CENTRAL NERVOUS SYSTEM NUTRIENT-SENSING AND THE REGULATION OF ENERGY AND GLUCOSE HOMEOSTASIS

by

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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 lactate metabolism regulates hepatic glucose and lipid homeostasis, however it remains unclear whether hypothalamic lactate also controls energy homeostasis. Furthermore, the precise downstream molecular and signaling pathway(s) involved in hypothalamic lactate-sensing is yet to be fully elucidated. To specifically address these two questions, we tested the hypothesis that hypothalamic lactate metabolism regulates energy homeostasis (Study 1) and assessed whether the activation of N-methyl-D-aspartate (NMDA) receptors in the nucleus of the solitary tract (NTS) of the brainstem is required for hypothalamic lactate, and sufficient *per se*, to regulate glucose homeostasis (Study 2). In an *in vivo* rat model, we reported in Study 1 that central lactate lowers food intake and body weight through its metabolism into pyruvate. In Study 2, we identified that hypothalamic lactate metabolism requires the activation of NMDA receptors in the NTS to lower hepatic glucose production. Moreover, we showed that the activation of NTS NMDA receptors *per se* lowers hepatic glucose production. In summary, these findings advance the understanding of central nutrient-sensing in the regulation of energy and glucose homeostasis, which is critical in bridging the therapeutic gap of obesity and type 2 diabetes.

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way to thank them than to love them and care for them as they have for me. Second, I thank my heavenly Father, who knows me better than I know myself. I praise Him for allowing me to experience Him in miraculous ways as I unveil this perfect plan that He has for me. May my mouth continue to speak of His glory, my hands do His work, and my life presented as a living sacrifice for Him.

Trust in the Lord with all your heart and lean not on your own understanding; in all your ways acknowledge Him and he will make your paths straight.

(Proverbs 3:5-6)

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2DG	2-deoxy-D-glucose
ACC	Acetyl-CoA carboxylase
AgRP	Agouti-related peptide
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ARC	Arcuate nucleus
BBB	Blood brain barrier
BW	Body weight
CCK	Cholecystokinin
CMV	Cytomegalovirus
CNS	Central nervous system
CPT-1	Carnitine palmitoyltransferase 1
CTB	Cholera toxin B-subunit
DCA	Dichloroacetate
FAS	Fatty acid synthase
FI	Food intake
GLP-1	Glucagon like peptide 1
GP	Glucose production
ICV	Intracerebroventricular
IH	Intrahypothalamic
IL	Interleukin
IP	Intraperitoneal
IR	Insulin receptor
IRS	Insulin receptor substrate
IV	Intravenous

Jak2	Janus kinase 2
K_{ATP} channel	ATP-sensitive potassium channel
LCFA	Long-chain fatty acid
LCFA-CoA	Long-chain fatty acyl-coenzyme A
LDH	Lactate dehydrogenase
LH	Lateral hypothalamus
LRb	Leptin receptor (long form)
MCD	Malonyl-coenzyme A decarboxylase
MCR	Melanocortin receptor
mTOR	Mammalian target of rapamycin
NIRKO	Neuron-specific insulin receptor disrupted
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
PDH	Pyruvate dehydrogenase
PI_3K	Phosphatiylinositol-3 kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
R_a	Rate of glucose appearance
RIA	Radioimmunoassay
S6K	S6 kinase
SD rat	Sprague Dawley rat
SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
STZ	Streptozotocin

T2DM	Type 2 diabetes
UCP2	Uncoupling protein 2
VMH	Ventromedial hypothalamus
VMN	Ventromedial nucleus
α-MSH	α-melanocyte stimulating hormone

PUBLICATIONS THAT CONTRIBUTED TO THIS THESIS

1. **Lam CK**, Chari M, Wang PY, Lam TK. Central lactate metabolism regulates FI. *Am J Physiol Endocrinol Metab*. 295(2):E491-6 (2008). (Used with permission)

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1
INTRODUCTION

1.1 OBESITY AND DIABETES

Obesity is increasingly prevalent worldwide and has reached epidemic proportions. It is tightly associated with type 2 diabetes (T2DM) (99), cardiovascular disease (109), and various cancers (44). Obesity is characterized by a disruption in energy homeostasis, whereby energy (food) intake exceeds energy expenditure.

Normally, our body is equipped with physiological and biochemical responses to counteract day-to-day fluctuations in food intake (FI). For instance, acute elevations in insulin and leptin induced by nutrients (235) decrease FI and increase energy expenditure (164). Conversely, the fasting state shifts the energy balance such that energy stores are maintained by an increase in FI (156). As a result of these energy status feedback signals, the caloric storage and body weight (BW) are generally stable for most humans over long periods of time despite the wide variations in day-to-day FI patterns. Unfortunately, chronic exposure to high caloric diets combined with reduced physical activity lead to a disruption in this precise energy homeostatic control (164).

Since the aforementioned defect lies in the increase in FI and decrease in energy expenditure, it is thus important to understand the underlying mechanisms that control both of these parameters in the normal setting, and subsequently to evaluate whether such mechanisms are impaired in obesity. *One of the goals of this thesis is to dissect the regulatory mechanisms of FI.*

In addition to the control of energy homeostasis, the body also adapted precise mechanisms to regulate glucose levels. Diabetes, affecting approximately 170 million

individuals worldwide and expected to more than double within 30 years (291), is a disease characterized by an impairment in glucose homeostatic control leading to hyperglycemia.

There are two distinct forms of diabetes, namely Type 1 and Type 2. Type 1 diabetes is an autoimmune disease marked by absolute insulin deficiency due to destruction of insulin-producing beta cells of the pancreas (61). On the other hand, T2DM, which is tightly linked to obesity, is due to a combination of insulin resistance and inadequate insulin secretion. Insulin resistance is defined as the inability of insulin to (i) stimulate glucose uptake into muscle and fat and (ii) suppress glucose production (GP) in the liver in response to a glucose load (30) This, together with inadequate insulin secretion, causes chronic hyperglycemia, leading to diabetic complications (43; 279). Some of the T2DM complications include retinopathy (134), nephropathy (185), neuropathy (1; 258), heart attacks, strokes (43), cardiovascular diseases (186),

With the extensive list of secondary health concerns, combating the adverse impact of uncontrolled diabetes is a tremendous struggle, especially for developing countries (180). Therefore, attempts to address the underlying cause of diabetes to develop more long-term treatments are critical. As diabetes is characterized by hyperglycemia, *the second goal of this thesis is to further dissect the underlying mechanisms that regulate glucose levels*.

1.2 THE ROLE OF THE HYPOTHALAMUS: REGULATION OF ENERGY AND GLUCOSE HOMEOSTASIS

The central nervous system (CNS) has been documented to regulate whole body homeostasis. Ranging from the respiratory system to the circulatory system, thermoregulation to energy expenditure, the CNS plays a fundamental role in these homeostatic controls. Of the entire CNS, the hypothalamus in particular is generally accepted to mediate the day-to-day regulation of a number of factors including body temperature (96), blood pressure (108), thirst (12) and hunger (247), and is a fundamental structure for the integration of the nervous and endocrine systems.

The first demonstration of the hypothalamus serving as a satiety centre was conducted six decades ago, whereby hyperphagia and obesity resulted after the ventromedial nucleus (VMN) of the hypothalamus was subjected to bilateral lesions (115). In parallel, the first hint at hypothalamic control of glucose homeostasis was via lesion of the floor of the fourth ventricle, which resulted in glucosuria (24). It was not until the recent decades, however, that the boom in the field of CNS regulation of energy and glucose homeostasis occurred. To date, the hypothalamus has been extensively shown to sense hormones and nutrients in order to regulate not only energy homeostasis (65; 166; 188; 280; 295) but also glucose homeostasis (63; 95; 124; 136; 142; 151; 206; 208; 213). Based on these rather remarkable experimental findings of the recent past, the arcuate nucleus (ARC) has emerge as the ‘master’ nuclei for the

regulation of energy and glucose homeostasis. *The goal of this thesis is to further dissect the CNS mechanisms that control both energy and glucose homeostasis.*

1.2.1 Hypothalamic Neuropeptide Expression

From an anatomical perspective, it can be seen why the ARC is the key site for hormonal- and nutrient-sensing mechanisms to regulate energy and glucose homeostasis. The ARC is situated around the base of the brain's third ventricle and lies immediately above the median eminence where the capillary endothelium lacks tight junctions (293), forming an incomplete blood-brain barrier (BBB). Therefore, the ARC is more accessible to circulating hormones and nutrients. The ARC contains an array of neuronal subtypes that are involved in energy and glucose homeostatic regulations, of which two are most widely studied. The first are neurons that express the anorexigenic products of the peptide pro-opiomelanocortin (POMC). POMC is post-translationally cleaved to a series of smaller peptides such as α-melanocyte stimulating hormone (α-MSH), which by binding the melanocortin receptor 4 (MC4R) inhibits feeding (248). Belonging to the second subtype are neurons that co-express the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP). The activation of these orexigenic neurons stimulates feeding via (i) an increase in NPY/AgRP signaling (247), and (ii) antagonism of the anorexigenic effect of POMC through direct synapses with POMC neurons or competitive binding of AgRP to the α-MSH binding site at MC4R (Figure 3).

Indeed, increasing the expression of the orexigenic neuropeptides NPY (56) or AgRP (209) increases FI and BW while central POMC or its analogues administration reduces FI, increases energy expenditure and promotes weight loss (84; 278). In

accordance, antagonism of the melanocortin signalling pathway, which is important for POMC action, resulted in obesity (5). Additional support for a critical role of NPY/AgRP and POMC neurons in energy homeostasis comes from the fact that gene expression of AgRP, NPY and POMC changes acutely, and correspondingly, to adjustments in energy status triggered by caloric deprivation or excess (19; 80; 81).

The aforementioned interplay between the orexigenic NPY/AgRP and anorexigenic POMC neuronal subsets and their downstream effector signalling form the melanocortin signalling system. It is the activation of this "hypothalamic melanocortin tone" (25) that is instrumental in the regulation of glucose homeostasis as well (Figure 3). Indeed, direct activation of the central melanocortin system by central administration of melanocortin agonists improves glucose homeostasis (85; 207). Oppositely, intracerebroventricular (ICV) administration of NPY or melanocortin receptor antagonist causes insulin resistance independent of changes in FI (5; 170; 282). Interestingly, rates of peripheral glucose disposal and lipolysis were unaffected in the NPY-treated group (282).

Based on the above findings, the hypothalamus, especially the ARC, is poised to integrate a variety of hormonal and metabolic signals to regulate energy and glucose homeostasis. And as obesity and diabetes are characterized by hyperphagia and hyperglycemia, the characterization of these hormone- and nutrient-sensing pathways in the hypothalamus that regulate energy and glucose homeostasis is critical in understanding the defects that underlie the disrupted homeostatic controls in obesity

and diabetes. *The focus of this thesis is to dissect the underlying mechanisms of CNS nutrient-sensing that regulate both energy and glucose homeostasis.* However, to first gain an overall perspective of how the CNS regulates FI and glucose levels, we will first review the relevant literature that highlights the role of CNS-sensing mechanisms that are activated by hormones.

1.2.2 Hormonal Action in the Hypothalamus

Insulin

The well-studied and extensive actions of insulin in the periphery, from altering hepatic glucose metabolism to modifying extrahepatic functions, converge at one aim – to ensure that glucose homeostasis is maintained. In the recent decades, the action of insulin has been uncovered to extend beyond the periphery and the realm of glucose regulation. The seminal study by Woods *et al*. illustrated for the first time that increase in central insulin reduced FI and BW in baboons (297), a finding later demonstrated in both rodents (177) and humans (110). These anorectic effects of insulin require its downstream signaling including the binding to insulin receptors (IR) (8; 39; 265) and activation of phosphatidylinositol-3 kinase (PI_3K) (200) (Figure 1a).

The central action of insulin is not reserved for energy homeostatic control as neuron-specific insulin receptor disrupted (NIRKO) mice were found to develop mild insulin resistance and elevated plasma insulin levels in association with obesity (54). This suggested, for the first time, that neuronal insulin signaling regulates peripheral glucose homeostasis. Indeed, ICV infusion of insulin or its mimetic, Cpd-1, suppressed hepatic GP independent of alterations in BW or changes in circulating levels of insulin and other glucoregulatory hormones (208). As with the regulation of energy homeostasis, an intact insulin-signaling cascade is critical in such regulation. This cascade involves the binding of insulin to its receptor (205; 208), as well as the activation of insulin receptor substrate (IRS) (95), PI_3K (208) and protein kinase B (PKB, or Akt) (95) (Figure 1a).

Further delineating the mechanism downstream of central insulin-signaling, the activation of central ATP-sensitive potassium (K_{ATP}) channels (205; 224) and efferent vagal fibres (224) appears to be required. Such activations likely trigger an interleukin (IL)-6/ signal transducer and activator of transcription (STAT) 3 signaling cascade in the liver to lower GP (124). It remains to be determined how the insulin-signalling cascade (i.e. IR → IRS-2 → PI_3K → PKB) leads to the activation of K_{ATP} channels. However, the involvement of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) has been suggested. Constitutive activation of PI_3K-PIP_3 signalling in POMC neurons increase K_{ATP} channel conductance, which hyperpolarizes neurons to result in a hyperphagic phenotype (223). Interestingly, seemingly contradictory to its anorectic nature, insulin actually profoundly activates PIP_3 formation leading to POMC cell hyperpolarization (53; 223). This strongly argues that the appetite-suppressive effects of insulin is unlikely mediated by the POMC neurons (60) (Figure 1a). Indeed, IR knockout mice in specific neuronal populations indicate that only AgRP-IR knockout mice, but not POMC-IR knockout mice, failed to suppress hepatic GP in response to elevated circulating insulin and had reduced insulin-stimulated hepatic IL-6 expression independent of changes in energy homeostasis (145). Moreover, ICV infusion of a potent MC3/4R antagonist did not alter the effect of circulating insulin to inhibit hepatic GP (208) but ICV NPY infusion precludes the inhibition of GP elicited by circulating insulin. Together, these data suggest that central insulin operates on a melanocortin-independent pathway, signaling through NPY/AgRP and not POMC, to regulate not only energy homeostasis, but also hepatic GP (Figure 1a).

Leptin

As with insulin, the discovery of leptin (302) was indeed another milestone in obesity and diabetes research. A deficiency of leptin – seen in leptin deficient *ob/ob* or leptin-receptor deficient *db/db* mice (90) – causes severe hyperphagia and obesity (247). Selective leptin replacement in uncontrolled, insulin-deficient diabetic rats was sufficient to prevent diabetic hyperphagia (257). It is important to note that obesity due to defective leptin-signaling might in part be contributed to by a reduction in energy expenditure. Deficiency of leptin or its receptor decreases physical activity, a phenotype that is normalized with restoration of leptin signaling (63; 220). Localization was further made to the ARC since selective restoration of leptin-signaling in the ARC of leptin receptor null mice significantly increased locomotor activity (63).

In both rodents and humans, deficiency in leptin or its functional receptors not only leads to profound obesity but also insulin resistance and other endocrine deregulations (6; 58; 90). In fact, observations strongly suggest that leptin, just as insulin, can regulate glucose homeostasis independent of changes in BW. Chronic increases in plasma leptin, independent of changes in BW, enhances insulin action, reverses insulin resistance and improves glucose homeostasis in lipodystropic rodents (20; 73; 254). Furthermore, leptin-treated ob/ob mice had a 40% reduction in glucose and insulin levels compared to pair-fed ob/ob mice (245). The hypothalamic ARC has been spotlighted as the key site for these glucose homeostatic effects. Restoration of leptin signaling in the ARC was sufficient to dramatically improve hyperinsulinemia and normalize blood glucose levels while only modestly reducing FI and body fat mass (63).

In addition, leptin receptor restoration improved insulin sensitivity in leptin receptor-deficient Koletsky (fa^k/fa^k) rats (189).

It has now come to be known that leptin, upon binding to its receptor in the ARC, activates two independent intracellular signaling cascades, which work in concert to regulate glucose homeostasis (Figure 1b). The first of the two is the well-established STAT3-dependent pathway. Upon binding of leptin to the long form of leptin receptor (LRb), Janus kinase 2 (Jak2) is activated, leading to the phosphorylation of cytoplasmic targets such as STAT3 (192). The activation of STAT3 is required for the regulation of energy (22) and, more recently considered, glucose homeostasis (41) of leptin. In line with this, the inactivation or deficiency of a negative regulator of the JAK-STAT pathway, suppressor of cytokine signaling (SOCS) 3, in selective brain regions and neurons increase leptin sensitivity and improve glucose homeostasis (136; 301) (Figure 1b).

While the aforementioned STAT3-dependent pathway is imperative, it does not stand solo in mediating the effect of central leptin. Knowing that leptin, like insulin, requires the activation of hypothalamic PI_3K to reduce FI (201), it seems that the binding of leptin to its receptor to activate PI_3K is a likely candidate. Indeed, hypothalamic infusion of PI_3K inhibitor curtailed the improvement in insulin sensitivity elicited by restoration of functional ARC leptin receptors in leptin receptor deficient fa^k/fa^k rats (189). This suggests that hypothalamic leptin, like insulin, activates PI_3K to regulate glucose homeostasis. However, it is highly plausible that the activation of PI_3K by leptin and insulin to regulate glucose homeostasis occurs in different neuronal populations

since leptin activates PI_3K in POMC but not NPY/AgRP neurons (300), whereas insulin signaling in AgRP but not POMC neuron regulates glucose homeostasis. Consistent with this view, selective deletion of SOCS3 in POMC neurons enhances leptin action and improves glucose homeostasis (136) (Figure 1b). Nonetheless, the role of the downstream effectors of leptin-PI_3K signaling cascade that regulate glucose homeostasis remains to be elucidated.

Glucagon Like Peptide – 1 (GLP-1)

GLP-1 is a potent hormone secreted by the L-cells of the intestines (284) and discrete populations of neurons (129). Traditionally, this gut hormone is thought to regulate glucose homeostasis via its incretin effects, namely by directly acting on the beta cells to stimulate insulin secretion and biosynthesis, decrease glucagon secretion and promote pancreatic beta cell growth (71). However, with the discovery of GLP-1 receptor mRNA widely present in the brain, including, but not limited to, the hippocampus, amygdale, hypothalamic nuclei such as the ARC and paraventricular nucleus (PVN), and the hindbrain (181), the action of central GLP-1 is now widely established as a controller of FI. ICV injection of GLP-1 causes anorexia (270; 280). However, the validity of the findings were quickly questioned following the observation that the same dose of ICV GLP-1 that reduced feeding also developed strong conditioned taste aversion (283), suggesting that the anorectic effect of ICV GLP-1 might be an aversive side-effect. However, based on the observation that selectively blocking

endogenous GLP-1 release causes hyperphagia in physiologically healthy animals (30) and the subsequent recognition of the central nucleus of the amygdale as the site responsible for the visceral illness response of GLP-1 (139), the PVN and hindbrain have now come to be known as mediators of the anorectic effect of CNS GLP-1 (100; 179) (Figure 1c).

It was traditionally thought that action of CNS GLP-1 was exclusively reserved for the regulation of FI and BW. Rebutting this initial attribution, however, emerging studies are pointing at the direct regulation of peripheral glucose homeostasis by CNS GLP-1. Of note, utilizing ICV infusion of GLP-1 antagonist and agonist, it was found that CNS GLP-1 signaling is involved in regulating peripheral insulin secretion and partitioning of glucose disposal (142). These changes collectively increase hepatic glycogen storage in preparation for the next fasting state (142). A similar increase in insulin secretion upon an intravenous (IV) glucose tolerance test was observed with direct ICV GLP-1 administration (239). Interestingly, while GLP-1 receptors are found in the ARC and do not regulate FI (239), they do mediate GLP-1 action to regulate peripheral glucose homeostasis (239) (Figure 1c). Administration of GLP-1 in to the ARC effectively lowered hepatic GP, a phenomenon not reproducible with GLP-1 administration into the PVN (239). While it remains unknown to date how the CNS GLP-1 system is activated and the mechanism(s) behind CNS GLP-1 regulation of glucose homeostasis is yet to be clarified, the activation of K_{ATP} channels represent a possible candidate. Co-infusion of K_{ATP} channel blocker prevented the GLP-1-induced suppression of GP (239). Furthermore, this GP-suppressing effect of central GLP-1 appears to be POMC-mediated as GLP-1

receptors largely co-localizes with POMC, and not NPY/AgRP neurons in the ARC (239) (Figure 1c).

In essence, hormones such as insulin, leptin and GLP-1 act centrally to regulate energy and glucose homeostasis. These hormones act on respective receptors in the CNS and exert their effects via potentially shared or distinct signaling pathways. However, much is still to be studied and evaluated to identify the potential convergence or divergence.

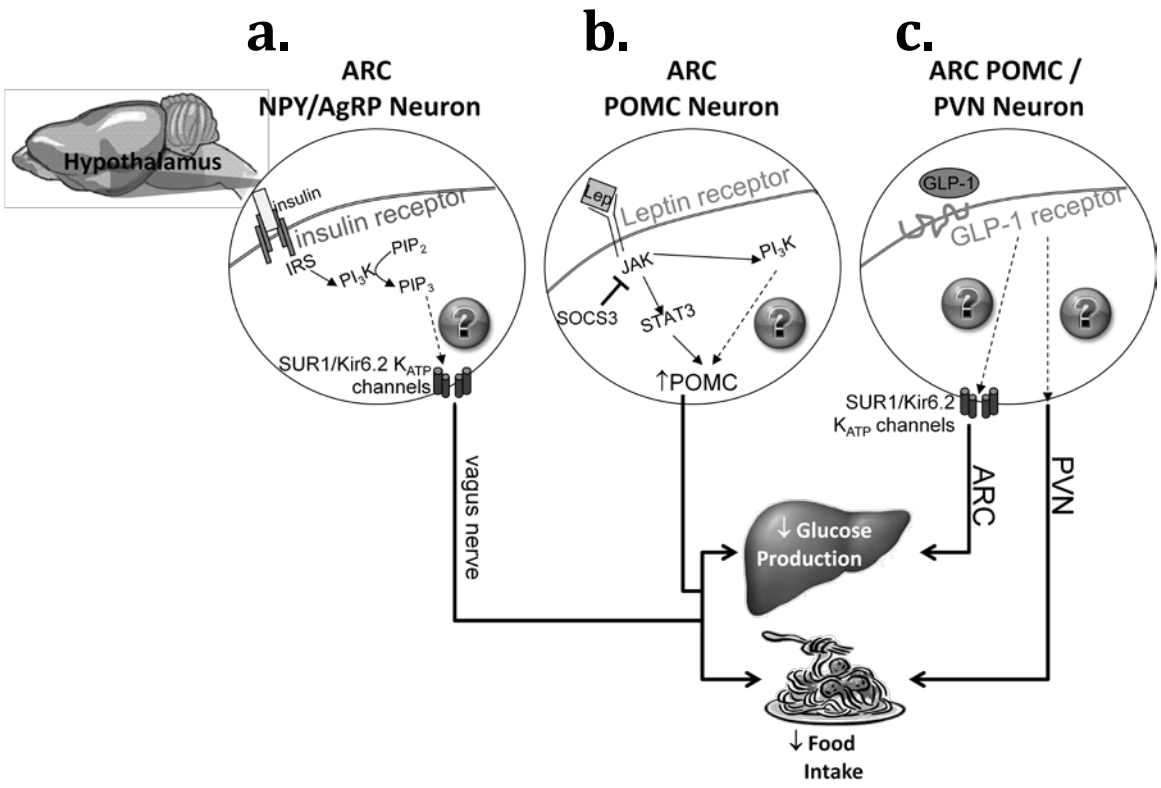


Figure 1 Hormonal action in the hypothalamic arcuate nucleus regulates energy and glucose homeostasis

a: Insulin, binding to its receptor, activates insulin receptor substrate (IRS) and phosphatidylinositol-3 kinase (PI_3K). PI_3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), which subsequently activates the SUR1/Kir6.2 K_{ATP} channels to alter signaling in neurons such as the NPY/AgRP neuron. Via a melanocortin-independent pathway which is relayed through the vagus nerve, hepatic glucose production and food intake are decreased. **b:** Leptin, upon binding to the long form of the leptin receptor, activates the Janus kinase 2 (JAK)/ signal transducer and activator of transcription (STAT) 3 pathway to increase POMC and decrease NPY/AgRP (not shown here) neuropeptide expressive, summating to a decrease in FI. In addition, Leptin has more recently been found to activate PI_3K. Together with the JAK/STAT3 pathway, these signaling cascades increase POMC neuron activity, which results in a decrease in hepatic glucose production. **c:** Paraventricular nucleus (PVN) and hindbrain (not shown here) GLP-1 has been found to decrease FI. More recently, ARC GLP-1 is indicated to decrease hepatic glucose production, likely through SUR1/Kir6.2 K_{ATP} channel-dependent mechanisms in the POMC neurons.

1.2.3 Hypothalamic Nutrient Sensing

In addition to processing input from hormones, the hypothalamus senses nutrients to initiate metabolic responses to regulate energy and nutrient homeostasis. Proposing a role of "nutrient-sensing", i.e. the acute accumulation of nutrients, *per se* in the regulation of homeostasis was not a recent development. In fact, over 50 years ago, the glucostatic (173) and lipostatic (135) hypotheses proposed that circulating nutrients generated in proportionate amounts to storage depots serve as signals to the brain to initiate alterations in FI and energy expenditure. However, only recently has the notion of direct hypothalamic nutrient-sensing to regulate energy and glucose homeostasis been directly demonstrated in the literature.

Glucose and Lactate

Glucose is an important source of energy for the majority of mammalian cell types, and is particularly vital for the brain given its role as the sole substrate for brain energy metabolism. It was, however, the observation that severe hypoglycemia or hyperglycemia can drastically alter FI (173) – an idea which ultimately gave birth to the glucostatic hypothesis – that hinted at potential physiological roles of central glucose utilization (159) beyond serving as a fuel. The discovery of glucose-sensing neurons in satiety and feeding centres of the hypothalamus (10; 211) further encouraged a link between hypothalamic glucose-sensing and the regulation of energy homeostasis. Since those seminal studies, implications that central glucose-sensing/-metabolism is an

essential component in the regulation of feeding has sparingly appeared in the literature. Of note, central administration of 2-deoxy-D-glucose (2DG), a non-metabolized glucose analog that inhibits glucose utilization, increased FI and BW (27) while conversely, central administration of glucose potently reduced FI and BW (70) (Figure 2). Recently, it was demonstrated that a rise in central glucose levels alone decreases α2-adenosine monophosphate-activated protein kinase (AMPK) activity in the hypothalamus (183). This was of great significance as AMPK was identified in the same study to regulate FI by responding to other known anorectic signals such as leptin and a refeeding protocol (183). Alas, *it appears that no further support other than these initial observations have convincingly confirmed that changes in CNS glucose-derived energy might regulate FI*.

Recent work has also suggested a direct link between central glucose-sensing and the regulation of peripheral glucose levels. Specifically, an acute increase in central glucose resulted in a decrease in blood glucose and insulin levels, and a suppression of hepatic GP via a curtailing of both gluconeogenesis and glycogenolysis (151). Interestingly, while neuronal activity is coupled to glucose utilization (132; 218; 236; 285), neurons actually preferentially utilize lactate, metabolized from glucose in the glial cells, as an oxidative fuel (169). This proposal forms the basis of the astrocyte-neuron lactate shuttle hypothesis (219). Indeed, the infusion of ICV lactate was able to recapitulate the effects of central glucose on blood glucose levels and hepatic GP (151). Furthermore, the effects of both ICV lactate and glucose were nullified when lactate metabolism to pyruvate was prevented with the co-infusion of an inhibitor of lactate

dehydrogenase (LDH), oxamate (151). It is important to note that oxamate is a competitive inhibitor of both the lactate-generating LDH-A (the muscle isoform that, within the brain, is expressed exclusively in the glial cells (29)) and the pyruvate-generating LDH-B (the heart isoform, and the only isoform found in neurons (29; 38)). Therefore, while the aforementioned finding suggests that metabolism of glucose to lactate and subsequently to pyruvate in the hypothalamus is an essential biochemical step in the regulation of glucose homeostasis (Figure 2), isoform-specific inhibition of LDH would certainly be most powerful to convincingly prove this hypothesis. This set of elegant studies remains to be tested. Nevertheless, furthering the notion of CNS glucose-/lactate-metabolism to regulate GP is the suppression of GP resulting from the hypothalamic administration of dichloroacetate (DCA) (151), which inhibits pyruate dehydrogenase (PDH) (127) to promote the conversion of pyruvate to acetyl-CoA.

The importance of this metabolic coupling between neurons and glia via the generation and intracellular trafficking of lactate in the CNS regulation of glucose homeostasis has also been demonstrated in a few other notable studies. Specifically, the perfusion of the ventromedial hypothalamus (VMH) with lactate was sufficient to severely blunt the counterregulatory hormone response to hypoglycemia, with a marked suppression of both glucagon and epinephrine release during a hypoglycemic clamp (33). Importantly, this finding is also seen when glucose was perfused into the VMH (33). The caudal hindbrain has also been established as a sensor of local deprivation of glucose, which subsequently activates a response to restore glycemia. Interestingly, infusing an inhibitor of monocarboxylate transporters, which lactate

transport is dependent on, into the caudal fourth ventricle of rodents resulted in increased blood glucose levels (214). Conversely, an increase in caudal hindbrain lactate worsened insulin-induced peripheral hypoglycemia (214). Together, these studies collectively demonstrate the importance of CNS metabolism of glucose into lactate in the CNS regulation of glucose homeostasis. *However, the precise downstream pathway(s) involved in this glucose-/lactate-sensing mechanism to regulate glucose homeostasis remains to be determined.*

An acute elevation in circulating glucose is known to markedly suppress liver GP (182; 236; 274). When plasma glucose levels were doubled in the presence of a concurrent intrahypothalamic (IH) infusion of oxamate, this inhibitory action of acute hyperglycemia on GP was blunted by 40% (151). This study reveals that the activation of hypothalamic lactate metabolism is a critical component of the effectiveness of circulating glucose *per se* to control glucose levels. In parallel, circulating lactate has also been demonstrated to regulate hepatic glucose fluxes (128), and it was recently shown that the inhibition of either hypothalamic LDH or K_{ATP} channels during a physiological increase in circulating lactate led to an increase in hepatic GP (143) (Figure 2). Interestingly, elevation in circulating lactate levels also negatively regulates FI and BW (193; 255). *However, it still remains unknown whether, like that of glucose homeostatic regulation, the CNS holds a critical role in mediating the FI- and BW-lowering effects of circulating lactate.*

Few articles to date have studied glucose-sensing in specific neuronal cell types. However, seeing that ARC NPY/AgRP and POMC neurons are fundamental to hypothalamic regulation of energy and glucose homeostasis, it might not be too far-fetched to hypothesize that these neurons might be potential glucose sensors. Of particular note, it was recently demonstrated that the POMC neuron-specific expression of a mutated K_{ATP} channel subunit Kir6.2 was sufficient to impair glucose homeostasis, as determined by an oral glucose tolerance test (213). Furthermore, electrophysiological analyses determined that a high-fat diet was able to impair glucose sensing by POMC neurons, and this impairment was linked to an upregulation in the mitochondrial uncoupling protein (UCP) 2 (213). Classification of ARC glucose-sensing neurons warrants further investigation. Generally speaking though, these neurons are thought to monitor and integrate changes in central glucose concentration, most likely resultant of substantial peripheral increase or decrease in glucose levels, and appropriately regulate their own neuronal activity and neurotransmitter release (65). *In summary, the underlying mechanisms of CNS glucose/lactate-sensing that regulate glucose homeostasis remain to be fully dissected*.

Fatty Acid

While the brain does not, to our knowledge, use fatty acids as a primary source of energy, it has been recently demonstrated that select enzymes and intermediates of fatty acid metabolism contribute to the hypothalamus' ability to serve as a monitor of

energy status to regulate energy and glucose homeostasis. A look into the biochemical fate of glucose and fatty acid reveals convergence between these two seemingly distinct metabolic pathways at the level of acetyl-CoA (see Figure 2), hinting at potential convergence in their roles as energy and glucose homeostatic regulators in the CNS. Given such, we will review the relevant literature that highlights the role of CNS fatty acid-sensing mechanisms in the regulation of energy and glucose homeostasis. This will allow us to better understand the potential mechanisms that underlie CNS glucose-/lactate-sensing in the control of energy and glucose homeostasis.

ICV oleic acid, a long-chain fatty acid (LCFA), reduces FI, hypothalamic NPY mRNA levels, plasma insulin and glucose levels as well as GP (206). The latter parameter was demonstrated to be via a hypothalamic K_{ATP} channel-dependent mechanism (206), which is in line with a later demonstration that alterations in hypothalamic K_{ATP} channel activity *per se* regulates GP (224) (Figure 2). Adding to the physiological relevance of the previous finding, blockade of central K_{ATP} channels during IV lipid infusions resulted in significant elevation of GP (152). In the same study, circulating LCFAs was found to regulate glucose homeostasis via a hypothalamically-triggered mechanism that is dependent on 1) the esterification of LCFAs to LCFA-CoAs, 2) the opening of K_{ATP} channels and 3) neural transmission via the vagus nerve (152). Recently, our lab also demonstrated that increase in circulating LCFAs lowers GP via a hypothalamic protein kinase C (PKC)-dependent mechanism which is upstream of K_{ATP} channels (234). While the importance of the hypothalamic LCFA in producing a surfeit signal to regulate energy and glucose homeostasis is clearly established, the role of fatty acid oxidation in this

signal remains unclear. It is known, however, that inhibition of carnitine palmitoyltransferase-1 (CPT-1), a key determinant in the level of cytosolic pool of LCFAs through regulating the transport of LCFAs into the mitochondria for β-oxidation (Figure 2), markedly reduce FI, down-regulate NPY and AgRP mRNA levels and decrease GP (204).

In order to fully appreciate the importance of central fatty acid metabolism in initiating the hypothalamic behavioural and metabolic responses necessary to regulate energy and glucose homeostasis, we must look at the upstream biochemical processes that are involved in the formation of LCFA-CoAs. These are all ultimately responsible for the generation of the hypothalamic surfeit signal.

AMP-Activated Protein Kinase (AMPK)

AMPK is an evolutionarily conserved energy sensor that in essence acts as a fuel gauge of mammalian cells (111). It operates by phosphorylation of various targets and by responding to an increasing cellular AMP: ATP ratio. Under normal physiological conditions, hypothalamic AMPK activity increases during fasting and decreases upon refeeding (183). Conversely, modulation of hypothalamic AMPK activity *per se* changes feeding behavior. Activation of AMPK raises FI while inhibition decreases FI and BW (13; 122; 138; 183) (Figure 2). These changes in feeding patterns are accompanied by changes in neuropeptide expressions. Particularly, hypothalamic dominant negative AMPK decreased neuropeptide NPY and AgRP mRNA levels whereas constitutively active AMPK increased both (13; 122; 138; 183). Selective genetic knockout of AMPK in either

NPY/AgRP or POMC neurons in mice disrupted energy homeostasis (55). These studies, together with the recent finding that an obesity-resistant hypophagic rat strain (Lou/C) exhibited impaired AMPK responses to starvation (267), collectively established that AMPK signaling is necessary for proper energy balance.

Interestingly, hormonal signals and circulating nutrients, both known to control energy homeostasis, also alter hypothalamic AMPK activity. Central glucose (183), α-lipoic acid (a short chain fatty acid that is a cofactor of mitochondrial enzymes with anorectic properties) (138), leptin (13; 183), insulin (183) and GLP-1 (251) all decrease hypothalamic AMPK activity. Interestingly, leptin's alteration of AMPK activity was absent in a mouse model with MC4 receptor knockout, indicating that the melanocortin receptor-signaling mediates the effect of leptin on AMPK activity (183). Opposite to these anorectic signals, orexigenic peptides such as ghrelin (13; 144), AgRP (183), adiponectin (an adipocyte-secreted hormone) (148) and orexigenic cannabinoids (144) stimulated hypothalamic AMPK activity. Taken together, large amount of evidence point to AMPK as a downstream enzyme that converges and coordinates various nutrient and hormonal signals, both anorexigenic and orexigenic, in the hypothalamus to regulate energy homeostasis. However, whether hypothalamic AMPK is sufficient and necessary for nutrient-sensing mechanisms to regulate glucose homeostasis remain to be evaluated.

It is perhaps interesting to note that the pattern of neuropeptide regulation elicited by the manipulations of hypothalamic AMPK activity, i.e. changes in NPY/AgRP

but not POMC levels (183), is similar to that seen with central fatty acids administration (206) or manipulation of hypothalamic lipid metabolism by inhibition of CPT1 (204). This hints at a possible parallel or converging mechanism at work bridging AMPK to fatty acid sensing in the brain. In fact, one of the best characterized actions of AMPK is the phosphorylation, and hence inhibition, of acetyl-CoA carboxylase (ACC) (190; 256). This inhibition hinders the conversion of acetyl-coA to malonyl-coA, the latter which is known to inhibit CPT1 action (176). It is therefore reasonable to postulate that the effects of energy regulation elicited by AMPK are mediated by the downstream fatty acid sensing mechanism via the 'malonyl-coA→CPT1 inhibition→LCFA-CoA accumulation' hypothesis of appetite regulation.

Acetyl-CoA Carboxylase (ACC)

Fatty acids that are present in the cells are either imported from the circulation or generated *de novo*. The committed step of *de novo* fatty acid biosynthesis is the initial conversion of acetyl-coA to malonyl-coA, which is mediated by the enzyme ACC (286). It was first identified that genetic knockout mice lacking ACC2 had significantly lower fat mass and BW than their wildtype counterparts although they had higher food consumption (2). This unmatched FI and BW might in part be accounted for by increased energy expenditure in ACC2 knockout mice (52). To compliment this whole-body knockout model, a few important recent studies have highlighted the importance of hypothalamic ACC in energy homeostasis. Of note, while central leptin inhibits AMPK, it activates ACC (91). In fact, blockage of central ACC activity, which prevented the IV

leptin-induced rise in malonyl-coA, eliminated the anorectic effect of leptin and the accompanying drop in NPY mRNA level (91).

While most studies thus far have focused on energy homeostasis, a recent study pointed at the possible involvement of ACC in glucose homeostasis. Citrate, an intermediate metabolite produced in the mitochondria in the citric acid cycle, is an allosteric effector of ACC activity. Central citrate not only decreased FI and BW (232), but also resulted in lower blood glucose levels and increased glucose uptake (46).

Fatty Acid Synthase (FAS) and the Malonyl-CoA Hypothesis

FAS catalyzes the synthesis of LCFA-CoAs from malonyl-CoA in a downstream reaction of the reductive synthesis of long-chain fatty acids. The potential role for fatty acid intermediates in energy homeostatic regulation is supported by the finding that systemic or central treatment of mice with the FAS inhibitor cerulenin or C75 led to marked inhibition of feeding and weight loss in a leptin-independent, NPY-dependent manner (163). C75-induced weight loss was shown to partly arise from an increase in energy expenditure (149). ICV C75 caused a significant increase in whole-body and skeletal muscle fatty acid oxidation, as well as a concomitant increase in the expression of PPARα, which activates expression of genes encoding enzymes of fatty acid oxidation (47). Indeed, the importance of hypothalamic FAS in the regulation energy homeostasis was made indisputable with the use of brain and beta cell specific FAS knockout mice. These tissue-specific FAS knockout mice exhibited hypophagia and increased energy expenditure compared to control animals independent of changes in beta cell function

(48). Interestingly, central inhibition of ACC, which prevents malonyl-CoA accumulation, abolished C75-induced anorexia and neuropeptide mRNA changes (121). This highlights the importance of malonyl-CoA with the regulation of feeding behavior.

Indeed, hypothalamic concentration of malonyl-CoA falls during fasting and rises after refeeding (121). Overexpression of malonyl-coenzyme A decarboxylase (MCD) in the hypothalamus of rats, which lowers hypothalamic malonyl-coA level by its conversation back to acetyl-coA, not only increased FI and BW (113) but also impaired the suppression of GP during insulin-clamped settings (113). Notably, these MCD-overexpressed animals had a significant reduction in hypothalamic LCFA-CoA, to a level that is highly comparable to those seen in animals with hypothalamic inhibition of LCFA esterification that also exhibited impaired glucose homeostasis (152).

Malonyl-CoA is also involved in nutrient and hormonal sensing in the hypothalamus. Specifically, central glucose and leptin has recently been confirmed to positively regulate hypothalamic malonyl-CoA concentrations (294). A single dose of ICV leptin induces a sustained increase in hypothalamic malonyl-CoA level, in line with previous findings that central leptin lowers hypothalamic AMPK activity (13; 183) and augment hypothalamic ACC activity (91). These complimenting kinetic changes in AMPK, ACC, FAS and malonyl-CoA are perhaps not merely coincidental and strongly supports that there exists an underlying link between these individual factors (Figure 2).

Amino Acid

The mammalian target of rapamycin (mTOR) is a regulator of cell growth, and much like AMPK, it is a cellular energy sensor whose kinase activity varies with nutritional status (281). Its activity is sensitive to various nutrients, including glucose and some fatty acids, and particularly the branched-chain amino acid leucine (298). Interestingly, when a low-dose of leucine was administered into the third cerebral ventricle of rodents, central mTOR signaling was activated and a marked decrease in short-term FI and BW was observed (65). This leucine-mediated anorectic effect was nullified when hypothalamic mTOR activity was inhibited via rapamycin treatment (65). The anorectic effects mediated by central leucine administration correlate with an increase in the phosphorylation of hypothalamic S6 kinase (S6K), an effector of mTOR activity (65), in a dose-dependent manner (233).

Potentially extending the physiological relevance of CNS amino acid-sensing is a recent report showing that high-protein meals, which effectively promote weight loss (25; 31; 221), selectively activates POMC neurons in the ARC (83). However, no conclusive statements could be drawn in regards to hypothalamic amino-acid sensing as no measurements of CNS amino acid levels, leucine for example, were made. While the role of central amino acid-sensing in the regulation of energy homeostasis has been shown, its role in controlling circulating glucose levels remains unknown. As such, evaluating the possibility for amino acids to regulate glucose homeostasis and determining whether this regulation occurs via mTOR pathway-dependent or -independent mechanisms remains an important question.

The metabolism of glucose and fatty acids are perhaps of opposite importance with respect to fueling local energy supply for the brain. But when it comes to maintenance of whole-body homeostasis, however, these nutrients appear to form a united front and collectively serve as a nutrient surfeit signal, activating hypothalamic pathways which ultimately initiate the CNS-mediated regulation of energy and glucose homeostasis (Figure 2). LCFA-CoA and malonyl-CoA have emerged as the molecules of focus which are poised to integrate the activation of glucose and fatty acid sensing mechanisms in the hypothalamus. However, the extent to which hypothalamic nutrient-sensing pathways interact with those of the previously detailed hormone-sensing is uncertain. As such, the integration and possible co-dependence of central nutrient- and hormones-sensing pathways remains an area of interest that is open to further scrutiny.

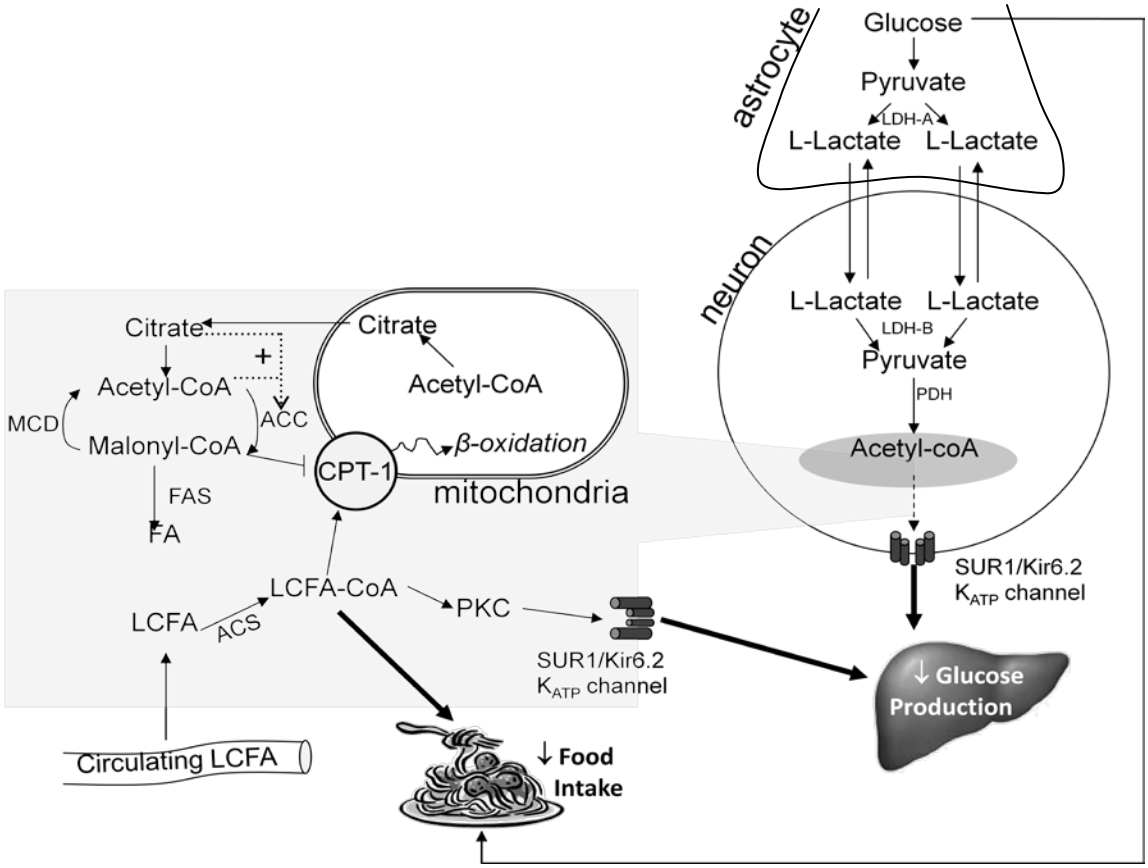


Figure 2 Glucose and fatty-acid sensing pathways in the hypothalamus

Glucose is taken up astrocytes where it is glycolytically metabolized to pyruvate. In astrocytes, pyruvate is preferentially converted to L-lactate by lactate dehydrogenase A (LDH-A). Lactate is then taken up by neurons and is preferentially converted back to pyruvate by means of LDH-B. Finally, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase (PDH). (Inset) Extracellular long chain fatty acids (LCFAs) are immediately esterified to LCFA-CoAs upon entry into cells via acyl-CoA synthetases (ACS), and LCFA-CoAs gain access to the mitochondria to undergo β-oxidation via the acyltransferase carnitine palmitoyltransferase-1 (CPT-1), located on the outer mitochondrial membrane. Cellular fat oxidation is regulated by the availability of malonyl-CoA, which potently inhibits CPT-1 activity. Malonyl-CoA, in turn, is mainly derived from acetyl-CoA via the enzyme acetyl-CoA carboxylase (ACC), which marks a point of convergence between glucose and fatty acid sensing mechanisms. Finally, ACC is allosterically inhibited by AMP-activated protein kinase (AMPK)-mediated phosphorylation. The increased flux through hypothalamic fatty acid metabolism has been shown to lower hepatic GP and food intake via a K_{ATP} channel-dependent mechanism. Hypothalamic glucose has been shown to lower hepatic GP and FI, the former known to be mediated by downstream lactate metabolism.

1.3 HYPOTHALAMIC NUTRIENT-SENSING AND ITS IMPLICATIONS FOR DISEASE

The recent past has yielded novel and exciting experimental data that have furthered the concept that the hypothalamus is a key regulator of energy and glucose homeostasis. While this has revealed new metabolic and hormonal signaling pathways in CNS-sensing mechanisms, a necessary step is to flesh out these findings in models of metabolic disease (obesity, insulin resistance and/or diabetes) in order to gain an appreciation of what is disrupted in pathology.

LCFAs serve as a central signal of nutrient abundance, in turn triggering the series of neuronal signaling cascades necessary to regulate nutrient intake and production. Shortly after the effects of ICV oleic acid were recognized (206), it was evaluated whether short term alterations in nutrient availability affect the ability of central oleic acid to regulate energy and glucose homeostasis. In rats overfed on a 3-day high fat diet, an ICV oleic acid bolus was unable to neither inhibit FI nor suppress GP under conditions of a pancreatic basal insulin clamp. Interestingly, by pair-feeding rats on the high-fat diet the ability of ICV oleic acid to suppress hepatic GP was restored (187). This provides compelling evidence that the hypothalamic responses triggered by an acute increase in central LCFAs are nutritionally regulated. Along with the fact that hypothalamic insulin resistance develop in rodents fed 1 day of high-fat diet (210), this data presents a startling reality in terms of how rapidly intrinsic homeostatic mechanisms can fail. As the rise in central LCFA-CoAs is a critical initiator of the fatty

acid-mediated homeostatic regulation, the authors specifically postulated that the increase in lipid availability by overfeeding fails to translate into this increase in the intracellular pool of LCFA-CoA (225). This is indeed the case: when a systemic lipid emulsion designed to double plasma LCFAs and hypothalamic LCFA-CoAs (152) was administered to overfed rats, the circulating lipids failed to increase hypothalamic LCFA-CoAs (225). An impeded BBB LCFA transport cannot account for this effect, as no increase in hypothalamic LCFA-CoAs was also observed in overfed rats when oleic acid was directly infused intrahypothalamically (225). Hypothalamic CPT1 activity was significantly increased in the overfed rats, and remarkably, by hypothalamically inhibiting CPT1 activity or expression, the authors were able to suppress FI as well as GP in overfed rodents (225). Thus, inhibiting hypothalamic lipid oxidation via the inhibition of CPT1 hyperactivity is sufficient to restore energy balance as well as glucose homeostasis in overfed rodents.

Long known to serve as a cellular energy sensor, even in the simplest of organisms, AMPK has recently been shown to have a regulatory role in the hypothalamus-mediated control of energy balance (183). With the fatty acid biosynthetic pathway in mind, the accumulation of intracellular malonyl-CoA and LCFA-CoA would be antagonized by AMPK hyperactivity. Indeed, a number of studies have examined this potential dysregulation of AMPK activity in obese or diabetic models. Streptozotocin (STZ)-induced diabetic rats are characterized by marked hyperphagia, and compared with control rodents, it has been found that hypothalamic AMPK activity is higher in diabetic rats. This hyperactivity (and the accompanying increase in FI) is

normalized by the ICV administration of an AMPK inhibitor, as well as insulin and leptin (194). Additionally, in a 12-week model of diet-induced obesity, the ability of leptin to inhibit AMPK activity in various hypothalamic nuclei, including the ARC, was lost (171). Conversely, it was recently found that mice absent in CaMKK2, a regulator of AMPK activity, were protected from diet-induced obesity, insulin resistance, and glucose intolerance (11).

While each of these observed deregulations hint at varying defects mediating the loss in CNS effectiveness to regulate energy and glucose homeostasis, a very recent publication hints that there may indeed be a common impairment underlying them all. It was observed in rats selectively bred to develop diet-induced obesity that they had defective ARC neuronal projections (35). Furthermore, leptin, which is essential for the normal development of ARC projections, was found to be ineffective in activating ARC neuron signalling in diet-induced obese neonates (35). Thus, it is quite likely that the genetically-governed structural defects persist into adulthood and play a key role in initiation and progression of ineffective hypothalamic surfeit signalling.

However, not all central sensing mechanisms are disrupted in models of obesity and/or diabetes. For example, an acute increase in central or hypothalamic lactate has been shown to regulate glucose homeostasis by suppressing GP in normal rodents (151). Interestingly, we have also observed that administration of central lactate, at the same dose used in normal rodents, is able to lower GP in an early-onset model of STZ-Diabetes (50). Furthermore, central lactate was able to suppress GP in normal rodents

with experimentally-induced hypoinsulinemia, and more significantly, in diet-induced insulin resistance resulting from a 3 day high-fat diet (50). It is intriguing that in a similar model of acute diet-induced insulin resistance, activation of PKC in the hypothalamus (234), but not central oleic acid, was also effective in suppressing GP (187).

Clearly, further investigation is necessary to elucidate the exact mechanism underlying this selective preservation in central nutrient sensing in models of metabolic disease.

1.4 THE ROLE OF THE NUCLEUS OF THE SOLITARY TRACT: CNS REGULATION AS AN INTEGRATIVE SYSTEM

A strong focus thus far has been placed on the hypothalamic ARC in the regulation of energy and glucose homeostasis – this has perhaps presented a somewhat biased view of the central control of energy and glucose homeostasis. While it is true that the ARC is a master site of CNS control of energy and glucose homeostasis, other regions of the brain are also involved. In terms of energy homeostasis, for instance, the hypothalamic ARC projects to an assortment of second order neurons within other hypothalamic sites such as the PVN and lateral hypothalamus (LH) (16; 21; 76; 77; 79; 164). These sites then subsequently synapse with other brain regions. Ascending projections to the cortical, limbic and thalamic systems (26; 228) mediate behavioural changes related to FI and energy expenditure via cognition and memory (57; 94; 161; 231; 271) while descending projections to the brainstem (167; 240; 266) elicit autonomic control towards the periphery (103) (Figure 3). While the above portrayal already appears complex, in reality it only provides a largely simplified glimpse of the diversity and intricacy of the actual network.

As it is unrealistic to cover the CNS network in its entirety, focus hereafter will be placed on the brainstem, with a particular interest in the nucleus of the solitary tract (NTS) which is held to be another critical site of CNS control of the periphery.

Just as the ARC is naturally poised at the prime loci in the hypothalamus to sense and integrate circulating factors to respond accordingly to deviations from homeostasis, the NTS within the brainstem (or medulla), is ideally set at the interface between the brain and periphery, facilitating its role as a go-between of the CNS and periphery. Indeed, anatomically, the NTS is situated in the dorsal brainstem and is the primary site for termination of peripheral sensory afferents, prominently from the vagus nerve. Also known as the cranial nerve X, the vagus nerve innervates a large selection of peripheral structures, including but not limited to the airways, lungs, heart, gastrointestinal tract, liver and portal vein (28; 49). A majority of the nerve fibers in the vagus nerves are afferent nerves, such as baroreceptor, chemoreceptor, mechanoreceptor as well as lung and gastrointestinal fibres. These afferent nerve fibers collectively travel along the tractus solitarus towards the NTS and form synapses with neurons within the NTS to effectively convey the state of the periphery to the brain (141). Given this unique feature, the NTS is understandably the key site for the autonomic reflex pathways which control cardiovascular (14), respiratory (34), gastric (275), ingestive (15; 68; 191; 242) and metabolic (273) functions.

The projection of abdominal visceral afferents to the NTS is now amply documented (49; 114; 230). Studies have revealed the remarkable ability of the NTS to regulate energy homeostasis in response to these gastrointestinal signals, a response pathway termed the “vago-vagal reflex”. Indeed, selective disruption of gut vagal afferent signals to the NTS abolish the negative-feedback control of FI (87; 244; 260) while activation of gut afferent fibers by increased gastric volume (172), small intestinal

nutrient concentration (241) and administration of peptide cholecystokinin (CCK) (243) leads to reduction in meal size. While these gut vagal signals are important in regulating energy homeostasis, the NTS also receives vagal afferents from the hepatic branch (3; 114; 230). Through glucosensors within the hepatoportal regions, this connection has been shown to inform the brain of peripheral glucose concentration (3; 196) in order to decrease feeding (237; 238), stimulate glucose utilization (42; 92), reduce hepatic glucose output (253) and regulate pancreatic hormone secretion (273). These responses, which are effected through efferent signals towards the pancreas, adrenal glands, adipocytes, skeletal muscle and liver (45), summate to one end: to minimize fluctuations in plasma glucose levels after feeding for the maintenance of glucose homeostasis (197). Recently, our lab has identified a novel gut-brain-liver circuitry which indicates that duodenal lipids lower hepatic GP via (i) stimulation of gut vagal afferents, (ii) integration of signals in the NTS, and (iii) activation of the hepatic branch of the vagus nerve (288). Not only does this study provide the strongest evidence thus far that the NTS can relay vagal signals to alter hepatic GP, it also pinpoint the N-methyl-D-aspartate (NMDA) receptors as the mediator of these effects (Figure 3).

Indeed, glutamate, which can bind to NMDA receptors, is the primary neurotransmitter released by sensory afferents projecting to the NTS (268). Via binding of glutamate released from the presynaptic terminal to its receptor in the postsynaptic membrane, glutamatergic signals are transduced into electrical and biochemical events in the recipient neurons (252). Glutamate receptors are gated ion channels and are classified into three families based on the pharmacological nature of their ligand: the

[alpha]-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, the kainite receptors and, of most relevance to us, the NMDA receptors (174). Structurally, all NMDA receptors are composed of an obligatory NR1 subunit (133; 155) in combination with one or more NR2 subunits (174) and occasionally an NR3 subunit (69). In response to the binding of glycine and glutamate to the NR1 and NR2 subunit, respectively, NMDA receptors open (133) to transduce excitatory neurotransmission.

As mentioned, NMDA receptors within the NTS are important for afferent signal transduction (32) and thus it follows that modulation of NMDA receptor activity in the NTS affects systems which rely on afferent signals, including but not limited to cardiovascular functions (14; 147; 158; 268), energy homeostasis (66; 67; 106; 107; 123; 276; 303), gastric function (275) and even hepatic GP (288). Interestingly, however, it remains unknown whether the NMDA receptors in the NTS are important for non-afferent-dependent signal transduction. *There are no studies to date that have shown that the NMDA receptors in the NTS can regulate peripheral homeostasis independent of the vagal afferent, but rather via CNS sensing per se*. This proposition is perhaps not too farfetched given that the NTS, aside from receiving inputs from the periphery via the vagal afferents, also extensively synapse with other CNS regions, including the ARC of the hypothalamus which is the known site of CNS-sensing. In line with this, it is well-established that the NTS actually receives direct (54; 97; 188) as well as indirect (167; 240; 266) signals from the ARC in regards to regulating energy homeostasis (Figure 3). *However, no studies to date have provided direct evidence of such a hypothalamic-medullary crosstalk for the control of glucose homeostasis.*

Putting together these two interesting pieces of information, namely that (1) the NMDA receptors in the NTS are needed by the vagal afferents to control energy and glucose homeostasis and that (2) the NTS, aside from receiving afferent input from the periphery, also receives neuronal projections from the hypothalamus to control energy homeostasis, *it would be of significant interest to determine whether the NTS can receive hypothalamic signals to control glucose homeostasis, and whether this regulation requires the NMDA receptors in the NTS*.

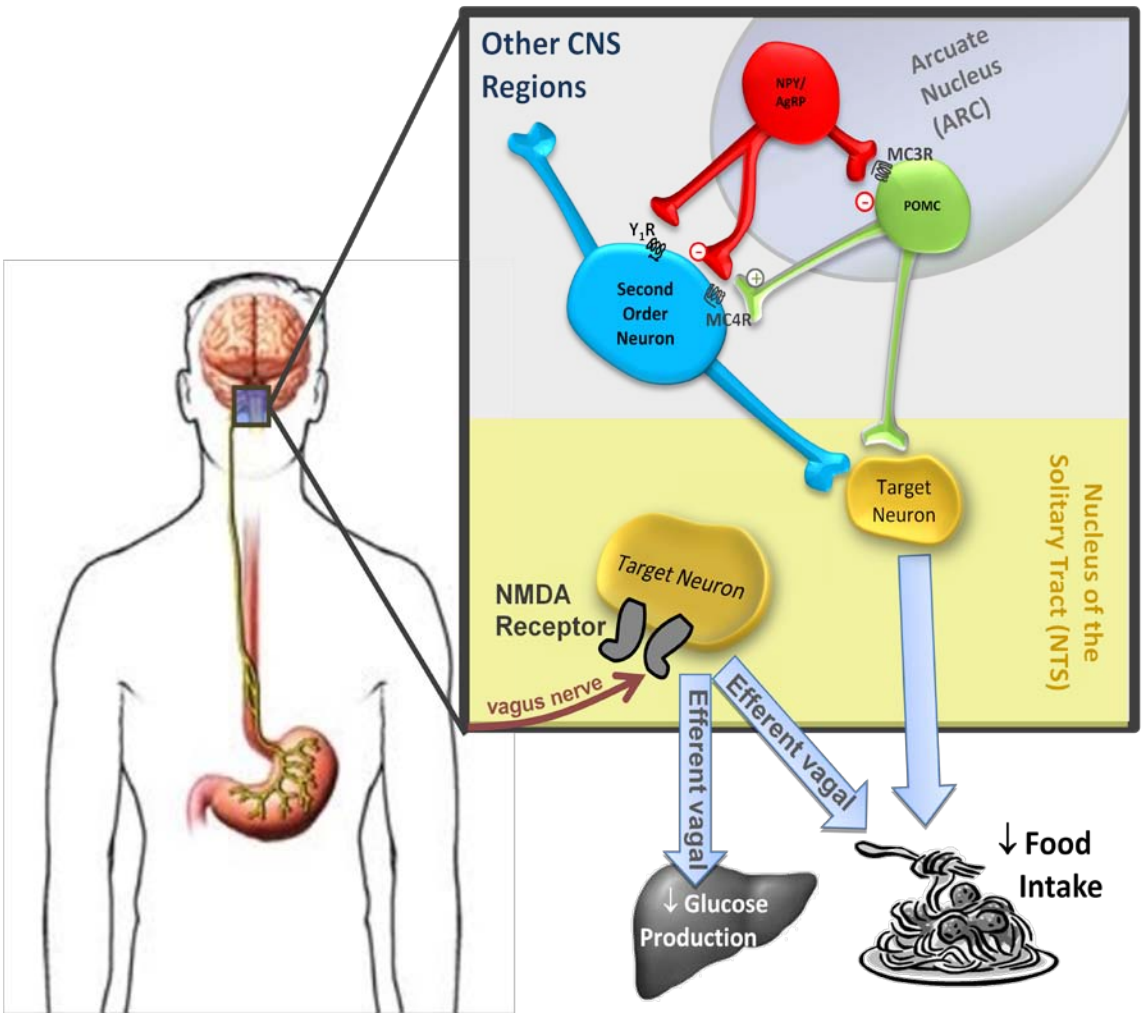


Figure 3 Integrative capacity of the nucleus of the solitary tract to control food intake

Visceral afferents of the vagus nerve transmit meal-related signals to the nucleus of the solitary tract (NTS), which via activation of glutametergic receptors such as the N-methyl-D-aspartate (NMDA) receptors regulate food intake and hepatic GP. In addition to these vagus-dependent stimulation, the NTS also receives both direct and indirect projections from the arcuate nucleus (ARC) to control FI.

2

General Hypothesis and Aims

To date, it remains largely unclear what precise neuronal pathway(s) mediates the hypothalamic regulation of energy and glucose homeostasis, and what biochemical and signaling effectors are responsible for the control. Research with these particular foci will reveal new molecular targets to lower appetite and plasma metabolite levels in obesity and T2DM.

Given that hypothalamic lactate metabolism has been shown to regulate peripheral glucose and lipid homeostasis *in vivo* (150; 151), we hypothesize that central lactate-sensing, via downstream lactate metabolism to pyruvate, lowers FI and BW to regulate energy homeostasis. Furthermore, given that the NTS actually receives direct (54; 97; 188) and indirect (167; 240; 266) signals from the ARC regulating energy homeostasis, and that the activation of NTS NMDA receptors is required for a gut-brain-liver axis to lower GP (288), we postulate that the GP-lowering effect of hypothalamic lactate requires a hypothalamic-medullary pathway that consists of the activation of NMDA receptors in the NTS.

The overarching aim of this current thesis is to extend the quest to uncover novel CNS biochemical pathways and molecules in the regulation of energy and glucose homeostasis. With a particular focus on hypothalamic lactate-sensing, the two specific aims of the thesis are:

I. To determine whether lactate-sensing in the hypothalamus controls energy homeostasis (Study 1)
II. To delineate the downstream mechanism of lactate-sensing in the regulation of glucose homeostasis (Study 2)

3

General Materials and Methods

*Note: All study protocols described hereafter were reviewed and approved by the Institutional Animal Care and Use Committee of the University Health Network.

Model

8-week-old male Sprague-Dawley (SD) rats, weighing between 240-280g (Charles River Laboratories, Montreal, QC) were used for our studies. Rats were housed in individual cages and maintained on a standard 12-12h light-dark cycle with access to standard rat chow (Harlan Teklad 6% Mouse/Rat Diet; composition: 52% carbohydrate, 31% protein and 17% fat; total calories provided by digestible nutrients: 3.83 kcal/g) and water *ad libitum*.

Surgical Procedure

Stereotaxic Surgery

According to the atlas of the rat brain, rats were stereotaxically implanted with indwelling cannula (Plastics One Inc., Roanoke, VA) as previously described (206; 234; 288). In brief, rats were anesthetized with intraperitoneal (IP) ketamine (Ketalean; Bimeda-MTC, Cambridge, Ontario) and xylazine (Rompun; Bayer) then fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with ear bars and a nose piece set at +5.0 mm. 22-gauge stainless steel single guide cannulae were used for third cerebroventricular implantations and 26-gauge stainless steel double guide cannulae were used for mediobasal hypothalamic or nucleus of solitarary tract implantations, using the following coordinates:

Third cerebral ventricle (206)	2.5 mm posterior of bregma 0.0 mm lateral from midline 8.0 mm below skull surface
Mediobasal hypothalamus (234)	3.1mm posterior of bregma 0.4mm lateral from midline 9.6mm below skull surface
Nucleus of solitary tract (288)	0.0mm on occipital crest 0.4mm lateral to midline 7.9mm below skull surface

Rats were given a period of recovery time post-stereotaxic surgery in individual cages, maintained on a standard 12h-12h light-dark cycle with access to standard rat chow and water *ad libitum*. Only rats whose daily FI and BW had recovered to baseline values underwent subsequent surgical or experimental procedures.

Intravenous Catheterization

5 days post-stereotaxic surgery, a subset of recovered rats (monitored by daily FI and BW) that were to receive systemic circulating nutrients as described in Study 1.4 then underwent intra-jugular venous catherization for infusion purposes.

For another subset of rats that were to undergo the pancreatic (basal insulin) – euglyceamic clamp experiments, as described in Study 2, both the right internal jugular vein and right carotid artery were catheterized for infusion and sampling purposes (50; 234; 288) 1 week post-stereotaxic surgery.

In brief, rats were anesthetized with IP ketamine and xylazine and the right internal jugular vein and/or right carotid artery were cathetherized with polyethylene

catheters (PE 50, Clay Adams) extended a 15 mm cuff-extension of Silastic tubbing (Corning). Both catheters were tunneled subcutaneously and exteriorized. The catheters were filled with 10% heparinzed saline to maintain patency, then closed at the end with a metal pin. Recovery from IV catheterization was monitored by measuring daily FI and BW gain, ensuring that only animals that have at least attained FI and BW within 10% of baseline were used in subsequent experimental procedures.

Statistical Analysis

Data were presented as means ± standard error. Statistical analysis was done by unpaired Student's *t*-test or Fisher's analysis of variance (ANOVA) where appropriate. In specific, *t*-test was used to compare between two treatment groups and one-way ANOVA was used to compare between three or more treatments groups. For analysis of experiments with two independent variables, factorial ANOVA was used. Statistical significant in ANOVA was followed by Turkey's *post hoc* test as a follow-up test to compare between all treatment groups in order to pinpoint where the significance lies. Statistical analysis was accepted as significant with $P < 0.05$. For pancreatic clamp experiments, the time period t = 60 – 90 minute (min) was averaged as the basal condition and the time period t = 180 = 210 min was averaged as the clamp condition.

4

STUDY 1

CENTRAL LACTATE METABOLISM REGULATES ENERGY HOMEOSTASIS

4.1 ABSTRACT

The CNS regulates FI and BW, but the associated mechanisms remain to be elucidated. We tested the hypothesis that hypothalamic metabolism regulates energy homeostasis. We first established that ICV injection of saline 1h before the dark cycle had no effect on FI (~27g) and BW (~268g) 1 or 2 days post-injection in rats. In contrast, ICV injection of equivolume of lactate reduced FI and BW after 1 day by 58±9% and 10±2%, respectively. BW remained lower after 2 days. Having reported for the first time to our knowledge that central injections of lactate reduced FI and BW in rodents, we then inhibited central lactate metabolism to pyruvate with the use of a lactate dehydrogenase inhibitor, oxamate. Co-administration of lactate with oxamate at a dose which had no effect alone abolished the central effects of lactate on FI and BW. Conversely, central injections of pyruvate recapitulated the effects of lactate, lowering FI from 27 ± 2 to 12 ± 4 g and BW by 6 ± 2% 18h post injection. Finally, the role of the CNS to peripheral nutrient-sensing is demonstrated: 3-h IV infusion of lactate, which doubled plasma lactate level, curtailed FI for 1 day and BW for 2 days, respectively. Inhibition of hypothalamic lactate metabolism prevented the ability of circulating lactate to lower FI and BW. Together, our data indicate that activation of hypothalamic lactate metabolism via (A) direct hypothalamic injection of lactate or glucose and (B) IV elevation of circulating lactate, lowers FI and BW.

4.2 MATERIALS AND METHODS

Intracerebroventricular Administration

5 days post-ICV surgery, rats whose daily FI and BW had recovered back to baseline (FI ~26 – 30 g/day; BW ~260 – 280 g) and had been stable for a minimum of 3 consecutive days underwent the feeding protocol. Baseline measurements of FI and BW were taken on day 0 (10 AM, time -6h). Water and food were then withdrawn from the animals. Rats received treatments 1h before the dark cycle (time 0h), involving ICV injection of 3 µl of one of the following solution over a period of 30s:

a) Saline
b) D-lactate, non-active, non-metabolized isoform of L-lactate (Sigma; 5 mM, dissolved in saline)
c) L-lactate (Sigma; 5 mM, dissolved in saline)
d) Oxamate (Sigma; 50 mM, dissolved in saline)
e) Oxamate (50 mM) + L-lactate (5 mM)
f) Pyruvate (Sigma; 5 mM, dissolved in saline)

The concentrations of the above ICV treatments were based on previous studies indicating that CNS lactate metabolism regulates glucose and lipid production (150; 151). A dose-response curve for the effects of ICV lactate (0.5, 1 and 2.5 mM) injections was also performed. Water and standard diet chow were given back to the rats at the onset of the dark cycle. FI and BW were monitored at 10 AM for the subsequent 2 days (t = 18h, 42h).

Intracerebroventricular and Intravenous Administration (for Systemic Circulating Nutrient)

7 days post-iv surgery, rats whose daily FI and BW had recovered back to baseline and had been stable for a minimum of 3 consecutive days underwent the feeding protocol. Baseline measurements of FI and BW were taken on day 0 (10 AM, time -6h). Water and food were then withdrawn. At 4h before the dark cycle (time -3h), animals received an ICV bolus of oxamate (50 mM: 5µl in 1 min) over 1 min. After the bolus, animals began receiving 3h of continuous ICV administration of oxamate at a rate of 5 µl/h and IV administration of lactate at a rate of 100 $\mu mol \cdot kg^{-1} \cdot min^{-1}$. This continuous infusion ended 1h before the onset of the dark cycle (time 0h). At the end of the 3h, animals received an additional ICV bolus of oxamate (5µl in 1 min). Water and standard rat chow were given back to the animals at the onset of the dark cycle. FI and BW were monitor at 10 AM for the subsequent 2 days (t = 18h, 42h).

4.3 RESULTS

Central Administration of Lactate Lowers Food Intake and Body Weight

The metabolic fate of brain glucose has been largely clarified by the proposal of the astrocyte-neuron lactate shuttle (219), which indicate that neurons preferentially utilize glial derived-lactate as an oxidative fuel (169). We therefore first determine whether lactate in the CNS controls FI and BW. We first subjected male SD rats to ICV surgeries and allowed them 1 week of recovery. Only fully recovered rats, marked by a complete return to baseline FI (~26 – 30 g regular chow diet / day) and BW (~260 – 280 g) for a minimum of 3 days, were subjected to further experimentations, namely ICV injections. To develop a protocol for FI- and BW-monitoring in response to single bolus ICV injections, we first injected ICV saline (3 µl over 30 s) in rats 1 h before the dark cycle (i.e. 6 h after initial FI and BW measurements at 10 a.m.) and monitored their FI and BW for 2 subsequent days (Figure 4b). We found that ICV saline injections had minimal effect on FI and BW at 18 (1 day) or 42 (2 days) h (Figure 5a, b) post-injection. Similarly, ICV injection of a non-active isoform of lactate, D-lactate (5 mM), had minimal effects on FI and BW (Figure 5a, b). We then injected equivolume of L-lactate (5 mM) 1 h before the dark cycle and saw reductions in both FI and BW. ICV L-lactate decreased FI from 29 ± 1 to 12 ± 3 g (a 58 ± 9% decrease) 18 h post-injection (Figure 5a). FI was not significantly different after 42 h (Figure 5a). This finding is consistent with previous studies indicating that a single ICV bolus injection of another nutrient, L-leucine, reduces FI for up to 24 h (65) but is in contrast to the finding that ICV oleic acid, a LCFA, lowers FI

for up to 48 h (206). Accompanying this FI reduction, ICV lactate also decreased BW by 28 ± 5 g (a 10 ± 2% decrease) after 18 h and remained significantly lower 42 h post ICV L-lactate injection (Figure 5b). Thus, we report for the first time to our knowledge that a selective rise in central lactate levels decreases FI and induces weight loss.

We next performed a dose-response curve for the anorectic effect of single ICV bolus of L-lactate which allowed us to determine whether the effect of ICV L-lactate is dose-dependent, and permitted succeeding studies to use the lowest dose that can generate the maximal effect. At both 0.5 and 1 mM of L-lactate, we saw minimal effects of FI and BW at 18 or 42 h post-injection (Figure 5c, d). On the other hand, ICV L-lactate at 2.5 mM lowered FI 18-h post-injection from 28 ± 0.9 to 22 ± 1.5 g ($P < 0.05$), a drop which was fully reversed to baseline by 42 h (Figure 5c). This partial decrease in FI was not accompanied by a drop in BW at either 18 nor 42 h post-injection (Figure 5d). As it appears that the anorectic effect of L-lactate was strongest at 5 mM in accordance with the dose-response curve, Subsequent experiments characterizing the underlying mechanisms of CNS lactate-sensing used ICV L-lactate at 5 mM, ensuring a relatively maximal stimulation on the downstream biochemical/ signaling pathway(s) of CNS lactate sensing.

Central Lactate Metabolism Mediates the Food Intake and Body Weight-Lowering Effects of Lactate

The activation of hypothalamic lactate metabolism regulates peripheral glucose (151) and lipid (150) homeostasis. As such, we wanted to examine whether central lactate metabolism mediates central lactate in the regulation of energy homeostasis. To do so, we first pharmacologically inhibited central lactate metabolism with the use of lactate dehydrogenase inhibitor, oxamate, which when co-injected with lactate will prevent the latter from being metabolized into pyruvate (Figure 4a). Central injections of oxamate (50 mM) alone did not affect FI and BW up to 42 h (Figure 6b, c). Yet, when oxamate was coinjected ICV with L-lactate, it completely abolished the 18-h effect of central lactate on FI (Figure 6b), as well as both the 18- and 42-h effects on BW (Figure 6c). These data indicate that central lactate metabolism to pyruvate is required for central lactate to regulate energy homeostatic parameters such as FI and BW.

Central Administration of Pyruvate Lowers Food Intake and Body Weight

If central lactate metabolism to pyruvate represents an important biochemical pathway to lower FI and BW, it follows that direct ICV injection of pyruvate should recapitulate the anorectic effects of central lactate (Figure 4a, 8a). ICV pyruvate (5 mM; 1 molecule of lactate generates 1 molecule of pyruvate) injections lowered FI from 27 ± 2 to 12 ± 4 g (Figure 7b), with an accompanying 18-h BW reduction of 6 ± 2% (Figure 7c). It is important to note also that the anorectic effects of ICV pyruvate closely resemble

those observed with ICV lactate injections. Together, these data suggested that hypothalamic lactate metabolism to pyruvate regulates FI and BW.

Central Lactate Metabolism Is Required for Circulating Lactate to Lower Food Intake and Body Weight

Direct sensing of glucose (70), fatty acids (206), amino acids (65), and lactate (as demonstrated in the aforementioned experiments) by the brain regulates FI and BW. However, it remains unknown whether these CNS nutrient-sensing mechanism(s) takes part in mediating circulating nutrients' ability to regulate FI and BW. With that in mind, we next tested whether a selective and sustained doubling of plasma lactate levels for 3 h regulates FI and BW, and whether the associated effects are in part centrally regulated: i.e. whether the effect on FI and BW of peripheral lactate are dependent on CNS lactate metabolism (Figure 8a). We subjected rats to ICV surgeries on day -12 and then IV surgeries on day -7 for central and peripheral substance administration, respectively. Only rats with FI and BW that returned to baseline levels by day -3 were used for experimentation. After consistent baseline FI and BW readings for 3 days (d -3 to 0), we performed *in vivo* infusion experiments on day 0 (Figure 8b). In brief, IV lactate (100 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) was infused for 3 h, terminating 1 h before the dark cycle (Figure 8b) to increase plasma lactate levels from 0.9 ± 0.1 mM (*n* = 5, IV saline) to 2.1 ± 0.4 mM (*n* = 6, IV lactate), as is consistent with previous studies (150). This IV lactate infusion reduced FI from 27 ± 2 to 1 ± 0.5 g (98 ± 2% decrease) and BW by 43 ± 6 g (15 ± 3%) at

18 h, both of which returned to baseline after 42 h (Figure 9a, b). To determine whether these effects on FI and BW induced by circulating lactate are, at least in part, centrally regulated, ICV oxamate was injected in a second set of rats to inhibit central lactate metabolism to pyruvate in the presence of IV lactate infusion (Figure 8a, b). ICV oxamate administration in addition to IV lactate partially reduced the FI- and BW-lowering effect of doubling levels of circulating lactate: FI was only lowered from 28 ± 3 to 18 ± 2 g (35 ± 7% decrease) after 18 h, returning to slightly higher than baseline after 42 h (Figure 9a). BW was unchanged (Figure 9b) at both 18- and 42-h post-infusion. It is critical to state that central administration of oxamate was unable to fully nullify the anorectic effects of circulating lactate (Figure 9a), indicating that other regions of the brain or other parts of the body are involved in this appetite regulation. Nonetheless, we have demonstrated that central lactate metabolism to pyruvate is required for circulating lactate to regulate FI and BW.

4.4 FIGURES

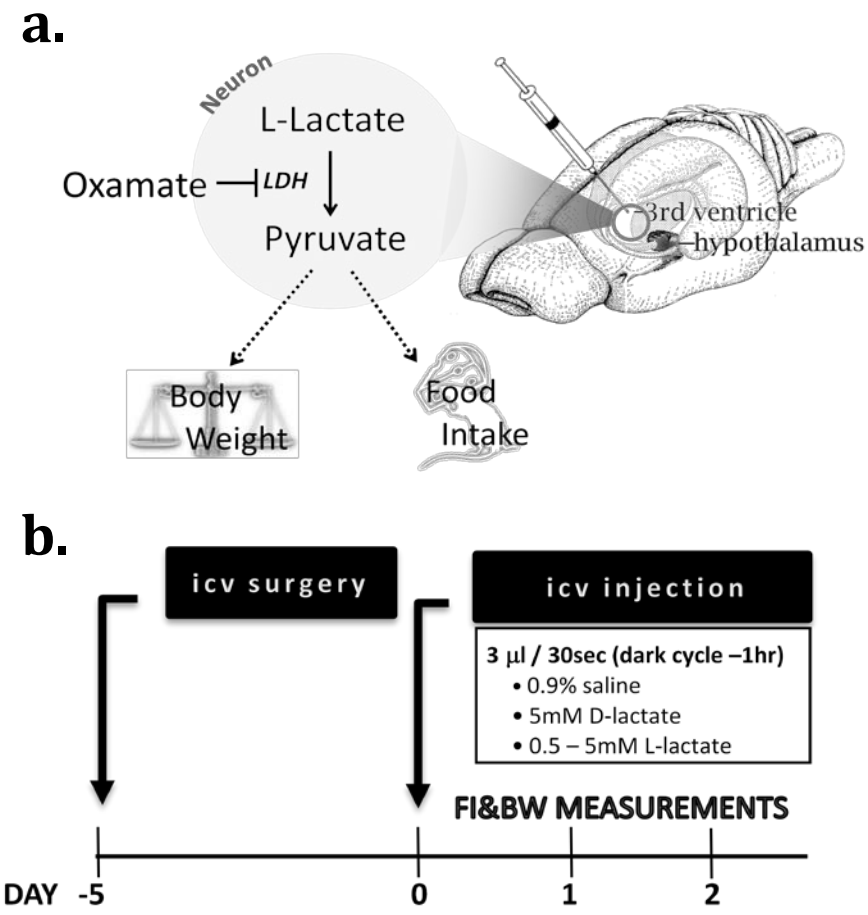


Figure 4 Schematic representation of working hypothesis and experimental design for the energy homeostatic regulation by CNS lactate metabolism

a: Proposed model for food intake and body weight regulation by CNS lactate metabolism. Oxamate is an inhibitor of LDH, which blocks the interconversion between L-lactate and pyruvate. **b:** Schematic representation of experimental design. ICV (3rd ventricle) stereotaxic surgery was performed on male Sprague Dawley rats (~260 – 280 g) and given 5 days of recovery for food intake and body weight to stabilize before ICV administration. Baseline food intake and body weight were measured (Time -6 h). Rats were injected at Time 0 h (i.e. 1 h before the dark cycle) with 3 µl of saline, D-lactate (5 mM), L-lactate (0.5, 1, 2.5, 5 mM), . Food intake and body weight were monitored for 2 subsequent days.

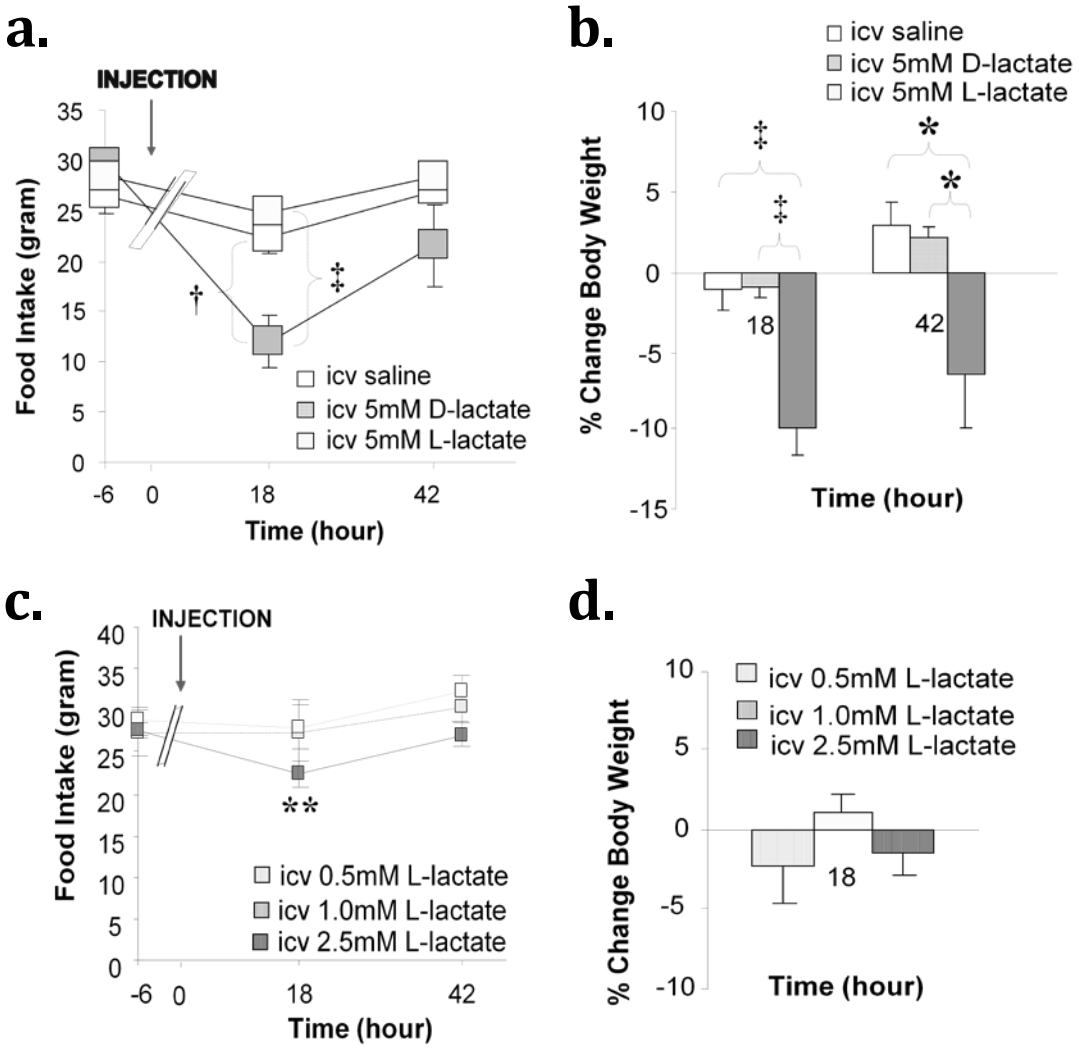


Figure 5 Central injections of lactate lowers food intake and body weight

a & b: Effect of ICV saline (*n* = 10), D-lactate (5 mM, *n* = 7), and L-lactate (5 mM, *n* = 9) on food intake (a) and % change in body weight (b). **c & d**: Effect of ICV L-lactate (0.5 mM, *n* = 4; 1 mM, *n* = 5; 2.5 mM, *n* = 5) on food intake (c) and % change in body weight (d). **P* < 0.05, ICV lactate vs. ICV saline/D-lactate; †*P* < 0.01 ICV lactate vs. ICV saline/D-lactate; ‡*P* < 0.001, ICV lactate vs. ICV saline/D-lactate; ***P* < 0.05, ICV 2.5 mM lactate vs. basal.

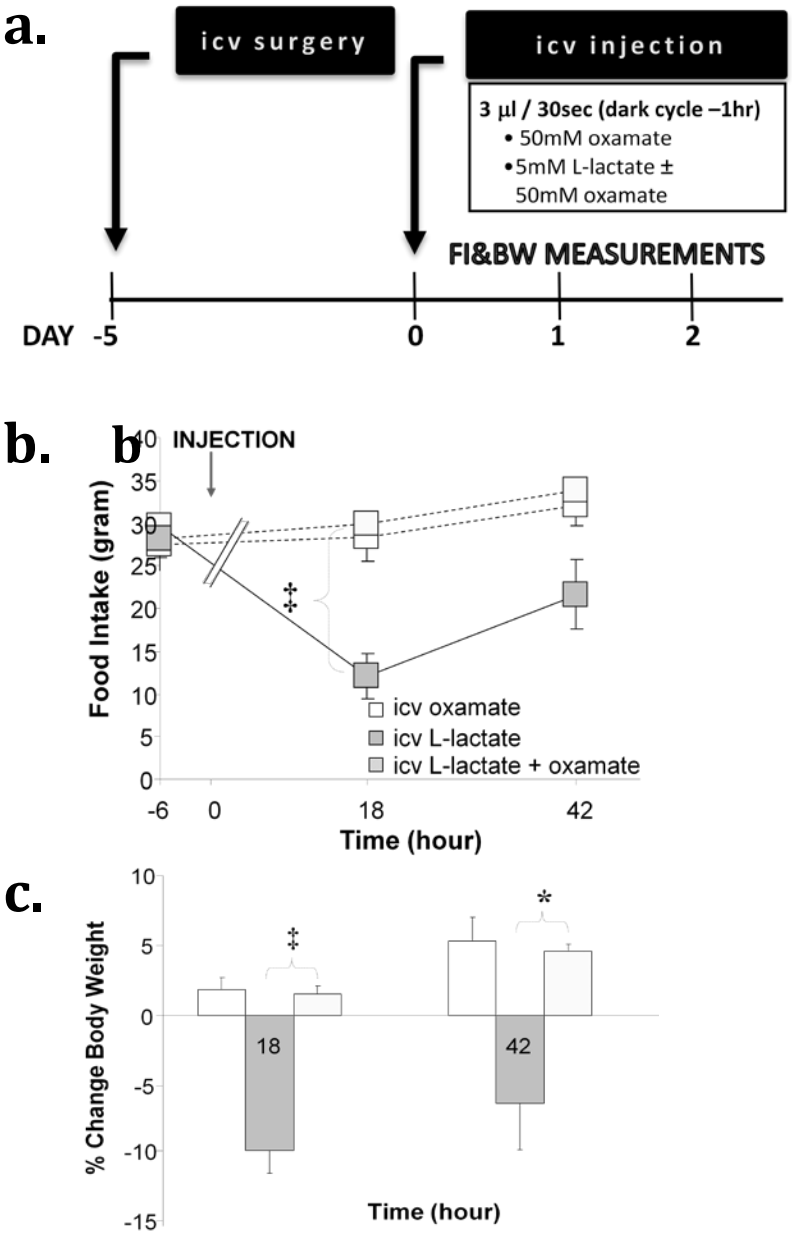


Figure 6 Central co-injections of oxamate with lactate block the anorectic effects of lactate

a: Schematic representation of experimental design. Rats were injected at time 0 h (i.e., 1 h before dark cycle) with 3 µl of oxamate (50 mM), L-lactate (5 mM), or L-lactate with oxamate. Food intake and body weight were monitored for 2 subsequent days. **b & c**: Effect of ICV injections of oxamate (n = 7), L-lactate (n = 9), and L-lactate with oxamate (n = 6) on food intake (b) and % change in body weight (c). *P < 0.01, ICV lactate vs. ICV lactate with oxamate; ‡P < 0.001, ICV lactate vs. ICV lactate with oxamate.

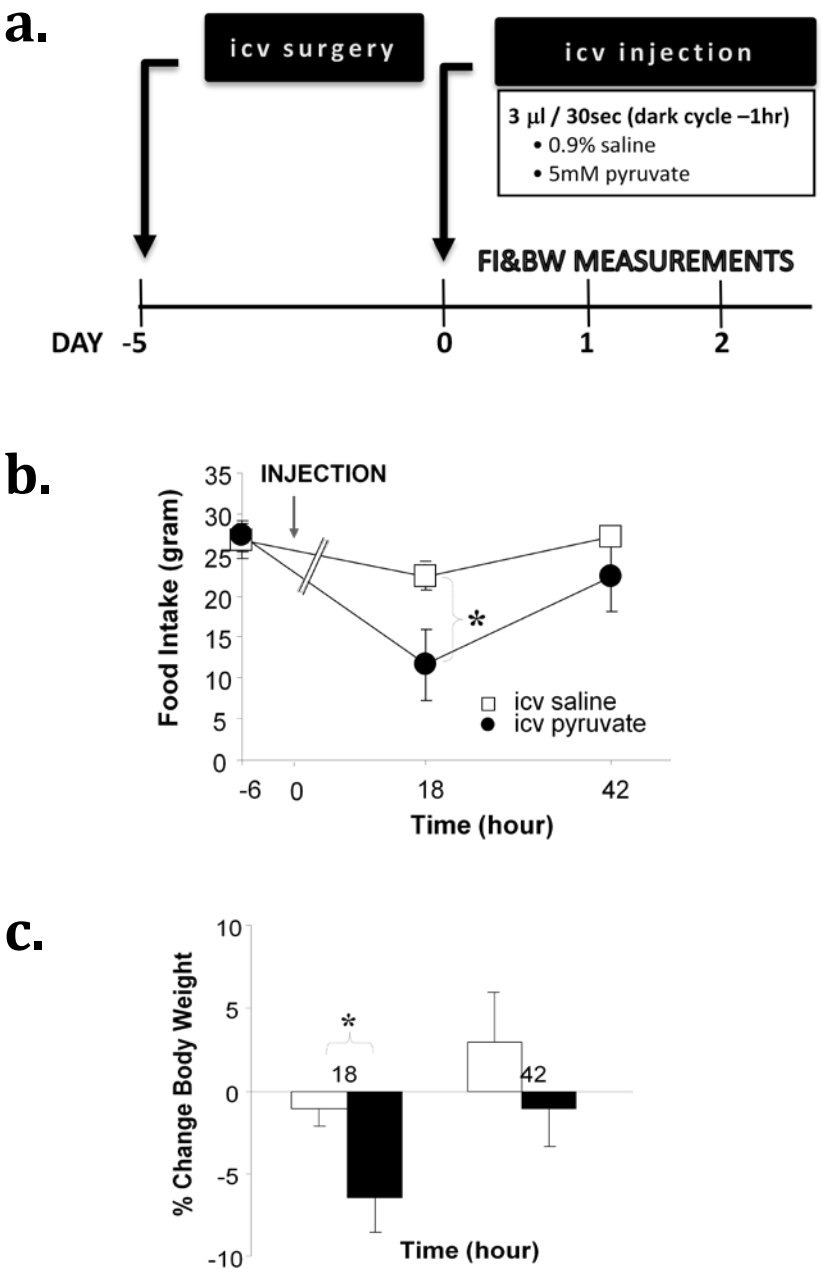


Figure 7 Central injections of pyruvate lower food intake and body weight

a: Schematic representation of experimental design. Rats were injected at time 0 h (i.e., 1 h before dark cycle) with 3 µl of lactate or pyruvate (5 mM). Food intake and body weight were monitored for 2 subsequent days. **b & c**: ICV pyruvate (*n* = 7), a product of lactate metabolism, recapitulated the anorectic effect of ICV lactate, affecting food intake (b) and % change in body weight (c). **P* < 0.05, ICV pyruvate vs. ICV saline.

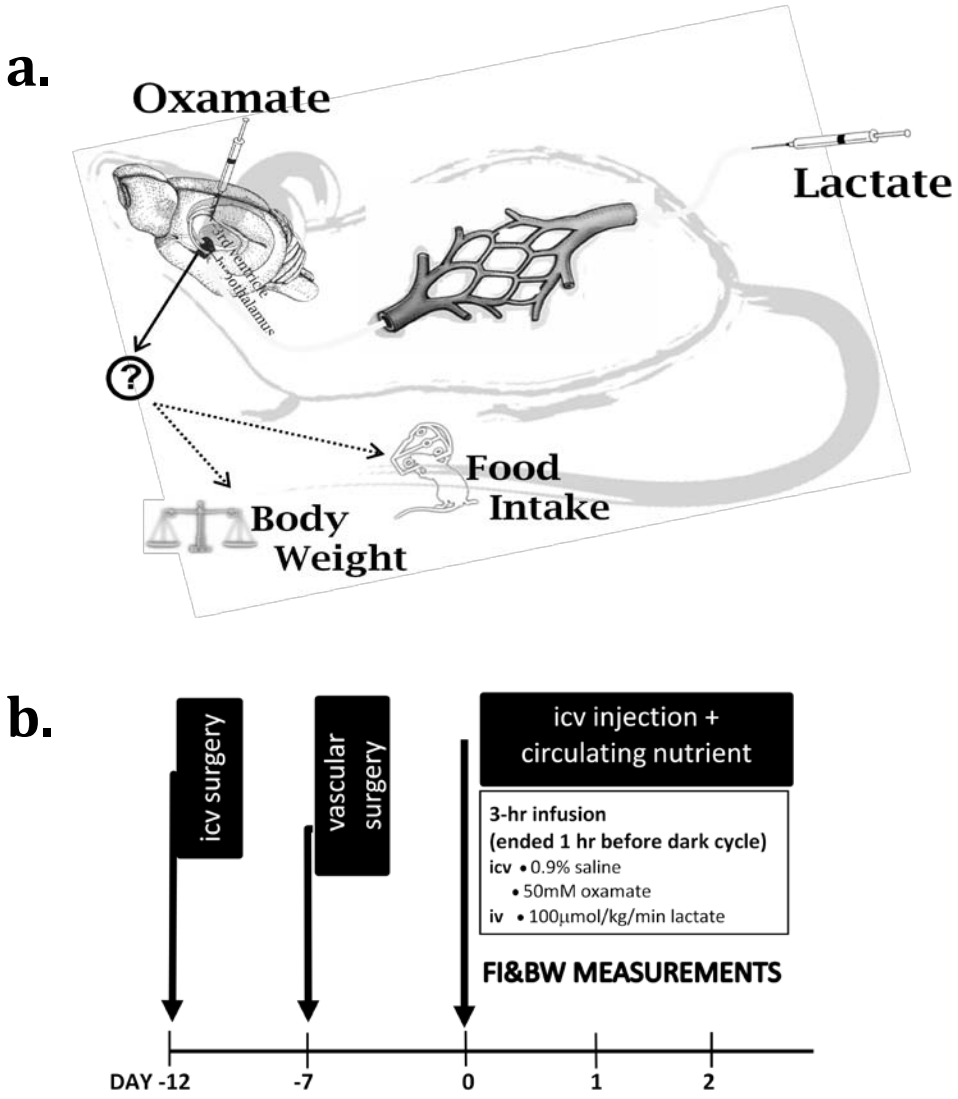


Figure 8 Schematic representation of working hypothesis and experimental design for the involvement of the central nervous system in peripheral nutrient-sensing

a: Proposed model for food intake and body weight regulation on ICV oxamate co-administered with IV lactate. If central lactate metabolism mediates the anorectic effect of circulating lactate, central administration of LDH inhibitor, oxamate, should abolish the FI- and BW-lowering effects of IV lactate infusion. **b**: Schematic representation of experimental design. ICV (3rd ventricle) surgery was performed on male Sprague Dawley rats (~260 – 280g). Rats were given 5 days of recovery before IV surgery, after which 7 days were given for food intake and body weight to stabilize before feeding protocol. Baseline food intake and body weight were measured (time -6 h). Rats were administered ICV saline or oxamate (50 mM at 5 μl/h) together with IV lactate (100 μmol· kg^{-1}·min^{-1}) for 3 h, ending 1 h before the onset of the dark cycle (time -4 h to -1 h); ICV bolus of saline or oxamate (50 mM) was given over a 1-min period at the beginning and end of the 3-h continuous infusion. Food intake and body weight were monitored for 2 days.

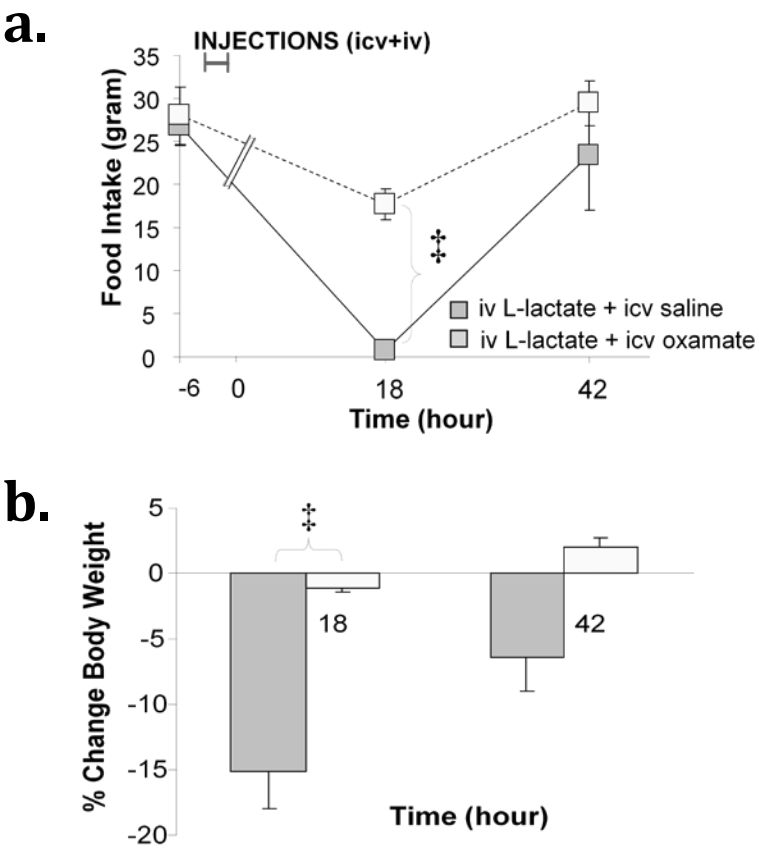


Figure 9 Central administration of oxamate blocks the anorectic effect of circulating lactate

a & b: Effect of ICV saline with IV lactate (n = 6) and ICV oxamate with IV lactate (n = 5) on FI (a) and % change in body weight (b). ‡P < 0.001, ICV saline with IV lactate vs. ICV oxamate with IV lactate

5

STUDY 2

ACTIVATION OF NMDA RECEPTORS IN THE NUCLEUS OF THE SOLITARY TRACT REGULATE GLUCOSE HOMEOSTASIS

5.1 ABSTRACT

While the hypothalamus is known to sense nutrients, including lactate metabolism-mediated sensing, to regulate peripheral glucose, energy and lipid homeostasis, the downstream pathway(s) involved in such sensing remains unclear. To assess whether the brain stem, in particular the NMDA receptors in the NTS, is required for hypothalamic nutrient-sensing mechanisms to lower GP, we first developed a rodent model with bilateral catheters in the MBH and NTS. [3-^{3}H] glucose was infused either into the MBH or NTS of rats at the same rate and duration as in our normal MBH or NTS infusion studies. It was found that MBH administration of tracer selectively increased radioactivity in the MBH but not in the NTS of the same rats. The reverse was true of NTS tracer administration. Having developed an *in vivo* model which enabled us to (A) concurrently but independently deliver substances into the MBH and NTS, and (B) examine the neuronal communication between the hypothalamus and the NTS, we then activated hypothalamic nutrient-sensing by direct delivery of lactate into the MBH. Activation of hypothalamic mechanisms by lactate markedly lowered GP. More importantly, inhibition of NTS NMDA receptors with MK-801 blocked this effect. NMDA receptors are composed of NR1 and NR2 subunits which are activated by glycine and glutamate, respectively. We showed that activation of NMDA receptors in the NTS *per* se via direct NTS delivery of glycine markedly lowered GP. Inhibition of NTS NMDA receptors with MK-801 abolished this GP-suppression induced by NTS glycine. Together, our data suggest that the activation of NTS NMDA receptors is necessary for hypothalamic signals to control GP and can be directly activated with glycine.

5.2 MATERIALS AND METHODS

MBH/NTS Administration in Pancreatic (Basal Insulin)-Euglycemic Clamp Procedure

4 days post-iv catherization, animals whose daily FI and BW had recovered back to baseline underwent the clamp studies. Rats were restricted to 20g of food the night before the experiment, equivalent to removing food for 5 h before the beginning of *in vivo* studies, to ensure the same nutritional status during the clamp procedure, which lasted a total of 210 min. At t = 0 min, central infusion of various treatments into the MBH, NTS or both locations via indwelling cannulae were initiated and maintained throughout the entire duration of the clamp at a constant infusion rate of 0.33 μl/h (all central infusion performed with CMA/400 syringe microdialysis infusion pumps). Briefly, the treatment groups were:

a) MBH saline + NTS saline
b) MBH saline + NTS MK-801 (Sigma; 0.06ng/min, dissolved in saline)
c) MBH L-lactate (5mM, dissolved in saline) + NTS saline
d) MBH L-lactate (5mM) + NTS MK-801 (0.06ng/min, with 2 h pre-infusion starting at t = -120 min).
e) NTS saline
f) NTS MK-801 (0.06 ng/min)
g) NTS glycine (Fisher Chemicals; 10μM, dissolved in saline)
h) NTS glycine (10μM) + NTS MK-801 (0.06 ng/min)

Animals in treatment groups a to d had both MBH and NTS cannulae and those in treatment groups e to h had only NTS cannulae. A primed-continuous IV infusion of [3-^{3}H] glucose (Perkin Elmer; 40μCi bolus; 0.4 μCi/min; all infusion performed with Harvard Apparatus PHD 2000 infusion pumps) was also initiated at the onset of the

procedure (t = 0 min) and maintained for the entire duration of the clamp to assess glucose kinetics based on the tracer-dilution methodology. A pancreatic (basal insulin)-euglycemic clamp was started at t = 90 min until 210 min to evaluate the effect of treatments on glucose metabolism independent of changes in glucoregulatory hormones at basal levels. To achieve this, somatostatin (3μg/kg/min) was continuously infused to inhibit endogenous insulin and glucagon secretions. Insulin (0.8mU/kg/min) was replaced back to near basal levels (see Tables 1, 2). During this period, a variable infusion of 25% glucose solution was started and periodically adjusted to maintain the plasma glucose concentration at the basal state (see Tables 1, 2 for basal and clamp glucose levels).

Plasma samples were collected to determine glucose levels and the specific activities of [3-^{3}H]glucose and tritiated water at regular intervals throughout the basal (t 0 – 90 min) and clamp period (t 90 – 210 min). Plasma samples were also collected at regular intervals for determination of plasma insulin and glucagon levels. At the end of the experiment, rats were anesthetized and tissue samples were freeze-clamped *in situ* and stored at -80°C for later analysis. Time between the injection of anesthesia and the freeze-clamping of tissue samples was less than 60 seconds (sec). Of note, the MBH was sampled by dissecting a wedge of tissue including the entire mediolateral and dorsoventral extent of the arcuate nuclei while minimizing ventromedial nucleus tissue. The NTS was sampled by injecting 2μl (volume found to be restricted locally to the NTS) of bromophenol blue via the NTS cannulae and obtaining the BPB-stained portion.

Tracer Verification of Cannulae Placement

To verify the anatomical placement of the bilateral cannulae, and to confirm that infusion in the MBH was localized (i.e. did not spread to the NTS) and vice versa, radioactive tracer [3-^{3}H] was administered via the bilateral cannulae in the MBH or NTS at the same infusion rate (0.33μl/h) and duration (210 min) as in the pancreatic-euglycemic clamp procedure. By the end of the 210-min infusion, MBH and NTS tissue samples were obtained as previously discussed and were homogenized for counting.

Biochemical Analysis

Plasma glucose

Plasma glucose levels were measured with the use of a glucose analyzer (Glucose Analyzer GM9, Analox Instruments, Lunenbertg, MA). The analyzer was calibrated before usage in each experiment. To measure plasma glucose concentration, plasma was obtained from a blood sample (~0.1 ml) of rat through centrifugation at 6000 rpm. A 10μl sample of plasma was immediately pipetted into the glucose analyzer to determine plasma glucose cencentration. The glucose analyzer determines glucose concentrations by the glucose oxidase method. In brief, the glucose analyzer measures the rate of oxygen consumption in the reaction between plasma sample and glucose oxidase, as in the following reaction:

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{D-Gluconic acid} + H_2O_2$$

Under the assay conditions, the rate of the enzymatic reaction is directly proportional to the glucose concentration of the plasma sample. The oxygen consumption, which is proportional to the glucose concentration, is determined with a polarographic oxygen sensor. Specifically, Clark-type amperometric oxygen electrodes are immersed in the solution of interest with a potential applied between them that is sufficient to reduce dissolved oxygen at the working electrode. Through this, the partial pressure of oxygen in the solution is determined given that it is proportional to the limiting current.

Plasma glucose tracer specific activity

The radioactivity of [3-^{3}H] in plasma was determined using 50 µl aliquots of plasma. The plasma aliquot was first deproteinized with $Ba(OH)_2$ and $ZnSO_4$, then centrifuged (7 min at 6000 rpm and 4°C)to separate precipitate from supernatant. The protein-free supernatant was kept. Since tritium on the C-3 position of glucose is lost to water during glycolysis, the supernatant was evaporated to dryness for the removal of tritiated water to ensure that liquid scintillation counting of the evaporated supernatant would represent radioactivity from [3-^{3}H] glucose in the plasma only.

Plasma insulin

Plasma insulin levels were determined by radioimmunoassay (RIA) using a 2-d commercial rat insulin RIA kit (100% specificity) from Linco Research (St. Charles, MO). The principle of RIA is based on antigen-antibody binding where a fixed concentration of

labeled tracer antigen (^{125}I-labeled insulin) is incubated with a constant dilution of antiserum (guinea pig anti-rat insulin serum) such that there remains excess antigen unbound to antibody. When unlabeled antigen from the plasma sample is added to this system, there is competition between the labeled and unlabeled antigen to bind to the limited amount of antibodies. Based on the principle of substrate binding, the percent binding of the labeled and unlabeled antigen is proportional to their relative concentrations. By separating the antibody-bound tracer from the unbound tracer then counting the radioactivity of the fractions, the concentration of insulin in the sample can then be determined based on a predetermined standard curve.

A 2-d protocol provided by the supplier was used. In brief, a standard curve was determined in duplicates using the provided insulin standards (0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/mL). ^{125}I-labeled insulin (50 µL) and rat insulin antibody (50 µl) were added to the aforementioned standards, as well as the experimental plasma samples (50 µl), followed by vortexing. After an overnight incubation at 4°C, 1.0 ml of precipitating reagent was added to each tube followed by vortexing and 20-min incubation at 4°c. The samples were centrifuged to pellet the bound insulin. The radioactivity of the pellet was then counted by a gamma counter (Perkin Elmer 1470). The counts (B) for the samples and standards were expressed as a percentage of the mean counts of the total binding reference tubes (B_0):

$$\% \text{ total binding} = \% \frac{\text{B}}{\text{B}_0} = \text{sample} \therefore \text{standard} \div \text{B}_0 \times 100\%$$

The percent activity bound for each standard was plotted against the known concentration (0.1 to 10 ng/mL) to construct the standard curve. Finally, the plasma insulin concentrations of the experimental samples were then determined by interpolation.

Plasma glucagon

Plasma glucagon levels were determined by RIA using a 3-d commercial kit from Linco Research (St. Charles, MO). The principle of RIA is based on antigen-antibody binding where a fixed concentration of labeled tracer antigen (^{125}I-labeled glucagon) is incubated with a constant dilution of antiserum (guinea pig anti-glucagon serum) such that there remains excess antigen unbound to antibody. When unlabeled antigen from the plasma sample is added to this system, there is competition between the labeled and unlabeled antigen to bind to the limited amount of antibodies. Based on the principle of substrate binding, the percent binding of the labeled and unlabeled antigen is proportional to their relative concentrations. By separating the antibody-bound tracer from the unbound tracer, then counting the radioactivity of the fractions, the concentration of glucagon in the sample can then be determined based on a predetermined standard curve.

A 3-d protocol provided by the supplier was used. The specific protocol was similar to that of plasma insulin determination with minimal changes: plasma glucagon determination has an additional overnight incubation of the standards (20, 50, 100, 200 and 400 pg/ml) and samples at 4°c with the glucagon antibody alone.

Calculations

To determine GP and uptake in our experiment animals, the technique of using [3-^{3}H] glucose (9), and the steady state formula (263) were employed. [3-^{3}H] glucose tracer was infused at a constant rate into the rat and allowed for sufficient equilibration period of the radioactive tracer. Thereafter, using the steady state formula, the rate of glucose appearance, R_a, which is equal to the rate of glucose disappearance, R_d, is determined by dividing the [3-^{3}H] glucose infusion rate by the specific activity of the plasma [3-^{3}H]glucose:

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

Under steady-state basal condition, the rate of glucose uptake (R_d) equals the rate of glucose appearance (R_a), which is the same as the rate of the endogenous GP. During the pancreatic clamp setting where exogenous glucose was infused to maintain euglycemia, the rate of endogenous GP was therefore obtained from the difference between R_d and the rate of glucose infusion.

5.3 RESULTS

NTS NMDA receptors integrate hypothalamic lactate-sensing signals to control glucose production

The hypothalamus' nutrient-sensing ability, in particular that of lactate metabolism-mediated-sensing, to regulate glucose homeostasis has been identified (151). However, the downstream effectors of such hypothalamic nutrient-sensing remain largely unidentified. Interestingly, while the neuronal communication between the hypothalamus and the NTS has been evaluated in the control of various homeostatic mechanisms such as the cardiovascular system (108) and osmoregulatory circuits (36), it is currently unknown whether and how the NTS integrates hypothalamic nutrient-sensing neuronal signals to regulate glucose homeostasis *in vivo*. As such, the first aim of this study is to evaluate whether the NTS relays hypothalamic lactate-sensing signals to lower GP *in vivo*.

To assess whether the NTS NMDA receptors are required for hypothalamic nutrient-sensing mechanisms to regulate glucose homeostasis (Figure 10a), we first developed a rat model that received bilateral catheters into both the MBH and NTS. Upon recovery from stereotaxic surgery on day 7, measured by a return to basal FI and BW for at least 3 days, rats were administered [3-^{3}H] glucose at 0.33 µl/ h for 3.5 h, the same rate as normally in our hypothalamic or NTS infusion studies, either into the MBH or NTS (Figure 11a, b). Upon completion of the tracer infusions, MBH and NTS wedges were harvested, homogenized and counted for radioactivity. MBH administration of [3-^{3}H] glucose selectively increased radioactive counts (dpm/mg) in the MBH but not in the

NTS of the same rats (Figure 11a). Conversely, NTS administration of [3-^{3}H] glucose selectively increased radioactive counts in the NTS but not in the MBH (Figure 11b). Cortical tissue samples were found to have no radioactivity and were used as a negative control for both groups (Figure 11a, b). Thus, having developed an *in vivo* model which enabled us to (A) concurrently but independently, without direct contact between the two regions, deliver substances into the MBH and NTS, and (B) examine the neuronal communication between the hypothalamus and the NTS, we then activated hypothalamic nutrient-sensing by direct delivery of lactate into the MBH.

Male Sprague Dawley rats were subjected to stereotaxic surgery to implant bilateral catheters in the MBH and NTS on day 0. Upon recovery from surgery on day 7, measured by a return to basal FI and BW for at least 3 days, recovered rats then underwent vascular surgery with their jugular vein and carotid artery cannulated for infusion and sample purposes, respectively. These rats were then allowed to recover, and on day 11 they underwent the pancreatic (basal insulin) euglycemic clamp technique to assess glucokinetics: the use of the pancreatic (basal insulin) euglycemic clamp technique and tracer-dilution methodology allows for the assessment of the effects that treatments have on the rate of hepatic GP and peripheral glucose uptake independent of differences among groups in peripheral circulating insulin and glucose levels (50; 234; 288) (Figure 10b). We first co-infused MBH and NTS saline into the same rat and saw no effect on glucose infusion rate, hepatic GP or utilization. Next, we activated hypothalamic nutrient-sensing mechanisms by direct delivery of lactate into the hypothalamus as previously described (150; 151), with the co-infusion of NTS saline.

During the clamps, glucose had to be infused systemically to prevent hypoglycaemia in hypothalamic lactate-administered rats (comparing to hypothalamic saline), indicating an enhancement of whole body insulin sensitivity independent of differences among groups in peripheral circulating insulin and glucose levels (Table 1). Based on the steady-state tracer dilution methodology, the increased glucose infusion (Figure 12a) was due entirely to a suppression of GP (Figure 12b, c), rather than a change in glucose uptake (Figure 12d). Thus having confirmed previous findings that hypothalamic lactate-sensing lowers hepatic GP, we next evaluated whether inhibition of NTS signals, namely the NMDA receptors in the NTS, would negate the ability of hypothalamic nutrient-sensing signals to lower GP *in vivo*. We inhibited NTS NMDA receptors via NTS delivery of a high affinity blocker of the NMDA receptor, MK-801 (133). We infused MK-801 at a concentration that alone did not have effect glucose kinetics parameters (0.06ng/min) into the NTS and first activated hypothalamic nutrient-sensing mechanisms by lactate delivery in the same rats (Figure 10a, b). In the presence of similar plasma insulin levels (Table 1) and glucose uptake (Figure 12d), NTS MK-801 completely abolished the ability of hypothalamic lactate to increase glucose infusion rate (Figure 12a) and decrease GP (Figure 12b, c). These data suggest that NTS NMDA receptors mediate hypothalamic lactate metabolism to lower GP.

NTS glycine activates NMDA receptors to lower glucose production

Given that the NTS NMDA receptors mediate hypothalamic nutrient sensing signals, it should follow that direct activation of the NMDA receptors in the NTS should recapitulate the effect induced by hypothalamic-relayed signals. Furthermore, it is not illogical to postulate that perhaps the NTS can directly sense nutrients to regulate peripheral glucose homeostatic parameters. Interestingly, glycine, a nutrient in itself, is a ligand for the NMDA receptor. In fact, an elevation of glycine concentration in hippocampal slices (with glycine at 10 μM) or cerebral cortex is sufficient to augment NMDA receptor function (23; 140). Furthermore, glycine potentiates the NMDA-mediated neuronal firing rate of the dorsal horn *in vivo* (40). Therefore, we set out to first examine whether direct activation of NMDA receptor in the NTS via NTS glycine administration regulates glucose homeostasis *in vivo* (Figure 13a). We infused the amino acid glycine at 10 μM, ~1.5 fold higher than the CSF [glycine] in both humans and rodents (175; 290)**,** directly into the NTS of conscious unrestrained rats, and evaluated the gluco-regulatory effects of NTS glycine with the pancreatic clamp technique (Figure 13b).

During the pancreatic clamp when circulating insulin and glucose were maintained at near basal levels (Table 2), NTS glycine administration markedly increased the rate of exogenous systemic glucose infusion required to maintain euglycemia compared to NTS saline (Figure 14a), indicating an enhancement of whole body insulin sensitivity independent of differences among groups in peripheral circulating insulin and

glucose levels (Table 2). It was determined that the increase in glucose infusion rate induced by NTS glycine was accounted for entirely by an inhibition of GP (lowered to ~4.9 mg/kg.min) (Figure 13b, c), rather than an increase in glucose uptake (Figure 13d). We also administered NTS glycine at 5 μM (instead of 10 μM) and GP during the clamp was lowered partially to 6.9 mg/kg.min. Under non-clamped physiological settings, NTS glycine (10 μM) administration for 90 min was sufficient to lower plasma glucose levels by ~1.5 mM (Figure 15). Together, we have demonstrated that direct NTS glycine administration lowered GP and plasma glucose levels.

To verify whether NTS glycine administration lowered GP/levels through the activation of NTS NMDA receptors, we co-infused NMDA receptor ion channel blocker MK-801 with glycine directly into the NTS (Figure 13a, b). During the clamps, it was first noted that NTS MK-801 alone did not affect any of the glucose kinetics parameters compared to NTS saline (Figure 14a-d). However, co-administration of NTS MK-801 with glycine was sufficient to fully negate the increased glucose infusion rate (Figure 14a) and the decreased GP (Figure 14b, c) induced by NTS glycine independent of differences among groups in peripheral circulating insulin and glucose levels (Table 2). In addition, NTS MK-801 fully reversed the plasma glucose-lowering effect induced by glycine under the unclamped conditions (Figure 15). These data indicate that glycine activates NMDA receptors in the NTS to lower GP and plasma glucose levels.

5.4 FIGURES

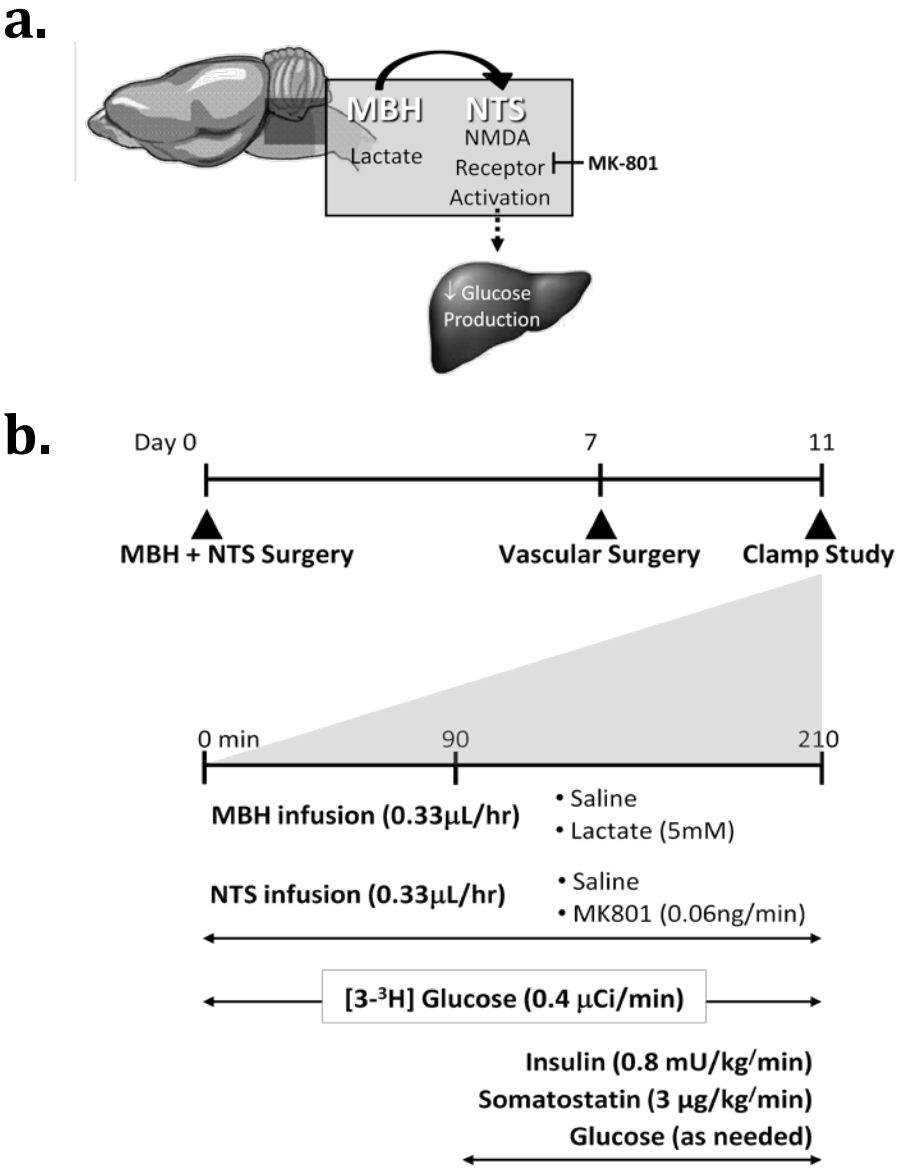
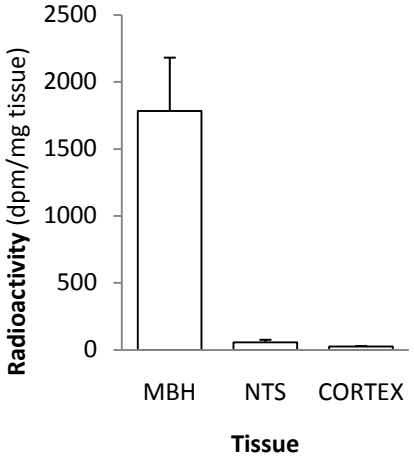


Figure 10 Schematic representation of hypothesis and experimental design for the hypothalamic-medullary neurocircuitry to regulate glucose production

a: Proposed model for a hypothalamic (MBH) lactate- medullary (NTS) NMDA receptors pathway to lower hepatic GP. Hypothalamic lactate lowers hepatic GP. MK-801 is a potent NMDA receptor antagonist that prevents the activation of the receptors. NTS administration of MK-801 abolishes the GP-lowering effect of hypothalamic lactate. **b**: Schematic representation of experimental design: MBH and NTS stereotaxic surgery were performed on male Sprague Dawley rats (~240 – 280 g). After 1 week of recovery (d 7), vascular surgery (i.e. intravenous catherization) was performed. Rats were given 5 recovery days until clamp studies (d 11), upon which NTS infusion of saline or MK-801 were given to MBH saline- or lactate-infusing animals.

a.



b.

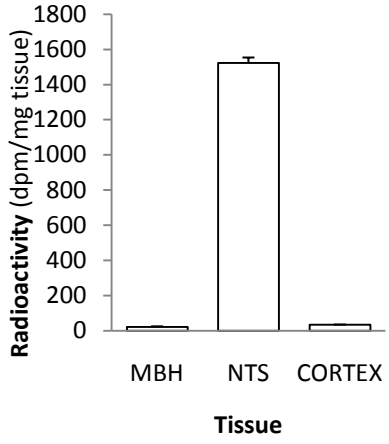


Figure 11 Verification of mediobasal hypothalamus and nucleus of the solitary tract infusion specificity

a & b: To verify the anatomical placement of cannulae and to ensure that infusion was localized, radioactive tracer administered in the mediobasal hypothalamu (a, MBH, n = 4) or nucleus of the solitary tract (b, NTS, n = 4) via the bilateral cannulae was confirmed to be contained within the respective tracer-infused tissues.

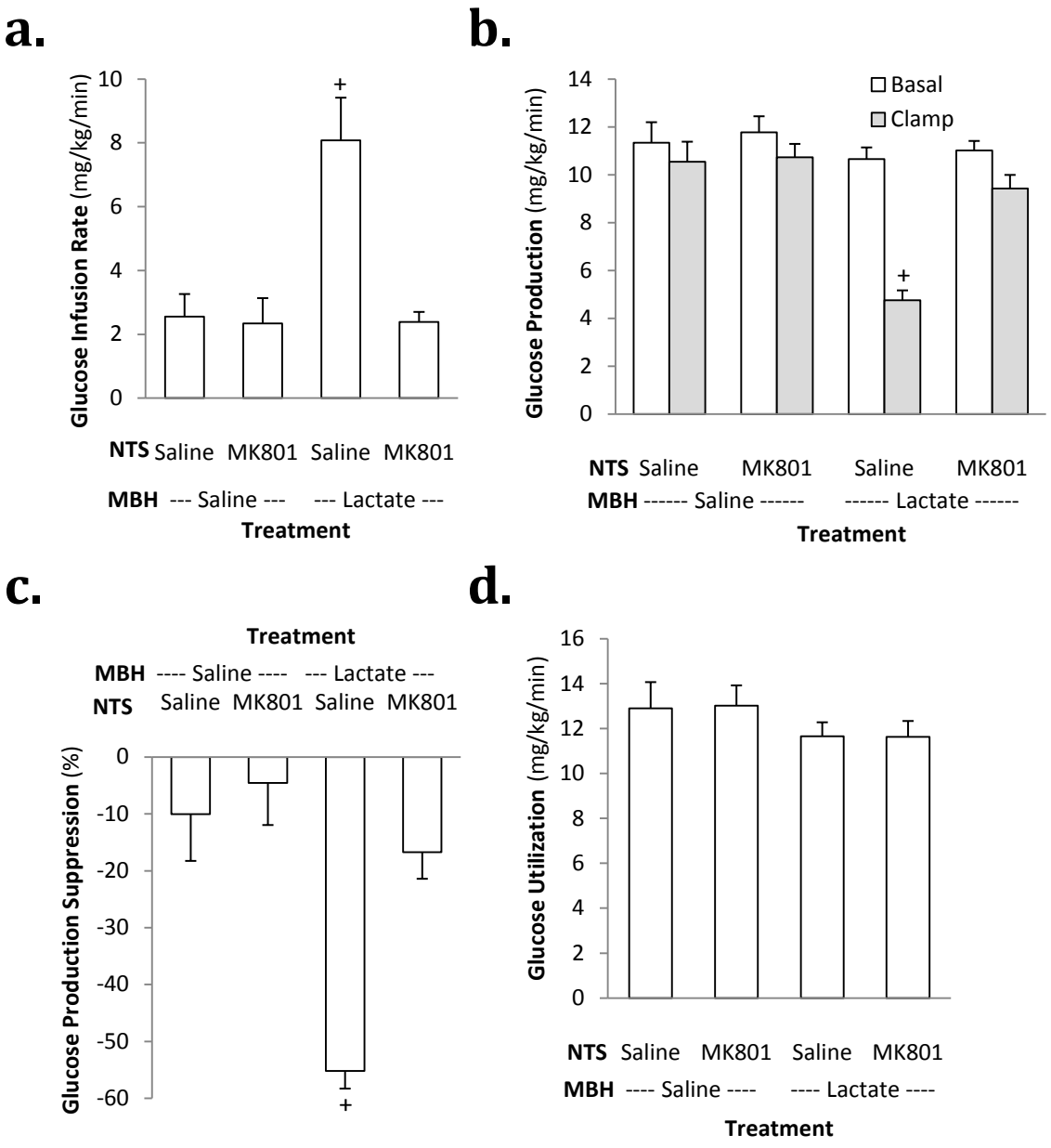


Figure 12 MK-801 in the nucleus of the solitary tract abolished the glucose production-lowering effect induced by hypothalamic delivery of lactate

a & b: NTS administration of MK-801 in lactate-infused animals prevented the expected increase in glucose infusion rate (a) and lowering in GP (b) found to be elicited by hypothalamic lactate treatment. **c:** Suppression of GP during the clamp period was expressed as % decrease from basal GP. **d:** Glucose utilization was comparable in all groups. MBH saline + NTS saline (n=5), MBH saline + NTS MK-801 (n=5), MBH lactate + NTS saline (n=5), MBH lactate + NTS MK-801 (n=6), + $P < 0.001$ (ANOVA).

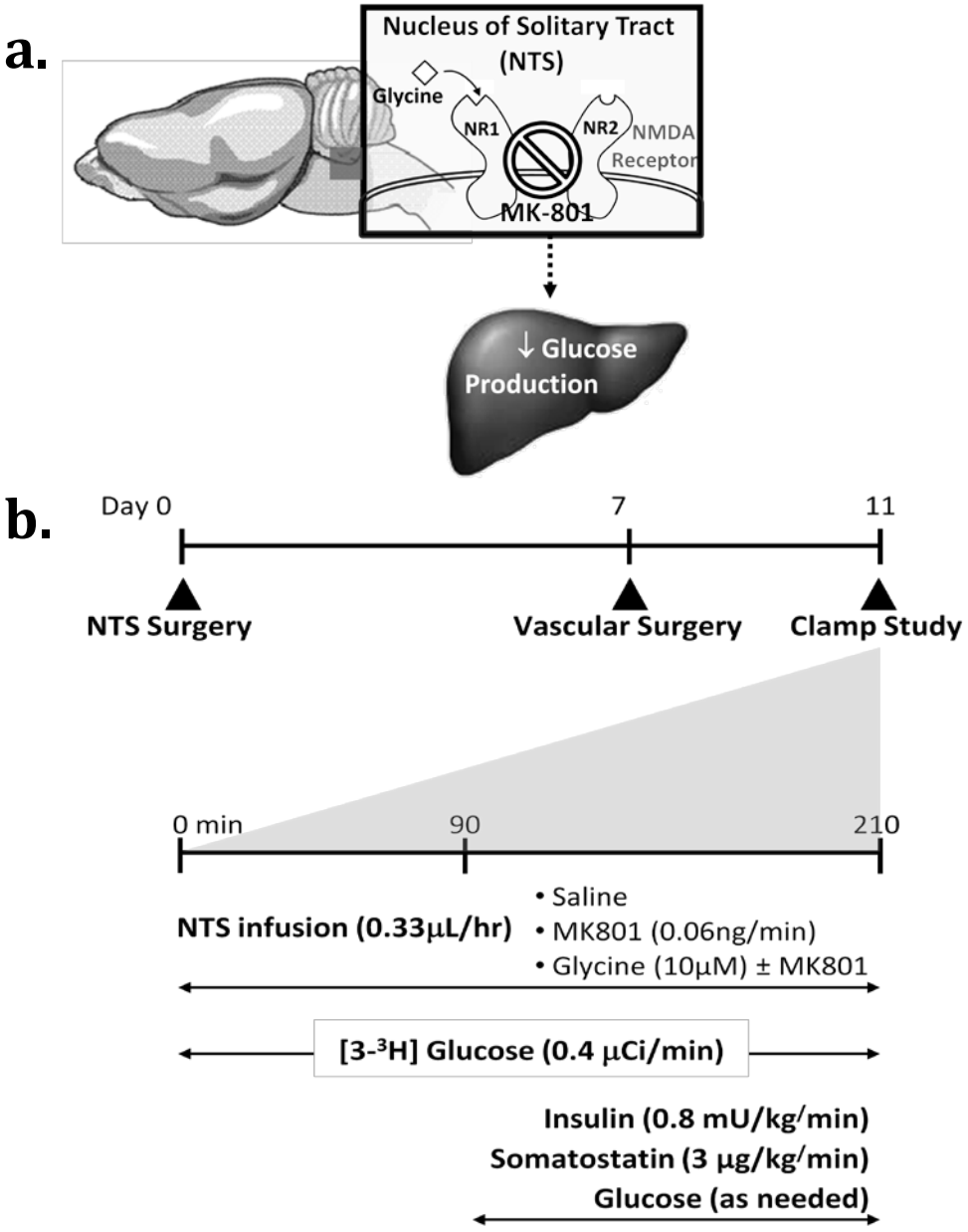


Figure 13 Schematic representation of working hypothesis and experimental design for the regulation of hepatic glucose production by N-methyl-D-aspartate receptors in the nucleus of the solitary tract

a: Proposed model for the activation of NMDA receptors *per se* to lower hepatic GP. NTS glycine binds to the NR1 subunit of the NMDA receptor, leading to a decrease in hepatic GP. NMDA receptor antagonist MK-801 binds to the NMDA receptor, preventing ion flow through these channels and blocks the GP-lowering effect of glycine. **b**: Schematic representation of experimental design. NTS stereotaxic surgery was performed on male Sprague Dawley rats (~240 – 280g). After 1 week of recovery (d 7), vascular surgery (i.e. intravenous catherization) was performed. Rats were given 5 recovery days until clamp studies (d 11).

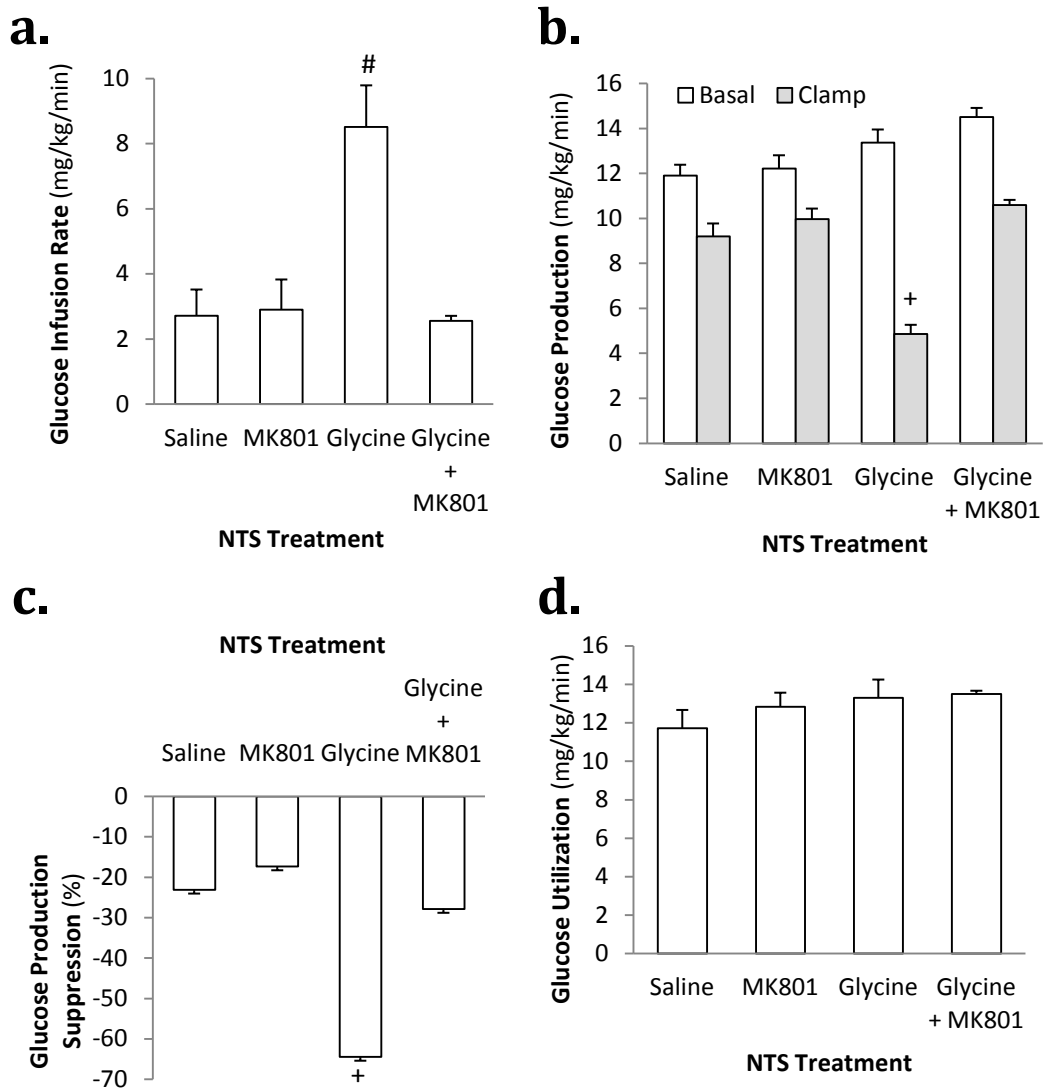


Figure 14 Glycine in the nucleus of the solitary tract lowers hepatic glucose production, an effect that is negated by N-methyl-D-aspartate receptor antagonist MK-801

a & b: During the pancreatic-euglycemic clamp procedure, NTS administration of glycine increased glucose infusion rate (a) and lowered GP (b), effects which are fully negated by co-administration with MK-801 (a,b) **c:** Suppression of GP during the clamp period was expressed as % decrease from basal GP. **d:** Glucose utilization was comparable in all groups. NTS saline (n=6), NTS MK-801 (n=5), NTS glycine (n=6), NTS glycine + MK-801 (n=5). # $P < 0.01$, + $P < 0.001$ (ANOVA).

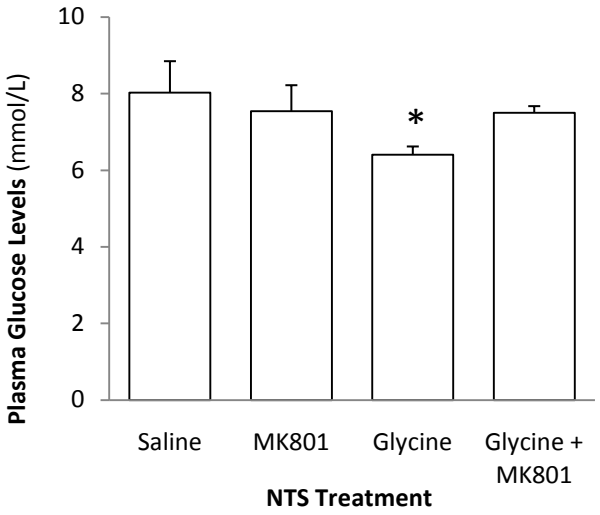


Figure 15 Glycine in the nucleus of the solitary tract lowers plasma glucose levels, an effect that was fully negated by co-administration with MK-801

Under non-clamped settings, NTS glycine infusion lowered plasma glucose levels within 90 min, an effect that was fully negated by co-administration with MK-801. NTS saline (n=6), NTS MK-801 (n=5), NTS glycine (n=6), NTS glycine + MK-801 (n=5). * $P < 0.05$, # $P < 0.01$, + $P < 0.001$ (ANOVA).

	MBH Saline + NTS Saline (n=4)	MBH Saline + NTS MK-801 (n=4)	MBH Lactate + NTS Saline (n=5)	MBH Lactate + NTS MK-801 (n=5)
Basal (t=0):				
Insulin (ng/ml)	1.0 ± 0.2	0.7 ± 0.3	0.9 ± 0.3	0.9 ± 0.1
Glucose (mM)	7.7 ± 0.2	7.6 ± 0.2	8.7 ± 0.6	7.5 ± 0.3
Clamp (t180-210):				
Insulin (ng/ml)	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Glucose (mM)	7.4 ± 0.3	7.5 ± 0.4	5.8 ± 1.1	7.6 ± 0.3

Data are means ± SEM. Medialbasal hypothalamus (MBH). Nucleus of the solitary tract (NTS).

Table 1 Plasma insulin and glucose concentrations of the groups during basal and clamp conditions

	NTS Saline (n=6)	NTS MK-801 (n=5)	NTS Glycine (n=6)	NTS Glycine + MK-801 (n=5)
Basal (t=0):				
Insulin (ng/ml)	1.1 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	1.1 ± 0.2
Glucose (mM)	8.0 ± 0.2	7.7 ± 0.2	7.5 ± 0.8	7.7 ± 0.4
Clamp (t180-210):				
Insulin (ng/ml)	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.2	0.9 ± 0.2
Glucose (mM)	7.6 ± 0.4	7.5 ± 0.4	5.8 ± 0.4	7.9 ± 0.2

Data are means ± SEM. Nucleus of the solitary tract (NTS).

Table 2 Plasma insulin and glucose concentrations of the groups during basal and clamp conditions

6
DISCUSSION

6.1 STUDY 1

In the past few decades, research in the field mainly focused on dissecting the neuronal circuit that is triggered by hormones such as leptin and insulin to regulate FI and BW (39; 59; 89; 222; 246). In comparison, little effort had been put into evaluating CNS nutrient-sensing in the regulation of energy homeostasis. It had been demonstrated that hypothalamic mechanisms associated with the regulation of energy homeostasis are disrupted in obesity, causing an elevation of appetite and BW (95; 120; 150; 225; 247). Although increased efforts have been placed on uncovering the neuronal networks underlying the control of these two parameters, much work remains in probing the precise biochemical and signaling cascade(s) that are responsible. Indeed, research with particular focus on the mechanistic pathway of CNS regulation of energy homeostasis will reveal new molecular targets to lower appetite and BW. And in doing so, these efforts should also advance T2DM management given the close association between the two rising health issues of obesity and T2Dm. The current study extends the quest to uncover novel CNS biochemical pathways and molecules in the regulation of energy balance with a focus on hypothalamic lactate-sensing.

Neuronal uptake of glucose-derived lactate provides fuel for neurons (169). Furthermore, hypothalamic lactate metabolism has been shown to regulate peripheral glucose and lipid homeostasis *in vivo* (150; 151). On the basis of these independent yet parallel findings, we postulated that the CNS senses lactate and via lactate metabolism regulates energy homeostasis. To our knowledge, we here provided the first direct

evidence that activation of central lactate metabolism to pyruvate with two independent approaches – ICV or IV lactate infusion – reduces FI and BW. This is further supported by the finding that direct central delivery of pyruvate, a metabolite of lactate metabolism fully recapitulated the anorectic effect of lactate.

Our single bolus injection of lactate into the third cerebral ventricle reduced FI for 1 day in a dose dependent manner. This finding is in agreement with the few studies which have also indicated that direct administration of nutrients *per* se in the CNS directly lower FI and BW. Specifically, Cota *et al*. demonstrated that single ICV bolus injections of an essential amino acid, L-leucine, reduces FI for up to 24 h (65) in rats fasted for 24 hrs and Obici *et al*. indicated that ICV oleic acid, a LCFA, lowers FI for up to 48 h in a protocol similar to ours (206). These data, in conjunction with various other reports which have noted changes in energy homeostasis through indirect alterations of hypothalamic nutrient availability (113; 137; 184; 204) cumulatively stress the ability of the CNS to sense nutrients to regulate energy homeostasis. Fittingly, one of the major targeted sites of our ICV (3^{rd} ventricle) administration is the ARC of the hypothalamus. Being one of the most widely studied and accepted brain regions in regards to energy homeostatic control, the ARC is in all probability the mediator of our observed lactate-sensing effects upon ICV administration. However, in order to localize the central lactate-induced anorectic effect to the ARC, we must perform the same experiments with direct IH injections into the mediobasal hypothalamus. Compared to ICV injections, IH administrations more specifically targets the ARC. Nonetheless, with the strong parallel effects seen with ICV and IH lactate in the regulation of GP (151), and the

anatomical support that the ARC is positioned to effectively sense circulating nutrients due to lack of an effective BBB (293), we are convinced that our effects are ARC-mediated.

The ARC, as previously described, is densely populated by NPY/AgRP and POMC neurons which jointly control energy homeostasis (247). Alteration in the levels of neuropeptides produced by these neurons modifies FI and BW (5; 85; 170; 207; 282). With that in mind, the current study does not distinguish the types of neurons (i.e. NPY/AgRP or POMC neurons) that are involved in central lactate-metabolism-mediated decrease in FI and BW. Quantification of neuropeptide expression by real time RT-PCR will potentially shed light on the neurons that are involved. Both ICV administration of L-leucine and oleic acid produce a significant decrease in orexigenic neuropeptide NPY expression accompanying the drop in FI and BW (65; 206). To more precisely evaluate the involvement of specific populations of neurons, we would need to utilize genetic approaches that target selective neuronal populations. Via the manipulation of associated molecules involved in neuronal lactate-sensing within these selected populations, we could potentially identify the neurons that are involved in mediating our observed effects. Indeed, emerging studies are showing that genetic disruptions of CNS nutrient and hormonal sensing mechanisms in AgRP or POMC neurons impair energy and glucose homeostasis (17; 55; 136; 145; 213).

Despite day-to-day fluctuations, the body is actually highly efficient in controlling BW within a precise range over a long-term period (74; 247). BW is determined by a

tight balance between FI and energy expenditure. Obesity is thus a manifestation of an excess in ingested calories above the ongoing requirements (188; 247). Upon ICV administration of lactate, we observed a decrease in BW after 18 h, which remained significantly lower 42 h post-injection. Although the change in BW might have largely been due to the concomitant drop in FI, we cannot exclude the possibility that an increase in energy expenditure might attribute partially to the decrease in BW. Indeed, the output of ARC is widespread with efferent projections accessing a vast array of brain areas, including those that influence cognitive, reward, emotion as well as nuclei controlling motor and autonomic functions (26). More importantly, the ARC has been shown to regulate energy expenditure (146; 203; 247). Therefore, it remains to be determined whether ICV lactate regulates energy expenditure. This parameter could be tested by measuring oxygen consumption rate or by evaluating physical activity using infrared sensing techniques in our experimental animals as previously described (48). Moreover, the phenotype of our observed weight loss is unknown, i.e. fat mass versus lean mass. Therefore, adiposity analysis would need to be performed by methods such as dual energy X-ray absorptiometry (DEXA) or fat pad quantification to provide insight into the contribution of adipose tissue mass versus lean body mass in the BW reduction.

According to the astrocyte-neuron lactate shuttle hypothesis, the use of extracellular lactate by neurons requires its metabolism to pyruvate by the enzyme LDH (169). To determine whether central lactate metabolism was downstream of, and required for, the anorectic effect of ICV lactate, we co-injected oxamate at a dose which had no effect alone together with lactate. Oxamate is a competitive inhibitor of LDH (38)

and therefore acts as an excellent tool to inhibit lactate metabolism. Previous studies by Lam *et al*. demonstrated that co-administration of ICV oxamate with lactate abolished the metabolic effects of the latter on blood glucose and lipid levels as well as decreases in hepatic glucose and lipid production (150; 151). We also noted that the FI- and BW-lowering effect of ICV lactate was fully abolished with the co-administration of oxamate. This is further supported by the finding that ICV pyruvate injection, at the same dose as our ICV lactate injection since each molecule lactate converts to one molecule of pyruvate, elicited similar anorectic effects as ICV lactate administration. Together, these data provide evidence that lactate metabolism is involved in the anorectic effect of CNS lactate. Moreover, by demonstrating that lactate-metabolism is coupled to neuronal activity to lower FI and BW, our study has further validated astrocyte-neuron lactate shuttle hypothesis, which remains controversial and the debatable (216).

Certainly, to strongly make a claim in support of the astrocyte-neuron lactate shuttle hypothesis, molecular approaches to specifically and selectively knock down LDH in astrocytes or neurons would be extremely useful. There exist two distinct isoforms of LDH, namely LDH-A (or –M)and LDH-B (or –H), which are found predominantly in muscle and heart, respectively (261). Classification in the brain correlates LDH-A with glial cells (29; 305) and LDH-B with neuronal cells (217). In line with the astrocyte-neuron lactate shuttle hypothesis, the A isoform preferentially converts pyruvate to lactate while the B isoform reconverts lactate back to pyruvate. Although oxamate is not an isoform-specific inhibitor of LDH, it would seem likely that it is the blockade of LDH-B by oxamate that prevents the exogenous load of central lactate from metabolizing into pyruvate,

hence impeding the downstream cascade that would otherwise result in the anorectic effect of lactate. In theory, molecular knock down of LDH-A found in the glial cells would not abolish the anorectic effect of ICV lactate since it would promote lactate metabolism into pyruvate. On the other hand, molecular knock down of LDH-B found in the neurons should prevent the FI- and BW-lowering effect of ICV lactate, as was observed with oxamate injection. In this way, isoform-specific inhibition of LDH would provide strong support for the astrocyte-neuron lactate shuttle hypothesis.

Interestingly, in the aforementioned study by Lam *et al*., central lactate metabolism was in fact required downstream of central glucose's regulation of glucose and lipid homeostasis. Both ICV glucose and lactate similarly lowered blood glucose and lipid levels, as well as hepatic glucose and lipid production, effects which were prevented with the blockade of lactate metabolism (150; 151). This also is in accordance with the astrocyte-neuron lactate shuttle, which stresses that usage of glucose as fuel in neurons requires its conversion to lactate in glial cells and subsequent metabolism of lactate back to pyruvate. Certainly, it remains to be determined whether central glucose lies upstream of the effect of central lactate in lowering FI and BW. Indeed, it has been demonstrated over a quarter of a century ago that hypothalamic glucose administration lowers FI and BW (70) while glucose deprivation in the hypothalamus increases FI and BW (184). These findings are still thought to be rather contentious by some. However, taken together with strong support that there are definite glucose-sensing nuclei within the hypothalamus holding distinct glucose-responsive and –sensitive neuronal populations (159), it seems highly possible that the brain, in particular glucose-sensing

nuclei such as the ARC, can sense glucose to lower FI and BW via a biochemical pathway involving lactate metabolism.

It is reported that IV administration of nutrients (i.e., lactate, glucose, lipid, amino acids) negatively regulates FI and BW (154; 193; 255; 287; 299). However, the site(s) of the anorectic actions were not investigated. Furthermore, hypothalamic nutrient sensing mechanisms were never evaluated with respect to their ability in detecting *circulating* nutrients to regulate energy balance. Here we report that central lactate metabolism to pyruvate is required for circulating lactate to lower BW and FI in conscious, unrestrained rodents. We first established an *in vivo* model that illustrated that systemic elevation of plasma lactate levels by ~2.5 fold for 3 h lowered FI and BW. In the presence of this systemic elevation of lactate, we then negated central lactate metabolism to pyruvate with central injections of the lactate dehydrogenase inhibitor oxamate at the same dose that abolished the anorectic effects of central lactate injections. Central administration of oxamate was sufficient to reverse the anorectic effects of circulating lactate. These data suggest that, similar to the regulation of glucose and lipid homeostasis (150; 151), CNS lactate metabolism is required for circulating lactate to regulate energy homeostasis. It is important to note that the present study as a whole is pharmacological in nature and is designed to evaluate the role of the biochemical metabolism of lactate to pyruvate in the regulation of FI. The physiological relevance of CNS lactate metabolism to the regulation of FI remains to be clarified.

Taken together, our results provide support that activation of hypothalamic lactate metabolism negatively regulate FI and BW. In light of the fact that CNS lactate metabolism controls plasma glucose and lipid levels (150; 151), these studies position the biochemical metabolism of lactate to pyruvate in the hypothalamus as a novel biochemical target to reverse obesity. Therapeutic strategies designed to modulate CNS lactate metabolism may prove useful in lowering BW, glucose and lipid levels in obesity and type 2 diabetes mellitus.

6.2 STUDY 2

Supported by an enormous body of evidence, the importance of the ARC in control of glucose homeostasis appears indisputable. While this hypothalamic site has emerged as the 'master' nuclei of relevance to glucose homeostasis, it is hard to believe that it works in solo in the CNS regulation of glucose homeostasis. Given the fact that central energy and glucose homeostatic controls share much in common, we can perhaps gain valuable insights into the latter by reviewing what is known about the former. Review of the literature supports that the recipients of ARC-initiated neuronal projections to control energy balance consist of various brain regions, including the NTS of the brainstem (26; 99; 157; 165). Is a comparable hypothalamic-medullary network from the ARC to the NTS also present for CNS-regulated glucose homeostasis? To address this question in specific, we concentrated on hypothalamic lactate-sensing. While hypothalamic lactate metabolism-dependent-sensing has elegantly been demonstrated in Study 1 of this thesis and other previous studies to regulate peripheral energy, glucose and lipid metabolism (150; 151), the downstream effectors have not been completely examined.

In the second portion of this thesis, we first demonstrated that pharmacological inhibition of the NTS NMDA receptor via direct delivery of receptor blocker MK801 into the NTS in fact negated the GP-lowering effect of hypothalamic lactate. This provides the first direct evidence *in* vivo suggesting a downstream neuronal pathway by which hypothalamic nutrient exerts its effect on the liver to lower GP and presents functional

evidence of the hypothalamic-medullary control of peripheral glucose homeostasis. Of note, our novel discovery stresses once again the resemblance between central control of glucose and energy homeostasis, the complexity of the network involved, and most importantly, functionally demonstrated for the first time to our knowledge the neuronal network involving a hypothalamic-medullary crosstalk that maintains glucose homeostasis.

In retrospect, hypothalamic-medullary networks are not new to the realm of neuroregulation nor are they exclusive to glucose and energy homeostatic controls. In fact, complex neuronal networks involving hypothalamic projections to other brain regions to produce alterations in peripheral outputs have been well-documented in the control of the cardiovascular system (108) and osmoregulatory circuits (36) as well. That these hypothalamic-medullary pathways, in particular those connecting the MBH-NTS, are also involved in energy and glucose homeostasis should not be surprising given the respective role of each of the two regions. The ARC, a prominent nucleus in the MBH, is one of the first contact sites of peripheral substances that pass through the median eminence (293) and a needed integration centre for these signals (198; 212). The NTS in the brainstem is deemed the prime outlet to the periphery via motor output circuits of the autonomic system (14). It is reasonable that signals received, processed and integrated by the ARC are projected to the NTS so that necessary changes to the periphery can be exerted. One point that was not addressed in this study, however, was whether the identified MBH-NTS modulatory pathway engages direct neuronal connections between the MBH and NTS or involves intermediate brain regions and

nuclei. Referring once again to the known pathways for energy homeostasis, it is not unreasonable to postulate that both options are possible. Based on anterograde tracing with PHA-L, efferent projections of ARC neurons are found to strongly project to various other hypothalamic nuclei (26). Of significant relevance to the present study are the orexigenic NPY/AgRP and anorexigenic POMC neurons in the ARC which synapse onto second order neurons in regions including, but not limited to, PVN and LH (16; 21; 76; 77; 79; 164) to regulate FI and BW. These second order neurons in the PVN or LH then extensively project to an array of brain regions, ranging from the cortical regions involved in memory, emotions and reward such as the hippocampus and extended amygdala as well as to the midbrain, brainstem and spinal cord (26). Taken together, this extended network effectively controls the ongoing energy status of the periphery via the receipt and transmission of signals. Interestingly, the aforementioned NPY/AgRP and POMC neurons or their associated neuropeptides have also been extensively implicated in CNS regulation of glucose homeostasis as well (5; 85; 120; 170; 207; 282). Thus, these indirect networks from the ARC to NTS might also be utilized in the control of glucose homeostasis, forming an overlapping neurocircuitry involving second- or third-order neurons like that of the control of energy homeostasis. Alternatively, ARC neurons can and, in fact, do directly project to the NTS. For instance, hormonal action of leptin in the ARC has been cited to involve projections to the NTS (188) and electrophysiological findings indicate that ARC NPY and POMC efferents terminate at the NTS (54; 97). More recently, retrograde tracing with tracer cholera toxin B-subunit (CTB) has identified anatomically hypothalamic POMC projections to the dorsal vagal

complex, which contains the NTS (78; 304). Furthermore, NTS injection of NPY or melanocortins, which are the released from NPY/AgRP and POMC neurons, respectively, have been found to affect FI accordingly (64; 101; 292). These findings strengthen the claim that direct NPY/AgRP and POMC neuron projections toward the NTS regulate peripheral homeostasis. However, it still remains to be determined whether the effect noted in our studies reflect direct, indirect or a combination of both types of MBH-NTS circuits.

While the aforementioned NPY/AgRP and POMC neurons mediate glucose homeostasis, it is unclear which population is involved in this hypothalamic lactate-NTS NMDA receptor pathway. Of note, the activation or opening of the SUR1/Kir6.2 K_{ATP} channel is required for hypothalamic lactate to lower GP as blockade of the SUR1/Kir6.2 K_{ATP} channels nullifies the effects of hypothalamic lactate (151). Since the opening of the K_{ATP} channel leads to silencing of neurons, we postulate that this hypothalamic lactate effect is mediated by the silencing of the orexigenic NPY/AgRP neurons. This results in an overall increase in the melanocortin tone to decrease GP. To make this distinction, however, genetic approaches that manipulate targeted neuronal populations are necessary.

For the first portion of Study 2, we primarily focused on the NTS as a relay centre for ARC-sensed signals – in actuality, the NTS is also much of a 'sensing' region of the brain. In fact, pioneering work by Grill and colleagues showed that the caudal brainstem alters ingestive behavior to regulate energy homeostasis even in the absence of

hypothalamic or cerebral cortical inputs (102). Therefore, the next question that we set out to address was whether activation of the NTS NMDA receptors *per se* is sufficient to regulate hepatic GP.

We identified that NTS NMDA receptors not only act as a switchboard integrating hypothalamic signals to regulate GP, but provided evidence that selective activation of the NTS NMDA receptors by delivery of the amino acid, glycine, is sufficient to lower GP and plasma glucose levels in normal rodents. Consistently, inhibition of the NTS NMDA receptors with the blocker, MK-801, abolished the effect of glycine to lower GP and plasma glucose levels.

The field of CNS control of peripheral glucose homeostasis was sparked with the initial observation by Claude Bernard that punctures in the floor of the fourth ventricle resulted in glucosuria (24). We now come to appreciate that this lesion experiment of a site in proximity to the NTS in the brainstem is perhaps the first evidence that the NTS is directly involved in glucose homeostatic regulation. Our present findings demonstrate, for the first time to our knowledge, that the NTS can directly sense nutrient glycine to exert regulation of hepatic GP. From an anatomical point of view, it can be seen why the NTS is an area that is capable of direct sensing of hormones and nutrients. Just like the ARC, the NTS is one of the few areas within the brain that lacks an effective BBB (165), thus exposing the neurons of the NTS to primary contact with circulating factors. In fact, injection of the hormone leptin into the fourth ventricle of rats, which contains leptin-responsive neurons (78), reduces FI (104) and inhibits gastric emptying (259). Direct

insulin injection into the NTS of anesthetized rats attenuates the baroreceptor reflex important for the control of the cardiovascular system (178). Furthermore, in support of NTS nutrient-sensing, both glucose-sensitive and –responsive neurons have been characterized in the NTS (4). More importantly, injection of glucose into the NTS of anesthetized rats decreases gastric motility and intragastric pressure in these animals (86). NTS injection of lactate, the neuronal energy substrate derived from glucose, alters the firing rate of NTS glucose-sensing neurons similarly as that of NTS glucose injections (116). It must be stressed, though, that the aforementioned studies mainly associated NTS-sensing with energy homeostasis, and to date nominal gains hae been made regarding whether the NTS can sense nutrients directly to control glucose homeostasis. Perhaps the most notable study in this regard reported that lactate injection into the fourth ventricle of rats delays recovery from insulin-induced hypoglycemia, while inhibition of lactate-metabolism via administration of monocarboxylate transporter (MCT) inhibitor in the NTS increases plasma glucose levels (214). This study does not actually probe into identifying which parameters, i.e. glucose utilization or production, NTS lactate-sensing might potentially be affecting. The novelty of our studies partly arises from the fact that we pinpointed hepatic GP, and not utilization, as the mediator of the effects observed under our experimental settings. However, we acknowledge that the pancreatic clamp technique has its limitations in terms of representing a truly physiological setting , therefore, we also evaluated the ability of NTS NMDA receptors to control glucose homeostasis in an unclamp setting. 90 min of glycine administration *per*

se was sufficient to lower plasma glucose levels by ~1.5 mM. This drop in plasma glucose levels is nullified by co-administration of a potent NMDA blocker, MK-801.

In line with this, the GP-lowering ability of NTS glycine, like that of hypothalamic lactate, was abolished by co-administration of MK-801 into the NTS, indicating that the activation of NMDA receptors in the NTS is both necessary and sufficient in lowering hepatic GP. NMDA receptors are composed of NR-1 and -2 subunits, which bind glycine and glutamate respectively (133). Our data are consistent with reports indicating that the binding of glycine to the NMDA receptor potentiates NMDA receptor-mediated neurotransmission (18; 23; 140) and strengthens the claim that the glycine binding site on the NMDA receptor is not chronically saturated as has been previously been assumed. Rather, glycine binding can actually act as a modulator to NMDA transmission in the NTS (18; 40). A search into the existing literature indicates that a majority of focus on the NMDA receptors has been in relation to cognitive function (155). Our study presents a glimpse of a much broader scope of function for NMDA receptors. Certainly, the diverse roles of NTS NMDA receptors make sense given that as the NTS is a principle input centre of visceral afferents including, but not limited to, the aortic (215), gastric and hepatic branch of the vagal nerve (49; 86). More importantly, NMDA receptor immunoreactivity is present on almost all vagal afferent terminals in the NTS (3; 7; 106; 202; 230; 238). Activation and inhibition of NTS NMDA receptors modulate cardiovascular functions (14; 147; 158; 268) and inhibition of NTS NMDA receptors with the antagonist, MK-801, increases FI (66; 67; 106; 107; 123; 276; 303) and regulates gastric function (275). It is pertinent to note that while fluctuations in FI and gastric

function indirectly alter glucose homeostasis, our studies were carefully designed to ensure independence from these parameters. While a single central or NTS bolus injection of MK-801 has been shown to increase liquid sucrose intake (66; 67; 276), the dose that caused such increases was at least four fold higher (~50 ng) than the dose employed in our NTS continuous infusion glucose regulation studies (~ 12 ng). Furthermore, a single bolus injection of MK-801 into the fourth ventricle at a much higher dose (2 μg) fails to alter gastric emptying but increases sucrose intake (66). As such, we precluded potential concern that our results were due to alterations in FI or gastric function due to NTS NMDA receptor activation or inhibition.

Perhaps another point to address arises from the fact that the NTS NMDA receptors, as previously alluded to, are present on vagal afferent terminals. Recently, we and others have pinpointed that the NTS NMDA receptors respond to lipid-sensing mechanisms initiating from the gut to regulate GP and energy homeostasis (68; 288). To ensure that our results are not intermingled with vagal afferent activation effects, but are fully representative of a hypothalamic signal-relay or NTS-sensing *per se*, we performed our studies on rats in the post-absorptive state (i.e. animals were fasted for 5 h prior to experimentation) where the energy status of the subjects were comparable with the same amount of FI. As such, we precluded any potential concern that our changes were due to varying hepatic or gastrointestinal vagal afferent activation. Cumulatively, the effects we observed with our dose of MK-801 and chosen protocol are independent of changes in FI, gastric function or vagal afferent activity. If the NTS NMDA receptors that are required and sufficient to lower GP and plasma glucose levels are not

those present on vagal afferent terminals, then it remains to be determined where these NMDA receptors are located as well as the characterization of the responsible neurons mediating these effects.

NMDA receptor-mediated biochemical and signaling pathways have been targeted for therapeutic developments (133) largely because NMDA receptors in the CNS play an important role in excitatory neurotransmission and synaptic plasticity. They have been implicated in learning and memory (155) and have therefore been of interest in a number of psychiatric and neurological disorders such as Alzheimer's disease (250), Parkinson's disease (272), schizophrenia (160), depression (112) and mental retardation (75). A reduction in the glutamatergic system may be integral to the etiology of many of the listed cognitive disorders (133). The fact that glycine potentiates NMDA receptor-mediated transmission suggests that administering glycine or its analogues to patients with cognitive disorders may be beneficial. Indeed, studies have reported that supplementing glycine or other NR1 glycine-binding site agonists with antipsychotic therapy improves cognitive function in schizophrenia (269; 277). Of note, the most commonly prescribed class of antipsychotics, the atypical antipsychotics has been clinically documented to induce significant weight gain and glucose intolerance (131), the latter initially seen as secondary to weight gain. More recently though, acute diabetogenic effects independent of weight gain have been reported with the use of atypical antipsychotics (51; 119; 227; 229). Modulation of NTS glutamatergic neurotransmission through glycine delivery may have therapeutic values beyond

cognitive benefits, bypassing some of the adverse diabetogenic effects of the present treatments.

It is important to point out that our observed GP- and plasma glucose-lowering effects of NTS NMDA activation were seen in normal rodents. However, is the same effectiveness present in settings of insulin resistance and uncontrolled diabetes? In actuality, various central sensing mechanisms are disrupted in models of obesity and/or diabetes. Some of these include the well-defined leptin resistance (62; 88) whereby leptin fails to decrease FI and BW in obese subjects. Furthermore, while ICV infusion of insulin lowers glucose production in normal rodents (208), this regulatory ability was lost after merely one day of high-fat feeding (210). The same inability for ICV oleic acid to lower GP was seen with rats overfed 3 days of high fat-diet (187). However, not all of these central sensing mechanisms are disrupted in models of obesity and/or diabetes. For example, central administration of lactate, at the same dose used in normal rodents, is still able to lower GP in an early-onset model of STZ-diabetes (50). In a similar model of acute diet-induced insulin resistance, activation of PKC in the hypothalamus (234) was also effective in suppressing GP . Therefore, to extend the therapeutic values and feasibility of our findings, it might be just as critical to identify whether NTS NMDA activation can lower GP and plasma glucose levels in settings of insulin resistance and uncontrolled diabetes. These studies remain to be conducted.

7

FUTURE DIRECTIONS

7.1 STUDY 1

1. We have identified, for the first time to our knowledge, that hypothalamic lactate metabolism is critical in the regulation of FI and BW in male SD rats. It has been previously shown that the K_{ATP} channels are, in turn, downstream of hypothalamic lactate metabolism to lower hepatic glucose and lipid production (150; 151). Logically, the follow-up work of this study will be to examine whether the hypothalamic K_{ATP} channels are involved in the downstream mechanistic pathway of FI and BW control of hypothalamic lactate-metabolism. Using both pharmacological (i.e. glibenclamide) and molecular (i.e. adenoviral infection) approaches to inhibit the hypothalamic SUR1/Kir6.2 K_{ATP} channels, which will essentially prevent hyperpolarization of hypothalamic neurons, we shall attempt to examine how the over-activation of hypothalamic neurons affects lactate-sensing and energy homeostasis. If the regulation of FI and BW parallels that seen with the regulation of glucose and lipid homeostasis, we expect to see that blockade of the hypothalamic K_{ATP} channels would nullify the effect of lactate-sensing on FI and BW. It is important to note that our proposed mechanism of K_{ATP} channel in the brain is different but not contrary to that in the beta cell: unlike in the brain where lactate lies downstream of glucose, in the beta cell lactate is not downstream of glucose-sensing to influence insulin release under normal physiological conditions (125). Indeed, our hypothesis is in line with a recent study indicating that activation of the AgRP K_{ATP}

channels by insulin signaling inhibits NPY/AgRP release and controls of peripheral glucose metabolism (145).

2. While much of the present work is focused on the acute effects of nutrient-sensing on energy homeostasis, it is important to acknowledge that much of energy homeostasis is chronically regulated. Therefore, it will be worthwhile in the future to investigate the chronic effects of modulations in hypothalamic nutrient-sensing pathways. Previous reports indicate that disruption of the SUR1 subunit of the SUR1/Kir6.2 K_{ATP} channels from birth (i.e. SUR1 knockout mice) do not affect energy balance (152; 224; 249). However, the concern of compensatory mechanisms of at-birth knockouts is one of validity. For instance, studies indicate that AgRP neurons ablation in adult mice (105; 168), but not AgRP knockout mice from birth (168; 226), exhibit an obese phenotype. Therefore, examining adult-onset knockdown of the nutrient-sensing pathway and its control of FI and BW is of potential interest. In order to address this, however, one would require a chronic (i.e. 3 – 4 months) knockdown of the SUR1/Kir6.2 K_{ATP} channels, which could only be achieved by adeno-associated virus and not adenovirus delivery (289).

3. As alluded to in the discussion, our study does not define the neuronal population that is involved in the reduction in FI and BW of hypothalamic lactate. In order to do so, we must utilize genetic approaches which target specific neuronal subgroups, namely NPY/AgRP or POMC neurons, to determine how such modifications affect energy homeostasis. However, with the currently available resources at our

laboratory, this might not be feasible. An alternative means would be to determine the changes in neuropeptide levels via real-time RT-PCR to elucidate the neuropeptides that might be mediating such effects.

7.2 STUDY 2

1. Thus far, our approaches in this study have been highly pharmacological in nature: to supplement our pharmacological inhibition of NTS NMDA receptors, a molecular approach can strengthen the findings – in particular, a molecular knockdown of the NMDA receptor *in vivo* would best illustrate our hypothesis. The NMDA receptor is composed of NR1 and NR2 subunits which are activated by glycine and glutamate, respectively (174). The NR1 subunit is an ubiquitous, as well as necessary, component of functional NMDA receptor channels (162), and logically, disruption of the NR1 subunit would essentially abolish NMDA function. Indeed, previous studies indicate that NMDA-induced increases in neuronal intracellular calcium and membrane currents are abolished in NR1-null mice (195). In parallel, selective knockdown of the NR1 subunit of the NMDA receptor in the spinal cord dorsal horn was sufficient to reduce NMDA-induced neuronal current (262). These data indicate that a selective knockdown of NR1 of the NMDA receptor is sufficient to negate NMDA receptor-induced neurotransmission. In addition to its necessity for function, the advantage of knocking down the NR1 subunit over the other two subunits – NR2 and 3 – lies in the higher feasibility to knock down one as supposed to multiple genes: NR1 is encoded by a single gene (117) whereas the heterogeneity of the other subunits occurs due to the existence of multiple related genes (82; 126; 199). Taken together, to alternatively evaluate whether hypothalamic-lactate or NTS-glycine regulate glucose homeostasis through the activation of NMDA receptor, we shall develop a molecular approach to knock down the NR1 subunit of the NMDA

receptor in the NTS *in vivo*. For a short-term knockdown, adenoviral infection is a feasible option: adenoviral vector expressing the shRNA of NR1 under the cytomegalovirus (CMV) promoter has been previously described (93). For a more chronic expression, we would need to construct an adeno-associated virus expressing the shRNA-NR1. This has also been previously described and infection with the adeno-assiciated virus in the spinal cord dorsal horn decreases NR1 protein levels by ~60% and fully reversed the injury-induced pain in rodents (93).

2. A previously discussed limitation in our study is the relatively weak direct physiological support of our findings. A common and relatively practicable experiment that could significantly strengthen the physiological basis of our finding is the fasting-refeeding episodes. Normally, excessive increases in blood glucose levels of animals being refed after a prolonged fasting period are prevented via restriction of gluconeogenesis (72). We would expect that if CNS nutrient-sensing is important in normal physiology, it would be triggered by a physiological stimulus such as the fasting-refeeding episode. More precisely, if nutrient-sensing related signals activated by a refeeding protocol are integrated at the NTS NMDA receptors, then inhibition of the NTS NMDA receptors would negate the glucose homeostatic control and consequently result in a rise in plasma glucose levels.

3. Following then, if the NTS NMDA receptors play a role physiologically, then it is critical to determine whether NTS glycine can lower GP in high fat diet-induced obesity. It has been shown recently by our laboratory that central lactate

administration lowers GP in rodents with diet-induced insulin resistance (50). If it is true that NTS NMDA receptors lie downstream of hypothalamic-lactate, then it should follow that NTS glycine administration would also bypass the CNS defect to lower hepatic GP in the same diet-induced insulin resistance model. Uncovering this will enable us to further position the CNS nutrient-sensing defects along the nutrient-sensing cascade.

4. While the neuronal communication between hypothalamic lactate-sensing and the NTS NMDA receptors are identified here, the full mechanistic picture is still to be elucidated. Two major questions are: how hypothalamic lactate metabolism subsequently activates the K_{ATP} (151) channels; and, what acts as a convergence between lactate- and lipid-sensing. Hypothalamic AMPK appears to be a likely mediator – it has been implicated in mediating the hormonal- and nutrient-sensing-regulated energy homeostasis (130). Specifically, activation of hormonal and nutrient neuronal sensing mechanisms is associated with a reduction in hypothalamic AMPK activity (91; 183), and alterations of hypothalamic AMPK activity regulate FI and BW (183; 264). A reduction of AMPK activity activates acetyl-coA carboxylase and increases the formation of malonyl-CoA from acetyl-CoA (which is derived from pyruvate). A rise in malonyl-CoA inhibits CPT-1 and elevates long chain fatty acyl (LCFA)-CoA levels. The accumulation of malonyl-CoA/LCFA-CoA levels *per se* or via leptin signaling has been demonstrated to suppress FI and BW (91; 113; 153; 204; 296). The role of AMPK in the GP-lowering effects warrants future investigations. If hypothalamic malonyl-CoA/LCFA-CoA accumulation due to AMPK

inactivation is shown to lower GP, it could potentially provide a mechanistic link between lactate-pyruvate metabolism *and* the activation of K_{ATP} channels. This is based on the fact that LCFA-CoA in the hypothalamus and the beta cells activates K_{ATP} channels (37; 98; 150-152); therefore, by alternating AMPK activity (i.e. activating or inactivating), we can investigate its role in CNS nutrient-sensing mediated glucose homeostatic control.

5. As with study 1, our data do not define the neuronal population that is involved in the hypothalamic-medullary crosstalk. In order to do so, we must utilize genetic approaches which target specific neuronal subgroups, namely NPY/AgRP or POMC neurons, to determine how such modifications affect glucose homeostasis. However, with the currently available resources in our laboratory, this might not be feasible. Furthermore, neither the neurocircuitry linking the hypothalamus and NTS, nor loci of the NMDA receptors in the NTS which mediate our observed effects, were identified. Immunohistochemical studies localizing NMDA receptors on neuronal populations and retrograde tracing might prove useful in this regard.

8

CONCLUSION

Much progress in the field of CNS regulation of energy and glucose homeostasis has been made since Claude Bernard first hinted at the then-underappreciated CNS as a key player in peripheral glucose homeostatic control over century ago. In fact, numerous landmark studies have made significant contributions to the field, and have collectively established that certain hypothalamic nuclei respond to shifts in energy and nutrient status relayed by circulating factors, including metabolites, as well as hormones, to initiate behavioural and metabolic responses to restore energy and glucose homeostasis. Thus, the CNS is inarguably linked to obesity and diabetes, diseases that at first glance might seem like primarily peripheral metabolic diseases.

But the puzzle is far from complete. The nature of the input signal required for the hypothalamic regulation of nutrient and energy homeostasis, and the related biochemical mechanisms, still remain largely unclear. Specifically, it can still be argued that the efferent mechanisms by which hypothalamic signaling is linked to metabolism in peripheral tissues remain largely unknown (118; 246), and while the bulk of research has focused on the ARC in particular, other brain nuclei are likely to be involved in the processing of peripheral hormone and nutrient signals (Schwartz et al. 2005). Indeed, the present thesis set out to address some of these unknowns.

Focusing specifically on hypothalamic lactate-sensing, we determined that lactate metabolism-mediated pathways not only regulate peripheral glucose (151) and lipid (150) homeostasis, but is also involved in the control of FI and BW. Furthermore, we identified a hypothalamic-medullary connection, involving the NMDA receptors in

the NTS, as being required to relay hypothalamic lactate-sensing to lower hepatic GP. Lastly, our data suggest that activation of the NTS NMDA receptors is sufficient to lower GP. Indeed, the present thesis opens up a new array of questions and future studies directed to clarifying this complex neuronal cross-talk between the hypothalamus and the NTS. Answers to these questions should, in turn, shed light on the discovery of novel molecules in various brain regions that can control glucose homeostasis.

Continued efforts are necessary in order to fully characterize the nutrient, nervous, or hormone-mediated responses in the brain, and the extent to which they may be altered in the diseased metabolic state. This will undoubtedly lead to a prioritization of possible central targets when it comes to developing novel therapeutics to combat the rapidly increasing obesity and diabetes epidemic.

9

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