Hypothalamic AMP-Activated Protein Kinase Regulates Glucose Production

by

Shuo Yang

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Physiology
University of Toronto

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General Abstract

Hypothalamic AMP-activated protein kinase (AMPK) regulates energy homeostasis in response to nutritional and hormonal signals. However, its role in glucose production regulation remains to be elucidated. Here, we tested the hypothesis that bidirectional changes in hypothalamic AMPK activity alter glucose production in rodents. First, we found that knocking down hypothalamic AMPK activity in an in vivo rat model led to a significant suppression of glucose production independent of changes in food intake and body weight. Second, we showed that activation of hypothalamic AMPK negated the ability of hypothalamic glucose- and lactate-sensing to lower glucose production. Collectively, these data indicate that changes in hypothalamic AMPK activity are sufficient and necessary for hypothalamic nutrient-sensing mechanisms to alter glucose production in vivo, and highlight the novel role of hypothalamic AMPK in the maintenance of glucose homeostasis in addition to energy balance.
Acknowledgments

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<tbody>
<tr>
<td>α-MSH</td>
<td>α-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acyl-CoA synthetase</td>
</tr>
<tr>
<td>Ad-CA AMPK</td>
<td>Adenovirus expressing the constitutively active form of AMPK</td>
</tr>
<tr>
<td>Ad-DN AMPK</td>
<td>Adenovirus expressing the dominant form of AMPK</td>
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<tr>
<td>Ad-GFP</td>
<td>Adenovirus tagged with green fluorescent protein</td>
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<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>AICAR</td>
<td>Aminoimidazole carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’ adenosine monophosphate (AMP)-activated protein kinase</td>
</tr>
<tr>
<td>AMPKK</td>
<td>AMP-activated protein kinase kinase</td>
</tr>
<tr>
<td>Arc</td>
<td>Arcuate nucleus</td>
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<tr>
<td>BAT</td>
<td>Brown adipose tissues</td>
</tr>
<tr>
<td>CaMKKK</td>
<td>Calmodulin-dependent protein kinase kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CPT-1</td>
<td>Carnitine palmitoyl transferase-1</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DYRK</td>
<td>Dual specificity tyrosine-phosphorylation-regulated kinase</td>
</tr>
<tr>
<td>ERK8</td>
<td>Extracellular signal-regulated kinase 8</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
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<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
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<td>GLUT1</td>
<td>Glucose Transporter-1</td>
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<td>GLUT4</td>
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<tr>
<td>HMGR</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA reductase</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRF-3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>K$_{ATP}$ channel</td>
<td>ATP sensitive potassium channel</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
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<tr>
<td>LCFA-CoA</td>
<td>Long chain fatty acyl-Coenzyme A</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MBH</td>
<td>Mediobasal hypothalamus</td>
</tr>
<tr>
<td>MCD</td>
<td>Malonyl-CoA decarboxylase</td>
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<tr>
<td>MELK</td>
<td>Maternal embryonic leucine zipper kinase</td>
</tr>
<tr>
<td>MNK1</td>
<td>Mitogen-activated protein kinase 1</td>
</tr>
<tr>
<td>MT-II</td>
<td>Melanocortin receptor agonist</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
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<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
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<tr>
<td>PP2C</td>
<td>Protein phosphatase 2C</td>
</tr>
<tr>
<td>PVC</td>
<td>Paraventricular nucleus</td>
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<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling-3</td>
</tr>
<tr>
<td>SRC</td>
<td>Sarcoma kinase</td>
</tr>
<tr>
<td>SREBP1c</td>
<td>Sterol regulatory element binding protein 1c</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
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<tr>
<td>ZAPK</td>
<td>Zeta-chain-associated protein kinase</td>
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1 Introduction

1.1 Diabetes Mellitus

Diabetes mellitus, which affects more than 170 million people world-wide today, is characterized by a disruption in glucose homeostasis that leads to chronic hyperglycemia [1]. This disease is divided into two categories: Type 1 and Type 2. Type 1 diabetes is characterized by the near absolute deficiency in insulin secretion due to the underlying autoimmune destruction of the insulin-producing pancreatic β-cells [2]. The more common Type 2 diabetes mellitus (T2DM), which is the focus of this thesis, accounts for >90% of all diabetic cases, and is caused by a combination of insulin resistance and the inability of the β-cells to secrete sufficient insulin to compensate for the insulin resistance [3]. It is the result of complex interactions of multiple factors including obesity, a sedentary lifestyle and genetic predisposition, which eventually leads to the deterioration of glucose homeostasis [3]. When circulating fatty acids are elevated, which is commonly seen in obesity, insulin resistance can occur at the level of the muscles to take up glucose, and at the liver to suppress glucose production [3]. If the β-cells can no longer keep up with this increasing demand for insulin secretion, the dysregulation of glucose homeostasis occurs, leading to chronic hyperglycemia.

Multiple devastating complications can result from hyperglycemia-induced oxidative stress and intracellular reactive oxygen species production, such as retinopathy, nephropathy, and cardiomyopathy [4]. If left untreated, T2DM can eventually lead to blindness, renal failure and
increased risks of cardiovascular disease and stroke [5]. Furthermore, the prevalence of T2DM is predicted to double and reach 366 million people world-wide by 2030 [1], thus, effective anti-diabetic treatments are urgently required. Given that one of the key contributing factors leading to hyperglycemia is the elevation of hepatic glucose production [6], it is imperative to gain a better understanding of the mechanisms that regulate glucose production. With this in mind, the goal of this thesis is to reveal novel signaling molecules in the hypothalamic sensing pathways that regulate glucose production.
1.2 Regulation of Glucose Homeostasis by the Hypothalamus

The idea of the central nervous system (CNS) control of peripheral glucose homeostasis was first introduced by Claude Bernard in 1855, who found that punctures in the floor of the fourth ventricle resulted in glycosuria [7]. However, it was only in the recent decade that significant understandings in the CNS, in particular the hypothalamic control of glucose homeostasis began to take place. Peripheral hormones such as insulin, leptin and glucagon-like peptide-1 (GLP-1) [8-12], as well as nutrients such as glucose and fatty acids [13-15] directly activate signaling pathways in the hypothalamus to regulate hepatic glucose production. In contrast, inhibiting the central signaling of these molecules leads to a disruption in glucose production regulation and glucose homeostasis [8, 15-18]. More importantly, in rodent diabetes and obesity models, a number of these central signaling pathways are impaired [9, 19-21]. Thus, it is clear that diabetes is not merely a peripheral metabolic disease; there is also a significant central component, which needs to be taken into account. The focus of this thesis is to continue to dissect the underlying mechanisms of CNS-sensing in the regulation of glucose production and homeostasis.

Hormone Signaling in the Hypothalamus

Since the discovery of insulin in 1921, its effects on glucose homeostasis regulation were thought to be restricted primarily to the peripheral organs [22]. The direct effect of insulin in the CNS has long been linked to the modulation of feeding behavior [23, 24]; however, it was not until a decade ago that the central action of insulin was extended well beyond the governing of
exogenous energy intake to the regulation of endogenous hepatic glucose metabolism and peripheral glucose homeostasis. The first group that hinted at this showed that neuron-specific knockout of insulin receptor (IR) in mice elevates plasma insulin and induces mild insulin resistance in association with obesity [25]. Subsequently, another study substantiated this new role of central insulin by demonstrating that the direct administration of insulin into the third cerebral ventricle suppresses hepatic glucose production independent of changes in body weight and circulating insulin and other glucoregulatory hormones [8]. It was then further elucidated that the central action of insulin requires the downstream signaling of phosphatidylinositol-3 kinase (PI3K) and the activation of ATP sensitive potassium (K\textsubscript{ATP}) channels [8, 16]. The signal is relayed by the hepatic vagal nerve to the liver [16], where it leads to the decrease in gluconeogenesis, likely through an increase in the interleukin -6/ signal transducer and activator of transcription (STAT) 3 signaling [26]. In streptozotocin-induced uncontrolled diabetes, this hypothalamic insulin signaling pathway is markedly reduced and further inhibiting it via the intracerebroventricular (ICV) infusion of a PI3K inhibitor blunts the improvement in glycemic response upon systemic insulin treatment, whereas enhancing central insulin signaling improves it [20]. This highlights the significance of central insulin signaling as a major determinant of responses to insulin treatments in uncontrolled diabetes [20]. Furthermore, one day of high-fat diet was sufficient to disrupt the regulatory ability of hypothalamic insulin to lower glucose production [21], which also indicates a potential role of central insulin resistance in the pathogenesis of T2DM.

Leptin is an adiposity signal from the adipocytes, and similar to insulin, it is another peripheral signal that has been shown to trigger the hypothalamus to regulate glucose homeostasis. Acute ICV infusion of leptin in diet-induced insulin resistant rats and in lipodystrophic mice restored hepatic insulin sensitivity [10, 27]. In addition, ICV infusion of
leptin is also sufficient *per se* to suppress hepatic glucose production by decreasing both glycogenolysis and gluconeogenesis at basal circulating insulin levels and independent of changes in body weight [11]. Furthermore, blocking STAT3 activation via either the ICV infusion of the STAT3 peptide inhibitor or the hypothalamic injection of a dominant-negative form of STAT3, prevented the effect of ICV leptin on hepatic glucose production, suggesting a STAT3-dependent mechanism [11]. However, STAT3 signaling is not the only pathway mediating CNS leptin’s effect; hypothalamic infusion of a PI3K inhibitor attenuated the improvement in insulin sensitivity elicited by the restoration of functional leptin receptors in the hypothalamic arcuate nucleus (Arc) of the leptin receptor-deficient fa^k/fα^k rats [17]. These data collectively suggest that in addition to regulating energy homeostasis, the central action of leptin also extends to the regulation of glucose production and homeostasis.

GLP-1 is a hormone secreted by the L-cells of the intestines [28] and discrete populations of neurons [29]. In the periphery, GLP-1 acts as an incretin by promoting insulin secretion and biosynthesis, inhibiting glucagon secretion and enhancing β-cell proliferation [30]. In the CNS, GLP-1 receptor mRNA are found but not restricted to the Arc and paraventricular nucleus (PVN) of the hypothalamus [31]. ICV administration of GLP-1 effectively inhibits feeding in fasted rats [32], which links central GLP-1 action with energy homeostasis regulation. A more recent study also implicated hypothalamic GLP-1 in the regulation of glucose homeostasis. Importantly, administration of GLP-1 directly into the Arc lowers hepatic glucose production, which similar to the effect of central insulin, was dependent on the activation of K_{ATP} channels [12]. However, the precise downstream mechanisms mediating the effects of GLP-1 remain to be fully elucidated. Together, these studies show that the hypothalamus plays an important role in controlling glucose production and homeostasis. Subsequent findings also zoomed in further to
the hypothalamic Arc as a major site of central sensing mechanisms that regulate glucose production.

**Hypothalamic Arcuate Nucleus (Arc) in the Regulation of Glucose Homeostasis**

The Arc is well known as an integration centre in the mediobasal hypothalamus (MBH) that mediates hormonal and nutrient signals to regulate appetite and body weight [33, 34]. Adjacent to the third ventricle and the median eminence, it consists of at least two populations of neurons that are extensively studied in the regulation of energy homeostasis: the neuropeptide Y (NPY) and Agouti-related peptide (AgRP)-containing neurons, which stimulate appetite and increase body weight, and the proopiomelanocortin (POMC)-expressing neurons, which is the precursor for the anorexigenic α-melanocyte-stimulating hormone (α-MSH) that inhibits food intake and decreases body weight [35].

Recent evidence strongly suggests an important role of the Arc in the regulation of glucose homeostasis [22]. Receptors for both insulin and leptin are found with high expression in the Arc [22]. A selective decrease in the IR expression in the Arc leads to insulin resistance in rats [36] and restoring leptin receptors in the Arc in leptin-receptor-deficient fa^k/fa^k rats improved insulin sensitivity [17]. In addition, as mentioned previously, the selective administration of GLP-1 in the Arc specifically, lowered glucose production [12]. Together, these data suggest that the central effects of peripheral hormones to regulate glucose homeostasis reside in the Arc.

Neuron-specific knockouts have also allowed a closer look at the neuronal types mediating each hormone’s central effects. Circulating insulin fails to suppress glucose
production in NPY/AgRP neuron-IR knockout mice independent of changes in body weight, whereas POMC neuron-IR knockout mice retain normal glycemic response to insulin infusion [37], indicating that it is the NPY/AgRP neurons that mediate the effect of central insulin to regulate glucose homeostasis. Further supporting this, is the finding that central NPY infusion prevents the central effect of insulin, which suggests that down-regulating NPY release is likely required for insulin’s ability to inhibit glucose production [38]. In contrast to the effect of central insulin, the regulation of glucose homeostasis by central leptin signaling seems to be mediated by the POMC neurons. POMC neuron-specific deletion of suppressor of cytokine signaling-3 (SOCS3), which negatively regulates leptin downstream signaling via STAT3, improves leptin action, insulin sensitivity and glucose homeostasis [39]. Furthermore, leptin also activates PI3K only in the POMC neurons [40], which has been implicated to improve insulin sensitivity [17]. Similarly, the effect of Arc GLP-1 infusion to suppress glucose production seems to be POMC-mediated as GLP-1 receptors are largely co-localized with POMC and not NPY/AgRP [12].

Recently, evidence also suggests glucose-sensing by POMC neurons as POMC-specific expression of a mutated K_{ATP} channel subunit Kir6.2 impaired glucose tolerance [41].

Taken together, the POMC and NPY/AgRP neurons of the hypothalamic Arc mediate the central effects of hormones to regulate glucose production and glucose homeostasis. This suggests that the CNS controls the availability of nutrients through parallel modulation of both energy balance and glucose production, thus the underlying molecules that mediate this central regulation need to be thoroughly examined in the interest of understanding the pathogenesis and treatments of T2DM and obesity.
Nutrient-Sensing in the Hypothalamus

In addition to integrating hormonal signals, the hypothalamus directly senses an elevation in the circulating and hypothalamic levels of nutrients and metabolites, namely long chain fatty acids (LCFA) [14, 15], glucose and lactate [13, 42, 43]. The increase in these nutritional signals triggers a neuronal network to regulate hepatic glucose production.

Fatty Acids
(Modified from the review by Breen et al. Diabetes Metab Res Rev. 27(2):p.113-9, 2010)

In the CNS, although fatty acids are not the primary fuel, they serve as energy ‘surfeit’ signal to inhibit endogenous glucose production from the liver. The first group that demonstrated this phenomenon showed that ICV administration of the LCFA oleic acid lowers plasma insulin and glucose levels, which represents an improvement in insulin sensitivity [14]. More importantly, at basal circulating insulin level during the pancreatic euglycemic clamp studies, ICV oleic acid significantly decreases the rate of hepatic glucose production. In support of the role of central LCFA sensing in the regulation of glucose production is another study showing that the hypothalamic sensing of an elevation in the circulating LCFA is required to counteract the direct stimulatory effect of the LCFA on hepatic gluconeogenesis [15].

Circulating LCFA are taken up by the brain and as they enter the cells, they are quickly esterified into LCFA-Coenzyme A (LCFA-CoA) by acyl-CoA synthetase (ACS) [44] (Figure 1). The accumulation of hypothalamic LCFA-CoA is a key step required for the hypothalamic sensing of circulating LCFA, since inhibiting hypothalamic ACS increases liver glucose production in the presence of elevated circulating LCFA [15]. Supporting this is the finding that inhibition of carnitine palmitoyl transferase I (CPT-1), which transports LCFA-CoA from the
cytosol into the mitochondria for β-oxidation, increases hypothalamic LCFA-CoA concentration and recapitulates the effect of ICV LCFA infusion [45]. Further along this line of thoughts, decreasing malonyl-CoA, a competitive inhibitor of CPT-1 via the over-expression of malonyl-CoA decarboxylase (MCD) in the hypothalamus, decreases LCFA-CoA accumulation and prevents the hypothalamic sensing of circulating fatty acids to lower glucose production [46].

Similar to effects of central insulin and GLP-1 infusions, hypothalamic K<sub>ATP</sub> channel activation is required for hypothalamic LCFA to lower glucose production as shown using both pharmacological and genetic approaches to knock down hypothalamic K<sub>ATP</sub> channel [14, 15]. The activation of K<sub>ATP</sub> channels subsequently signals the liver through the hepatic branch of the vagus nerve and the surgical resection of this nerve also negates the hypothalamic lipid sensing mechanism to lower hepatic glucose production [15]. A recent study has provided insights in the potential pathways and effectors downstream of hypothalamic lipid that regulates glucose homeostasis. Hypothalamic infusion of the protein kinase C (PKC) activator lowers glucose production [47]. However, this effect was negated by the co-infusion of the PKC-δ isoform-specific inhibitor and the blocking of the K<sub>ATP</sub> channel. Furthermore, inhibition of hypothalamic PKC abolished the effect of hypothalamic lipid infusion to lower glucose production [47]. These data suggest that PKC-δ lies downstream of hypothalamic lipid and upstream of K<sub>ATP</sub> channels to regulate glucose homeostasis, although the precise mechanism remains to be fully elucidated.
Figure 1. Fatty Acid Sensing in the Hypothalamus.

Long chain fatty acids (LCFA) are taken up by the hypothalamus and are quickly esterified into LCFA-Coenzyme A (LCFA-CoA) by acyl-CoA synthetase (ACS). Carnitine palmitoyl transferase I (CPT-1) is a mitochondrial outer membrane transporter that catalyzes the rate-limiting uptake of LCFA-CoA into the mitochondria, where LCFA-CoA provides the substrate for β-oxidation. Malonyl-CoA, which is derived from acetyl-CoA via acetyl-CoA carboxylase (ACC), competitively inhibits CPT-1 and promotes the accumulation of intracellular LCFA-CoA. AMP-activated protein kinase (AMPK) phosphorylates and inhibits ACC and malonyl-CoA formation. The accumulation of hypothalamic LCFA-CoA lowers hepatic glucose production through a PKC- and $K_{ATP}$ channel-dependent manner.
Glucose

Glucose is the primary source of energy for the brain to maintain normal function [48]. However, more recently, evidence suggests that not only does glucose provide a source of fuel to support neuronal activity, it also acts as a signaling molecule to regulate peripheral glucose balance [49].

Circulating glucose crosses the blood brain barrier primary via the 55kDa isoform of glucose transporter-1 (GLUT1) [48]. Once inside the brain, the 45kDa isoform of GLUT1 facilitates the uptake of glucose into the glia [48, 50]. Evidence supports the existence of an astrocyte-neuron lactate shuttle and the coupling of neuronal activity to glial glucose utilization [51] (Figure 2). Studies show that neurons preferentially utilize glial glucose-derived lactate as an oxidative fuel [52, 53]. In the astrocyte, glucose is converted to pyruvate through glycolysis, and subsequently converted to lactate via lactate dehydrogenase (LDH)-A [54]. Lactate gets shuttled across to the neurons via monocarboxylate transporters (MCT) [51, 55] and converted back to pyruvate in the neurons by LDH-B [56]. Both ICV and hypothalamic infusion of glucose lead to a decrease in blood glucose levels and the suppression of hepatic glucose production during the pancreatic euglycemic clamps [13]. This appears as a result of a suppression of both gluconeogenesis and glycogenolysis [13]. Moreover, the central infusion of lactate recapitulates the effect of central glucose on lowering glucose levels and hepatic glucose production [13]. The inhibition of LDH using oxamate abolishes the effects of both central glucose and lactate infusion [13], which suggests that the metabolism of glucose to lactate and the subsequent conversion to pyruvate in the hypothalamus is required to regulate glucose production and homeostasis. Further extending downstream, the hypothalamic infusion of dichloroacetate (DCA), which favors the conversion of pyruvate to acetyl-CoA by increasing the activity of pyruvate dehydrogenase (PDH), also suppressed glucose production [13]. In addition,
hypothalamic K\textsubscript{ATP} channel blocker negates the effects of central glucose/lactate [13], which again confirms the critical role of the K\textsubscript{ATP} channel in generating the signal to decrease hepatic glucose production.

To place the hypothalamic glucose-sensing pathway into perspective in the regulation of glucose homeostasis, the inhibition of hypothalamic LDH blunts 40% of the inhibitory action of an elevation in circulating glucose on hepatic glucose production [13]. In addition, inhibiting hypothalamic LDH or K\textsubscript{ATP} channels in the presence of a physiological increase in the level of circulating lactate increases glucose production, which suggest that the hypothalamic lactate-sensing mechanism also provides a restraint on the direct effects of systemic lactate to increase glucose production, thereby maintaining glucose homeostasis [43].

Taken together, these data implicate the glucose metabolic pathway in the regulation of glucose production. However, the downstream signaling pathways of acetyl-CoA that may lead to the activation of the K\textsubscript{ATP} and the lowering of hepatic glucose production remain to be elucidated.
Glucose is taken up by glucose transporter-1 (GLUT1) into the astrocytes where it is metabolized to pyruvate through glycolysis. Pyruvate is then preferentially converted to L-lactate by lactate dehydrogenase-A (LDH-A) in the astrocyte and shuttled across to the neurons by monocarboxylate transporters (MCT). In the neurons, lactate is converted back to pyruvate via lactate dehydrogenase-B (LDH-B) and then to acetyl-CoA via the pyruvate dehydrogenase (PDH) complex. Central glucose/lactate lowers hepatic glucose production through a $K_{\text{ATP}}$ channel-dependent mechanism.

Figure 2. Glucose Sensing in the Hypothalamus.
In summary, the metabolic pathways of nutrients and metabolites such as LCFAs, glucose or lactate in the hypothalamus can activate a negative feedback system to prevent further endogenous glucose release from the liver; however, the mechanisms that mediate this glucose production-lowering effect remain to be fully unveiled. Therefore, the goal of this thesis is to identify novel molecules involved in the CNS nutrient-sensing mechanisms in the regulation of glucose production. Given the role of 5′adenosine monophosphate (AMP)-activated protein kinase (AMPK) as a master regulator of cellular nutrient metabolism and energy balance (Figure 1), we predict that hypothalamic AMPK is a novel mediator of CNS nutrient-sensing in the regulation of glucose production.
1.3 Introduction to AMPK

AMPK was first described by Carling et al. [57] over 20 years ago when they discovered that the same AMP- stimulated kinase was associated with the inactivation of both acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR), which are the rate-limiting enzymes of fatty acid and cholesterol synthesis respectively. Since then, it has been well recognized as a regulator of cellular energy balance that is activated by energy deficiency (high AMP:ATP ratio) and in turn promotes energy conserving processes while inhibiting energy consuming processes [58]. In the following years, heightened interests in this highly conserved kinase have brought about discoveries of its roles that expand much beyond a simple energy sensor at the cellular level. Of special importance to this thesis, a number of groups around the world have established an important role of AMPK in the mammalian hypothalamus in mediating hormonal and nutrient signals to regulate whole-body energy metabolism [59-68]. Yet the glucoregulatory function of hypothalamic AMPK remains to be fully unveiled.

Structure and Regulation of AMPK

AMPK is a highly conserved serine/threonine protein kinase consisting of a catalytic α subunit and regulatory β and γ subunits [58]. Homologues of all three subunits have been found in a wide variety of eukaryotic organisms ranging from the single celled yeast Saccharomyces cerevisiae and protist Giardia lamblia to the multicellular plants and mammals [69]. In mammals, each subunit is encoded by distinct genes, forming two α isoforms (α1, α2), two β isoforms (β1, β2), and three γ isoforms (γ1, γ2, γ3) [58]. Further increasing the complexity of the
protein, several of the AMPK subunits (α1, γ2, γ3) can also undergo alternative splicing and initiation, thereby generating more varieties in the heterotrimer [70]. The catalytic domain, which includes the first 312 residues of the α subunit, is common to both AMPKα1 and AMPKα2 [71, 72] and the phosphorylation of threonine 172 (Thr172) in this catalytic domain is required for the enzymatic activity of AMPK [69]. The γ subunit is made up of two Bateman domains that can each bind either an AMP or ATP molecule in a mutually exclusive manner [73]. The β subunit holds the heterotrimeric complex in place by providing the scaffold that binds the α to the γ subunit [69].

The activity of AMPK is regulated intricately at the level of each subunit. Direct phosphorylation of Thr172 on the α subunit by upstream kinases (AMPKK), which include the tumor suppressor LKB1 and Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase-β (CaMKKβ), provides the greatest increase in AMPK enzymatic activity [74, 75]. In addition to activation via direct phosphorylation of the catalytic domain, binding of AMP to the regulatory γ subunit can allosterically increase AMPK activity [76]. Furthermore, AMP binding also prevents the dephosphorylation of Thr172 by protein phosphatase 2Cα (PP2Cα) [76-78], thereby enhancing AMPK activation. Conversely, the binding of ATP to the same site on the γ subunit, prevents AMP binding and activation of AMPK. Thus, these multiple mechanisms of control ensure that AMPK is sensitive to small changes in the intracellular AMP: ATP ratio, and readily respond to promote catabolic processes (such as fatty acid oxidation and glycolysis) that generate ATP and inhibit anabolic reactions (such as fatty acid and cholesterol synthesis) that consume ATP [58, 69]. Myristoylation on the β subunit is another site of regulation of AMPK activity, since removal of the myristoylation site not only led to the relocation of AMPK to the cytoplasm from the membrane, but also significantly increases the basal AMPK activity [79]. However, it is currently unclear what signals the myristoylation process [70].
AMPK in Regulation of Intracellular Fatty Acid Metabolism

The role of AMPK in maintaining intracellular energy balance has long been established before the discovery of its effects on whole-body energy homeostasis. One of the most well known intracellular functions of AMPK is its phosphorylation of ACC and the regulation of fatty acid oxidation. As mentioned above, the rate-limiting step of fatty acid oxidation involves the transport of the substrate, LCFA-CoA into the mitochondria and is catalyzed by the mitochondrial outer membrane transporter CPT-1 (Figure 1). CPT-1 is competitively inhibited by malonyl-CoA [80], which is directly derived from acetyl-CoA via the action of ACC. Two forms of ACC exist (ACC1 and ACC2), which regulate fatty acid metabolism [70]. Following AMPK activation by signals such as an increase in the ratio of intracellular AMP:ATP ratio, it inhibits ACC activity by phosphorylating ACC1 at serine 79 and ACC2 at serine 221 [70]. The resultant decrease in ACC activity leads to the decrease in the levels of malonyl-CoA and lessens the inhibition on CPT-1. This increases the uptake of LCFA-CoA into the mitochondria for β oxidation to generate ATP. In addition to promoting energy production, AMPK activation also inhibits energy consuming anabolic processes such as fatty acid and cholesterol synthesis. Fatty acid synthase (FAS) is the enzyme that catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA [70]. Older studies have shown that AMPK inhibits FAS transcription [81] possibly through decreased expression of the transcription factor sterol regulatory element binding protein 1c (SREBP1c) [82, 83]. More recent evidence suggests that AMPK may also regulate FAS post-transcriptionally in the 3T2-L1 adipocytes [84]. In addition, AMPK also phosphorylates serine 872 of HMGR, the rate-limiting enzyme of cholesterol synthesis, thereby inhibiting its catalytic activity [85]. In response to intracellular energy
deficiency, the action of AMPK on FAS and HMGR helps to conserve ATP while its regulation of ACC promotes the generation of ATP, thus keeping cellular energy balance.

Hypothalamic AMPK in the Regulation of Food Intake and Whole-Body Energy Balance

In the recent decade, studies around the world have independently shown that in mammals, AMPK not only acts as a cellular energy sensor but a regulator of whole-body energy homeostasis. In the physiological setting, hypothalamic AMPK is activated by fasting and inhibited by refeeding [64]. Furthermore, it mediates and integrates anorexigenic and orexigenic signals to regulate exogenous food intake and body weight.

Hypothalamic AMPK is inhibited by anorexigenic signals such as leptin, insulin and glucose [64]. Leptin inhibits the activity of AMPKα2 specifically in the Arc and PVN of the hypothalamus, whereas insulin and glucose seem to exert a wider effect by reducing AMPKα2 activity in all hypothalamic regions [60, 64]. In mice administered with the constitutively active regulatory γ1 subunit of AMPK, the anorexigenic effect of leptin is largely abolished, which suggests that the inhibition of hypothalamic AMPK is required in mediating at least a major part of leptin’s effect on food intake [64]. Furthermore, in agreement with the intracellular function of AMPK to inhibit ACC, leptin leads to an increase in ACC activity and the level of malonyl-CoA [86]. Given the previously described role of malonyl-CoA in affecting fatty acid metabolism, this suggests that the modulation of the lipid metabolic pathway in the hypothalamus by AMPK may mediate at least part of the effects of hormones to regulate feeding and energy balance. In addition, other anorexigenic signals such as GLP-1, α-lipoic acid, ciliary neurotrophic factor (CNTF) and the melanocortin receptor agonist MT-II, also decrease hypothalamic AMPK
activity [62, 64, 67, 87]. In contrast, orexigenic signals such as cannabinoids, ghrelin, adiponectin and AgRP increase the activity of hypothalamic AMPK [59, 60, 63, 64, 68]. Importantly, the effect of adiponectin to stimulate food intake was largely dependent on the increase in AMPK activity specifically in the Arc, as the dominant negative AMPK effectively attenuated the orexigenic effect of adiponectin [59]. In contrast, adiponectin-deficient mice had decreased AMPK activity in the Arc and were resistant to high fat diet-induced obesity [59]. These findings suggest a potential convergence of hormones and nutrients at the level of hypothalamic AMPK, to modulate energy balance.

In line with these ideas, changes in the activity of hypothalamic AMPK per se are sufficient to regulate feeding and body weight: dominant negative AMPK expression in the hypothalamus decreases food intake and body weight whereas the constitutively active AMPK increased both [64]. Moreover, selective knockout of AMPKα2 in the AgRP or POMC-containing neurons of the Arc disrupts energy balance in mice [65], further supporting the important role of AMPK in the Arc to maintain energy homeostasis. Recent studies also show that the decrease in AMPK activity in the ventromedial nucleus of the hypothalamus (VMH) mediates the central effects of thyroid hormones to increase the expression of thermogenic markers in the brown adipose tissues (BAT) and decrease body weight independent of changes of food intake [66]. This suggests that hypothalamic AMPK not only may modulate feeding but also energy expenditure in the regulation of energy balance. Collectively, these studies solidly establish an important role of hypothalamic AMPK as a master regulator of energy homeostasis at the whole-body level.
1.4 Parallel Hypothalamic Nutrient-Sensing Pathways in the Regulation of Energy and Glucose Homeostasis

Given the well characterized role of AMPK in governing fatty acid metabolism, and the more recent discoveries that hypothalamic AMPK mediates hormone- and nutrient-sensing to regulate energy homeostasis, it is reasonable to postulate that hypothalamic fatty acid metabolism could regulate energy homeostasis. In fact, accumulating evidence indicate that brain lipid metabolism regulates food intake and body weight [88]. The first line of evidence was the discovery that the FAS inhibitor C75 decreases feeding, body weight and the mRNA level of the orexigenic peptide NPY in mice [89]. This effect is dependent on the accumulation of brain malonyl-CoA, since the co-infusion of the ACC inhibitor TOFA into the third cerebral ventricle, which prevents the formation of malonyl-CoA, abolishes the anorectic effect of C75. In agreement with this finding, the over-expression of MCD, which decreases the level of malonyl-CoA, increases food intake [46] and reverses the effect of C75 to suppress food intake in mice [61]. Furthermore, as previously mentioned, leptin’s anorectic effect is coupled with an elevation of the level of malonyl-CoA in the hypothalamic Arc [86]. These data strongly suggest that malonyl-CoA, the intermediate of the fatty acid metabolism is a signaling molecule in the hypothalamus to control appetite and body weight.

Further strengthening the involvement of hypothalamic fatty acid metabolism in the regulation of food intake and energy balance was the discovery that ICV injection of the LCFA oleic acid decreases food intake and the NPY mRNA expression in rats [8]. LCFAs are taken up by the brain where they are esterified and equilibrate with LCFA-CoA [44]. Since LCFA infusion into the brain directly provides the substrate for LCFA-CoA formation and as
mentioned previously, C75 increases the level of malonyl-CoA, which is in turn also expected to increase the level of LCFA-CoA by inhibiting CPT-1, it suggests that perhaps the anorectic effects of both C75 and central oleic acid infusion are due to the accumulation of brain LCFA-CoA [15]. Consistent with this idea, the central administration of CPT-1 inhibitors increases the level of LCFA-CoA in the Arc and decreases food intake. Together, these data suggest that the accumulation of the lipid derivative LCFA-CoA in the brain serves as a signal of nutrient-abundance, which activates a negative feedback mechanism to restrict further intake of exogenous fuel into the body to maintain energy balance.

Interestingly, as mentioned before, the regulation of glucose homeostasis by the hypothalamus seems to share a similar pathway involving lipid metabolism. It appears that in response to the accumulation of malonyl-CoA and LCFA-CoA, the negative feedback system activated in the hypothalamus not only restricts exogenous fuel intake, but also the endogenous glucose output by the liver, which regulates glucose homeostasis [14, 15, 45, 46]. Therefore, considering the role of hypothalamic AMPK in affecting ACC activity and consequently the levels of hypothalamic malonyl-CoA and LCFA-CoA, it is possible that changes in hypothalamic AMPK activity can alter glucose production (Figure 1). It is also of interest to note that, as mentioned previously, the metabolism of hypothalamic glucose/lactate to acetyl-CoA also regulates glucose production [13]. Since acetyl-CoA directly provides the substrate for malonyl-CoA production via ACC and consequently leads to the accumulation of LCFA-CoA (Figure 2), there may be a potential convergence in the hypothalamic glucose/lactate and fatty acid-sensing pathways to regulate glucose production. AMPK directly modulates ACC activity, thus, changes in hypothalamic AMPK activity may affect glucose/lactate-sensing to suppress glucose production.
The Following Sections are Adapted from

2 Hypothesis and Aims

The hypothalamus lowers hepatic glucose production by directly sensing the increase in hypothalamic and circulating nutrient (i.e. glucose and lipid) and metabolite (lactate) levels \([15, 42, 49, 90]\). The downstream metabolic pathways of these nutritional signals that regulate glucose production remain to be fully elucidated. The general aim of this thesis is to identify novel molecules involved in the hypothalamic nutrient-sensing mechanism that regulates glucose production in the interest of revealing novel therapeutic targets to restore glucose homeostasis in T2DM. Given the role of hypothalamic AMPK in the regulation of CNS lipid metabolism \([58]\), which is implicated in the regulation of glucose production \([14, 15, 45, 46]\), we predict that hypothalamic AMPK is a novel molecule mediating hypothalamic nutrient-sensing mechanism in the regulation of glucose production.

AMPK inhibits ACC and thus the conversion of acetyl-CoA to malonyl-CoA, a competitive inhibitor of CPT-1 activity that prevents the uptake of LCFA-CoA into the mitochondria for \(\beta\)-oxidation. Since in the hypothalamus, an accumulation of LCFA-CoA lowers glucose production \([15, 45]\), it is reasonable to postulate that changes in hypothalamic AMPK activity may regulate glucose production. Moreover, hypothalamic AMPK’s effects on the malonyl-CoA and LCFA-CoA levels have been implicated in mediating the effects of central glucose and hormones (such as leptin, insulin, and GLP-1) in regulating feeding and body weight \([64, 67, 91]\). It is possible that a parallel pathway exists to regulate glucose production. We
hypothesize that since inhibition of AMPK lead to an increase in intracellular LCFA-CoA, it would be sufficient by itself to decrease hepatic glucose production (Figure 3A).

In the liver, β-cells and muscles, glucose flux increases the levels of malonyl-CoA and LCFA-CoA. In parallel, the conversion of glucose/lactate to pyruvate and then acetyl-CoA is required for hypothalamic glucose-sensing to suppress glucose production [13]. Thus, there exists a possibility of a potential convergence between CNS lipid sensing and CNS glucose sensing in regulating glucose production. It is possible that by generating acetyl-CoA and thus promoting malonyl-CoA formation, the glucose production-lowering effect of hypothalamic glucose/lactate was mediated by the intracellular accumulation of LCFA-CoA. Since AMPK inhibits ACC and consequently blocks the conversion of acetyl-CoA to malonyl-CoA, we hypothesize that activating hypothalamic AMPK would prevent the effects of hypothalamic glucose/lactate to lower glucose production (Figure 8A).

We restricted our manipulation of AMPK activity to the MBH containing the Arc given: 1) the critical role of the Arc in mediating peripheral signals to regulate glucose production and homeostasis [12, 17, 22, 36, 37, 39, 41]; 2) the disruption of energy homeostasis by the selective knockout of AMPK in the AgRP/POMC neurons of the Arc [65] and 3) the potential parallel hypothalamic pathways that regulate both energy and glucose homeostasis [14, 45, 46].
3 Materials and Methods

3.1 General Materials and Methods

3.1.1 Experimental Animal Model and Surgical Procedures

Animal Model

Adult 8-week-old male Sprague-Dawley rats, weighing between 280-310g were used for all in vivo experiments (Charles River Laboratories, Montreal, Quebec). Rats were housed in individual cages and maintained on a 12 hr/12 hr light-dark cycle with access to regular chow (Teklad 6% Mouse/Rat Diet with a composition of 52% carbohydrate; 31% protein and 17% fat, and a total caloric content of 3.83kcal/g) and water ad libitum. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University Health Network.

Stereotaxic Surgery

Rats were stereotaxically implanted with a bilateral cannula into the mediobasal hypothalamus (MBH) using the atlas of the rat brain. Briefly, rats were anesthetized with intraperitoneal injection of ketamine (60mg/kg; Ketalean; Bimeda-MTC, Cambridge, Ontario) and xylazine (8mg/kg; Rompun; Bayer) and mounted onto the stereotaxic apparatus by placing the ear bars into the ear canal and securing the nose with the anterior nose piece. The skull is then implanted with a 26-gauge stainless steel double guide cannula using the following coordinates for the MBH: 3.1mm posterior of bregma, 0.4mm lateral from midline, and 9.6mm
below skull surface. Instant adhesive and dental cement were used to secure the implants in place. Recovery of the rats following surgery was assessed with daily monitoring of food intake and body weight.

**Vascular Surgery**

Five days following stereotaxic surgery, rats that have recovered (in food intake and body weight) were again anesthetized with intraperitoneal ketamine (60mg/kg; Ketalean; Bimeda-MTC, Cambridge, Ontario) and xylazine (8mg/kg; Rompun; Bayer). Indwelling catheters were inserted into the right internal jugular vein and the left carotid artery for infusion and sampling purposes during the pancreatic euglycemic clamp studies. Briefly, polyethylene catheters (PE-50; Cay Adams, Boston, MA) extended with a segment of silastic tubing (length of 2cm, internal diameter of 0.02 inches; Dow Corning, Midland, MI) were used. Both catheters were tunneled subcutaneously and exteriorized. A 10% heparinized saline solution was used to fill the catheters to maintain the patency. Finally, the catheters were closed at the end with a metal pin. The rats were given 3 days of recovery following the surgery before the pancreatic euglycemic clamp studies.
3.1.2 Pancreatic (Basal Insulin) Euglycemic Clamp Procedure

The *in vivo* experiments were carried out in rats whose food intake and body weight had recovered back to the normal baseline level. Rats were restricted to ~60kcal of food the night before the experiment to ensure the same nutritional status. Infusion studies lasted a total of 210 minutes. At $t = 0$ min, a MBH infusions were initiated and maintained throughout the experiments at a rate of 0.006µl/min using the CMA/400 syringe microdialysis infusion pumps. A primed continuous intravenous infusion of $\text{3-}^{3}\text{H-glucose (40\mu Ci bolus, 0.4\mu Ci/min; Perkin Elmer; infused with Harvard Apparatus PHD 2000 infusion pumps)}$ was also initiated at 0 min and maintained throughout the study to assess glucose kinetics. At $t = 90$, the pancreatic clamp was initiated to assess the effect of MBH treatments on glucose metabolism independent of differences in the glucoregulatory hormones. To do this, somatostatin (3μg/kg/min) was continuously infused intravenously to inhibit endogenous insulin and glucagon secretions, and exogenous insulin (0.8mU/kg/min) was infused to maintain the glucoregulatory hormones at near basal levels. A 25% glucose solution was infused intravenously at variable rates and adjusted periodically to maintain the plasma glucose levels at comparable near basal levels among the groups.

Plasma samples for determination of $\text{3-}^{3}\text{H-glucose specific activity and plasma glucose}$ levels were collected in 10-min intervals to assess the glucose kinetics under basal (60-90 min) and clamped (180-210) conditions. Plasma samples for plasma insulin and glucagon measurements were also taken at regular intervals. At the end of the infusion studies, rats were anesthetized and manually injected with 3 µl of diluted bromophenol blue on each side of the MBH cannula to ensure correct placement of the cannula. Subsequently, to obtain the MBH samples, a wedge of tissues including the entire mediolateral and dorsoventral extent of the
arcuate nuclei (which contains the bromophenol blue staining) were dissected and freeze-clamped in situ. The tissues were stored at -80°C for subsequent AMPK activity assay.
3.1.3 Biochemical Analysis

Plasma Glucose

Plasma glucose concentrations were measured using the glucose analyzer (Glucose Analyzer GM9, Analox Instruments, Lunenbertg, MA), which was calibrated before each infusion study. Plasma samples of rats were obtained by centrifuging the blood samples at 6000 rpm. To measure the plasma glucose concentration, 10µl of the plasma sample was pipetted into a solution containing oxygen and glucose oxidase in the glucose analyzer. The glucose in the plasma reacts with oxygen in the following reaction catalyzed by glucose oxidase:

\[ \text{D-glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2 \]

A polarographic oxygen sensor is used to detect oxygen consumption, which is directly proportional to the glucose concentration in the plasma sample.

Plasma Glucose Tracer Specific Activity

Plasma samples (50µl) were first deproteinized with Ba(OH)\(_2\) and ZnSO\(_4\), and then centrifuged for 5 minutes at 6000 rpm at 4°C. The protein-free supernatant containing 3-\(^3\)H-glucose was obtained. Since the supernatant also contains tritiated water from the glycolysis of 3-\(^3\)H-glucose, the supernatant was first evaporated to dryness to remove the tritiated water to ensure that the liquid scintillation counts would only represent the radioactivity of 3-\(^3\)H-glucose in the plasma samples.
Plasma Insulin Assay

Plasma insulin levels were determined by a double antibody radioimmunoassay (RIA) kit specific for rat insulin (Linco Research Inc, St. Charles, MO). The general principle of the RIA is as follows: insulin in the sample competes with a fixed amount of $^{125}$I- labeled insulin for binding sites on the specific antibodies; bound and free insulin are separated by the addition of a second antibody immunosorbent followed by centrifugation and aspiration of the supernatant; the radioactivity of the pellet is measured and is inversely proportional to the amount of insulin in the sample.

First, a standard curve was constructed using nonradioactive insulin standards with known concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 ng/ml) in duplicate. Samples (50 µl) were then pipetted into appropriate tubes followed by the addition of $^{125}$I-insulin (50 µl) then the rat insulin antibody (50 µl). The tubes were then vortexed to ensure mixing and were incubated overnight (18-24 hrs) at 4°C. On the following day, 1.0 ml of precipitating reagent was added to all tubes followed by vortexing and incubating for 20 minutes at 4°C. The tubes were then centrifuged at 2500 rpm for 40 minutes. The supernatant was aspirated and the radioactivity in the pellet was counted in a gamma counter (Perkin Elmer 1470). The counts (B) for each of the standards and unknown were expressed as a percentage of the mean counts of the total binding reference tubes ($B_0$):

$$\% \text{ Activity Bound} = \frac{B \text{ (Standard or Sample)}}{B_0} \times 100\%$$

The % activity bound was plotted against the known concentration of the standard. The unknown concentration of the samples was determined by interpolation of the standard curve.
Plasma Glucagon

Plasma glucagon levels were determined using the double antibody radioimmunoassay (RIA) kit specific for rat glucagon (Linco Research Inc, St. Charles, MO). The principle of the glucagon RIA is similar to the insulin RIA described above with slight differences: standards (20, 50, 100, 200 and 400 pg/ml) and samples were first incubated alone with the glucagon antibody at 4 °C overnight. $^{125}$I-glucagon was then added to all tubes on the following day and incubated overnight at 4 °C. The unknown concentration of the samples was determined by interpolation of the standard curve.
3.1.4 Calculations

Tracer Dilution Methodology

The $^{3-3}\text{H}$-glucose dilution methodology and the steady state formula were used to determine the glucose uptake ($R_d = \text{disappearance rate of glucose}$) and glucose production ($R_a = \text{appearance rate of glucose}$):

$$R_a = R_d = \frac{\text{Constant tracer infusion rate (\(\mu\text{Ci/min}\))}}{\text{Specific activity (\(\mu\text{Ci/mg}\))}}$$

$^{3-3}\text{H}$-glucose was infused intravenously at a constant rate and allowed sufficient time to equilibrate. At basal steady state, $R_a$ (GP) is equal to $R_d$, which is determined by dividing the $^{3-3}\text{H}$-glucose infusion rate by the specific activity of the plasma $^{3-3}\text{H}$-glucose. During the pancreatic clamp studies where exogenous glucose was infused to maintain euglycemia, the rate of glucose production is the difference between $R_d$ and the rate of glucose infusion:

$$R_a = R_d - \text{Glucose Infusion Rate}$$
3.1.5 Statistical Analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) to test for significant differences between groups and the post hoc comparisons were performed with Tukey’s t-test. Differences were accepted as significant with p < 0.05. Values at t = 60-90 min during the pancreatic clamp studies were average to represent the basal condition and values at t = 180-210 min were averaged to represent the clamped condition.
3.2 Inhibition of Hypothalamic AMPK (Figure 3B)

3.2.1 Molecular Approach

Adenovirus tagged with green fluorescent protein (Ad- GFP) and adenovirus expressing the dominant negative form of AMPKα2 with an Asp-157-to-Ala mutation (Ad- DN AMPKα2 [D^{157}A]) were provided by our collaborators Dr. Guy A. Rutter’s laboratory from Imperial College, UK.

Immediately following the MBH surgeries, a group of rats were injected on each side of the MBH cannula, 3µl of one of the following adenoviruses:

1. **Ad- GFP (1.4×10^9 plague-forming units/ml)**

2. **Ad- DN AMPK (1.1×10^{13} plague-forming units/ml)**

Body weight and food intake were monitored each day following MBH surgeries and viral injections. Vascular surgeries, as described in the General Materials and Methods section, were done five days after MBH surgeries. Three days following the vascular surgeries, rats that have fully recovered underwent the pancreatic euglycemic clamp studies as described in the General Materials and Methods section.
3.2.2 Pharmacological Approach

MBH and vascular surgeries were performed as described in the General Materials and Methods section. During the pancreatic euglycemic clamp studies, recovered rats were treated with one of the following MBH treatments throughout the entire duration of the clamp (t = 0 – 210 min):

1. 5% Dimethyl Sulfoxide (DMSO)

2. Compound C (50µM, dissolved in 5% DMSO; Calbiochem, USA)
3.2.3 AMPK Activity Assay.

AMPK activity was determined by our collaborators from Dr. Guy A. Rutter’s laboratory in Imperial College, UK. In brief, MBH wedge samples were lysed in 200-500µl ice-cold lysis buffer [50mM Tris_HCl (pH 7.4, 4°C), 250mM sucrose, 50mM NaF, 1mM Na pyrophosphate, ethylenediaminetetraacetic acid (EDTA), 1mM ethylene glycol tetraacetic acid (EGTA), 1mM Dithiothreitol (DTT), 0.1mM benzamidine, and 0.1mM phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF), 5µg/ml soybean trypsin inhibitor, and 1% (vol/vol) Triton X-100] and centrifuged at 4°C, at 13200 rpm for 5 min to remove cell debris. Protein concentration was determined using a bicinconinic acid-based protein assay kit (Pierce, UK). Total extract (10µg protein) was used to determine AMPK activity, which was done by measuring phosphotransfer, with synthetic “SAMS” peptide (HMRSAMSLHLVKRR) as the substrate (24). Results were analyzed by linear regression using Graphpad™ software and were expressed in counts per minute (CPM). Non-AMPK dependent background (lysis buffer only) incorporation of radioactivity was subtracted from all values. Assays were performed in triplicate.
3.2.4 Immunohistochemistry

Eight days following Ad-GFP injections in the MBH, rats were anesthetized and perfused transcardially with 40ml of saline and then 35ml of 4% paraformaldehyde. Brains were removed and 4-mm-thick coronal sections containing the MBH were embedded and frozen in optimal cutting temperature compound (Tissue-Tek) and stored at -80°C. Frozen brain sample was cut into 10-µm-thick coronal sections via cryostat sectioning then mounted on glass slides. GFP was co-stained with either AgRP or POMC. Briefly, tissues were first blocked for one hour with 10% normal goat serum and 0.2% Triton X-100 dissolved in phosphate buffered saline (PBS) and then incubated overnight at 4°C with a combination of either chicken anti-GFP (1:1800; Abcam) and rabbit anti-POMC (1:1500; Phoenix Pharmaceuticals) antibodies or chicken anti-GFP (1:1800; Abcam) and rabbit anti-AgRP (1:200; Phoenix Pharmaceuticals) antibodies. On the next day, tissues were washed with PBS and incubated at room temperature with goat anti-chicken IgG (1:1000; Alexa-Flour 488) and goat anti-rabbit IgG (1:1000 for POMC and 1:700 for AgRP; Alexa-Flour 546) secondary antibodies. A fluorescence microscope was used to view the slides. The percent co-localization was roughly estimated by counting the total number of GFP-positive cells and the number of GFP-positive cells that that were also AgRP or POMC-positive.
3.3 Activation of Hypothalamic AMPK (Figure 8B)

3.3.1 Pharmacological Approach

MBH and vascular surgeries were performed as described in the General Materials and Methods section. During the pancreatic euglycemic clamp studies, recovered rats were treated with one of the following MBH treatments throughout the entire duration of the clamp (t = 0 – 210 min):

1. Vehicle (saline or 25mM aminoimidazole carboxamide ribonucleotide [AICAR] dissolved in saline; Sigma)

2. Glucose (2mM, dissolved in saline)

3. Lactate (5mM, dissolved in saline)

4. AICAR (25mM, dissolved in saline) + Glucose (2mM, dissolved in saline)

5. AICAR (25mM, dissolved in saline) + Lactate (5mM, dissolved in saline)
3.3.2 Molecular Approach

Adenovirus expressing truncated, constitutively active AMPKα1 \(^{312}\) (residues 1-312) with a Thr-172-to-Asp mutation (Ad- CA AMPKα1 [T\(^{172}\)D]) were provided by our collaborators Dr. Guy A. Rutter’s laboratory from Imperial College, UK.

Immediately following the MBH surgeries, a group of rats were injected on each side of the MBH cannula, 3µl of one of the following adenoviruses:

1. **Ad- GFP (1.4X10\(^9\) plague-forming units/ml)**

2. **Ad- CA AMPK (3.83 X10\(^{10}\) plague-forming units/ml)**

Body weight and food intake were monitored each day following MBH surgeries and viral injections. Vascular surgeries, as described in the General Materials and Methods section, were done five days after MBH surgeries. Three days following the vascular surgeries, rats that have fully recovered underwent the pancreatic euglycemic clamp studies. Throughout the entire duration of the clamp study (t = 0 – 210 min), adenovirus-injected rats were treated with one of the following MBH treatments:

1. **Saline**

2. **Glucose (2mM, dissolved in saline)**

3. **Lactate (5mM, dissolved in saline)**
4 Results

4.1 Molecular and Pharmacological Inhibition of Hypothalamic AMPK Lower Glucose Production

The hypothalamus has been increasingly recognized as a major site of glucose homeostasis regulation in response to nutrients, such as lipids. Specifically, an accumulation in hypothalamic lipid-derived LCFA-CoA results in a significant decrease in glucose production. Considering the important role of AMPK in regulating fatty acid oxidation by modulating the levels of malonyl-CoA and LCFA-CoA, perhaps changes in hypothalamic AMPK activity may regulate glucose production (Figure 3A). Therefore, we first tested the hypothesis that inhibiting hypothalamic AMPK will be sufficient to lower glucose production.

Adenovirus expressing the dominant negative form of AMPK (Ad-DN AMPK) was injected immediately following MBH surgeries and bilateral MBH cannula implantation (Day 0). Vascular surgeries and infusion clamp studies were carried out on Day 5 and Day 8 respectively (Figure 3B). On the morning of the clamp studies, we observed a 40.7 ± 15.6 % decrease in the daily food intake (p<0.05) and a trend toward lower body weights in the Ad-DN AMPK injected rats (p=0.07) comparing to the Ad-GFP injected controls (Table 1). Moreover, direct injection of Ad-DN AMPK into the hypothalamus also led to a significant (~50%) reduction in hypothalamic AMPK activity compared to Ad-GFP injection immediately following the clamp studies (Figure 4A).
Using the tracer dilution method in combination with the pancreatic (basal insulin) euglycemic clamp technique, we assessed the effects of Ad-DN AMPK on glucose kinetics in vivo. During the clamp, independent of significant differences in the circulating insulin and glucagon levels between the Ad- DN AMPK and Ad- GFP treated groups (Table 1), the exogenous glucose infusion rate needed to maintain euglycemia was ~3-fold higher in the Ad-DN AMPK injected rats comparing to the Ad- GFP injected controls (p<0.05) (Figure 4B). The increase in the glucose infusion rate was fully accounted for by a decrease in the rate of glucose production (5.0 ± 0.7 mg kg\(^{-1}\) min\(^{-1}\)) with respect to control (10.6 ± 0.4 mg kg\(^{-1}\) min\(^{-1}\), p<0.05) (Figure 4C, D) and not due to a change in glucose uptake (Figure 4E). These data show, for the first time, that molecular inhibition of hypothalamic AMPK activity is sufficient to suppress glucose production in vivo.

Immunohistochemistry staining for GFP in rat hypothalamus injected with Ad-GFP showed dense localization of GFP in the mediobasal hypothalamic regions and ~40% co-localization of GFP with AgRP-positive neurons and another ~40% co-localization with POMC-positive neurons (Figure 5, 6). This indicates minimal diffusion of our adenovirus from the site of MBH injection and the main target of our adenovirus being the Arc, which has been identified as a major site that regulates glucose homeostasis.

Since Ad- DN AMPK led to hypophagia and a trend toward lower body weights comparing to the Ad- GFP on the day of the clamp, it could not be ruled out that its effects on lowering glucose production during the clamp may be secondary to its effects on the adipose mass/energy storage of the rats. Therefore, next we tested the effects of acute inhibition of hypothalamic AMPK on glucose production using the pharmacological inhibitor of AMPK, compound C.
Rats were subjected to MBH bilateral cannula implantation on Day 0 and vascular catheter insertion on Day 5 (Figure 3B). A group of recovered rats with similar body weights were restricted to ~60 kcal of food the night before the infusion studies (Day 8) to ensure the same nutritional status. During the clamp, direct infusion of 50µM compound C led to a significant increase in the glucose infusion rate needed to maintain euglycemia comparing to the 5% DMSO control (p<0.05) (Figure 7A). Similar to the Ad- DN AMPK group, this increase was independent of significant differences in the circulating insulin/glucagon concentrations during the clamp (Table 2) and was due completely to a decrease in the rate of glucose production (4.1 ± 0.5 mg kg⁻¹ min⁻¹) comparing to control (9.7 ± 1.6 mg kg⁻¹ min⁻¹, p<0.05, Figure 3B, 3C) (Figure 7B, C) without a significant change in glucose uptake (Figure 7D). This suggests that inhibiting hypothalamic AMPK acutely can lower glucose production without changing the adiposity/body weight of the animal. To test this hypothesis further, we also performed pancreatic euglycemic clamp studies in another group of Ad- GFP injected rats that matched the Ad- DN AMPK injected group in average body weight and food intake. As predicted, the rate of glucose production during the clamp was not significantly decreased (10.4 ± 2.0 mg kg⁻¹ min⁻¹, n=4) comparing to that of Ad- GFP injected rats with higher body weight and food intake (10.6 ± 0.4 mg kg⁻¹ min⁻¹, n= 6). These data collectively indicate that the inhibition of hypothalamic AMPK is sufficient to lower glucose production independent of changes in body weight and adiposity.
A: A schematic representation of the working hypothesis: inhibition of hypothalamic AMPK activity by the dominant negative form of AMPK (DN AMPK) or compound C leads to the lowering of hepatic glucose production. B: Experimental procedure and clamp protocol. A bilateral mediobasal hypothalamic (MBH) catheter was implanted on day 0. Adenovirus tagged with GFP (Ad-GFP) or adenovirus expressing DN AMPK (Ad-DN AMPK) was injected into the MBH of a group of rats immediately after MBH catheter implantation. Venous and arterial cannulations were done on day 5, and the pancreatic clamp protocol was performed on day 8. In the Ad-GFP and Ad-DN AMPK-injected rats, no MBH infusions were given during the clamp experiments. IN rats with no adenovirus injection, 5% DMSO control or compound C was infused into the MBH during the clamps.
Figure 4. Molecular knockdown of hypothalamic AMPK by DN AMPK is sufficient to lower glucose production.

A: Hypothalamic AMPK activity was significantly diminished in animals injected with Ad-DN AMPK, compared with control animals with injection of Ad-GFP (*P < 0.001). Hypothalamic injection of Ad-DN AMPK led to an increase in glucose infusion rate (B) (*P < 0.01) and a decrease in glucose production (C) (*P < 0.001) compared with the GFP control. D: Suppression of glucose production during the clamp period (180-210 min) expressed as percentage reduction from basal steady state (60-90 min) (*P < 0.01 vs. GFP control). E: Glucose uptake was not significantly different from that of GFP control. Values are shown as means ± SEM.
Table 1. Body weights and plasma insulin, glucagon, and glucose concentrations of rats treated with Ad-GFP or Ad-DN AMPK in the mediobasal hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight</th>
<th>Insulin (ng/ml)</th>
<th>Glucagon (pg/ml)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ad-GFP (n=6)</strong></td>
<td>Basal</td>
<td>0.8 ±0.2</td>
<td>60 ±2</td>
<td>146 ±4</td>
</tr>
<tr>
<td></td>
<td>0.282 ±0.004</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clamp</td>
<td>0.8 ±0.1</td>
<td>53 ±4</td>
<td>140±6</td>
</tr>
<tr>
<td><strong>Ad-DN AMPK (n=14)</strong></td>
<td>Basal</td>
<td>0.8 ±0.1</td>
<td>82 ±9 *</td>
<td>153 ±8</td>
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<td></td>
<td>0.254 ±0.012</td>
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</tr>
<tr>
<td></td>
<td>Clamp</td>
<td>0.8 ±0.1</td>
<td>54 ±5</td>
<td>128 ±7</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Basal (t=0). Clamp (t=180-210). *p<0.05 versus Ad-GFP at basal.
Figure 5. Co-localization of GFP with AgRP and POMC in the mediobasal hypothalamus of Ad-GFP injected rats.

A, D: Representative immunofluorescence images showing GFP expression (green) in the mediobasal hypothalamus. B, E: Immunofluorescence staining for AgRP and POMC (tinted white) respectively in the mediobasal hypothalamus. C, F: Merged images (A and B, D and E) show that GFP is co-localized with AgRP-positive and POMC-positive neurons of the arcuate nucleus. 3V, third ventricle.
Figure 6. Percent co-localization of GFP with AgRP and POMC in the mediobasal hypothalamus of Ad-GFP injected rats.

Percentage of GFP-positive neurons that express AgRP or POMC (n=4 for each group).
Table 2. Body weights and plasma insulin, glucagon, and glucose concentrations of rats treated with 5% DMSO or compound C in the mediobasal hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight</th>
<th>Insulin</th>
<th>Glucagon</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(kg)</td>
<td>(ng/ml)</td>
<td>(pg/ml)</td>
<td>(mg/dl)</td>
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<tr>
<td><strong>5% DMSO</strong></td>
<td>Basal</td>
<td>0.9 ± 0.1</td>
<td>58 ± 3</td>
<td>144 ± 10</td>
</tr>
<tr>
<td></td>
<td>0.298 ± 0.014</td>
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</tr>
<tr>
<td>(n=6)</td>
<td>Clamp</td>
<td>0.7 ± 0.1</td>
<td>51 ± 3</td>
<td>124 ± 16</td>
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<tr>
<td><strong>Compound C</strong></td>
<td>Basal</td>
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</tr>
<tr>
<td></td>
<td>0.306 ± 0.004</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>Clamp</td>
<td>0.7 ± 0.1</td>
<td>47 ± 1</td>
<td>110 ± 7</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Basal (t=0). Clamp (t=180-210).
Direct infusion of compound C (Cmpd C), the pharmacological inhibitor of AMPK, into the MBH significantly increased the glucose infusion rate (A) (*P < 0.001) and decreased the glucose production (B) (*P < 0.05) during the clamps compared with the 5% DMSO control group. C: Suppression of glucose production during the clamp period (180-210 min) expressed as the percentage reduction from the basal steady state (60-90 min) (*P < 0.001). D: Glucose uptake in the compound C-treated group did not differ significantly from that of the 5% DMSO-treated control group. Values are shown as means ± SE.

Figure 7. Hypothalamic administration of compound C, the pharmacological inhibitor of AMPK, lowers glucose production.
4.2 Pharmacological and Molecular Activation of Hypothalamic AMPK Negate the Ability of Hypothalamic Glucose/Lactate to Lower Glucose Production

In addition to lipids, the hypothalamus can also directly sense an elevation in the levels of hypothalamic glucose and its metabolite lactate to lower hepatic glucose production. Given that the levels of malonyl-CoA and LCFA-CoA in the hypothalamus regulates glucose production, and that glucose and lactate metabolism provides the substrate for acetyl-CoA generation, which is in turn converted to malonyl-CoA through the action of ACC, there exists a possibility of a potential convergence between CNS lipid sensing and CNS glucose sensing to regulate glucose production. Since AMPK inhibits ACC, which should prevent the generation of malonyl-CoA from acetyl-CoA, we next hypothesized that hypothalamic AMPK activation would reverse the effects of hypothalamic glucose/lactate to lower glucose production (Figure 8A).

Using the pancreatic euglycemic clamp studies, we first confirmed that MBH administration of glucose/lactate increased the glucose infusion rate (p<0.05) (Figure 9A) and lowered glucose production (p<0.05) (Figure 9B, C) independent of significant differences in the levels of circulating hormones (Table 3), as previously reported. However, when the pharmacological AMPK activator AICAR was co-infused with glucose/lactate into the MBH of another group of rats with similar body weights, the increase in glucose infusion rate (Figure 9A) and the decrease in glucose production (Figure 9B, C) were completely abolished at comparable insulin, glucagon and glucose levels (Table 3). AICAR infusion alone (n=3) did not have any significant effects on the basal glucose production (14.2 ± 2.0 mg kg⁻¹ min⁻¹), clamp glucose production (10.8 ± 0.3 mg kg⁻¹ min⁻¹) and glucose uptake (13.2 ± 0.9 mg kg⁻¹ min⁻¹) comparing to the saline treatments (n=3) (12.4 ± 0.7 mg kg⁻¹ min⁻¹, 11.5 ± 1.3 mg kg⁻¹ min⁻¹,
12.7 ± 1.0 mg kg\(^{-1}\) min\(^{-1}\) respectively) \textit{in vivo} (Figure 9B, C). Therefore, MBH AICAR and saline infusions were grouped together as a single vehicle group. Together, these data show that pharmacological activation of hypothalamic AMPK negates the ability of hypothalamic glucose/lactate to lower glucose production.

Next, we alternatively activated hypothalamic AMPK with a molecular approach using an adenovirus expressing the constitutively active form of AMPK (Ad-CA AMPK). In this specific experimental protocol, Ad-CA AMPK injection did not have a significant effect on the body weight and food intake comparing to Ad- GFP in contrast to previous findings (Table 4). In addition, no significant differences in the basal plasma concentrations of glucose, insulin and glucagon as well as the basal glucose production rate were observed between the Ad-CA AMPK and the Ad- GFP groups (Table 4).

During the clamp, at comparable circulating insulin and glucagon levels (Table 4), MBH glucose/lactate infusion in the Ad- GFP injected rats significantly increased the glucose infusion rate needed to maintain euglycemia (p<0.05) comparing to the saline/Ad- GFP group (Figure 10A). Again, this was in association with a reduction in the rate of glucose production (p<0.05) (Figure 10B, C) and not a change in glucose uptake (Figure 10D). However, in rats injected with Ad- CA AMPK, MBH infusion of glucose/lactate during the clamp failed to increase the glucose infusion rate (Figure 10A) and lower glucose production (Figure 10B, C). Activation of hypothalamic AMPK by Ad- CA AMPK injection alone, like AICAR, did not affect the glucose kinetics in this protocol (Figure 10A-D). Together with the pharmacological gain-of-function data, these results indicate that selective activation of hypothalamic AMPK negates the ability of hypothalamic glucose/lactate sensing to regulate glucose production.
4.2.1 Figures and Tables

Figure 8. Schematic representation and experimental protocol of Section II.

**A:** Schematic representation of the working hypothesis: activation of hypothalamic AMPK by AICAR or the constitutively active form of AMPK (CA AMPK) prevents the ability of hypothalamic glucose/lactate to decrease glucose production. **B:** Experimental procedure and clamp protocol. A bilateral MBH catheter was implanted on day 0. Adenovirus tagged with GFP (Ad-GFP) or adenovirus expressing CA AMPK (Ad-CA AMPK) was injected into the MBH of a group of rats immediately after MBH catheter implantation. Venous and arterial cannulations were done on day 5, and the pancreatic clamp protocol was performed on day 8. In rats with no adenovirus injection, AICAR, saline, glucose, lactate, AICAR+glucose, or AICAR+lactate was infused into the MBH during the clamp experiments. In rats injected with Ad-GFP or Ad-CA AMPK, saline, glucose, or lactate was infused into the MBH during the clamp studies.
Figure 9. Hypothalamic administration of AICAR, the pharmacological activator of AMPK, negates the ability of hypothalamic glucose/lactate-sensing mechanisms to decrease glucose production.

A: Direct MBH infusion of glucose or lactate during the clamps increased glucose infusion rate (*P < 0.001) and lowered glucose production (B) (*P < 0.001) compared with those of MBH vehicle (AICAR/saline) treatments. MBH glucose or lactate co-infused with AICAR failed to increase glucose infusion rate (A) and lower glucose production (B) compared with those of vehicle treatments. C: Suppression of glucose production during the clamp period (180-210 min) expressed as the percentage reduction from the basal steady state (60-90 min) (*P < 0.05 vs. other groups). D: Glucose uptake was comparable in all groups. Values are shown as means ± SE.
Table 3. Body weights and plasma insulin, glucagon, and glucose concentration of rats treated with vehicle, glucose, lactate, AICAR + glucose or AICAR + lactate in the medibasal hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (kg)</th>
<th>Insulin (ng/ml)</th>
<th>Glucagon (pg/ml)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong>&lt;sup&gt;y&lt;/sup&gt; (n=6)</td>
<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>0.291 ± 0.010</td>
<td>1.0 ± 0.1</td>
<td>63 ± 5</td>
<td>144 ± 2</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.9 ± 0.1</td>
<td>56 ± 5</td>
<td>146 ± 6</td>
<td></td>
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<tr>
<td><strong>Glucose</strong> (n=5)</td>
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</tr>
<tr>
<td>Basal</td>
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<td>145 ± 9</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.9 ± 0.1</td>
<td>49 ± 4</td>
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<td><strong>Lactate</strong> (n=5)</td>
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<tr>
<td>Basal</td>
<td>0.295 ± 0.014</td>
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<td>58 ± 3</td>
<td>143 ± 3</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.9 ± 0.1</td>
<td>45 ± 3</td>
<td>140 ± 9</td>
<td></td>
</tr>
<tr>
<td><strong>AICAR + Glucose</strong> (n=5)</td>
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<tr>
<td>Basal</td>
<td>0.306 ± 0.002</td>
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<td>56 ± 7</td>
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</tr>
<tr>
<td>Clamp</td>
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<td>42 ± 7</td>
<td>152 ± 7</td>
<td></td>
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<td><strong>AICAR + Lactate</strong> (n=5)</td>
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<td>Basal</td>
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<td>Clamp</td>
<td>0.8 ± 0.1</td>
<td>49 ± 3</td>
<td>140 ± 8</td>
<td></td>
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</tbody>
</table>

Data are means ± SEM. Basal (t=0). Clamp (t=180-210).<sup>y</sup> Vehicle includes saline or AICAR infusion alone.
Direct MBH administration of glucose or lactate to the GFP treatment groups increased glucose infusion rate (A) (*P < 0.01) and lowered glucose production (B) (*P < 0.001) compared to those of GFP/saline and CA AMPK/saline groups during the clamps. Direct MBH administration of glucose or lactate during the clamps to the CA AMPK treatment groups failed to increase glucose infusion rate (A) and lower glucose production (B). C: Suppression of glucose production during the clamp period (180-210 min) expressed as the percentage reduction from the basal steady state (60-90 min) (*P < 0.01 vs. other groups). D: Glucose uptake was comparable in all groups. Values are shown as means ± SE.

Figure 10. Hypothalamic administration of the constitutively active form of AMPK (CA AMPK) negates the ability of hypothalamic glucose/lactate-sensing mechanisms to decrease glucose production.
Table 4. Body weights and plasma insulin, glucagon, and glucose concentrations of rats treated with Ad-GFP plus saline, Ad-CA AMPK plus saline, Ad-GFP plus glucose, Ad-GFP plus lactate, Ad-CA AMPK plus glucose, or Ad-CA AMPK plus lactate in the mediobasal hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (kg)</th>
<th>Insulin (ng/ml)</th>
<th>Glucagon (pg/ml)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ad- GFP + Saline (n=5)</strong></td>
<td>Basal</td>
<td>0.8 ±0.2</td>
<td>60 ±2</td>
<td>146 ±4</td>
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<td></td>
<td>0.270 ± 0.014</td>
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</tr>
<tr>
<td></td>
<td>Clamp</td>
<td>0.9 ±0.1</td>
<td>54 ±5</td>
<td>141 ±5</td>
</tr>
<tr>
<td><strong>Ad- CA AMPK + Saline (n=6)</strong></td>
<td>Basal</td>
<td>0.7 ±0.2</td>
<td>54 ±4</td>
<td>127 ±14</td>
</tr>
<tr>
<td></td>
<td>0.273 ± 0.017</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clamp</td>
<td>0.7 ±0.1</td>
<td>46 ±2</td>
<td>121 ±9</td>
</tr>
<tr>
<td><strong>Ad- GFP + Glucose (n=6)</strong></td>
<td>Basal</td>
<td>0.8 ±0.2</td>
<td>54 ±4</td>
<td>152 ±10</td>
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<td>0.283 ± 0.016</td>
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<td>Clamp</td>
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<td>52 ±3</td>
<td>144 ±11</td>
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<td><strong>Ad- GFP + Lactate (n=6)</strong></td>
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<td>58 ±5</td>
<td>142 ±4</td>
</tr>
<tr>
<td></td>
<td>0.284 ± 0.013</td>
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</tr>
<tr>
<td></td>
<td>Clamp</td>
<td>0.9 ±0.1</td>
<td>46 ±2</td>
<td>136 ±7</td>
</tr>
<tr>
<td><strong>Ad- CA AMPK + Glucose (n=6)</strong></td>
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<td>0.5 ±0.1</td>
<td>59 ±5</td>
<td>144 ±8</td>
</tr>
<tr>
<td></td>
<td>0.262 ± 0.014</td>
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<tr>
<td></td>
<td>Clamp</td>
<td>0.5 ±0.1</td>
<td>46 ±2</td>
<td>125 ±4</td>
</tr>
<tr>
<td><strong>Ad- CA AMPK + Lactate (n=7)</strong></td>
<td>Basal</td>
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<td>56 ±4</td>
<td>156 ±13</td>
</tr>
<tr>
<td></td>
<td>0.278 ± 0.024</td>
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<tr>
<td></td>
<td>Clamp</td>
<td>0.7 ±0.1</td>
<td>54 ±4</td>
<td>136 ±10</td>
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</table>

Data are means ± SEM. Basal (t=0). Clamp (t=180-210).
In the recent decade, AMPK has become increasingly well recognized as not only a simple intracellular energy sensor but also a master regulator of whole-body energy homeostasis [58]. Numerous groups around the world have independently shown that in the hypothalamus, nutrients and anorexigenic signals such as glucose, insulin, leptin and GLP-1 inhibit AMPK [64, 67, 91], whereas orexigenic signals such as ghrelin, adiponectin and cannabinoids activate it [59, 63]. In agreement with these findings suggesting that central AMPK mediates the regulation of energy balance, increasing the activity of hypothalamic AMPK per se via the administration of the pharmacological activator AICAR or the constitutively active regulatory subunit γ1 of AMPK increases food intake and body weight in rodents [60, 64], whereas inhibiting the AMPK catalytic α domains by the dominant negative mutant administration significantly lowers food intake and body weight [64]. Furthermore, a study done by Claret et al. using neuron-selective knockout of AMPK in the Arc also gave hint to the potential hypothalamic neuronal types mediating the effect of AMPK on energy intake [65]. Specifically, POMC neuron-specific AMPKα2 knockout mice displayed dysregulated food intake and decreased energy expenditure, which led to an obese phenotype. Together, these data highlight the importance of hypothalamic AMPK in the maintenance of energy homeostasis.

Accumulating evidence have illuminated the role of the hypothalamic Arc in regulating not only energy homeostasis, but also glucose balance [49]. Given the well characterized role of hypothalamic AMPK in the regulation of exogenous fuel intake, it is logical to ponder whether
hypothalamic AMPK also regulates endogenous glucose production in parallel. Although studies have shown that downregulating VMH AMPK suppresses counterregulatory glucagon and epinephrine responses to acute hyperinsulinemia-induced hypoglycaemia [92], it remained unclear whether changes in hypothalamic AMPK are sufficient _per se_ to regulate glucose production at basal levels of circulating glucoregulatory hormones. Furthermore, given the ability of the hypothalamic nutrient-sensing mechanisms in regulating glucose homeostasis independent of changes in the circulatory hormone levels, it is reasonable to postulate that changes in hypothalamic AMPK activity may be necessary for the ability of central nutrient-sensing to alter glucose production. Proceeding with these questions in mind, in the current study, we find that inhibiting hypothalamic AMPK activity was sufficient to lower glucose production at basal levels of insulin, and activating hypothalamic AMPK negated the ability of hypothalamic glucose/lactate sensing to decrease glucose production. These findings provide direct evidence for an extended metabolic regulatory role of hypothalamic AMPK to glucose production regulation.

Complementary pharmacological and molecular approaches were employed in the current study to manipulate hypothalamic AMPK activity. In light of findings that leptin, glucose and refeeding significantly reduce the activity of hypothalamic AMPK, and the knockdown of both α1 and α2 catalytic subunits of hypothalamic AMPK was sufficient per se to lower food intake and body weight [64], we first knocked down total hypothalamic AMPK activity by ~50% using adenovirus expressing the dominant negative form of AMPKα2 (Ad- DN AMPKα2 with a D^{157}A mutation), as provided by our collaborator Dr. Guy Rutter’s lab from Imperial College, UK. The aspartate 157 lies in the conserved DFG motif that is essential for protein kinase activity [93]. The mutation to alanine leads to an inactive kinase which inhibits total hypothalamic AMPK activity, likely by binding and sequestering AMPK β subunits, which leads
to the destabilization and decrease in the levels of both α1 and α2 [72]. In our experimental protocol, the suppression of glucose production during the pancreatic euglycemic clamps by Ad-DN AMPK injection was accompanied by hypophagia only on the night before the clamp studies and a trend toward a decrease in body weight comparing to the Ad-GFP injected controls. The modest effect of Ad-DN AMPK on food intake and body weight comparing to the previous studies done by Minokoshi et al [64] may be due to the confounding effects of the additional vascular surgeries of our protocol, leading to a greater variation in the rates of recovery of the rats. More chronic studies using adeno-associated virus may be able to reveal a more significant change in body weight over a longer period of time. In addition, future measurements of adiposity by assessing the changes in subcutaneous fat storage in response to Ad-DN AMPK may reveal a more prominent change in body fat composition that may not have translated to a significant change in body weight in our protocol. It is also interesting to note that Ad-DN AMPK did not significantly change the basal glucose production rate, which may be partly explained by the significant increase in the basal level of circulating glucagon, possibly as an adaptive response to the chronic effect of a decrease in hypothalamic AMPK activity. Next, due to the possibility that the slight decrease in food intake and body weight in our Ad-DN AMPK treated rats comparing to the controls may have partly contributed to the suppression of glucose production we saw during the clamps, we examined the acute infusion of the pharmacological inhibitor of AMPK, compound C on glucose production regulation.

Compound C or 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine, is a potent reversible inhibitor of AMPK that is competitive with ATP [83], which upon binding to the regulatory γ subunit, prevents the allosteric activation of AMPK by AMP [76]. During the clamp, in rats with comparable body weights and food intake, acute hypothalamic infusion of compound C significantly decreased glucose production to a similar
extend as the Ad-DN AMPK, independent of changes in the circulating glucoregulatory hormone levels. These data suggest that a decrease in hypothalamic AMPK activity is sufficient by itself to lower glucose production under basal insulin levels. In addition, the Ad-GFP injected rats with similar body weight and food intake as the Ad-DN AMPK injected rats also did not show a significant suppression of glucose production similar to the Ad-GFP injected rats that were heavier than the Ad-DN AMPK injected group, which further confirms our hypothesis that this effect on glucose production was independent of any changes in body weight or endogenous fuel intake. It should be noted however, that although compound C showed minimal activity towards some protein kinases such as zeta-chain-associated protein kinase (ZAPK), spleen tyrosine kinase (SYK), PKCθ, protein kinase A (PKA), Janus kinase (JAK) 3, glycogen phosphorylase, interferon regulatory factor 3 (IRF-3) and sarcoma kinase (SRC) [68, 83], it has been demonstrated to inhibit other kinases such as extracellular signal-regulated kinase 8 (ERK8), mitogen-activated protein kinase 1 (MNK1), maternal embryonic leucine zipper kinase (MELK) and dual specificity tyrosine-phosphorylation-regulated kinase (DYRK) in vitro [94], which may have potentially contributed to the effects seen in our study.

AMPK inhibition leads to the increase in the activity of ACC and a consequent increase in malonyl-CoA level, which in turn promotes the accumulation of LCFA-CoA by inhibiting CPT-1 and the uptake of LCFA-CoA into the mitochondria [58] (Figure 11). The observed effects of hypothalamic AMPK inhibition to lower glucose production is consistent with the ability of hypothalamic CPT-1 inhibition and the increase in hypothalamic malonyl-CoA and LCFA-CoA levels to lower hepatic glucose production [45]. It may also explain the recent finding that the whole-body deletion of the β1 regulatory subunit of AMPK in mice (AMPK β1-/-) improved insulin sensitivity and prevented high fat diet-induced insulin resistance [95]. In this study, total AMPK activity was significantly decreased in a number of tissues including the liver,
adipose tissues, and the hypothalamus. Importantly, these AMPK β1/- mice displayed a significantly lower level of glucose-6-phosphatase (G6Pase), the enzyme that catalyzes the last step of both gluconeogenesis and glycogenolysis, after overnight fasting [95]. This corresponded to a significant lowering of hepatic glucose production in response to insulin during the hyperinsulinemic euglycemic clamp studies. High fat diet feeding did not prevent the effect of insulin to lower glucose production in these AMPK β1/- mice, which suggests that inhibition of AMPK exerts a favorable effect on glucose homeostasis regulation. Interestingly, in contrast to this in vivo decrease in glucose production, in isolated hepatocytes from AMPK β1/- mice, gluconeogenic enzyme expression was elevated [95]. This suggests an important extrahepatic control over glucose production in the whole-body AMPK β1/- mice [95], which may potentially be the decrease in hypothalamic AMPK activity that lowers glucose production. Future measurements of ACC activity and the levels of malonyl-CoA and LCFA-CoA in the hypothalamus in the Ad-DN AMPK and compound C treated groups will help elucidate the potential metabolic pathway mediating the inhibition of hypothalamic AMPK to decrease glucose production. In addition, as we did not assess the activities of the enzymes involved in glucose production such as G6Pase and phosphoenolpyruvate carboxykinase (PEPCK) specifically in the hepatocytes, we cannot rule out the potential contributions of the kidney [96] and small intestines [97] to the changes in endogenous glucose production seen in our studies, although these contributions are expected to be minor in the short-term food-restricted states in our studies.

An elevation in the levels of circulating and hypothalamic glucose and its metabolite lactate is sensed by the hypothalamus, which in response activates a neuronal network to decrease hepatic glucose production [13]. Since glucose and lactate metabolism directly provides the substrate for the formation of acetyl-CoA (Figure 12), a substrate for AMPK, we next
investigated whether activating hypothalamic AMPK will negate the ability of hypothalamic glucose/lactate-sensing to lower glucose production. The pharmacological activator of AMPK, AICAR (5-amino-4-imidazole carboxamide riboside), has been extensively used to elucidate the role of hypothalamic AMPK in regulating food intake and body weight [60-62, 98]. AICAR is a cell-permeant adenosine analogue that is rapidly taken up into cells and phosphorylated to form ZMP (zinc metalloproteinase), an AMP mimetic that usually does not change the intracellular levels of AMP or ATP [99]. Co-infusion of AICAR with glucose/lactate in the hypothalamus during the pancreatic-euglycemic clamp effectively abolished the effects of glucose/lactate to lower glucose production. Since AICAR has also been shown to affect other AMP-sensitive metabolic enzymes such as glycogen phosphorylase in addition to AMPK, which could have confounding effects [100], we also complemented our studies with the molecular activation of hypothalamic AMPK via an adenovirus expressing a truncated constitutively active Ad-CA AMPKα1\(^{312}\) [T\(^{172}\)D] that is common to both AMPKα1 and α2 [101]. Threonine 172 of the catalytic subunits is the major site of phosphorylation and activation by AMPK kinases [102] and the mutation to aspartate prevents the dephosphorylation and inactivation by protein phosphatases [103]. This effectively leads to a substantial increase in the total activity of AMPK [72]. Similar to the effect of AICAR, Ad-CA AMPK injection prevented the effect of central glucose/lactate to lower glucose production during the clamp studies. This is consistent with hypothesis that glucose/lactate-sensing shares a parallel biochemical pathway as LCFA-sensing in the hypothalamus. It is possible that an increase in hypothalamic glucose/lactate metabolism accumulates acetyl-CoA, which promotes the formation of malonyl-CoA and LCFA-CoA accumulation; AMPK activation inhibits ACC activity and blocks the increase in malonyl-CoA and LCFA-CoA thereby negating the central glucose sensing mechanisms to lower glucose production. However, this biochemical pathway remains to be validated through future
assessment of ACC activity and malonyl-CoA and LCFA-CoA levels. It is interesting to note that activation of hypothalamic AMPK did not by itself increase glucose production, but negated central nutrient-sensing mechanism to lower glucose production. This observation also warrants future exploration, however, it is worth pointing out that a lowering of hypothalamic malonyl-CoA through the overexpression of MCD by itself also did not increase glucose production, but negated the hypothalamic nutrient-sensing mechanism to lower glucose production [46]. Considering that T2DM is a chronic disease, the effects of chronic activation of hypothalamic AMPK over a few months in the context of glucose homeostasis regulation may provide more insights into the role of hypothalamic AMPK in the development of T2DM.

As stated earlier, the NPY/AgRP and POMC neurons are implicated in sensing hormonal and nutritional signals to regulate both energy and glucose homeostasis [33, 49]. Although in our study, we did not investigate whether changes in hypothalamic AMPK in the NPY/AgRP and POMC neurons specifically regulate glucose production, we did observe that our Ad-GFP injections into the mediobasal hypothalamus were largely co-localized with either AgRP or POMC. This raises the possibility that changes in hypothalamic AMPK activity in the NPY/AgRP and POMC neurons regulate glucose production under our experimental protocol. In connection with this hypothesis, a recent study showed that POMC neuron-specific deletion of LKB1 (one of the AMPK kinases that activate AMPK), in female mice resulted in impaired glucose tolerance and increased hepatic glucose production during the hyperinsulinemic euglycemic clamps [104]. It should be noted however, that co-localization analysis software such as ImarisColoc should be employed in the future to more accurately assess the percent co-localization of the adenovirus to the specific neuronal types. Direct manipulations of AMPK activity in these neuronal types are also required to determine the specific roles they play in mediating the effect of hypothalamic AMPK in glucose production regulation. Additionally with
regards to glucose production regulation, we also cannot rule out the potential effects of our adenoviruses on other areas of the MBH in particular the VMH neurons, in which hypothalamic AMPK is required for the counterregulatory hormone responses to hypoglycemia.

In summary, our study provides evidence that changes in hypothalamic AMPK activity not only modulates exogenous fuel intake but also regulate endogenous glucose production. Although this suggests the possibility that inhibiting hypothalamic AMPK may represent a novel therapeutic means to lower glucose production and plasma glucose levels in diabetes and obesity, future experiments will be required to assess the chronic effects of inhibiting hypothalamic AMPK over a longer period. It should also be noted that in the peripheral tissues, activation and not inhibition of AMPK lowers plasma glucose levels and improves glucose homeostasis [105].

Studies have implicated AMPK in the skeletal muscles in mediating the beneficial effects of exercise-induced carbohydrate and fatty acid metabolism, mitochondrial biogenesis, and an enhancement in insulin stimulated glucose uptake [58]. AICAR-induced activation of AMPK increases the expression, translocation and fusion of the insulin-sensitive glucose transporter 4 (GLUT4) to the plasma membrane [106-111]. When AMPKγ3 was knocked out, expression of GLUT4 in response to exercise was blunted [112]. In the adipose tissues, AMPK activation have been shown to inhibit lipolysis possibly through direct phosphorylation of hormone-sensitive lipase, preventing its activation by protein kinase A [113]. As T2DM is also characterized by increased circulating lipid levels, due partly to the impaired insulin-mediated suppression of lipolysis from adipocytes [3], and circulating fatty acids can lead to insulin resistance, AMPK activation in adipocytes is also favorable in improving glucose homeostasis.

In another peripheral glucoregulatory organ, the liver, AMPK activation suppresses transcription of the gluconeogenic genes G6Pase and PEPCK [114]. Liver-specific knockout of
AMPKα2 displayed glucose intolerance and fasting hyperglycemia, likely due to the increase in gluconeogenesis and glucose production [105, 115]. In addition to the favorable effects to lower glucose production, liver AMPK activation also inhibits lipogenesis by suppressing glucose-induced-expression of genes such as fatty acid synthase, ACC and pyruvate kinase [81, 83]. Taken together, AMPK activation in the liver lowers both glucose and lipid production. It is interesting to note that in the liver, an accumulation of LCFA-CoA increases glucose production under hyperinsulinemic-euglycemic clamp conditions [116]. Given that AMPK activation decrease LCFA-CoA concentration, it is reasonable to postulate that AMPK activation in the liver lowers glucose production via modulating the fatty acid metabolism pathway as hypothesized in the current study in the hypothalamus. The interesting observation that the hypothalamus and peripheral tissues seem to share similar biochemical pathways, but with different physiological outcomes may suggest a unique mammalian evolved trait to prevent a system from over-activation or great fluctuations in order to maintain whole-body homeostasis.

AMPK is activated by metformin and thiazolidinediones, two of the widely used treatments for T2DM [58, 117] and given the mounting interest in targeting AMPK to alleviate hyperglycemia [100], differential role of hypothalamic AMPK in the regulation glucose production unveiled in our study indicate caution be taken in developing drugs that target AMPK to treat diabetes and obesity.
6 Future Directions

This thesis identified a novel role of hypothalamic AMPK in the regulation of glucose production and glucose homeostasis. Our findings open up several potential lines of investigation for future work, as discussed below (Figure 11, 12):

1. Although we have shed light on the role of hypothalamic AMPK in glucose production regulation under basal insulin euglycemic clamp conditions, it remains unclear the physiological circumstances in which this pathway is involved. During physiological fasting and subsequent refeeding, plasma glucose level rises rapidly, but this rise is restrained due to an important physiological mechanism to suppress hepatic gluconeogenesis and glucose production in the early post-prandial state [118]. Studies show that refeeding can reduce the activity of hypothalamic AMPK [64]. If this reduction in hypothalamic AMPK activity is required for the physiological suppression of glucose production, then activating hypothalamic AMPK during refeeding may negate the ability of nutrient influx to lower glucose production and thereby leading to a greater elevation in blood glucose level.

2. In continuation from the previous hypothesis that an inhibition in hypothalamic AMPK may be required for the physiological refeeding to restrain glucose production, it will be of interest next, to investigate whether this pathway is dysfunctional in models of T2DM. In the early onset (3 day) high-fat diet-induced insulin resistance model, central fatty acid fails to lower glucose production [19]. Since in this thesis, we have predicted that an
inhibition of hypothalamic AMPK in the normal rodents lower glucose production by modulating hypothalamic fatty acid metabolism, investigating whether inhibition of hypothalamic AMPK activity will similarly lower glucose production in this disease model may help to pinpoint the step that is defective in high-fat diet-induced insulin resistance.

3. The observed effects of hypothalamic AMPK inhibition to lower glucose production is consistent with the ability of hypothalamic CPT-1 inhibition and the increase in hypothalamic malonyl-CoA and LCFA-CoA levels to lower hepatic glucose production [45]. Moreover, since hypothalamic AMPK activation prevented central glucose/lactate sensing’s ability to lower glucose production, it hints at the possibility that the glucose production-lowering effects of glucose/lactate sensing pathway may also depend on an accumulation of hypothalamic LCFA-CoA. However, these hypotheses regarding the downstream mechanisms through which central glucose/lactate sensing and AMPK inhibition lowers glucose production remain to be confirmed. A recent study reported that hypothalamic protein kinase C-δ (PKC-δ) lies downstream of lipids and upstream of the hypothalamic $K_{ATP}$ channels to lower glucose production [47]. It is of interest to investigate, in future follow-up studies, whether activation of hypothalamic PKC-δ (or another PKC isoform) is also required for the ability of central glucose and lactate-sensing/AMPK inhibition to lower glucose production. Furthermore, since the hypothalamic $K_{ATP}$ channel is indispensable in mediating nutrients and hormones to send signals via the hepatic vagus to lower glucose production, it should be investigated whether its activation is also required for hypothalamic AMPK inhibition to lower glucose production.
4. Our study has provided a hint at the hypothalamic neuronal types in which changes in AMPK activity can alter glucose production. Specifically, MBH injection of adenovirus expressing GFP largely co-localized AgRP with and POMC in the Arc. In the future, AgRP or POMC neuron-specific knockouts or over-expression of AMPK using recombinant techniques will allow a more close examination of the roles of hypothalamic AMPK in the different neuronal types in regulating glucose production. In fact, AgRP and POMC neuron-specific knockouts of AMPK have been studied by Claret et al. in regards to food intake and energy homeostasis regulation [65]. Given the role of the AgRP and POMC neurons in mediating the effects of glucoregulatory hormones such as insulin, leptin and GLP-1 to lower glucose production, the future investigation of AMPK in AgRP and POMC neurons in glucose production regulation will be especially relevant to research on central control of glucose balance.

5. Although we have implicated in this study, the role of hypothalamic AMPK in mediating the central nutrient-sensing mechanism, it is currently unclear whether AMPK plays a role in the central hormone-sensing pathways in the regulation of glucose homeostasis. Extensive research has been conducted implicating hypothalamic AMPK in mediating the central effects of hormones to regulate food intake and body weight. Specifically, anorexigenic signals such as insulin, leptin and GLP-1 inhibit AMPK [64, 67, 91], and activating it prevents their anorexigenic effects [64]. Given the central role of these same hormones in lowering glucose production, it follows logically to next investigate whether an inhibition of hypothalamic AMPK is also necessary in mediating the effects of these hormones to regulate glucose production and glucose homeostasis.
The downstream pathway mediating the effect of DN AMPK/compound C to lower glucose production remains to be investigated. We hypothesize that inhibiting hypothalamic AMPK via DN AMPK or compound C leads to an increase in ACC activity and hypothalamic malonyl-CoA level; malonyl-CoA inhibits CPT-1 and the uptake of LCFA-CoA into the mitochondria for β-oxidation, thereby leading to the accumulation of hypothalamic LCFA-CoA. LCFA-CoA signals the liver to decrease glucose production via hypothalamic PKC and K<sub>ATP</sub> channels. Future studies also include: investigating whether hypothalamic AMPK inhibition mediates the effects of refeeding and anorexigenic hormones to lower glucose production; investigating whether high fat diet will impair the ability of AMPK inhibition to lower glucose production and determining whether the effect of AMPK inhibition was mediated by AgRP and POMC neurons.
Figure 12. Future direction following Aim 2.

The downstream mechanism of hypothalamic glucose/lactate sensing to lower glucose production remain to be dissected. We hypothesize that glucose and lactate provides the substrates for acetyl-CoA generation, which in turn leads to an increase in the levels of hypothalamic malonyl-CoA and LCFA-CoA to decrease glucose production. Activation of hypothalamic AMPK via CA AMPK or AICAR leads to the inhibition of ACC and prevents formation of malonyl-CoA from acetyl-CoA, thereby inhibiting the effect of glucose/lactate to lower glucose production. It also remains to be investigated whether this effect of hypothalamic AMPK activation was mediated by AgRP/POMC neurons.
7 References


77. Davies, S.P., et al., 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially


