often altered in various metabolic abnormalities including obesity, insulin resistance and NAFLD (Adams et al., 2005; Diraison et al., 2002). In fact, it has been speculated that heightened *de novo* lipogenesis is the major cause of NAFLD as it has been positively correlated with NAFLD severity in humans (Diraison et al., 2002; Liu et al., 2010). In addition, intricate isotope studies have found that *de novo* lipogenesis is the major contributing source of fat accumulation in the liver given that the rate of *de novo* lipogenesis is 3-fold higher in subjects with elevated hepatic lipid levels after being

matched for similar adiposity and blood lipid levels (Donnelly et al., 2005; Lambert et al., 2014).

De *nov*o lipogenesis is tightly regulated by the transcription factor sterol regulatory element-binding protein-1 (SREBP1). In *vitro* studies have found that fluoxetine activates SREBP1 in both gila cells and hepatocytes (Feng et al., 2012; Raeder et al., 2006). Furthermore, fluoxetine exposure in primary hepatocytes has been shown to increase lipid accumulation (Feng et al., 2012; Xiong et al., 2014) by activating SREBP-1 through the suppression of AMP-activated protein kinase (AMPK) signaling. Given the impact of de *nov*o lipogenesis on hepatic lipid accumulation, we sought to identify whether this pathway was activated in our model of fetal and neonatal exposure to fluoxetine. When activated, SREBP1 translocates to the nucleus promoting lipogenesis by increasing the transcription of several of its target genes including acetyl-coA carboxylase (α) (*acc1*), Fatty Acid Synthase (*fasn*), elongation of very long chain fatty acids (*elovl*), stearoyl-CoA-1 (*SCD-1*) and diglyceride acyltransferases (*dgat1/2*) (Postic and Girard, 2008). In the present study, we investigated the enzymes involved in lipogenesis. We observed that the steady-state mRNA levels of *elovl6* and *dgat1* were significantly elevated in the liver of male fluoxetine-exposed offspring at 26 weeks of age. Given that both DGAT1 and Elov6 are regulated by the transcription factor SREBP1 (Kumadaki et al., 2008), these two enzymes may contribute to the observed increase in hepatic triglyceride accumulation.

Dgat1 is responsible for catalyzing the conversion of diacylglycerol and fatty acyl CoA to a triacylglycerol moiety while Elovl6 is an enzyme responsible for catalyzing the elongation of unsaturated and monounsaturated fatty acid chains C:12-C:18 (Jakobsson et al., 2006). DGAT1 overexpressing hepatocytes increase the synthesis of triglycerides, without affecting the output of microsomal lipids from the liver (Millar et al., 2006). Therefore, increased expression of DGAT1 results in increased hepatic lipid accumulation. Elovl6 knock out mice (Elovl6^{-/-}) are obese and develop NAFLD when challenged with a high fat diet (Matsuzaka et al., 2012; 2007). Furthermore, evidence from Elovl6^{-/-} mice also suggests that Elovl6 increases the hepatic lipid accumulation of longer chain fatty acids (Shimano, 2012). Elovl6 may also be mediating hepatic insulin resistance in part due to the inactivation of Akt signaling which could explain previous studies which demonstrated that SSRI-exposed offspring exhibited hyperinsulinemia and insulin resistance (De Long et al., 2015a; Matsuzaka et al., 2012). As a result, increased expression of *dgat1* and *elovl6* trigger increased accumulation of hepatic lipid levels, with a greater ratio of long chain fatty acids (carbon length C18:C16 ratio); both of which are directly related to hepatic insulin resistance and NAFLD progression (Shimano, 2012). Therefore, our data of heightened *elovl6* expression is consistent with the existing literature with the exception that we did not see a concomitant up regulation of SREBP1 transcript levels. Thus we next decided to investigate the possible epigenetic modifications involved that may influence transactivation of *elovl6* and *dgat1* gene expression.

A critical indication of exposure to an altered in *utero* environment is delivering a baby small for gestational age (SGA). This, followed by a shift to a plentiful environment results in significant catch-up growth and increases the risk of post-metabolic disease (Heerwagen et al., 2010; Ma et al., 2014; Sohi et al., 2011). An altered in *utero* environment can ensue due to chemical insults (i.e., nicotine, bisphenol A, SSRIs) during intrauterine life (i.e., a period of developmental plasticity). It has been suggested that many of these changes may be epigenetic in nature (Barker et al., 1993; De Long et al., 2015a; Ma et al., 2014). In fact, chronic and acute exposure to fluoxetine in an adult rodent model alters histone acetylation leading to chromatin silencing in the brain (Cassel et al., 2006; Robison et al., 2014). In addition, altered DNA methylation patterns were observed at weaning in the brains of the offspring exposed to fluoxetine during fetal and neonatal life (Toffoli et al., 2014). Furthermore, previous data from our research group found that exposures to other drugs (i.e., nicotine) during pregnancy lead to postnatal hypertriglyceridemia through enhanced acetylation of histone H3 [K9,14] surrounding the proximal promoter region of FAS, a key lipogenic enzyme (Ma et al., 2014). However, to date, there are no data to suggest that fetal and neonatal fluoxetine exposure to SSRIs leads to long-term epigenetic modulations, nor that they occur in the peripheral tissues. Therefore we sought to investigate whether fluoxetine exposure led to posttranslational histone modifications and whether acetylated histone H3 [K9,14] could be a putative mechanism(s) to explain the increased hepatic lipid accumulation in our model. Here we demonstrate that fetal and neonatal exposure to fluoxetine results in increased global acetylated histone H3 in the liver of male offspring at 26 weeks of age. Moreover, we

have evidence that there is increased acetylated histone H3 specifically at the proximal promoter region of *elovl6*, a critical gene involved in the regulation of lipid composition in hepatocytes. Taken together, these studies suggest that fetal and neonatal fluoxetine exposure may lead to increased histone H3 acetylation, favouring Elov16 transcriptional activation and ultimately, increased hepatic de *nov*o lipogenesis and elongation of fatty acids. In order to investigate whether fetal and neonatal exposure to fluoxetine directly led to the long-term alterations in the liver, we sought to measure both the steady state mRNA expression of critical enzymes within the lipogenesis pathway along with the global expression of acetylated histone H3 in the liver of PND 21 male rat livers. It is noteworthy that PND 21 represents the time of the maximal fluoxetine exposure in the offspring. There were no significant changes observed in the mRNA expression of lipogenic genes or acetylated histone H3 at PND21 in the livers of offspring exposed to fetal and neonatal fluoxetine, implying that perinatal fluoxetine exposure likely programs long-term intrahepatic fat accumulation via an indirect mechanism(s). We currently do not understand why the changes in intrahepatic triglyceride accumulation do not become apparent until later in life. Further studies investigating the metabolic profile over the life-course are required to understand the underlying causes.

In summary, our findings demonstrate that fetal and neonatal fluoxetine exposure leads to increased Elov16 expression. This may in part be due to elevated acetylated histone H3 [K9,14] at its proximal promoter region, although it did not reach significance ($p=0.09$). Currently, there are no studies to our knowledge that have investigated the long-term

metabolic outcomes of antenatal SSRI use in humans or rodents. While this study was performed in rats, our results raise the possibility that similar changes in hepatic lipid accumulation may also occur in children who were exposed to SSRI antidepressants during perinatal life. Consequently, future follow-up studies should consider investigating hepatic lipid levels in children with prenatal exposure to SSRI antidepressants.

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Declaration of interest

The authors declare no conflicts of interest in the work reported in the present article.

Contribution

All authors contributed substantially to the conception and design of the study, collection and analysis of the data, along with the drafting and final editing of the manuscript.

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Figure Legend

Figure 1: The effect of fetal and neonatal fluoxetine exposure on hepatic steady-state mRNA levels of *acc1*, *fasn*, *scd1*, *elovl6*, *dgat1* and *dgat2* in the male rat offspring at 26 weeks of age. Data are presented as fold change from the controls and results are expressed as the mean $\pm$ SEM. *Statistically different ($p < 0.05$) from control offspring. N=9-10 per group.

Figure 2: The effect of fetal and neonatal fluoxetine exposure on hepatic steady-state mRNA levels of key lipogenic transcription factors *lxra* and *srebp1c* in the male rat offspring at 26 weeks of age. Data are presented as fold change from the controls and results are expressed as the mean $\pm$ SEM. * Statistically different ($p < 0.05$) from control offspring. N=9-10 per experimental group

Figure 3: The effect of fetal and neonatal fluoxetine exposure on A) Total hepatic acetylated histone H3 B) Acetylated Histone H3 [K9,14] at the proximal promoter site of *elovl6* and C) *dgat1* in male offspring at 26 weeks of age. Data are presented as fold change from the controls and results are expressed as the mean $\pm$ SEM. * Statistically different ($p < 0.05$) from control offspring

Figure 4: The effect of fetal and neonatal fluoxetine exposure on hepatic steady-state mRNA levels of total hepatic acetylated histone H3 in the male rat offspring at 3 weeks of age. Data are presented as fold change from the controls and results are expressed as the mean $\pm$ SEM. *Statistically significant = $p < 0.05$. N=9-10 per experimental group.

Figure 1

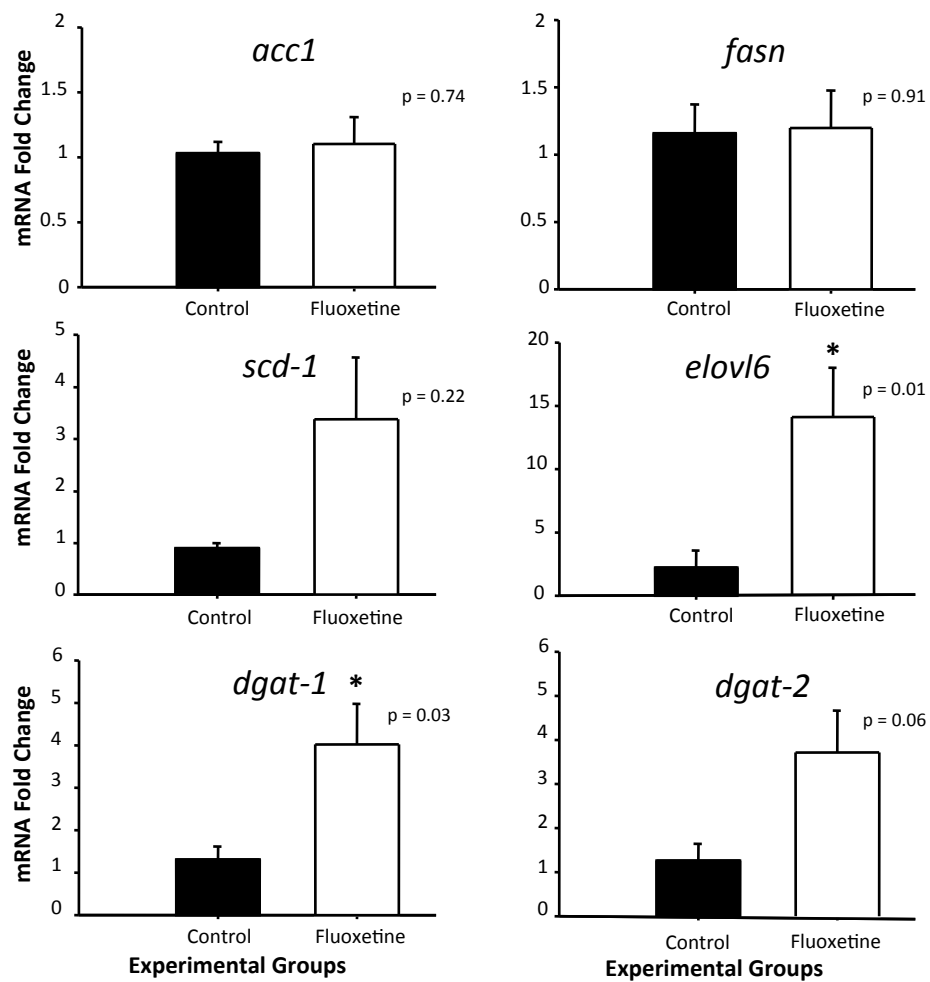


Figure 2

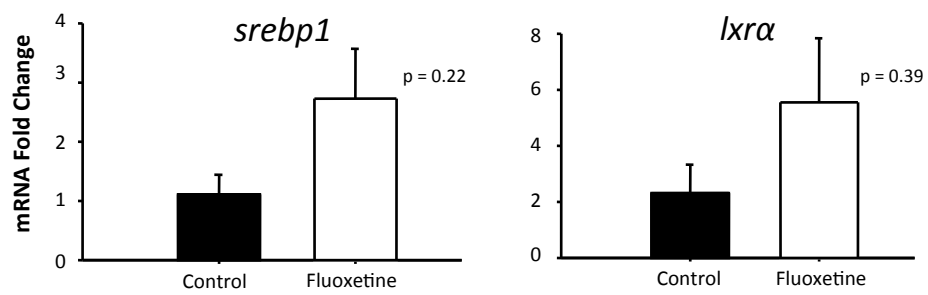


Figure 3

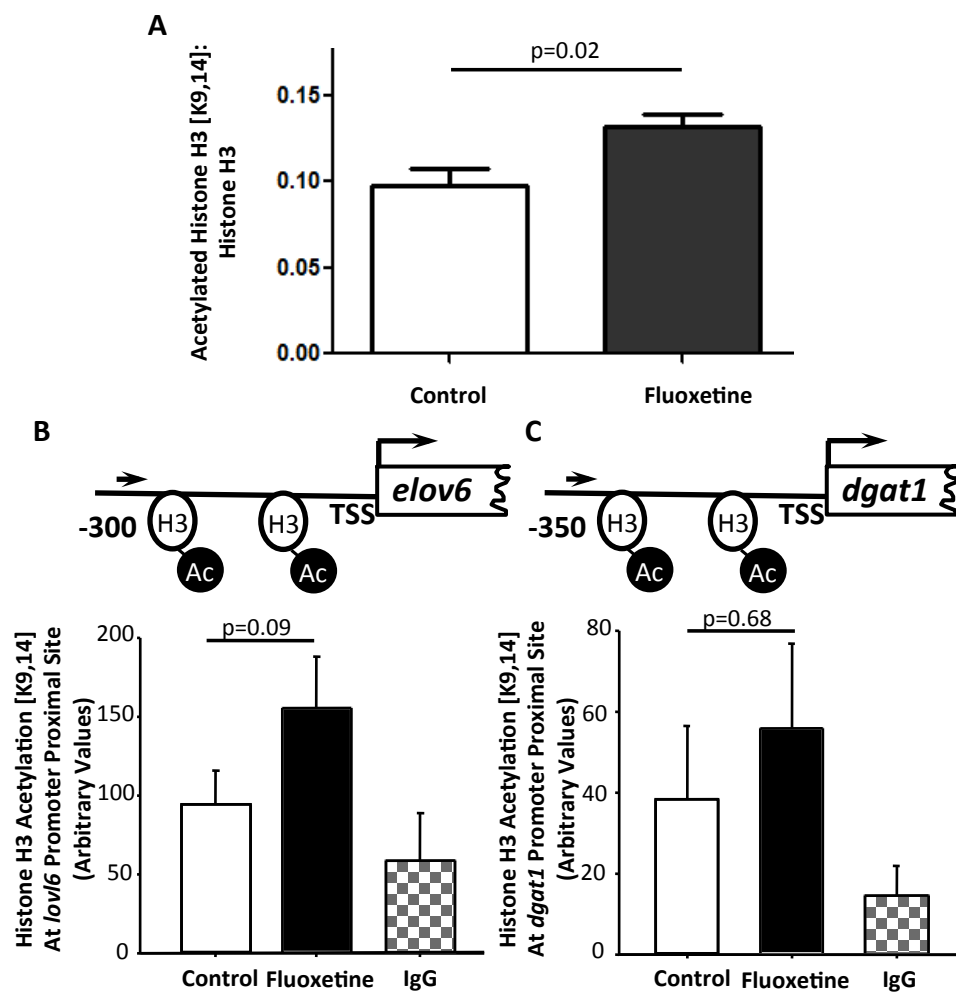


Figure 4

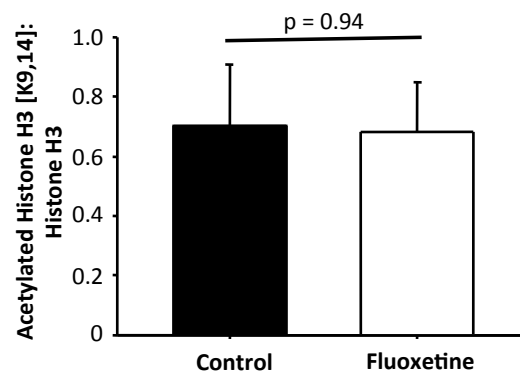


Table 1

RT-PCR Primers

Table 1

	Forward	Reverse
hprt	5' – GCA GTA CAG CCC CAA AAT GG – 3'	5' – GGT CCT TTT CAC CAG CAA GCT – 3'
cyc	5' – CCG CTG TCT CTT TTC GCC – 3'	5' – GCT GTC TTT GGA ACT TTG TCT GC – 3'
lxra	5' - ACA ACC CTG GGA GTG AGA G - 3'	5' - TAG CAT CCG TGG GAA CAT - 3'
srebp1c	5' - AGC CGT GGT GAG AAG CGC AC - 3'	5' - ACT GCT GCT GCC TCT GCT GC - 3'
acc1	5' - ATT GGG CAC CCC AGA GCT A - 3'	5' - CCC GCT CCT TCA ACT TGC T - 3'
fasn	5' - GAG TCC GAG TCT GTC TCC CGC TTG A - 3'	5' - GCC GTG AGG TTG CTG TTG TCT GTA - 3'
scd1	5' - CTG ACC TGA AAG CTG AGA AG - 3'	5' - ACA GGC TGT GCA GGA AAG TT - 3'
elovl6	5' - GGT CGG CAT CTG ATG AAC AAG - 3'	5' - CGA ATA TAC TGA AGA CCG CAA GAG - 3'
dgat1	5' - CAG ACC AGC GTG GGC G - 3'	5' - GAA CAA AGA GTC TTG CAG ACG ATG - 3'
dgat2	5' - GGA ACC GCA AAG GCT TTG TA - 3'	5' - AAT AGG TGG GAA CCA GAT CAG C - 3'

Table 2

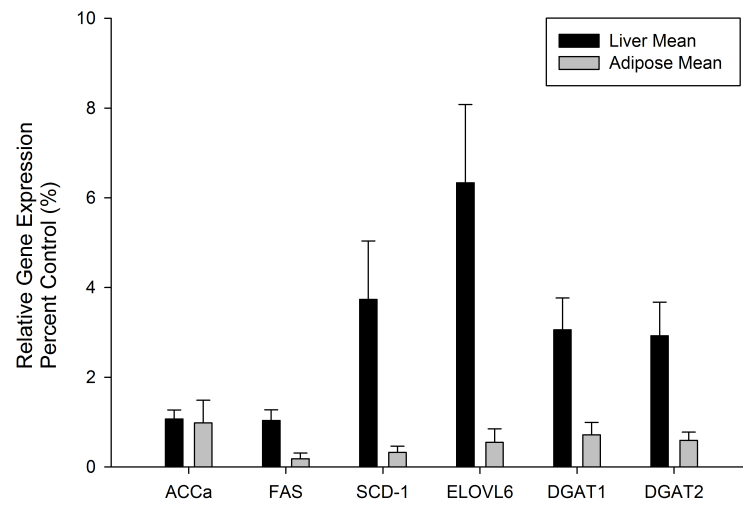
Effects of fetal and neonatal fluoxetine exposure on steady state mRNA levels of genes involved in hepatic lipogenesis at 3 weeks of age

Table 2

	PND21		
	Control	Fluoxetine	p-value
<i>acc1</i>	1.12 ± 0.15	0.84 ± 0.09	0.12
<i>fasn</i>	1.38 ± 0.29	0.82 ± 0.13	0.10
<i>scd1</i>	2.02 ± 0.63	0.58 ± 0.15	0.15
<i>elovl6</i>	1.31 ± 0.25	0.77 ± 0.11	0.12
<i>dgat1</i>	1.07 ± 0.12	1.00 ± 0.11	0.67
<i>dgat2</i>	1.03 ± 0.08	0.82 ± 0.09	0.08

Supplemental 1: The effect of fetal and neonatal fluoxetine exposure on hepatic and adipocyte steady-state mRNA levels of key lipogenic transcription factors *lxra* and *srebp1c* in the male rat offspring at 3 weeks of age. Data are presented as hepatic and adipocyte percent control of fold change from fluoxetine-exposed offspring. Results are expressed as the mean $\pm$ SEM. * Statistically different ($p < 0.05$) from control offspring. N=9-10 per experimental group

Supplemental Figure 1



Chapter 6: Discussion

Summary of the work

The overall goal of this dissertation was to contribute to the literature regarding selective serotonin reuptake inhibitor (SSRI) use and its association with an increased risk Type 2 Diabetes Mellitus (T2DM). Additionally, we wanted to explore the long-term metabolic consequences of fetal and neonatal SSRI exposure and to identify mechanisms underlying the development of such metabolic disturbance. Here we show that SSRIs have a direct effect on isolated pancreatic beta cells (chapter 2) and that these pancreatic deficits can be replicated in an animal model of fetal exposure to SSRIs (chapter 3). Lastly, we have shown that fetal and neonatal exposure to the SSRI fluoxetine alters glucose homeostasis in the adult offspring; an effect that appears to be mediated via post-translational epigenetic modifications promoting hepatic lipid accumulation in the offspring (chapters 4 and 5).

SSRIs & Beta Cell Function

Oxidative Stress

Long-term use of SSRIs in adult populations increases the risk of developing T2DM (RR: 1.49; 95% CI, 1.29 to 1.71) (Yoon et al., 2013). Beta cell dysfunction is central to the onset of T2DM (Kahn et al., 2008; Leahy, 2005). We have data to support the notion that SSRIs affect beta cell function (insulin secretion) both in *vitro* (chapter 2), and in *vivo* (chapter 3). Consistent with our findings in Chapter 2, a recent study has reported that fluoxetine has deleterious effects on beta cell function (i.e., GSIS) (Cataldo et al., 2015).

Notably, this study suggests that fluoxetine may have a direct effect on beta cell function in contrast to an effect due to the modulations of the extracellular serotonin levels. Similarly, work from my thesis suggests that fluoxetine can directly affect beta cell function, an effect due, in part to increased production of reactive oxygen species (ROS) (DeLong et al., 2014). ROS are reactive oxygen molecules that are central in cellular signaling. Under normal physiological conditions, these free radicals are neutralized by antioxidant enzymes found within the cell such as superoxide dismutase 1/2, glutathione peroxidase and catalase (Pi et al., 2010). Beta cells inherently have a suppressed antioxidant defense system making them particularly susceptible to ROS damage (Grankvist et al., 1981; Lenzen et al., 1996). Increased cellular ROS results in oxidative damage to proteins, of which the mitochondria are particularly susceptible (Pi et al., 2010). When mitochondria are damaged, there is a significant proton leak between the mitochondrial membranes, decreasing the ATP production and increasing the release of protein from the inner mitochondrial membrane into the cytosol (Maechler et al., 2010). These two events initiate a cascade of events that result in beta cell dysfunction and ultimately beta cell apoptosis (Maechler et al., 2010). We have demonstrated that fluoxetine exposure to INS-1E cells increased oxidative damage, and decreased mitochondrial activity *in vitro* (chapter 2). The observation that SSRI treatment induced oxidative stress and/or mitochondrial dysfunction is not exclusive to our group. Similar results were found with fluoxetine (Abdel-Razaq et al., 2011; Djordjevic et al., 2011; Hroudová and Fišar, 2012; Inkielewicz-Stêpniak, 2011; Souza et al., 1994), sertraline (Battal et al., 2014; Li et al., 2012), and escitalopram (Kassan et al., 2013) albeit the

majority of the studies were also done in vivo. Furthermore, studies from our group (chapter 2) and others (Sakr et al., 2015) have demonstrated that while fluoxetine exposure can induce oxidative stress, its effects can be rescued with an antioxidant therapy (i.e., folic acid or resveratrol). While we have shown that SSRIs impair beta cell function and that this can be rescued through the use of an antioxidant, it still remains to be determined whether it is the direct effect of the SSRI, or the indirect effect of the increased serotonin metabolism. Future directions should focus on determining the role of intracellular serotonin production and/or selectively blocking serotonin reuptake on beta cell function. Likewise, the contribution of serotonin degradation by monoamine oxidase (MAO) to the production of intracellular ROS should be determined and whether SSRI treatment alters MAO expression levels in these cells.

In Utero SSRI exposure and postnatal metabolic outcomes

Perinatal exposure to SSRIs in rodents has been shown to alter key components of the serotonergic signaling pathway in the brain (Homberg et al., 2010; Anderson et al., 2004; Borue et al., 2007). Therefore I hypothesized that antenatal SSRI-exposure may also alter the serotonergic pathways in the pancreas; an effect that may impact pancreatic development (i.e., establishment and maintenance of beta cell mass) and/or function. First I sought to identify if, in a pregnancy model, whether fetal exposure to an SSRI antidepressant caused aberrant pancreatic development including deficits in beta cell mass. Beta cell mass is determined by a balance of beta cell replication, neogenesis and apoptosis. In animal models, a significant loss of beta cell mass at birth increases the risk

of T2DM into adulthood (Simmons et al., 2001). For the most part, the beta cell loss is due to increased beta cell apoptosis (Dumortier et al., 2007; Petrik, 1999; Snoeck et al., 1990). However, here we demonstrate a model of fetal exposure to sertraline, that there is a beta cell deficit at birth, in part due to decreased islet vascularization with no change in proliferation or apoptosis (chapter 3). Recent data from our laboratory suggest that the altered expression of VEGF may be due to epigenetic modifications. We have shown that fluoxetine treated INS-1E cells have increased expression of miR-29b. MicroRNAs are a post-transcriptional mechanism to control translation of genes. MicroRNAs are small non-coding RNA strands (approximately 22 base pairs long) that bind to transcripts and direct them for degradation (Cai et al., 2009). Importantly for this study, miR-29b targets VEGF, and the increase in its expression in fluoxetine-treated INS-1E cells parallels the decrease in VEGF transcript levels (unpublished data, De Long NE et al.,). Further experiments are needed to establish whether the relationship between fluoxetine induced changes in miR-29b and VEGF is causal or correlative.

Once I had determined that fetal exposure to an SSRI caused pancreatic deficits, I wanted to know if these changes had long-lasting functional consequences for glucose homeostasis in adulthood. Therefore, for my third objective I performed metabolic phenotyping of SSRI-treated offspring up to 6 months of age. Results from this study confirmed that offspring exposed to fluoxetine exhibited abnormal glucose homeostasis and decreased insulin sensitivity (De Long et al., 2015). It is important to note here that these effects were not mediated via a beta cell defect as beta cell mass was not impacted

at either birth (as seen with sertraline exposure) or at 26 weeks of age. Metabolic deficits in this model included increased hepatic triglycerides, increased abdominal adiposity, and a greater proportion of offspring with mild to moderate fatty liver scoring (chapter 4). It is conceivable that SSRIs could have direct effects on the hepatocytes to increase lipid accumulation. We and others have shown *in vitro* that SSRIs directly increase lipogenesis and lipid accumulation in hepatocytes (De Long unpublished; Feng et al., 2012; Xiong et al., 2014). Interestingly, evidence from the literature and unpublished data from our lab suggest that SSRIs may be promoting the co-activation of estrogen related receptor α (ERR α) and peroxisome proliferator-activated receptor gamma, coactivator 1 α (PGC-1 α) (De Long unpublished; Jeong et al., 2015; Luo et al., 2003; Schreiber et al., 2003), leading to the increased expression of lipogenic enzymes including fatty acid synthase. However, further experiments are required to establish unequivocally the role of ERR α in fluoxetine-induced hepatic lipid accumulation.

The Model

Route of Administration

SSRI antidepressants are used for a wide range of psychiatric disorders including a number of off-label uses (Stone et al., 2003). SSRIs are effective and commonly prescribed for eating disorders due to their appetite suppressing effects (Appolinario and McElroy, 2004; McElroy et al., 2000). However, translating this to a perinatal animal model of fluoxetine exposure presents a significant concern. Altered maternal nutrition during pregnancy is known to alter metabolic profiling in the offspring (reviewed in

Braun et al., 2013). Studies in the past have shown that SSRI exposure during pregnancy decreased maternal food consumption (Anelli et al., 1992; Byrd and Markham, 1994; da Silva et al., 1998; Uphouse et al., 2006). These studies administered the SSRIs via intragastric gavage or via injection. It is our opinion that both of these processes increase the stress on the dam which may have led to diminished appetite or have led to glucocorticoid-mediated reductions in birth weight which are also associated with abnormal metabolic outcomes in adulthood (Franko et al., 2010; Nyirenda et al., 2009; Reusens et al., 2011). One of the strengths of our model is that we have found a safe, non-stressful way to administer SSRIs throughout the antenatal period, without causing subsequent changes in food consumption and/or maternal weight gain (De Long et al., 2015).

Critical Windows of Exposure

When developing a model of perinatal chemical exposure, a variable of consideration is the length of exposure. Studies from our lab with maternal nicotine exposure have shown that for this chemical at least, life-long pancreatic deficits only occur when maternal exposure extends from prior to pregnancy until the end of lactation (Bruin et al., 2007). With respect to perinatal SSRI exposure, discrepancies are found in the literature regarding critical windows. For example, da Silva et al., (2015) found that fluoxetine decreased body weight, increased mitochondrial function (i.e., citrate synthase activity, oxygen consumption) in both the muscle and the hypothalamus and increased energy expenditure (da Silva et al., 2015). However this group exposed their offspring to fluoxetine via subcutaneous injection, solely throughout lactation (from postnatal day 1 to

21). To date it is undefined whether there are critical windows of exposure for SSRIs but clinical data may suggest that there are. For instance, SSRI exposure during the early stages of pregnancy may increase the risk of congenital anomalies (Knudsen et al., 2014; Wemakor et al., 2015) while late stage SSRI exposure increases the risk of persistent pulmonary hypertension of the newborn (Huybrechts et al., 2015). Therefore, future studies should focus on determining what are the critical windows of exposure (as outlined in Bruin et al., 2007). In addition, these studies should be repeated with fluoxetine in order to compare our metabolic outcomes with the ones seen with only exposure during lactation.

Not All SSRIs are Equivalent

Although the available SSRIs [fluoxetine (Prozac®), sertraline (Zoloft®), paroxetine (Paxil®), fluvoxamine (Luvox®) and citalopram (Celexa®)] are all used to treat MDD, they all have different pharmacological effects due to their varying structures. Importantly these drugs also have a variable risk of teratogenicity. For example paroxetine is known to cause atrial septal defects (OD: 1.8, 95% CI: 1.1 - 3.0) right ventricular outflow tract obstruction defects (OR: 2.4, 95% CI: 1.4 - 3.9) (Reefhuis et al., 2015) whereas fluoxetine has negative outcomes with respect to persistent pulmonary hypertension in newborns (OR:1.28; 95% CI: 1.01-1.64) (Huybrechts et al., 2015). There are no known teratogenic effects related to the use of sertraline, citalopram and escitalopram during pregnancy (Reefhuis et al., 2015). These outcomes may be a result of drug-specific receptor interactions and binding efficiencies as outlined in Appendix A. These details may

explain some the inconsistency of results in my thesis with respect to the effects of fetal SSRI exposure on pancreatic development. In chapter 3 I found that sertraline exposure during pregnancy decreased beta cell mass at birth, however I did not see a similar effect following exposure to fluoxetine. While these divergent outcomes may be due to the change in route of administration, it is more likely due to the differences in structure of the drugs themselves as SSRIs themselves have shown to have varying effects in specifically testicular tissues, even though they are in the same class (Erdemir et al., 2014).

Since not all SSRI antidepressants perform the same, it should be avoided to group these pharmaceuticals into one class because of the risk of losing pertinent information regarding drug-specific outcomes. This applies to both adult and antenatal SSRI exposure studies. Many of the serotonin modulating medications bind to other receptors/transporters in a stimulating or antagonizing manner and may have distinct effects on cellular function. Future studies should consider regrouping these pharmaceuticals, not merely by their associated class, but rather by their primary receptor target. Accounting for these additional unintended targets could explain observed differences beyond serotonin modulation.

Limitations/Future Directions

We provide data here to suggest that it may in fact be the drugs used to treat depression that increase the risk of endocrine and metabolic perturbations associated with T2DM.

However, we cannot ignore the contribution of the depression itself. It is well established that there is a bidirectional relationship between depressive symptomatology and T2DM (Chapter 1). Due to the increased co-occurrence of these two diseases, there has been discussion regarding whether a specific subtype of depression that is more susceptible to developing metabolic dysfunction exists. While some subtypes of depression are at a higher risk of obesity (i.e., atypical, undifferentiated, melancholic) (Cizza et al., 2012; Levitan et al., 2012) a more specific distinct clinical entity of depression that includes metabolic disturbances as a central pathological process termed “metabolic syndrome type II” has been proposed (McIntyre et al., 2007). However this area still remains controversial and further studies are needed to address whether a metabolic subtype of depression exists. In fact, if a metabolic subtype of depression can be established, these findings will be fundamental in teasing out the contribution of the depression, and/or the drugs used to treat the depression (SSRI therapy) and their effects on metabolic function.

Aside from the effects of depression on adult metabolic function, untreated depression during pregnancy poses additional risks for both mother and child (chapter 1) (Gavin et al., 2005; Wisner et al., 2009) and therefore can have a significant impact on postnatal metabolic disorders later in life. As such, the effects of SSRI use during pregnancy would optimally be tested in an animal model of depression to more closely mimic the human situation. The main limitation to this experimental strategy lies in the lack of a suitable animal model of depression. Since depression is a multivariate disorder with a spectrum of diagnostic criteria, it is hard to develop an animal model that effectively represents

such a heterogeneous phenotype (Nestler and Hyman, 2010). Most animal models are achieved and evaluated through construct validity whereby the animals are exposed to a variety of acute and/or chronic stressors such as environmental stresses (i.e., chronic mild stress, learned helplessness, social stress) or traumatic life events (i.e., maternal separation) (Deussing, 2006). Despite the lack of an animal model that completely recapitulates the spectrum of symptoms associated with the human disease, future studies should test the effect of fetal and neonatal exposure to SSRIs in at least one animal model of depression. This will allow us to unravel the contributions of drug vs. underlying disease. Furthermore, this study should be performed using more than one drug within the SSRI class to determine what are the specific long-term effects of each drug individually.

Contribution of Sex

In chapter 3 we have shown differences in the transcriptional and biochemical pathways are related to the underlying sex differences. Future studies should continue to consider sex with respect to depression $\pm$ SSRI use during pregnancy. These studies will be clinically relevant to the human population as there is now data to suggest that based on the sex of the child, the untreated depression and/or SSRI use, differentially affects postnatal metabolic health in children (Grzeskowiak et al., 2013).

Maternal Metabolic Profile

Other than weight gain, this dissertation did not investigate the effects of SSRI use on maternal metabolism. Given that SSRIs cause metabolic dysfunction, it is reasonable to suggest that SSRI-induced changes in maternal metabolism could indirectly affect the metabolic fate of the offspring. However, there is insufficient data in the literature to

suggest that depression and/or SSRI use during pregnancy increases the risk of gestational diabetes and therefore requires further attention (Lopez-Yarto et al., 2012).

Serotonergic System & Maternal Beta Cell Function

While most of the body's 5-HT is produced in the gut (98%), more recently it has been discovered that the entire serotonergic biosynthetic and signaling pathways are present in other peripheral organs including the pancreas. (Paulmann et al., 2009). It has been established that the serotonergic pathway is found in the beta cells including the serotonergic gene *fev*, which transcribes the transcription factor *Pet1*. Perturbations in *Pet1* can lead to altered beta cell function through impaired insulin production and secretion (Huang et al., 2005; Kutlu et al., 2009; Ohta et al., 2011; Paulmann et al., 2009; Richmond et al., 1996; Schraenen et al., 2010). Serotonin is also present in the pancreatic beta cells as it is stored within the secretory β -granules and has been implicated in the process of insulin release (Paulmann et al., 2009). Indeed, *Tph1*^{-/-} mice which have only 10% the serotonin levels of the wild type animals, are diabetic and have impaired insulin secretion; these deficits in pancreatic function were rescued by serotonin supplementation (Paulmann et al., 2009). There are many metabolic changes that occur during typical pregnancy including insulin resistance during the second half of pregnancy thereby causing a compensatory action of beta cell expansion (Barbour et al., 2007). During pregnancy, human placental lactogen increases up to 30-fold and is also responsible for the upregulation of *Tph1* (Barbour et al., 2007; Kim et al., 2010; Schraenen et al., 2010). This observed expansion in beta cell mass is a result of serotonin signaling through the 5-

HT_{2b} receptor which mediates by the activation of Jak2/Stat5, Erk, and PI3K pathways (Iida et al., 2015; Kim et al., 2010). Furthermore, paracrine/autocrine signaling via the 5-HT₃ receptor appears to decrease the threshold for glucose-stimulated insulin secretion, increasing insulin availability during pregnancy (Ohara-Imaizumi et al., 2013). Similar perturbations in the serotonergic system have proven to be harmful on the maternal heart (Park et al., 2015). Therefore, disruptions within the peripheral serotonin-signaling pathway during pregnancy (i.e., the administration of SSRIs) may significantly alter maternal beta cell physiology and cardiac function. However there is very limited data surrounding the effect of SSRIs on the maternal metabolic profile during pregnancy. In fact, one systematic review investigating the effects of antidepressants on maternal metabolic outcomes identified only two studies with the appropriate controls making it difficult to draw any conclusion on the subject matter (Lopez-Yarto et al., 2012). Further studies should focus on how maternal SSRI use during pregnancy may impact maternal physiology and how these influences may impact fetal and neonatal environment. This is important because exposure during fetal and neonatal life are known to predispose the offspring to adult onset pathologies.

Clinical Significance

From animal studies it is evident that fetal and neonatal SSRI use can disrupt postnatal metabolic homeostasis. This is important because approximately 10% of women experience depressive symptoms during pregnancy and as a result approximately 1 in 10 pregnancies may be complicated with antidepressant use (Andrade et al., 2008; S. M.

Marcus, 2009). This is of concern since there are no epidemiological studies to describe the long-term consequences and/or disease risk for these children into adulthood. While we present data suggesting SSRI use during pregnancy and lactation may increase the risk of endocrine and metabolic perturbations associated with T2DM, we are not advising mothers with depression to discontinue medication. While these findings suggests a significant outcome in rodents, further investigations are critical to understanding the complexities within this field before suggesting similar outcomes in humans.

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Appendix A - Receptor interactions

	SSRI						Bupropion
	Prozac®	Zoloft®	Paxil®	Celexa®	Lexapro®	Luvox®	
	Fluoxetine	Sertraline	Paroxetine	Citalopram	Escitalopram	Fluvoxamine	
SERT inhibitor	x	x	x	x	x	x	x
Norepinephrine Transporter inhibitor	x	x		x	x	x	x
Dopamine Transporter (SLC6A3)	x	x		x	x	x	x
5HT1B				x	x		
5HT1D				x	x		
5HT2A	x						
5HT2C	x		x	x	x		
5HT3	x	x		x	x		x
Alpha 1 Adrenergic	x	x		x	x	x	x
Alpha 2 Adrenergic	x						
Muscarinic Receptor 1	x	x		x	x	x	x
Muscarinic Receptor 2	x	x		x	x	x	x
Muscarinic Receptor 3	x						
Muscarinic Receptor 4	x						
Muscarinic Receptor 5	x						
Histamine Receptor 1	x	x		x	x		x
Histamine Receptor 3	x						
muscarinic acetylcholine receptor	x	x					x
cholinergic muscarinic 5	x	x					x
dopamine receptor D1							
dopamine receptor D2	x	x		x	x		x
dopamine receptor D3				x	x		
cytochrome P450 2C8	x						
cytochrome P450 2C9						x	
Voltage-gating potassium channel	x			x	x		
sigma opioid receptor	x					x	
cholinesterases	x						

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