Assessing the role of PTEN in cholinergic neurons in glucose and energy homeostasis

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

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Master of Science
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University of Toronto
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Abstract

Insulin resistance and insulin deficiency are key determinants of type 2 diabetes. Insulin resistance is manifested by attenuation of insulin signaling. A major insulin signaling pathway is mediated by phosphatidylinositol kinase (PI3K), which is negatively regulated by phosphatase and tensin homologue (PTEN). Neuronal PI3K-mediated insulin action plays a critical role in the regulation of systemic insulin resistance. The dorsal ventral complex (DVC) is the control centre of vagal activity and utilizes acetylcholine, a neurotransmitter, which is synthesized by the enzyme choline acetyl transferase (ChAT). To assess the role of PTEN in cholinergic neurons, we generated mice deficient of PTEN in these neurons using the cre-loxP system. These mice were protected against high fat diet-induced weight gain and glucose intolerance. They also had less inflammatory adipose tissue. Overall, PTEN deletion in the cholinergic neurons provides protection against diet-induced obesity and diabetes.
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<tr>
<td>AAV</td>
<td>Adeno-associated viruses</td>
</tr>
<tr>
<td>AchE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
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<tr>
<td>α-MSH</td>
<td>Alpha melanocyte-stimulating hormone</td>
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<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>ARH</td>
<td>Arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>ATM</td>
<td>Adipose tissue macrophages</td>
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<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
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<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CHT</td>
<td>Choline transporter</td>
</tr>
<tr>
<td>CLAMS</td>
<td>Comprehensive lab animal monitoring system</td>
</tr>
<tr>
<td>CLS</td>
<td>Crown-like structure</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine 3</td>
</tr>
<tr>
<td>DMV</td>
<td>Dorsal motor nucleus of vagus</td>
</tr>
<tr>
<td>DREADD</td>
<td>Designer receptors exclusively activated by designer drugs</td>
</tr>
<tr>
<td>DVC</td>
<td>Dorsal vagal complex</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1/2</td>
</tr>
<tr>
<td>eWAT</td>
<td>Epididymal white adipose tissue</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G6pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HGP</td>
<td>Hepatic glucose production</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IML</td>
<td>Intermediolateral nucleus</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>K(_{\text{ATP}})</td>
<td>Potassium ATP channel</td>
</tr>
<tr>
<td>LDT</td>
<td>Laterodorsal tegmental</td>
</tr>
<tr>
<td>LHA</td>
<td>Lateral hypothalamic area</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBH</td>
<td>Mediobasal hypothalamus</td>
</tr>
<tr>
<td>MC4R</td>
<td>Melanocortin-4 receptors</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>NCD</td>
<td>Normal chow diet</td>
</tr>
<tr>
<td>NG</td>
<td>Nodose ganglion</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>Nse</td>
<td>Neuron-specific enolase</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>p-AKT</td>
<td>Phosphorylated AKT</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pepck</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>Phox2b</td>
<td>Paired-like homeobox 2b</td>
</tr>
<tr>
<td>PIP(_2)</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP(_3)</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PNS</td>
<td>Parasympathetic nervous system</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>pS6</td>
<td>Phosphorylated ribosomal protein 6</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>Rip2</td>
<td>Rat insulin promotor 2</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic factor-1</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>sWAT</td>
<td>Subcutaneous white adipose tissue</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>VAchT</td>
<td>Vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>vDB</td>
<td>Diagonal band of Broca</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
</tr>
<tr>
<td>VNS</td>
<td>Vagus nerve stimulation</td>
</tr>
</tbody>
</table>
Chapter 1 – Introduction
1.1 Type 2 diabetes

Type 2 diabetes (T2D) has shown a marked increase in incidence in recent decades which is associated with the global obesity epidemic (Barnes, 2011). In Canada, the percentage of adults with diabetes is expected to rise from 9.3% in 2015 to 12.1% in 2025 (Canadian Diabetes Association, 2016). This represents a critical burden that will continue to strain the Canadian economy and the health care system. T2D represents 90-95% of all diabetes (American Diabetes Association, 2018). The chief determinants of T2D are insulin resistance and impaired insulin release from the pancreatic β-cells (C. R. Kahn, 1994) leading to hyperglycemia (American Diabetes Association, 2018). Over a sustained period, hyperglycemia leads to micro and macrovascular complications (Fowler, 2008). Microvascular complications include retinopathy, nephropathy, and neuropathy (Fowler, 2008). In fact, T2D is the leading cause of blindness in the Western world and is the major cause for end stage kidney disease, necessitating dialysis and renal transplantation (American Diabetes Association, 2018). Macrovascular complications include ischemic heart disease, cerebrovascular disease, and peripheral arterial disease.

Individuals may be predisposed to T2D due a combination of environmental and genetic factors (S. E. Kahn et al., 2014). The two main environmental determinants for T2D are an unhealthy diet, with increased caloric intake particularly of processed foods with high refined carbohydrate content and decreased consumption of fresh vegetables and vegetables, and a sedentary lifestyle causing decreased energy expenditure (Marín-Peña et al., 2016). Additionally, in utero exposure to environmental chemicals and an
altered microbiome have been linked to T2D pathogenesis (S. E. Kahn et al., 2014). Genes that are known to contribute to the development of T2D are associated with β-cell dysfunction and adiposity (S. E. Kahn et al., 2014). The onset of T2D can be delayed with lifestyle modifications aimed to reduce body weight (Marín-Peñaiver et al., 2016). The first-line medical treatment for T2D is metformin, which belongs to the biguanide family, with a mode of action to prevent hepatic glucose production (HGP). If metformin alone does not achieve target plasma glucose levels, additional drugs to consider include sulfonylureas, sodium-glucose transporter-2 inhibitors, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor agonists, and/or insulin. The underlying causes of insulin resistance are unclear. A variety of processes including inflammation and lipo/glucotoxicity have been implicated in the disruption of insulin action (Shimomura et al., 1998; Shoelson et al., 2006). Much research is under way to elucidate the mechanisms leading to insulin resistance.

1.2 Insulin Signal Transduction

In response to glucose, the pancreatic β-cells secrete insulin, which activates the insulin receptor (IR) within target metabolic tissues, including muscle, liver and adipose, to exert its biological effects (Boucher et al., 2014) (Figure 1). Insulin activates phosphoinositide 3-kinase (PI3K), an enzyme that phosphorylates cytoplasmic bound phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-trisphosphate (PIP₃). PIP₃ in turn binds to the pleckstrin homology (PH) domains to promote phosphorylate AKT. Activated AKT can phosphorylate a wide array of target proteins to
mediate metabolic effects in a tissue-specific manner (Alsadat & Khorami, 2015). AKT-mediated insulin signaling can lead to phosphorylation of forkhead box protein O1 (FOXO1), which dampens HGP and can increase glucose transporter 4 (GLUT4) translocation to the plasma membrane in skeletal muscle (Zhang et al., 2006; Alsadat & Khorami, 2015). Additionally, AKT can phosphorylate mammalian target of rapamycin complex 1 (mTORC1), which can control cell growth by enhancing protein translation, ribosome biogenesis, and lipid biosynthesis (Düvel et al., 2011). The PI3K signaling pathway is highly evolutionarily conserved (Hay, 2011). Disruptions in PI3K activity have been shown to aggravate T2D by inducing insulin resistance. For example, pharmacological inhibitors of PI3K, have shown to worsen insulin and glucose tolerance in mice (Smith et al., 2012). Additionally, in humans, specific polymorphisms of the p85α regulatory subunit of PI3K have been linked to reductions in glucose homeostasis (Hansen et al., 1997). In obesity, there is a transition of macrophages from their M2 anti-inflammatory state to M1 pro-inflammatory state, which can lead to insulin resistance (Olefsky & Glass, 2010). PI3K activity is also implicated in cancer, since phosphorylated (p)-AKT can mediate cellular proliferation, inactivation of pro-apoptotic proteins, and potentiation of metabolic signaling, permitting dysregulated cell growth and tumorigenesis (Simpson & Parsons, 2001).

Phosphatase and tensin homologue (PTEN) is an endogenous negative regulator of PI3K. It is a lipid phosphatase that dephosphorylates PIP3 to PIP2 to effectively inhibit PI3K activity (Stambolic et al., 1998). PTEN is located on chromosome 10 in humans and
was originally discovered as a tumor suppressor (Li et al., 1997). Mutations in the PTEN have been linked to rare autosomal dominant hamartomous cancer syndromes including Cowden’s disease and Bannayan–Zonana syndrome in humans (Simpson & Parsons, 2001). PTEN activity can be regulated at transcriptional, translational, and post-translational levels (Kreis et al., 2014). Intense research is ongoing to understand how post-translational phosphorylation, acetylation, oxidation, nitrosylation, and ubiquitination of PTEN can alter its phosphatase activity (Kreis et al., 2014). In the context of metabolism, PTEN’s role in modulating insulin signaling has uncovered its critical regulatory roles in the pathogenesis of T2D.
**Figure 1:** PI3K-PTEN Signaling Pathway. In response to glucose, the pancreatic β-cells secrete insulin, which activates the insulin receptor (IR) of target tissues, including muscle, liver, and adipose to exert its biological effects. IR is a receptor tyrosine kinase which can phosphorylate key substrates in the cytoplasm, which then activate phosphoinositol 3-kinase (PI3K), which can phosphorylate cytoplasmic membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$). PIP$_3$ then binds to AKT for phosphorylation. Activated AKT can in turn phosphorylate a wide array of downstream target proteins to mediate various biological effects.

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1.3 The Role of PTEN in Metabolic Tissues

To study the essential role of PTEN in mediating glucose homeostasis, various mouse models harboring PTEN null mutation in a wide array of tissues and cell types have been generated. Since whole body PTEN deletion in mice leads to lethality by embryonic day (E) 7.5. (Cristofano et al., 1998), other strategies are used to genetically knock it down post development. This includes antisense oligonucleotides, which successfully reduced PTEN transcription in the liver and fat in mice. These mice showed increased p-AKT levels in the liver and improved glucose homeostasis (M. Butler et al., 2002). Using the cre-lox recombination system in mice also showed important essential roles of PTEN in various metabolic tissues. Hepatocyte-specific deletion of PTEN, driven by the albumin promoter cre led to a marked increase in systemic insulin sensitivity (Horie et al., 2004). A similar increase in insulin sensitivity and protection from diabetes was seen in mice with PTEN disruption in adipose tissue, using adipocyte protein 2 or adiponectin cre promoters (Kurlawalla-Martinez et al. 2005; Morley et al., 2015).

Furthermore, skeletal muscle deletion of PTEN, mediated by cre under the control of muscle creatine kinase promoter, protected mice from high fat diet (HFD)-induced insulin resistance and diabetes (Wijesekara et al., 2005). Additionally, mice with pancreatic β-cell specific deletion of PTEN, using the rat insulin promoter (Rip)2 -mediated cre, were protected from developing T2D in both a HFD-fed or genetic leptin receptor-deficient (db/db) model of diabetes (L. Wang et al., 2010).
PTEN deletion, however, does not lead to beneficial effects in all tissues. For example, liver specific deletion of PTEN, while enhancing insulin sensitivity at 12-weeks of age, leads to spontaneous development of hepatocellular carcinoma by 44 weeks of age (Horie et al., 2004). Taken together, this evidence shows the possible therapeutic benefits of targeting PTEN in T2D with caution for potential oncogenic effects. Thus, understanding and targeting tissue and cell specific strategies for PTEN is paramount for therapeutic consideration for T2D.

1.4 Neuronal Control of Glucose Homeostasis

The nervous system plays a critical role in the maintenance of glucose homeostasis and energy balance (Figure 2). Earlier studies demonstrated that intracerebroventricular (ICV) injection of insulin into baboons led to major suppression of food intake and subsequent weight loss (Woods et al., 1979). Further research has outlined that regions such as the hypothalamus and hindbrain contain intricate circuitries of neurons, which respond to peripheral signals to modulate glucose homeostasis (Ruud et al., 2017). A critical region within the hypothalamus is the mediobasal hypothalamus (MBH), which contains the arcuate (ARH) and the ventromedial hypothalamic neurons (VMH). Inhibition of insulin signaling within the MBH has been shown to lead to dysregulation of glucose homeostasis in mice (Ono et al., 2008). Importantly, the MBH has been shown to respond to insulin in a PI3K-dependant matter to suppress HGP via the activation of potassium ATP (K\textsubscript{ATP}) channels (Pocai et al., 2005). The ARH contains orexigenic (appetite increasing) agouti-related peptide
(AgRP) and anorexigenic (appetite suppressing) pro-opiomelanocortin (POMC) neurons. Studies have demonstrated that the excitation of these neuronal subtypes in mice by chemogenetics and optogenetics can directly influence feeding and glucose homeostasis (Ruud et al., 2017). Another important hypothalamic region within the MBH is the VMH, which is a major satiety centre (Klöckener et al., 2012). The VMH contains a special subtype of neurons that express steroidogenic factor (SF-1). Recently, direct activation of VMH SF-1 neurons in mice chemogenetically using designer receptors exclusively activated by designer drugs (DREADDs), was shown to improve peripheral insulin sensitivity (Coutinho et al., 2017).

Leptin, which is released from the adipose tissue, is another hormone that is important in regulating energy expenditure and metabolism (Y. Zhang et al., 1994). In the hypothalamus, leptin binds to its receptor and causes dimerization of Janus kinase 2 (JAK2) and phosphorylation of signal transducer and activators of transcription 3 (STAT3), which translocates to the nucleus to alter gene expression of orexigenic and anorexigenic factors (Sahu, 2003). Leptin, like insulin, can also induce PI3K signaling in the hypothalamus to improve insulin sensitivity in rodents (Kim et al., 2000; Sivitz, et al., 2009). Importantly, mice with a mutation in the leptin receptor gene (db/db) develop spontaneous hyperglycemia, and hyperinsulinemia with massive obesity (Kobayashi et al., 2000).
Figure 2: Mouse brain regions that modulate glucose homeostasis. Sagittal section of a mouse brain with important areas highlighted. The hypothalamus contains the paraventricular nucleus (PVH), the lateral hypothalamic area (LHA), the ventromedial nucleus of the hypothalamus (VMH, containing the SF-1 neurons), and the arcuate nucleus of the hypothalamus (ARH, comprised of POMC and AgRP neurons). The dorsal vagal complex (DVC) is located in the medulla within the brainstem. 3V, third ventricle; 4V, fourth ventricle; LV, lateral ventricle.

These hypothalamic regions regulate glucose homeostasis in a dual manner. Firstly, the neurons project signals to higher order structures within the cortex, which dictate feeding behaviours. MBH neurons project towards other regions of the hypothalamus, including the lateral hypothalamic area (LHA), which then relay information through the thalamus, to cortical regions, such as the anterior cingulate and insular cortex (Kampe et al., 2009). Current research is underway to fully elucidate how this complex hypothalamic-cortical circuitry, together with the mesolimbic “reward pathway”, influences feeding (Kampe et al., 2009). Secondly, the hypothalamus regulates glucose homeostasis by relaying information to the autonomic nervous system (ANS). The ANS consists of the sympathetic and parasympathetic nervous systems (PNS). Broadly, the sympathetic nervous system (SNS) is catabolic and promotes glucose production from the liver for immediate uptake by the skeletal muscle. SNS innervation can also activate brown adipose tissue (BAT) to increase thermogenesis and suppress insulin secretion from the pancreas (Thorp & Schlaich, 2015). The sympathetic control of glucose homeostasis involves direct connections from the paraventricular nucleus (PVN) within the hypothalamus to the intermediolateral nucleus (IML) in the spinal cord (Badoer, 2001). Conversely, the PNS provides anabolic signals via the vagus to counteract the SNS to lower endogenous glucose production (Ruud et al., 2017). The dorsal vagal complex (DVC) within the hindbrain mediates the PNS or vagal response (Figure 3). The DVC contains the nucleus of the solitary tract (NTS), which receives information from the vagal afferent nerves, such as the nodose ganglion (NG) in the cervical vertebrae of the spine (Mizuno & Oomura, 1984; Grabauskas, et al., 2010). The vagal afferents
respond to peripheral signals, including glucose, insulin, leptin, glucagon-like peptide-1 (GLP-1), and cholecystokinin (CCK), which collectively signal the nutritional status of the body (Yi et al., 2010). The DVC contains the dorsal motor nucleus of vagus (DMV), which is the initiating point of the efferent vagus nerve (Yi et al., 2010). The efferent vagus nerve primarily suppresses HGP, but also promotes pancreatic insulin secretion and glycogenesis in the muscle and liver (Nelson et al., 1967; Shimazu & Fujimoto, 1971; Matsuhisa et al., 2000). The vagus nerve suppresses HGP by increasing STAT3 signalling in the liver, which downregulates critical gluconeogenic genes, namely phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6pase) (Inoue et al., 2006). Importantly, the DVC, like most neurons, contains the insulin receptors and has its activation mediated through N-Methyl-d-aspartate (NMDA) and K\textsubscript{ATP} channels (Filippi et al., 2012; Lam et al., 2010; Thomzig et al., 2005). Similar to the ARH, the DVC also contains POMC neurons. The acute chemogenic activation of these POMC neurons in the DVC can suppress food intake in mice (Zhan et al., 2013). Vagus nerve stimulation (VNS), in which electrical impulses are applied to the vagus nerve, is a medical therapy that has been used to treat seizures and major depressive disorders (Handforth et al., 1998; Rush et al., 2000). Studies have shown that VNS also improves glucose homeostasis in obese rodents and causes weight loss in humans with depression and obesity (Gil, Bugajski, & Thor, 2011; Pardo et al., 2008). Interestingly, individuals with obesity and diabetes are shown to have impaired vagal nerve activity (Carnethon et al., 2003; Richterm et al., 1996).
In T2D and obesity, the central response to insulin and leptin is compromised leading to disruptions in glucose and energy homeostasis. This central insulin and leptin resistance have been attributed to local inflammation in the hypothalamus following HFD feeding (Souza et al., 2005; Olofsson, et al., 2013). HFD feeding also causes gliosis, the inflammation of glial cells, in the MBH (Thaler et al., 2012). As well, HFD feeding is associated with insulin resistance in the DVC, which is associated with dysregulated food intake and HGP. However, the underlying mechanisms remain unclear (Abraham et al., 2014).
Figure 3: Schema of the vagal control of glucose homeostasis. The dorsal vagal complex (DVC) within the hindbrain contains the nucleus of the solitary tract (NTS), the dorsal motor nucleus of vagus (DMV), and the area postrema (AP). The afferent vagus nerve responds to peripheral state in the gastrointestinal tract and hepatic portal system via glucose, leptin, glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK), and lipids, to signal the metabolic status of the body to the NTS. The DVC then projects to the hypothalamus and to higher cortical structures to regulate satiety and feeding behaviour. These regions project back to the DVC and activate the efferent vagus nerve via the DMV, to regulate glucose homeostasis by suppressing HGP, promoting insulin secretion, and increasing glycogenesis.
1.5 Neuronal PTEN

PTEN is highly expressed in neurons and plays a critical role in brain development and homeostasis (Perandones et al., 2004). In keeping with PTEN’s role as a negative regulator in PI3K-mediated cell growth and proliferation in neurons, humans with PTEN mutations have commonly presented with sporadic brain tumours and macrocephaly (Simpson & Parsons, 2001). Moreover, PTEN mutations have recently been linked to autism and neurodegenerative disorders, including Alzheimer’s Disease (M. G. Butler et al., 2005; Knafo & Esteban, 2016). Studies have uncovered that PTEN controls axonal growth within the neuronal growth cone during neurogenesis. As such, neuronal PTEN deletions in rodents cause an overgrowth of axons, disrupting normal brain development (Jaworski et al., 2005; Kwon et al., 2006). In post-mitotic adult neurons, PTEN is present in many subcellular regions, including the mitochondria and the nucleus, in addition to the dendrite, where it plays a unique role in neuronal plasticity (Kreis et al., 2014).

Neuronal PTEN has also been shown to control metabolism in mouse models (Table 1). Initial studies showed ICV injection of PI3K inhibitors, wortmannin or LY294002, reverse the effects of central insulin in suppression of food intake and weight gain (Niswender et al., 2003). PTEN deletions in various hypothalamic neuron subpopulations have shown different phenotypes in mice, which underscores the heterogeneity and complexity of this brain region. PTEN deletion in leptin-sensitive hypothalamic neurons in mice improved whole-body insulin sensitivity, and promoted the
“beiging” of WAT with increased uncoupling protein 1 (UCP-1) expression (Plum et al., 2007). Conversely, PTEN deletion in either POMC or SF-1 expressing neurons in the hypothalamus led to increased eating (hyperphagia) and obesity in mice (Plum et al., 2006; Klöckener et al., 2012). Interestingly, rat insulin promoter Rip2-cre mice, originally designed to drive gene deletion in pancreatic β-cells, also showed widely expressed cre in varying regions of the brain, including the hypothalamus (Wicksteed et al., 2010). As such, Rip2-cre PTEN\textsuperscript{fl/fl} mice were shown to have increased hypothalamic PI3K signaling, along with increased c-fos expression within the DVC, which suggested increased vagal activation. The HFD-fed Rip-cre PTEN\textsuperscript{fl/fl} mice had improved glucose homeostasis, which was abolished with left vagotomy or mecamylamine, a non-selective, non-competitive nicotinic acetylcholine receptor antagonist (L. Wang et al., 2014). This suggested that the phenotype may be dependent on increased vagal activity. While these data suggest an important role of PTEN in the ANS, direct genetic data are lacking and is the subject of this thesis.
<table>
<thead>
<tr>
<th>Location (cre promoter)</th>
<th>Effect of PTEN deletion on glucose homeostasis and metabolism</th>
<th>Source</th>
</tr>
</thead>
</table>
| POMC expressing neurons in the ARH (POMC Cre) | (↑) PI3K activity  
(↑) Visceral adiposity on HFD  
(↑) Body weight in ♀ on NCD  
(↑) Food intake  
(=) Energy expenditure | (Plum et al., 2006) |
| Leptin-sensitive hypothalamic nuclei (ObRb Cre) | (↑) PI3K activity  
(↑) Peripheral insulin sensitivity  
(↓) Visceral adiposity on NCD  
(↓) Body weight on NCD  
(=) Food intake  
(↑) Energy expenditure  
(↑) UCP-1 activation in WAT | (Plum et al., 2007) |
| SF-1 expressing neurons in the VMH (SF-1 Cre) | (↑) PI3K activity  
(=) Adiposity on HFD  
(↑) Body weight on NCD  
(↑) Food intake | (Klöckener et al., 2012) |
| Rip2 expressing neurons in the hypothalamus (Rip2 Cre) | (↑) PI3K activity  
(↑) Peripheral insulin sensitivity  
(↑) Visceral adiposity on HFD  
(↑) Subcutaneous adiposity on HFD  
(↑) Body weight on HFD  
(↑) Energy expenditure  
(↑) Vagal outflow to the spleen | (L. Wang et al., 2014) |

**Table 1:** The impact of PTEN deletion in various neuronal populations on glucose and energy homeostasis in different transgenic mouse models.  
(↑) – Increase, (↓) – Decrease, (=) – No change.
1.6 Cholinergic Neurons

Acetylcholine is a major neurotransmitter with many functions, including facilitating learning and memory in the central nervous system (CNS), controlling involuntary bodily functions in the ANS and triggering skeletal muscle contractions at the neuromuscular junction (Picciotto et al., 2012). The enzyme choline acetyltransferase (ChAT) synthesizes acetylcholine from choline which is translocated into the neuron by the high-affinity sodium coupled choline transporter (CHT; Vizi et al., 2008; Figure 4). Although ChAT is required for acetylcholine synthesis, its rate limiting step is the uptake of the choline precursor by CHT (Dobransky & Rylett, 2005). Choline that is used in acetylcholine synthesis is primarily derived from phosphatidylcholine, a component of the cell membrane, or from previously hydrolyzed acetylcholine (Ulus et al., 1989). ChAT enzymatically adds an acetyl group to choline from acetyl-CoA that originates from the mitochondria (Vizi et al., 2008). As such, neurons that utilize acetylcholine in their synapses and highly express ChAT are considered to be cholinergic (Hedrick et al., 2016). Active ChAT is mostly found in the cytoplasm of the neuron, although there are traces of ChAT bound to neuronal membranes and the nucleus (Benishin & Carroll, 1981). ChAT mRNA expression begins during development in different regions of the CNS at varying times. In rodents, ChAT transcription in the spinal cord is evident at embryonic day (E)13 and in the brain at E17 (Ibanez et al., 1991). ChAT activity increases after birth, into adulthood, when cholinergic pathways to the cortex,
hippocampus and striatum develop rapidly (Aznavour et al., 2005; Mechawar & Descarries, 2001; Van Vulpen & Van Der Kooy, 1996).

Vesicular acetylcholine transporter (VACHT or VAT) loads acetylcholine to its vesicles, preparing it for release into the synaptic cleft (Prado et al., 2013). VACHT exchanges two luminal protons for each ACh molecule and has also been proposed to be a rate-limiting step for acetylcholine release (Nguyen et al., 1998; Prado et al., 2013). Interestingly, VACHT and CHAT are both in a common locus, referred to as “the cholinergic gene locus”, often resulting in co-expression of these two genes (Weihe et al., 1998). Once released, acetylcholine binds to nicotinic receptors or muscarinic receptors to exert its effects (Picciotto et al., 2012). Nicotinic receptors (nAChRs) are non-selective, fast-acting excitatory cation channels, which are made up of an alpha (α2-9) and beta (β2-β4) subunits (Role & Berg, 1996). The main subtypes of nAChRs in the brain are α4β2 and α7, while α3β4 is found in the PNS (Role & Berg, 1996). These receptors respond to many agonists, including nicotine. Interestingly, nicotine administration to genetically diabetic (db/db) mice improved glucose homeostasis, decreased body weight, and suppressed inflammation cytokine production (X. Wang et al., 2014). Unlike nAChRs, muscarinic receptors (mAChRs) are coupled to G-proteins and can be both excitatory (M2 and M4) and inhibitory (M1, M3, and M5, Kruse, et al., 2014). Interestingly, M3 mAChRs are shown to be essential for vagal stimulation of insulin secretion in the pancreatic β-cells (Gautam et al., 2006). Acetylcholine levels are highly regulated as it is rapidly metabolised to choline and acetate by acetylcholinesterase
(AchE; Vizi et al., 2008). Cholinesterase inhibitors, which reversibly prevent AchE-mediated breakdown of acetylcholine, have been developed for the treatment of myasthenia gravis and Alzheimer’s disease (Knapp et al., 1994; Mantegazza et al., 2011).
Figure 4: Schema of acetylcholine synthesis, degradation, and function. Choline is transported into the neuron by choline transporter (CHT). Choline acetyltransferase (ChAT) then adds an acetyl group to the choline. Next, the acetylcholine is loaded into its vesicle by the vesicular acetylcholine transporter (VAT or VAChT). It is then released at the synapse in a calcium-dependent manner and binds to either pre or post-synaptic receptors. AChE rapidly hydrolyzes acetylcholine to choline and acetate.

Acetylcholine is released mostly in the CNS, ANS, and neuromuscular junctions. In the CNS, cholinergic projections mainly stem from the basal forebrain, the brainstem and local interneurons (Figure 5; Vizi, 2008). The basal forebrain is adjacent to the striatum and innervates many brain regions, including the cortex, hippocampus, olfactory bulb, and amygdala via specific cholinergic pathways Ch1-8 (Mufson & Levey, 1983). The cholinergic basal forebrain system contains the diagonal band of Broca (vDM), nucleus basalis of Meynert (NBM), substantia innominate (SI) and the medial septum (MS); and the cholinergic brainstem system contains the laterodorsal tegmental (LDT) and pedunculopontine tegmental nuclei (PPT), in addition to the DVC (Paul et al., 2015). The DMV of the DVC consists of more than 95 percent ChAT neurons (Armstrong et al., 1990). The cholinergic brainstem system projects to hypothalamus and thalamus as well.

Both the PNS and SNS use acetylcholine in their preganglionic neurons. However, only the postganglionic neurons of the PNS use acetylcholine, whereas the SNS utilize catecholamines, either norepinephrine or epinephrine. While the CNS contains the majority of cholinergic structures, other non-neuronal cells types, including epithelial, endothelial and immune cells can also release acetylcholine during certain conditions and mediate auto- and paracrine functions (Wessler & Kirkpatrick, 2008). For example, in response to hypothermia, acetylcholine is produced by endothelial cells and binds to M1 mAChRs to produce nitric oxide and mediate vasodilation (Evora et al., 2007; Wessler & Kirkpatrick, 2008). Furthermore, activated T lymphocytes can also be induced to express ChAT and release acetylcholine (Fujii et al., 1998).
lymphocytes can produce acetylcholine to restrict neutrophil recruitment, independent of the vagus nerve (Reardon et al., 2013). Trace amounts of acetylcholine are found in circulation, which are thought to originate from ChAT expressing endothelial cells and lymphocytes (Kawashima et al., 1997).
Figure 5: Major cholinergic structures within the mouse brain. The basal forebrain cholinergic system contains the diagonal band of Broca (vDB), nucleus basalis of Meynert (NBM), substantia innominata (SI), and the medial septum (MS). The brainstem cholinergic system contains the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei, in addition to the dorsal vagal complex (DVC). The two main cholinergic systems project to other regions of the brain including the cortex, hypothalamus, thalamus, and hippocampus.

Chapter 2 – Objectives and Hypothesis
2.1 Objectives and Hypothesis

Our objective is to determine the role of PTEN in cholinergic neurons in the regulation of glucose and energy homeostasis in mice using a ChAT-PTEN mouse model. We hypothesize that PTEN deletion in cholinergic neurons will improve glucose homeostasis with less weight gain in mice fed a HFD, thereby providing protection against T2D.
Chapter 3 – Materials & Methods
3.1 Mice

Cholinergic neuron-specific PTEN-deficient mice were generated by breeding *ChAT(IRES)-Cre*+ mice (*Cre* recombinase fused with an optimized internal ribosome entry sequence, IRES, inserted downstream of the ChAT stop codon, Jackson Laboratory, Bar Harbor, ME, USA, stock #006410) with mice harbouring exons 4 and 5 of *PTEN* gene flanked by loxP sites by homologous recombination (*PTEN*fl/fl, Stambolic et al., 1998). Their offsprings, *ChAT (IRES)-Cre*+-*PTEN*+/fl, were interbred to generate *ChAT (IRES)-Cre*+-*PTEN*+/+ control (herein referred to as *ChATcre*+-*PTEN*+/+ mice) and *ChAT (IRES)-Cre*+-*PTEN*fl/fl knockout (herein referred to as *ChATcre*+-*PTEN*fl/fl) littermates. Vagal- and hindbrain-specific PTEN-deficient mice were generated by breeding mice that expressed *cre* recombinase under paired-like homeobox 2b (Phox2b) promoter (Jackson Laboratory, Bar Harbor, ME, USA, stock #016223) with *PTEN*fl/fl mice. Similarly, these *Phox2b-Cre*+-*PTEN*+/fl were interbred to generate *Phox2b-Cre*+-*PTEN*+/+ wildtype and *Phox2b-cre*+-*PTEN*fl/fl knockout littermates. To obtain imaging for *cre*-expression, *ChATcre*+-*PTEN*fl/fl and *ChATcre*+-*PTEN*+/+ mice were bred with mice harbouring a ROSA26 reporter, which expresses β-galactosidase in *cre*-expressing cells (Jackson Laboratory, Bar Harbor, ME, USA, stock #003309). Mice were housed in a pathogen-free facility at the Toronto Medical Discovery Tower (Toronto, ON, Canada) with a 12 h light–dark cycle and free access to water and standard irradiated rodent chow (5% energy from fat; Harlan Teklad). Both male and female experimental groups were generated and littermates were used for controls. Animal protocols were approved and
performed in accordance with regulations established by the Toronto General Hospital Research Institute Animal Care Committee.

Prior to weaning, ear clips were obtained from pups and were digested overnight in 47µL ear buffer (25 mM Tris pH 7.5; 50 mM EDTA, 1% SDS) and 3µL of 0.5mg/mL proteinase K (Merck, Kenilworth, NJ, USA) in a 55°C water bath. DNA digest was then diluted by adding 950µL of deionized water. For cre genotyping, the forward 5’-GGC-AGT-AAA-AAC-TAT-CCA-GCA-’3 and reverse 5’-GTT-ATA-AGC-AAT-CCC-CAG-AAA-TG-’3 primers were used. The melting temperature was 95°C for 5 minutes, the annealing temperature was 60°C for 30 seconds, and the primer extension phase was 72°C for 45 seconds, for 35 cycles. The final PCR product is 280 bp. For PTEN genotyping, the forward 5’-CTC-CTC-TAC-TCC-ATT-CTT-CCC-’3 and reverse 5’- GTT-ATA-AGC-AAT-CCC-CAG-AAA-TG-3’ primers were used. The melting temperature was 94°C for 5 minutes, the annealing temperature was 62°C for 1 minute, and the primer extension phase was 72°C for 1 minute, for 40 cycles. The final PCR product is 228 bp for the wildtype allele and 350 bp for the mutant allele. For Rosa26 genotyping, the mutant reverse 5’-GCG-AAG-AGT-TTG-TCC-TCA-ACC-3’, common 5’-AAA-GTC-GCT-CTG-AGT-TGT-TAT-3’, and reverse 5’-GGA-GCG-GGA-GAA-ATG-GAT-ATG-’3 primers were used. The melting temperature was 94°C for 3.5 minutes, the annealing temperature was 65°C for 1 minute, and the primer extension phase was 72°C for 1 minute, for 30 cycles. The final PCR product is 350 bp for the mutant allele, and
~650 bp for the wildtype allele. All the primers were purchased from Integrative DNA Technologies, Skokie, IL, USA.

After 8 weeks of normal chow diet (NCD, 5% fat), random cohorts of mice were given a high fat diet (HFD; 60% fat, 24% carbohydrates and 16% protein based on caloric content; F3282; Bio-Serv, Flemington, NJ, USA) and for 12 weeks.

3.2 Immunofluorescent Staining

Mice expressing the ROSA26 (β-galactosidase) reporter were used for immunofluorescent staining. These mice were anesthetized and transcardially perfused with phosphate buffered saline (PBS), then by freshly made 4% paraformaldehyde, followed by extraction of the whole brain which were gently removed and cryoprotected in a 25% sucrose gradient overnight. Brains were then cut on a 1mm adult coronal brain matrix (Kent Scientific, Torrington, CT, USA) and snap frozen in optimal cutting temperature compound (OCT, Tissue-Tek, Torrance, CA, USA) in liquid nitrogen. A Cryostar NX70 sliding frozen microtome (Thermo Fischer Scientific, Waltham, MA, USA) was used to cut 8µM thick hindbrain and forebrain sections.

Prior to immunofluorescent staining, a light antigen retrieval was done in a ~95°C sodium citrate dehydrate buffer for 10 minutes. Slides were then washed in TBSTriton (20 mM Tris-Cl, 136 mM NaCl with 0.025% Triton X-100, Thermo Fischer Scientific) for 15 minutes. Sections were incubated in 10% donkey serum (Cell Signaling Technology, Beverly, MA, USA) diluted in TBSTriton for 1 hour at room temperature
Next, slides were immunostained with primary antibodies for β-galactosidase (1:250, Abcam), phospho-S6 (1:250, Cell Signaling Technology), or c-fos (1:250, Santa Cruz Technology, Santa Cruz, CA, USA) overnight at 4°C. Slides were washed in TBST for 15 minutes. Sections were then incubated with fluorescein isothiocyanate (FITC) and cyanine 3 (Cy3) secondary antibodies, for 35 minutes away from the light. Slides were then washed in TBS for 15 minutes and cover slips added using a DAPI mounting medium. Slides were then imaged on a Zeiss AxioObserver inverted fluorescent microscope with ZEN software (ZEISS, Oberkochen, Germany). The number of β-gal and pS6 expressing neurons in the DVC were counted to assess the percentage of β-gal cholinergic neurons co-expressed pS6 using ~4 fields using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3.3 In Vivo Metabolic Analyses

Body weights were measured at 8 weeks of age (baseline) and 12 weeks later on a NCD or a HFD. Blood glucose were measured using an Accu-Chek® Aviva glucometer (Roche Laboratories, Indianapolis, USA) in venous tail blood. Following an overnight fast of 16 hours, glucose tolerance tests were done, with glucose at a dose of 1.0 g/kg, body weight injected intraperitoneally and blood glucose measured at 0, 15, 30, 45, 60, and 120 minutes after injection. Insulin tolerance tests were performed on mice fasted for 4 hours using Humalog insulin (Eli Lilly and Company, Indianapolis, IN, USA) at a dose of 0.50 units per kg body weight injected intraperitoneally and blood glucose measured at 0, 15, 30, 45, 60, and 120 minutes after injection. Metabolic caging, Comprehensive Lab
Animal Monitoring System (CLAMS, Columbus instruments, Columbus, OH, USA) was used to assess metabolic parameters, including food and water intake, energy expenditure, oxygen consumption (VO\textsubscript{2}), carbon dioxide production (VCO\textsubscript{2}), respiratory exchange rate (RER), and locomotion in the X and Z planes. The mice were given 24 hrs to acclimate to the apparatus and then had data measurements collected for 24 hours. The light cycle was from 6 am to 6 pm and the dark cycle was from 6 pm to 6 am the following day. Mice were weighed prior to metabolic caging. Analysis was undertaken by CLAX software, and with parameters normalized to body weights.

### 3.4 Histology and Immunohistochemistry

Upon sacrificing, epididymal white adipose tissue (eWAT), subcutaneous white adipose tissue (sWAT), brown fat (BAT), liver, and pancreas were fixed in 4% paraformaldehyde in PBS, then processed into paraffin blocks. The blocks were sectioned onto slides at 150µM intervals by the Pathology Research Program (PRP) at the University Health Network, Toronto, ON. These sections were hematoxylin and eosin (H&E) stained to assess general morphology. For H&E staining, sections were deparaffinised in xylene twice for 5 min, then rehydrated in decreasing series of ethanol of 100%, 95%, 90% and 70% for 4 min each followed by running tap water bath for 30 seconds. Next, sections were stained with hematoxylin (Sigma Aldrich, St. Louis, MO, USA), then placed for 30 seconds in 70% ethanol with 1% hydrochloric acid. Sections were then rinsed in a running tap water bath for 15 seconds then placed in eosin (Sigma-
Aldrich) for 2 min and rinsed with tap water for 40 s. Sections were dehydrated in an increasing series of ethanol of 70%, 90%, 95% and 100% ethanol for 4 min each then xylene twice for 5 min. Finally, coverslips were added to the slides using xylene-based SHUR/Mount (VWR, Radnor, PA, USA). To visualize tissue morphology and to assess adipocyte areas and diameters, H&E sections of ~10 fields at 200x were captured with CellSens software (Olympus, Tokyo, Japan). Oil red O staining was performed by PRP on sections from flash frozen fresh livers in OCT (Tissue-Tek).

Immunohistochemistry for F4/80 was done to visualize macrophage infiltration in eWAT. eWAT sections were deparaffinized and rehydrated as mentioned above, then placed in a hydrogen peroxide (H₂O₂, 30% vol/vol in water) block for 10 minutes, then rinsed in water twice for 5 minutes and PBS with 0.1% Tween-20 (PBST) for another 5 minutes. An antigen retrieval was performed in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween-20, pH 9.0) in a pressure cooker. Slides were then left in water for 10 minutes and washed in PBST 3 times. Blocking was done using Avidin/Biotin Blocking Kit as per manufacturer protocol (Invitrogen, Carlsbad, CA, USA). Following a wash in PBST, the samples were blocked in 5% goat serum in PBST for 30 minutes and then incubated in an F4/80 antibody (1:200 dilution, Santa Cruz Biotechnology) for 2 hours at RT. F4/80 was detected using goat anti-rat IgG-biotinylated (Santa Cruz Biotechnology) at a 1:200 dilution for 30 minutes at RT followed by 3 rinses with PBST. The secondary antibody was detected with streptavidin horseradish peroxidase (HRP, Vector labs, Burlingame, CA, USA) for 30 min at RT. After a subsequent rinse in PBST 3 times,
colour development was done using Vector NovaRED Peroxidase Substrate Kit (Vector labs) as per protocol. Next, the slides were rinsed in water 3 times and stained in hematoxylin for 30 seconds, rinsed again in water once and then placed in 0.2% HCl in water, rinsed in water and dipped in PBS. Sections were then dehydrated as mentioned above and had coverslips added using SHUR/Mount (VWR). Histological and immunohistochemical imaging was done using a Leica DM1000 microscope (Wetzlar, Germany) with Olympus DP72 camera. IHC for F4/80 sections were analyzed for crown like structures in ~10 fields at 100X.

3.5 Liver Triglyceride Measurement

50 mg of liver was homogenized in 700µL of lysis buffer consisting of 1% nonidet-p40 (NP-40) dissolved in water and a protease inhibitor cocktail (Roche Laboratories). Liver triglyceride concentration was then assessed using a Triglyceride Colormetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) as per manufacturer’s instructions.

3.6 Western Blots

Tissue lysates for Western blots were made by mechanically homogenizing tissue in cold lysis buffer, consisting of RIPA buffer (Sigma-Aldrich) and a protease inhibitor cocktail (Roche Laboratories). Protein concentrations were then measured using a Bradford protein assay (Biorad Laboratories, Hercules, CA, USA) with a microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). 30µg of total protein,
together with Laemmli buffer (Biorad Laboratories) was prepared for loading. The proteins were run with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a running buffer solution (5 mM tris base, 192 mM glycine and 0.1% SDS). Proteins were then transferred to a methanol-hydrated polyvinylidene fluoride (PVDF) membrane using a Trans-Blot Turbo Transfer System (Biorad Laboratories, CA, USA). Membranes were then blocked at RT in 5% non-fat dry milk diluted in tris-buffered saline solution (TBST, 20 mM Tris-Cl, 136 mM NaCl with 0.1% Tween-20) for one hour. Membranes were then washed three times with TBST for fifteen minutes, then incubated overnight at 4°C with primary antibodies, PTEN (1:1000 dilution, Abcam, Cambridge, UK), phospho-STAT3 (Y705, 1:1000), total STAT3 (1:1000) phospho-AKT (S473; 1:1000), total AKT (1:1000), and GAPDH (1:5000). All primary antibodies for Western blots, except for PTEN, were purchased from Cell Signaling Technology (Danvers, MA, USA). After overnight incubation, membranes were washed three times with TBST for fifteen minutes then incubated in HRP-conjugated polyclonal goat anti-rabbit antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% non-fat dry milk diluted in TBST at RT. Membranes were then washed in TBST for 15 minutes and then incubated in chemiluminescent ECL-plus reagent (PerkinElmer Inc., Waltham, MA, USA) at RT and imaged using MicroChemi 4.2 (DNR Bio-Imaging Systems, Mahale HaHamisha, Jerusalem, Israel). Western blot signals were quantified using ImageJ software. Protein levels were normalized to GAPDH and expressed relative to littermate control levels.
3.7 Reverse Transcriptase Quantitative PCR

RNA was extracted from tissue with TRIzol reagent (Invitrogen). Tissue was lysed in 1 mL of Trizol by a model 150VT ultrasonic homogenizer (BioLogics, Manassas, VA, USA) and then incubated at RT for 5 minutes. The lysate was then combined with 200 μL chloroform. Samples were then manually shaken for 15 seconds and incubated at RT for 3 minutes. Following this, samples were centrifuged at 11,900 g for 10 minutes at 4°C. The upper clear layer was then moved and combined with 500 μL isopropanol, and stored in the -20°C freezer overnight. Samples were centrifuged at 11,900 g for 10 minutes at 4°C. Subsequently, the supernatant was removed from the tube, with the pellet remaining. The pellet was then washed twice with 1mL of cold 75% ethanol, then dried. The pellet was then dissolved in DEPC-treated water. RNA concentrations were measured in a microplate spectrophotometer with a Take3 Micro-Volume Plate (BioTek Instruments). 1µg of RNA was used to prepare cDNA using an M-MLV Reverse Transcriptase kit (Thermo Fisher) as per manufacturer’s instructions. Each sample was run in triplicates of 10 μL, including 1µL of the forward primer, 1µL of the reverse primer, 5µL of SYBR green mixture (Applied Biosystems, Carlsbad, CA, USA), and 3µL of the sample using standard conditions in a 7900HT Fast-Real-Time PCR System (Applied Biosystems) on a 384 well plate. Prior to qPCR, the plate was centrifuged at 3000rpm for 2 minutes. Transcription levels were normalized against β-actin and expressed in arbitrary units. The qPCR primers used are listed in Table 2 and were ordered from Eurofins (Louisville, KY, USA), formerly MWG-operon.
Table 2: Primer sequences for Real Time qPCR.

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<th>Reverse(5’ – 3’)</th>
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<tr>
<td>Ucp-1</td>
<td>ACTGCCACACCTCCAGTCATT</td>
<td>TTTGCGCTCACTCAGGATTGG</td>
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</table>

3.8 Plasma Insulin Measurement

Plasma insulin measurements were measured using an ultrasensitive mouse insulin ELISA kit (Elk Grove Village, IL, USA).
3.9 Statistics

All data are presented as mean ± standard errors of the mean (S.E.M.). Two-tailed independent-sample Student’s t-tests and two-way ANOVA with Sidak’s multiple comparisons test using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) were performed. Statistical significance was defined as p-value < 0.05. *p< 0.05; **p< 0.01.
Chapter 4 – Results
4.1 ChATcre\textsuperscript{+}-PTEN\textsuperscript{fl/fl} Mice

In order to study the role of PTEN in cholinergic neurons, we generated mice with \textit{Pten} deletion in their ChAT expressing neurons, \textit{ChATcre\textsuperscript{+}-PTEN\textsuperscript{fl/fl}}, and \textit{ChATcre\textsuperscript{+}-PTEN\textsuperscript{+/+}} control littermates (Figure 6A—B). Mice were genotyped for their \textit{cre} and \textit{PTEN} alleles (Figure 6C) and weaned at ~21 days of age (Luk et al., 2016). At 8 weeks of age, mice were randomly assigned to a NCD or HFD for 12 weeks. \textit{ChATcre\textsuperscript{+}-PTEN\textsuperscript{fl/fl}} were born in Mendelian ratio and appeared healthy, with minor fur loss.
Figure 6: (A—B) Generation of ChATcre \textsuperscript{+/-} -PTEN\textsuperscript{0/0} mice using cre-loxP recombination system. (C) PCR genotyping for cre (left), and PTEN\textsuperscript{+/+}, PTEN\textsuperscript{+/-}, and PTEN\textsuperscript{0/0} (right). Representative PCR genotyping from ear clip DNA samples shows cre\textsuperscript{+} (280bp), and the heterozygous (PTEN\textsuperscript{+/-} ~228 and 350 bp), wildtype, (PTEN\textsuperscript{+/-} ~228 bp) and knockout (PTEN\textsuperscript{0/0} ~350bp), figure adapted from Luk et al., 2016.
4.2 ChATcre\(^{+}\)-PTEN\(^{0/\text{fl}}\) mice have enhanced PI3K signaling in their DVC cholinergic neurons

In order to identify cholinergic neurons, we crossed our ChATcre\(^{+}\)-PTEN\(^{0/\text{fl}}\) and ChATcre\(^{+}\)-PTEN\(^{+/+}\), with mice expressing the ROSA26 reporter. (Figure 7A-B). These mice express β-galactosidase (β-gal) under the control of cre promoter, allowing for the identification and visualization of ChATcre\(^{+}\) neurons. Furthermore, our immunofluorescent staining demonstrated strong β-gal expression in DVC neurons. This finding is similar to a previous study that used a GFP reporter, and showed that the same ChATcre\(^{+}\) mice have cre expression within DVC neurons of the hindbrain (Rossi et al., 2011). There was not any visible β-gal staining in other non-cholinergic regions, including the hippocampus and cortex. Our immunofluorescent imaging demonstrated greater colocalization of pS6 to β-gal expressing cholinergic neurons in the DVC of the hindbrain of ChATcre\(^{+}\)-PTEN\(^{0/\text{fl}}\) mice when compared to their controls (Figure 7C). As pS6 is downstream of PI3K, its expression is indicative of enhanced PI3K signaling in response to PTEN deletion. Thus, these results suggest that PTEN has been deleted within the β-gal expressing cholinergic neurons of the DVC. We next wanted to ensure that PTEN deletion was specific to neurons. Indeed, western blots of non-neuronal tissues including eWAT and liver did not show differences in PTEN protein levels when comparing ChATcre\(^{+}\)-PTEN\(^{0/\text{fl}}\) and their littermate controls (Figure 7D-E).
Figure 7: Immunofluorescent staining of the hindbrain with anti-β-gal indicating ChAT-cre+ expressing neurons, pS6 and merged (Scale 100µM). (B) Anatomy of hindbrain: Fourth ventricle (4V), nucleus tractus solitarius (NTS), dorsal motor nucleus of vagus (DMV), twelfth cranial nerve (12N). (C) Percentage of β-gal expressing neurons in the DVC which co-express pS6, for ChATcre+ -PTENfl/fl (n=3) and ChATcre+ -PTEN+/+ controls (n=4), unpaired t test, Western blot for PTEN of protein lysates of (D) eWAT and (E) liver from HFD-fed ChATcre+ -PTENfl/fl mice and ChATcre+ -PTEN+/+ controls (top panel) with quantification of the fold change over control (bottom panel), unpaired t tests, n=4 per group. Mean±SEM; *p<0.05, **p<0.01
4.3 HFD-fed ChATcre\textsuperscript{+}-PTEN\textsuperscript{fl/fl} mice have improved glucose and insulin tolerance

To assess whole-body glucose homeostasis, we performed glucose and insulin tolerance tests on mice on NCD or HFD starting at eight weeks of age. No difference in glucose or insulin tolerance was seen between the ChATcre\textsuperscript{+}-PTEN\textsuperscript{fl/fl} and their controls at baseline, 4, 8 or 12 weeks on NCD in males and females (data not shown). However, both male and female ChATcre\textsuperscript{+}-PTEN\textsuperscript{fl/fl} mice on 12 weeks of HFD demonstrated improved glucose and insulin tolerance compared to controls with varying significance (Figure 8A-B). In addition, HFD-fed ChATcre\textsuperscript{+}-PTEN\textsuperscript{fl/fl} had trending lower plasma insulin levels compared to controls, which further suggests their role in the regulation of insulin sensitivity (Figure 8C). Overall, this indicates that PTEN in cholinergic neurons play an important role in the regulation of glucose homeostasis in response to a HFD challenge.
Figure 8: Glucose (top) and insulin (bottom) tolerance tests of HFD-fed (A) female and (B) male ChATcre\(^+\)-PTEN\(^{+/+}\) (n=6 for females and n=8 for males) mice and ChATcre\(^+\)-PTEN\(^{+/+}\) controls (n=10 for females and n=12 for males) and respective area under the curve (AUC), two-way ANOVA. (C) Plasma insulin levels after 12 weeks of HFD, unpaired \(t\) test, Mean±SEM, * \(p<0.05\), ** \(p<0.01\).
4.4 *ChATcre*<sup>+</sup>*-PTEN<sup>fl/fl</sup> mice were protected against HFD-induced weight gain with decreased adiposity*

*ChATcre*<sup>+</sup>*-PTEN<sup>fl/fl</sup> mice on NCD had similar weight compared to controls at both 8 and 20 weeks of age (data not shown). However, female *ChATcre*<sup>+</sup>*-PTEN<sup>fl/fl</sup> mice gained significantly less body weight than their controls after twelve weeks of HFD (Figure 9A) with no significant difference in males (Figure 9B). Body composition analysis showed that *ChATcre*<sup>+</sup>*-PTEN<sup>fl/fl</sup> and their controls on HFD had similar weights of most organs (Figure 9C) with the exception of adipose tissue, whereby *ChATcre*<sup>+</sup>*-PTEN<sup>fl/fl</sup> mice had less percent epididymal WAT (eWAT) weight compared to their controls more significantly in females (Figure 9D). Additionally, *ChATcre*<sup>+</sup>*-PTEN<sup>fl/fl</sup> mice on HFD had trending less percent weights of subcutaneous fat depots in both males and females (Figure 9D).
Figure 9: Body weights of female (A) and male (B) $\text{ChATcre}^+\text{-PTEN}^{+/+}$ mice (n=6 for females and n=8 for males) and $\text{ChATcre}^+\text{-PTEN}^{+/+}$ controls (n=10 for females and n=12 for males) at baseline and after 12 weeks of HFD feeding and (C) organ weights and (D) fat depot weights of mice, unpaired t tests, mice and their controls, Mean±SEM; * p<0.05
4.5 Decrease in eWAT adipocyte size and increased adipogenesis in HFD-fed ChATcre⁺-PTEN⁺/⁻ mice

We next assessed the morphology of eWAT adipocytes of ChATcre⁺-PTEN⁺/⁻ and control mice on HFD. We found that ChATcre⁺-PTEN⁺/⁻ mice had smaller adipocytes in diameter and area compared to their controls (Figure 10A—C). To further understand the cellular processes potentially responsible for these findings, we measured the gene expression of adipogenesis markers in eWAT (Figure 10D). Quantitative RT-PCR analysis demonstrated a trending increase in adipogenic markers, adiponectin and peroxisome proliferator-activated receptor gamma (PPARγ). In keeping with decreased fat mass, HFD-fed ChATcre⁺-PTEN⁺/⁻ mice had a significantly decreased expression of leptin mRNA levels than their wildtype controls. There were also significant alterations in some lipogenesis (fatty acid synthase, FasN) and lipolysis (adipose triglyceride lipase, ATGL) markers in ChATcre⁺-PTEN⁺/⁻ mice, likely reflective of dynamic changes in the fat tissue.

Previous studies have shown that 8 weeks of HFD causes an increase in BAT adipocyte size (Swift et al., 2017). We therefore also examined the histology of BAT in ChATcre⁺-PTEN⁺/⁻ and control mice using H&E staining (Figure 9A). HFD-fed ChATcre⁺-PTEN⁺/⁻ mice had BAT with smaller adipocytes than their controls (data not shown). This attenuation in the enlargement of adipocytes in BAT of ChATcre⁺-PTEN⁺/⁻ suggests that the PTEN deletion in cholinergic neurons may also affect BAT adipocyte size. Despite the differences in adipocyte size, UCP-1 gene expression levels in BAT of
HFD-fed $ChATcre^{+}$-$PTEN^{fl/fl}$ and their wildtype controls were similar (Figure 9E). Interestingly, $UCP-1$ gene expression in eWAT was trending higher in HFD-fed $ChATcre^{+}$-$PTEN^{fl/fl}$ mice, suggesting increased “beiging” of eWAT in these mice.
Figure 10: (A) Representative H&E stained image of eWAT sections for HFD-fed ChATcre+\textsuperscript{*}-PTEN\textsuperscript{fl/fl} mice and ChATcre+\textsuperscript{*}-PTEN\textsuperscript{+/+} controls (Scale: 100µm), (B) quantification and (C) distribution of eWAT adipocyte size, unpaired t tests, n=5 per group. (D) q-RT-PCR analysis of eWAT mRNA for adipogenesis, lipogenesis and lipolysis markers for HFD-fed ChATcre+\textsuperscript{*}-PTEN\textsuperscript{fl/fl} mice (n=6) and ChATcre+\textsuperscript{*}-PTEN\textsuperscript{+/+} controls (n=9), unpaired t tests. (E) q-RT-PCR analysis of eWAT and BAT for UCP-1 mRNA, for HFD-fed ChATcre+\textsuperscript{*}-PTEN\textsuperscript{fl/fl} mice (n=5) and ChATcre+\textsuperscript{*}-PTEN\textsuperscript{+/+} controls (n=8), unpaired t tests. Data are obtained from males and females combined. Mean±SEM; * p<0.05, p<0.01
4.6 Less macrophages in eWAT of HFD-fed ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) mice

We next assessed for resident adipose tissue macrophages (ATMs), as they have been implicated in insulin resistance and obesity (Lumeng, et al., 2007). Immunohistochemistry for F4/80, a macrophage marker, showed that HFD-fed ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) mice have a trending decrease in the amount of crown-like structures (CLS) in their eWAT (Figure 11A-B). CLS are known to represent macrophages that have clustered around dead adipocytes, a phenomenon that may occur during obesity (Murano et al., 2008). Furthermore, quantitative RT-PCR of eWAT mRNA showed a decrease in F4/80 gene expression in the HFD-fed ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) compared to controls, further suggestive of less macrophage infiltration (Figure 11C). Moreover, eWAT of HFD-fed ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) demonstrated less gene expression of some M1 pro-inflammatory (inducible nitric oxide synthase – iNOS) as well as some M2 anti-inflammatory (arginase 1 – Arg1 and mannose receptor C type 2 – Mrc2) markers (Figure 11C). Together, the decrease in mRNA expression of these markers suggests lower eWAT inflammation and less macrophage infiltration in the ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) mice (Figure 11C). We next wanted to assess for insulin signaling within the eWAT that may be implicated in the changes in glucose and insulin tolerance. Western blots did not reveal any changes in the basal levels of p-AKT protein in the eWAT of HFD-fed ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) mice compared to controls (Figure 10D).
Figure 11: (A) F4/80 immunostaining in eWAT of HFD-fed ChATcre<sup>-</sup>-PTEN<sup>fl/fl</sup> mice and ChATcre<sup>-</sup>-PTEN<sup>+/+</sup> controls (Scale: 100µm) and (B) quantification of crown-like structures (CLS) per field, unpaired t test, n=4 per group. (C) qRT-PCR analysis of eWAT mRNA for macrophage and inflammation markers, for HFD-fed ChATcre<sup>-</sup>-PTEN<sup>fl/fl</sup> mice (n=6) and ChATcre<sup>-</sup>-PTEN<sup>+/+</sup> controls (n=9), unpaired t tests. (D) Western blot of eWAT lysates for basal p- and total (t)-AKT (top panel) and quantification (bottom panel), unpaired t test. Data were obtained from males and females combined. Mean±SEM; *p<0.05, ** p<0.01.
4.7 Decreased fat deposition in livers of HFD-fed ChATcre<sup>+</sup>-PTEN<sup>-/-</sup> mice

We next assessed for fat deposition in liver by H&E staining which showed that HFD-fed ChATcre<sup>+</sup>-PTEN<sup>-/-</sup> mice have less lipid droplets than their controls, suggestive of less fatty liver or hepatosteatosis (Figure 12A). Oil Red O staining of liver sections also qualitatively showed HFD-fed ChATcre<sup>+</sup>-PTEN<sup>-/-</sup> to have less hepatosteatosis than their controls (Figure 12A). Moreover, triglyceride concentration measurements showed ChATcre<sup>+</sup>-PTEN<sup>-/-</sup> mice to have a trending decrease in their livers compared to controls (Figure 12B). Next, we assessed for the inflammatory state of the liver. Quantitative RT-PCR analysis of liver mRNA demonstrated trending decreases in pro-inflammatory cytokine genes, including tumor necrosis factor alpha (Tnfa), inducible nitric oxide synthase (iNOS), and interleukin-1 beta (IL1β) in HFD-fed ChATcre<sup>+</sup>-PTEN<sup>-/-</sup> mice compared to their controls (Figure 12C). We next assessed for insulin signaling by western blot of liver lysates which showed no change in basal p- or total AKT in our HFD-fed ChATcre<sup>+</sup>-PTEN<sup>-/-</sup> mice compared to controls (Figure 12D). Given that vagal activation can suppress HGP via STAT3 signaling (Inoue et al., 2006) through α7 nAChRs (Marrero et al., 2010), we next assessed for p- and total STAT3 by western blot of liver lysates, which showed no difference between ChATcre<sup>+</sup>-PTEN<sup>-/-</sup> and control mice (Figure 12E).
Figure 12: (A) Representative H&E Staining (top) and Oil Red O staining (bottom) of livers of HFD-fed ChATcre$^{+}$-PTEN$^{+/+}$ and ChATcre$^{+}$-PTEN$^{+/+}$ controls (Scale: 100µm). (B) Quantification of liver triglyceride concentration for HFD-fed ChATcre$^{+}$-PTEN$^{+/+}$ (n=5) and ChATcre$^{+}$-PTEN$^{+/+}$ controls (n=8), unpaired t test. (C) qPCR for macrophage and inflammation markers in the liver for HFD-fed ChATcre$^{+}$-PTEN$^{+/+}$ (n=6) and ChATcre$^{+}$-PTEN$^{+/+}$ controls (n=9), unpaired t test. (D) Western blot of liver lysates for phosphorylated AKT (p-AKT) and total AKT (t-AKT, left) and quantification (right). (E) Western blot of liver lysates for phosphorylated STAT3 (p-STAT3) and total STAT3 (t-STAT3, left) and quantification (right). Data shows males and females combined. Mean±SEM.
4.8 No significant changes in energy expenditure, yet increased locomotion in HFD-fed ChATcre⁺-PTENfl/fl mice

We utilized CLAMS metabolic cages to further delineate how cholinergic PTEN deletion impacts energy expenditure and behaviour. Mice were subjected to CLAMS following 12 weeks of HFD. CLAMS analysis demonstrated that HFD-fed ChATcre⁺-PTENfl/fl mice have similar food intake, oxygen consumption, heat production, carbon dioxide production, and respiratory exchange ratio (RER) when compared to their controls (Figure 13A-B). This suggests that ChATcre⁺-PTENfl/fl mice have similar energy expenditure to their controls. Interestingly, the HFD-fed ChATcre⁺-PTENfl/fl mice showed more X-ambulatory, X-total and Z-total locomotion only during dark cycle, compared to their controls (Figure 13C). This finding is similar with previous findings, which demonstrates that mice with neuronal PTEN deletions in the hippocampus, cortex, and cerebellum using a neuron-specific enolase (Nse) promoter-driven cre mouse have increased locomotor activity, as assessed by open-field testing (Kwon et al., 2006; Lugo et al., 2014). This suggests that the PTEN deletion in cholinergic neurons may induce behavioural changes.
Figure 13: (A) Energy expenditure parameters, including VO\(_2\), VCO\(_2\), Heat, and RER for HFD-fed ChATcre\(^{+}\)-PTEN\(^{+/}\) (n=4) and ChATcre\(^{+}\)-PTEN\(^{+/}\) controls (n=5). (B) Food intake per day measurement. (C) Locomotion of the mice during the light and dark cycles. Data shows males and females combined, unpaired t tests, Mean±SEM; \(^*\)p<0.05,
4.9 HFD-fed Phox2Bcre⁺-PTEN⁰/⁻ mice have improved glucose homeostasis, less weight gain and adiposity

A potential caveat with using ChATcre⁺ mice to target PNS/vagal neurons, is the widespread nature of cholinergic neurons. As noted earlier, many neuronal populations and brain regions largely utilize acetylcholine at their synapses. Importantly, sympathetic preganglionic neurons, which counteract PNS/vagal activity, are also cholinergic. For this reason, we also generated mice with PTEN deficiency in paired-like homeobox 2b (Phox2b) expressing neurons using mice, which express cre under this promoter which is specifically within cholinergic neurons of the hindbrain. These mice have been shown to express cre within the preganglionic neurons of the DMV in the DVC, but not in sympathetic regions such as the IML (Rossi et al., 2011). Hence, we bred Phox2b-cre mice with PTEN⁰/⁻ mice to generate Phox2B-PTEN⁺/⁺ wildtype and Phox2b-Cre⁺-Phox2B-PTEN⁰/⁻ knockout mice. We fed these Phox2b-PTEN⁰/⁻ and control littermates HFD for 12 weeks, following an initial NCD diet for 8 weeks. Similar to ChATcre⁺-PTEN⁰/⁻ and control mice, we assessed for the various metabolic parameters, including glucose and insulin tolerance, and body weight and composition. Initial glucose and insulin tolerance tests have demonstrated that female Phox2b-PTEN⁰/⁻ fed a HFD for 12 weeks had improved glucose tolerance, while males did not (Figure 14A-B).

Furthermore, females and males did not show any changes in their insulin sensitivity, as assessed by an insulin tolerance test. Additionally, HFD-fed Phox2b-PTEN⁰/⁻ male mice gain significantly less weight than their controls, when females did not (Figure 14C-D). Male and female Phox2b-PTEN⁰/⁻ mice also had significantly smaller eWAT and sWAT
depots per % body weight respectively, while they had no changes in their BAT weights (Figure 14E). These mice also had no changes in the weights of any other organs (Figure 14F).
Figure 14. Glucose (top) and insulin (bottom) tolerance tests of HFD-fed (A) female and (B) male Phox2B-PTENfl/fl mice (n=3 for males and females) and Phox2B-PTEN+/+ controls (n=7 for males and n=6 for females) and respective area under the curve (AUC), two-way ANOVA, effect of genotype. Weights of female (C) and male (D) mice at baseline and after 12 weeks of HFD and (E) organ weights and (F) fat depot weights of mice, unpaired t tests. Mean±SEM; * p<0.05, **p<0.01.
Chapter 5 – Discussion, Limitations and Future Directions
5.1. Summary

Our study demonstrates that PTEN deletion in cholinergic neurons improves glucose homeostasis in mice fed a HFD. Furthermore, these mice have smaller depots of eWAT with less adipose tissue macrophages and less gene expression of markers of inflammation along with increase in some markers of adipogenesis. Interestingly, female ChATcre⁺-PTEN⁺/- mice have demonstrated a more significant phenotype than the males in this respect. Females ChATcre⁺-PTEN⁺/- mice presented with significantly enhanced insulin sensitivity, less eWAT weight and body weight, while the male knockouts only demonstrated trends. This suggests that there is a potential sex difference in the phenotype. HFD-fed ChATcre⁺-PTEN⁺/- mice also had less fatty liver and had more locomotion, with no change in energy expenditure. These results together show a novel role of enhanced PI3K insulin signaling within the cholinergic system to play a potential beneficial role in providing protection against HFD-induced peripheral metabolic abnormalities.
5.2 Discussion

5.2.1 The role of PI3K in the DVC

There has been a growing interest in insulin signaling in the cholinergic structures of the brainstem in the recent years. Initial studies examine the autonomic role of insulin-mediated PI3K-AKT signaling in the NTS in controlling cardiovascular parameters such as blood pressure and heart rate (Huang et al., 2004). Additionally, loss of function manipulations of PTEN using small interfering RNA (siRNA) in the brainstem were shown to increase AKT phosphorylation, cause hypotension, and suppress baroreflex-mediated sympathetic vasomotor tone (Tsai et al., 2017). Other investigators used patch-clamp recording on the DMV to find that PI3K activity reduced the firing of neurons involved with gastric motility (Blake & Smith, 2012). This effect was also dependant on K\textsubscript{ATP} channels, which are widely expressed in the DVC and are necessary for vagal mediated HGP suppression (Blake & Smith, 2012; Pocai et al., 2005).

In our model, PTEN deletion in ChAT-cre neurons in the DVC caused increased PI3K signaling within these neurons, with improvement in glucose homeostasis after being subjected to a HFD. Research by Dr. Tony Lam’s group has provided an alternate explanation for how insulin signaling in the DVC affects glucose metabolism. Using insulin infusion and pharmacological inhibitors, his lab demonstrated that insulin signaling in the DVC functions to suppress HGP through a PI3K-independent pathway. Furthermore, they suggest that insulin action in the DVC lowers blood glucose and
improves energy expenditure via extracellular signal-regulated kinases 1/2 (ERK1/2), via mitogen-activated protein kinase (MAPK, Filippi et al., 2012, 2014). Interestingly, many *in vitro* studies have proposed that PTEN indirectly negatively regulates ERK1/2 activity (Bouali et al., 2009; Chetram et al., 2012). Moreover, PI3K has been shown to stimulate ERK1/2 in *Xenopus laevis* oocytes and *Simia aethiops* kidney cells. (Hu et al., 1995; Lopez-Illasaca et al., 1997). However, PI3K has also been linked to inhibition of ERK1/2 in neuroblastoma cells, underscoring the complexity of these signaling pathways (Heide et al., 2003). Overall, the PTEN deletion and increased PI3K signaling we see in the DVC, may prompt the ERK1/2 pathway to suppress HGP.

PTEN deletion in *ChATcre*+/PTEN<sup>fl/fl</sup> mice may cause enhanced activation of the neurons in the DVC. *In vivo* PTEN knockdown in the dentate gyrus of mice via retroviral injection caused neuronal hyperactivity due to hypertrophy and a higher density of synapses (Williams et al., 2015). Chronic stimulation of the vagal nerve has been shown to reduce feeding, suppress weight gain, and improve glucose homeostasis in rats and pigs (Gil et al., 2011; Malbert et al., 2017) Thus, *ChATcre*+/PTEN<sup>fl/fl</sup> mice may be protected from development of T2D from hyper-activation of the vagus nerve.

### 5.2.2 *Comparisons to Rip2cre*+/PTEN<sup>fl/fl</sup> mice

Given our initial implication of *Rip2cre* neurons in the anti-inflammatory involving the vagus (L. Wang et al., 2014), we wanted to further refine a specific role of PTEN in cholinergic neurons. We observed interesting parallels and contrasts between our
Both 

\( \text{ChATcre}^+\text{-PTEN}^{\text{fl/fl}} \) and the \( \text{Rip2 cre}^+\text{-PTEN}^{\text{fl/fl}} \) mice. While both 

\( \text{ChAT-PTEN}^{\text{fl/fl}} \) and 

\( \text{Rip2cre}^+\text{-PTEN}^{\text{fl/fl}} \) on a HFD showed improved glucose homeostasis, less fatty liver, and smaller eWAT adipocytes, \( \text{Rip2cre}^+\text{-PTEN}^{\text{fl/fl}} \) on a HFD had a marked expansion of sWAT with increased M2 polarized macrophages and massive weight gain. In contrast, our 

\( \text{ChATcre}^+\text{-PTEN}^{\text{fl/fl}} \) had a modest attenuation in weight gain after a HFD. Importantly, both strains of mice demonstrated eWAT with smaller adipocytes with enhanced adipogenesis markers. This can likely be attributed to vagal outflow, since previous studies have shown vagal stimulation to enhance WAT adipogenesis and insulin sensitivity (Kreier et al., 2002).

Another study has demonstrated that activation of \( \text{Rip2-cre} \) neurons increases SNS outflow to WAT to promote browning, with no change to the BAT (B. Wang et al., 2018). This corresponds with the trending enhancement of UCP-1 expression we noted in WAT of our \( \text{ChATcre}^+\text{-PTEN}^{\text{fl/fl}} \) mice, while it was unchanged in the BAT. \( \text{Rip2-cre} \text{PTEN}^{\text{fl/fl}} \) mice on a HFD have also demonstrated a suppression of M1 macrophages, compared to wildtype controls, yet unlike our \( \text{ChAT-PTEN} \) mice, they had a marked increase of M2 macrophages. This change in inflammatory profile of the macrophages was dependant on \( \alpha7 \) nAChR signaling from the vagus nerve. Altogether, our study helps to distinguish how PTEN deletion in the cholinergic neurons leads to a different metabolic outcome compared to a more broad PTEN deletion in the hypothalamus and pancreatic \( \beta \)-cells in the \( \text{Rip2cre}^+\text{-PTEN}^{\text{fl/fl}} \) mice.
5.2.3 Comparing PTEN’s role in Phox2B and ChAT neurons

Cholinergic neurons in the DVC has been shown to regulate glucose homeostasis through melanocortin-4 receptors (MC4R), which have been linked to obesity and insulin resistance in genome wide association studies (Loos et al., 2008). The MC4R responds to alpha melanocyte-stimulating hormone (α-MSH), a cleaved product of the POMC protein, which is synthesized in the hypothalamus and DVC (Guan et al., 2017). Studies by the lab of Dr. Joel Elmquist have demonstrated localization of MC4R on the ChATcre+ cholinergic neurons in the DMV, in which its deficiency in ChAT neurons leads to diminished insulin sensitivity and impaired glucose tolerance (Rossi et al., 2011). When these mice had MC4R re-expressed in ChAT neurons they had an improvement in their energy expenditure, hyperglycemia and hyperinsulinemia. Since ChAT is expressed in both sympathetic and parasympathetic neurons, this group also generated Phox2B-cre mice to target brainstem parasympathetic DVC while sparing sympathetic regions. When MC4R was re-expressed in the Phox2b expressing neurons, mice they had less hyperinsulinemia, but no change in hyperglycemia and energy expenditure. This highlighted how ChATcre+ expression in the SNS may alter phenotypes. We therefore also used these Phox2B-cre mice to generate Phox2Bcre+-PTEN0/0 mice, with PTEN deletion in DVC without affecting SNS. Our preliminary data show that Phox2Bcre+-PTEN0/0 mice have similar improvements in their glucose homeostasis and changes in adiposity compared to ChATcre+-PTEN0/0 mice. These results suggest that PTEN deletion in the PNS is responsible for the metabolic phenotype observed in ChAT-PTEN.
mice. Our \textit{ChATcre}^{+}\textit{-PTEN}^{0/0} mice show an increase in UCP-1 and some markers of lipolysis in their eWAT, both which are known to be stimulated by the SNS (Ghorbani et al., 1997; Weiss & Maickel, 1968). However, parasympathetic stimulation through the nicotinic receptors of the vagal nerve has also been implicated in WAT lipolysis (Andersson & Arner, 1995).

5.2.4 \textit{Role of cholinergic neurons outside of the DVC}

A recent study has shown that the basal forebrain, a major cholinergic brain structure, can influence appetite and feeding through connections to the hypothalamus. Specifically, when the \textit{ChAT} neurons in diagonal band of broca (vDM) in the basal forebrain, were ablated stereotaxically using an adeno-associated virus (AAV), mice became obese and hyperphagic (Herman et al., 2016). Interestingly, a small population of \textit{ChAT} neurons are also found within the hypothalamus which regulate food intake and BAT thermogenesis (Jeong et al., 2015, 2017). Since we saw extremely few β-gal expressing hypothalamic neurons, none of which co-expressed pS6, it is likely that findings from our \textit{ChATcre}^{+}\textit{-PTEN}^{0/0} mice are likely not due to changes in the hypothalamus. We noted that some of our \textit{ChATcre}^{+}\textit{-PTEN}^{0/0} mice had slight fur loss. Past research has demonstrated that hair follicles and the epidermis express ChAT (Hanada, et al., 2013).
5.3 Limitations and Future Directions

5.3.1 Measurement of Vagal Activity

Our current method of quantifying immunofluorescent stained neurons within DVC using ImageJ through manual counting is a limitation. For future experiments, we can use a stereological approach for quantification through unbiased cell counting in a 3D plane and can assess brain-wide distributions for pre-specified neurons (C. Zhang et al., 2017). Stereology would allow for more objective counting of the number of neurons co-expressing pS6 and β-gal in differing brain regions.

We can also characterize signaling within the DVC by immunostaining for c-fos and ERK1/2, to assess for neuronal activation and alternative insulin signaling respectively (Ayush et al., 2015; Bullitt & Carolina, 1990; Filippi et al., 2012; L. Wang et al., 2014). We can also use different strategies to inhibit vagal activity, to assess the role of the vagal activity in the improvement of glucose homeostasis we observed in our ChATcre+·PTEN0/− mice. We can use mecamylamine, a non-selective nAchR antagonist, to block vagal activity (L. Wang et al., 2014). We can also subject mice to a subdiaphragmatic vagotomy and assess whether this can attenuate the phenotype that we see in our ChATcre+·PTEN0/− mice (Edvell & Lindstrom, 1998).

Additionally, we can assess for suppression of HGP by measuring gene expression of gluconeogenic enzymes, such as Pepck and G6pase, in the liver by qRT-
PCR. Also, we can subject our mice to a pyruvate tolerance test, to further assess HGP (Miyake et al., 2002; Tamura et al., 2007). We noticed a trending decrease in the concentration of triglycerides in the liver HFD-fed ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) mice and thus would be interested in to further understanding the fat profile of the livers. We can investigate to see if there are alterations in the amount of saturated, monounsaturated, polyunsaturated, highly unsaturated, omega-6, and omega-3 fatty acids (Desai et al., 2017). We can also measure aspartate aminotransferase and alanine aminotransferase in the plasma to better assess fatty liver and liver injury (Sivasubramaniyam et al., 2017).

Lastly, we can measure for different circulating inflammatory cytokines (Tnfα, IL6, IL10) and adipokines (leptin, adiponectin, resistin, plasminogen activator inhibitor-1).

### 5.3.2 Further assessment of insulin signaling

We have shown no changes in the basal levels of p-AKT in the eWAT and liver of ChATcre\(^{+}\)-PTEN\(^{fl/fl}\) mice. It would be informative to dedicate an experiment to stimulate another cohort of mice with insulin (with saline vehicle control) prior to their sacrifice, to assess for acute effects of insulin stimulation on p-AKT. Other insulin signaling proteins downstream of PI3K and AKT including mTORC and FOXO1 can be examined by western blotting following insulin stimulation (Hay, 2011).
5.3.3 Potential role of the SNS

Given that ChAT neurons are found in the preganglionic neurons of the SNS, this may have contributed to the phenotype of our ChAT-PTEN mice. To investigate this we can measure plasma norepinephrine, the catecholamine utilized at the postganglionic neuron by the SNS, and assess the tissue content of norepinephrine in WAT using an ELISA (B. Wang et al., 2018). Furthermore, we can immunostain WAT sections for tyrosine hydroxylase, the rate limiting enzyme in norepinephrine synthesis, to further assess for sympathetic activity (B. Wang et al., 2018). We can compare between Phox2Bcre+ -PTEN$^{fl/fl}$ and ChATcre+ -PTEN$^{fl/fl}$ mice for differences in UCP-1 expression in their BAT and WAT after NCD and HFD. Since Phox2Bcre+ -PTEN$^{fl/fl}$ mice do not have cre expression in their preganglionic SNS neurons, this will allow us to dissect which findings are contributed by SNS versus PNS. We can also assess for changes in insulin levels between the two strains, as the SNS outflow suppresses insulin secretion (Zern, 1980). We can also measure UCP-1 expression in the inguinal sWAT (Shabalina et al., 2013).

5.3.4 Alternate Mouse Models

PTEN has a significant role in neurodevelopment. Therefore, it may be important to develop alternative mouse models with inducible PTEN knockdown in target brain regions. Researchers have developed AAVs encoding short-hairpin RNA (shRNA) that target PTEN for suppression, which they have injected into the brains of rodents
(Lewandowski & Steward, 2014; Zukor et al., 2013). We can use this technology to disrupt PTEN within the DVC and study its corresponding effect on glucose homeostasis. Other groups have used pharmacological injections of bisperoxovanadium compounds to inhibit PTEN. However, they are injected systemically, and therefore manifest in non-specific effects (Christie et al., 2010; Scrivens et al., 2003).

In order to assess the specific role of insulin signaling in the PI3K pathway, we can generate mice with deletion of the insulin receptor in their cholinergic neurons (ChATcre⁺-IRfl/fl). This mouse model would have a disruption in PI3K signaling in the cholinergic neurons, which is theoretically the opposite model of our ChAT-PTEN model. We can assess the metabolism and glucose profiles of these mice to see if these mice have an impairment of insulin signaling, causing them to be insulin resistant in their DVC. Lastly, we can inject an LY294002 and MEK1-DN, antagonists of PI3K and ERK1/2 respectively, into the DVC of our ChATcre⁺-PTENfl/fl mice and their controls (Filippi et al., 2012; Niswender et al., 2003). If this abolishes the phenotype, we could further conclude that the PI3K and ERK1/2 signaling in the DVC is indispensable. This would be helpful in understanding which is the dominant insulin signaling pathway in the DVC that improves glucose homeostasis. We are currently developing ChATcre⁺-PTENfl/fl mice and their controls with db/db mutation. These mice have a point mutation in their leptin receptor gene which causes spontaneous development of chronic hyperglycemia, obesity and hyperinsulinemia within six weeks of age on NCD (Kobayashi et al., 2000). It would be interesting to assess ChAT-cre⁺PTENfl/db/db mice
and compare with $ChAT-cre^+PTEN^{0/0}$ on HFD with their metabolic parameters. This will allow for a genetic model of diabetes to be compared with a HFD model and also assess leptin versus insulin signaling pathways utilized by the PI3K signaling.

A potential limitation is that we cannot rule out that $cre$ expression itself influences the phenotype of our mice. $Cre$ expression has been shown to cause $cre$ toxicity in mouse models and lead to various effects, including DNA damage, growth inhibition, and apoptosis (Loonstra et al., 2001; Naiche et al., 2007). $Cre$ expression has also been reported to alter the metabolic phenotype. For example, $cre$ expression under the control of nestin promoter has been shown to enhance insulin sensitivity and decrease lean mass (Harno et al., 2013). Therefore, to rule out any unintended effects of Cre in our mice, we can examine $ChATcre^+PTEN^{0/0}$ mice and compare to $ChATcre^+PTEN^{+/+}$ mice. Similar findings between these mice would ensure that $cre$ expression alone does not impact our phenotype.

5.3.5 Mouse Behaviour

The changes that we are seeing in locomotion in $ChATcre^+PTEN^{0/0}$ mice suggests that there may be an underlying behavioural phenotype in these mice. Another mouse model of PTEN deletion in the hippocampus and cortex using Nse-cre demonstrated increased locomotion, in association with neurodevelopment and behavioural abnormalities (Kwon et al., 2006). Moreover, acetylcholine signaling is necessary for learning and memory; as such, it would be of interest to assess these
parameters using Morris water or Barnes Maze (Atri et al., 2004; Barnes, 2011; Morris et al., 1982). PTEN mutations have also been implicated in autism spectrum disorders, in which there are pronounced social deficits and repetitive behaviour (Lugo et al., 2014; Ravizza et al., 2013). Hence, tests for autism-like behaviour, including social preference and marble burying tests can also be considered (Angoa-Pérez et al., 2013; Moy et al., 2004). We can also subject our Phox2B mutant mice to CLAMS to assess specifically for parasympathetic cholinergic neuronal PTEN in the regulation of metabolic parameters.

5.3.6 Anti-Inflammatory Reflex

The vagus nerve innervates the spleen and can initiate the “anti-inflammatory reflex”, in which vagal outflow suppresses pro-inflammatory cytokines release via α7 nAChRs (Borovikova et al., 2000; Tracey, 2007). The anti-inflammatory reflex was enhanced in Rip2cre⁺-PTENflo/fl mice and was attributed to their decrease in systemic inflammation and enhanced insulin sensitivity (L. Wang et al., 2014). Whether this also holds for ChATcre⁺-PTENflo/fl mice remains to be tested. Firstly, we can treat ChATcre⁺-PTENflo/fl mice with methyllycaconitine, an α7 nAChR antagonist, which would target the receptors involved in mediating the anti-inflammatory reflex (Davies et al., 1999; P. S. Olofsson et al., 2012). Additionally, we can breed our ChATcre⁺-PTENflo/fl mice with mice harbouring an α7 nAChR null mutation (H. Wang et al., 2003). If the α7 nAchR antagonist and mouse model abolishes the improvement in glucose homeostasis that we saw in our ChATcre⁺-PTENflo/fl mice, this would indicate that the anti-inflammatory reflex is critical in determining our phenotype. Also, we can isolate
peritoneal macrophages and measure the expression of M1 and M2 markers, to see if there is an enhancement in M2 macrophage differentiation, as was seen in the Rip2-
\[PTEN^{fl/fl}\] mice. Additionally, we can use flow cytometry to measure monocyte CD14 and toll-like receptor 4, two important factors in inflammation-driven IR, which are known to be suppressed by the anti-inflammatory reflex (Fernandez-Real et al., 2011; Hamano et al., 2006).

### 5.4 Final Remarks

Taken together, the results from our study have shown the importance of PTEN and insulin signaling in the cholinergic neurons in the regulation of diet-induced obesity and T2D. For many of the experiments, increased n-values are needed to reach statistical confidence and to analyze all the data separately in males and females. Additional work discussed can continue to clarify the importance of the cholinergic system in metabolic disease states and can aid in the future development of therapies aimed to thwart these insidious conditions.
Chapter 7 – References


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6.1 Statement of Contributions:

J.A.R. designed and performed the experiments, compiled and analyzed data. A.J.E. performed the brain cryosectioning. H.D. helped with transcardial perfusion. S.S. and M.J.K. helped perform the plasma insulin measurements. S.E.P. obtained data for preliminary glucose and insulin tolerance tests, and body compositions. I.D. and E.P. helped with genotyping. M.W. designed experiments, guided the project and aided in the interpretation of the data and preparation of the thesis.