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HYPOTHALAMIC REGULATION OF THE PANCREATIC ISLET OF LANGERHANS

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List of Abbreviations

3V……Third Ventricle
4V……Fourth Ventricle
Ach……...Acetylcholine
AD……....Alzheimer's Disease
ADP……Adenosine Diphosphate
AMP……....Adenosine Monophosphate
AMPK……..Adenosine Monophosphate Kinase
ARC……Arcuate Nucleus
AS160……..Akt Substrate of 160 KiloDaltons
ATF6……....Activating Transcription Factor 6
ATP……..Adenosine Triphosphate
BBB……..Blood Brain Barrier
BDNF……...Brain Derived Neurotropic Factor
BiP……....Binding Immunoglobulin Protein
CA^{2+}……Calcium
cAMP……...Cyclic Adenosine Monophosphate
CART……...Cocaine Amphetamine Regulated Transcript
CeA……..Central Amygdala
CMV……Cytomegalovirus
CNO……Clozapine-N-Oxide
CNS……..Central Nervous System
CRE…….....Cre Recombinase
CRR……..Counter-Regulatory Response
DAG……Diacylglycerol
DIO…….....Diet Induced Obesity
DMN……....Dorsomedial Nucleus
DMX……Dorsal Motor Nucleus of the 10^{TH} Nerve
DREADD……Designer Receptor Exclusively Activated by Designer Drugs
EDCs……Endocrine Disrupting Chemicals
ER……Endoplasmic Reticulum
ERα……...……Estrogen Receptor Alpha
$FADH_2$……..Flavin Adenine Dinucleotide
FFA……...Free Fatty Acids
FOXO……...Forkhead Box Proteins
GDP…….……Guanosine Diphosphate
GLP-1…….……..…Glucagon Like Peptide 1
GLP-1R……....Glucagon Like Peptide 1 Receptor
GLUT 1,2,3,4……..Glucose Transporter 1, 2, 3, 4
GPCR……...G-Protein Coupled Receptor
GSIS…….……Glucose Stimulated Insulin Secretion
GTP……..Guanosine Triphosphate
GTT……..Glucose Tolerance Test
HGP…….....Hepatic Glucose Production
HIER……Heat Induced Antigen Retrieval
HK1……..Hexokinas

HTRC……..Human Tissue Resource Center
IAPP……....Islet Amyloid Polypeptide
ICV……..Intracerebroventricular
IGT……..Impaired Glucose Tolerance
IKK……..…IκB Kinase
IP……..…Intraperitoneal
IP_3…….....Inositol Triphosphate
IP_3R…….....Inositol Triphosphate Receptor
IR…….....Insulin Receptor
IR-A…….....Insulin Receptor A Chain
IR-B…….....Insulin Receptor B Chain
IRE1α……...Inositol Requiring Enzyme 1 Alpha
IRS1/2……..….Insulin Receptor Substrate 1/2
ITT……...Insulin Tolerance Test
JNK……..….c-Jun-N-Terminal Kinase
K^+……..…Potassium
LHA……..…Lateral Hypothalamus
LoxP……Locus of X-Over P1
M3AChR…….Muscarinic 3 Acetylcholine Receptor
MAP3K…….…....Mitogen Activated Protein Kinase Kinase Kinase
MAPK……..….Mitogen Activated Protein Kinase
MeHab……...….Medial Habenula
MEK…….....Mitogen Activated Protein Kinase Kinase
MIP-Cre……...Mouse Insulin Promoter Driven Cre Recombinase
mM……...……...Millimolar
mRNA……..….Messanger Ribonucleic Acid
NADH……..….Nicotinamide Adenine Dinucleotide
Neurog3……...……....Neurogenin 3
NIRKO……..…Neuron Specific Insulin Receptor Knockout
NOS1…….....Nitric Oxide Synthase 1
NPY/AgRP……Neuropeptide Y/Agouti Related Protein
OCT……...Optimal Cutting Temperature Compound
PAG……..…..Periaqueductal Grey
PC1/3…….....Protein Convertase 1/3
PC2…….Protein Convertase 2
PDX……Pancreatic and Duodenal Homeobox 1
PERK…….....PKR like Endoplasmic Reticulum Kinase
PHA-L…… .Phytohaemagglutinin leucoagglutinin
PI3K……...Phosphoinositide 3 Kinase
PIP2……Phosphotidylinositol-3,4-Bisphosphate
PKB……Protein Kinase B
PLC……Phospholipase C
POMC……Proopiomelanocortin
PP……...Pancreatic Polypeptide
PP2A……..Protein Phosphatase 2A
PRV…….....Pseudorabies Virus

PRV-Ba2001……..Pseudorabies Virus Bartha 2001
PRV-BaBlu……...….Pseudorabies Virus Bartha Blue
PTEN…….....Phosphatase and Tensin Homalog
PTP1B……...….Protein Tyrosine Phosphatase 1B
PVN……Paraventricular Nucleus
Raf…….....Rapidly Accelerated Fibrosarcoma
RaM……..….Raphe Magnus
Ras……..…...Rat Sarcoma
RER……...…Respiratory Exchange Ratio
RIP……...…….Rat Insulin Promoter
ROS……...……...…Radical Oxygen Species
RRP……...……...…Ready Releasable Pool
SCN……..……Suprachiasmatic Nucleus
SF-1……..……Steroidogenic Factor 1
SN…….……....Substantia Nigra
T1DM…….…….…Type 1 Diabetes Mellitus
T2DM…….……Type 2 Diabetes Meillitus
TCA…….……Tricarboxylic Acid
VDCC…….…….....Voltage Dependent Calcium Channel
VMH…….……...Ventromedial Hypothalamus
VMN…….……...Ventromedial Nucleus
VMNdm…….……..Dorsomedial Ventromedial Nucleus
VTA…….…….....Ventral Tegmental Area
α2R…….….Alpha Adrenergic 2 Receptor
α-MSH…….....Alpha Melanocortin Stimulating Hormone
β-Gal……....Beta Galactosidase
βIRKO……..Beta Cell Specific Insulin Receptor Knockout

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Abstract

Type 2 Diabetes Mellitus (TD2M), characterized by fasting and post-prandial glucose intolerance and β-cell dysfunction, is an epidemic in the United States that continues to worsen. Despite the various therapeutics that successfully treats T2DM, the rate of this disease continues to rise. The Central Nervous System (CNS) has been implicated in controlling metabolic homeostasis; however, much is still unknown about the various ways the CNS, more specifically the hypothalamus, regulate whole body metabolic homeostasis. Our research investigated the role of insulin signaling in the hypothalamus on pancreatic islet cell function. From previous work, we established a circuit that connects the hypothalamus to the pancreatic islet, and utilized this map to investigate the activation of the circuit in the presence of insulin signaling in the different areas of the hypothalamus. We came upon the discovery that insulin significantly activates the CNS-Islet circuit in the Ventromedial Nucleus (VMN) compared to the other areas of the hypothalamus.

With this information, we decided to investigate the role of insulin signaling in the VMN on pancreatic islet function by knocking out the insulin receptor in the VMN of Insulin Receptor floxed mice. Using the stereotaxic apparatus, we delivered an Adenovirus carrying Cre-Recombinase or β-galactosidase into the VMN of these mice (VMN-Cre or VMN-LacZ) and conducted metabolic tests, which showed mild glucose intolerance with a robust counter-regulatory response, but no change in insulin or glucagon levels. These mice also seemed to conserve glucose reserves and utilize fat, based on the Respiratory Exchange Ratio (RER). However, in a pilot study, we showed that if we extended the study on these mice, they eventually became glucose intolerant with severe hyperinsulinemia. Further study needs to be done to investigate long term insulin resistance in the VMN on pancreatic islet cell function.

Chapter I

Type 2 Diabetes Mellitus and the Brain: A Brief Overview

1.1 Summary

Obtaining nutrients and food is vital for any organism to survive. Normally, the carbohydrate component of food and nutrients is mostly broken down into monosaccharides and delivered to cells in order to create the energy needed for the cell, and therefore the organism, to live. There are a variety of checks and balances to ensure a physiological level of glucose is continuously circulating throughout the organism, with a special emphasis on providing a constant glucose supply to the brain, which is that organ's sole fuel source under normal conditions. Two major pancreatic hormones insulin and glucagon that work in partnership to either deliver glucose from the bloodstream to tissues or increase circulating glucose levels, respectively. Maintaining a physiological level of glucose is necessary and essential for survival of an organism, for too much or too little glucose (hyperglycemia or hypoglycemia) can become lethal.

In the case of Type 2 Diabetes Mellitus (T2DM), as well as Type 1 Diabetes Mellitus (T1DM), it is the chronic hyperglycemic state that has been a major culprit for causing diabetic complications such as blindness, kidney failure, neuropathy, which can lead to lower leg amputation, and cardiovascular disease (Wallia 2014, Ziegler-Graham 2008). T2DM has been a worldwide epidemic that continues to rise. In the United States alone, there are 30.3 million people, or 9.4% of the US population, who have a diagnosis of T2DM in the year 2015. It is estimated, however, that 8.1 million Americans are not aware that they have T2DM, and 84.1 million people have what is known as "pre-diabetes", which describes a person likely to become

diabetic within 5 years of a pre-diabetes diagnosis (Center for Disease Control, 2017). T2DM, however, is not confined to the adult population. Studies have shown a 21% increase of T2DM in children 10-19 years old. Although the overall numbers are still quite low, it is an alarming rate of increase that cannot be ignored (Hamman 2014). The major players in the pathogenesis of T2DM range from a genetic susceptibility component, to a correlative rise in obesity as well socio-economic and environmental factors. Diabetes has a detrimental physiological consequence in the individual, which in turn presents a burden in the population. In 2017, the United States population spent a total of $327 billion dollars, a 26% increase from a 2012 report. The largest components of those economic costs are because of hospital inpatient care (30%), prescription medications to treat T2DM complications (30%), anti-diabetic agents and supplies (15%) and physician office visits (13%), and including cardiovascular complications, which is prevalent in patients with T2DM, more than doubles those costs (Diabetes Care 2017, Einarson 2018).

As mentioned earlier, there are a variety of checks and balances that promotes glucose homeostasis, including insulin and glucagon. Insulin and glucagon both originate from a cluster of cells called the Islet of Langerhans, which accounts for just 1% of the pancreatic mass and encompasses the 2 major contributors of the endocrine pancreas, while the remaining 99% of the pancreas is made up of ductal and acinar cells that aid in digestion through secretion of digestive enzymes and bicarbonate. Insulin is produced and secreted by a cell type called the β-cell and glucagon is produced and secreted by a cell type called the α-cell. The inability for the β-cell to produce a sufficient amount of insulin, or stay alive is fundamental to the pathogenesis and progression of T2DM. This chapter aims to discuss in great detail the discovery, secretion, regulation and function of insulin and its role in T2DM.

T2DM is a metabolic disease which is characterized by dysfunctional glucose homeostasis due to a breakdown of endocrine hormone function. The Central Nervous System (CNS) relies heavily on glucose to maintain function and therefore contributes greatly to the influx and regulation of nutrients, which makes the CNS likely to have an important role in maintaining glucose homeostasis. This project focuses on the role of the CNS in its regulation of those checks and balances, specifically the pancreatic Islet of Langerhans, that allows an organism to maintain a safe level of circulating glucose.

1.2 Islet of Langerhans

As mentioned previously, The Islet of Langerhans comprises of 1% of the pancreatic mass. It is a sphere of cells that all secrete hormones into the blood stream. It was first discovered in 1869 by a German medical student by the name of Paul Langerhans. The discovery was made when Langerhans fragmented the pancreas which was isolated from numerous species, including rabbit, salamander, guinea pig, dog, cat, pigeon, snake, frog, hen and human, and observed the fragments under a microscope. He then discovered a cell that was a "small irregularly polygonal structure, with brilliant cytoplasm; free of any granules with distinct round nuclei of moderate sizes. [They] lie together in considerable numbers, diffusely scattered in the parenchyma of the gland" **(**Barach 1952). Future observers of this cluster of cells, which were first described by Paul Langerhans, discovered that they secrete various factors internally, rather than externally as did the rest of the pancreas, and were named the Islet of Langerhans by a renowned French scientist named Laguesse (Barach 1952).

The pancreatic Islet of Langerhans is comprised of 5 main cell types: α-cells, β-cells, δ-cells, ε-cells and PP-cells. All of these cell types secrete a hormone into the blood stream of the

pancreas that is carried away from the islet, and all contribute either directly or indirectly to the homeostasis of circulating glucose. δ-cells are not only found in the pancreatic Islet, but also in the stomach lining and intestines. They secrete a 28 amino acid hormone called somatostatin in the pancreatic islet that largely serves as an inhibitory hormone for both α- and β-cell secretion. δ-cells in the stomach and gastrointestinal tract secrete somatostatin in order to slow down digestion by inhibiting acid secretion in the stomach lining as well as inhibiting pro-digestive hormones to essentially slowdown the digestive process in the intestine. Gastric somatostatin secretion is regulated through neural inhibition δ-cells through the vagus nerve, which releases acetylcholine (ACh) onto δ-cells to inhibit secretion of somatostatin. However, studies have shown that neural release of δ-cells in the pancreatic islet increase secretion of somatostatin. This is believed to happen in order to indirectly regulate β-cell secretion of insulin, since somatostatin has been shown to be an inhibitor of insulin release (Debas 1994, Molina 2014). ε-cells, the rarest cell type in the islet, release a hormone called ghrelin, which is also secreted by cells in the brain and stomach in much larger amounts than the pancreatic islet (Ariyasu 2001, Ferrini 2009). The role of ghrelin is to increase hunger and acid secretion in the stomach to prepare the organism for food intake by signaling to distinct regions of the CNS, mainly the hypothalamus and Ventral Tegmental Area (VTA), to increase hunger related signals as well as increase dopamine release in the reward center of the brain (Schwartz 2000). Pancreatic Polypeptide cells, or PP cells, release pancreatic polypeptide which reduces food intake and is found at high concentrations in patients with anorexia nerviosa and low concentrations in obese patients (Batterham 2003). Pancreatic Polypeptide binds to its receptors, which are located in the hypothalamus and brain stem, and effectively decrease food intake with the help of a second hormone which is secreted from the intestine called PYY (Batterham 2003, Hankir 2011).

Interestingly, PP cells controls exocrine function by inhibiting digestive juices to enter the gastrointestinal tract through CNS signaling (Williams 2014). These three cell types, while important in their own regard, only make up 10-25% of an islet. The majority of the islet is comprised of 2 major cell types that have a strong connection to one another and a stronger implication in the pathogenesis of T1DM and T2DM.

In humans, the α- and β-cells comprise about 90% of the total islet (40% α-cells and 50% β-cells) with the remaining 10% being a combination of δ, ε and PP cells (Steiner 2010). The dysfunction of both these cell types has been implicated in the pathogenesis of T1DM as well as T2DM (Müller 1973, Unger 1981). The α-cell secretes a 3.4 kDa hormone called glucagon, which is converted from a 160 kDa proglucagon peptide. Mature glucagon is produced and secreted by cleavage of this proglucagon peptide by Prohormone convertase 2 (Bell 1983, Arhén 2015). Glucagon's role in glucose homeostasis is to prevent the occurrence of hypoglycemia by increasing the production of glucose. Glucagon binds to the glucagon receptor, a G-Protein Coupled Receptor (GPCR), which is expressed in the liver, brain, kidney, gastrointestinal tract, adipose tissue and heart. Glucagon action in the liver directly increases circulating glucose levels by mobilizing glucose, which was stored as glycogen, into the bloodstream. The secretion of glucagon is regulated mainly by glucose, where the absence of glucose electrically activates α-cells and causes a depolarization of the cell membrane, which leads to an influx of Ca^{2+} through voltage gated calcium channels (VDCC) and facilitates exocytosis of glucagon granules (Walker 2011). There are a variety of other factors that facilitate and regulate glucagon secretion, such as the sympathetic and parasympathetic nervous system as well as paracrine signaling from the β-cell and δ-cell that modulate glucagon secretion (Walker 2011).

The α-cell is only half the story with regards to glucose homeostasis. The β-cell works in concert with the α-cell by secreting a hormone called insulin to decrease circulating blood glucose levels in order to facilitate the delivery of glucose to insulin sensitive tissues like the adipose tissue and muscle and prevent hyperglycemia. The β-cell has been widely studied due to its implication in the development of T1DM and T2DM. Both are a result of the destruction of the β-cell, however, the method and severity of the destruction is what differentiates the two diseases. T1DM is characterized as an autoimmune disease, where the host immune system identifies and destroys its own β-cells. T2DM is characterized as a metabolic disease where the inability of the β-cell to compensate for the increased demand of insulin leads to an increase in β-cell death. This high demand leads an increase in reactive oxygen species and a clogging of the endoplasmic reticulum with misfolded proteins which, if not alleviated, leads to β-cell apoptosis, or a cell-programmed death. Insulin secretion, like glucagon secretion, is predicated on the ratio of ATP/ADP. Unlike glucagon secretion, high levels of ATP promotes cascade of cellular events that eventually leads to the exocytosis of insulin granules into the circulation. The exact mechanism of how a rise in ATP levels lead to a rise in insulin secretion will be discussed later in this chapter, as well as further exploring a common theme among the cell types of the Islet of Langerhans which is a neuronal component that directly innervates these cell types to fine tune the secretory amount of hormone entering the circulation.

1.2.1 The Pancreatic β-cell

During early embryogenesis in mice, the pancreas, which is derived from the endoderm, expresses a transcription factor called Pancreatic and Duodenal Homeobox 1 (PDX1) that is used to identify the pancreas molecularly by embryonic day 8.5. These cell types will eventually

mature and develop and be part of either the exocrine, endocrine or ductal system. The endocrine islet in mouse develops from the trunk of the pancreas when transient expression of a transcription factor called Neurogenin3 (Neurog3) diverts cell types towards the endocrine fate, while Neurog3 negative cells develop into the ductal fate (Romer 2015). The first endocrine cell type to emerge is the a α-cell, followed by the β-,δ-,ε- cells during a secondary transition. And finally the PP cell forms during late embryogenesis (Romer 2015). The β-cell forms after numerous transcription factors are either expressed or silenced, and in both mice and humans, the cells aggregate into its islet structure shortly after birth (Romer 2015). The β-cell expands rapidly through cellular replication almost immediately postnatally. A study examined the number of replicating β-cells in humans from ages 2 weeks to 21 years. Cellular replication was determined through nuclear presence of a protein called Ki-67. The number of Ki-67+/insulin+ cells are high during the first 3-4 years of birth, but then there is a rapid and steep drop of cellular replication, and is maintained at a low level throughout adolescence and adulthood. Thus, an individual's β-cell mass is created and maintained during the early years of their life (Meier 2008). In mice, the exception to this is if there is an increased demand for insulin, which happens during pregnancy as well as obesity. A pregnant mouse's β-cell mass expands 3-4 times the size of non-pregnant mice, as well as an expansion of mass during high fat or high nutrient diet, and both of these expansions are due to an increase in cell replication (Rieck 2010, Linnemann 2014). Limited human studies show, however, the increase in replication post adolescence is relatively non-existent in both cases of pregnancy and obesity (Butler 2003, 2010). Instead, there is an increase in β-cell neo-genesis that leads to an increase in number of β-cells, but the degree to which the number of cells increase is marginal compared to rodents. This alludes to a discrepancy in the manner in which the two species responds to an increased demand of insulin levels.

β-cells are required to increase its production and secretion of insulin during times of high demand. The major contributor to the increase in production of insulin is a metabolic phenomenon known as insulin resistance, which is a driving force to the pathogenesis of T2DM. Insulin resistance is a term that describes the inability of insulin to illicit a downstream cellular response. To overcome insulin resistance, there is an increased production and secretion of insulin to initiate a signaling cascade within the cell. This increase in demand causes the β-cell to increase its production of insulin, which in turn causes an increase in negative factors that can be detrimental to the survival of the β-cell such as Reactive Oxygen Species (ROS), which can cause damage to a variety of proteins and can induce detrimental DNA damage (Pi 2010). An increase in production of insulin also leads to an increase in protein translation and production. The cell is an imperfect structure where mistakes can happen. However, the cell has developed a technique to mitigate these mistakes, as is the case when proteins become misfolded and are needed to be cleared in order to prevent aggregation and blocking of the normal protein processing pathway. When there is a high demand for insulin, the β-cell increases its production of misfolded proteins, to the extent at which the endoplasmic reticulum (ER) cannot clear out the amount of misfolded proteins in a timely manner. Three proteins, IRE1α (inositol requiring 1), PERK (PKR like ER kinase) and ATF6 (activating transcription factor 6 alpha), are the main sensors in the endoplasmic reticulum. As the amount of misfolded proteins increase, an inhibitor of the ER Stress Response, BiP, becomes inhibited itself, thus activating the ER Stress response, which will cause an increase in mRNA degradation, as well as a downregulation of protein synthesis in order to clear up the high levels of misfolded proteins. If, however, this does not relieve the ER of the amount of misfolded proteins, then the pro-apoptotic pathway is initiated,

which induces high levels of ROS to essentially kill the cell to prevent further damage to neighboring cells (Scheuner 2008).

As the production of insulin continues to increase to overcome the levels of insulin resistance, the final blow to the β-cell occurs by a protein that is created and secreted by the β-cell with insulin. Insulin is co-housed in the same granule with another protein called Islet Amyloid PolyPeptide (IAPP). IAPP has been shown to be an inhibitor of insulin secretion, but is secreted at a much less volume than insulin (1-2:250, IAPP: Insulin) (Westermark 2011). As the demand for insulin increases, the β-cell increases its secretion of insulin, which in turn increases the secretion of IAPP. IAPP is part of a family of amyloid proteins, which are notorious for aggregating and inhibiting function, as is the case in Alzheimer's disease. The increase in IAPP production causes an extracellular increase in IAPP aggregates, which can bury itself into the cellular membrane, causing a perforation to occur in the plasma membrane. There is an increase in a IAPP in the ER in parallel to the increase in insulin production, as all proteins are processed in the ER before being stored into secretory granules. The increase in IAPP levels in the ER promotes more ER stress and eventually β-cell apoptosis (Butler 2004, Matveyenko 2006, Westermark 2011, Alarcon 2012).

The initial driver of the inevitable death of β-cells is the high demand for insulin brought upon by system insulin resistance, thus removing of the insulin resistant state alleviates the β-cell from the exhaustion that occurs from producing and secreting inordinate amounts of insulin. In order to understand insulin resistance, the secretion, targeting and regulation of insulin must be understood.

1.3 Insulin

Since its discovery in 1922 by Fredrick Banting, Charles Best, John Macleod and James Collip, purified insulin has saved the lives of millions of T1DM and T2DM patients. Diabetes of any kind was a death sentence before insulin could be purified and administered to patients in hospitals. Banting, a surgeon, and Best, his assistant, worked together at the University of Toronto, where they would perform pancreatomies on dogs, and minced and ground up the pancreas. They then injected the extract back into the dog to keep it alive, and observed that the injection seemed to lower the dog's blood glucose levels. James Collip then was given the task of purifying the ingredient in the extract that was responsible for lowering blood glucose. After many failed attempts, he decided to use 90% ethanol and successfully precipitated the anti-diabetic agent they called "insulin". Today, pharmaceutical companies have created a more pure and humanly relevant insulin synthetic based on the findings of Donald F Steiner M.D. of the University of Chicago, who was the first person to discover that insulin was part of a longer chain that was cleaved in order to create insulin and another peptide called C-peptide (Steiner 1967, Phillipson 2015). The work done by Dr. Steiner created a paradigm shift into the way scientists thought about the processing of hormones before being secreted in their mature state.

Monomeric insulin consists of a 21-amino acid "A" chain, and a 30-amino acid "B" chain linked together by disulfide bonds (Fu 2013). Insulin is packed into its secretory granules at a concentration of about 40 milliMolar (mM), and exists in the granule as a hexamer consisting of a triplet of insulin dimers held together by Zn^{2+} (Weiss 2000, De Meyts 2004). Although insulin is a 51 amino acid structure that weighs about 5.8 kDa, the insulin gene encodes a protein that is 110 amino acids long, which is known as preproinsulin. Preproinsulin gets cleaved into proinsulin in the ER and is transported into the secretory vesicle by the Golgi Apparatus. Within the vesicle, proinsulin is cleaved into insulin and C-peptide by Prohormone Convertase 1/3 (PC

1/3) and Prohormone Convertase 2 (PC2), which is stimulated by the acidification of the insulin granule itself. (Steiner 1967, Rhodes 1987, Fu 2013). The granules are then segregated into two distinct pools, one being the readily releasable pool (RRP) which sits about 100-200 nanometers away from the inner surface of the plasma membrane, and a second pool termed the newcomer pool, which sits deeper in the cell (Kalwat 2017). There are many potentiators and initiators of insulin secretion, but none more important and powerful than glucose itself.

On the β-cell, there is an isoform of a glucose transporter termed GLUT2. GLUT2 has a high Km of 15-20 mM, which, in combination with glucokinase, makes it a suitable glucose sensor, since transport rates of glucose vary directly in parallel with changes in physiological blood glucose concentrations. Glucose enters the cell via GLUT2 and is immediately trapped inside the cell through phosphorylation of the 6-Carbon, which converts it to glucose-6-phosphate. The main kinase to catalyze this phosphorylation event in the pancreatic β-cell is glucokinase. Glucokinase, also called Hexokinase IV, is an isozyme of hexokinase and has a lower affinity for glucose than the other hexokinase isozymes. It is also expressed in the liver, intestine and distinct areas of the hypothalamus. Once glucose is trapped in the cytosol of the cell, it undergoes a catabolic process known as glucose metabolism. Glucose first enters the glycolytic pathway and is broken down and modified into pyruvate. During this event, 2 co-factors are produced, Adenosine Tri-Phosphate (ATP) and Nicotinamide Adenine Dinuleotide (NADH). As pyruvate enters into the mitochondria, it becomes modified and metabolized into Acetyl CoA through Pyruvate Dehydrogenase, which gets shunted into the Tri-Carboxylic Acid (TCA) cycle and binds with Oxaloacetate to create citrate. Pyruvate can directly turn into oxaloacetate through pyruvate carboxylase, which enters the TCA cycle. This mode of entering the TCA, called the anaplerotic pathway, has shown to be important for β-cell function (Schuit

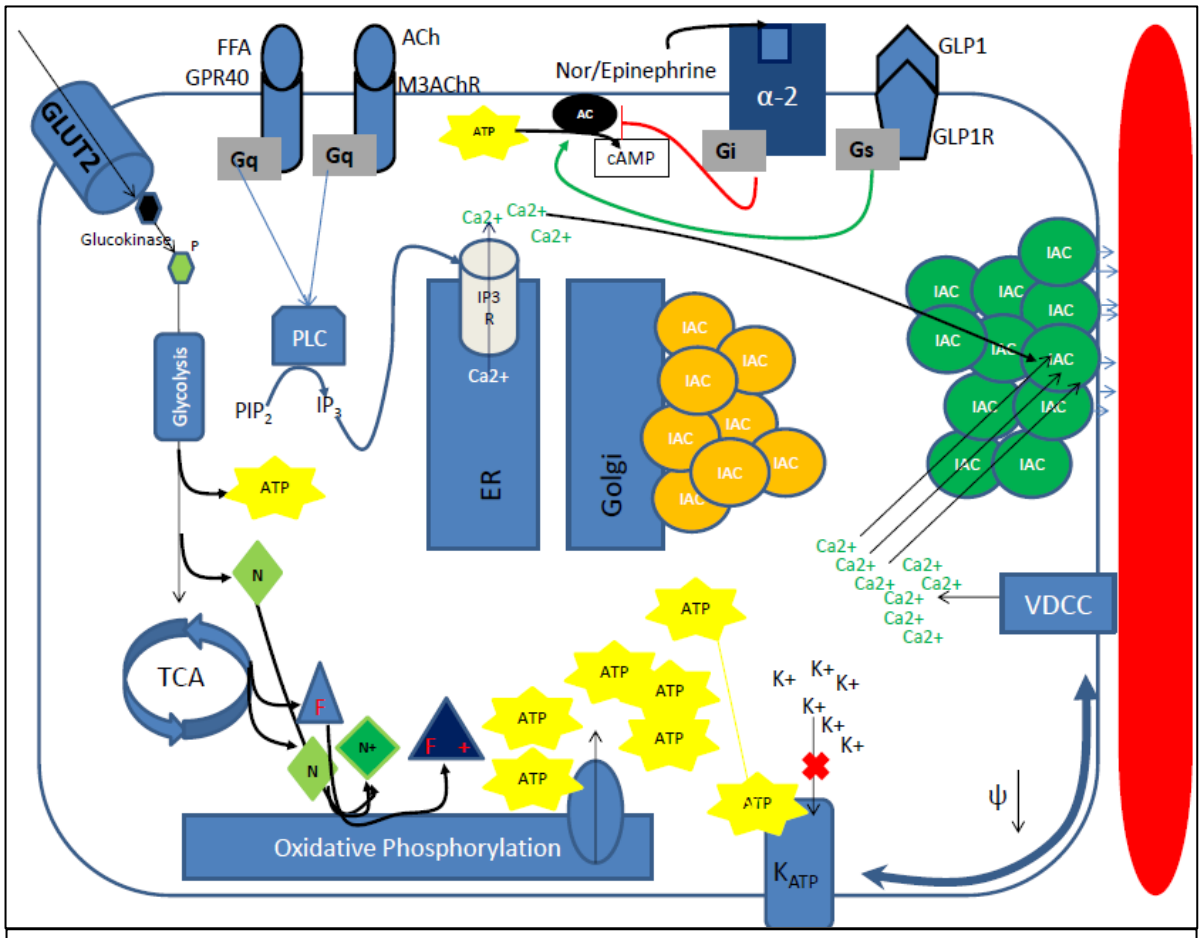

Figure 1.1: Schematic shows various ways pancreatic β-cell is regulated. Glucose is the main regulator of insulin secretion. Glucose is transported through GLUT2 and is quickly phosphorylated by glucokinase. Glucose-6-phosphate then enters the glycolytic pathway and is further metabolized to create ATP. ATP then binds to ATP sensitive potassium channels, leading to membrane depolarization and opening of voltage dependent calcium channels, leading to an influx of calcium and assists in exocytosis of insulin granules into circulation. Other factors, such as long-chain fatty acids (such as palmitate) and Acetylcholine bind to GPR40 receptor and Muscarinic 3 Receptor (M3AChR), which are G-protein coupled receptors (GPCR) coupled with the Gq protein. Downstream activation of Phospholipase C (PLC) cleaves PIP2 into DiAcylGlycerol (DAG, not shown) and IP3, which enters the ER to bind to the IP3 receptor, which empties ER stores of Ca2+ to facilitate exocytosis of insulin granules. GLP-1 receptor and α2 receptor are both GPCRs that have opposing G-proteins, with GLP-1R signaling through Gs (stimulating) and α2R signaling through Gi (inhibitory). GLP-1R and α2R bind GLP-1 and norepinephrine/epinephrine, respectively, which either stimulates or inhibits cAMP production. cAMP production activates Protein Kinase A to facilitate insulin secretion.

1997). As citrate cycles through the TCA cycle, more of the NADH co-factor is produced, along

with a third cofactor called $FADH_2$. NADH and $FADH_2$ are now used to create more ATP by depositing their electrons in specialized proteins that are lined along the inner membrane of the mitochondria. 1 molecule of NADH and $FADH_2$ creates 3 and 2 molecules of ATP, respectively. As electrons are deposited into the electron transport chain, an electrochemical gradient is formed with protons being shuttled in the outer mitochondrial membrane space. In order to alleviate this build-up of protons, the protons are shunted through an enzyme complex called the ATP synthase channel. Protons entering and exiting the channel creates mechanical energy and causes the turning of the ATP synthase, which creates more ATP (Itoh 2004). This creation of ATP leaves the mitochondria and provides the energy for other cellular processes to occur. With respect to insulin secretion, ATP binds to K+ATP channels and closes the channel. Potassium is at a higher concentration in the cell than out of the cell, which drives potassium outside the cell. After closing of the channels, there is a buildup of positive ions inside the cell which raises the membrane potential. Rising of the membrane potential activates VDCC, which opens up calcium channels which allows calcium to enter the cell. Calcium then enters the endoplasmic reticulum and empties out ER calcium stores to further drive up the membrane potential. Calcium also facilitates the release of those RRP vesicles, in what has been termed the "first-phase" of insulin secretion. Newcomer vesicles then fuse to the plasma membrane within 50 milliseconds, and is released for the "second-phase" of insulin secretion (Figure 1.1) (Curry 1968, Kalwat 2017).

The β-cell maintains a secretory polarity, where vesicles are released towards the venous islet microcirculation and moved away from the islet milieu (Rhodes 2013). The first organ that insulin binds to is the liver. Insulin comes into the portal vein from the pancreas at 5 nmol/L and binds to the insulin receptor (IR), which is composed of a pair of homodimeric tyrosine kinase transmembrane receptor (Song 2000). After binding of insulin to the IR, an auto-phosphorylation

of tyrosine residues on the tail of the receptor occurs and activates a scaffolding protein called Insulin Receptor Substrate 1/2 (IRS 1/2), which signals a downstream cascade that causes upregulation and downregulation of glucose metabolism specific genes. The liver is a rich source of stored glucose, which is kept in the form of glycogen. It is also responsible for de novo glucose production, through a process called gluconeogenesis. During a low glucose environment, the liver is activated by glucagon and other catecholamines to increase circulating blood glucose by breaking down glycogen, through a process called glycogenolysis, into individual glucose molecules, which is then transported out of the cell through GLUT2. The liver also creates glucose by taking the end product of glycolysis, pyruvate, and converting it back into glucose through gluconeogenesis. After the binding of insulin to the IR in hepatocytes, insulin then suppresses glucose entering the bloodstream from the liver by downregulating expression and production glycogenolytic and gluconeogenic enzymes.

1.3.1 Expression of the Insulin Receptor

Along with hepatocytes, IR is expressed in adipose tissue, skeletal muscle, cardiomyocytes, epithelial cells, kidney cells and distinct areas of the brain. The adipose tissue is responsible for storage of energy in the form of fat. It takes up lipid molecules as well as glucose molecules and converts them into triglyceride. Insulin regulates and promotes the storage of excess energy, particularly glucose, into lipid droplets. Binding of insulin to its receptor on the adipocyte membrane initiates an intracellular signaling cascade, which starts with IRS 1/2 binding to the auto-phosphorylated tyrosine residues on the receptor tail, followed by activation of Phosphoinisotol-3-Kinase (PI3K), which in turn activates a multifaceted protein called Akt, sometimes called Protein Kinase B (PKB). Akt is a serine/threonine kinase that initiates the translocation of a GLUT isoform called GLUT 4 in adipocytes. Once GLUT4 is present on the

cell membrane, glucose then enters the cell and becomes stored as fat (Summers 2000). GLUT4 is an insulin sensitive transporter that is translocated to the cell surface solely through the actions of insulin signaling. GLUT4 is also expressed in the heart and skeletal muscle. Skeletal muscle translocation of GLUT4 is also initiated through an enzyme called Adenosine Mono-Phosphate Kinase (AMPK), which becomes activated through Adenosine Mono-Phospahte (AMP). AMP is created when ATP is hydrolyzed into AMP during strenuous physical activity, when the cell is in need of energy. AMPK works in concert with Akt to inhibit a protein called Akt Substrate of 160 kDa (AS160), which inhibits the translocation of GLUT4 to the cell surface (Koistinen 2003). The anti-diabetic drug metformin has been shown to increase AMPK activity in liver and skeletal muscle in T2DM patients, and is used to help lower circulating glucose levels independent of insulin (Musi 2002, Treebak 2006).

The IR is widely distributed in the brain with highest concentrations in the olfactory bulb, ventral tegmental area (VTA), cerebral cortex, cerebellum, hippocampus and hypothalamus (Hörsch 1999, Plum 2005). The brain is an insulin-responsive organ, however does not require insulin for glucose uptake. Neurons, astrocytes and glial cells all express a variety of glucose transporters, namely GLUT1 and GLUT3, which are constitutively expressed on the plasma membrane. There is a small amount of insulin that enters the CNS through the choroid plexus, however, the predominant way insulin enters the brain is through the blood brain barrier (BBB) (Strubbe 1988, Banks 2012). The BBB is a lining of endothelial cells that separate the circulating blood from the brain. The BBB also has insulin receptors that connect the basal side to the central side of the BBB, where insulin binds to the insulin receptor on the peripheral side and gets transported through the BBB and towards the CNS, where it binds to neurons and astrocytes that express the insulin receptor (Figure 1.2).

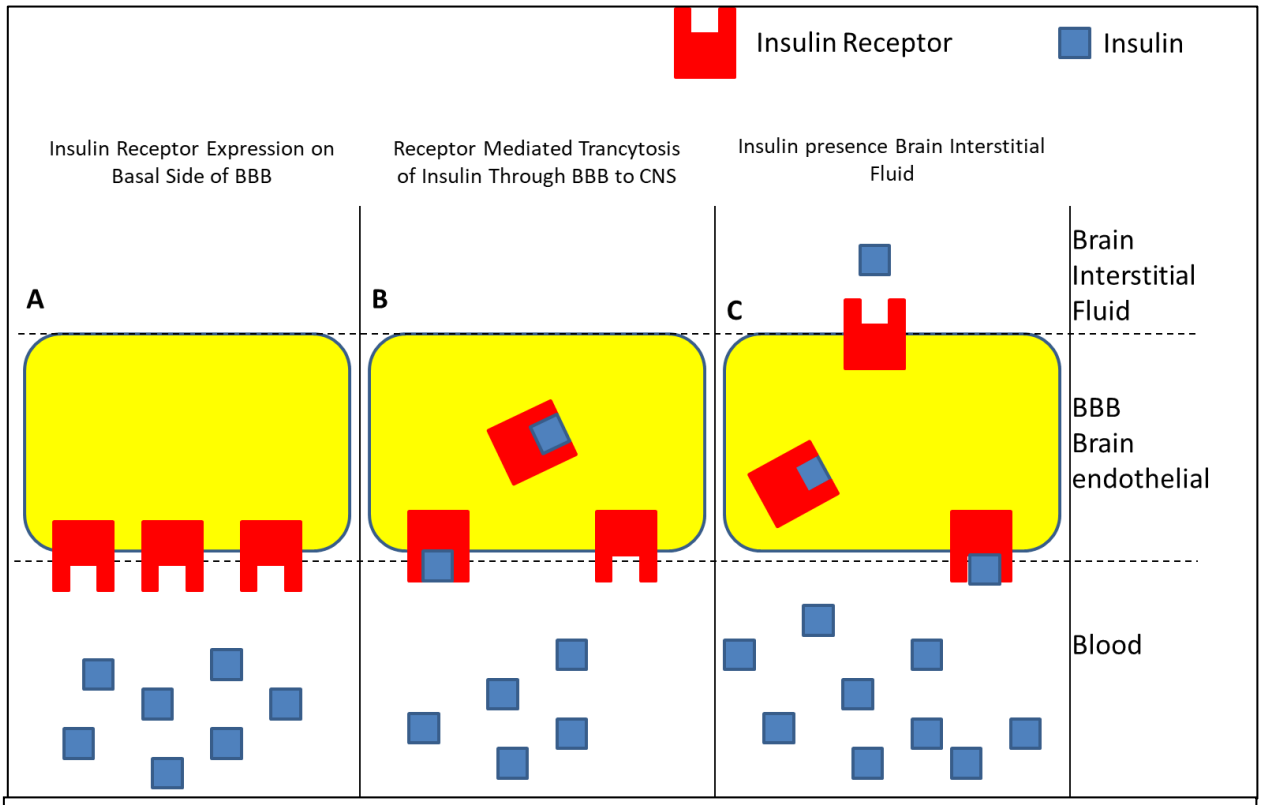

Figure 1.2: Schematic of transcytosis of insulin passing through the blood brain barrier from periphery to CNS, by insulin binding to its receptor in the peripheral side and facilitating the transport of insulin through the epithelium and to the CNS.

The olfactory bulb is responsible for sense of smell and connects to various areas of the brain responsible for emotions and learning. Insulin signaling in the olfactory bulb has been shown to desensitize sense of smell in both rodents and humans, where it reduces the rodent's ability to smell food (Aimé 2012, Brünner 2013). The VTA is the reward center of the brain that is dense in dopaminergic neurons. It signals to a plethora of areas and regions in the brain to control reward seeking behavior. Insulin signaling in the VTA reduces hedonic feeding, as shown in an elegant study that reduced a mouse's desire to eat high-fat sweetened food after a satiated state when the investigators administered insulin into the VTA (Mebel 2012). The hippocampus is responsible for consolidation learning and spatial memory that is used for navigation. Insulin signaling in this area has been implicated in contributing to increase learning

and spatial memory. Insulin signaling has also been associated in preventing Alzheimer's disease (AD) through degradation of potential plaque forming proteins (Bedse 2015). Intriguingly, a variety of studies have shown that T2DM patients are at a significantly higher risk of AD (Baker 2011, Roberts 2014, Willette 2015).

The hypothalamus has been considered the metabolic regulatory center of the brain. It sits below the thalamus and is part of the limbic system. The hypothalamus has numerous areas that are responsible for a variety of functions and is split into 3 distinct regions, the anterior, tuberal and posterior hypothalamus. Insulin receptor expression is highly enriched in the Arcuate Nucleus (ARC), Ventromedial Nucleus (VMN) in the anterior hypothalamus (Storlien 1975, Havrankova 1978). Both the ARC and VMN are in a prime location to bind central insulin. The both sit right above the BBB to receive insulin that comes through to the CNS via transcytosis and also sit directly medially to the third ventricle (3V), where insulin that enters the CSF through the choroid plexus. The ARC is a major site to integrate a variety of nutrient and hormones to give either a satiety or hunger signal. These two signals are driven by 2 major subsets of neurons: Proopiomelanocortin (POMC) and Neuropeptide Y/Agouti-Related Protein (NPY/AgRP) neurons. Insulin signaling in these neurons promote distinct effects that reduce circulating glucose levels. Insulin signaling in NPY/AgRP neurons decrease hepatic glucose production (HGP), while insulin signaling in POMC neurons reduces adipocyte lipolysis, which prevents buildup of lipid accumulation in the liver (Figure 1.3) (Könner 2007, Shin 2017).

The VMN is responsible for various functions that include thermoregulation, sexual activity, fear and hunger. Insulin receptors in the VMN is confined to a specific area of the VMN called the dorsomedial VMN (VMNdm) in a subset of neurons that contain Steroidogenic Factor-1 (SF-1) which is responsible for sex differentiation and metabolism. There has been

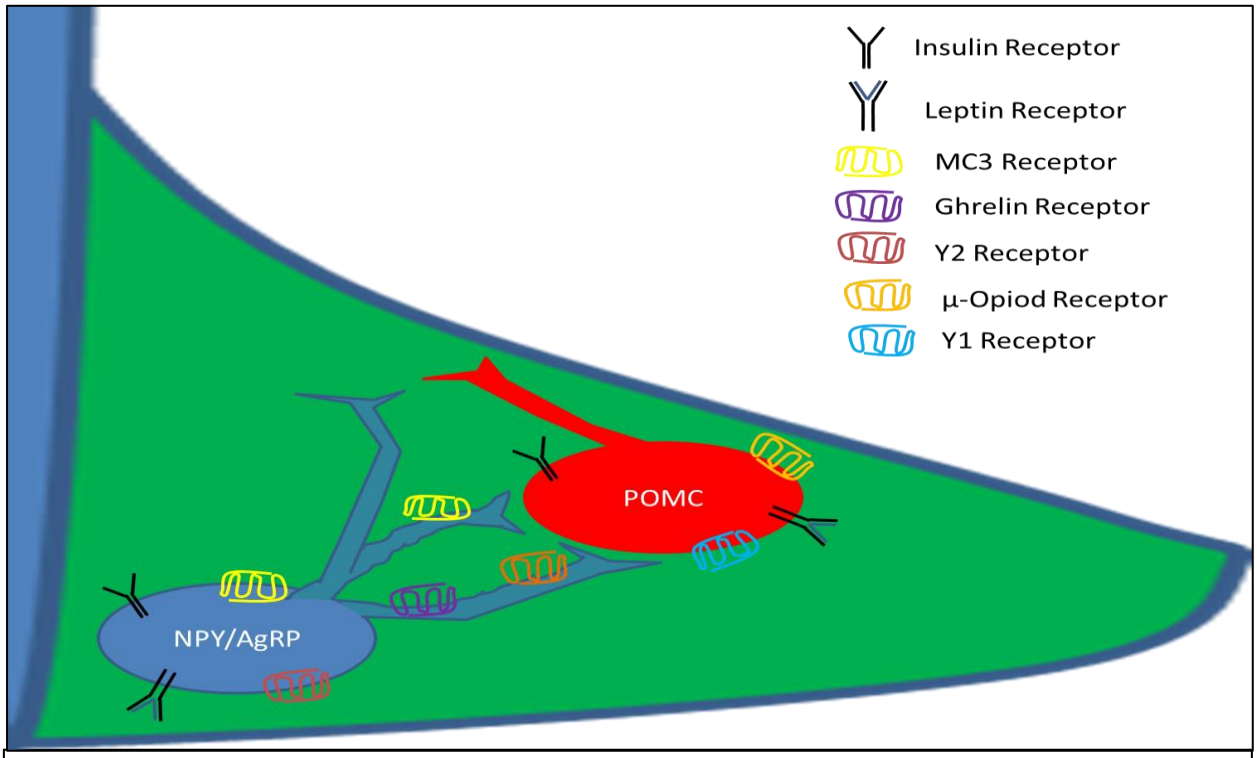

Figure 1.3: Schematic of two neuronal cell types in ARC. NPY/AgRP neurons and POMC neurons signal to one another and propagate their signal to other hypothalamic areas. Insulin and Leptin signaling, as well as MC3 Receptor, and μ-opoid Receptor in these neurons decrease hunger and increase satiety in organisms, while interruption of insulin and leptin signaling in these neurons cause hyperphagia and obesity. Activation of Ghrelin, Y2 Receptor and Y1 Receptor increase hunger and decrease satiety (Cone 2001,Belgardt 2009, Gough 2012,Sohn 2015)

some controversy in terms of the exact function of insulin signaling in this area of the hypothalamus. Investigators have used a transgenic model to knockout the insulin receptor in SF-1 neurons in the VMN by using a Cre-Lox system. SF-1, however, is not confined to the VMN, and is also expressed in the pituitary gland as well as the testis of male mice. (Klöckener 2011, Yi 2011). Other bodies of work have used techniques, such as stereotaxic surgery, to specifically target areas of interest, and are more reliable than Cre-Lox Systems (Harno 2013). Investigators have tried to knockout and inhibit the insulin receptor in the VMN using this stereotaxic method in rats. Their results show islet cell dysfunction in the absence of a high fat diet challenge

(Paranjape 2010, 2011). However, further examination of the articles show lack of injection site verification. Additionally, the stereotaxic coordinates provided by the investigators implicate the A13 region, not the VMN (Paxinos 2006). Therefore, insulin signaling in the VMN has been an area of interest to properly elucidate its exact function. In order to understand what insulin resistance is, one must first understand the insulin signaling mechanisms and the intracellular regulatory system that is in place to assure proper signaling of insulin.

1.3.2 Insulin Signaling

The insulin receptor is a tetrameric protein that has 2 extracellular α-subunits and 2 transmembrane β-subunits. The two subunits originate from one long mRNA transcript that is alternatively spliced in different tissue types, where the variation comes from the exclusion or inclusion of exon 11. IR excluding exon 11 is referred to as Insulin Receptor isoform A (IR-A) and those including exon 11 is referred to as Insulin Receptor isoform B (IR-B). IR-A is expressed in fetal tissue and the brain, and has a higher affinity for insulin along with a higher internalization rate, while IR-B expression is highest in the liver (Frasca 1999, Boucher 2014). After binding of the insulin to its receptor, IR undergoes a conformational change that results in the auto-phosphorylation of tyrosine residues on the β-subunit. The insulin signaling pathway splits from the post auto-phosphorylation of the tyrosine residues into a metabolic pathway. The metabolic pathway consists of recruitment of IRS 1/2, which is a scaffolding protein that recruits the downstream actors of this pathway, which was discussed earlier in this chapter. The activation is PI3K initiates the subsequent activation of a series of kinases that lead to activation of Akt. In the liver, Akt activation causes the multi-phosphorylation of Forkhead box O (FOXO) protein, which is a transcription factor responsible for the expression of lipogenic and gluconeogenic proteins. Phosphorylation of the FOXO protein excludes this transcription factor

from the nucleus and prevents expression of glucose producing proteins. The cell growth and differentiation pathway consists of recruitment of a Guanine Nucleotide Exchange Factor called SOS-Grb2, which is recruited by IRS1 and Shc. This complex activates Ras, which is a small GTP exchange factor, by exchanging the Guanine Di-Phosphate (GDP) bound to Ras with Guanine-TriPhosphate (GTP). Activated Ras then activates Raf, which is also called the mitogen-activating protein kinase kinase kinase (MAP3K). Raf then phosphorylates Mitogen-activating, ERK activating kinase (MEK), which then activates ERK, otherwise known as MAPK (Manchester 1994, Seger 1995, Boucher 2014). This pathway plays a direct role in cell proliferation and differentiation, regulation of gene expression as well as organization of extra-nuclear events, such as cytoskeletal reorganization (Boucher 2014). The products of these two pathways act as negative regulators of insulin signaling to either dampen further activation of the receptor, or preventing phosphorylation of its downstream targets (Boucher 2014). One major negative regulator of insulin signaling is the Protein Tyrosine Phosphatase 1B (PTP1B), which dephosphorylates the insulin receptor as well as IRS proteins. A second major phosphatase and negative insulin signaling regulator is the Protein Phosphatase 2A (PP2A), which is a serine/threonine phosphatase, and is responsible for dephosphorylating a variety of downstream targets of insulin signaling, such as Akt and ERK. Another important player in regulating insulin signaling is a phosphatase called Phophatase and Tensin Homolog (PTEN), which dephosophorylates PIP_3, the product of PI3K. (Boucher 2014).

There is an essential need to regulate insulin signaling. Aberrant insulin signaling can cause a life-threatening metabolic state in an organism called hypoglycemia, which can lead to seizures, loss of consciousness, coma and death. Therefore, dampening of insulin signaling, or insulin resistance, is a protective mechanism against hypoglycemia in an acute time frame. The

IR itself has a mechanism to prevent hypoglycemia, which is receptor internalization. The insulin receptor is internalized through a regulatory mechanism called endovdcccytosis. A protein called clathrin coats the area of the plasma membrane that contains the receptor, which cause the plasma membrane to undergo a conformational change and create a pit with the receptor at the bottom of the pit. It will eventually form a vesicle and fuse with a lysosome, which is responsible for degradation of intracellular proteins. Endocytosis of the insulin receptor can either lead to degradation of receptor by fusing with the lysosome, or it can be recycled after the ligand, insulin, is removed from the receptor and degraded (Goldstein 1979, Trischitta 1989). Acute insulin resistance is a mechanism to protect the organism from hypoglycemia. However, in the instance of T2DM and obesity, chronic insulin resistance becomes a detriment rather than a benefit.

Obesity is one of the leading causes of T2DM, and is a major contributor to insulin resistance. Excess ectopic accumulation of lipids, that is lipid accumulation outside of the adipocyte, has been shown to increase insulin resistance. Obese patients have high levels of circulating free fatty acids (FFA) that have been shown to induce activation of inflammatory related proteins called c-Jun-N-terminal kinases (JNK) and IκB Kinases (IKK) that block insulin signaling in the cell. Also, FFAs increase activation of Protein Kinase C, which phosphorylates IRS-1 on Serine-307, leading to its de-activation (Schenk 2008). Obesity causes a low-grade inflammation throughout the organism, leading to activation and increased secretion of pro-inflammatory cytokines, such as TNF-α, IL1β, or IL-6, by both the immune cell and adipocyte. These cytokines increase the insulin resistance in multiple tissues, including the brain. Insulin resistance has been characterized by the location of the resistance, and investigators have separated it into either peripheral insulin resistance or central insulin resistance. There is

mounting evidence that obesity causes insulin resistance at the level of the CNS. Inflammatory consequences of obesity have been shown to affect neurons in the hypothalamus that control feeding behavior. JNK and IKK activation in AgRP neurons in the ARC caused weight gain as well as peripheral insulin resistance. Activation of JNK in these neurons increases Leptin resistance, while IKK activation increases peripheral insulin resistance. Leptin is a hormone secreted by adipocytes to signal to the hypothalamus of a satiated feeling. Resistance to leptin blocks that satiated signal, which leads to overeating and weight gain, which perpetuates the resistance (Tsaousidou 2014).

There is a large volume of knowledge about the origin, function and secretion of insulin, as well as the regulatory mechanisms that prevent aberrant insulin signaling. The signal to stop insulin secretion, however, still remains a mystery and controversy. It is well known that the element that primarily stops further insulin secretion is the same element that starts it off: glucose. Once circulating glucose levels have dropped after a meal, GLUT2 and Glucokinase would not be able to sense and trap glucose inside the β-cell and therefore decrease the levels of ATP. This allows K_{ATP} channels to open and a re-polarization of the plasma membrane occurs and Ca^{2+} is prevented from entering the cell, which decreases secretion of insulin granules. In obesity and T2DM, however, insulin secretion seems to deviate from the control of glucose, where patients have higher basal insulin levels, and aberrant insulin secretion to overcome the insulin resistance (Corkey 2012). There must be another factor involved in regulating insulin secretion, albeit, to a lesser extent than glucose itself. As previously discussed, the organism protects itself from hypoglycemia and has at its disposal a variety of checkpoints to ensure that insulin signaling is heavily regulated. So it would make physiological sense, that insulin itself

controls its secretion. However, there has been much debate as to how insulin regulates its own secretion.

1.3.3 Regulation of Insulin: A Short Historical Perspective

Charles Best, one of the pioneers that discovered insulin, and his colleague performed an experiment where rats were injected with either a constant dose of protamine zinc insulin for a 1 week period, or increasing dose of insulin throughout the week. Rats that were given a high dose of insulin (13 units) died at the end of the experiment from hypoglycemia, which provides further evidence to the dangers of hypoglycemia. Best and colleagues then measured endogenous insulin levels at the end of the experiment on the remaining rats and found that with increased injection of insulin, the rats decreased the secretion of endogenous insulin, compared to rats that did not receive a protamine zinc insulin injection (Best 1941). This was one of the first studies to support the idea that insulin downregulates further insulin secretion. Subsequent studies used more advanced and clever techniques to understand how insulin regulates its own secretion, only to come to an impasse. There have been several studies that have supported the idea that insulin negatively regulates further insulin secretion (DeFronzo 1981, Elahi 1982, Waldhäusl 1982, Garvey 1985, Ratzmann 1985, Stagner 1986, Argoud 1987, Boden 1993), as well as a few studies that have concluded that insulin increases its own secretion (Aspinwall 1999, Leibiger 2000, Bouche 2010, Halperin 2012). Nonetheless, there is a controversy on a fundamental question of how insulin is regulating itself.

The idea that insulin perpetuates its own secretion was highlighted in a set of studies that used human subjects, 8 with Impaired Glucose Tolerance (IGT), 11 with T2DM and 8 healthy controls. Each was administered dextrose for 80 minutes and then given a 4 hour infusion of

either saline or insulin. Afterword they measured Glucose Stimulated Insulin Secretion (GSIS) and showed an increase in insulin secretion in insulin sensitive and T2DM subjects, but not in IGT subjects. In another study with a similar experimental model, they measured C-peptide levels as well. Their conclusion on both studies was that insulin potentiates GSIS (Bouche 2010, Halperin 2012). Based on various studies that used mice with the insulin receptor on the β-cell knocked out (βIRKO mice), they concluded that insulin signals to the β-cell in an autocrine fashion to "prime" the β-cell and potentiate GSIS (Kulkarni 1999). In order for the β-cell to have an autocrine effect of insulin, insulin must be secreted and present in a sufficient amount in order for enough extracellular binding of insulin to elicit an intracellular response. As discussed earlier, the β-cell maintains a polarity in terms of insulin secretion, which directs insulin granules to exocytose its content into the venous islet microcirculation towards the hepatic portal vein, and away from the islet milieu. The other way insulin can have an autocrine effect is that it returns to the islet after it has passed through the liver, adipose and skeletal environment. Insulin was measured in the portal vein at a concentration of about 5 nmol/L, and when it circulates back, insulin can be measured to be present at a concentration of 1-10 pmol/L. In order for insulin to elicit a response, there must be a concentration of at least 1 nmol/L range (Tsunekawa 2011, Rhodes 2013). Even if one assumes that the β-cell is exposed to a sufficient amount of insulin, the insulin receptor on the β-cell will continue to behave accordingly, and become permanently downregulated if insulin is not cleared from the islet. Furthermore, if insulin potentiates and increases its own secretion, the question then becomes what stops this positive feedforward loop. Insulin induced insulin secretion would induce systemic insulin resistance and would leave an individual susceptible to T2DM and obesity (Corker 2012, Rhodes 2013). This

suggests that the stipulations for autocrine signaling of insulin are anatomically and physiologically improbable, if not detrimental.

The conclusion of insulin inhibiting its own secretion, however, has been demonstrated in various studies. However, for the same reasons insulin is unlikely to potentiate its own secretion through autocrine mechanisms, it is unlikely to inhibit insulin secretion via autocrine signaling. A few studies have introduced the possibility of a distal signal to the pancreatic β-cell to inhibit further insulin secretion. These authors have implicated the central nervous system as the main player in inhibiting further insulin secretion. Studies were done through a variety of subjects, including rodents, dogs and humans, where the main nerve connecting the CNS to the pancreas (vagus nerve) was severed in dogs and a lack of suppression was seen in vagotomized dogs when injected with insulin, compared to controls (Stagner 1986). When examining insulin regulation in human subjects, investigators injected insulin into 5 patients, categorized as healthy, kidney transplant and pancreas/kidney transplant. Insulin failed to suppress C-peptide levels in the pancreas/kidney transplant, compared to the other 2 groups. This failure was assigned to the fact that pancreas/kidney transplant subjects had a denervated pancreas, and therefore, were not able to receive any neuronal signals to inhibit insulin secretion (Boden 1993). The idea that the CNS is responsible for inhibiting further insulin secretion has been supported by various other investigators who have come to the conclusion of a negative feedback loop (DeFronzo 1981, Elahi 1982, Garvey 1985, Argoud 1987). It is therefore imperative that the matter of the CNS regulating hormone secretion be explored further.

1.4 Central Nervous System

The CNS has been implicated in glucose metabolism for over a century, beginning with renowned French scientist Claude Bernard who introduced the role of the CNS in glucose metabolism when he stimulated the floor of fourth ventricle (4V) in the brainstem of a rabbit and observed an increase in glucose being excreted from the animal through urine (glycosuria). He detailed this in his seminal work, and called this phenomenon *Piqûre diabétique* (Bernard 1849). About 100 years later, scientists observed this same phenomenon when soldiers of war came home after suffering gunshot wounds to the head and had an increase in glycosuria (Huntingter 1950, Sturm 1953).

Since then, there has been a plethora of studies linking the CNS, more specifically, the hypothalamus to the pancreatic islet. Lesion studies from the 1980s in the Ventromedial Hypothalamus (VMH) of rats showed an increase in serum insulin levels, to present day where investigators mapped a circuit that directly connects the pancreas to the hypothalamus, as well as established a functionality of the circuit for glucose-sensing neurons (Tokunaga 1986, Rosario 2016). Rosario et al was the first to establish this circuit using an engineered retrograde virus, allowing for increased accuracy, called pseudorabies virus (PRV), which travels through a neuron in the opposite direction as a propagated (Card 2014). Pseudorabies virus is a part of the α-herpessimplex virus family and has genetically modified for usage as a tool to trace a neural circuit. In the study conducted by Rosario et al, the strain of PRV used was called PRV-Bartha Blu, which expresses β-galactosidase as a reporter gene, and was injected in the pancreas of 16-18 week old mice. Infected mice were then sacrificed 24, 48, 72 and 96 hours post infection and their brains were harvested, paraffin embedded and sectioned at a 10 micron thickness (Rosario 2016). Immunofluorescence staining had detected β-galactosidase (β-gal) 24 hours post infection in the brainstem, mainly in the dorsal motor nucleus of the 10^{th} nerve (DMX) and the nuclear

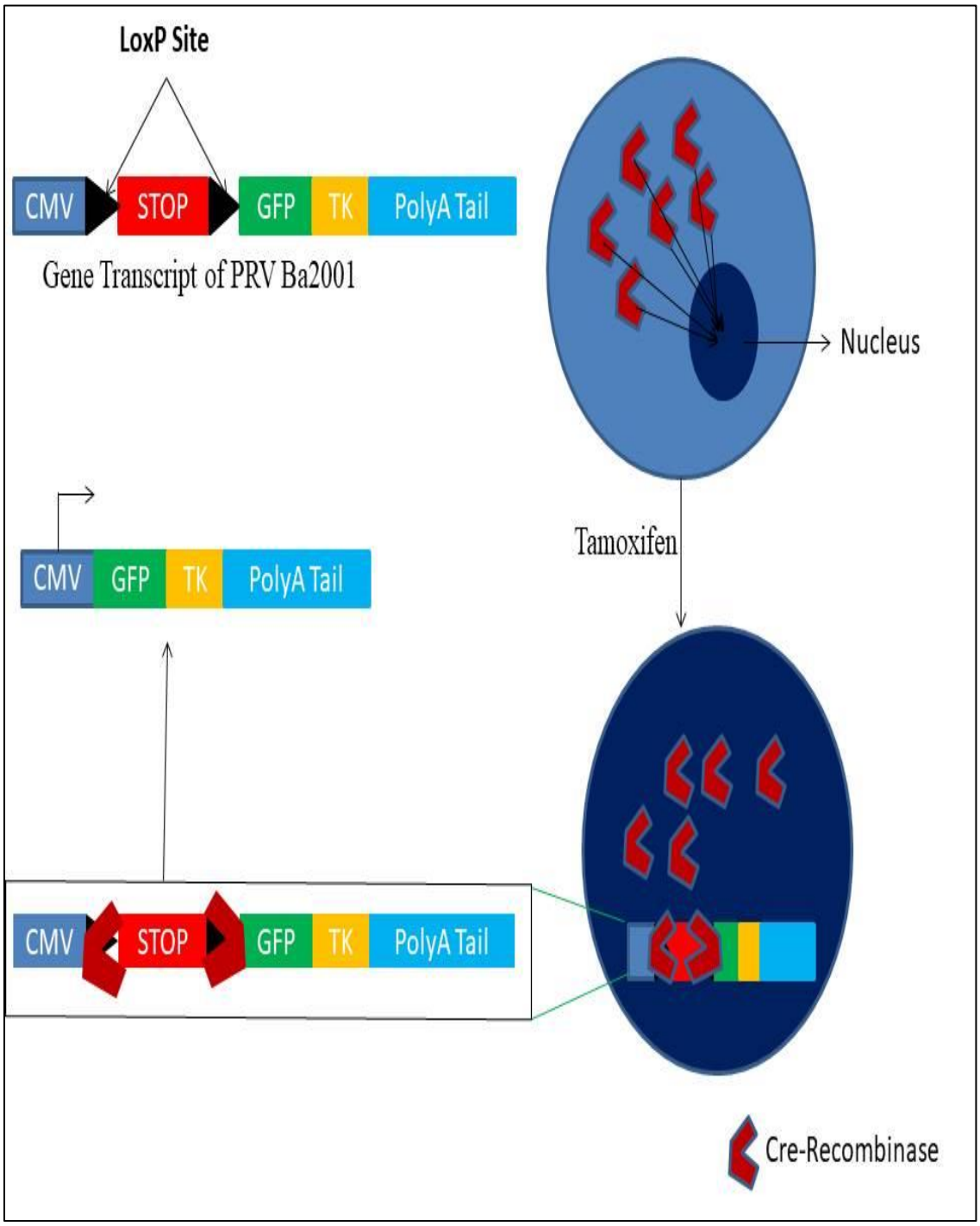

Figure 1.4: Schematic of PRV-Ba2001 Mapping. Tamoxifen administration relocates Cre-Recombinase to nucleus. After PRV infection, viral DNA enters nucleus and Cre-Recombinase recognizes LoxP site and excises out stop sequence, which allows GFP and TK expression to occur (Rosario 2016).

solitary tract (NTS). After 48 hours, there was an enrichment of signal in the DMX and NTS as

well as expression of β-gal in the Cerebellum. Signal was detected in midbrain regions of the Raphe Magnus (RaM) Periaqueductal Grey (PAG) and the A5 region. After 72 hours, β-gal was detected in the Central Amygdala (CeA), Medial Habenula (MeHab) and the Hypothalamus, specifically the Lateral Hypothalamic Area (LHA), Dorsomedial Nucleus (DMN), Ventromedial Nucleus (VMN), Arcuate Nucleus (ARC), Paraventricular Nucleus (PVN) and the Suprachiasmatic Nucleus (SCN). After 96 hours, an enrichment of β-gal signal in these areas of the hypothalamus, however no signal was detected anterior to the PVN and SCN (Rosario 2016).

As stated earlier, the pancreas is comprised of both exocrine and endocrine cells, with both cell types known to have a connection to the CNS. In order to parse the connection to the Islet of Langerhans from the exocrine acinar cells, the investigators used a transgenic mouse model that expresses Cre-Recombinase under the control of the Mouse Insulin Promoter (MIP-Cre) and infected the mice with another strain of PRV called PRV-Bartha 2001 (PRV-Ba2001) that conditionally expresses the reporter gene Green Fluorescent Protein in the presence of Cre Recombinase (Figure 1.4) (Card 2014). Cre-Recombinase is an enzyme that recognizes a sequence of DNA called a locus of X over P1 (loxP), which is a 34 base pair sequence that is derived from a bacteriophage P1 (Araki 1997). After recognition, Cre-Recombinase cuts and excises the loxP site, and anneals the end strands together. PRV Ba2001 is packaged with DNA that contains a Cytomegalyvirus (CMV) promoter as a start site for transcription, followed by a Stop sequence that is flanked by 2 loxP sites. Following the stop sequence is the GFP cassette and a thymidine kinase cassette. When Cre Recombinase recognizes and excises the loxP sites, the Stop sequence is then removed from the sequence, which allows GFP expression to occur, as well as thymidine kinase, which is an enzyme that allows for replication and packaging of new virus that does not include the stop sequence (Callaway 2008, Card 2014). In the MIP-Cre

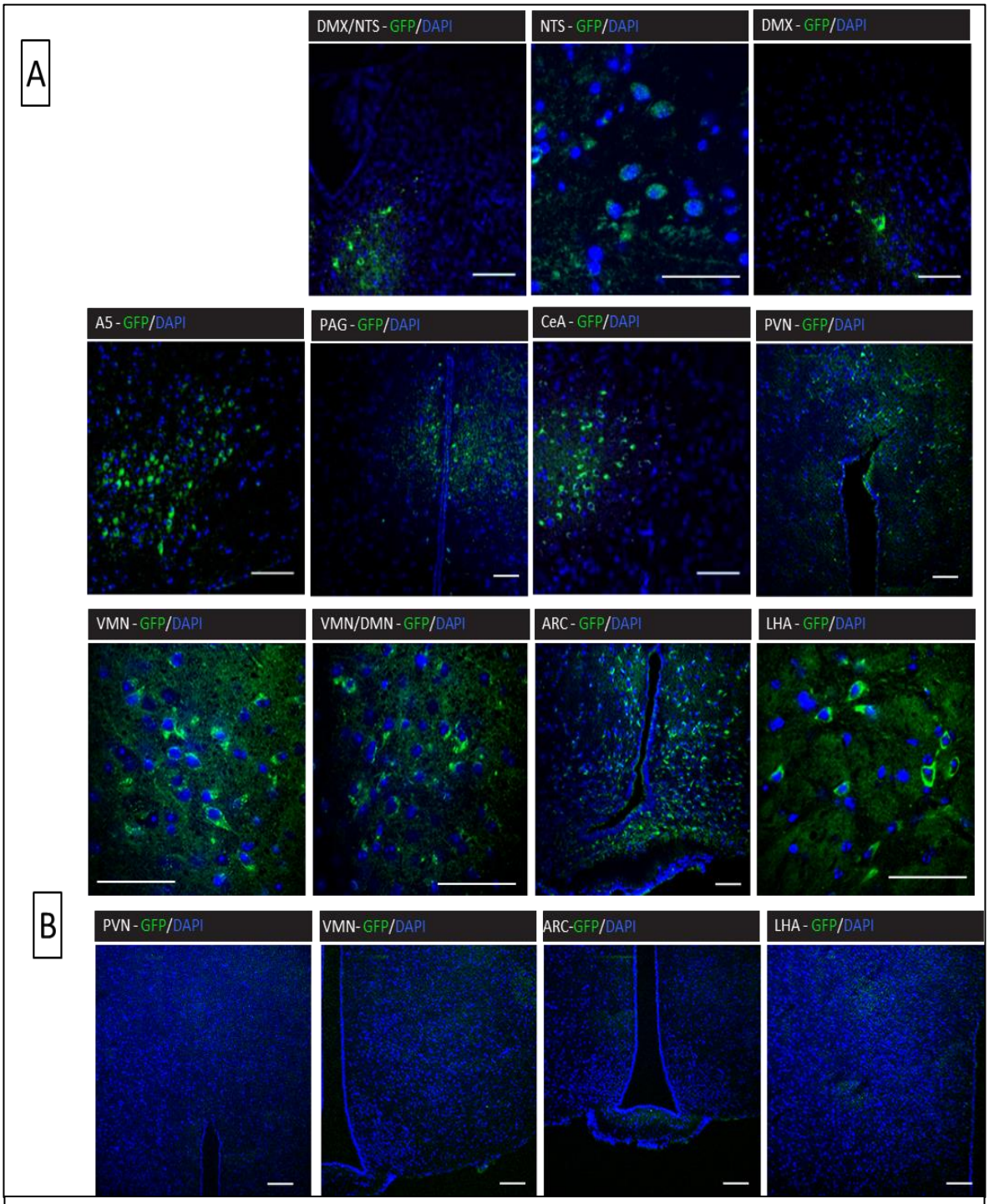

Figure 1.5: Map shows areas of the CNS that have a connection to the pancreatic Islet. PRV-Ba2001 were injected into MIP-Cre mice after 3 doses of **(A)** tamoxifen or **(B)** tap water was administered via oral gavage over 5 days. GFP (green) was stained for via immunofluorescence, which signifies neurons in the CNS that communicates to the pancreatic islet (Rosario 2016). (Work conducted by Inderroop Singh)

mouse, the Cre-recombinase has an addition to the enzyme called and estrogen receptor. This

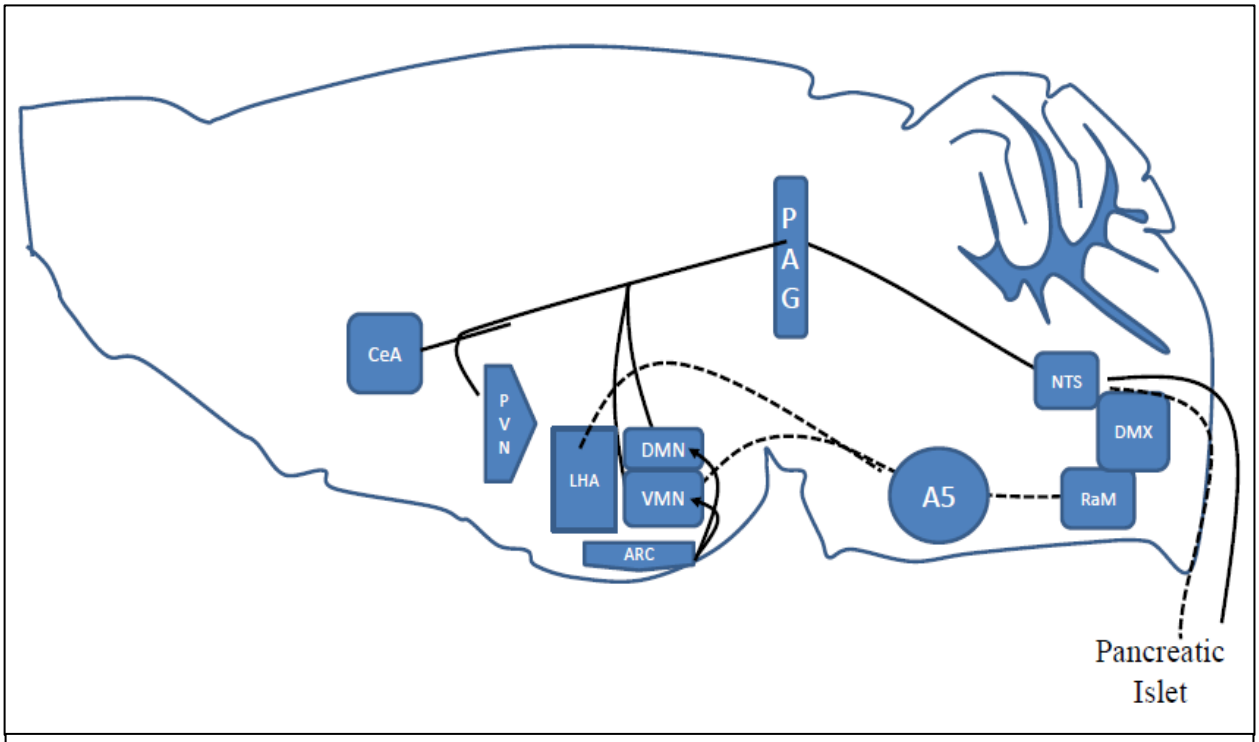

Figure 1.6: Schematic of the MIP-Cre map showing possible areas of activation and propagation of signal. Nutrients and hormones enter the blood brain barrier and CSF to activate cell surface receptors on the Arcuate (ARC) or Ventromedial Nucleus (VMN). Signal either enters the PAG down to the brainstem to propagate a parasympathetic signal (solid line) or goes through the A5 region and goes through the brainstem to send a sympathetic signal (dotted line) (Rosario 2016, Pozo 2018).

transcript allows translocation of the Cre-Recombinase into the nucleus of the cell when bound to an agonist, which in this study, was a breast cancer therapeutic drug called Tamoxifen. MIP-Cre mice were given 3 oral gavage doses of Tamoxifen or tap water as control, and then infected with PRV-Ba2001 the day after the last dose. Mice were then sacrificed 96-120 hours post infection of PRV-Ba2001, and brains were harvested and processed as previously described and the circuit connecting the hypothalamus to the pancreas was reconstructed (Figure 1.5) (Rosario 2016). GFP expression would only occur if there was Cre-Recombinase present in the same cell as the viral DNA. Because we infected MIP-Cre mice, Cre-Recombinase was only expressed under the control of the Mouse Insulin Promoter, which means that GFP would only be

expressed if the virus entered a post-synaptic terminal that was innervating a cell from the Islet of Langerhans. This section of the study revealed that most of the areas that were highlighted in the PRV-BaBlu map were also highlighted in this PRV-Ba2001 map, with the exceptions being the Cerebellum, SCN and MeHab. This suggests that outside of the 3 regions mentioned, the other regions that were highlighted in the PRV-BaBlu and PRV-Ba2001 map. This also suggests that the CNS heavily prefers to innervate the endocrine pancreas, rather than the exocrine pancreas (Figure 1.6) (Rosario 2016).

Finally, Rosario et al investigated whether glucose sensing in the CNS regulated hormone secretion in the pancreatic islet of Langerhans. As is the case in the liver and β-cell, the main glucose sensor in the neuron is glucokinase, and therefore the group looked for expression of glucokinase in the brain, and found that the ARC, VMN and LHA had the highest amount of glucokinase expression compared the other areas in the CNS-Islet circuit (Rosario 2016). In order to establish a functionality of the glucose sensing in the CNS regulating islet cell function, the group stereotaxically injected an adenovirus carrying the gene that expresses Hexokinase I (HK1), which as stated previously is an enzyme with a much higher affinity for glucose than glucokinase. Therefore, expressing HK1 in the areas that express glucokinase would shift the glucose sensing curve to the left. Shifting the glucose curve in the ARC and LHA perturbed islet cell function, where injection HK1 in the ARC created a glucose intolerant environment that decreased β-cell function, and increased α-cell function when HK1 was injected into the LHA. The VMN, however, showed no significant change in islet cell function when HK1 was injected (Rosario 2016). This study was the first of its kind to demonstrate that nutrient sensing in the CNS can regulate pancreatic islet cell function without any secondary perturbations in feeding or

drinking behavior, as well as activity levels, respiratory exchange rate and heat production (Rosario 2016).

This study elegantly set up the following thesis project, where we will now investigate the role of insulin signaling in the CNS on islet cell function. We hypothesize that insulin inhibits its own secretion by activating neurons in the CNS that sends an inhibitory signal to the pancreatic islet to decrease β-cell function.

Chapter II

Functionality of CNS-Islet Circuit

2.1 Introduction

Rosario et al established a neuro-circuit connecting the hypothalamus to the pancreas, and reconstructed the map to show that most of those connections to the pancreas were specific to the Islet of Langerhans and established functionality of the CNS-Islet circuit by showing that perturbations of glucose sensing in areas of the hypothalamus modulating pancreatic islet cell function (Rosario 2016). One of the many functions of the hypothalamus, is to be the metabolic center of the CNS, integrating information related to the status of the levels of nutrients circulating and executing a function to maintain homeostasis. Hypothalamic neurons can sense the levels of glucose, fructose, long-chain and short-chain fatty acids as well as a variety of amino acids, and send a signal towards the brainstem and down to the spinal cord or vagus nerve to innervate or inhibit cells in the pancreas, stomach, liver and adipose tissue (white and brown) (Blouet 2010). The hypothalamus is also able to innervate or inhibit centers of the brain that control food intake, most notably, the connection between the LHA and VTA, along with the Substantia Nigra (SN), regulate hedonic feeding and reward seeking behavior in order to maintain nutrient homeostasis (Blouet 2010, Stuber 2016).

Executions of these functions are based upon not just information regarding nutrient availability, but also level of circulating hormones. Hypothalamic neurons express a variety of hormone receptors, including the Insulin Receptor, where it is thought to be expressed in the ARC and the VMN (Paranjape 2010, 2011, Klöckener 2011). It has been known that insulin signaling in these two areas send projections to other hypothalamic areas. It is also known that

insulin signaling in the hypothalamus also regulates hepatic glucose production, as well as regulates adipose tissue function (Konner 2007, Shin 2017). There has been some controversy, however, over the effect of insulin signaling in the hypothalamus on pancreatic islet cell function, and a consensus has yet to be reached on the effect insulin signaling in the hypothalamus has on islet cell function and systemic insulin sensitivity (Paranjape 2010, 2011, Klöckener 2011). In order to answer this question, one must first establish whether the neurons involved in insulin signaling are part of the circuit that connects the CNS to the islet cells. We proceeded to accomplish this by infecting the pancreas of C57Bl/6 mice with PRV-BaBlu and analyzing areas of the hypothalamus to find what areas of the hypothalamus are part of the CNS-Islet circuit and significantly activated in the presence of insulin.

2.2. Materials and Methods

Cell Prep of PRV-BaBlu

PRV-BaBlu (PRV Bartha Blue, Beta Galactosidase) and PK15 cell lines were obtained from Lynn Enquist at Princeton University. In vitro propagation of PRV-Ba2001 was done by infection of PK15 cell line, which were grown in 25 mM DMEM (Gibco 11965) with 10% FBS (HyClone SH30910.03) and 1% Pen-Strep (Invitrogen 15140). Cells were then split after 90% confluency (10 cm dishes) via trypsinization (0.25%, Gibco 15400). Split cells were infected with PRV-Ba2001 for 1 hour with tilting of plates every 15 minutes (2 µlof 10^8 pfu/ml +200 µlDMEM with 0.5% FBS). Infection medium was then aspirated after 1 hour and fresh DMEM with 2% FBS and 0.5% Pen-Strep was applied. PRV-Ba2001 propagated on cells until signs of cytopathic destruction was apparent. Cells and media were then harvested via cell scraper in a 50 ml conical tube and 1 ml of mixture was aliquoted to 10 1.5 ml centrifuge tubes. The remaining

solution was then stored at -80 for further usage. Each 1.5 ml tube was sonicated at 80% amplitude for 10 pulse periods of 10 seconds each in an ice water bath sonicated (Diagenode Bioruptor UCD-200), and then centrifuged at 2,000 g for 5 minutes to remove any cellular debris. 100-200 µl of supernatant was then aliquoted into 0.5 ml centrifuge tubes and snap frozen on dry ice. They were then stored in -80. Viral titer was confirmed in 200 µl. PRV-Ba2001 infection dilutions in duplicated 6-well plates in dilutions from purified lysates obtained in 1% methyl ceullulose (Sigma M0152) and 1% Sodium carbonate (Fisher BP328). They were then supplemented in DMEM with 2% FBS to observe plaque formation in confluent PK15 cells in 6-well plates after 48 hours incubation. Viral plaques were visualized by staining the wells with 1 ml 0.5% methylene blue in 70% ethanol. Viral titer is calculated by counting total countable plaques on all plates, dividing by the total volume plated based on the lowest possible dilution giving countable plaques, and multiplied by reciprocal of the lowest dilution that gave countable plaques.

In Vivo Injections

PRV-BaBlu was injected into 12-14 week C57Bl/6J mice from Jackson Labs. 10 µl of virus was injected into the pancreas in 3-4 µl aliquots. The incision was then sutured and given a dose of meloxicam twice over a 24 hour period and buprenorphine twice over a 12 hour period post-surgery. After 120 hours post infection, mice were placed on a 3 hour fast in the morning (starting at 6:00 am) and then given an injection of either 1mU of Insulin (Novolog, Novo Nordisk) or a volume equivalent of Saline as control. Mice were then euthanized and tissue was fixed in the same manner above.

Sectioning

Brains were first cut into multiple coronal sections using a brain matrix (Kopf). The brain were submitted to the University of Chicago Human Tissue Resource Center (HTRC) to be fixed in paraffin blocks and sectioned onto slides at a thickness of 10 µm.

Immunofluorescence

Pancreas slides were deparrafinized by being submerged for 2 changes in Histoclear (National Diagnostics) for 10 minutes each. They were then rehydrated by being submerged in various concentrations of alcohol, starting with 2 changes of 100% EtOH for 2 minutes each, 2 changes of 95% EtOH for 2 minutes each, 2 changes of 80% EtOH for 2 minutes each, and then 2 changes of 1X PBS for 5 minutes each. Brain slides were deparrafinized and rehydrated in a similar manner, with the exception of 2 additional rehydration steps of being submerged in 50% EtOH and 30% EtOH for 2 minutes each after the 2 changes of 80% EtOH and prior to the 1x PBS submerge. Slides then underwent an antigen retrieval technique called Heat Induced Antigen Retrieval (HIER), where they were placed into a Pyrex Glass container with 10 mM of Sodium Citrate Buffer with EDTA at a pH 6.2. The Pyrex Glass Container with the Buffer and slides were then placed in a microwave with the power level at 10. The Pyrex lid was then placed on the container with the vent hole open. It was then heated 3 times for 5 minutes with a 30 second interval between each 5 minute session and cooled until at room temperature. Slides were then blocked by adding 50 µlof 10% Donkey Serum for 1 hour. Anti-Beta Galactosidase (1:2000) or Anti-cFos (1:500) Overnight at Room Temperature. Each antibody was aliquoted into 0.3 % Tween-20 PBS with 1 %BSA. Slides were then washed in 1X PBS in 4 changes for 5 minutes each before the secondary antibodies (1:400) were placed on each respective slide for 1 hour at room temperature in dark. Excess secondary was then washed off for 4 changes of 1X PBS for 5 minutes each and then 1 change of diH2O for 5 minutes. 7-10 µl Slow-Antifade Dapi

was then added to the tissue and a coverslip was then placed and sealed onto the slides with clear nail polish. Slides were ready to view right away, or placed in a 4 C fridge.

Imaging and Processing

Images were taken via Olympus DSU "fixed cell" Spinning Disk Confocal located at the Integrated Light Microscopy Core Facility at the University of Chicago. Images were taken at 10x and 20x magnification, and anatomical structures and GFP-positive areas were identified using the Franklin and Paxinos (The Mouse Brain in Stereotaxic Coordinates) Atlas. Raw images were processed using ImageJ (NIH).

Statistical Analysis

All results in this chapter were analyzed using an unpaired t-test in the GraphPad Prism program to assess any significant differences between groups.

2.3 Results

Experimental Design

C57Bl/6 mice were ordered from Jackson Labs at 10-12 weeks of age. After acclimation to the animal facility, mice then underwent pancreatic surgery, and given an injection of PRV-BaBlu and after 120 hours, put on a 3 hour fast, where they were given an intraperitoneal (IP) injection of Insulin at 1mU/kg body weight or an equivalent volume of saline as control. Mice were then euthanized 1 hour post IP injection and their brains were harvested. Brain tissue then underwent immunofluorescence to detect β-galactosidase and cFos, which is a transcription factor that is also used as a universal marker of an activated neuron when present in the nucleus (Roux 1990) (Figure 2.1).

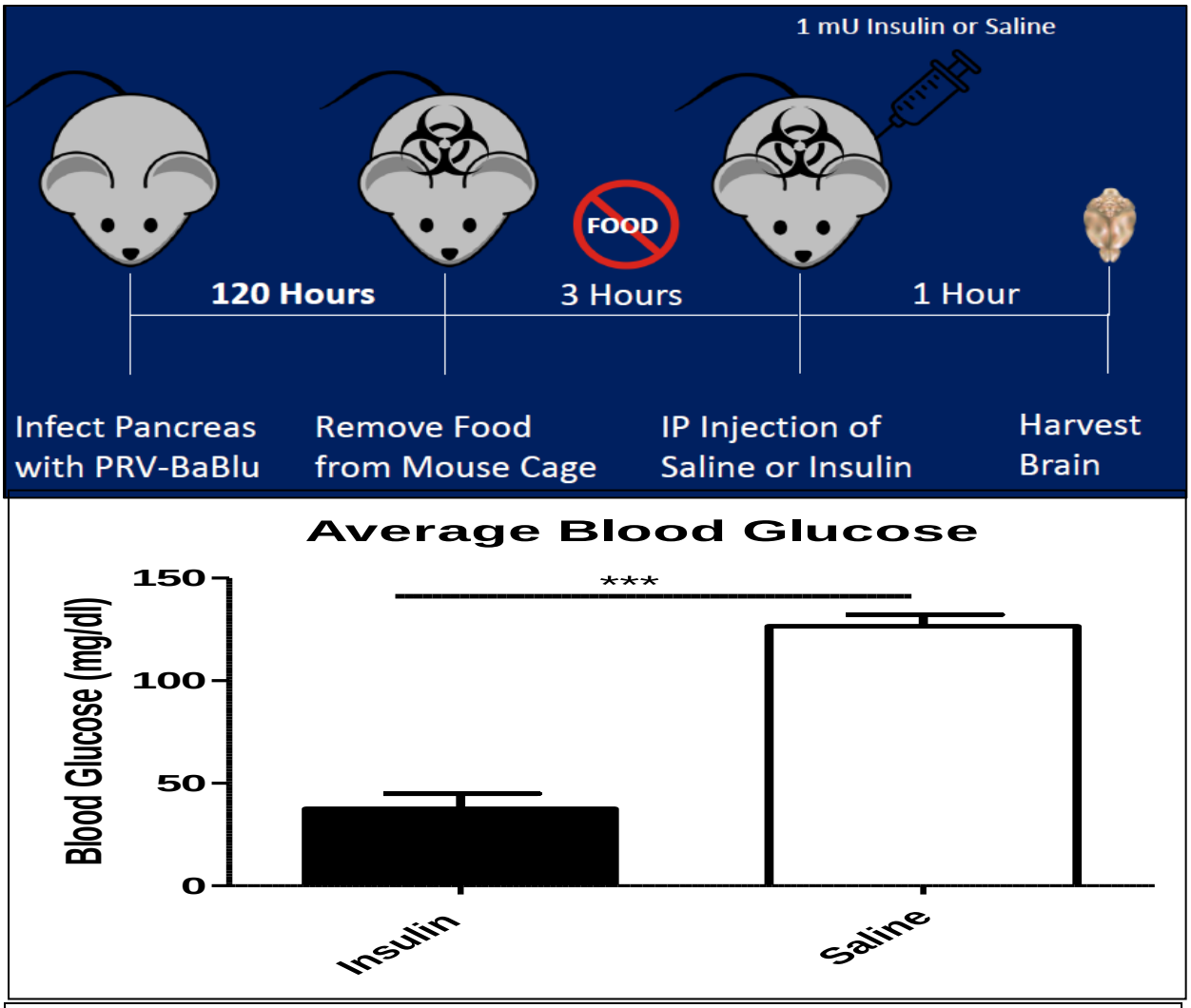

Figure 2.1: The top panel shows a schematic of the experimental design to address which areas of the hypothalamus does insulin significantly activate the circuit. The bottom panel is the average blood glucose of the animals either injected with insulin (37.50 mg/dl ± 7.489 N=4) or saline (126.5 mg/dl ± 5.694 N=4) at the time of euthanization(1 hour post injection). Data was analyzed using unpaired t-test. $p < 0.0001$. *** denotes extreme significance

VMN Significantly Activated in Presence of Insulin

Images were then quantified for activated neurons that were either part of or apart from the CNS-Islet circuit, which was denoted as β-gal (+) or β-gal (-), with either an insulin (n=4) or saline (n=4) injection. When examining the ARC, there is a slight decrease in neurons that are double positive for β-gal and cFos, however it remained statistically insignificant (p=0.1250) (Figure

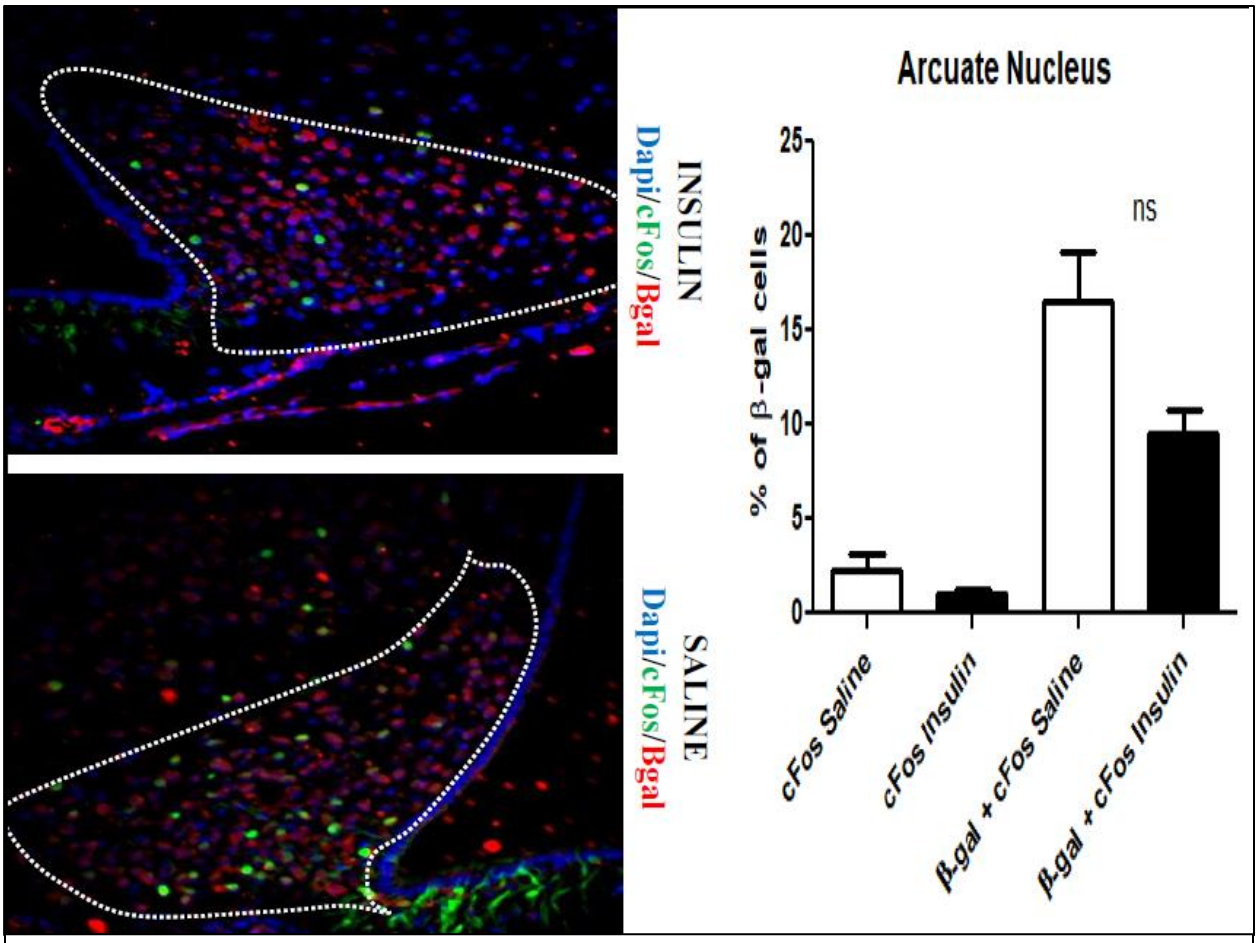

Figure 2.2: Bar graphs on the right side of panel represents number of activated neurons either within or outside of the CNS-Islet circuit in the presence of saline (white bars) or insulin (black bars). The top left panel is a representative image of the ARC in the presence of Insulin and the bottom left panel is a representative image of the ARC in the presence of Saline. Nuclear stain DAPI is shown in blue, cFos in green and β-gal in red. "ns" denotes "not significant". An Unpaired t-test was performed on "c-Fos Saline vs. cFos Insulin" (p=0.1583) and "β-gal + cFos Saline vs. β-gal + cFos Insulin" (p=0.0508).

2.2). Similar observations were seen in the LHA, DMN as well as the PAG, nor was there a significant number of neurons activated outside the CNS-Islet circuit [cFos (+), β-gal (-)] in any of the areas ((LHA p=0.3231, p=0.5933; DMN p=0.6137, p-0.1854; PAG p=0.2713, p=0.7537) (Figure 2.4, 2.5, 2.6).

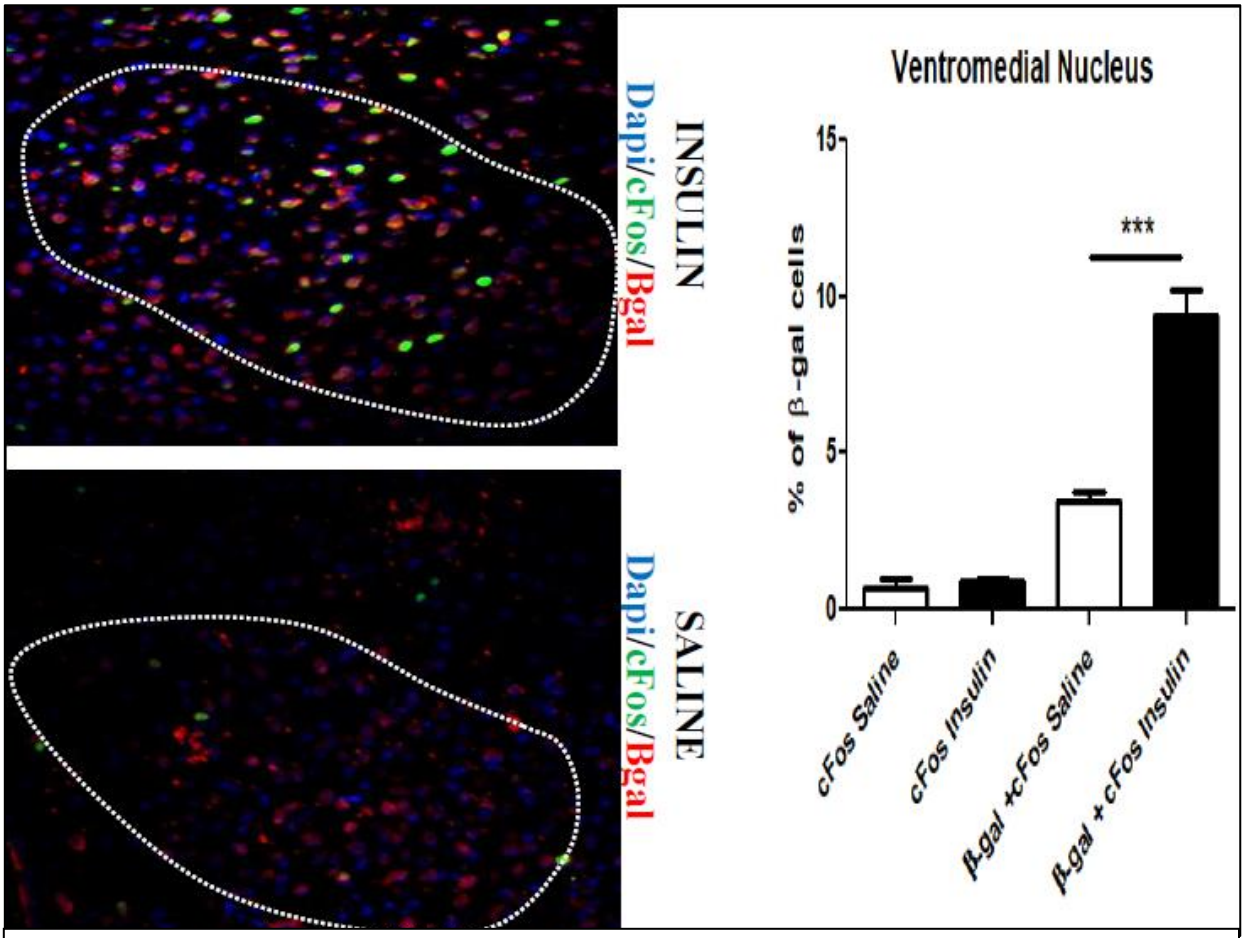

Figure 2.3: Bar graphs on the right side of panel represents number of activated neurons either within or outside of the CNS-Islet circuit in the presence of saline (white bars) or insulin (black bars). The top left panel is a representative image of the VMN in the presence of Insulin and the bottom left panel is a representative image of the VMN in the presence of Saline. Nuclear stain DAPI is shown in blue, cFos in green and β-gal in red. An Unpaired t-test was performed on "c-Fos Saline vs. cFos Insulin" (p=0.5754) and "β-gal + cFos Saline vs. β-gal + cFos Insulin" (p=0.0005). *** denotes extreme significance

However, there was an overwhelmingly significant difference in the VMN between the number of activated neurons in the circuit when stimulated with insulin compared to saline (***p=0.0005) (Figure 2.3). The PVN was excluded from this analysis due to the fact that it has been implicated as an area involved in pain detection, where the injection itself would activate neurons in the PVN, and therefore cannot be distinguished from the effect of insulin or saline. In the hypothalamus, the ARC and VMN are the two areas that have insulin receptor present on the

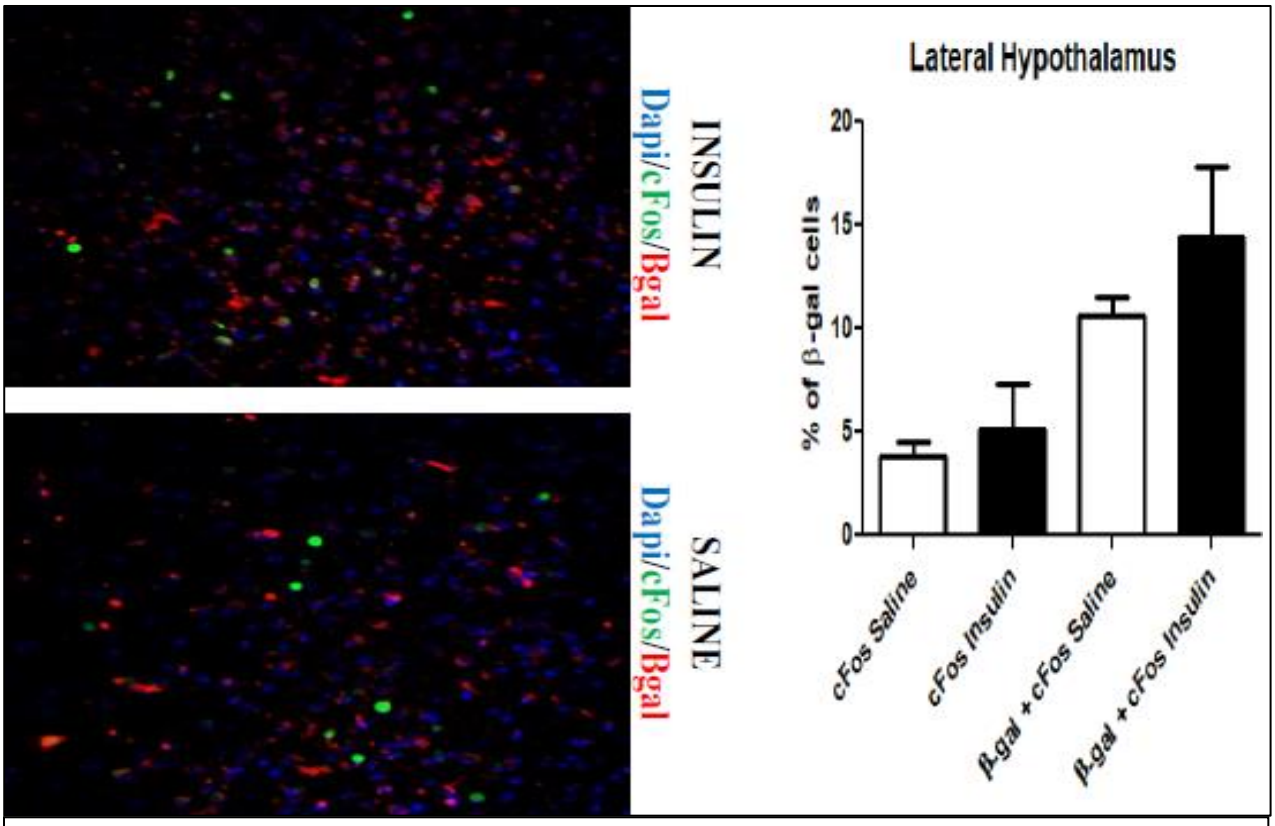

Figure 2.4: Bar graphs on the right side of panel represents number of activated neurons either within or outside of the CNS-Islet circuit in the presence of saline (white bars) or insulin (black bars). The top left panel is a representative image of the LHA in the presence of Insulin and the bottom left panel is a representative image of the LHA in the presence of Saline. Nuclear stain DAPI is shown in blue, cFos in green and β-gal in red. An Unpaired t-test was performed on "c-Fos Saline vs. cFos Insulin" ($p=0.5933$) and "β-gal + cFos Saline vs. β-gal + cFos Insulin" ($p=0.3231$).

neuronal cell surface. Any activation in the presence of insulin must be due to the propagation of signal from the original source of the ARC or VMN. The VMN has been shown to express the insulin receptor in neurons that also co-express Steroidogenic Factor 1 (SF-1), therefore the insulin receptor is expressed in SF-1 neurons. The injection itself would activate neurons in the PVN, and therefore cannot be distinguished from the effect of insulin or saline. In the hypothalamus, the ARC and VMN are the two areas that have insulin receptor present on the neuronal cell surface. Any activation in the presence of insulin must be due to the propagation of

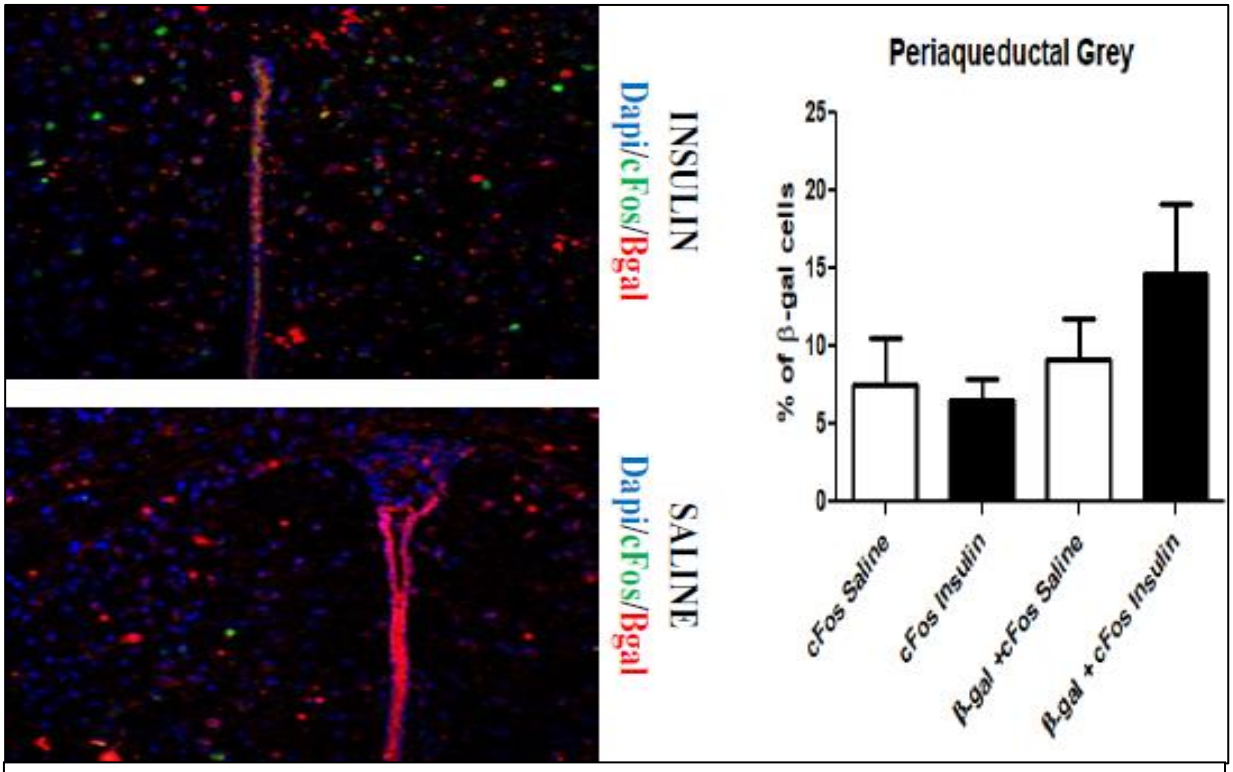

Figure 2.5: Bar graphs on the right side of panel represents number of activated neurons either within or outside of the CNS-Islet circuit in the presence of saline (white bars) or insulin (black bars). The top left panel is a representative image of the PAG in the presence of Insulin and the bottom left panel is a representative image of the PAG in the presence of Saline. Nuclear stain DAPI is shown in blue, cFos in green and β-gal in red. An Unpaired t-test was performed on "c-Fos Saline vs. cFos Insulin" ($p=0.7537$) and "β-gal + cFos Saline vs. β-gal + cFos Insulin" ($p=0.2713$).

signal from the original source of the ARC or VMN. The VMN has been shown to express the insulin receptor in neurons that also co-express Steroidogenic Factor 1 (SF-1), therefore the insulin receptor is expressed in SF-1 neurons. The Lateral Hypothalamus (LHA), Dorsomedial Nucleus (DMN) and Periaqueductal Gray (PAG) was also analyzed and no significant difference between the saline and insulin groups comparing activated neurons in and out of the circuit

2.4 Discussion

Rosario et al had established a circuit that connects the endocrine pancreas to the mediobasal hypothalamus. They then found glucose sensing areas in the hypothalamus that

regulates pancreatic islet cell function (Rosario 2016). We then investigated the possibility of insulin activating the circuit in specific areas of the hypothalamus. We infected 12-14 week old C57Bl/6 mice with PRV-BaBlu in order to recreate the circuit. PRV-BaBlu is a non-specific tracer, however when using a Cre-inducible tracer (PRV-Ba2001), the same areas that was implicated with PRV-BaBlu were also highlighted by PRV-Ba2001 (Rosario2016). PRV-BaBlu expresses β-galactosidase, which can be detected via immunofluorescence with an antibody that is much stronger than the GFP antibody, which is expressed by PRV-Ba2001. After 120 hours, the virus is shown to be fully expressed in the hypothalamus when starting from the pancreas, therefore we waited the allotted time before we injected the mice with insulin or saline. The mice were put on a 3 hour fast that started at 6:00 AM, which is when mice typically stop eating. After 3 hours, they were given a 1mU/kg dose of insulin or the volume equivalent of saline via intraperitoneal injection. Blood glucose readings showed the effect of insulin through a drop in glucose levels compared to saline. Mice were euthanized an hour after injection in order to allow c-Fos protein to accumulate and enter the nucleus of the neuron. It has been shown that mRNA levels of c-Fos peak 30-60 minutes post stimulus, while protein levels peak at 1-3 hours post stiumulus (Sharp 1991) When insulin was injected, the circuit was increasingly activated in the ARC, but not significantly activated. The ARC is comprised of orexigenic and anorexigenic neurons, which both express the insulin receptor (Belgardt 2009). AgRP/NPY (orexigenic) and POMC/CART (anorexigenic) propagate differential signals when activated by insulin, where AgRP/NPY neurons are inhibited, while POMC/CART neurons are activated (Belgardt 2009). Although there was no significant difference in overall activation of the circuit in the ARC, both inhibited and activated neurons were quantified. One could potentially co-stain tissue samples by α-Melanocortin Stimulating Hormone (α-MSH) in order to quantify POMC neurons that are part

of the circuit, which has not been investigated to this date, nor has the role of insulin signaling in AgRP neurons on β-cell function been investigated, which allows for future experiments to elucidate its function. There was also a lack of significant activation of the circuit in the DMN, LHA and PAG, potentially due to the lack of evidence of insulin receptor expression in these areas. When analyzing the VMN, there was a significant difference in activation of neurons in the circuit when injected with insulin versus saline. The VMN has been implicated in the counter-regulatory response therefore a potential activator of these neurons could be the hypoglycemia that occurs when insulin is injected intraperitoneally. However, protein c-Fos localization in the nucleus occurs 1-3 hours post stimulus, and hypoglycemia, along with glucagon secretion, occurs 45 minutes to 1 hour after insulin is injected intraperitoneally (Rosario 2016). Other investigators have also studied the effect of insulin on neuronal activation, particularly Foster et al, who performed a hyperinsulinemic clamp in rats and analyzed c-Fos activity 90 minutes post infusion (Foster 2016). Their findings included a large decrease in c-Fos activity in the VMN, but attribute this decrease in activation to a drop in glucose, rather than a direct effect of insulin (Foster 2016). Other investigators have done patch clamp studies on insulin responsive neurons in the VMN, and show an inhibition in the firing rate (Spanswick 2000, Klöckener 2011). However, these studies added 5-300 nM and 200 nM of insulin, respectively. As stated previously, physiological secretion of insulin is around 5 nM when secreted into the hepatic portal vein (Rhodes 2013). This inhibition could be due to an increase in insulin receptor downregulation, which could also be a reason for the inhibition of neurons that express the insulin receptor when bathed in high concentrations of insulin. When adding a lesser amount of purified insulin (20 nM) investigators showed an increase in activation of POMC neurons, and an inhibition of AgRP/NPY neurons, as opposed to previous studies

which exposed arcuate neurons to 100 nM of insulin and saw an inhibition (Spanswick 2000, Qiu 2014).Investigators also use manufactured insulin that contains Zn^{2+} which has been shown to inhibit and hyperpolarize neurons (Qiu 2014). Zinc is expected to be secreted at a concentration of 1.5-150 nM, and is expected to significantly decrease before reaching then CNS (Easley 2009).

Further studies are needed to elucidate the role of insulin signaling on neurons in the hypothalamus. Activation of the circuit in the VMN in the presence of insulin is likely due to insulin receptors on Steroidogenic Factor-1 (SF-1) neurons, which possess the majority of insulin receptors in the VMN. Further experiments can be conducted in order to validate c-Fos data, such as crossing SF-1 Cre mice with IR floxed mice to knockout the insulin receptor in SF-1 neurons. One would reconstruct the map and inject insulin intraperitoneally and analyze activated neurons in the VMN. If insulin truly activated the circuit in the VMN, there would be very little activation of neurons in the presence of insulin compared to control mice. Nevertheless, the circuitry in the VMN was significantly activated in the presence of insulin. The next chapter will focus on knocking out the insulin receptor in the VMN to elucidate its role in pancreatic islet cell function.

Chapter III

Role of Insulin Signaling in the VMN on Pancreatic Islet Cell Function

3.1 Introduction

The previous chapter detailed the investigation of activation of the circuit in the presence of insulin. We discovered that the circuit is significantly activated in the presence of insulin in the Ventromedial Nucleus. The Ventromedial Nucleus has been implicated in a variety of functions such as eliciting the counter-regulatory response to hypoglycemia, contributing to sexual behavior and differentiation in rats as well as playing a role in defensive behavior (Paranjape 2010, Flanagan-Cato 2011, Chan 2013, Yates 2015). The VMN is comprised of 3 main neuronal types: Steroidogenic Factor-1 (SF-1) neurons, Brain Derived Neurotropic Factor (BDNF) neurons, and Estrogen Receptor α (ERα) neurons. SF-1 neurons contain a variety of receptors, including the insulin receptor, which has been implicated in regulating metabolic homeostasis. There have also been studies that indicated about 70% of neurons that express the insulin receptor also express the GLUT4 glucose transporter (Levin 2009). SF-1 has been shown to be expressed in other areas as well, particularly adrenocortical cells, Leydig cells, ovarian theca and granulosa cells (Ikeda 1993).

There have been a plethora of studies that analyze the role of the insulin receptor in the brain, using mice which have their insulin receptor knocked out at birth through the nestin-cre promoter (NIRKO mice), as well as mice with perturbed insulin signaling by knocking out IRS-2 through the Rat Insulin Promoter (RIP). These studies have contributed a great deal of knowledge towards the significance of insulin signaling in the brain on peripheral glucose homeostasis. NIRKO mice displayed sex differences, where female mice showed a larger

increase in body weight, plasma insulin levels and white adipose tissue and similar increases with male mice in food intake and plasma leptin levels (Brüning 2000). RIP-Cre mice, which knocked out IRS-2 in both the pancreatic β-cell and hypothalamus, showed differential phenotypes amongst 3 papers, that have showed increased leptin levels, appetite, body mass, however they were still protected from diabetes, due to the increase in pancreatic β-cells that did not have their insulin receptor knocked out (Choudhury 2014, Kubota 2014, Lin 2014). These studies set the stage for area specific knockouts of the insulin receptor. Cre technology has provided crucial insight into functionality of insulin signaling in POMC and AgRP neurons (Könner 2007), as well as insulin signaling in SF-1 neurons of the VMN (Klöckener 2011), which is the area we found to be significantly activated in the presence of insulin. The insulin receptor was knocked out in SF-1 neurons through the crossing of SF-1 Cre mice to IR flox mice. However, as stated previously, SF-1 is expressed in a variety of tissue, and no direct evidence of VMN specific knockout of the insulin receptor was given (Klöckener 2011). Finally, other investigators have either tried to knockout or block insulin receptor activity through stereotaxic surgery, which has been shown to be the best way to knockout proteins in the brain in an area specific manner (Harno 2013). Investigators injected either a lentivirus containing an antisense to the insulin receptor or an anti-insulin antibody into the VMN of rats (Paranjape 2010, 2011). However, the stereotaxic coordinates given in the Paranjape et al 2011 paper implicated the A13 region, rather than the VMN (Paxinos 2006, Paranjape 2011), and the investigators do not provide adequate confirmation of the injection site. The role of the insulin signaling in the Ventromedial Nucleus remains a controversial mystery. This chapter will outline the role of the insulin receptor in the VMN, using proper stereotaxic coordinates of the mouse

brain as well as injection confirmation of the adenovirus carrying either a Cre-Recombinase construct, or a β-galactosidase into IR flox mice.

3.2 Materials and Methods

Ventromedial Hypothalamus Injections

12-14 week old Insulin Receptor Floxed mice (Jackson Labs, 0069S) were anesthetized via 3% Isoflurane and placed on a stereotaxic apparatus (David Kopf Instruments) with a heating pad placed underneath the mouse. The mouse skull was secured with ear bars on either side of the mouse, and artificial tears were used to lubricate mouse's eyes throughout surgery, and the Isoflurane was then reduced to 2%. Betadinine and 70% Ethanol swabs were used to sterilize surgical sight via alternating method and Buprenorphine along with Meloxicam was injected subcutaneously behind shoulder blades of mouse prior to incision. A 5 mm incision was to reveal Bregma and lambda line and a cotton swab applicator was pressed against top of skull to allow the Bregma lambda line to show, as well as to test the fixation of the mouse's head in the apparatus. Once Bregma point was identified and mouse's skull was shown to be aligned using alignment indicator (David Kopf Model 1905), the manipulator was then set to a Bregma of -1.700, Medial/Lateral point of ±0.350. A #77 drill bit was used to burr a hole at the M/L point, which was done very slowly, and once the dura became visible, a syringe with a flexible needle was used to detect the depth from the top of the hole to the top of the dura, which was measured. A syringe with a 33 gauge needle (Hamilton 7000.5) was then loaded with 400 nanoliters of virus and 200 nanoliters were aliquoted per injection site. The needle went 0.1 millimeters lower than the target depth in order to create a pocket for the virus to reside (D/V coordinates. Injection site: 5.350-5.450, Pocket: 5.450-5.550). The virus was injected at a rate of 20 nl/min and the

syringe was left in the injection site for 10 minutes after 200 nanoliters were injected to prevent spread of virus to areas outside of Ventromedial Hypothalamus. The syringe was then slowly taken out of the brain, and melted bone wax was used to seal the burr holes. The incision site was then sutured; the mouse was given 300 microliters of saline intraperitoneally and placed back in its cage for recovery.

Glucose Tolerance Test

Mice were handled every day for 5 days prior to glucose tolerance test (GTT) to diminish effects of stress on glucose readings. Their weights were measured and they were given an injection of saline with a volume ten times that of their body weight every other day to simulate the effect and volume of the injection of glucose. On the day of the GTT, mice were put on a 4 hour fast starting at 6:00 AM, and a 2 mg/kg injection of glucose was given. Their blood glucose readings were measured at time point 0 (before the injection is given) 5, 10, 20, 40, 60 and 120 minutes post injection. 20 μl of blood was collected in a microvette tube (Sarstedt Ag & Co) at time points 0,5,10,40 and 120, and centrifuged at 4°C at 2,000 G for 10 minutes, and the serum was then collected and stored at -80°C until an Insulin ELISA (ALPCO mouse ultrasensitive insulin ELISA kit 80-INSMSU-E01) was conducted.

Insulin Tolerance Test

Mice were handled every day for 5 days prior to insulin tolerance test (ITT) to diminish effects of stress on glucose readings. Their weights were measured and they were given an injection of saline at a volume twice their body weight every other day to simulate the effect and the volume of an insulin injection. On the day of the Insulin Tolerance test, mice were fasted for 3 hours and given a 0.5 mU/kg insulin injection (Humalog, Eli Lilly), and their blood glucose was measured

at 0 (prior to insulin injection), 15, 30, 60, 90 and 120 minutes post injection. A vial of glucose was kept in case of hypoglycemic shock.

Glucagon ELISA

In accordance with IACUC guidelines, blood collections for glucagon measurements were done separately from actual ITT. At least one week after ITT, mice were fasted for 3 hours, and 100 μl of blood was collected. Five days after fast, mice were again fasted for 3 hours and administered 0.5 mU/kg of insulin. 100 μl of blood was then collected at the 60 minute time point. Blood was centrifuged at 4°C at 2,000 G for 10 minutes, and serum was collected and frozen at -80°C until the Glucagon ELISA (ALPCO 48-GLUHU-E01).

Metabolic Cage Assessment

Feeding and drinking behavior, along with energy expenditure, were measured using a 2 cage system, with VMN-Cre mouse in one cage and VMN-LacZ mouse in another. The cages were part of a combined indirect calorometry system (TSE Systems, Bad Homburg, Germany). Mice were given 48 hours to be acclimated to the new environment, then metabolic parameters were taken 48-72 hours subsequent to the acclimation period.

Tissue Harvesting/Sectioning

Animals were anesthetized with 3% Isoflurane and then euthanized via cervical dislocation. The brain was then quickly harvested and a section of the brain containing the hypothalamus was sectioned using microtome blades (Leica) and a brain matrix (David Kopf) and submerged in a disposable base mold (Fisher) filled with OCT (Tissue Tek). The mold with OCT and brain slice was then placed on a flat block of dry ice where it was kept on until completely frozen. Mold

was then wrapped in plastic wrap and aluminum foil and kept in -80°C for at least 24 hours. Tissue sections were cut at 10 μm using a Cryocut 1800 (Leica) and sections were placed on a Superfrost plus slide (Fisher) and stored at -80°C for Immunofluorescent staining.

Immunofluorescence Staining

Slides were taken out of -80°C and brought to room temperature and allowed to air dry. A Pap pen (Daido Sangyo, Japan) was used to outline the tissue and 50 μl of 4% PFA (Biotium) was placed on the tissue for 20 minutes. Slides were then washed 3 times with 1X PBS for 5 minutes each and then 10% Donkey Serum was then used as a blocking buffer for 1 hour at room temperature. Blocking buffer was then suctioned off and 50 μl of either Anti-β-galactosidase (abcam, 1:1,000) or Anti-Cre Recombinase (Novagen, 1:500) was aliquoted onto the tissue. Antibodies were diluted in 1X PBS and contained 0.3% Tween 20 (Fisher), 2% BSA (Roche) and 10% Donkey Serum. Antibodies were kept on for 3 hours at room temperature and then washed off with 4 washes of 1X PBS at 5 minutes each wash. Cy3 Anti-Chicken or Cy3 Anti-Mouse secondary antibodies were then diluted at 1:400 in 1x PBS and 2% BSA. 50 μl of the secondary was placed on either tissue containing the Chicken Anti-β-galactosidase or Mouse Anti-Cre Recombinase primary antibody for 1 hour at room temperature. Slides were then washed 4 times in 1x PBS and once in diH_20 for 5 minutes each. 10 μl of *SlowFade* Gold Antifade Mountant (Invitrogen) was place directly on tissue and a coverslip (Corning) was used to cover the tissue and was sealed with clear nail polish (NYC). Fluorescence could be viewed right away or slides stored at 4°C where fluorescence could be viewed for no longer than a week after staining procedure.

3.3 Results

Confirmation of Injection Site

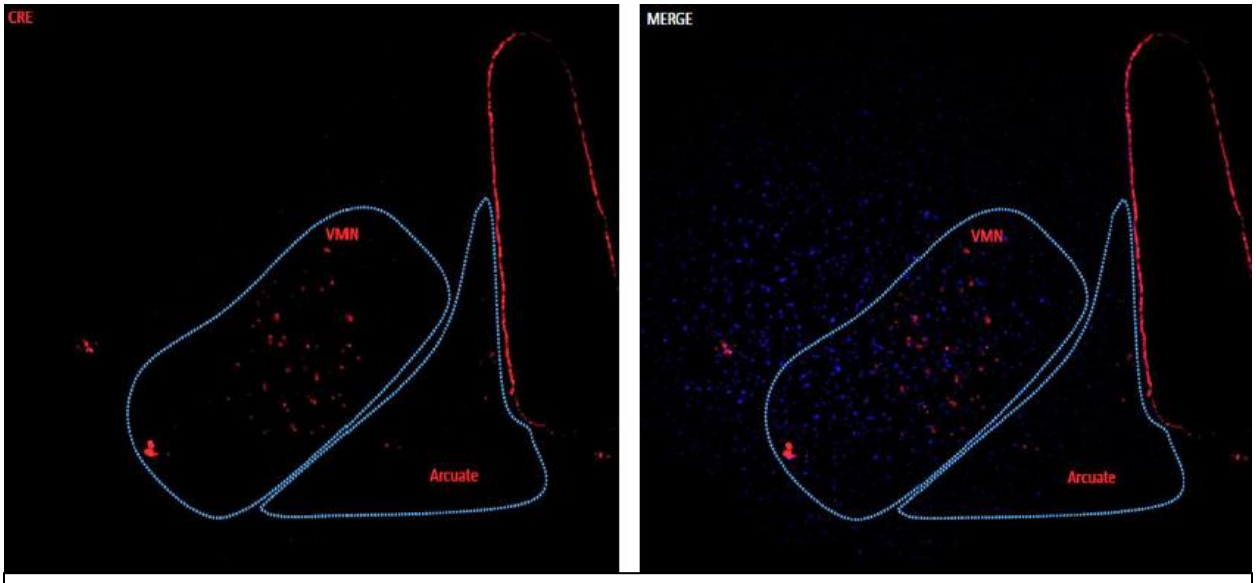

Figure 3.1: Injection confirmation verifies that virus was injected into the intended target (VMN) with minimal leakage into the ARC (Both VMN and ARC are outlined in blue). Left panel shows CRE signal (red), while right panel shows overlap of Cre signal to nuclear stain DAPI in blue. Pictures were taken at 20x magnification.

IR flox mice were injected with adenovirus carrying the Cre-Recombinase construct that was driven by the Insulin Receptor promoter. Confirmation of the injection site was needed in order to verify the coordinates used were the correct coordinate that would be used to inject the VMN. The coordinates used from the mouse brain atlas were Bregma: -1.700 mm; M/L 0.350 mm; D/V -5.350 to -5.450 mm. Mice were then sacrificed a week after injection and brains were harvested and frozen in OCT. Immunofluorescence staining of Cre verified the coordinates did in fact lead to the VMN, and were therefore used throughout the study (Figure 3.1).

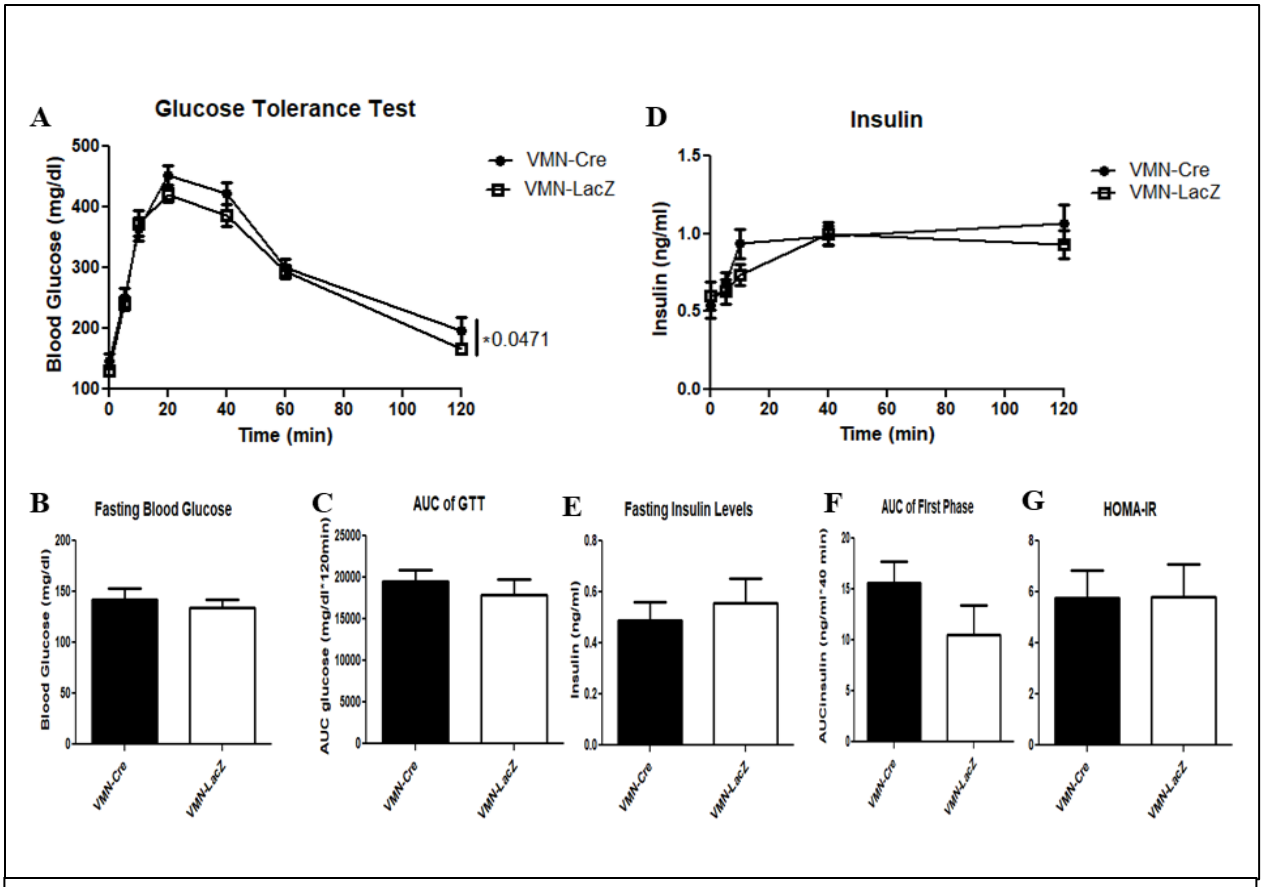

Figure 3.2: **A**. Glucose tolerance test on knockout (VMN-Cre) vs. control (VMN-LacZ) shows difference in tolerance level of glucose perturbed in knockout mice compared to control (Cre (n=5) vs Lac Z (n=5), Source of Variation: Column Factor p=0.0471) **B.** No difference in fasting blood glucose levels, or **C.** insulin levels. **D.** Shows insulin levels corresponding to the GTT. There seems to be a slight increase in insulin levels, particularly during the first phase (**F**) however, this is not statistically significant (Insulin: Cre (n=5) vs Lac Z (n=5), Source of Variation: Column Factor p=0.2542). There is no difference in HOMA-IR levels, which measures insulin resistance **(G)**. * signifies P<0.05

VMN^{Cre} Mice are Glucose Intolerant with Slight Increase in Insulin Secretion

When Cre-Recombinase was expressed in the VMN of IR flox mice, a Glucose Tolerance Test (GTT) was conducted a week post injection, which showed VMN^{Cre} mice being glucose intolerant compared to control mice, which had an Adenovirus containing Lac-Z injected (Cre (n=5) vs Lac Z (n=5), Source of Variation: Column Factor p=0.0471), with no difference in fasting glucose levels. Even though there was a slight increase in the first phase of insulin

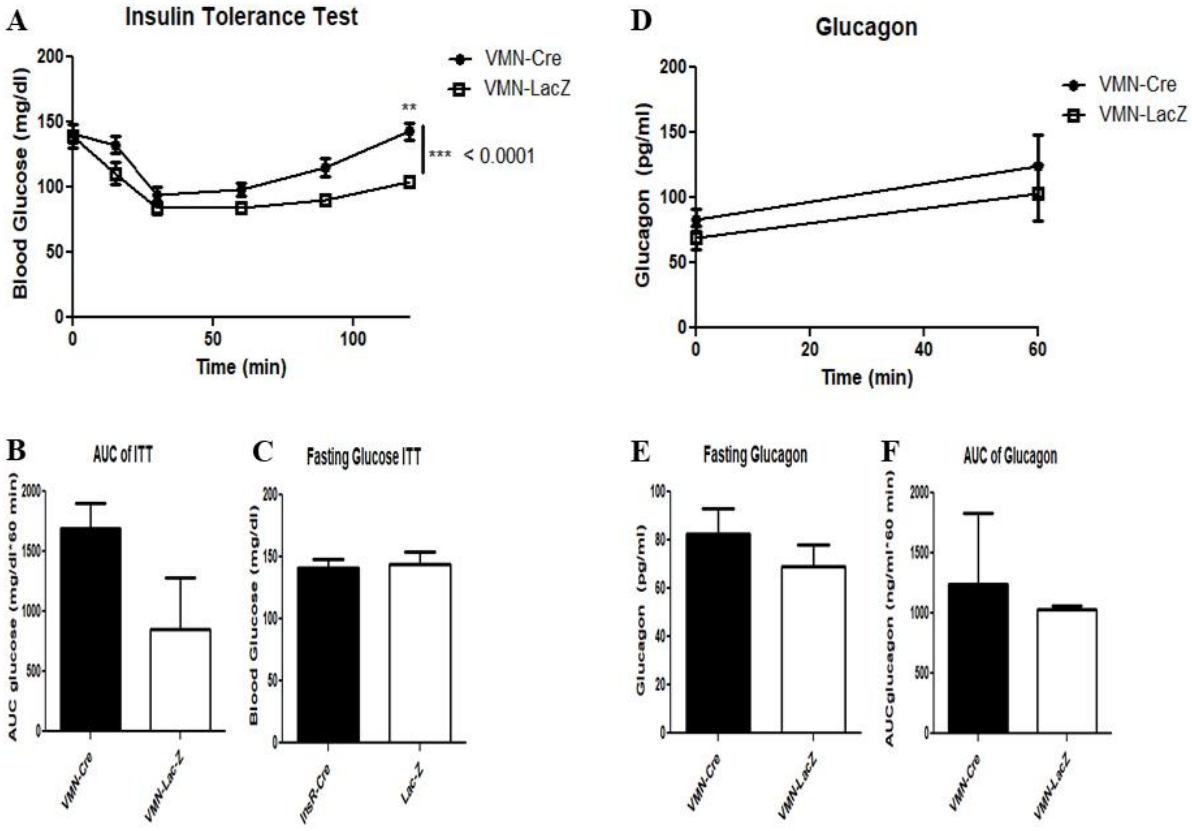

Figure 3.3: **A**. Insulin Tolerance Test on VMN-Cre (black) vs. VMN-LacZ (white) shows extremely significant difference in counter-regulatory response Cre (n=5) vs Lac Z (n=4), Source of Variation: Column Factor p<0.0001***, Cre (n=5) vs. Lac-Z (n=4), Bonferroni Post Test, 120 minute time point, 142.7 $\pm$ 6.766 vs. 104.5 $\pm$ 3.614, p<0.01**). **B,C.** Shows no statistical difference in Area Under the Curve (p=0.1491).There was also no difference in fasting glucose. **D.** Corresponding glucagon was not different between two groups, with no change in fasting glucagon levels **(E)** and AUC **(F)**.

secretion, which was measured via Insulin ELISA (ALPCO), the difference was not statistically significant (Cre (n=5) vs Lac Z (n=5), Source of Variation: Column Factor p=0.2542)), nor was there a significant difference in fasting insulin levels and first phase insulin secretion between the two groups. Although there is no statistical significance in overall levels of insulin, there is a trending increase in insulin levels in the knockout mice. One way to mitigate this would be to

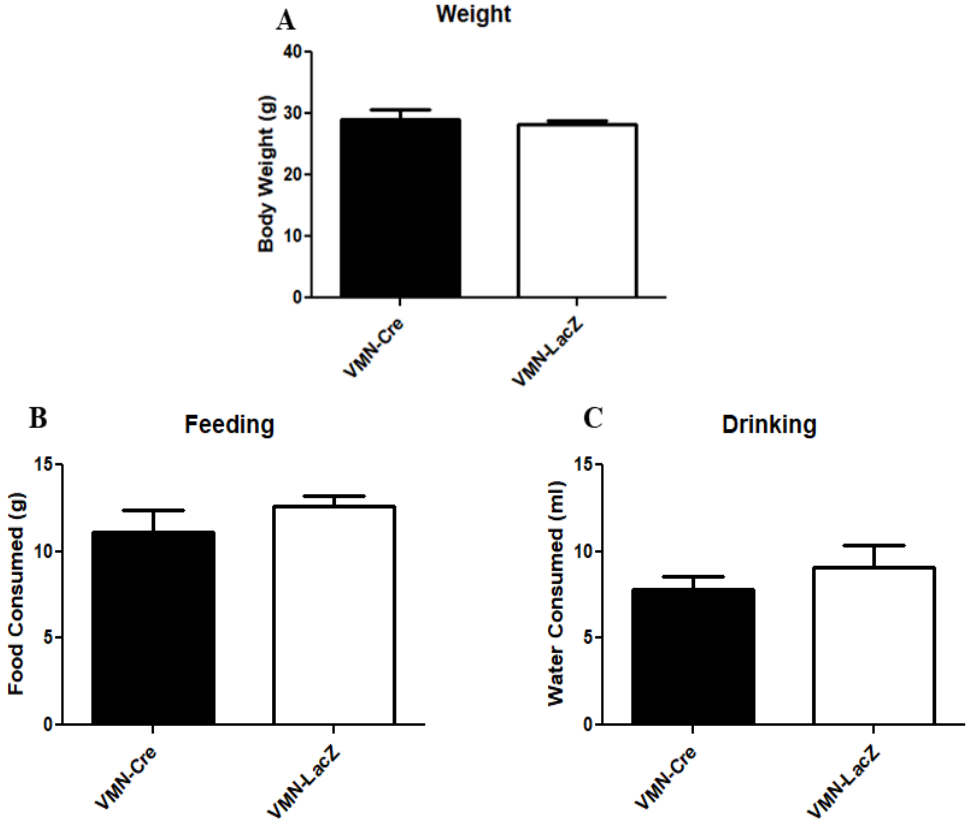

Figure 3.4: VMN-Cre and VMN-LacZ showed no difference in body weight **(A)** nor feeding and drinking behavior **(B,C)** Statistical Analysis for all three measurements were conducted using an unpaired t-test.P=0.7113 for "Weight" P=0.3410 and P=0.4273 for "Feeding" and "Drinking", respectively.

increase the number of mice per group, as the technique of handling mice had improved over time, and this data set includes animals that were experimented on early during training (Figure 3.2).

Robust Counter-regulatory Response in VMN-Cre Mice with No Change in Glucagon Levels

Insulin sensitivity and counter-regulatory response was measured via Insulin Tolerance Test. Mice were put on 3 hour fast and injected intraperitoneally with 0.5 mU/kg of insulin (Novalog). Insulin sensitivity is measure in the first 60 minutes of the ITT, which shows no apparent

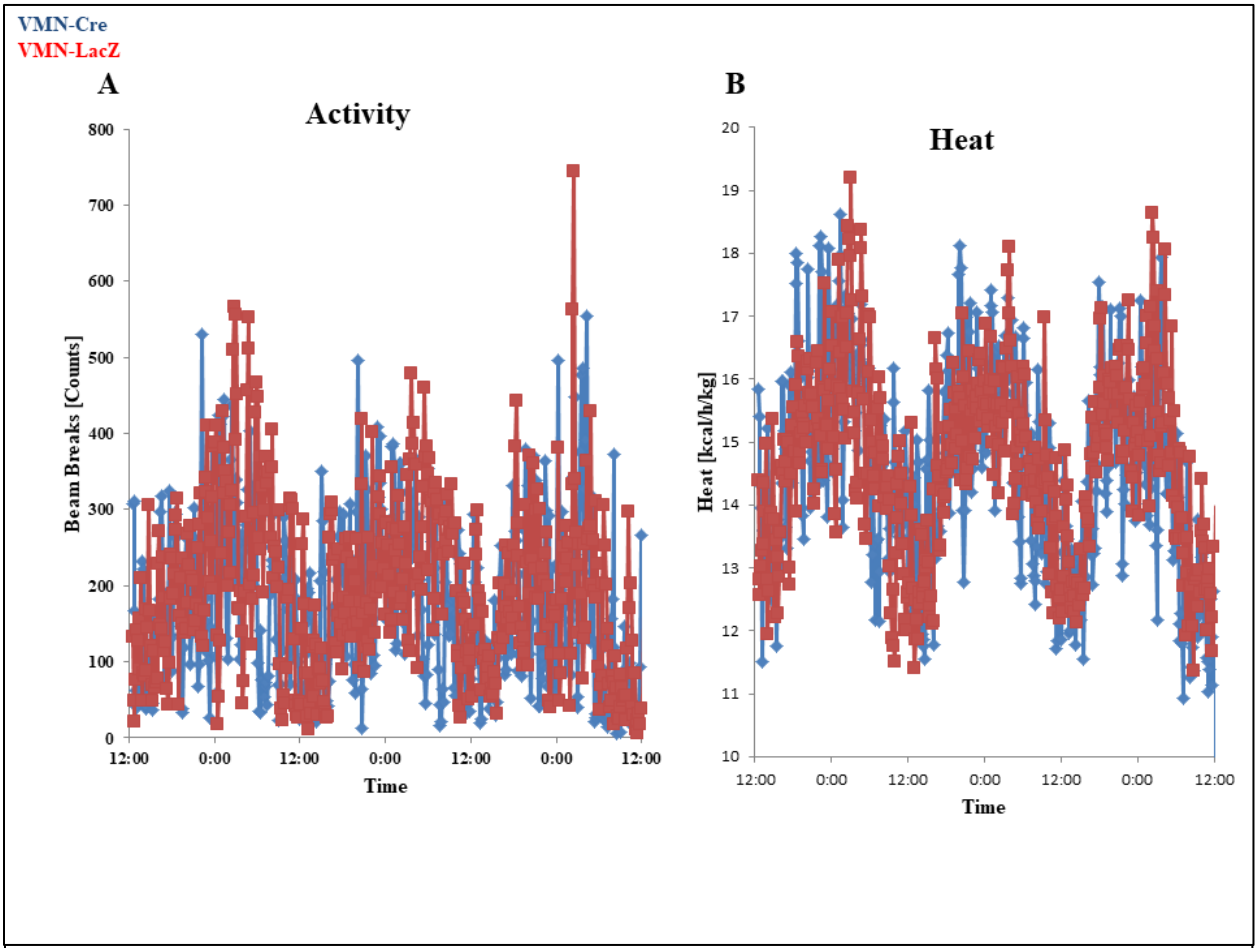

Figure 3.5: There was no significant difference in Movement **(A)** and Heat produced **(B)** between VMN-Cre mice (Blue) and VMN-LacZ mice (Red).

difference in insulin sensitivity, however, the latter 60 minutes signify the counter-regulatory response to hypoglycemia, in which there was an extremely significant difference between the VMN-Cre mice and the VMN-Lac-Z mice, with knockout mice having a robust counter-regulatory response compared to control mice (Cre (n=5) vs Lac Z (n=4), Source of Variation: Column Factor $p<0.0001$***, Cre (n=5) vs. Lac-Z (n=4), Bonferroni Post Test, 120 minute time point, 142.7 ± 6.766 vs. 104.5 ± 3.614, $p<0.01$**) (Figure 3.3).

When analyzing glucagon levels, however, there was no significant difference in glucagon from the 0 minute time point to 60 minute time point. Further work needs to be conducted, specifically in measuring catecholamine levels, which also assist in the counter-

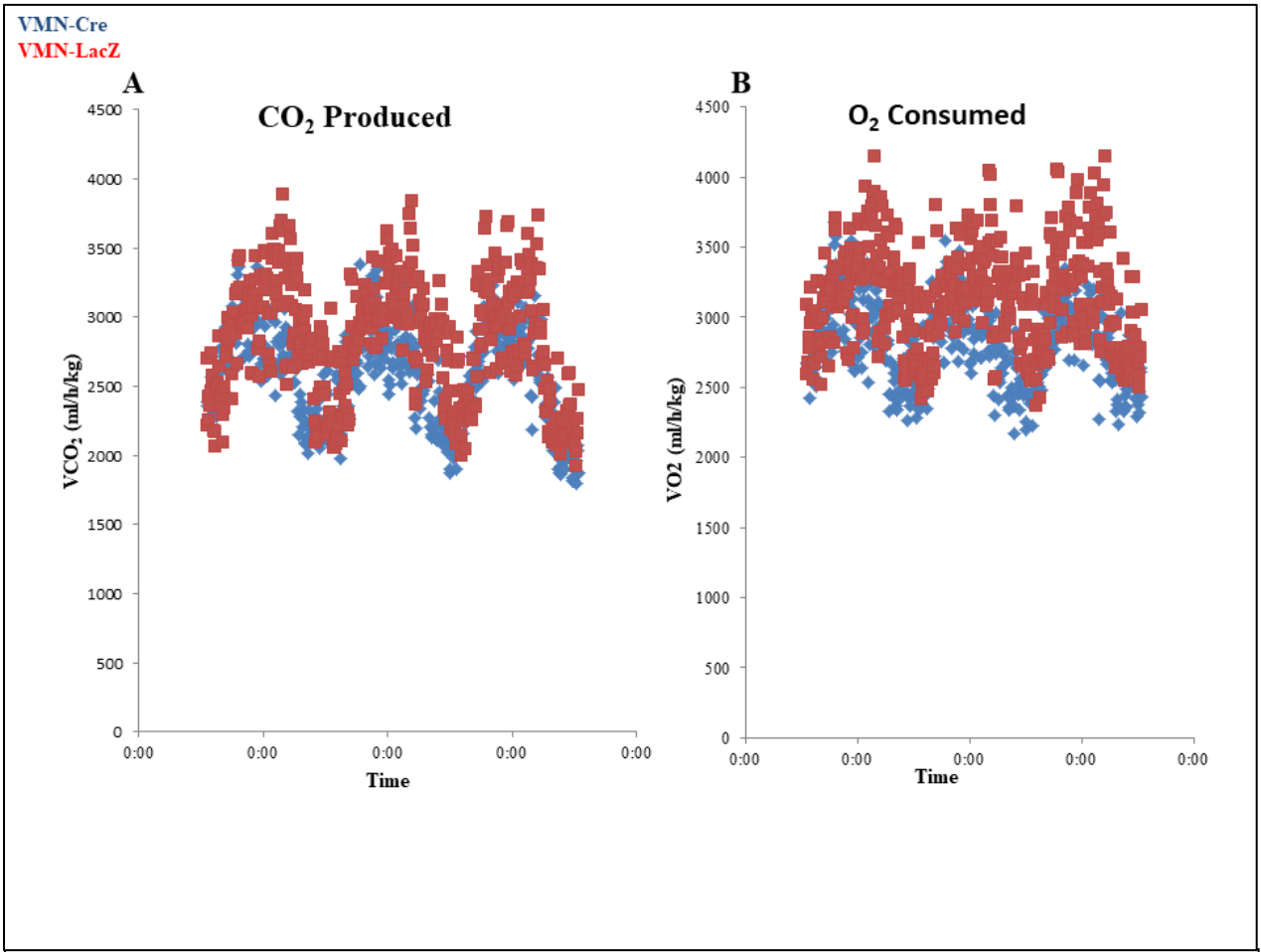

Figure 3.6: VMN-Cre mice produce less carbon dioxide than VMN-LacZ mice **(A),** and there was a decrease in amount of oxygen consumed **(B).**

regulatory response. Disruption of insulin signaling has no apparent effect on α-cell function, nor does it have an effect on peripheral insulin sensitivity. Other factors, such as norepinephrine, should be analyzed for future experiments (Figure 3.3).

VMN-Cre Mice Preferentially Utilizes Fat Compared to VMN-LacZ Mice

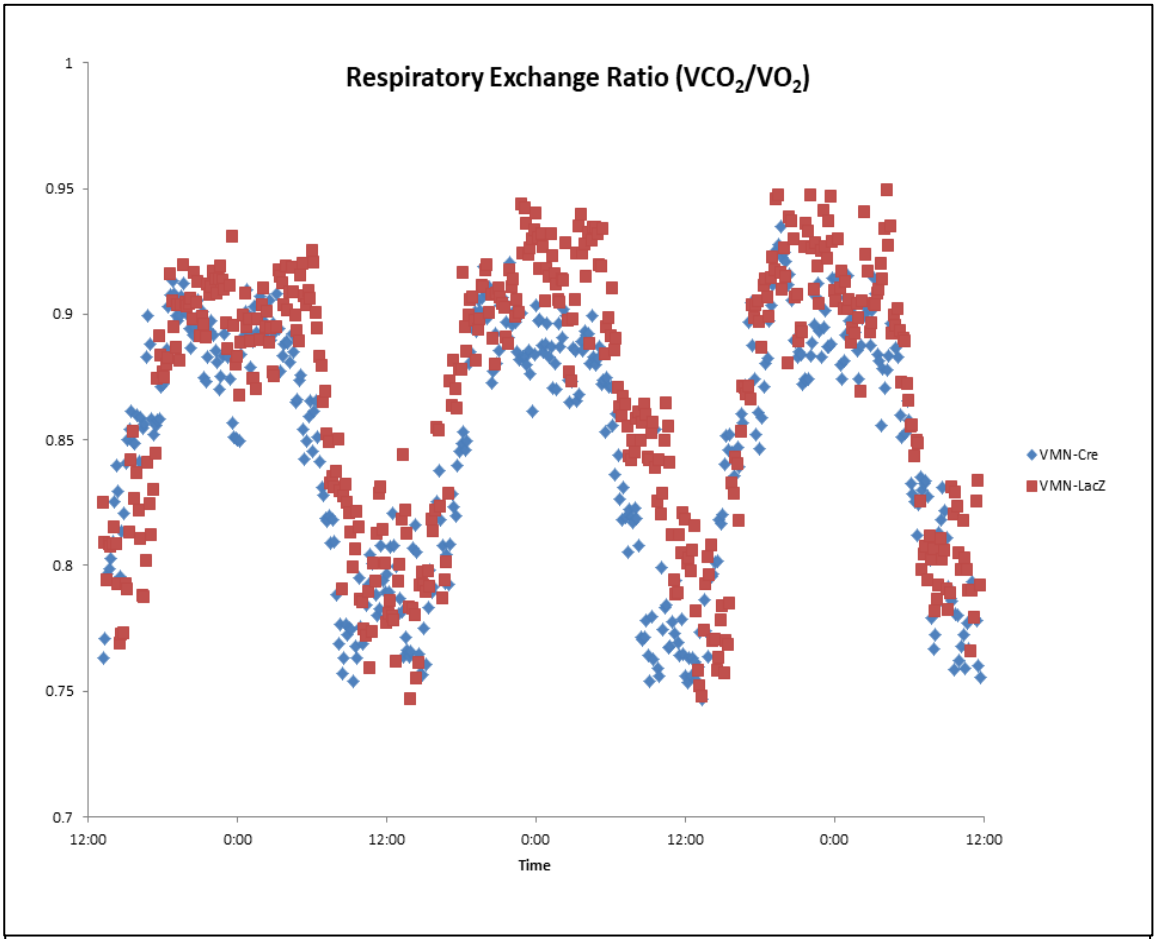

Figure 3.7: The ratio of the volume of carbon dioxide produced to the amount of oxygen consumed indicates the respiratory exchange ratio. VMN-Cre mice tend to have a lower RER than VMN-LacZ mice, which indicates VMN-Cre mice have more of a preference for fat as a fuel source rather than carbohydrates.

After analyzing islet function, mice were inserted into a metabolic cage to assess drinking and feeding behavior along with respiratory exchange rate (RER), activity and basal metabolic rate. Mice were given 2 days to acclimate to new environment, and measurements after acclimation period were assessed. There was no difference in feeding and drinking behavior (Figure 3.4), nor was there a difference in activity levels and production of heat (Figure 3.5). VMN-Cre mice had a consistently lower CO_2 production and O_2 consumption compared to control (Figure 3.6), which points to a lower Respiratory Exchange Ratio (RER) in VMN-Cre, which alludes to a

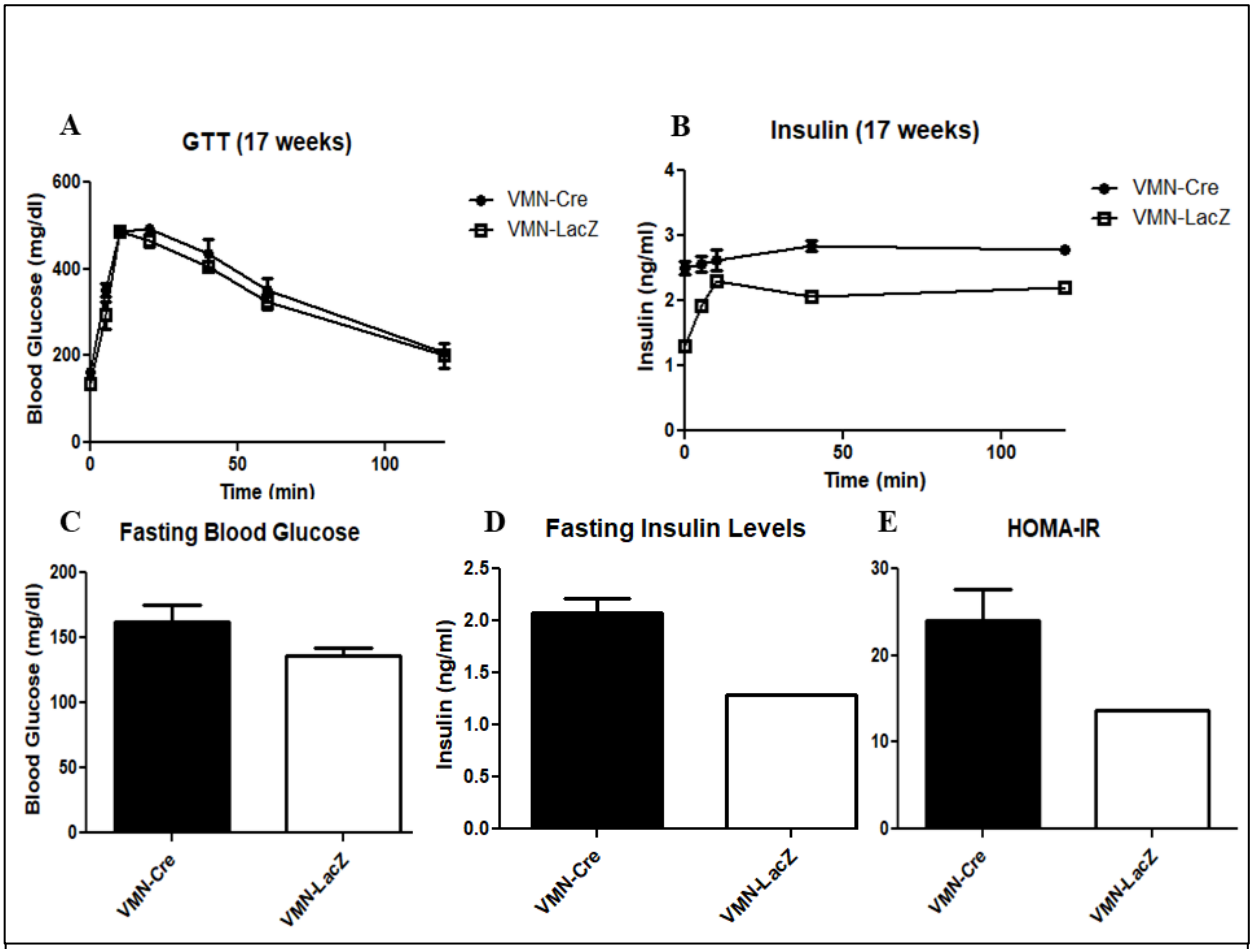

Figure 3.8: **A.** shows slight glucose intolerance in VMN-Cre mice vs. VMN-LacZ(P=0.0237, Source of Variation: Column Factor, 2Way ANOVA VMN-Cre n=2, VMN-LacZ n=3) with statistically insignificant increase in fasting blood glucose **(C)** (P=0.1351, Unpaired T-test). **B.** Insulin levels indicate severe hyperinsulinemia, with diminished biphasic secretion in VMN-Cre mice (n=2) and VMN-LacZ (n=1) and increased fasting insulin levels **(D)** and increased insulin resistance in VMN-Cre mice**.** Statistical analysis was done using 2Way ANOVA **(A,B)** and Unpaired T-test **(C,D,E).**

preferential utilization of fat rather than carbohydrates (Figure 3.7). This data, along with the previous Insulin Tolerance Test data suggests that VMN-Cre mice have perturbed glucose sensing in which the CNS is signaling a lower glycemic environment than systemically present. This concept will be discussed further in the discussion section.

Pilot Study Implicates VMN-Cre Mice Become Severely Hyperinsulinemic Over Time

During our initial stereotaxic experiments, 3 mice (2 VMN-Cre, 1 VMN-LacZ) were injected with their respective virus, and underwent metabolic assessments, such as GTT and ITT. More mice were then injected and the initial 3 mice were left and their timeline was unintentionally extended. When we were ready to euthanize mice in order to confirm injection sites, we observed that the 2 VMN-Cre mice looked much heavier than the lone VMN-LacZ mouse (34.5 $\pm$ 0.5 g .vs. 29.0 g). We decided to then re-conduct a glucose tolerance test and measured their insulin levels. VMN-Cre mice still maintained their glucose homeostasis compared to VMN-LacZ, however insulin levels showed glucose levels were maintained through severe hyperinsulinemia while on a normal chow diet. These results suggest the continuation of disrupted insulin signaling in the CNS, particularly the VMN, could lead to marked levels of insulin resistance throughout the periphery, as shown by the increase in HOMA-IR in VMN-Cre mice. More work is needed to confirm this phenomenon; however, this would indicate central insulin signaling, if perturbed, can lead to systemic insulin resistance (Figure 3.8).

3.4 Discussion

This chapter highlighted the role of insulin signaling in the VMN on pancreatic islet cell function. When we knocked out the insulin receptor in the VMN, there was an increase in glucose intolerance in VMN-Cre mice compared to the VMN-LacZ mice, however, there was a trending, yet statistically insignificant, increase in insulin secretion in the knockout mice. When conducting the Insulin Tolerance Test, there was no difference in peripheral insulin sensitivity, however there was a robust increase in the counter-regulatory response in VMN-Cre mice compared to VMN-LacZ mice, however analysis of fasting glucagon and glucagon secretion in the presence of insulin showed no difference. This suggests an over-activation of other counter-regulatory mechanisms such as norepinephrine, cortisol and growth hormone. The VMN has

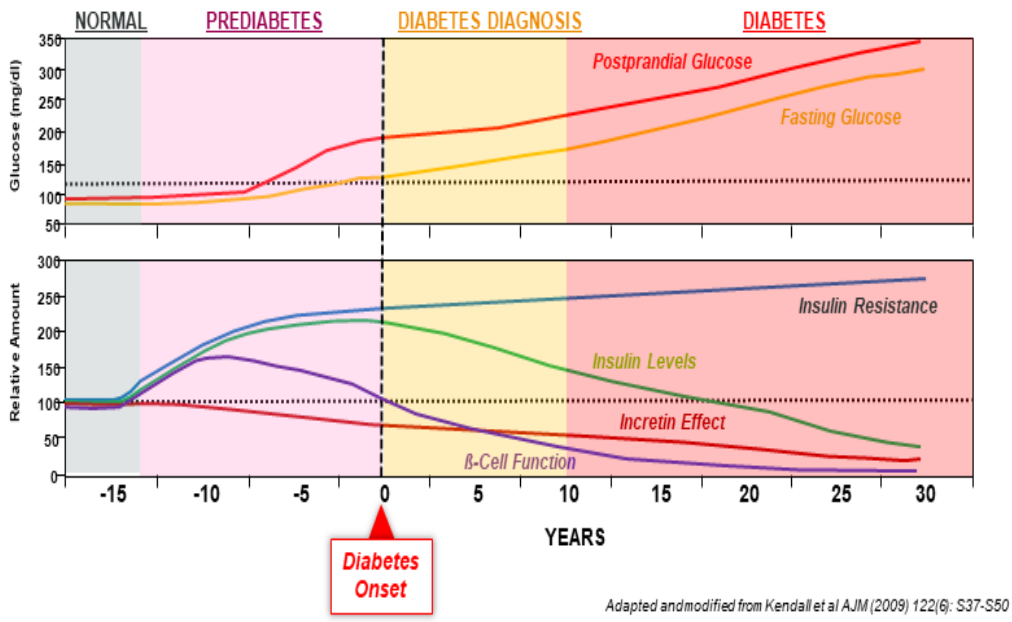

Figure 3.9: This figure shows a schematic of the progression of T2DM. Top panel represents the level of fasting and post prandial glucose (orange and red line), and the bottom panel represents the relative amount of insulin resistance (blue), insulin level (green), effect of incretin (red) and β-cell function (purple). As insulin resistance increases due to acquired defects (such as increase in fat intake), this increase forces an increase in insulin levels and β-cell function to maintain normal fasting and post-prandial glucose levels. Incretin levels start to fall at the onset of insulin resistance and continue to decline in the presence of insulin resistance. As β-cell function decreases due to overuse, insulin levels start to plateau. Insulin resistance, however, continues to rise, which causes a rise in fasting and post-prandial glucose into hyperglycemic levels. The sharp decline in β-cell function followed by the decline insulin levels indicates β-cell failure, which causes unregulated fasting and post-prandial glucose and T2DM.

been implicated in the counter-regulatory response through a variety of studies (Beverly 1995, de Vries 2005, Chan 2013, Reno 2017). It has also been suggested that the counter-regulatory response in the VMN that elicits the increase in norepinephrine is dictated by the level of ambient glucose present in the presence of the VMN (de Vries 2005). As mentioned previously, VMN neurons express the insulin receptor, and 70-75% of insulin receptor positive neurons in

the VMN also express GLUT4, an insulin responsive glucose transporter (Levin 2009). A decrease in insulin receptor expression in the VMN would also indicate a decrease in GLUT4 expression on the neuronal cell surface. The VMN neurons inability to properly sense glucose could be the reason there is an exaggerated counter-regulatory response in VMN-Cre mice, as the VMN has been shown to have connections to the A5 region of the CNS, which is an area that contains noradrenergic neurons (Rosario 2016, Pozo 2018). This notion that insulin dependent glucose sensing is perturbed in the VMN is further supported by the fact that VMN-Cre mice have a lower RER than VMN-LacZ mice, which indicates a preference for fat as a primary source of fuel, rather than carbohydrates. Fat is used when there is little glucose present, which is sensed through the CNS. Further studies need to be conducted to measure the various counter-regulatory hormones that could be released when insulin receptor is knocked out in the VMN.

Even though there was no initial effect of disrupted insulin signaling in the VMN on Islet cell function, we discovered that if the study was extended for 4 months post knockout, VMN-Cre mice (n=2) tended to gain more weight than VMN-LacZ mice (n=1). This observation led us to conduct a pilot study to investigate glucose tolerance and insulin secretion. We observed slight glucose intolerance with severe hyperinsulemia in the background. The reason for the increase in body weight and severe hyperinsulinemia could be due to an increase feeding, or a broken feedback loop that potentiates hyperinsulinemia and insulin resistance, which leads to an increase in feeding and weight gain. Metabolic Cage Assessment of mice was conducted, on average, 6 weeks after injection of the adenovirus which showed no increase in feeding or drinking behavior. However, a week after the initial injection of adenovirus, there was a trending increase in insulin secretion, which was collected simultaneously through tail snip while conducting a glucose tolerance test. A better, yet more difficult, method could be used to conduct

a glucose tolerance test, which consists of injecting glucose intravenously, rather than intraperitoneally, as well as administrating an oral glucose tolerance test to see the effects of incretin signaling with a deficiency in VMN insulin signaling. The trending increase in insulin secretion could have caused peripheral insulin resistance, and propagated further insulin secretion, thus creating a vicious cycle that perpetuates whole body insulin resistance. The latter would support other studies that have shown that a break in neural connections of the CNS and pancreas leads to hyperinsulinemia (DeFronzo 1981, Elahi 1982, Waldhäusl 1982, Garvey 1985, Ratzmann 1985, Stagner 1986, Argoud 1987, Boden 1993). These results are also in line with the idea of insulin induced insulin resistance, which is thought to be one of the driving forces of T2DM (Corkey 2012). There has been a new wave of thought that proposes Endocrine Disrupting Chemicals (EDCs) causes an increases in insulin levels, which could be a reason for the dramatic increase in the numbers of obese and diabetic patients in the last 30 years (Neel 2011, Corkey 2012, Holmes 2016, Bonini sf2018). This chapter also revisits the question of the driver of whole body insulin resistance: where does it start? The mechanism of peripheral insulin resistance particularly in the adipose tissue, skeletal muscle and liver, have been thoroughly studied on how it drives β-cell failure and subsequent T2DM (Hotamisligil 1993, Kubota 2000. DeFronzo 2009). The CNS has been thought of as a tool for "fine-tuning" various hormone secretions to maintain metabolic homeostasis. However, many others have shown throughout the years the importance of pancreatic hormone signaling in the CNS, particularly the hypothalamus, in whole body insulin sensitivity and pancreatic islet cell function (Brüning 2000, Obici 2002a, Obici 2002b, Imai 2008, Mighiu 2013).

Our data, taken with the figure above, indicates that insulin resistance and β-cell failure are the drivers of T2DM. Our data proposes a model that shows insulin signaling in the brain negatively

feeds back to the β-cell to inhibit further insulin secretion to not only prevent hypoglycemia, but also insulin resistance. When that feedback loop is broken, this causes a slight increase in insulin levels acutely, but can lead to severe hyperinsulinemia as this feedback loop remains broken. Insulin signaling in the VMN also allows for insulin responsive neurons that co-express GLUT4 to properly sense glucose in order to sense and react to low glucose levels. Continued work on this subject could lead to a definitive answer on insulin signaling dependent counter-regulatory response, the regulation of insulin secretion through the CNS, and the importance of central insulin signaling in maintaining whole body insulin sensitivity.

Chapter IV

Additional Studies and Future Direction

4.1 Introduction

The question of how insulin regulates itself has been split through a plethora of studies, either through the insulin induced insulin secretion model, or the insulin induced insulin inhibition model (DeFronzo 1981, Elahi 1982, Waldhäusl 1982, Garvey 1985, Ratzmann 1985, Stagner 1986, Argoud 1987, Boden 1993, Aspinwall 1999, Leibiger 2000, Bouche 2010, Halperin 2012). The argument that insulin induces its own secretion through autocrine mechanisms does not make sense anatomically nor physiologically. The microvasculature and the secretory architecture of the β-cell does not allow for proper autocrine signaling to occur. Insulin induced insulin secretion suggests a feedforward mechanism that cannot be stopped, and can induce whole body insulin resistance to protect the organism against hypoglycemia. Insulin induced insulin inhibition, particularly with the CNS as the mediator, makes sense from an anatomical and physiological perspective. The question is which part of the CNS connects to the pancreas to initiate this feedback mechanism. We have shown through retrograde tracing that there is indeed a circuit that connects the hypothalamus to the pancreatic Islet of Langerhans (Rosario 2016), as well as the area of the hypothalamus that could potentially be responsible for regulating insulin secretion: the Ventromedial Nucleus (VMN). Through the stereotaxic apparatus, we injected an Adenovirus containing either Cre-Recombinase or β-galactosidase into the VMN of Insulin Receptor Floxed mice. Acutely, Insulin and glucagon secretion remained intact, with insulin secretion being slightly elevated, however the counter-regulatory response was particularly robust in VMN-Cre mice, which could indicate a role of noradrenergic neurons in responding to

a lack of insulin signaling in the VMN. When the experiment is prolonged, the mice become slightly glucose intolerant, but extremely hyperinsulinemic in order to maintain their glucose levels. Insulin sensitivity and the counter-regulatory response of these mice, however, have not been measured. This chapter will focus on additional studies that can be done for further validation of the aforementioned experiments, and also future experiments to further along the knowledge of CNS regulation of the pancreatic islet as well as its role in T2DM.

4.2 Validation of the CNS-Islet map

Pseudorabies Virus (PRV) is a powerful tool to unearth potential circuits that can connect various areas of the CNS to one another, as well as various organs in the periphery to their CNS regulators. Rosario et al uncovered the hypothalamus as the initiator of signals to the pancreas, and through validation of the initial map, discovered a majority of the CNS-Pancreas connections were Islet specific. This CNS-Islet map was the first of its kind to establish the CNS and the pancreatic islet as direct connects to one another. However, this map was established through a virus going the opposite direction of a propagated signal. The only way to re-establish the map is to connect the CNS to the Islet in an anterograde fashion, and there are a variety of tools to accomplish this.

One of which is through a protein found in red kidney beans called Phytohaemagglutinin leucoagglutinin (PHA-L). PHA-L is used to trace axons from cell body to pre-synaptic terminals, and can be stereotaxically injected into various areas of the hypothalamus to trace their circuits to the brainstem and periphery. PHA-L travels through first-order neurons and will be expressed in the area that is directly innervated by neurons in the site of injection. Once the next area is highlighted, PHA-L is then injected in that area to highlight the next step in the circuit, until the

brainstem is highlighted. In order to validate this circuit, one could also co-inject these mice with what is called a Designer Receptor Exclusively Activated by Designer Drugs (DREADD). DREADD is a synthetic G-Protein Coupled Receptor (GPCR) that couples to either Gq, Gs, or the inhibitory Gi protein. Co-infection of the hypothalamic areas with PHA-L and HM3D(Gq)-mCherry fusion protein would trace the neuronal projections of the site of infection as well as validate that neuronal connection through activation of the DREADD and co-labeling of cFos in the area or projection. Injection of the Designer Drug Clozapine-N-Oxide (CNO) intraperitoneally can cross the blood brain barrier and activate the DREADD, leading to the subsequent activation of downstream neurons, where there would be PHA-L co-expressed along with cFos.

4.3 Validation of the Neuronal Activation of the VMN in the Presence of Insulin

Chapter II had established that there is a significant increase in CNS-Islet circuit activation of the VMN. We established this by recreating the retrograde map injecting PRV-BaBlu in the pancreas of C57Bl/6 mice and injecting them intraperitoneally with insulin 120 hours later. We concluded that this was due to a direct effect of insulin rather than a drop in glucose levels due to the timing at which these mice were sacrificed as well as other studies that prolonged the time mice were hypoglycemic showed a suppression of cFos in the VMN (Foster 2016). Other studies have injected insulin through intracerebroventricular (ICV) injections, however, this is not the natural route of insulin, and therefore does not accurately reflect the activation pattern of neurons in the presence of insulin (Ishihara 2009). To validate the activation pattern of the circuit, further experiments need to be conducted.

Insulin receptor is abundantly expressed in the VMN, specifically in SF-1 neurons. In order to validate the activation pattern of VMN neurons that are part of the circuit, one could first cross SF-1-Cre mice x IRfloxed mice to produce mice that have their insulin receptors deleted in all cells expressing SF-1 (Klöckener 2011), which would be gonadal cells as well as neurons in the VMN. SF-$1^{\Delta IR}$ mice as well as control mice would then have their pancreas infected with PRV-BaBlu and have the CNS-Islet circuit recreated. These mice would then be put on a 3 hour fast 120 hours post infection and then IP-injected with insulin followed by sacrifice 1 hour post insulin injection. One would then compare neuronal activation of the circuit in the VMN for mice with and without the insulin receptor. If there is a significant increase or no difference in how activated the circuit is in the VMN in SF-$1^{\Delta IR}$ mice compared to control, then this would indicate that insulin did not directly activate the neurons in the VMN. However, if there is a significant decrease in activation of the circuit in the VMN, then this would indicate that the significant decrease must be due to a loss of insulin signaling in the VMN, thus the activation seen previously must be due to a direct effect of insulin. This method, unlike injecting Cre into IR-Floxed mice, ensures that all insulin receptors are deleted from neurons containing SF-1, which express the majority of the insulin receptor in the VMN (Klöckener 2011), rather than just the area injected with Cre-Recombinase, in order to get a better indication of the type of effect insulin has on the VMN.

It would also be interesting to see if there is a difference in neuronal activation in the presence of obesity. Recreating the circuit in Diet Induced Obese (DIO) mice and injecting insulin intraperitoneally would allow us to investigate the profile of neuronal activation of the circuit in obesity.

4.4 Neurotransmitters Involved in the CNS-Islet Circuit

The CNS-Islet circuit was able to highlight various areas of the hypothalamus that connect the hypothalamus to the pancreatic islet. It does not, however, inform of which type of neurons are also in the circuit. The Arcuate nucleus consists of orexigenic (POMC/CART) and anorexigenic neurons (AgRP/NPY), both of which express various receptors, including the insulin receptor (Cone 2001). As mentioned earlier, the VMN contains SF-1 neurons, along with Brain Derived Neurotropic Factor (BDNF) and Estrogen Receptor α (ERα) neurons, DMN contain NPY neurons while the PVN contain a host of neurons, including Nitric Oxide Synthase 1 (NOS1) neurons. The LHA contain Orexin, Melanocortin, and Neurotensin neurons. Co-staining of β-galactosidase and these neuronal types would indicate the profile of neurons inside and outside the circuit. Any neuronal cell type that is significantly expressed in the circuit could be manipulated by either increasing its activation or decreasing it using the aforementioned DREADD receptors that are expressed through the specific neuronal cell type promoter (i.e. HM3D(Gq) expressed through the NPY promotor) in order to investigate their role in the regulation of the pancreatic Islet of Langerhans.

4.5 Insulin Signaling in the VMN on Counter-Regulatory Response (CRR)

A plethora of studies have implicated the involvement of the VMN in the CRR (de Vries 2005, Paranjape 2010, Chan 2013). The VMN has been shown to increase glucagon secretion as well as secretion of norepinephrine (Borg 1994, de Vries 2005, Paranjape 2010, Chan 2013). Our data shows that there is indeed an exaggerated counter-regulatory response when insulin signaling is knocked out, however, there is no change in glucagon secretion. This leads us to believe that other CRR hormones are affected by insulin signaling in the VMN. In order to parse out which ones are affected, we would conduct a catecholamine ELISA (ABNOVA) on mice given an IP injection of insulin. We suspect that norepinephrine is the main player in increasing

the CRR in VMN-Cre mice, due to the connection that has been established between the VMN and the A5 region, which consists of noradrenergic cell groups. Studies have established that the A5 region sends its projections to the VMN to regulate hypoglycemia as well as other reproductive behaviors (Kitaoka 2010, Miyaki 2011, Barnes 2011), however there have been suggestions that the VMN also projects to the A5 region (Rosario 2016, Pozo 2018). A retrograde study implicated a connection between the VMN and the A5 (Rosario 2016), but no anterograde studies have been conducted to trace the projections from the dorsomedial VMN, where a majority of insulin receptor positive cells are expressed to the hindbrain. I propose injecting PHA-L into the dorsomedial VMN and track its projections, while injecting fluorogold (a retrograde tracer) into the A5, to see if the connections are bi-directional. The next step would be to inject HM3D(Gq) into to VMN driven by the Insulin Receptor promoter and IP inject CNO in order to activate the DREADD and analyze the A5 region for c-Fos levels in order to establish functionality in the projection from the VMN to the A5.

4.6 Prolonging and Fine-Tuning the Knockout of the Insulin Receptor and Continuing Metabolic Measurements

Data outlined in Chapter III showed that VMN-Cre mice had a robust CRR, but did not show any significant change in pancreatic islet function. Acute insulin levels were trending upward in the VMN-Cre mice, but no increase in glucagon levels. However, when we conducted metabolic tests on mice after they had been injected 17 weeks ago, VMN-Cre (n=2) mice showed slight glucose intolerance with extreme hyperinsulinemia compared to VMN-LacZ mice (n=1). The difference was stark, and should be followed up by increasing the “n” per group to at least an n>5 per group. We also would follow up the study by conducting an insulin tolerance test on these mice, along with glucagon levels to measure their insulin sensitivity and CRR. This would

be done in increments of 1 month, where GTT, ITT and their respective insulin and glucagon levels would be measured 3 times over a 3 month span, along with metabolic cage assessment. This would allow us to parse the onset of hyperinsulinemia with the increase in weight gain, as well as measure feeding and drinking behavior once a month. We would also be able to establish when the onset of insulin resistance occurs through hyperinsulinemic-euglycemic clamps. We would also measure pancreatic α- and β-cell mass to see if the increase in weight and hyperinsulinemia causes an increase in mass to compensate for the presumable insulin resistance.

The stereotaxic apparatus allows for spatial precision in knocking out a protein, and much time and effort was utilized in delivering the virus to knockout the insulin receptor in the VMN. The VMN is situated between the ARC and the DMN, with very little room separating the areas. It is therefore plausible that virus could have leaked into the neighboring areas. Injection confirmations have showed that most, if not all the virus was shown to be expressed exclusively in the VMN, however ether were 2 examples (1 VMN-Cre and 1 VMN-LacZ) where most of the virus was in the VMN, but small traces of virus could be detected in the ARC. The specificity of the adenovirus expressing Cre under the control of the insulin receptor promoter made the virus specific to those areas expressing the insulin receptor. In order to create a more precise knockout, we would inject an adenovirus that carried Cre Recombinase that was driven by the SF-1 promoter. As stated previously, the VMN is the only hypothalamic area to express VMN, and a majority of insulin receptor expression in the VMN is on the surface of SF-1 neurons. Any leakage of virus to neighboring areas would not affect the expression of insulin receptor in those areas. To add another layer of confirmation, we would also utilize Laser Capture Microdissection in order to quantify how much protein was knocked out in the VMN and verify that neighboring areas did not have their insulin receptor expression perturbed due to the injections.

4.7 Investigating the Role of GLUT4 in Insulin Receptor Positive Neurons in VMN

Chapter III had established that knockout of the insulin receptor in the VMN leads to exaggerated CRR, as well as a lower RER, which signifies the mouse's preference for fat as a fuel source, rather than carbohydrates. Fat becomes utilized in the absence of circulating glucose, however, there is no difference in feeding nor was there a difference in fasting glucose levels. This could be due to an increase in sympathetic innervation of white and brown fat and alludes to a perturbation in glucose sensing in the CNS in the absence of insulin signaling in the VMN. In the periphery, insulin sensitive tissues, such as skeletal muscle and adipocytes, use GLUT4 as their main glucose transporter to uptake glucose. In the CNS, there is a variety of glucose transporters, such as GLUT1, GLUT2, GLUT3 and the fructose transporter GLUT5 (Ngarmukos 2001). GLUT4 is also expressed in discrete areas of the hypothalamus, particularly the VMN, where it is expressed on 70-75% of all insulin positive neurons in the VMN (Levin 2009). Studies have investigated the role of GLUT4 in whole brain knockout and have shown a perturbed CRR in the presence of hypoglycemia (Reno 2017). No one has yet to investigate the role of GLUT4 in the VMN, however. Therefore I propose to investigate the role of GLUT4 in insulin positive VMN neurons.

We would use GLUT4 Floxed mice and stereotaxically inject an adenovirus with Cre-Recombinase expressed under the control of the insulin receptor promoter, in order to knockout the GLUT4 in insulin receptor positive neurons in the VMN. We believe that the data shown in Chapter III regarding CRR perturbation and lower RER is an indirect effect of disrupted insulin signaling, while the trending increase in hyperinsulinemia is a direct effect of insulin signaling. In order to parse this out, we want to knockout the GLUT4 glucose transporter in the VMN and then conduct a GTT and ITT, with their respective insulin and glucagon ELISAs, as well as a

catecholamine ELISA to measure norepinephrine. We expect insulin levels to be similar, while we expect a robust CRR as well as catecholamine levels to be significantly increased. We could also recreate the circuit and double stain for β-galactosidase and GLUT4 to test how many GLUT4 positive neurons are part of the CNS-Islet circuit.

4.8 Investigate the Role of Insulin Signaling in the Ventral Tegmental Area/Substantia Nigra

The Ventral Tegmental Area/Substantia Nigra (VTA/SN) is an area of the midbrain that is the center of the reward system. Part of the reward system is hedonic feeding, which if gone awry, increases the risk of obesity and T2DM (Faulconbridge 2011, Yu 2015, Robinson 2015). The insulin receptor has been known to be expressed in these areas and have been implicated as a suppressor of hedonic feeding (Figlewicz 2016). Therefore we propose to investigate insulin resistance in this area by expressing Adenovirus-Cre in the VTA/SN through stereotaxic surgery in Insulin Receptor Floxed mice. We would then administer "palatable foods" such as sucrose and a high fat diet and measure feeding and drinking behavior as well as body weight of these mice, followed by glucose and insulin tolerance tests, with their respective insulin and glucagon ELISAs. We expect any change in hormonal levels to be secondary to the increase in feeding behavior and adiposity.

It would also be interesting to see if restoration of insulin signaling in Diet Induced Obesity (DIO) mice could reverse the obesity, making it a potential therapeutic target. We would restore this by increasing the expressing of HMD4i DREADD (inhibitory) in the VTA/SN using stereotaxic surgery under the control of the insulin receptor promoter, where insulin signaling in these neurons suppresses dopaminergic neurons, in DIO mice. We would then inject IP-CNO to

activate the inhibitory DREADD and then measure feeding and drinking intake of high fat diet and sucrose water.

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