FLUOXETINE MODULATES SEROTONIN AND HEDGEHOG SIGNALING

FLUOXETINE: EXAMINING THE SELECTIVE SEROTONIN REUPTAKE INHIBITOR'S EFFECTS ON SEROTONIN AND HEDGEHOG SIGNALING IN THE PANCREATIC BETA CELL

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Abstract

Major depressive disorder (MDD) is one of the most common psychiatric illnesses worldwide, with pharmacotherapy as a first-line option for the management of this illness. The National Center for Health Statistics found that the use of antidepressants has increased by more than 4 fold in the last 20 years. While SSRI's act centrally to treat MDD, their peripheral effects are often overlooked. Interestingly, components of the serotonergic system including the serotonin transporter (SERT), serotonin receptors, and enzymes important for serotonin synthesis (tryptophan hydroxylase 1 and 2; Tph1 and Tph2) are affected by SSRI treatment both centrally and peripherally. This disruption of serotonin signaling in the pancreas is of particular interest as there is a considerable link between the serotonin and hedgehog signaling pathways, both of which are important for pancreatic beta cell function. I hypothesize that pancreatic beta cell exposure to the SSRI fluoxetine in vitro will lead to altered hedgehog signaling ultimately resulting in a disruption in insulin secretion.

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List of Abbreviations

5-HT - 5-hydroxytryptamine; serotonin
5-HT1-7 - serotonergic receptors
5-HTP - 5-hydroxy-L-tryptophan
5-HTTLPR - serotonin-transporter-linked polymorphic region
ANOVA – Analysis of Variance
cDNA – complimentary DNA
CNS – central nervous system
Ct – comparative cycle times
DHh - desert hedgehog
DMEM – Dulbecco's Modification of Eagle's Medium
DSM-V - Diagnostic and Statistical Manual of Mental Disorder, 5th edition
DSP-4 - N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
Gli - glioma-associated oncogenes
HPA - hypothalamic-pituitary-adrenal
IDX 1- insulin promoter factor 1
IHh - indian hedgehog
MaO - monoamine oxidase
MDD – major depressive disorder
NaSSA - noradrenergic and specific serotonergic antidepressants
PCPA - para-chlorophenylalanine; 4-chloro-DL-phenylalanine
Ptch1 - patched
S.E.M – standard error of the mean
SARI - serotonin receptor antagonist and reuptake inhibitors
SERT - serotonin transporter
SHh - sonic hedgehog
SLC6A4 - serotonin reuptake transporter,
Smo - smoothened
SNRI - serotonin norepinephrine reuptake inhibitors
SSRI - selective-serotonin reuptake inhibitors
T2DM - type 2 diabetes
Tph - tryptophan hydroxylas

Chapter 1: Introduction

1.1Major Depressive Disorder

1.1.1 Impact

Major Depressive Disorder (MDD) has been increasing worldwide at staggering rates. Indeed, research from the National Ambulatory Medical Care Survey found that the number of people diagnosed with depression has increased by 450% between 1987-2007 (Marcus and Olfson, 2010; Olfson et al., 2002). Currently the World Health Organization estimates MDD affects over 300 million individuals worldwide with a 13.3-17.1% prevalence in the United States and Europe (Kessler et al., 2009). In Canada, it has been reported that 11.3 % of people aged 15 and older had symptoms consistent with depression, but this percentage could be much higher since symptoms of depression as dysphoria, fatigue, loss of appetite are often overlooked, with many cases of depression are underdiagnosed by primary health care providers (Government of Canada, 2013; VanItallie, 2005). The escalating prevalence of MDD is a huge concern as MDD has been described as a leading cause of disease burden in economically developed countries, and is associated with significant disability and mortality (Murray and Lopez, 1997). In addition to a reduced quality of life, MDD represents a significant economic burden to the healthcare system and families and individuals with this disease (Greenberg & Birnbaum, 2005). Estimates from the 1990's suggest over $51 billion had been spent on depression related treatment and disabilities in the United States, a cost that drastically increased by

150% to $83 billion over a 10 year period from 1990 to the year 2000 (Greenberg & Birnbaum, 2005). This increasing economic burden of depression related disability is a consistent trend worldwide (Greenberg and Birnbaum, 2005; Sobocki et al., 2006).

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

The Diagnostic and Statistical Manual of Mental Disorder, 5th edition (DSM-V) characterizes MDD as 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. Like many other chronic illnesses, MDD is a multifactorial disorder; MDD may be related to changes in brain structure, genetic polymorphisms, and decreased availability of monoamines.

Evidence for changes in brain structure playing a role in MDD comes from imaging studies. Neurophysiological measures identified a multitude of brain regions that are crucial to the emotional processing and stress regulation of which are affected by depression (Koolschijn et al., 2009). For instance, a reduction in grey matter volume, can be observed in the anterior cingulate cortex, prefrontal cortex, ventromedial striatum, amygdala and the hippocampal regions of individuals with early onset (<25 years of age) MDD. Reductions in neuronal pro-survival signals such as serotonin and brain derived neurotropic factor, along with a decrease in the volume and glial cell density of these

brain regions have also been examined (Martinowich and Lu, 2008; Russo and Nestler, 2013). Whether these reductions are due to genetic factors or early life events that precede depression onset, or if the depression brings about these neurophysiological changes is still unclear (Arnone et al., 2013). Further, there is an overall inconsistency in the literature concerning the exact structural changes in the brain of individuals with MDD. However, a combination of environmental factors such as the severity of allostatic load and timing of tragic life events along with neurophysiological factors such as the genetic predisposition, subtypes of depression and other neurodevelopmental diseases that may contribute to MDD make this multifactorial disorder difficult to delineate (Bijanki, Hodis, Brumm, Harlynn, & McCormick, 2014; Drevets, Price, & Furey, 2008; Sheline, Sanghavi, Mintun, & Gado, 1999). The heterogeneity in the neuropathology of depression and lack of a reliable research supporting neuroimaging measures of depression led researchers to investigate the expression of genes that play a role in the onset of MDD as biomarkers for this disorder.

The serotonin reuptake transporter, SLC6A4 is the target of many antidepressants; inhibition of SERT suppresses the reuptake of monoamines, thereby therapeutically increasing the monoamine concentration in the synaptic cleft. A considerable body of research has been dedicated to investigating the serotonin-transporter-linked polymorphic region (5-HTTLPR). This promoter region of the serotonin transporter gene has two allelic variants, a short (S) and long (L) (Bradley, Dodelzon, Sandhu, & Philibert, 2005). To date there has been no consensus with regards to effect of these allelic differences on

the likelihood of developing MDD. On one hand, studies linked the S allelic variant with decreased transcription of SERT, elevated hypothalamic-pituitary-adrenal (HPA) axis reactivity to adverse life events and an increased incidence of MDD (Bradley et al., 2005; Kiyohara & Yoshimasu, 2010; Vinberg, Miskowiak, & Kessing, 2014). Contrarily, a meta-analysis examining associations with the genetic polymorphisms of this gene in individuals with mental disorders such as MDD identified the L allele to be predictive of suicidal behavior (Bradley et al., 2005; de Medeiros Alves et al., 2015). Research demarcating the specific contribution of serotonin transporter polymorphisms contribution to the onset of MDD needs to be further examined.

The most commonly accepted hypothesis for the etiology of MDD is the monoamine hypothesis of depression. This hypothesis postulates a decreased bioavailability of neurotransmitter monoamines such as 5-hydroxytryptamine (5-HT; serotonin), dopamine and norepinephrine resulting in decreased central neural transmission. This hypothesis has been extensively studied, with an emphasis on the alteration of the serotonergic system on the etiology of MDD (Artigas, 2013; Nestler and Hyman, 2010; Nestler et al., 2002). Among the most intriguing findings, studies performed on patients who had unsuccessfully attempted suicide were found to have reduced serotonin levels in cerebral spinal fluid and a reduced serotonin transporter (SERT) binding potential in the midbrain and brain stem (J. J. Mann et al., 1996; Nye et al., 2013). In the brain, serotonin is synthesized from the amino acid L-tryptophan and is enzymatically converted to 5-hydroxy-L-tryptophan (5-HTP) by the rate limiting enzyme

tryptophan hydroxylase 2 (Tph 2) (Fidalgo et al., 2013). At the nerve terminals, serotonin is stored in synaptic vesicles, whereby it is released by exocytosis into the synaptic cleft upon membrane depolarization and subsequently binds to the serotonergic receptors (5-HT1-7) on the post synaptic neuron. Serotonin in the synaptic cleft is taken back into the presynaptic neuron via the serotonin transporter (SERT), and oxidized by monoamine oxidase to its metabolite 5-hydroxyindoleacetic acid (Ruddick et al., 2006). Antidepressants that target the serotonin transporter (i.e., selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors) reduce the ability of this transporter to remove serotonin from the synaptic cleft thus increasing the bioavailability of serotonin at the receptors on the postsynaptic neurons (Ruddick et al., 2006).

1.2Selective Serotonin Reuptake Inhibitors

1.2.1 Prevalence of Antidepressant Use

Pharmacotherapy is the leading option for treatment and management of moderate to severe MDD, with a marked upsurge of nearly 400% in the prescription of antidepressants in the past 20 years, a growing trend that is prevalent worldwide (Chien et al., 2007; Ilyas & Moncrieff, 2012; National Center for Health Statistics (US), 2011; Smith et al., 2008). Despite the plethora of medications for the treatment of depression, drugs that aim to modulate the serotonin signaling by targeting the serotonin reuptake transporter are most widely prescribed. Accounting for 90% of antidepressants prescribed, these drugs fall into a variety of classes which include selective-serotonin reuptake inhibitors (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), serotonin receptor antagonist and

reuptake inhibitors (SARIs), and noradrenergic and specific serotonergic antidepressants NaSSAs (Artigas, 2013). In particular, the SSRI's have been the leading class of drugs prescribed, a trend seen in many countries (Smith et al., 2008; Trifirò et al., 2013; Wemakor, Casson, & Dolk, 2014). Approved by the FDA in 1987, Fluoxetine rapidly became the most prescribed SSRI and psychotropic drug across the world (Leo, 1996; Pinna, 2015).

1.3 Depression and Type 2 Diabetes Mellitus

1.3.1 The Bidirectional Relationship

According to the World Health Organization, depression is the leading cause of disability worldwide (World Health Organization, 2018). While depression has been linked with a number of other illnesses, its association with metabolic disorders is quite evident. In particular, MDD is linked with a high risk of developing type 2 diabetes (T2DM) (Holt, Peveler, & Byrne, 2004; Newcomer, 2007; Vancampfort et al., 2014). T2DM is a chronic disease marked by insulin resistance and beta cell dysfunction leading to a fasting glucose level $\geq$ 7.0 mmol/L or $\geq$ 11.1 mmol/L 2 hour post oral glucose (75g) load. In T2DM, insulin resistance can lead to adverse metabolic outcomes such as hyperglycemia but dysfunction of the beta cell is fundamental to the progression of this disease. In addition to contributing to fatty liver, hepatic insulin resistance reduces insulin's ability to control the uptake of glucose into the hepatocyte, hindering the amount of hepatic glucose and overall lipid production. The resulting hyperglycemia triggers the

beta cells to secrete more insulin to compensate for this peripheral insulin resistance. This compensatory over production of insulin may result in dysfunction of the beta cells, ultimately leading to their demise.

A plethora of risk factors can contribute to an increased incidence of diabetes in individuals with MDD, these include but are not limited to familiality and genetic predisposition, socioeconomic status, sex, age as well as maladaptive behaviors that develop during a depressive episode such as binge eating and a reduction in physical exercise (Chien, Wu, Lin, Chou, & Chou, 2012; Katon, 2008). While the directionality of the relationship between MDD and T2DM is unclear, the association between these two disorders has been examined extensively (Hasan, Mamun, Clavarino, & Kairuz, 2015; Kan et al., 2013; Mezuk, Eaton, Albrecht, & Golden, 2008; A. Pan et al., 2012; An Pan et al., 2010; Renn, Feliciano, & Segal, 2011; Rotella & Mannucci, 2013). In 2013, Diabetes was predicted to affect 382 million people worldwide, a number expected to grow by 55% to 592 million by 2035, particularly in the developing countries (King, Aubert, & Herman, 1998; Zimmet, Alberti, & Shaw, 2001). These increasing rates of T2DM prevalence in developed countries are paralleled by similar trends in the prevalence of MDD, which has led some to suggest that MDD and T2DM may share a common pathophysiology. This association is strongly supported by a 42 study meta-analysis conducted by Anderson and colleagues that identified MDD to be approximately 2 times more likely in patients with diabetes compared with the general population (OR=2.0; 95% CI 1.8-2.2) (Anderson, Freedland, Clouse, & Lustman, 2001). It has been suggested that this increased likelihood of developing diabetes has been due to the related fundamental

biochemical changes that accompany diabetes, as well as the burden of the disease and its treatment (Mirjam J. Knol et al., 2007; Renn et al., 2011; Talbot & Nouwen, 2000). On the other hand, a number of studies suggested that individuals suffering from MDD are at an increased risk of new-onset T2DM, with some meta analyses reporting as much as a 60% (RR=1.60, 95% CI=1.37-1.88) increased risk of developing T2DM (Campayo et al., 2010; Engum, 2007; Hirschfeld, 2001; M. J. Knol et al., 2006). This body of research shows strong support for the link between MDD and diabetes, however, the mechanism(s) are poorly understood but may be related to the drugs used to treat MDD (Deuschle, 2013; A. Pan et al., 2012; Rubin et al., 2008; Yoon, Cho, Lee, & Park, 2013).

1.3.2 SSRIs Mechanism of Action

SERT plays a vital role in modulating serotonin signaling. Its ability to reuptake of serotonin from the synaptic cleft for its catabolism in the presynaptic neuron renders this transmembrane protein as a target for SSRI's. SSRIs act to inhibit the presynaptic SERT, thus increasing the serotonin levels in the synaptic cleft whereby it can exert its effect on the postsynaptic neuron (Fidalgo et al., 2013). Therapeutic effects of SSRI take time, with a period of 2-6 weeks before clinical efficacy. This latency has been hypothesized to be caused by time required to desensitize the 5-HT1A serotonin receptor. The 5-HT1A receptors act as an autoreceptor-mediated negative feedback loop, and can act to decrease the synthesis and packaging of the serotonin neurotransmitter. As a result of chronic exposure to SSRIs, the elevated and continuous stimulation by serotonin desensitizes 5-

HT1A receptors in the raphe nucleus, increasing the serotonergic neurotransmission by means of endocytosis or internalization of these receptors (Le Poul et al., 2000, 1995)

1.3.3 Are Antidepressants the Link?

SSRI's are known for their therapeutic effects regarding treatment of MDD as a result of their effects on inhibiting serotonin reuptake in the brain. However, these commonly prescribed serotonin targeting antidepressants may be resulting in the onset and development of T2DM (Deuschle, 2013; A. Pan et al., 2012; Rubin et al., 2008). In a recent meta-analysis of 8 different studies, Yoon and colleagues indicated that there was a notable heightened risk of developing T2DM among patients using serotonin antidepressants (RR: 1.35; 95% CI: 1.15-1.58) (Yoon et al., 2013).

Hallmarks of T2DM such as dysregulation of glucose homeostasis have been associated with the use of serotonin modulating antidepressants such as sertraline, fluvoxamine, paroxetine, venlafaxine, fluoxetine, citalopram, and mirtazapine (Derijks et al., 2008; Khoza & Barner, 2011). Additionally, a retrospective cohort study reported an increased odds ratio of developing T2DM in adolescents and children taking SSRIs (OR =1.37; 95% CI 1.10-1.71) (Jerrell, 2010). Andersohn and colleagues examined different factors that may contribute to the development of diabetes and identified, the length to time in which an individual is taking SSRI's, the mean daily dosage, and the type of SSRI to be significant contributors (Andersohn, Schade, Suissa, & Garbe, 2009). The risk of T2DM with SSRI use is more pronounced with long term use of antidepressants, as

individuals taking SSRI's for greater than 2 years had an incidence rate ratio of 2.06 for developing diabetes (95% CI=1.20-3.52) (Andersohn et al., 2009). Quite interestingly, the average absolute risk difference for new-onset diabetes between individuals taking any antidepressant medication (including participants using the most commonly prescribed SSRI's) and those without antidepressant treatment was 2.87 per 1,000 person-years (A. Pan et al., 2012). Although other factors may increase the risk of of new-onset diabetes in individuals suffering from MDD, 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, Gau, & Lai, 2014; Yoon et al., 2013). All in all, these studies provide a strong basis to support the hypothesis that SSRI use may play a role in the development of T2DM. Although the specific underlying mechanism has not been fully delineated, SSRI's modulate the serotonergic signaling which may in turn affect other signaling processes in the pancreatic beta cells which are important in the pathophysiology of T2DM (Dhavale, Panikkar, Jadhav, Ghulghule, & Agari, 2013; Gehlawat, Gupta, Rajput, Gahlan, & Gehlawat, 2013).

1.4 Pancreatic Beta Cell Function

1.4.1 Insulin Synthesis and Secretion

Pancreatic beta cells are localized in the islets of Langerhans and are important for the pancreas' endocrine function in synthesizing, packaging, and secreting insulin. This peptide hormone is composed of 51 amino acids and binds to insulin receptor on organs, facilitating the uptake of glucose. In the ribosomes of the rough endoplasmic reticulum of

beta cells, insulin is synthesized from preproinsulin. This is further cleaved into proinsulin, which is subsequently transported to the Golgi apparatus for packaging into secretory granules localized near the cell membrane. Decreased beta cell survival and/or perturbations in insulin synthesis or secretion may lead to the development of T2DM (Ahren, 2005). Given the previous research that found that SSRI exposure could increase the risk of T2DM and cause beta cell dysfunction, it is important to understand the mechanisms linking SSRI use to beta cell dysfunction (Derijks et al., 2008; Khoza & Barner, 2011).

1.4.2 Focus on the Serotonergic System

Serotonin, also known as 5-Hydroxytryptamine (5-HT) is widely known for its role in the central nervous system. Serotonin is responsible for brain functions including emotion, cognition, motor function, as well being as a regulator of neuroendocrine functions such as food intake, sleep and circadian rhythms (Martinowich and Lu, 2008). Despite serotonin's prominent role as a neurotransmitter, its importance extends beyond its neurological functions; serotonin also has a key role in a number of physiological processes peripherally. Quite surprisingly, only 5% of the body's serotonin is found in the brain (D.-Y. Kim & Camilleri, 2000). Peripheral serotonin production is mediated by the rate limiting enzyme, tryptophan hydroxylase 1 (Tph1), which converts L-tryptophan into 5-hydroxy-L-tryptophan (5-HTP) (Berger, Gray, & Roth, 2009). 5-HTP is subsequently converted to 5-HT (Serotonin) via aromatic l-amino acid decarboxylase, where by it can exert its effect both in the periphery. As serotonin is unable to cross the blood brain barrier, it is important to note that two distinct functional

pools of serotonin exist (Gaspar et al., 2003). Ever since the 1960's, the presence of serotonin in pancreatic beta cells has been known. It was later determined that all fundamental components of the serotonergic pathway are expressed in pancreatic beta cells. This includes but is not limited to serotonin receptors, plasma membrane serotonin transporter, tryptophan hydroxylase (Tph1 and Tph2), monoamine oxidase and aromatic L-amino acid decarboxylase (Huang, Ito, & Arai, 2005; D.-Y. Kim & Camilleri, 2000; Paulmann et al., 2009; Richmond, Codignola, Cooke, & Sher, 1996; Schraenen et al., 2010).

Serotonin is undoubtedly crucial for proper beta cell function. In addition to being co-localized with insulin in pancreatic beta cells, it is associated with the serotonergic gene *fev* (also known as the homologue of Pet-1). Pet-1 is a key molecule known for its function as a cascade factor required for the initiation of maturation of serotonergic neurons, as well as serotonin synthesis in both neurons and pancreatic beta cells (Ohta et al., 2011). Pet1 knockout mice have impaired insulin production and secretion, suggesting a link between serotonin and insulin signaling in the pancreatic beta cells (C. Liu et al., 2010; Ohta et al., 2011). Antidepressants that act to modulate the serotonergic system may contribute to T2DM by adversely affecting pancreatic beta cell function and survival, both of which are hallmarks of T2DM. While serotonin's co-localization with insulin in granules has been identified over four decades ago, serotonin's crucial role in the etiology of diabetes is poorly defined. A study conducted by Paulmann and colleagues reported that intracellular serotonin regulates insulin secretion, and is co-released upon glucose stimulation (Paulmann et al., 2009). In this study, *Tph1* deficient mice had a 90%

decrease in serotonin in the pancreatic beta cells compared to the wild type mice. This difference in serotonin was functionally evident as glucose tolerance tests revealed *Tph1* knockout mice had exhibited significantly higher blood glucose concentrations, a mild insulin deficiency, and beta cell dysfunction compared to wild type mice. Despite the dysglycemia and impaired insulin secretion, these *Tph1* deficient mice did not display any reduction in beta cell mass (Paulmann et al., 2009). Most notably, treatment with the *Tph1* product and serotonin precursor, 5-HTP normalized blood glucose levels and largely rescued the aberrant insulin secretion in the Tph1 deficient mice (Paulmann et al., 2009). Alternatively, pharmacological inhibition of serotonin catabolism with pargyline is known to contribute to a hypoglycemic and hyperinsulinemic response in mice (Paulmann et al., 2009). This restorative effect of pargyline induced hypoglycemia and increased plasma insulin exclusively in wild type, but not in *Tph1* deficient mice, further indicates serotonin's importance in the secretion of insulin from pancreatic beta cells (Paulmann et al., 2009). These findings were supported by the patch clamp experiments which found the secretory response in *Tph1* deficient beta-cells to be significantly impaired, only to be partially rescued by extracellular 5-HTP and completely restored by serotonin (Paulmann et al., 2009). Taken together these data demonstrate that perturbations in serotonin signaling adversely affect beta cell function by impairing insulin secretion and causing beta cell dysfunction leading to dysglycemia and other hallmarks of T2DM.

SSRIs can negatively affect insulin secretion in both animal and human studies. For example, studies in rats and humans have shown that SSRI antidepressant exposure

caused significant reductions in insulin response to an oral glucose tolerance test (Chen et al., 2007; Li et al., 2014). Beta cell mass, another significant contributor to the pathogenesis of diabetes may also be compromised by some SSRI's. *In vivo* experiments that disrupted the synthesis or degradation of serotonin and disturbed overall serotonin signaling have been shown to affect the beta cell mass (Edvell & Lindström, 1999; D.-Y. Kim & Camilleri, 2000). Indeed, SSRIs have also been shown to have effects on beta cell survival, and even increase beta cell apoptosis (N. E. De Long, Hyslop, Raha, Hardy, & Holloway, 2014; Isaac et al., 2013). In particular, De Long and colleagues reported that treatment of rat INS-1E with the SSRI fluoxetine led to adverse outcomes such as mitochondrial dysfunction and oxidative stress resulting in the ablation of glucose stimulated insulin secretion (N. E. De Long et al., 2014). A study investigating another insulin secreting cell line, Min6 cells treated with the SSRI sertraline resulted in impaired glucose-stimulated insulin secretion, endoplasmic reticulum stress, the unfolded protein response and increased beta cell apoptosis (Isaac et al., 2013). Furthermore, the SSRIs sertraline, paroxetine and fluoxetine have all been shown to affect tyrosine phosphorylation of IRS-2, with the sertraline displaying a drastic 40-60% reduction. This alteration of IRS signaling may have led to the observed inhibited glucose-stimulated insulin secretion as IRS signaling is an important contributor to insulin secretion (Hennige et al., 2003). SSRI's have further been shown to affect targets downstream of the insulin receptor such as inhibiting protein kinase B (PKB/Akt) activation (Isaac et al., 2013). This inactivation can result in the downstream inactivation of Akt, the key regulator of beta cell survival, proliferation and beta cell mass (Elghazi, Rachdi, Weiss, Cras-Méneur, &

Bernal-Mizrachi, 2007). It is thus suggested that SSRIs function as modulators of serotonin signaling may lead to a disruption of serotonergic pathways in beta cells, thus leading to beta cell dysfunction. However, the pathways by which serotonin perturbations can be linked to beta cell dysfunction still remain to be fully elucidated but may involve hedgehog signaling.

1.5 Serotonin and Hedgehog Signaling, what's the link?

In mammalian cells, three hedgehog homologs - *Sonic hedgehog* (*SHh*), *Indian hedgehog* (*IHh*), and *Desert hedgehog* (*DHh*) - have been previously identified, and play an key role in limb patterning, bone growth and nerve development respectively (Chiang et al., 1996; Parmantier et al., 1999; St-Jacques, Hammerschmidt, & McMahon, 1999). In vertebrates, these three homologs are similarly processed, modified, and released, exert their effect by inactivating the hedgehog signaling inhibitor, patched (Ptch1) (R. K. Mann & Beachy, 2004; Nakano et al., 1989). Binding of a hedgehog homologue to Ptch1 prevents Ptch1 from inhibiting Smoothened (Smo), the positive regulator of hedgehog signaling (van den Heuvel & Ingham, 1996). Once a hedgehog homolog relives Smo from its inhibition, this G-protein-coupled receptor activates genes in the downstream hedgehog signaling pathway(Beachy, Hymowitz, Lazarus, Leahy, & Siebold, 2010). Vertebrates have three glioma-associated oncogenes (Gli), namely Gli1, Gli2, and Gli3. Gli1, the ultimate effector molecule of the hedgehog pathway, is activated upon activation via Smo, whereby it activates transcription of downstream target genes and is itself a

transcriptional target of the pathway (Hynes et al., 1997). Figure 1 below depicts intercellular hedgehog signaling.

Although more commonly known for its role in mammalian development, hedgehog signaling is of particular importance to the insulinotropic function of beta cells (Thomas, Rastalsky, Lee, & Habener, 2000). Among the hedgehog homologs, SHh has been most extensively researched as it's been identified as a key morphogenetic factor and loss of function of SHh resulted in severe patterning defects such as cyclopia. It is important to note that in pancreatic beta cells, only the IHh and DHh homologs are present, and play a crucial role in healthy beta cell function and insulin release (Thomas et al., 2000). In the study conducted by Thomas and colleagues, the rat insulinoma cell line INS-1 was used to demonstrate that an inhibitor of hedgehog signaling pathway (cyclopamine) was able to inhibit insulin mRNA expression, promoter activation, content and secretion significantly (Thomas et al., 2000). Many studies that aim to diminish hedgehog signaling make use of a pharmacological agent known as cyclopamine, which exerts its inhibitory function by binding to Smo and impedes translocation of Gli proteins into the nucleus, leading to decreased expression of hedgehog target genes (Alam, Sohoni, Kalainayakan, Garrossian, & Zhang, 2016). Contrarily, cyclopamine, the hedgehog signaling inhibitor has also been shown to expand embryonic pancreas development in chick embryos (S. K. Kim & Melton, 1998). This led the authors to suggest cyclopamine as an agent to promote development of cell-replacement therapies for diabetes mellitus. While these two articles are conflicting regarding hedgehog signaling's importance to pancreatic tissue, hedgehog signaling undoubtedly has an

important role to play in pancreatic development and function (S. K. Kim & Melton, 1998; Thomas et al., 2000).

Previous research has identified an association between serotonin and hedgehog signaling, although the directionality of this relationship differs between cell types (Charytoniuk et al., 2002; Hynes et al., 1995; Laporta et al., 2014; Miao et al., 1997; Wang et al., 1995; Ye, Shimamura, Rubenstein, Hynes, & Rosenthal, 1998). While hedgehog signaling regulates the differentiation and survival of serotonergic neurons, a study published recently highlighted serotonin's role in activating hedgehog signaling in mammary epithelial cells (Laporta et al., 2014). Laporta and colleagues identified a correlation between serotonin and the epigenetic activation of hedgehog signaling genes. Using the *Tph1*-deficient mouse model, serotonin was found to induce hedgehog signaling by remodeling SHh promoter methylation which led to a new paradigm of serotonin mediated hedgehog signaling regulation. Furthermore, monoamines such as serotonin are widely known to be metabolized by monoamine oxidase (MaO). Although two isoforms of this enzyme exist, serotonin metabolism is primarily carried out by MaO-A. Importantly, acute treatment with the monoamine releasing agent, para-chloroamphetamine has been shown to upregulate Smo expression, a gene important in the hedgehog signaling pathway in rat brain (Rajendran et al., 2009). These results indicate that changes in serotonergic signaling and metabolism can regulate hedgehog signaling. It is well established that SHh also regulates the differentiation and survival of serotonergic neurons in the midbrain (Hynes et al., 1995a,b; Wang et al., 1995; Ye et al.,

1998). Despite the importance of hedgehog signaling in healthy insulin secretion, and the link between serotonin and hedgehog pathways, there are no studies to date investigating the effects of SSRI treatment on the regulation of hedgehog signaling in pancreatic beta cells, (Charytoniuk et al., 2002; Hynes et al., 1995; Miao et al., 1997; Wang et al., 1995; Ye et al., 1998). This thesis aims to examine this relationship further.

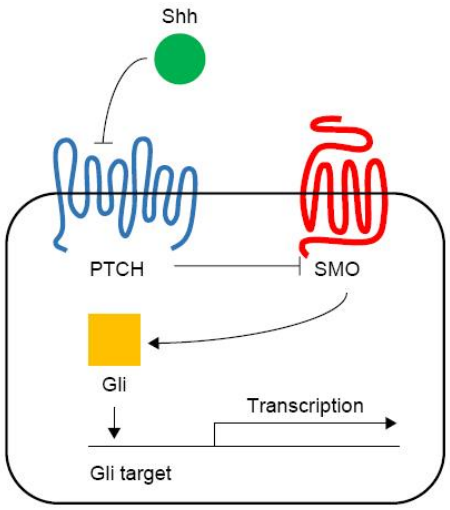

Figure I. A Visual Representation of Intercellular Hedgehog Signaling. Image adapted from "Jain, S., Song, R., & Xie, J. (2017). Sonidegib: mechanism of action, pharmacology, and clinical utility for advanced basal cell carcinomas. *OncoTargets and therapy*, *10*, 1645.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1 Rationale and Hypothesis

It is plausible that the correlation between the use of SSRIs and new-onset T2DM may be attributed to the decrease in beta cell function related to perturbations in pancreatic serotonergic signaling pathways. Indeed, our research group has previously demonstrated that acute fluoxetine treatment at concentrations representing the median and 85th percentile of human serum concentrations (1μM) resulted in diminished beta cell glucose-stimulated insulin secretion, decreased beta cell survival and impaired mitochondrial electron transport chain activity (N. E. De Long et al., 2014). However, despite the importance of hedgehog signaling in pancreatic beta cell function and identified links between hedgehog and serotonin signaling pathways there have been no studies which have ascribed SSRI-induced beta cell dysfunction to perturbations in hedgehog signaling. Therefore the goal of this study is to test the hypothesis that pancreatic beta cell exposure to the SSRI fluoxetine will lead to altered hedgehog signaling, ultimately resulting in a disruption in the insulinotropic function of pancreatic beta cells.

2.2 Specific Aims

1. Investigate the effects of the SSRI fluoxetine on hedgehog and serotonin signaling pathways important for beta cell function and insulin release.

2. Examine the effect of inhibition of serotonin signaling on altered hedgehog signaling *in vitro*, adversely affecting insulin content in pancreatic beta cells.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell Culture Maintenance and Treatment

Rat insulinoma 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. Cells were grown in 100mm x 20mm polystyrene tissue-culture treated plates (Corning, 353003, Corning, NY, USA). RPMI-1640 (RPMI; Sigma Aldrich, Oakville, ON) was supplemented with 1mM sodium pyruvate, 50μM β-mercaptoethanol, 1mM glutamine, 10% heat-inactivated fetal bovine serum (Gibco, 12483-020, Grand Island, NY, USA), 10mM HEPES and 1% penicillin streptomycin (Gibco, 15140-122). Unless otherwise noted, experimental protocols were carried out in RPMI media supplemented as described above.

INS-1E cells were seeded in 100mm dishes and grown until they reached 80% confluence. The rat insulinoma INS-1E cell line was ideal for this study they have morphological and physiological characteristics typical of native beta-cells such as glucose stimulated insulin release (Asfari et al., 1992). Cells were then washed with PBS (Lonza, Walkersville, MD) and then incubated with either 0 μM (vehicle control), 0.01, 0.05, 0.1, 0.5 or 1 μM fluoxetine hydrochloride (Toronto Research Chemicals, North York, ON) for 24hrs; these concentrations represent the range of physiologically relevant

doses (Keller et al., 2005; Reis, Aamo, Spigset, & Ahlner, 2009). The highest dose of Fluoxetine, 1µM concentration approximates the 90th percentile of human serum fluoxetine concentrations (i.e., 1.2 µM)(Keller et al., 2005; Reis et al., 2009). At this dose, our research group previously examined that beta cells displayed diminished glucose stimulated insulin secretion, mitochondrial dysfunction and decreased cell viability albeit at higher concentrations (N. E. De Long et al., 2015, 2014; N. De Long, E, Gutgesell, Petrik, & Holloway, 2015). When examining *Tph1,* INS-1E were treated with only the 0.5 and 1µM doses of Fluoxetine. To determine the effect of inhibiting serotonin production on beta cell function, cells were treated fluoxetine or 50 µM para-chlorophenylalanine (4-Chloro-DL-phenylalanine; PCPA) (Sigma-Aldrich, C6506) a serotonin synthesis inhibitor, which acts by inhibiting the rate limiting enzyme Tph (Koe & Weissman, 1966) for 24hr. Treatments were diluted in the supplemented media, prepared as described above.

3.2 RNA Extraction and Quantification

After 24hrs, INS-1E cells were harvested using TRIzolR Reagent (Ambion, 15596018, Carlsban CA, USA) and stored at -80°C until further extraction. Extracted RNA was homogenized with a 20G needle and incubated for 5-10 minutes at room temperature. Following the addition of 0.2 ml of chloroform (ACP Chemicals Inc., Montreal, Quebec), the solution was vortexed for 15 seconds and was incubated for 2-3 minutes at room temperature. After centrifugation at 4°C and 12, 000g for 15 minutes, a clear supernatant was collected and the remainder was discarded. RNA was precipitated by the addition of 0.5 mL of isopropanol (ACP Chemicals Inc., Montreal, Quebec) to the

collected supernatant and incubated at room temperature for 10 minutes. This solution was then centrifuged at 12,000 g for 10 minutes at 4°C. The RNA pellet was washed in 75% ethanol by homogenization and centrifuged further at 10, 000 g for 5 minutes at 4°C. This step was repeated twice. After the second ethanol wash and centrifugation, the supernatant was discarded and the RNA pellet was suspended in 30 µl of nuclease-free H2O water (Qiagen, 129115, Hilden, Germany). The concentration and purity of the extracted RNA was verified using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and stored at -80°C until further use.

3.3 Complimentary DNA (cDNA) Synthesis

As per the manufacturer's instructions 4 µg of cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814, Foster City, CA, USA). The iCycler thermocycler (BioRad Laboratories, Hercules, CA, USA) was used to make a total of 40 µL of cDNA. The iCycler Thermocycler synthesized the cDNA via the following protocol: 25°C for 10 min; 37°C for 120 min; 85°C for 5 min. A 1:40 dilution of cDNA samples was performed in nuclease-free H2O and all cDNA was stored at -20°C until further use.

3.4 Primer Design

Primers were designed using Primer-BLAST (National Center for Biotechnology

Information, Bethesda, MD, USA). PCR product size was set to 50-150 base pairs. The following melting temperature conditions were followed: Minimum: 58°C; Optimal: 60°C; Maximum: 60°C; Max difference: 2°C. Each primer sequence was run through OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA, USA) to check for hairpin, self-dimer and heterodimerization. Sequences selected had a ΔG (kcal/mol) > than -9 for all parameters. Primer sequences were synthesized by MOBIX Lab (Sanger Sequencing and Oligo Synthesis Facility, Hamilton, ON, Canada) and validated by examining melting point curves.

3.5 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

To determine the relative fold change of each gene transcript, RT-qPCR was performed using the Quantabio Perfecta SYBR Green FastMixR (95072-05K, Beverly, MA, USA) and LightCycler 480 384-Multiwell Plates (0472974900, Roche, Mannheim, Germany). Polymerase Chain Reaction was conducted using the Light Cycler 480 II (Roche, Mannheim, Germany) with one cycle of activation (95°C for 10 min); 50 cycles of denaturing (95°C for 10 sec); annealing (60°C for 10 sec); and elongation (72°C for 15 sec). Samples were run in triplicate and the expression of each gene was normalized to the geometric mean of HPRT (hypoxanthine phosphoribosyltransferase) and cyclophilin's expression. The relative fold changes were determined using the comparative double delta cycle times (Ct) method (2ΔΔCT), as previously described (Livak & Schmittgen, 2001). Table 1 below shows the list of primer sequences used for RT-qPCR.

3.6 Protein Extraction

INS-1E cells were seeded in 100mm dishes and grown until they reached 80% confluence. At confluency, the cells were treated with 0, 0.5μM and 1μM Fluoxetine and incubated at 37°C for 24 hours. After 24 hours, media was aspirated and the plates were washed with 2 mL of DPBS three times. 1000 μL of RIPA Lysis Buffer (150 mM sodium chloride (Bioshop, Burlington, ON, CA), 1% Triton X-100 (Sigma-Aldrich, St. Louis, NJ, USA), 0.5% sodium deoxycholate (Sigma-Aldrich, St. Louis, NJ, USA), 0.1% SDS (Bioshop, Burlington, ON, CA), 50 mM Tris HCL (Sigma-Aldrich, St. Louis, NJ, USA) and 1 EDTA free mini protease inhibitor tablet per 10 mL RIPA buffer (Roche, Mannheim, Germany) was added to each plate. Cell scrapers were used to mechanically detach the cells, and the suspensions were transferred into Eppendorf tubes. Samples were then sonicated using the Sonic Dismembrator Model 100 (Fisher Scientific, Waltham, MA, USA) for 10 seconds at 7 Hz. For samples separated by cytosolic and nuclear fraction, fractionation was performed as per Nuclear/Cytosol Fractionation Kit instructions (BioVision Inc., Milipitas, CA, USA).

3.7 Protein Quantification

Quantification of total protein was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) following manufacturer's instructions. Briefly, diluted albumin (BSA) standards were prepared ranging from 0 to 2000 μg/mL in concentration. Protein samples were also diluted 1:5 in RIPA buffer. 25 μL of each

standard and unknown sample was pipetted to a 96-well plate, and 200 µL of Working Reagent (50:1 Reagent A:B) was added to each well. The plate was incubated at 37°C for 30 minutes then its absorbance measured at 562 nm (Biotek Synergy H4 Hybrid Reader).

3.8 Insulin and Serotonin ELISA

Using protein samples from INS-1E treated with Fluoxetine and para-chlorophenylalanine (PCPA), insulin and serotonin quantification was performed using commercially available ELISA kits according to the manufacturer's instructions, at a dilution of 1:250 (insulin: Crystal Chem Inc. Downers Grove, IL, USA; LDN, Nordhorn, Germany). Absorbance of both ELISA's was read using a plate reader (Synergy™ H4) Hybrid Microplate Reader (BioTek Instruments, Winooski, VT, USA). Concentration of both insulin and Serotonin were then normalized to protein content obtained via BCA assay.

3.9 Protein Expression (Western Blotting)

Loading samples were prepared by diluting the sample cell suspensions in nuclease free water to 10 µg protein in a final volume of 25 µL. To each sample, 25 µL of 2x Laemmli Sample Buffer (BioRad, Mississauga, Canada) containing 5% β-mercaptoethanol (EMD, Gibbstown, NJ, USA) was added. Samples were then heated at 70°C for 10 minutes, and vortexed. Each sample was loaded into a 50 µL well of a Mini-PROTEAN SFX 10% Stain-Free Gel. Prism Ultra Protein Ladder 10 - 180 kDa ab116027

(abcam, Cambridge, MA, USA) was run in each gel as a standard for protein size. The gel was immersed in 1x Tris/Glycine/SDS Running Buffer (BioRad, Mississauga, Canada) and subjected to gel electrophoresis. Following gel electrophoresis, the gel was equilibrated in transfer buffer from the Trans-Blot Turbo RTA transfer kit (BioRad, Mississauga, Canada). Next, the protein was transferred to a PVDF membrane using the Trans-Blot Turbo RTA Transfer System and Kit (BioRad, Mississauga, Canada). After transfer, the membrane blots were blocked for an hour with 5% BSA in Tris Buffer Saline (BioRad, Mississauga, Canada) with 10% Tween 20 (BioRad, Mississauga Canada). Following blocking, the blot was incubated overnight at 4°C with primary antibody diluted in TBST containing 3% bovine serum albumin (Table 2.1). The next day, the membrane was subjected to 6 x 5 minute washes in TBST before being incubated for 1 hour with secondary antibody, also diluted in TBST containing 3% BSA (Table 2.2). After incubation, the membrane underwent 6 x 5 minute washes of TBST. Finally, the blots were soaked in 8mL of Clarity Western ECL solution (1:1 luminol enhancer solution to peroxidase solution) (BioRad, Mississauga, Canada) for 5 minutes then imaged using ChemiDoc XRS. Protein band densities were quantified using Image Lab v 6.0 (BioRad, CA, USA). Where required, Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific, Rockford, IL, USA) was used, then blots were blocked and reprobed with another primary antibody in order to quantify multiple proteins with relatively similar molecular weights.

Table 1: Primers used to detect target genes in INS-1E cells. All primers were ordered from MOBIX (McMaster University, Hamilton) at a concentration of 25 nM. F = Forward primer; R = Reverse Primer. All sequences are listed 5' to 3'.

Target Gene	***Accession Number***	***Primer Sequence***
HPRT	NM_012583.2	F: 5'-GCA GTA CAG CCC CAA AAT GG-3' R: 5'-GGT CCT TTT CAC CAG CAA GCT-3'
CYC	NM_017101.1	F: 5'-CCG CTG TCT CTT TTC GCC-3' R: 5'-GCT GTC TTT GGA ACT TTG TCT GC-3'
Gli1	NM_001191910.1	***F:*** 5'-TCC CCA TGA CTG TCT CTC GT-3' R: 5'-ACA ATT CCT GCT GCG ACT GA-3'
DHh	NM_053367.1	***F:*** 5'-AAC TTT GAG TGG GGT GGG AC-3' R: 5'-AAC TCC AAA TGG GTC GAG CA-3'
IHh	NM_053384.1	***F:*** 5'-AGC TGT GTC TGA CCA CCA TC-3' ***R:*** 5'-GCT GAG GGT ACC AGT GAA CA-3'
INS1	NM_019129.3	***F:*** 5'-CCC TAA GTG ACC AGC TAC AAT CAT AG-3' ***R:*** 5'-GGG CCA TGT TGG AAC AAT G-3'
INS2	NM_019130.2	***F:*** 5'-GCG GCA TCG TGG ATC AGT -3' ***R:*** 5'-GCC TAG TTG CAG TAG TTC TCC AGT T-3'
Tph1	NM_001100634.2	***F:*** 5'-GCG GCA TGA CCT CGA TGT-3' ***R:*** 5'-CTG GGC CAC CTG CTG ACT-3'
Tph2	NM_173839.2	***F:*** 5'-ACG CTG AAT CCA CCT GAC AA-3' ***R:*** 5'-GAA CCA CGG CAC ATC CTC TA-3'
Ptch1	NM_053566.1	***F:*** 5'-TGG AAG TTG GTG GAC GAG TG-3' R: 5'-TCT CCT ATC TTC TGG CGG GT-3'
Smo	NM_012807.1	***F:*** 5'-CGT GAG TGG CAT CTG CTT TG-3' ***R:*** 5'-GGG TGG TTG CTC TTG ATG GA-3'
Gli2	NM_001107169.1	***F:*** 5'-TGA GAT GAC ACG CTC AAC TTG TA-3' ***R:*** 5'-TCA GGA GAA AAG CAG ATC GAC A-3'
Hhip	NM_001191817.1	***F:*** 5'-GTG TGG TGG CTT CTA CCC AA-3' ***R:*** 5'-ATC TTG TTC TCC AGA CGC CC-3'

Table 2.1 Primary antibodies used to detect target protein.

Primary Antibody	*Company (Catalogue #)*	*Dilution*
Rabbit monoclonal [EP1192Y] to Ihh	Abcam (ab52919)	1:10,000
Rabbit polyclonal to Gli1	Abcam (ab151796)	1:3,000
Rabbit monoclonal [EPR16891] to GAPDH	Abcam (ab181602)	1:10,000
Anti-Dhh antibody	Abcam (ab97287)	1:3,000
Histone H3	Cell Signaling Technology (#4499)	1:2,000

Table 2.2: Secondary antibody.

Secondary Antibody	*Company (Catalogue #)*	*Dilution*
Goat anti-rabbit IgG H&L (HRP) preadsorbed	Abcam (ab7090)	1:10,000

3.10 Statistical Analysis

Statistical Analysis was conducted by SigmaPlot v11 (Systat Software Inc., San Jose, CA, USA) and graphs were created using GraphPad Prism 7.0c, (GraphPad Software Inc., San Diego, CA). Data were assessed for outliers using the Grubb's test (GraphPad QuickCalcs, GraphPad Software Inc.). Comparisons among two groups were analyzed using Student's t-test. Comparisons among multiple groups were analyzed using One-Way Analysis of Variance (ANOVA) followed by the Kruskal–Wallis post-hoc multiple comparisons test vs. control. When normality or equal variance failed, the Mann-Whitney Rank Sum Test or Kruskal-Wallis One-Way ANOVA on Ranks were used to determine significance. The level of significance was set at $\alpha = 0.05$. All data are presented with the standard error of the mean (S.E.M).

CHAPTER 4: Results

4.1 Treatment with Fluoxetine causes a reduction in Insulin Content in INS-1E.

To confirm that fluoxetine treatment results in impaired beta cell insulin production, we assessed insulin content in fluoxetine-treated INS-1E cells. Fluoxetine treatment (1µM) significantly reduced insulin content in INS-1E cells; this is in accordance with previous work from our group demonstrating that the same dose of fluoxetine resulted in impaired insulin secretion in the same cell line (Figure 1) (N. E. De Long et al., 2014).

4.2 Treatment with Fluoxetine causes a reduction in Rate Limiting Enzyme, *Tph1* and Serotonin Content.

To determine if fluoxetine treatment could alter key components of serotonin biosynthesis, we assessed expression of the rate limiting serotonin biosynthetic enzyme (*Tph1*) and serotonin content**.** Treatment of INS-1E cells with 1µM of fluoxetine for 24 hours significantly decreased *Tph1* mRNA at the 0.1µM, 0.5µM and 1µM doses of fluoxetine; these decreases in *Tph1* mRNA only influenced serotonin production in cells exposed to 1µM fluoxetine (Figure 2).

4.3 Treatment with Fluoxetine significantly decreases Hedgehog Signaling in INS-1E cells.

Cell viability was not significantly altered by fluoxetine treatment at any dose tested (all $p>0.05$; data not shown). Fluoxetine treatment significantly decreased key

components of the hedgehog signaling pathway. Specifically, a significant decrease of *DHh* at the 0.1µM and 0.5µM doses, *IHh* at the 0.1µM, 0.5µM and 1µM doses, *Gli1* at the 0.5µM and 1µM doses and *Gli2* at the 0.1µM and 1µM doses (Figure 3) were observed. A non-significant change in IHh and DHh protein from western blot analysis was obsererved (Figure 4 & 5). Gli1 translocation is indicative of overall hedgehog signaling activity, Figure 6 shows a no significant change in the Gli1 protein translocation from the cytoplasm to the nucleus.

4.4 Treatment with TPH Inhibitor, PCPA significantly decreases *Tph1* and serotonin content.

To explore whether or not fluoxetine-induced decreases in hedgehog signaling are mediated via impaired serotonin signaling, cells were treated with PCPA to inhibit serotonin biosynthesis. Treatment with 50µM of PCPA for 24 hours significantly decreased Tph1 mRNA expression (Figure 7A) and intracellular serotonin content (Figure 7B) thus validating the use of this inhibitor to block serotonin synthesis in this cell line.

4.5 Inhibition of serotonin biosynthesis significantly impairs insulin biosynthesis likely via decreased hedgehog signaling.

In order to determine if the *Tph1* inhibitor was also able to affect INS1/2 expression, INS-1E cells were treated with 50µM PCPA for 24hrs. The data in Figure 9A below shows a greater than 30% significant decrease in the *Ins1* gene. This decrease in transcription of the *Ins1* gene was functionally relevant as 24hr treatment of INS-1E with 50µM PCPA displayed a reduction in insulin content. Figure 9B shows a significant decrease of more than 50% of insulin content in treated INS-1E. Furthermore,

pharmacological inhibition of Tph1 results in decreased hedgehog signaling mRNA**.** INS-1E were treated with 50µM of PCPA for 24hrs in order to examine the effect of the Tph1 inhibitor saw a significant mRNA decrease in key components of the hedgehog signaling pathway including the ligands *IHh* and *DHh* as well as the primary signaling molecule *Gli1* (Figure 8).

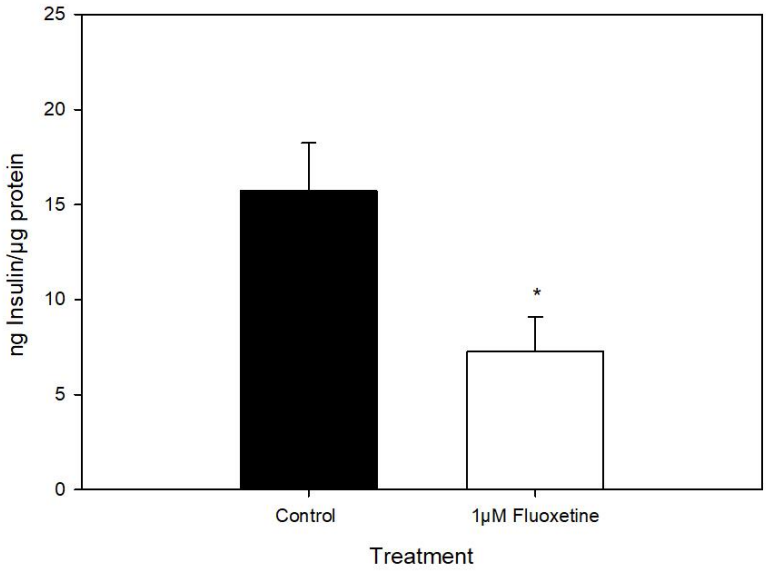

Figure 1. Fluoxetine treatment reduced INS-1E insulin content. Insulin quantification was performed using commercially available insulin ELISA (Crystal Chem, IL, USA). Data are presented as mean ± SEM. *$p \leq 0.05$ (n=5) relative to control calculated by a t-test.

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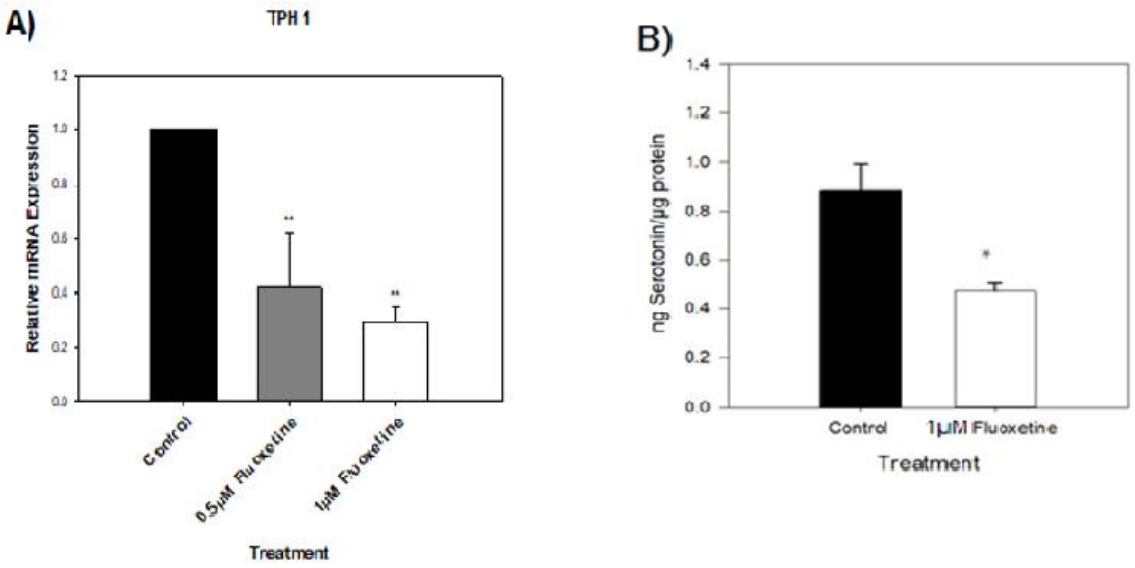

Figure 2. Fluoxetine treatment significantly reduced INS-1E Tph1 mRNA expression and intracellular serotonin content. A) mRNA levels were determined by RT-PCR of INS-1E treated with Fluoxetine for 24hrs. Data was normalized to the geometric mean of housekeepers *HPRT* and *CYC*. Data are presented as mean ± SEM. *$p \leq 0.05$ and **$p \leq 0.01$ relative to control calculated by a one way ANOVA, followed by the appropriate post hoc test. B) Serotonin quantification was performed using commercially available serotonin ELISA as per the manufacturer's instructions (LDN, Nordhorn, Germany). Data are presented as mean ± SEM. *$p \leq 0.05$ (n=5) relative to control calculated by a t-test.

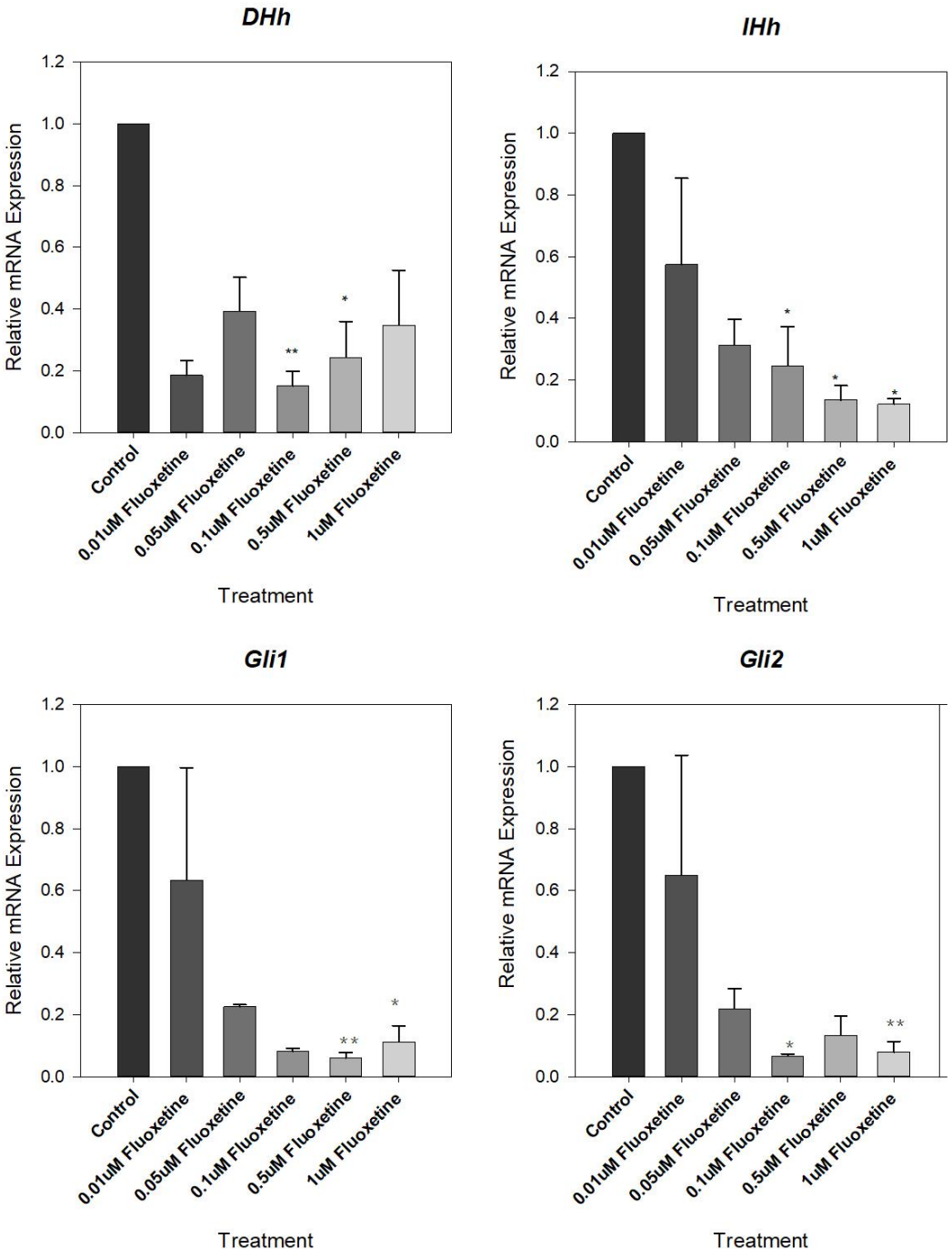

Figure 3. Relative expression of hedgehog signaling mRNA in INS-1E cells treated with fluoxetine for 24hrs. mRNA levels were determined by RT-PCR of INS-1E treated with Fluoxetine for 24hrs. Data was normalized to the geometric mean of housekeepers HPRT and CYC. Data are presented as mean ± SEM. *$p \leq 0.05$ and **$p \leq 0.01$ relative to control calculated by a one way ANOVA, followed by the appropriate post hoc test.

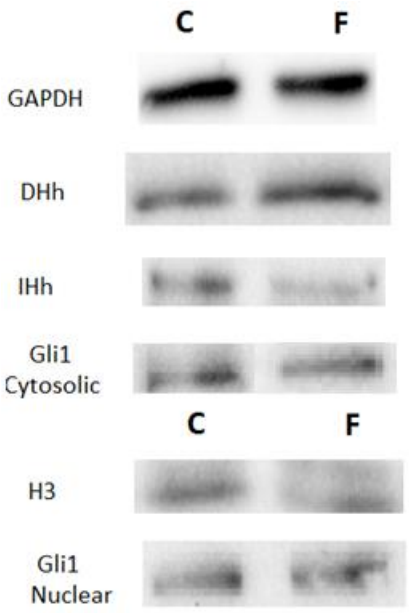

Figure 4. Representative western blot for DHh, GAPDH, IHh, H3, cytosolic and nuclear fraction Gli1. 'C' represents samples treated with control, and 'F' represents samples treated with 1µM fluoxetine. Protein expression of DHh, IHh and cytosolic Gli1 was quantified relative to GAPDH. Nuclear fraction Gli1expression was quantified relative to nuclear H3 expression. N=4 independent experiments.

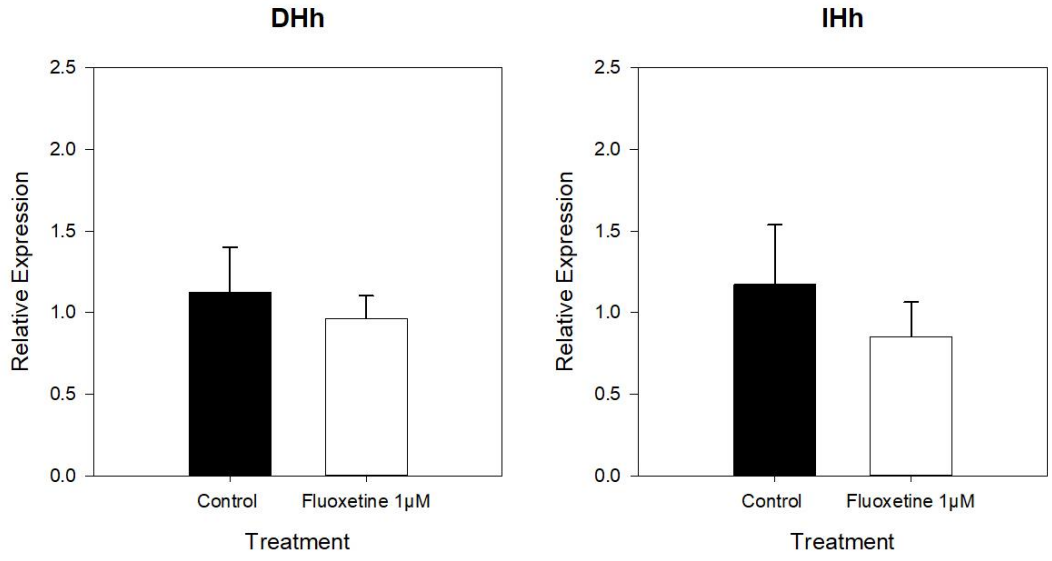

Figure 5. Western Blot analysis of DHh and IHh protein expression treated with 1µM fluoxetine. INS-1E were treated with 1µM Fluoxetine for 24hrs before fractionation into cytosolic and nuclear fraction (BioVision Inc., Milipitas, CA, USA). Western blot of cytosolic fraction shows Rabbit monoclonal antibody to Ihh (Catalog # ab52919) using a 1:10,000 dilution and Dhh antibody (Catalog # ab97287) at a 1:3000 dilution. Goat Anti-Rabbit IgG H&L (HRP) preadsorbed secondary antibody was used (Catalog # ab7090) at a 1:10,000 Dilution. IHh and DHh were normalized to Rabbit monoclonal GAPDH antibody (Catalog # ab181602) at a 1:10,000 dilution. Results are expressed as the mean ± SEM; n=4 independent experiments.

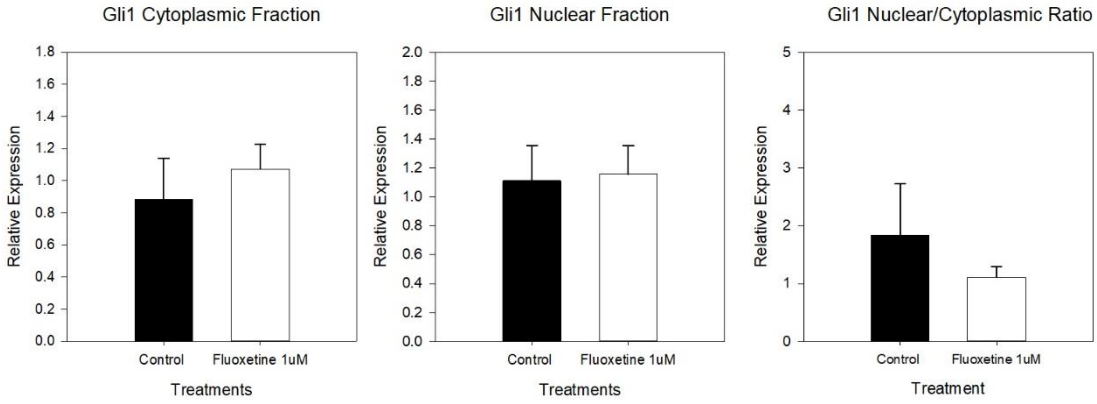

Figure 6. Western blot analysis of Gli1 protein translocation of INS-1E treated with 1µM fluoxetine. INS-1E (n=4) were treated with 1µM Fluoxetine for 24hrs before fractionation into cytosolic and nuclear fraction (BioVision Inc., Milipitas, CA, USA). Western blot shows Rabbit polyclonal to Gli1 antibody (Catalog # ab151796) at a 1:3000 dilution. Goat Anti-Rabbit IgG H&L (HRP) preadsorbed secondary antibody was used (Catalog # ab7090) at a 1:10,000 Dilution. Cytosolic Gli1 was normalized to Rabbit monoclonal GAPDH antibody (Catalog # ab181602) at a 1:10,000 dilution, while Nuclear Gli1 was normalized to Histone H3 (D1H2) XP® Rabbit mAb at a 1:2,000 dilution. Translocation of Gli1 Protein was calculated by dividing the nuclear fraction by the cytosolic protein expression in the control and treated samples. Results are expressed as the mean ± SEM; n=3 independent experiments.

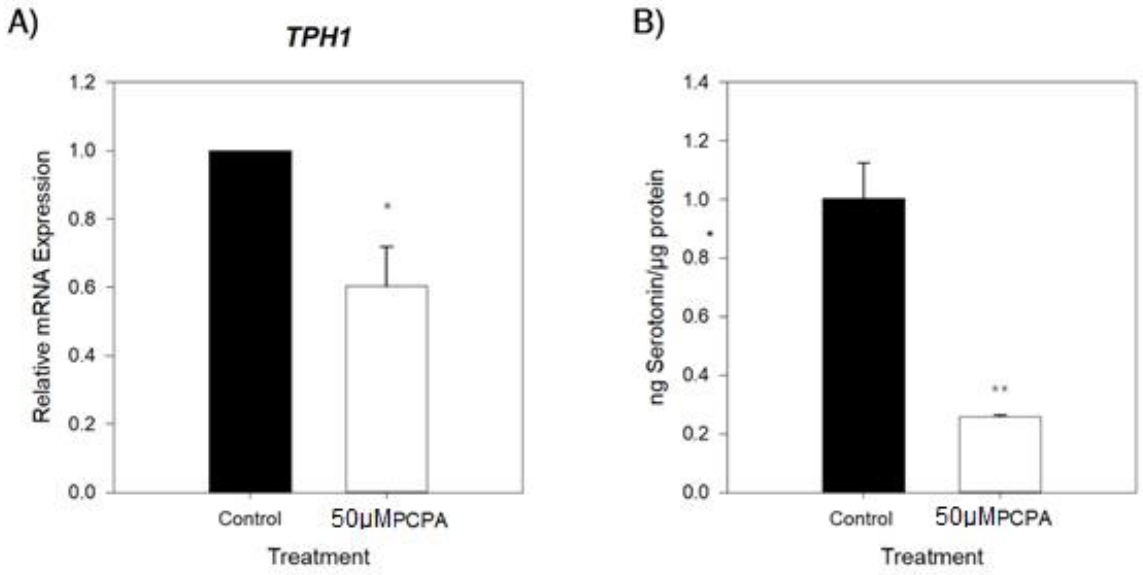

Figure 7. Reduced relative mRNA expression of *Tph1* and intercellular serotonin content of INS-1E treated with PCPA for 24hrs. A) mRNA levels were determined by RT-PCR in Rat INS-1E cells treated with 50µM PCPA for 24hrs. Data was normalized to the geometric mean of housekeepers HPRT and CYC. Data are presented as mean ± SEM. *$p \leq 0.05$ and (n=10) relative to control calculated by a t-test. B) Serotonin quantification was performed using commercially available serotonin ELISA as per manufacturers instruction (LDN, Nordhorn, Germany). Data are presented as mean ± SEM. *$p \leq 0.05$ and **$p \leq 0.01$ (n=5) relative to control calculated by a t-test.

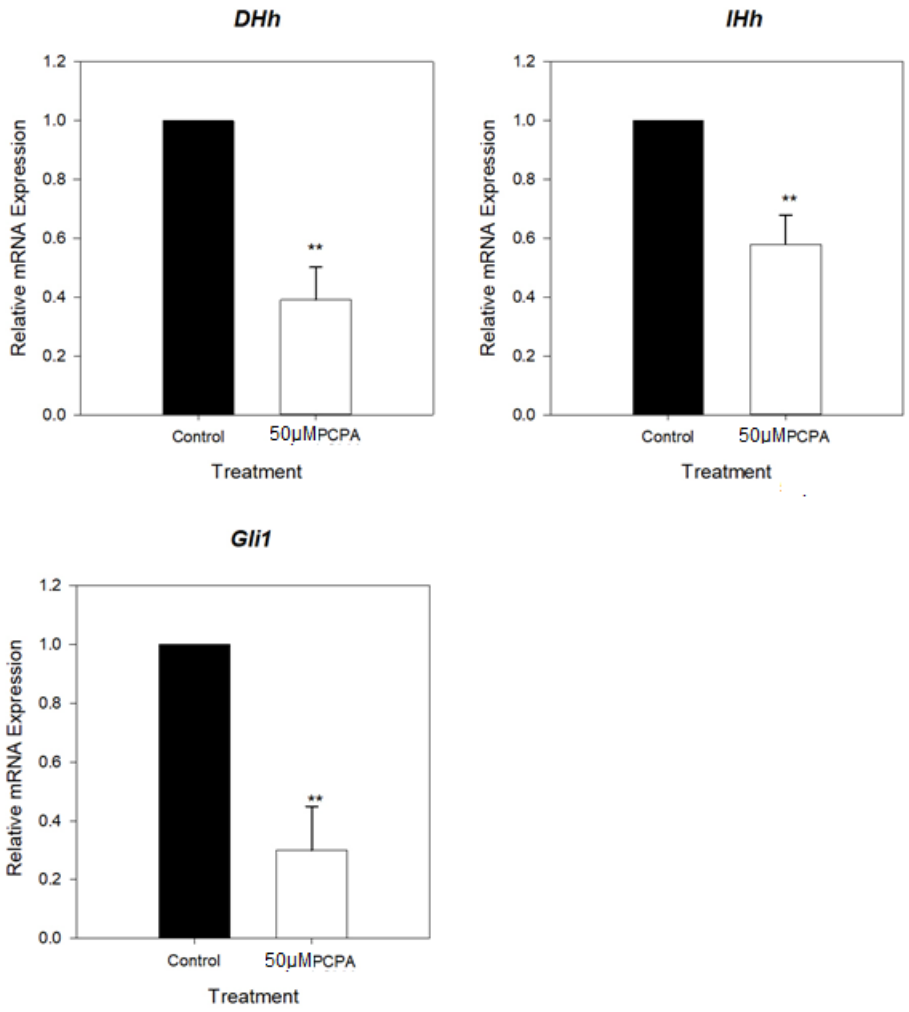

Figure 8. Relative mRNA expression of hedgehog signaling genes in INS-1E cells treated with PCPA for 24hrs. mRNA levels were determined by RT-PCR in Rat INS-1E cells treated with 50µM PCPA for 24hrs. Data was normalized to the geometric mean of housekeepers HPRT and CYC. Data are presented as mean ± SEM. **$p \leq 0.01$ (n=10) relative to control calculated by a t-test.

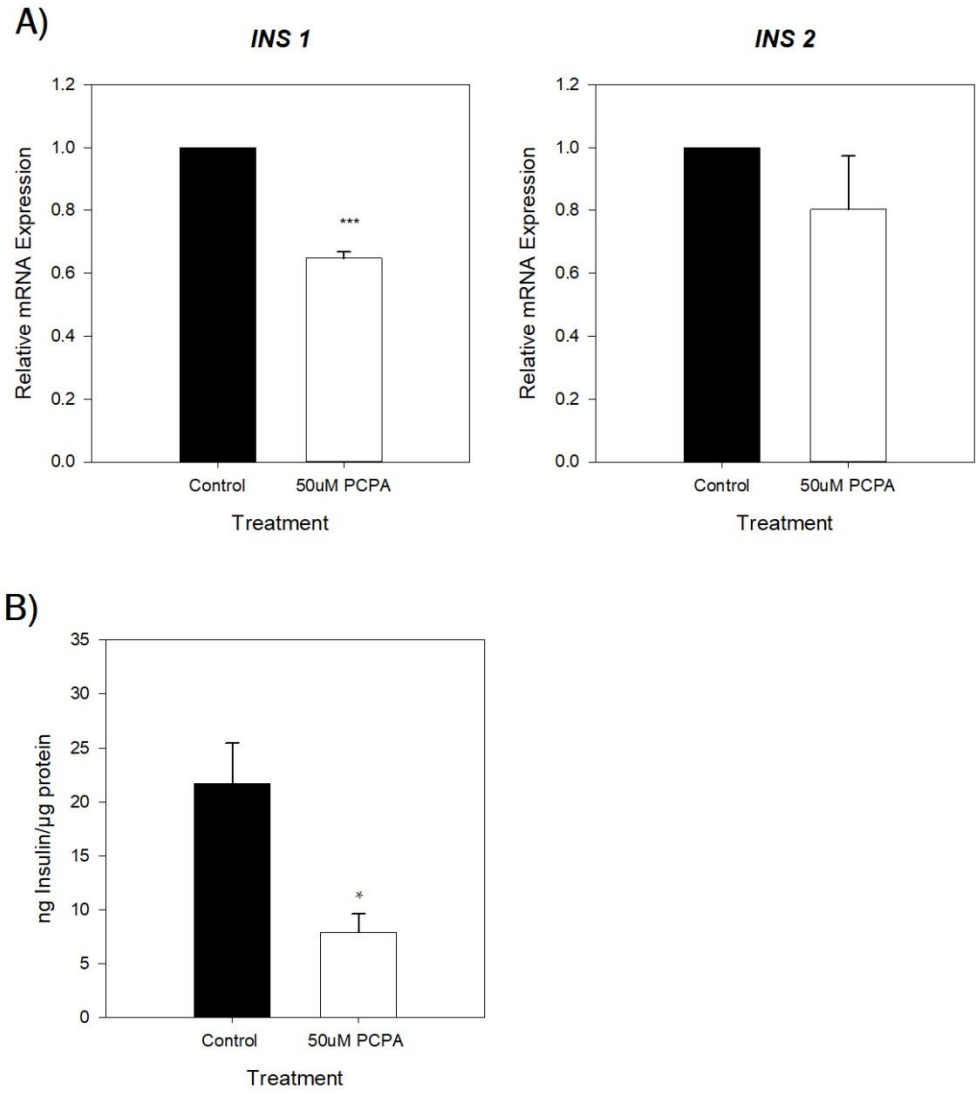

Figure 9. Relative mRNA expression of Ins1 was reduced, along with a significant reduction of insulin content in INS-1E cells treated with PCPA for 24hrs. A) mRNA levels were determined by RT-PCR in Rat INS-1E cells treated with 50µM PCPA for 24hrs. Data was normalized to the geometric mean of housekeepers HPRT and CYC. Data are presented as mean ± SEM. *$p \leq 0.05$ and ***$p \leq 0.001$ (n=10) relative to control calculated by a t-test. B) Insulin quantification was performed using commercially available insulin ELISA (Crystal Chem, IL, USA). Data are presented as mean ± SEM. *$p \leq 0.05$ (n=5) relative to control calculated by a t-test.

Chapter 5: Discussion

Long-term use of SSRIs in adults increases the risk of developing T2DM (RR: 1.49; 95% CI, 1.29 to 1.71) (Yoon et al., 2013). The goal of this thesis was to contribute to the literature regarding the potential mechanisms by which SSRI use is associated with an increased risk of T2DM. In particular, we wanted to examine the underlying mechanism(s) by which SSRIs may contribute to pancreatic beta cell dysfunction. To do so, we examined the effects of the commonly prescribed SSRI fluoxetine *in vitro* using the rat insulinoma cell line, INS-1E. Here we show that *in vitro*, fluoxetine adversely affects serotonin and hedgehog signaling, which appear to be directly related to the regulation of insulin content in the pancreatic beta cells.

There is considerable evidence from *in vivo* and *in vitro* studies to suggest that exposure to SSRI antidepressants, including fluoxetine (Prozac®), can negatively impact pancreatic beta cell function (Anderson et al., 2001; Carvalho et al., 2004; N. E. De Long et al., 2014; Isaac et al., 2013; Rickels & Schweizer, 1990; Yamada, Sugimoto, & Inoue, 1999). In particular reduced insulin synthesis/content appears to be a common feature of SSRI-induced pancreatic pathology. The following lines of evidence suggested that SSRIs could impact hedgehog signaling pathways in the pancreas to cause deficits in insulin production; 1) SSRIs are known to affect expression of key components of serotonin signaling pathways in the brain (Dygalo, Shishkina, Kalinina, Yudina, & Ovchinnikova, 2006; Mück-Šeler, Jevric-Causevic, & Diksic, 1996); 2) there is considerable cross-talk between serotonin and hedgehog signaling pathways in the body

including mammary and neuronal tissues (Laporta et al., 2014; Rajendran et al., 2009) and 3) serotonin and hedgehog signaling pathways have been shown to be important for maintaining insulin biosynthesis (Paulmann et al., 2009; Thomas et al., 2000). Therefore the goal of this study was to test the hypothesis that pancreatic beta cell exposure to the SSRI fluoxetine will lead to altered hedgehog signaling via disruption of the serotonergic system, ultimately resulting in a disruption in the insulinotropic function of pancreatic beta cells.

5.1 Importance of Serotonin in the Beta Cell

Quite remarkably, there are several similarities between pancreatic beta cells and the serotonergic neurons (Ohta et al., 2011). The shared ability of both neurons and pancreatic islet cells to import amine precursors, decarboxylate them, and concentrate the products, as well as identical transcription factors are examples of parallels between these two cell types (Cordes, 2005; Falck & Torp, 1961; Wilson, Scheel, & German, 2003). While the vast majority of serotonin is produced in the gut, it has been established that peripheral organs such as the pancreas express key components of the entire serotonergic biosynthetic and signaling pathways (Paulmann et al., 2009). In particular, the rodent *Pet-1* transcription factor (human orthologue, *Fev*) is required for serotonergic neuron maturation and transcriptional maintenance of the serotonergic system and is present in pancreatic betacells (Liu et al., 2010; Ohta et al., 2011). Disruption in Pet1 protein has been shown to impair pancreatic beta cell insulin production and secretion, indicating a convergence between serotonin and insulin signaling (Ohta et al., 2011). Further, serotonin and insulin are co-localized within secretory granules in the pancreatic beta cell,

which led researchers to suggest that serotonin may play a role in glucose stimulated insulin secretion; a fundamental aspect of beta cell function (Paulmann et al., 2009). This hypothesis has been supported by experiments whereby mice with the deletion of Tph1-/- were observed to have a diabetic phenotype and impaired insulin secretion, only to be rescued by supplementation with serotonin (Paulmann et al., 2009).

Recently, researchers have investigated the importance of pancreatic serotonin biosynthesis in the function of pancreatic beta cells, and suggested that that promoting Tph1 expression would serve as a new strategy for combating T2DM (Zhang et al., 2017). Transfection of pancreatic islets with a *Tph1* overexpressing lentivirus drastically increased Tph1 protein, followed by a significant increase in insulin mRNA expression, content and secretion in response to glucose stimulation (Zhang et al., 2017). Conversely, pharmacological inhibition of serotonin synthesis resulted in a significant decrease in insulin secretion in response to glucose stimulation (Zhang et al., 2017). To confirm that decreases in insulin secretion and content could be directly attributable to inhibition of *Tph1*, islets were transfected with *Tph1* shRNA lentivirus which decreased *Tph1* mRNA and protein expression by 50% as well significantly reducing insulin secretion and content (Zhang et al., 2017). Treatment of INS-IE cells with PCPA inhibited expression of *Tph1* and reduced intracellular serotonin content (Figures 7A and 7B). This decrease in serotonin content occurred in association with a significant reduction in *Ins1* mRNA, and insulin content (Figure 9). These data confirm that in INS-1E cells changes in serotonin synthesis are linked to alterations in insulin content. These results are in line with those of

Zhang et al. who demonstrated that PCPA significantly reduced serotonin production in isolated rat pancreatic islets (Zhang et al., 2017).

5.2 SSRIs Modulate Serotonin in INS-1E

Given that manipulation of serotonergic synthesis and signaling have been reported to alter beta cell function, an effect that I have confirmed in the INS-1E cells, the goal of my project was to determine if similar changes would be observed in pancreatic beta cells exposed to serotonin modulating antidepressants such as the SSRI fluoxetine. SSRIs such as fluoxetine are widely used for the treatment of depression. It is thought that use of SSRIs increases serotonin bioavailability in the CNS leading to improvement of depressive symptoms (Judd et al., 2004). However, several animal studies have shown that SSRIs such as fluoxetine decrease the overall levels of serotonin in the brain (Caccia, Fracasso, Garattini, Guiso, & Sarati, 1992; Dygalo et al., 2006; Thompson et al., 2004; Trouvin, Gardier, Chanut, Pages, & Jacquot, 1993). The mechanism by which serotonin levels are decreased by SSRIs appears to be via inhibition of its biosynthetic enzymes (i.e., TPH2) in the CNS (Dygalo et al., 2006). Dygalo and colleagues have shown that fluoxetine treatment decreases the expression of key components of serotonin signaling pathways in the brain (Cervo et al., 2005; Dygalo et al., 2006). In particular, both *Tph2*, the rate limiting enzyme in the synthesis of serotonin and *SLC6A4*, the gene that encodes the serotonin reuptake transporter (SERT) expression were reduced in the rat brainstem after a 2-week treatment with fluoxetine (Dygalo et al., 2006). These decreases in gene expression occurred in concert with significant reductions in serotonin expression (Dygalo et al., 2006).

We observed a similar decrease in *Tph1*, the isoform responsible for peripheral serotonin synthesis (Berger et al., 2009), in fluoxetine-treated INS-1E cells (Figure 2A). Like previous findings in the CNS of SSRI treated animals(Mück-Šeler et al., 1996), the decrease in *Tph1* mRNA in our study was associated with a subsequent decrease in intercellular serotonin content in fluoxetine treated INS-1E cells (Figure 2B). Our results are also consistent with a previous study that found a significant decrease in *Tph1* mRNA and serotonin in INS-1 cells treated with paroxetine, a different SSRI antidepressant (Li et al., 2014). Although researchers had previously hypothesized that the SSRI-induced decrease in intracellular serotonin levels in INS-1E could be attributed, in part, to inhibition of *Tph1* mRNA expression, the mechanism(s) by which SSRIs disrupt *Tph1* expression were not addressed (Li et al., 2014). While it is unclear how exactly SSRIs work to decrease *Tph* expression, a study investigating the effects of fluoxetine on the hypothalamus and telencephalon of female goldfish saw a significant decrease in the expression of estrogen receptor beta (Mennigen et al., 2008). Interestingly, ER beta has been shown to modulate *Tph1* expression in the dorsal raphe nuclei of mice (Gundlah et al., 2005). Mice exposed to selective estrogen receptor-beta agonists resulted in transcriptional induction of the *Tph2* isoform *in vivo* (Donner & Handa, 2009). These findings are supported by studies in which reduced serotonin levels and *Tph* mRNA expression was reported in estrogen receptor beta knockout mice (Imwalle, Gustafsson, & Rissman, 2005). Interestingly in my study, fluoxetine significantly decreased expression of estrogen receptor beta in INS-1E cells, which may play a role in the observed decrease of *Tph1* transcription (Appendix, Figure 10). However, it is important to consider

alternative mechanisms by which SSRIs may affect the intercellular serotonin levels in INS-1E cells.

The decrease in intercellular serotonin observed in INS-1E cells treated with SSRIs, may be in part due to their pharmacological action of inhibiting serotonin reuptake. By blocking SERT (Bismuth-Evenzal et al., 2012; Fuller & Wong, 1987); the reuptake of serotonin from the extracellular milieu would increase extracellular serotonin levels and decrease intracellular levels. The increased extracellular serotonin could then in turn bind to and activate the 5-HT1A receptor (Paulmann et al., 2009); activation of this receptor has been shown to further inhibit intracellular serotonin production (Paulmann et al., 2009). Taken together, the observed decrease in serotonin content observed in fluoxetine treated cells may be due to its action to block SERT and/or its ability to inhibit *Tph1* expression.

5.3 Serotonin and Hedgehog Signaling Cross-Talk:

The monoamine hypothesis of depression postulates that reduced monoamine concentration is the central reason for depression, and that treatment with antidepressants such as SSRIs work by elevating the monoaminergic activity in the brain (Dennis S. Charney, Menkes, & Heninger, 1981). However, there is debate surrounding the accuracy of this model as there are a number of pitfalls to this theory. For example, other drugs that block serotonin reuptake such as cocaine and amphetamine are not effective in treating depression as SSRIs and SSRIs can increase serotonin levels in hours, but the therapeutic effect takes weeks of continuous treatment including SSRIs (Andrews, Bharwani, Lee, Fox, & Thomson, 2015; Dennis S. Charney et al., 1981). Alternatively, the neurogenic

hypothesis of depression suggests that active neurogenesis in the hippocampus, a region of the brain involved in mood control, is necessary as new neurons in the adult brain are needed for proper mood control (Miller & Hen, 2015). Quite interestingly, humans with depression had decreased hippocampal volume, while SSRI treatment promoted neurogenesis, an effect that was observed in other species including adult rats and monkeys (Boldrini et al., 2009; J. E. Malberg, Eisch, Nestler, & Duman, 2000; Jessica E. Malberg & Duman, 2003; Perera et al., 2007; Small, Schobel, Buxton, Witter, & Barnes, 2011). It has thus been suggested that the decrease in monoamines observed in depressed individuals contributes to the stunted hippocampal neurogenesis, while treatment with antidepressants act to alleviate the reduced monoamine signaling and effectively support hippocampal neurogenesis(D. S. Charney, 1998). It is well known that Sonic Hedgehog (SHh) is a central regulator of adult hippocampal neurogenesis as it regulates the proliferation and differentiation of adult hippocampal neural progenitor cells (Lai, Kaspar, Gage, & Schaffer, 2003; Machold et al., 2003). Moreover, in male mice, genetic deletion of the SHh gene's isoform, desert hedgehog (DHh) gene- resulted in depressive behavior, supporting the notion that increased hippocampal neurogenesis is driven, at least in part, by hedgehog signaling (Umehara et al., 2006). Additionally, acute treatment with the monoamine releasing agent, para-chloroamphetamine has been shown to upregulate Smo expression, a gene important in the hedgehog signaling pathway in rat brain (Rajendran et al., 2009). Given that SSRI treatment and increased hedgehog signaling are associated with increased neurogenesis, this raises the interesting possibility that SSRIs may directly affect hedgehog signaling pathways.

The association between serotonergic and hedgehog signaling has been identified in other tissue types as well (Laporta et al., 2014). In support of this idea, Laporta and colleagues identified an association between serotonin levels and the epigenetic activation of hedgehog signaling genes in mammary epithelial cells (Laporta et al., 2014). Using the *Tph1*-deficient mouse model, serotonin was found to induce hedgehog signaling by remodeling SHh promoter methylation, indicating a novel cross talk between these two signaling pathways (Laporta et al., 2014). These results indicate that changes in serotonergic signaling can regulate hedgehog signaling. While an association between these two signaling pathways has been identified, their importance in the function of pancreatic beta cells remains unexplored.

5.3.1 Importance of Serotonin and Hedgehog Signaling Pathways for Insulin Biosynthesis

Inhibition of serotonin and hedgehog signaling have both been shown to disrupt insulin biosynthesis (Thomas et al., 2000; Yamada et al., 1999). Given that fluoxetine treatment reduced serotonin and insulin content, an effect that could be replicated by treatment with the TPH inhibitor PCPA, the goal of this thesis was to determine if changes in hedgehog signaling could be the pathway linking fluoxetine-induced perturbations in serotonin signaling to impaired insulin biosynthesis.

Studies have identified that inhibition of either *Tph1* or Hedgehog signaling in the pancreatic beta cells have led to a significant decrease in the insulin promoter factor 1 (IDX 1), while overexpression of the *Tph1* or *SHh* genes significantly increased the

expression of this transcription factor, and resulted in the subsequent increase in insulin content (Thomas, 2001; Zhang et al., 2017). To determine if changes in hedgehog signaling were downstream of SSRI-reductions in intracellular serotonin levels, I treated cells with the serotonin synthesis inhibitor PCPA and examined the mRNA expression of key hedgehog signaling genes. Treatment with PCPA at a concentration which reduced intracellular serotonin content led to a significant decrease in the mRNA expression of *IHh*, *DHh* and *Gli1*, all of which are crucial components of the pancreatic beta cell hedgehog signaling pathway (Figure 8). This finding matched a previous study conducted by Rajendran and colleagues who investigated if monoamines regulated SHh signaling in the adult rat hippocampus (Rajendran et al., 2009). In their study, treatment with PCPA resulted in a significant decrease in both *Smo* and *Ptc* mRNA, two components of the hedgehog signaling cascade (Rajendran et al., 2009). Authors of this study suggested that PCPA was acting as a non-selective inhibitor by reducing hippocampal serotonin and noradrenergic levels (Rajendran et al., 2009). However, neither 5,7-dihyrdroxytryptamine (a serotonergic inhibitor) nor N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), a noradrenergic inhibitor, altered expression of SHh or its co-receptors in the adult rat hippocampus (Rajendran et al., 2009). Moreover, Rajendran *et al*., did not find any significant effect of either chronic or acute fluoxetine treatment to significantly alter mRNA levels of hedgehog signaling genes in neurons (Rajendran et al., 2009). Although I found that fluoxetine significantly altered the steady-state mRNA levels of hedgehog signaling genes, this did not extend to differences in protein expression. Western blot analysis of fluoxetine-treated INS-1E cells did not find any differences in the nuclear

translocation of Gli1, the central signaling molecule to the hedgehog signaling pathway, or the expression of the DHh and IHh ligands (Figure 6). However, the sample size for this experiment was limited (N=3), so it is premature to conclude that hedgehog signaling is not involved in SSRI-induced changes in beta cell insulin biosynthesis.

Summary

I have shown that fluoxetine treatment reduced key components of hedgehog signaling in association with a decrease in intracellular insulin content (Figure 1 & 3). We predicted that perturbations in serotonin biosynthesis would be upstream of these alterations in hedgehog target genes. Indeed, when we inhibited serotonin synthesis with PCPA we saw decreased expression of the hedgehog pathway genes in association with decreased insulin content (Figure 8 & 9B). Thus treatment with the SSRI fluoxetine is believed to suppress serotonin synthesis in the pancreatic beta cell, leading to diminished hedgehog signaling and ultimately a dysfunctional beta cell. Similarly disruption of the serotonergic signaling via fluoxetine have also resulted in a significant decrease in the hedgehog signaling (Figure 2 & 3). Similar inhibitory effects were observed with respect to insulin in the pancreatic beta cells. PCPA treatment resulted in a decrease in *INS1* mRNA along with a significant decrease in insulin content (Figure 9). Similarly, the serotonin reuptake inhibitor, fluoxetine reduced insulin content in treated INS-1E cells (Figure 1).

Although my research suggests the hedgehog signaling may be an important mechanistic link by which SSRIs impact insulin biosynthesis, other studies have

identified additional pathways by which SSRIs can cause beta cell dysfunction. For example, despite intercellular serotonin's apparent role in promoting insulin secretion, extracellular serotonin treatment negatively modulates the release of insulin. Rat and mouse insulinoma cell lines, INS-1 and Min6 respectively displayed an inhibited secretory response to glucose stimulation when incubated with serotonin (Paulmann et al., 2009). This was hypothesized to be due to the increased stimulation of the autocrine 5-HT1A receptor (Paulmann et al., 2009). In addition, the decrease in intercellular serotonin and insulin observed in INS-1E treated with SSRIs may be in part due to their pharmacological action of inhibiting serotonin reuptake by blocking SERT (Bismuth-Evenzal et al., 2012; Fuller & Wong, 1987). Serotonin reuptake into the pancreas by SERT increases intracellular serotonin levels enabling serotonylation of Rab3a and Rab27a, both of which are key contributors to the signaling sequence involved in insulin exocytosis (Paulmann et al., 2009). Thus it was suggested that preventing the reuptake of serotonin via inhibition of SERT would decrease insulin exocytosis. Indeed, studies that blocked SERT, in turn preventing serotonin uptake into the cell have shown a decrease in intracellular serotonin levels in the pancreatic beta cells, resulting in an inhibition of glucose-stimulated insulin secretion (Li et al., 2014). Regardless, taken together these studies suggest that SSRIs may have profound impacts on beta cell function which may explain the increased risk of T2DM in patients taking these medications.

Chapter 6: Future Directions

There are a number of questions arising from the results of this study which remain to be addressed. The first question relates to the specificity of the response we observed; are these results translatable to all SSRI antidepressants. A wide range of SSRIs are available for treatment of MDD including fluoxetine (Prozac®), fluvoxamine (Luvox®), sertraline (Zoloft®), paroxetine (Paxil®) and citalopram (Celexa®). Although they all belong to the same class of drugs, each of these antidepressants differ in their structure and pharmacological effects. As such, they do not always have the same effects on any given tissue. For example, while rats treated with sertraline during pregnancy had decreased beta cell mass at birth, fluoxetine did not have a similar effect (N. E. De Long et al., 2014; N. De Long et al., 2015).

Likewise, Yamada and colleagues highlight this discrepancy in their research paper that investigated the differential effects of the SSRI fluoxetine and fluvoxamine induced hyperglycemia in mice, and observed that pretreatment with PCPA completely attenuated fluvoxamine-elicited hyperglycemia, although the hyperglycemia induced by fluoxetine was not affected by PCPA (Yamada et al., 1999). This discrepancy may be due to many of the serotonin modulating medications binding to other receptors/transporters in a stimulating or antagonizing manner and may have distinct effects on overall beta cell function (Yamada et al., 1999). Future research should aim to examine the effects of these SSRIs based on their primary receptor target rather than simply grouping them as SSRIs. For instance, the SSRI paroxetine is known to act as a SERT inhibitor as well as

interacting with the serotonin receptor 5HT2C, while the SSRI fluoxetine acts upon those receptors as well as the norepinephrine and dopamine transporter inhibitor, 5HT2A, 5HT3, as well as muscarinic and adrenergic receptors(Maj et al., 1996; Ni & Miledi, 1997). Taking into account the different receptors the SSRIs target may explain the discrepancy in adverse beta cell function observed by the treatment with these structurally different drugs.

In my research however, I focused on the effects of the SSRI Fluoxetine on INS-1E. Fluoxetine was the SSRI of choice for a multitude of reasons including it being the first and most commonly prescribed antidepressant, plenty of clinical research has been done on this SSRI and there are studies clearly identifying the serum concentrations of fluoxetine in rodents and humans doses (Keller et al., 2005; Reis et al., 2009). However, future studies should aim to examine if 1) other SSRIs exhibit the same decrease and link between hedgehog and serotonin signaling in the beta cells 2)Identify if this decrease in serotonin and hedgehog observed in beta cells treated with these SSRIs is due to the downregulation of TPH1. This can be done by the use of specific transcriptional silencing of TPH1 expression and examining the effect on hedgehog signaling in the beta cell (Zhang et al., 2017).

While previous studies have shown that SSRIs can cause beta cell dysfunction (Isaac et al., 2013; Long et al., 2015), future research should examine this association with respect to the pancreatic hedgehog signaling cascade. In order to delineate if SSRIs are causing dysfunction to the pancreatic beta cell via a disruption of the hedgehog signaling cascade, future experiments should aim to examine insulin secretion while

simultaneously treat these cells with the SSRI fluoxetine, whilst overexpressing hedgehog signaling using a hedgehog expression plasmid as did Thomas and colleagues (Thomas et al., 2000). This would then be followed by examining the effect of cyclopamine, the hedgehog signaling inhibitor on the serotonin content and insulin secretion. These experiments would help better delineate if the effects of SSRIs to influence insulin content and/or secretion are mediated via changes in serotonin and hedgehog signaling. Finally, although the INS-1E model has been extensively used as a model of pancreatic beta function, future experiments should aim to examine if this same link between serotonin and hedgehog signaling is present in pancreatic beta cell islets or *in vivo* studies.

Conclusion:

While SSRIs are widely prescribed for the treatment of depression, there is an association between SSRI use and metabolic diseases including T2DM (Anderson et al., 2001; Carvalho et al., 2004; Isaac et al., 2013; Jessica E. Malberg & Duman, 2003; Rickels & Schweizer, 1990; Yamada, Sugimoto, Yoshikawa, Kimura, & Horisaka, 1995). However the cellular and molecular mechanisms underlying this association are poorly understood and were the focus on this thesis. Central to the pathophysiology of T2DM is beta cell dysfunction which involved impairments in either insulin synthesis and/or secretion (Ahren, 2005). Importantly, SSRIs have been shown to inhibit insulin biosynthesis (Li et al., 2014). Our findings indicate that exposure to SSRIs results in significant impairment in insulin synthesis likely via their impact on serotonin and hedgehog signaling pathways. Although our data supports the notion that SSRI-mediated reductions in serotonin synthesis are upstream of altered hedgehog signaling, future

experiments should aim to inhibit hedgehog signaling via cyclopamine and examine if this leads to altered serotonin signaling in order to better determine the directionality of this association.

Chapter 7: Reference

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Chapter 8: Appendix

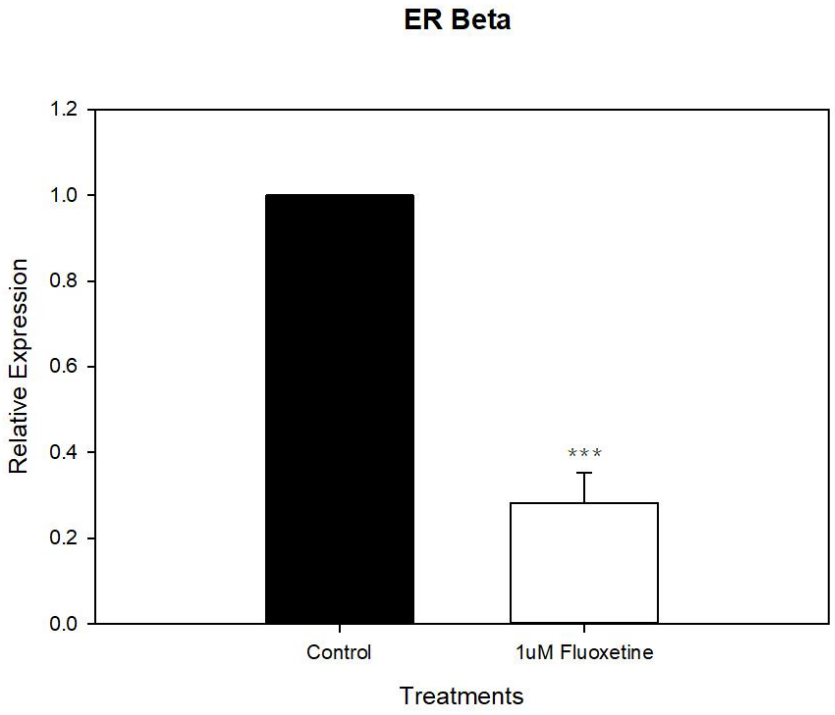

Figure 10. Relative expression of *ER Beta* mRNA in INS-1E cells treated with fluoxetine for 24hrs. mRNA levels were determined by RT-PCR of INS-1E treated with Fluoxetine for 24hrs. Data was normalized to the geometric mean of housekeepers HPRT and CYC. Data are presented as mean ± SEM. ***$p \leq 0.001$ relative to control calculated by a one way ANOVA, followed by the appropriate post hoc test.