CENTRAL NERVOUS SYSTEM (CNS) NUTRIENT SENSING IN DIABETES

by

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GENERAL ABSTRACT

An acute increase in hypothalamic glucose and its downstream metabolite lactate lower glucose production (GP) and plasma glucose (PG) levels in normal rodents. However, the effectiveness of this nutrient-sensing mechanism in metabolic disease is unknown. We assessed the effects of intracerebroventricular (i.c.v.) or intra-hypothalamic glucose and lactate on *in vivo* glucose kinetics in conscious rats. Study I revealed that i.c.v. lactate lowered PG via a suppression of GP in rodents with uncontrolled diabetes and diet-induced insulin resistance. Study II demonstrated that i.c.v. glucose was ineffective at suppressing GP in uncontrolled diabetic rodents or rodents with a prior 24 h whole-body or hypothalamic hyperglycemic insult. When PG levels *per se* were normalized in diabetic rodents hypothalamic glucose sensing to lower GP was rescued. As such, sustained hyperglycemia *per se* impairs hypothalamic glucose effectiveness in diabetes. Further studies are necessary to determine defective mechanisms upstream of lactate metabolism hindering CNS glucose sensing.

ACKNOWLEDGMENTS

Since my childhood, I have had a fascination and appreciation for science. A discipline of study so tangible and explanatory, it seemed like a natural fit for a perpetually curious individual with an inquiring mind. While my curiosity and determination has been imperative in achieving my academic success, I would be remiss if I did not highlight the contributions of several notable individuals who have helped make my graduate research training such an enriching experience.

Being the inaugural graduate student in the newly established lab of Dr. Tony Lam was initially intimidating. However, over the past two years, Tony has proven to be an effective supervisor and mentor who is knowledgeable beyond his years. His thorough and scrutinizing approach to research has, I believe, made me a better scientist and student. My progress has also been greatly facilitated by the talented members of our lab, particularly Penny Wang and Carol Lam who have been with me from day one. I would also like to give my regards to my advisory committee members Drs. Amira Klip and Dominic Ng, for taking time out of their busy schedules and providing feedback during the course of my project, as well as my undergraduate supervisor Dr. Geoff Werstuck, for supporting me during the graduate studies he so strongly advocated and encouraged me to pursue.

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As I embark upon my medical training and look back on my graduate years, I can say with confidence that my research training will not soon be forgotten; rather, it has yielded a skill set and a means of interpretation that will only be deepened and built upon. As such, I look forward to the next step and embracing the inevitable challenges that lie ahead.

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PUBLICATIONS THAT CONTRIBUTED TO THIS THESIS

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GENERAL INTRODUCTION

Diabetes

In normal individuals, the level of plasma glucose remains within a fairly tight range of 4 to 7 mM despite periods of feeding and fasting (172). However, the body's intrinsic insulin-sensitive ability to utilize glucose is disrupted in a group of heterogeneous metabolic disorders known as diabetes mellitus. As such, these diseases are ultimately characterized by chronic hyperglycemia, where, as per the clinical definition, the plasma glucose level exceeds 7.0 mM after fasting or 11.1 mM two hours post-glucose load (219). The two major forms of diabetes are type 1 (or insulin-dependent diabetes mellitus; IDDM) and type 2 (non-insulin dependent; NIDDM), which are characterized by the absolute or relative deficiency of insulin, respectively. Primarily driven by increasingly sedentary lifestyles and the escalating prevalence of obesity, the last few decades have witnessed an undeniable increase in the cases of type 2 diabetes in particular. Currently, diabetes affects approximately 170 million people (206), and the World Health Organization has predicted that this number will double by the year 2025 (212). The comorbidities associated with diabetes makes these numbers even more devastating: diabetes is a leading cause of heart disease, blindness, end-stage kidney disease, and non-traumatic loss of limb (77).

Type 1 diabetes results from the autoimmune-mediated destruction of the insulin-producing pancreatic β-cell islets, resulting in near-absolute insulin deficiency. Although its

pathogenesis is known to exploit genetic susceptibility in addition to the interaction of this genetic predisposition with environmental triggers, the precise etiology of the autoimmune mechanisms in type 1 diabetes remains a puzzle despite decades of research (8). Due the nature of the disease, individuals with type 1 diabetes are dependent on the injection of exogenous insulin as a primary therapy. However, excessive insulin treatment can lead to iatrogenic hypoglycemia, which is a cause of recurrent morbidity that can also affect individuals with type 2 diabetes (43).

Type 2 diabetes is the most common metabolic disease in the world. And although the primary trigger of type 2 diabetes is unknown, the disease is associated with insulin resistance (157), and it is quite evident that insulin resistance plays an early role in its pathogenesis. Thus, it is logical that the increasing incidence of type 2 diabetes is concurrent with the epidemic of obesity (158), which is also associated with insulin resistance. However, most obese and insulin resistant individuals do not develop hyperglycemia (91), as in these individuals, an increase in β-cell mass compensates for the increased metabolic burden and loss of sensitivity; however, when this β-cell adaptation fails, type 2 diabetes develops (28). In the insulin resistant state, the insulin-sensitive process of hepatic glucose production becomes dysregulated. Indeed, excessive glucose production is a primary contributor to the postprandial hyperglycemia that is observed in individuals with diabetes (48; 190). Therefore, insulin resistance as well as defective insulin secretion by pancreatic β-cells are both instrumental in the establishment of subsequent overt hyperglycemia which characterizes diabetes.

With the increased prevalence of obesity in the world, especially in developed and affluent societies, it is imperative to promote a shift away from the increasingly popular lifestyle of excessive caloric intake and decreased physical activity in order to diminish the related prevalence of type 2 diabetes. Once diabetes is established, the effective

management of hyperglycemia and other manifestations of diabetes via lifestyle changes and therapeutic interventions becomes critical. If this multifaceted metabolic disease is improperly managed, a wide array of devastating secondary complications can be promoted, which includes microvascular diseases such as renal failure, retinopathy and neuropathy, as well as macrovascular diseases such as CVD and stroke (61).

As such, it is important to develop an understanding of the pathways of hepatic glucose metabolism and fluxes, the mechanisms regulating these processes, and how disruptions in these regulatory mechanisms contribute to the hyperglycemia that is characteristic of diabetes. Advancements in therapeutic strategies targeting excessive hepatic glucose production are necessary to improve glycemic control in individuals with diabetes, and it is hoped that the findings of this thesis will contribute to this initiative.

Regulation of Hepatic Glucose Fluxes

In the liver, hepatocytes take up glucose in an insulin-independent manner by the high K_m GLUT2 (159). Its' high capacity, low-affinity nature means that GLUT2 is not saturated by higher concentrations of glucose, and as such, allows the rate of glucose transport to be proportional to the ambient glucose concentration. Interestingly, this isoform is abundantly expressed in tissues that are exposed to large glucose fluxes, including the liver, intestine, kidney, as well as pancreatic β-cells (19). Once in the liver, glucose is rapidly phosphorylated by glucokinase (GK), to form glucose-6-phopshate (G6P) (19). Acutely, GK activity is regulated by glucokinase regulatory protein (GKRP), which is normally sequestered in the nucleus at high glucose and insulin concentrations, but when glucose levels decrease (e.g. fasting), GKRP distribution becomes cytoplasmic, where it binds and inhibits GK, and sequesters it as an inactive form in the nucleus (23).

From here, G6P is directed into 1) the direct pathway of glycogen synthesis, via the formation of uridine diphosphate (UDP)-glucose or 2) glycolysis, which yields carbon-3 compounds like pyruvate and lactate (159). These carbon-3 compounds can undergo oxidation in the Krebs cycle or serve as gluconeogenic substrates for the de novo glucose and glycogen synthesis (indirect pathway) (105). There are numerous enzymes that regulate the cycling of glucose metabolites between gluconeogenesis and glycolysis (**Figure 1**); indeed, the control of metabolic flux between these two pathways can be largely influenced by the expression of genes encoding these enzymes, including phophoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-biphosphatase (gluconeogenic), as well as the glycolytic phosphofructo-1-kinase (PFK) and pyruvate kinase (PK) (129). Perhaps the most unique property of the liver, as far as glucose metabolism is concerned, is its' abundant expression of glucose-6-phosphatase (G6Pase) which dephosphorylates and effectively "frees" glucose into its releasable form. As well, G6Pase is not expressed in the muscle or

adipose tissue (198). This prevents the muscle and fat from producing glucose and makes the liver the primary endogenous producer of glucose. Total hepatic GP is determined by both the rates of gluconeogenesis as well as glycogenolysis.

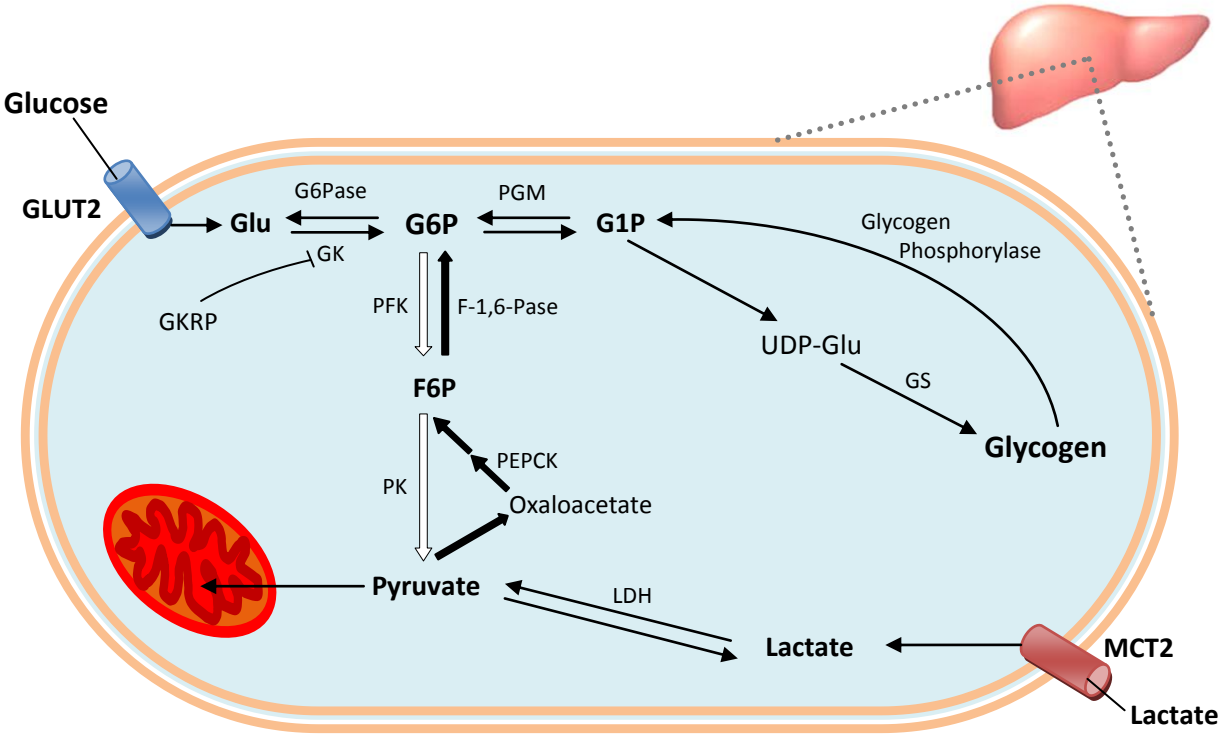

Figure 1. Biochemical Pathway of Liver Glucose Fluxes

Glucose is taken up by the facilitated glucose transporter GLUT2, and is subsequently phosphorylated to form glucose-6-phosphate (G6P) by glucokinase (GK). Glucokinase is inhibited and sequestered in the nucleus by glucokinase regulatory protein (GKRP) under periods of low glucose levels. In the setting of elevated insulin and glucose, G6P is primarily shunted into two metabolic fates: 1) glycolysis or 2) glycogen synthesis. After being isomerized into glucose-1-phosphate (G1P) by phosphoglucomutase (PGM) and converted to uridine diphosphate (UDP)-glucose, glucose is incorporated into glycogen for storage via glycogen synthase (GS). In glycogen breakdown, glycogen phosphorylase catalyzes the release of individual glucose units. The control of metabolic flux is heavily determined by the expression of glycolytic enzymes (white arrows), such as phosphofructokinase (PFK) and pyruvate kinase (PK), and gluconeogenic enzymes (thick black arrows), including phophoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-biphosphatase (F-1,6-Pase). Lactate, as part of the Cori cycle which aims to prevent lactic acidosis in active muscle, is taken up primarily by the monocarboxylate transporter (MCT)-2, where it is converted to pyruvate by lactate dehydrogenase (LDH) and can ultimately contribute to gluconeogenesis. The liver's unique property of strong glucose-6-phosphatase (G6Pase) expression confers its ability to be a glucose-producing (glycogenolysis + gluconeogenesis) organ.

The maintenance of glucose homeostasis in the body requires a dynamic equilibrium between the processes of hepatic glucose production as well as whole body glucose utilization. These processes are by and large regulated by signals initiated by circulating hormonal as well as nutrient factors, the most critical of which are outlined below.

Hormonal

A glucose load normally stimulates the release of insulin from the pancreas. Once released into the bloodstream, insulin is the primary regulator of blood glucose concentration, as it potently increases GLUT4-mediated glucose uptake in muscle and fat and inhibits hepatic GP. Insulin also promotes substrate storage across tissues by stimulating lipogenesis, glycogen and protein synthesis while inhibiting the corresponding reverse hydrolysis reactions (172).

Specifically, after binding the insulin receptor (IR) which leads to receptor tyrosine auto-phosphorylation, insulin signaling leads to the activation of insulin receptor substrates (IRS) which have been demonstrated as critical intracellular regulators of glucose homeostasis. Knockout mice which are deficient in IRS-1 displayed diminished hepatic insulin signaling, as well as peripheral insulin resistance and impaired glucose tolerance (7; 189), and IRS-2 knockout mice additionally had decreased β-cell mass leading to the development of type 2 diabetes (208). Further, insulin stimulates the long-term storage of glucose units as glycogen by promoting the activation of glycogen synthase, via the phosphatidylinositol-3 kinase (PI_3K) signaling pathway: here, protein kinase B (PKB a.k.a. Akt) is activated and in turn inhibits glycogen synthase kinase-3 (GSK-3) (42), which decreases the rate of the inhibitory phosphorylation by GSK-3 on glycogen synthase. Concurrently, insulin inhibits the production of glucose from the liver by blocking glycogenolysis as well as gluconeogenesis. Insulin directly regulates metabolic enzymes (**Figure 1**) by affecting their phosphorylation states, and additionally, can regulate their gene

expression (150). Insulin action inhibits the expression of PEPCK which catalyzes the rate-limiting step in gluconeogenesis (132; 186), and decreases the expression of other gluconeogenic enzymes including fructose-1,6-bisphosphatase and G6Pase. Conversely, insulin increases glycolytic enzyme expression including GK and pyruvate kinase (172).

Nutrient

In addition to its' potent control of glucose fluxes, another major action of insulin is to inhibit lipolysis, the breakdown of triglycerides into free fatty acids and glycerol. Adipocyte-derived circulating free fatty acids (FFA) are elevated in insulin-resistant states, and overall, have been suggested to contribute to the characteristic insulin resistance of diabetes and obesity via the inhibition of glycogen synthesis and glucose oxidation (160), and by increasing hepatic glucose output (11). In the liver, FFA increases gluconeogenesis, but under normal physiological conditions, hepatic autoregulation prevents an overall increase in hepatic GP; however, in type 2 diabetes, hepatic autoregulation is defective, which leads to an increase in GP in the presence of chronically elevated FFA (101).

Glucose regulates its hepatic metabolism via direct as well as indirect (through the action of hormones) mechanisms. In terms of the indirect mechanisms, glucose's most notable glucoregulatory action is its metabolism in the pancreatic β-cell, which is biochemically coupled to the secretion of insulin. Also, when blood glucose levels dip into the hypoglycemic range, there follows an activation of parasympathetic nerves which triggers a host of hormonal responses (123) – an inhibition of insulin release, as well as the secretion of epinephrine, growth hormone, glucagon, and cortisol – which aim at increasing both hepatic gluconeogenesis and glycogenolysis (21; 171; 204). With respect to the exact locus of glucose sensing responsible for coordinating this response, studies have established that the CNS holds an important position. In dogs, isolated brain hypoglycemia in the presence of peripheral euglycemia elicits a rapid counterregulatory response; conversely,

maintaining central euglycemia in the presence of peripheral hypoglycemia blunts this response (14). A few years later, similar bi-directional experimental designs revealed that this sensor is located in the ventromedial hypothalamus (VMH) (16; 18).

Directly, glucose is able to stimulate its own uptake and utilization in a concentration-dependant manner, known as its "mass effect", in just about all tissues with exception of the brain (215). However, glucose is quite clearly less potent than insulin in stimulating glucose metabolism, as insulin's unique anti-lipolytic ability reduces the use of FFAs for energy production (131), thus reducing the competition for and enhancing glucose oxidation. As well, an acute elevation in circulating glucose is known to markedly suppress liver GP in both man (170) and rodents (163). This "glucose effectiveness", or the ability of glucose to regulate its own production, was found to occur by means of a suppression of glycogenolysis, and consistent with this there was a measured decrease in hepatic glycogen phosphorylase and increase in glycogen synthase activities (163).

Glucotoxicity

Glycemia is a critical physiological parameter to monitor, as persistent elevations in blood glucose levels can have a multi-pronged, feed-forward effect to disrupt glucose homeostasis. This includes impairing insulin secretion, diminishing insulin sensitivity, and disrupting glucose effectiveness. Partially pancreatectomized rats, which develop marked hyperglycemia and hypoinsulinemia, displayed impaired *in vivo* glucose-stimulated insulin secretion (GSIS) in the presence of an acute elevation in plasma glucose levels (+100 mg/dL above baseline). However, when these diabetic rodents are treated with phlorizin, an inhibitor of renal glucose absorption that selectively lowers plasma glucose levels, this β-cell abnormality was corrected (165), demonstrating the deleterious role of sustained hyperglycemia *per se*, also known as "glucotoxicity" (164). What could be mediating this defect? It could be possibly due to a down-regulation of glucose transport, as the protein

levels of GLUT2 in the β-cells have been found to be markedly reduced in multiple diabetic models characterized by hyperglycemia and insulin deficiency, including Zucker fatty, BB, and STZ rodents (86; 140; 191). As well, extended hyperglycemia in man (214) as well in rodents (73) confers peripheral insulin resistance with a marked reduction in insulin-stimulated glucose utilization and glycogen synthesis.

As well, an acute increase in circulating glucose levels typically results in a marked suppression of GP in normal rodents, but in diabetic rodents, the GP rate remains elevated (163). However, it was shown with the use of T-1095, an orally bioavailable inhibitor of the sodium-dependent glucose transporter in the renal tubule, that the normalization of glycemia in diabetic rodents restored this regulation of GP (64). A similar impairment was also observed in humans with type 2 diabetes, where an increase in plasma glucose levels was unable to inhibit GP and stimulate peripheral glucose uptake, despite an overnight normalization of glycemia via low-dose insulin infusion (120). However, a three-day intensive glycemic control treatment via insulinization prior to experimentation was able to restore this acute ability, even in individuals with prolonged poorly controlled diabetes (75). Thus, the normalization of glycemia appears to be sufficient to rescue the glucotoxicity-induced impairments on glucose homeostasis.

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The various hormonal and other signals elicited by a nutrient load (hyperinsulinemia, hyperglycemia, suppression of glucagon secretion) (34) act in concert to suppress endogenous glucose production efficiently in normal subjects. This suppression is markedly impaired in patients with NIDDM and is the predominant cause of postprandial hyperglycemia (48). Until recently, it was generally accepted that the pathophysiology of type 2 diabetes primarily involves three organ systems: the liver, muscle, and adipose tissue. Defects across this triumvirate are proposed to work with each other to initiate and

progress the disorders in glucose as well as lipid homeostasis (47). Initial defects in insulin release could result in a disruption in glucose homeostasis via the decreased suppression of hepatic glucose production and reduced glucose uptake by muscle and fat. In this setting of diminished insulin effectiveness lipolysis is also promoted. Collectively, the chronic increase in FFAs and glucose can be deleterious and toxic to the health of the β-cell, eventually leading to the loss in β-cell number (28) and function (90) that is part and parcel with the progression of type 2 diabetes.

However, vast amounts of intriguing research that has amassed over the past decade in particular have revealed that a fourth organ be added to that list of systems: the brain. This research will be thoroughly reviewed in the subsequent section, with an emphasis on central nutrient sensing and the fate of central homeostatic mechanisms in the face of metabolic disease. In this thesis, our aim in particular was to evaluate the yet-to-be tested effectiveness of central glucose and lactate sensing to regulate glucose homeostasis in metabolic disease.

CNS Regulation of Glucose Homeostasis

(Adapted from Chari *et al. Physiology* **24**:159-70, 2009.)

The central nervous system (CNS) has been identified as a key regulator of whole body homeostasis, as it plays a fundamental role in regulation of the respiratory system, the circulatory system, digestion, thermoregulation, and energy expenditure, to name a few. Of the entire CNS, the hypothalamus in particular is generally accepted to mediate the day-to-day regulation of a number of physiological parameters including body temperature (69), blood pressure (71), thirst (6) and hunger (176), and is a fundamental structure for the integration of the endocrine and nervous systems.

The record of CNS control of glucose homeostasis began with the finding by Claude Bernard that punctures in the floor of the fourth ventricle results in glucosuria (12). However, the incredible growth in the field did not occur until over a century after Bernard's initial observation. Over the past decade, it has been demonstrated that the CNS senses A) hormones (40; 67; 80; 96; 97; 137), namely insulin, leptin, and glucagon-like peptide (GLP)-1, and B) nutrients (103; 136; 145), namely fatty acids and glucose, to regulate glucose homeostasis. As diabetes is characterized by hyperglycemia, the elucidation of defects in these hormone- and nutrient-sensing pathways in the hypothalamus that regulate glucose homeostasis will shed light on the central components that initiate and perpetuate this metabolic disease – and as such will serve as the basis of the following literature review. *This material is directly related to the studies that were performed in this thesis, which aimed to advance the literature on central nutrient-sensing mechanisms in various models of metabolic disease.*

CNS Hormone Action

Insulin

In the past decade, the action of insulin had been uncovered to extend beyond the periphery, when neuron-specific insulin receptor disrupted (NIRKO) mice were found to develop mild insulin resistance and elevated plasma insulin levels in association with obesity (24). This was the first demonstration that neuronal insulin signaling regulates peripheral glucose homeostasis. Indeed, it was subsequently shown that intracerebroventricular (i.c.v.) infusion of insulin or its mimetic into the third ventricle suppressed hepatic GP independent of alterations in body weight or changes in circulating levels of glucoregulatory hormones (137). In the same study, antagonizing insulin or its downstream signaling pathway in the brain, including the insulin receptor (IR) and PI_3K, impaired the ability of an acute elevation circulating insulin to suppress GP. Furthermore, decreasing IR expression selectively in the arcuate nucleus (ARC) of the hypothalamus elicited insulin resistance in rats (135), paralleling the finding in NIRKO mice (24). With respect to downstream insulin-signalling effectors, the hypothalamic overexpression of IRS-2, which is highly detectable in the hypothalamus including the ARC (144), and PKB/Akt via adenoviral gene therapy significantly improved the glycemic response to an insulin injection in diabetic rodents (67). And while constitutively active IRS-2 in the hypothalamus improved insulin sensitivity (67), insulin resistance develops in mice with a selective brain IRS-2 knockout (188). Collectively, these findings recognized the CNS as a site of insulin action, and implied the criticality of an intact insulin signaling cascade, involving the binding of insulin to its receptor and the subsequent activation of IRS, PI_3K and PKB, in regulating glucose homeostasis **(Figure 2a)**.

The ability of central insulin signaling to regulate peripheral glucose homeostasis appears to involve the activation of hypothalamic K_{ATP} channels. The GP-lowering effect of systemic or central insulin administration was abolished by the i.c.v. administration of the

sulfonylurea glibenclamide, which is a K_{ATP} channel blocker (137; 153). As well, hepatic branch vagotomy and selective vagal deafferentation indicated that the CNS-liver circuit requires efferent vagal fibres (153), and that it ultimately triggers an interleukin (IL)-6/ signal transducer and activator of transcription (STAT)-3 signaling cascade in the liver to lower GP (80). It remains to be ascertained how the insulin signalling cascade (i.e. IR → IRS-2 → PI_3K → PKB) leads to the activation of K_{ATP} channels in the hypothalamus, but the involvement of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) has been postulated. *In vitro,* PIP_3 directly activates K_{ATP} channels (113) and more significantly, the constitutive activation of PI_3K-PIP_3 signalling in pro-opiomelanocortin (POMC) neurons increase K_{ATP} channel conductance, which hyperpolarizes neurons to result in a hyperphagic phenotype (152) (**Figure 2a**).

SUR1/Kir6.2 K_{ATP} channels, which are characteristically found in pancreatic β-cells (3) and are critical in triggering insulin secretion, are also found in hypothalamic ARC neurons known to control energy and glucose homeostasis (51). The ARC contains an array of neuronal subtypes that are involved in the regulation of energy and nutrient homeostasis, of which two are most extensively studied. One subtype is POMC neurons, which express the anorexigenic products of the peptide. POMC is post-translationally cleaved to a series of smaller peptides; of note is α-melanocyte stimulating hormone (α-MSH), which inhibits feeding by binding the melanocortin receptor 4 (MC4R) (178). Belonging to the second subtype are neurons that co-express the orexigenic peptides agouti-related peptide (AgRP) and neuropeptide Y (NPY). The activation of these orexigenic neurons stimulate feeding by increasing NPY/AgRP signaling (176), and leads to a two-fold inhibition of anorexigenic signaling: directly synapsing with POMC neurons and competitive binding of AgRP to the α-MSH binding site at MC4R, collectively antagonizing POMC's anorexigenic effects. The interplay between these anorexigenic and orexigenic neuronal subsets and their downstream effector signalling form the melanocortin signalling system,

and it is the activation of this "hypothalamic melanocortin tone" (37) that is thought to be instrumental in the regulation of energy and glucose homeostasis.

Indeed, the i.c.v. infusion of NPY precludes the inhibition of GP elicited by circulating insulin, suggesting that the down-regulation of NPY release is likely a prerequisite for insulin's complete ability to suppress hepatic GP (196). Moreover, it was shown via the knockout of IR in specific neuronal subtypes that only in AgRP-IR knockout mice, and not POMC-IR knockout mice, did an elevation in circulating insulin fail to suppress hepatic GP and stimulate hepatic IL-6 expression independent of changes in energy homeostasis (99). In line with this, i.c.v. MC3/4R antagonist infusion did not alter the effect of circulating insulin to inhibit hepatic GP (137). Together, these data suggest that insulin operates on a melanocortin-independent pathway, signaling through NPY/AgRP and not POMC neurons, to regulate hepatic GP **(Figure 2a)**.

However, the results of brain insulin action studies completed in other species reveals a controversy as to whether direct or indirect (i.e. hypothalamic) insulin signaling on the liver dominate in the maintenance of glucose homeostasis. Using a normal dog model, Alan Cherrington's group demonstrated that a 4-fold rise in head insulin with the use of an intracarotid infusion failed to suppress net hepatic glucose output. In fact, a significant increase in endogenous GP was observed versus dogs receiving peripheral or portal insulin during the clamp (54). This increase is consistent with a previous finding from the same group, where a physiological increase in blood-borne insulin was thought to *augment* the counterregulatory response to hypoglycemia. In that study, despite equivalent peripheral insulinemia and hypoglycemia, the infusion of head insulin let to an increase in glucagon, cortisol and epinephrine levels along with an increase in hepatic glucose production versus peripheral insulin infusion (45). These findings contrast with an earlier study by a different group revealing that the direct i.c.v. administration of insulin in mongrel dogs actually *lowered* blood glucose levels and produced sustained hypoglycemia (2).

Leptin

As with insulin, the discovery of leptin (218) was indeed another milestone in the field of obesity and diabetes research. It is well-documented that this adipose tissue-derived hormone, which acts largely in the CNS (176), holds a critical role in the regulation of both energy and glucose homeostasis. In both rodents and humans, deficiency in leptin or its functional receptors leads to profound obesity and insulin resistance (4; 36; 63). Leptin replacement improves glucose homeostasis but this was initially attributed as secondary to reduced adiposity and feeding (29; 74; 149); however, later observations strongly suggest that leptin can also regulate glucose homeostasis independent of its effects on adiposity. A chronic increase in plasma leptin, independent of changes in weight, enhances both insulin sensitivity and glucose disposal in lipodystropic rodents (53; 180). Accordingly, another study found that leptin-treated ob/ob mice had a 40% reduction in glucose and insulin levels compared to pair-fed ob/ob mice (175). Direct stimulation of central leptin signalling also regulates glucose homeostasis. Of the various central loci, the hypothalamic ARC has been highlighted as the key CNS site for leptin's effects on glucose homeostasis, as the selective restoration of leptin receptors in the ARC of leptin receptor-deficient Koletsky (fa^k/fa^k) rats improved insulin sensitivity (126).

It is now known that leptin, upon binding to its receptor in the ARC, activates two independent intracellular signaling pathways, which work in concert to regulate glucose homeostasis **(Figure 2b)**. The first of the two is the well-established STAT3-dependent pathway, whereby leptin upon binding its' receptor activates Janus kinase 2 (Jak2), leading to the phosphorylation of cytoplasmic targets such as STAT3. Indeed, s/s mice, having lifelong obliteration in leptin-STAT3 due to a leptin receptor mutation, were found to be severely hepatic insulin resistant (25). The same study pharmacologically and adenovirally knocked-down central STAT3 activity to demonstrate that the GP-suppressing ability of i.c.v. leptin in overfed rats was nullified (25). As well, the inactivation or deficiency of a negative

regulator of the JAK-STAT pathway, suppressor of cytokine signaling (SOCS) 3, in selective brain regions was able to improve leptin sensitivity and glucose homeostasis (96; 216).

While this STAT3-dependent pathway is imperative, it may not be the sole mediator of CNS leptin's control of glucose homeostasis. Knowing that the activation of hypothalamic PI_3K is necessary for leptin, as well as insulin, to reduce food intake (128), it seems that the binding of leptin to its receptor to activate PI_3K and ultimately regulate glucose homeostasis is likely. Indeed, the intrahypothalamic infusion of a PI_3K inhibitor blunted the aforementioned improvement in insulin sensitivity elicited by restoration of functional ARC leptin receptors in leptin receptor deficient fa^k/fa^k rats (126), suggesting that hypothalamic leptin activates PI_3K to regulate glucose homeostasis. However, it is highly plausible that this activation of PI_3K by leptin to regulate glucose homeostasis is a pathway that is only relevant to certain neuron subtypes, as leptin activates PI_3K in POMC but not NPY/AgRP neurons (213), while insulin signaling in AgRP but not POMC neurons regulates glucose homeostasis (99). Consistent with this view, POMC-specific deletion of SOCS3 enhances leptin sensitivity and improves glucose homeostasis (96). Nevertheless, the role of the downstream effectors of the leptin-PI_3K signaling cascade that regulate glucose homeostasis remains to be elucidated.

Glucagon-Like Peptide 1

GLP-1, a potent hormone that is a mediator of the incretin effect, is secreted by the L-cells of the intestines (199) and discrete populations of neurons (85). Traditionally, this gut hormone is thought to regulate glucose homeostasis via directly acting on the β-cells to stimulate insulin secretion and biosynthesis, promote β-cell growth, and decrease glucagon secretion (50). GLP-1 also potently acts centrally to regulate food intake (194). However, emerging studies are hinting at the ability of central GLP-1 action to regulate peripheral

glucose homeostasis. Of note, with the use of GLP-1 antagonist and agonists administered i.c.v., it was found that CNS GLP-1 signaling is involved in regulating insulin secretion (97). Enhanced insulin secretion and an improved glucose disposal profile with direct i.c.v. GLP-1 administration were also determined with an i.v. glucose tolerance test (173).

GLP-1 receptor mRNA is detected widely across brain regions including, but not limited to, the hippocampus, the hindbrain and hypothalamic nuclei such as the ARC and PVN (119). Of these, the PVN and hindbrain in particular are known to mediate the anorectic effect of CNS GLP-1 action (70; 118; 194). Interestingly, while GLP-1 receptors are found in the ARC but are not involved in the regulation of food intake, they do mediate GLP-1's glucoregulatory action (173) **(Figure 2c)**. Administration of GLP-1 directly into the ARC lowers hepatic GP, a phenomenon not reproducible with direct GLP-1 administration into the PVN, where the anorectic effect is activated (173). To date, how the mechanism(s) behind CNS GLP-1 regulation of glucose homeostasis are yet to be clarified; however, the activation of K_{ATP} channels represents a possible candidate as the co-infusion of glibenclamide prevented the GLP-1-induced suppression of GP (173). Furthermore, this effect of central GLP-1 appears to be POMC neuron-mediated as GLP-1 receptors largely co-localizes with POMC, and not NPY/AgRP neurons, in the ARC (173).

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In essence, peripherally-derived hormones such as insulin, leptin and GLP-1 have been repeatedly demonstrated to act on their respective receptors in the CNS and exert their glucoregulatory effects via what seem to be distinct signaling pathways, perhaps converging at some downstream candidate(s). As mentioned earlier, the activation of K_{ATP} channels presents as a likely candidate, as not only is it a required step in the action of central insulin as well as GLP-1, but also in central *nutrient* sensing mechanisms which

regulate hepatic GP, which have been the focus of this thesis. *The relevant literature on central nutrient sensing will be reviewed in the following section.*

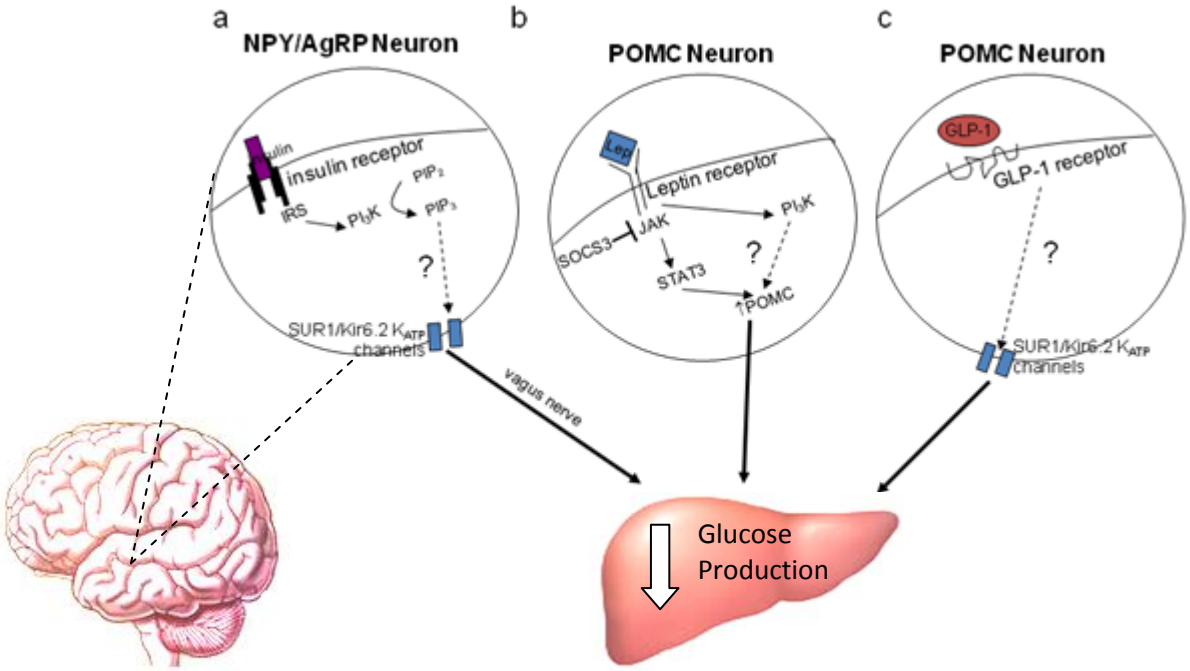

Figure 2. Hypothalamic Hormone Signaling Pathways Regulating Glucose Homeostasis.

(**a**) Insulin binds its receptor, leading to the activation of IRS and PI_3K. PI_3K subsequently phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate PIP_3, which in turn 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 ultimately relayed through the vagal afferent nerve, hepatic glucose production (GP) is decreased. (**b**) Leptin, upon binding to the long form of the leptin receptor activates two distinct pathways: (1) activation of the JAK/STAT3 pathway and (2) activation of PI_3K. Together, these signaling cascades lead to an increase in POMC neuron activity, which triggers a decrease in hepatic GP. (**c**) More recently, GLP-1 action in the arcuate nucleus has been shown to decrease hepatic glucose production, likely through SUR1/Kir6.2 K_{ATP} channel-dependent mechanisms in POMC neurons.

CNS Nutrient Sensing

In addition to processing input from hormones, the hypothalamus senses nutrients to initiate metabolic responses to regulate energy (41; 44; 100; 121; 136; 209) and nutrient (102; 103; 136) 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 (115) and lipostatic (95) hypotheses proposed that circulating nutrients generated in proportionate amounts to storage depots serve as signals to the brain to initiate alterations in energy intake and expenditure. However, only recently has the notion of direct hypothalamic nutrient sensing become a thoroughly demonstrated and credible means of controlling glucose homeostasis.

Glucose

An important source of energy for the majority of mammalian cell types, glucose is particularly vital for the brain where it is essentially the sole substrate for energy metabolism. The discovery of glucose sensing neurons in satiety and feeding centres of the hypothalamus (5; 139) hinted at potential physiological roles of central glucose utilization (108) beyond serving as a fuel. Indeed, since those seminal studies, central glucose sensing/metabolism has been established to be an essential component in the regulation of feeding (13; 44; 122) and the hypoglycemic counter-regulatory response (16; 18). 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 glucose production; this occurred via a curtailing of both gluconeogenesis and glycogenolysis (103). The metabolic fate of brain glucose has been largely clarified by the proposal of the astrocyte-neuron lactate

shuttle (148), which is supported by observations that neuronal activity is coupled to glucose utilization (94; 147; 168; 202) and that neurons preferentially utilize glial glucose-derived lactate as an oxidative fuel (114). Indeed, the infusion of i.c.v. lactate was able to recapitulate the effects of central glucose on blood glucose levels and hepatic GP (103). However, the effects of both i.c.v. lactate and glucose were nullified when co-infused with oxamate, an inhibitor of lactate dehydrogenase (LDH) activity (103), which antagonizes the preferentially lactate-generating LDH-A (the muscle isoform that, within the brain, is expressed exclusively in the glial cells (15)) and the preferentially pyruvate-generating downstream LDH-B (the heart isoform, and the only isoform found in neurons (15)) (22). This finding suggests that metabolism of glucose to lactate and subsequently pyruvate in the hypothalamus is an essential biochemical step in the regulation of glucose homeostasis **(Figure 3)**. Furthering this notion is the suppression of GP resulting from the hypothalamic administration of dichloroacetate (DCA) (103), which ultimately promotes the conversion of pyruvate to acetyl-CoA via the inhibition of PDH kinase, in turn activating PDH (82).

As discussed earlier, an acute elevation in circulating glucose is known to markedly suppress liver GP (120; 163; 192). When plasma glucose levels were doubled in the presence of a concurrent intrahypothalamic infusion of oxamate, this inhibitory action of acute hyperglycemia on GP was blunted by 40% (103), revealing that the activation of hypothalamic lactate metabolism is a critical component of the effectiveness of glucose *per se*. Circulating lactate has also been demonstrated to regulate hepatic glucose fluxes (84), 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 (98). *As such, the activation of central glucose and lactate sensing mechanisms is proven to be important in the regulation of GP in normal rodents – however, the effectiveness of these nutrient sensing mechanisms in settings of metabolic stress has yet to be determined.*

Few articles to-date have looked at glucose sensing in specific neuronal cell types. Of particular note, operating on the proposal that glucose sensing in the anorexigenic POMC neurons of the hypothalamus mechanistically mimics that of the pancreatic β-cell, 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 (145). 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 UCP2 (145). The discovery of hypothalamic AMPK activity as a critical integrator of nutrient and hormonal sensing in the regulation of energy homeostasis (121) warranted an examination into precise neuronal cell types and mechanisms involved, and to do this, Dominic Withers and colleagues generated mice that were AMPK-deficient in either the orexigenic agouti-related protein (AgRP) or anorexigenic POMC neurons (35). Here, electrophysiological studies revealed absent responses to alterations in extracellular glucose in AMPK-deficient AgRP as well as POMC neurons. However, both of the AMPK-deficient neuron populations responded normally to insulin or leptin action (35), potentially complicating the notion of a convergence of hypothalamic nutrient and hormonal signaling mechanisms.

However, one must exercise caution when extrapolating the cellular findings from the above two studies, as the glucose concentrations used in the electrophysiology of these studies far exceed hypothalamic glucose range, which is typically at 0.7-2.5 mM and thus only 15-30% of blood glucose levels (169; 181). As such, this limits the physiological impact of this recent work. Furthermore, the study by Withers and colleagues uses a subset of AgRP neurons which are glucose-responsive (i.e. glucose-excited), which is against the general consensus of the orexigenic AgRP/NPY neurons being by and large glucose-sensitive (i.e. glucose-inhibited) (27; 59; 127).

Fatty Acids

During the fasted state, fatty acids are primarily released as a means to provide an energy source for the body while conserving the glucose for neurons, which depend on carbohydrates as an energy source. The brain does not, to our knowledge, use fatty acids as a primary source of energy. However, they likely serve as a signal of nutrient abundance. Indeed, it has been demonstrated recently that select enzymes and intermediates of fatty acid metabolism contribute to the hypothalamus' ability to regulate glucose homeostasis **(Figure 3)**. In 2002, it was first demonstrated that the administration of LCFAs into the third cerebral ventricle triggers a neural pathway to regulate energy as well as glucose homeostasis. Of note, these rodents treated with i.c.v. oleic acid had lower plasma insulin and glucose levels indicating enhanced insulin sensitivity; with the use of a basal insulin pancreatic clamp this was confirmed, as i.c.v. oleic acid was found to markedly suppress hepatic GP (136). Interestingly, the infusion of the medium-chain fatty acid octanoic acid did not yield the same results, revealing specificity in the nature of the hypothalamic nutrient signal (136). The i.c.v. co-administration of the K_{ATP} channel blocker glibenclamide with oleic acid was able to nullify the GP-lowering effect of i.c.v. oleic acid alone (136). Moreover, this was in line with a later study demonstrating that hypothalamic K_{ATP} channel activity *per se* can regulate GP (153).

Based on the aforementioned observation that i.c.v. oleic acid but not octanoic acid, a medium-chain fatty acid which does not require CPT-1 for mitochondrial entry (136), has suppressive effects on GP, it was then tested if central CPT-1 activity-mediated changes in cytosolic long-chain fatty acids (LCFAs) can recapitulate the effects observed with i.c.v. administered LCFAs **(Figure 3)**. With the use of a CPT-1 ribozyme as well as pharmacological CPT-1 inhibitors, it was found that a decrease in CPT-1 activity was sufficient to lead to an increase in the concentration of LCFA-CoAs and, as a result, elicit a substantial suppression

of GP (134). Thus, the regulatory effects of cellular fatty acids in the hypothalamus are extramitochondrial and are likely cytosolic **(Figure 3)**.

The activity of CPT-1 is regulated by malonyl-CoA, which is generated from acetyl-CoA by ACC as the committed step of *de novo* fatty acid generation (203). Numerous studies have revealed a role of hypothalamic malonyl-CoA in the regulation of feeding and energy expenditure (32; 65; 76; 79; 109; 210), but to-date the application of the "malonyl-CoA hypothesis" to the regulation of glucose homeostasis remains limited. Citrate, an intermediate metabolite produced in the mitochondria in the citric acid cycle, is an allosteric effector of ACC activity; and it was recently demonstrated that i.c.v. citrate not only decreased food intake and body weight, but also resulted in lower blood glucose levels during a glucose tolerance test, increased glucose uptake during a hyperglycemic-euglycemic clamp, and increased liver glycogen synthesis (31). Thus, promoting the formation of hypothalamic malonyl-CoA is perhaps an important step in the regulation of glucose homeostasis.

Illustrating that circulating plasma fatty acids can access the brain and recapitulate the effect of central fatty acid sensing on glucose homeostasis should undoubtedly further its physiological relevance. Indeed, when an i.c.v. infusion of a K_{ATP} channel blocker was administered during an intravenous lipid infusion, there was a significant elevation in glucose production, which was attributed to an increase in glycogenolysis (104). The results of these pharmacological findings were confirmed with a genetic approach using mice deficient in the K_{ATP} channel subunit Sur1 (104). Pharmacological inhibition of hypothalamic acyl-CoA synthetases (ACS) by triacsin C, as well as a hepatic branch vagotomy negated the effects of circulating lipids on GP (104). Taken together, the study illustrates that circulating LCFAs can regulate glucose homeostasis via a hypothalamically-triggered mechanism that is dependent on 1) the esterification of LCFAs to LCFA-CoAs, 2) functional K_{ATP} channels and 3) neural transmission via the vagus nerve. Additionally, overexpressing malonyl-CoA

decarboxylase (MCD) in the hypothalamus of rodents negates the ability of circulating lipids to regulate glucose homeostasis (76). In accordance with the fatty acid sensing hypothesis, these rats not only had a marked reduction in hypothalamic malonyl-coA levels, but also a concomitant decrease in LCFA-CoA abundance (76). Recently, we have demonstrated that this increase in circulating lipids lowers glucose production via a hypothalamic protein kinase C (PKC)-dependent mechanism (162). Furthermore, the pharmacological activation of hypothalamic PKC was sufficient to lower hepatic GP, an effect which was nullified with the pharmacological or molecular disruption of hypothalamic K_{ATP} channels (162). Altogether, these results support the notion that the activation of hypothalamic PKC is necessary for central lipid sensing mechanisms to lower GP via the K_{ATP} channel-dependent pathway **(Figure 3)**.

Amino Acids

Recent studies have pushed for a physiological role of central amino acid sensing. 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 (195). Its activity is sensitive to various nutrients, including glucose and some fatty acids and particularly the branched-chain amino acid leucine (211); indeed, when a low-dose of leucine was administered into the third cerebral ventricle of rodents, leading to the activation of mTOR signaling, a marked decrease in short-term food intake and body weight was observed (41). This leucine-mediated anorectic effect was nullified when hypothalamic mTOR activity was inhibited via rapamycin treatment (41). The anorectic effects mediated by central leucine administration correlated with an increase in the phosphorylation of S6 kinase (S6K), an effector of mTOR activity, in the hypothalamus (41); these effects were confirmed in a recent study in a dose-dependent manner (161). However, the role of central amino acid sensing *per se* 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 utmost priority.

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The metabolism of different nutrients in the hypothalamus, particularly that of glucose and fatty acids, serves roles of polarizing 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 glucose homeostasis **(Figure 3)**. 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 interacts with those of the previously detailed hormone-sensing is uncertain. As such, the integration and co-dependence of central nutrient and hormone sensing pathways remains an area of interest that is open to further scrutiny. *The studies proposed and completed in this thesis aim to add to the growing literature and provide further insight into the nature and effectiveness of central nutrient sensing mechanisms.*

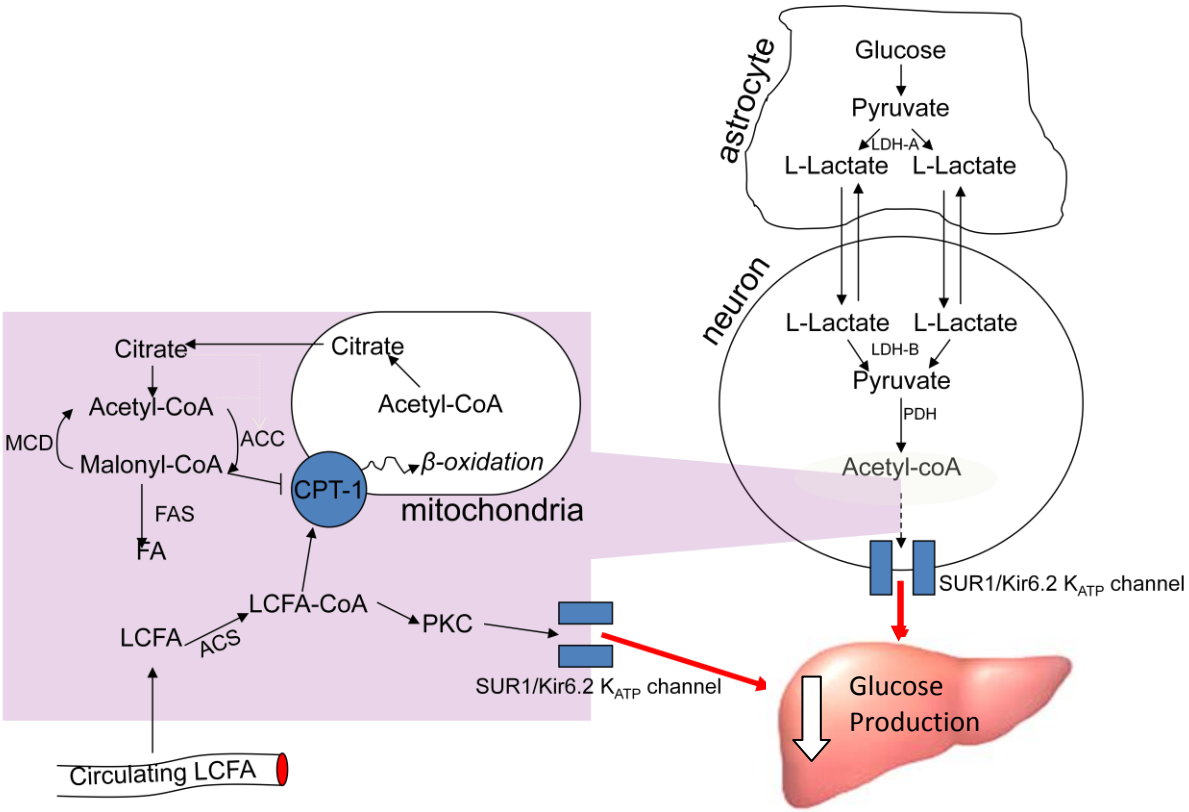

Figure 3. Glucose and Fatty Acid Sensing Mechanisms in the Hypothalamus

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 both hypothalamic glucose and fatty acid metabolism has been shown to lower hepatic glucose production via a K_{ATP} channel-dependent mechanism.

Implications for Diabetes and Obesity

The numerous studies outlined thus far have been instrumental in advancing the importance of brain, in particular the hypothalamus, in processing acute changes in hormonal signaling and nutrient availability and triggering a neuronal circuit to regulate glucose homeostasis. When it comes to this homeostatic regulation in the face of metabolic disease characterized by nutrient excess and/or dysregulated hormone action, experimental evidence, in general, points to a decreased effectiveness of this circuitry.

In mice that are genetically obese and lack functional leptin (ob/ob), the administration of leptin potently reverses obesity, lowering their food intake and body weight, and normalizing plasma glucose and insulin levels (149). However, leptin levels in obese humans (38) and obese and/or overfed rodent models (62) are paradoxically elevated. Leptin's effects are largely carried out in the brain (176), and experimental findings are consistent with the notion of impaired leptin access to the brain being a key component of leptin resistance. After chronic high-fat diet feeding, food intake and body weight become resistant to peripherally administered leptin. However, a single bolus of i.c.v. leptin in these mice maintained its robust suppressive effects on these parameters (197). Additionally, when 3-day overfed rodents received i.c.v leptin, there was a marked inhibition of glucose production (155). However, there is an upper limit to circumventing the resistance to peripherally-administered leptin via direct i.c.v. leptin administration, as during the course of chronic overfeeding the extent of hypothalamic STAT3 activation by both peripheral and i.c.v. leptin administration diminishes (57). Protein tyrosine phosphatase 1 B (PTP1B) has been shown to be involved in the blockade of leptin signalling, and indeed, neuron-specific PTP1B knockout mice on a chronic high-fat diet had lower fed blood glucose and serum insulin levels, and as such displayed improved insulin sensitivity and glucose tolerance (9). This finding was paralleled by a recent study which demonstrated

that the neuron-specific knockout of SOCS3, another suppressor of a leptin-signalling pathway (JAK-STAT), were resistant to chronic high-fat diet-induced weight gain and hyperleptinemia, and were more insulin sensitive as measured by glucose- and insulin-tolerance tests (125), observations that were similarly seen in mice with a haplosufficiency in whole-body SOCS3 that were maintained on a high-fat diet (78).

Endoplasmic reticulum (ER) stress, the cellular condition generally characterized by a disruption in protein synthesis and processing, and the subsequent activation of the compensatory unfolded protein response (UPR) has been demonstrated to hold an important role in the progression of obesity-induced peripheral insulin resistance and the establishment of type 2 diabetes (142). Very recently, it has been shown that mice on a chronic high-fat diet developed ER stress and subsequent UPR activation in the hypothalamus, and when mice were administered i.c.v. tunicamycin to selectively induce ER stress in the hypothalamus, leptin-induced hypothalamic STAT3 phosphorylation was abolished (141). XBP1 is a transcription factor that is activated in the UPR and upregulates genes encoding, among others proteins, ER chaperones which assist in protein folding and trafficking to combat ER stress. Indeed, mice displaying a neuron-specific knockout of XBP1 and placed on a high-fat diet exhibited hyperleptinemia, hyperinsulinemia and elevated fasted blood glucose (141). Additionally, these neuronal XBP1-deficient rodents had diminished glucose tolerance and insulin sensitivity (141). Overnutrition-induced hypothalamic ER stress has also been shown to activate a mediator of metabolic inflammation, IKKβ/NF-κB (217). This study also demonstrated that mice which had constitutively active IKKβ in the MBH impaired the 1) activation of Akt and the generation of PIP_3 in response to i.c.v. insulin, and 2) the phosphorylation of STAT3 in response to i.c.v. leptin. Furthermore, the suppressive effects of i.c.v. insulin and leptin on short-term food intake was blunted in these mice (217). Thus, the activation of IKKβ/NF-κB in the MBH causes both central leptin as well as central insulin resistance.

Furthering the role of central insulin resistance in the pathogenesis of diabetes, it was determined that hypothalamic insulin signalling via the PI_3K pathway was markedly reduced in rodents with STZ-induced uncontrolled diabetes (67). Furthermore, enhancing PI_3K signaling via adenoviral gene therapy was found to enhance the ability of peripherally-administered insulin to lower glucose levels, while pharmacological inhibition of hypothalamic PI_3K signalling blunted insulin-mediated glucose lowering (67). In normal rodents, the infusion of i.c.v. insulin lowers glucose production (137) However, this regulatory ability of hypothalamic insulin was lost after merely one day of high-fat feeding (138). The diminished phosphorylation of hypothalamic Akt and unchanged hepatic insulin signalling revealed that this acute overfed model had selective hypothalamic insulin resistance (138). Furthermore, these rodents had significantly increased S6K activity, and the adenoviral overexpression of dominant negative S6K in the MBH was able to reverse the observed hypothalamic insulin resistance in the high-fat diet-fed rats, restoring the ability of hypothalamic insulin to suppress GP (138).

LCFAs serve as a central signal of nutrient abundance, in turn triggering the series of neuronal signalling cascades necessary to regulate nutrient intake and production. Shortly after the effects of i.c.v. oleic acid were published, it was then evaluated whether short term alterations in nutrient availability affect the ability of central oleic acid to regulate energy and glucose homeostasis. In rats which overfed on a 3-day high fat diet, an i.c.v. oleic acid bolus was unable to not only inhibit food intake, but also suppress glucose production under conditions of a pancreatic basal insulin clamp; interestingly, by pair-feeding rats on the high-fat diet the ability of i.c.v. oleic acid to suppress hepatic GP was restored (124). This provides compelling evidence that the hypothalamic responses triggered by an acute increase in central LCFAs are nutritionally regulated, and along with the aforementioned hypothalamic insulin resistance that developed in rodents fed a high-fat diet for one day (138) 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 (154). This was indeed the case, as when a systemic lipid emulsion designed to double plasma LCFAs and hypothalamic LCFA-CoAs (104) was administered to overfed rats, the circulating lipids failed to increase hypothalamic LCFA-CoAs (154). An impeded blood-brain barrier 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 (154). 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 food intake as well as GP in overfed rodents (154). 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.

But interestingly, not all central nutrient sensing mechanisms are disrupted in models of metabolic disease. We recently demonstrated that the activation of hypothalamic PKC in the hypothalamus, a necessary mediator of hypothalamic lipid sensing to regulate glucose homeostasis, was sufficient to suppress hepatic GP in a 3-day overfed rodent model (162) in which hypothalamic lipid sensing *per se* was shown to be impaired (124).

To date, the effectiveness of hypothalamic glucose or lactate metabolism on the regulation of glucose homeostasis in models of diabetes or obesity has yet to be assessed. As described earlier, the acute infusion of glucose, as well as lactate, is able to serve as a signal of nutrient surfeit in the hypothalamus and trigger a circuitry to lower hepatic glucose production and plasma glucose levels in normal rodents (103). With the studies outlined in this thesis, we are, to our knowledge, making the first attempt to flesh out these hypothalamic nutrient-sensing mechanisms in multiple models of metabolic disease.

2

GENERAL HYPOTHESIS

The overall aim of this project is to evaluate CNS nutrient sensing (specifically that of glucose and its downstream metabolite lactate) in multiple metabolic disease models including diabetes, selective hyperglycemia, and insulin deficiency/resistance.

It has been previously shown that the direct administration of central and hypothalamic glucose can lower plasma glucose levels by means of inhibiting hepatic glucose production. By means of a facilitated transport system, circulating glucose can cross the blood brain barrier and readily gain central access. As a result, changes in blood glucose concentration result in corresponding changes in brain glucose concentration. The establishment of hyperglycemia in diabetes implies that glucose is unable to access or trigger the hypothalamic signal to lower glucose production that has been characterized in the normal, healthy setting. ***We hypothesized that CNS glucose sensing to lower glucose production is impaired in diabetes*.**

The chronic hyperglycemia observed in uncontrolled diabetes has established peripheral glucotoxic effects (i.e. that of elevated glucose *per se*), including insulin resistance, impaired GSIS, and an inability of circulating glucose to inhibit glucose production. Via the normalization of glucose levels, it has been shown that the latter impairment can be rescued in diabetes. ***We hypothesized in this project that glucotoxicity in the hypothalamus is, in part, responsible for the impairment of the CNS glucose sensing effect in uncontrolled diabetes.***

3

EXPERIMENTAL OBJECTIVES

** The treatment groups listed below have been evaluated for their effects on central (i.c.v.) and/or hypothalamic (i.h.) nutrient sensing with the use of the tracer (3-[^{3}H]glucose)-dilution methodology to assess in vivo glucose kinetics.*

<u>Study I</u> – CNS Lactate Sensing in Metabolic Disease

i) Direct administration of intracerebroventricular (third ventricle; i.c.v.) lactate (5mM) into STZ-diabetic rats to evaluate whether its ability to lower GP in normal rodents is effective in this model of early-onset uncontrolled diabetes.

ii) Subsequent administration of i.c.v lactate into experimentally-induced, selective model of hypoinsulinemia in the absence of hyperglycemia, as well as into high fat diet-induced insulin resistant rats to further examine the requirement for basal insulin levels/signaling for central lactate sensing to regulate GP.

<u>Study II</u> – Glucotoxicity and CNS Glucose Sensing

i) Direct administration of intracerebroventricular (third ventricle; i.c.v.) glucose (2mM) into STZ-diabetic rats to evaluate whether its ability to lower GP in normal rodents is effective in this model of early-onset uncontrolled diabetes.

ii) Recapitulating the impairment of CNS glucose sensing in an alternative model of *whole-body* glucotoxicity via a 24 h infusion of intravenous (i.v.) glucose (37.5%) to elevate blood glucose levels > 18 mM, as well as in a model of *hypothalamic* glucotoxicity via a 24 h infusion of i.h. glucose (4 mM). The effects of i.h. glucose on GP will be assessed.

iii) Administration of i.h. glucose (2 mM) to STZ-diabetic rats whose plasma glucose levels have been normalized via i.v. phlorizin treatment for a duration similar to the exposure of hyperglycemia in untreated STZ-rats. With this treatment, can CNS glucose sensing be restored in uncontrolled diabetes?

4

GENERAL METHODS AND MATERIALS

Animals

We studied 8-week-old male Sprague-Dawley rats (Charles River Laboratories, Montreal, QC) weighing 250-300g. Rats were housed in individual cages, subjected to a standard light-dark cycle, and were maintained on a regular chow Teklad 6% Mouse/Rat diet (#7002; Harlan Laboratories), unless otherwise stated, and had *ad libitum* access to distilled water. Recovery between surgical procedures was monitored by measuring daily food intake and body weight gain in the days preceding the infusion procedure. To ensure comparable postasbsorptive nutritional status, rats were limited to 20 g of food the day prior to experimentation. All studies were performed in conscious, unrestrained and unstressed rats. Further, all study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University Health Network (Toronto, ON).

Central and Hypothalamic Cannulations

At least ten days before the in vivo study, rats were implanted with a chronic catheter placed into the third cerebral ventricle (i.c.v.) or intrahypothalamically (i.h.) directly within the mediobasal hypothalamus (MBH). Rodents were anesthetized with a freshly made cocktail of ketamine (90 mg/kg Vetalar; Bioniche) and xylazine (10 mg/kg Rompun; Bayer) administered directly into the intraperitoneum (i.p.) and mounted into a stereotaxic apparatus (David Kopf Instruments, Model 900LS) with the ear bars set at 0.50 cm. For i.c.v. catheters, a 22-gauge stainless steel single guide cannula (C313G; Plastics One Inc., Roanoke, VA) was implanted into the third cerebral ventricle using the following

coordinates: 2.5 mm posterior from bregma (intersection between sagittal and coronal sutures) and 8.0 mm below the surface of the skull directly on the midsagittal suture. A dummy cannula (C313DC; Plastics One Inc.) was inserted to cap the guide cannula and prevent it from clogging. For i.h. catheters, a 26-gauge stainless steel bilateral guide cannula (C235G; Plastics One Inc.) was implanted into the mediobasal hypothalamus using the following coordinates: 3.1 mm posterior from bregma and 9.6 mm below the surface of the skull directly on the midsagittal suture. A bilateral dummy cannula (C235DC; Plastics One Inc.) was inserted to cap the guide cannula and prevent clogging. The implanted i.c.v./ i.h. catheter was secured to the skull using instant adhesive (Loctite) and dental cement.

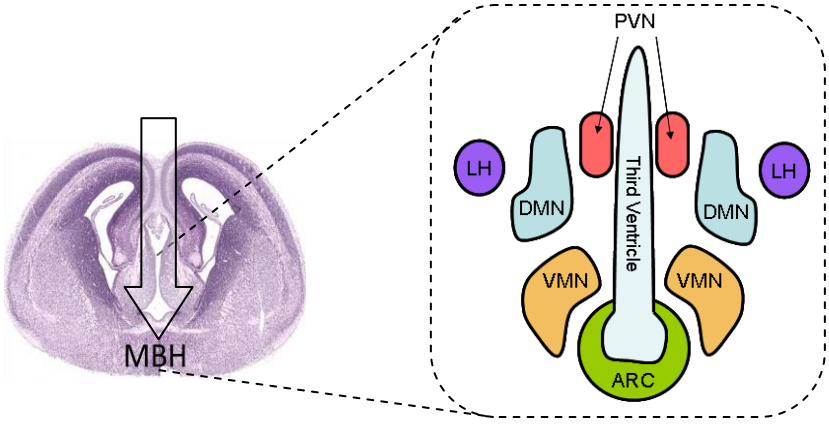

Figure 1 – Hypothalamic Administration and Nuclei. The coronal section (left) reveals the area of the brain that is targeted by the single intracerebroventricular (3rd ventricle) and bilateral intrahypothalamic (i.h.) cannulations. The i.c.v. cannula delivers substances into the base of the third cerebral ventricle, while the i.h. cannulae permits the more localized infusion of substances directly into the mediobasal hypothalamus (MBH) located at the base of the hypothalamus, which houses the arcuate nucleus (ARC). Other hypothalamic nuclei shown in the diagram (right) are the ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamus (LH) and the paraventricular nucleus (PVN).

Vascular Catheterization Surgery

After approximately one week after receiving the brain catheters, additional indwelling catheters were placed in the right internal jugular vein and left carotid artery for infusion and sampling purposes during the eventual *in vivo* procedures. Rodents were anesthetized with a freshly made cocktail of ketamine (90 mg/kg Vetalar; Bioniche) and xylazine (10 mg/kg Rompun; Bayer) administered directly into the intraperitoneum (i.p.). An ~1 inch

horizontal incision was made near the middle of the neck, while rodents were rested on their back, and the skin, fat, and connective tissue were cleared using blunt dissection. The right jugular vein was isolated and made taut with the use of 4-0 silk sutures and hemostat clamps. Micro-dissecting scissors were used to make an incision into the vessel, and a heparinzed saline-flushed catheter, made using 15 cm of polyethylene (PE-50) tubing capped with a 1.5 cm tip of silastic tubing, was inserted into the vessel. The catheter was advanced toward the superior vena cava, and a slight amount of negative pressure was applied using a heparinzed saline-filled syringe connected to the catheter to verify the withdrawing of venous blood. Silk sutures were double-knotted around the vessel to secure the catheter. The left common carotid artery was similarly isolated, cannulated and secured, with the catheter being advanced to the level of the aortic arch (~2 cm). Using a hemostat and a 16-gauge syringe, the catheters were tunneled subcutaneously and externalized on the backside of the rats, between the shoulder blades. Excess polyethylene tubing was cut off and the catheters were flushed with a 10% heparin solution to facilitate catheter patency. Finally, the catheters were plugged with blunted pins, and secured to the animal using masking tape.

Biochemical Analysis

Plasma glucose concentrations were measured via the glucose oxidase method, using an Analox Glucose Analyser (Analox, London, UK). Blood samples are spun down in a benchtop centrifuge, and a 10 µL plasma sample was pipetted into a solution which contains oxygen and glucose oxidase. The chemical reaction that is catalyzed by glucose oxidase (GOD) involves the oxidation of glucose to gluconic acid with the consumption of oxygen:

$$\text{D-glucose} + O_2 + H_2O \xrightarrow[\text{pH 6.0}]{\text{GOD}} \text{gluconic acid} + H_2O_2$$

GOD is highly specific for D-glucose with negligible cross-reactivity. Here, the maximum rate of oxygen consumption is directly proportional to the glucose concentration. The required oxygen is abstracted from the solution, and the resultant change in oxygen amount is monitored by an oxygen sensor. It is important to note that the reaction is sufficiently rapid to avoid any significant interference from atmospheric oxygen. The analyser was calibrated before use at the beginning of the day with the use of the 8.0 mM glucose standard in saturated benzoic acid. Given a 10 µL plasma sample, this method assures linearity of the reaction to at least 30.0 mM (or 540 mg/dL).

Plasma glucose tracer ([3-^{3}H]-glucose) specific activity was determined for each point by deproteinzing a 50 µL plasma sample with a 1:1 ratio of $ZnSO_4$ and $Ba(OH)_2$, pelleting the precipitate via centrifugation (6 min @ 6000 rpm), and counting a 75 µL aliquot of the supernatant, after the samples were evaporated, with a scintillation counter (Beckman Coulter LS6500). Prior to counting the samples, 7.0 mL of scintillation fluid (Budget-Solve; Research Products International, Mount Prospect, IL) was added to each sample vial.

Radioimmunoassays (RIA) were used to determine the plasma concentrations of insulin, glucagon and leptin (kits RI-13K, GL-32K, and RL-83K; Linco Research, St. Charles, MO). A radioimmunoassay system measures the concentration of a particular protein/antigen with the use of a specific antiserum to the antigen, a radioactive labeled (^{125}I in these kits) form of the antigen (tracer) to compete with cold antigen in the plasma sample, a means to separate unbound tracer from antibody-bound tracer, and an instrument to count the radioactivity.

A two-day protocol was utilized to determine plasma insulin concentrations (in ηg/mL). A standard curve was determined using the provided insulin standards at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ηg/mL in duplicate. The ^{125}I-labelled insulin (50 µL) and the rat insulin antibody (50 µL) were added to these standards as well as the experimental plasma samples

(50 µL) followed by vortexing and an overnight incubation at 4°C. It is important to note that the kit also exhibits a 100% specificity/selectivity for porcine insulin, which was exogenously provided during the pancreatic clamps. On day two, 1.0 mL of precipitating reagent were added to each tube followed by vortexing and a 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 'bound') for the samples and standards were expressed as a percentage of the mean counts of the total binding reference tubes (*Bo*):

$$\%\, total\ binding = \%\frac{B}{Bo} = (sample\ or\ standard)\ \div Bo\ \times 100\%$$

The % activity bound for each standard was plotted against the known concentrations (0.1 to 10 ηg/mL) to construct the standard curve, and through interpolation, the plasma insulin concentrations of the experimental samples was determined.

A similar procedure was carried out for the determination of the plasma glucagon (in ρg/mL) and leptin (in ηg/mL) concentrations, except these were three-day protocols which involved an additional overnight incubation of the samples and standards at 4°C with the glucagon or leptin antibody *alone*, before the ^{125}I-glucagon or ^{125}I-leptin tracer was added. Additionally, the provided standards were 20, 50, 100, 200 and 400 ρg/mL for the glucagon assay, and 0.5, 1, 2, 5, 10, 20, 50 ηg/mL for the leptin assay.

Calculations and Statistical Analysis

The calculation used to find out the rate of glucose turnover (*Ra*; rate of appearance of glucose), as determined using [3-^{3}H]-glucose was calculated using steady state formulas (185), was as follows:

$$Ra = \frac{I}{SA},\ Ra = Rd$$

where *Ra* = *total rate of glucose appearance (endogenous + exogenous glucose)*
Rd = *total rate of glucose disappearance*
I = *constant rate of tracer infusion (μCi/min)*
SA = *specific activity.*

In the basal period, the total *Ra* corresponds to the rate of endogenous glucose production (*GP*), which is also equivalent to the rate of glucose disappearance (*Rd*) or utilization during steady state. Both the kidney and the liver are able to be sources of endogenous glucose. In this project, rodents were assessed during the post-absorptive state – during which GP from the liver accounts for nearly the entire value of *Ra*; however, during the fasted state, the kidney can account for 20-25% of the whole body glucose turnover (56). As such, the values for *hepatic* GP and *endogenous* GP can be considered to be the same for the studies of this thesis. During the pancreatic clamp period, there is an additional source of *Ra* in the form of an exogenous infusion of cold glucose (glucose infusion rate; *GIR*), and as such the value of the total *Ra* includes not only the *GP* but also *GIR* (*Rd* = *Ra* = *GP* + *GIR*). Thus, to obtain *GP* rates during the clamp, the value of *GIR* must be subtracted from the value of *Ra* that is determined using the above equation.

Statistical analysis was done by unpaired Student's *t* test. Data are presented as means ± SEM (standard error of the mean). The definitions of the time periods and the time points encompassed for the presented average values are specified in the methods of the specific studies.

5

STUDY I: CNS LACTATE SENSING IN METABOLIC DISEASE

(Adapted from Chari *et al. Diabetes* **56**:836-40, 2008.)

Abstract

Hypothalamic lactate metabolism lowers hepatic GP and plasma glucose levels in normal rodents. However, it remains unknown whether activation of hypothalamic lactate metabolism lowers GP and plasma glucose levels in rodents with diabetes and obesity. We performed i.c.v. (3rd ventricle) administration of lactate (5 mM) to enhance central lactate metabolism in (A) the early-onset of STZ-induced uncontrolled diabetic rodents, (B) experimentally induced-hypoinsulinemic normal rodents (via somatostatin infusion), and (C) the early-onset of diet-induced insulin resistant rodents. The tracer-dilution methodology was used to assess the impact of i.c.v. lactate on the rate of GP in all three models. We first report that in the absence of insulin treatment, i.c.v. lactate administration lowered GP (by ~25%) and glucose levels (by >4 mM) in rodents with uncontrolled diabetes. Second, i.c.v. lactate administration normalized and prevented a rise in GP and glucose levels in normal rodents with experimentally-induced hypoinsulinemia. Finally, i.c.v. lactate administration lowered the rate of GP (by >45%) in normal rodents that acquired insulin resistance after 3 days of high-fat feeding to an extent similar to that seen in regular chow-fed normal rodents. These data suggest that basal insulin signaling is not required for central lactate to lower GP, and the activation of hypothalamic lactate metabolism could consequently bypass insulin resistance and lower glucose levels in the early-onset of diabetes and obesity.

Methods

Early-onset Uncontrolled Diabetic Model

Uncontrolled diabetes was induced with a single intravenous (i.v.) injection of streptozotocin (STZ; Sigma, St. Louis, MO; 60 mg/kg), a potent diabetogenic agent that is cytolytic against pancreatic β–cells, dissolved in sterile saline at the end of the vascular surgery. The glycemic status in diabetic rats was monitored daily using a hand-held glucometer (Accu-Chek Compact Plus, Roche Diagnostics, Laval, QC); only rats that were sufficiently hyperglycemic (blood glucose >15 mM) were included in the diabetic studies. After 54 hr to 60 hr of post-STZ injections, the *in vivo* i.c.v. infusion experiments were carried out and lasted a total of 3 hr in rats that were limited to 20 g of standard chow the previous night. We infused i.c.v. vehicle (0.9% w/v NaCl) or i.c.v. 5 mM L-lactate throughout the experiments using a microdialysis pump (CMA 400 syringe pump). A primed-continuous infusion of [3-^{3}H]glucose (Perkin Elmer; 40 μCi bolus; 0.4 μCi/min thereafter) was initiated at 90 min and maintained through the study with the use of the Harvard PHD 2000 pump to assess glucose kinetics via the use of the tracer-dilution methodology. Plasma samples were collected in the final 0.5 hr (150 to 180 min) for determination of [3-^{3}H]-glucose specific activity as well as hormone levels.

Experimentally-Induced Hypoinsulinemic Model

These *in vivo* infusion experiments lasted a total of 270 min, and were carried out in normal rats that were limited to 20 g of standard chow the previous night. A primed-continuous infusion of [3-^{3}H]-glucose (Perkin Elmer; 40 μCi bolus; 0.4 μCi/min thereafter) was initiated at the start of the protocol along with somatostatin (Sigma, St. Louis, MO; 1 μg/kg·min). An infusion of i.c.v. saline or 5 mM lactate was initiated at 90 min and maintained till the end of the experiments. Plasma glucose level was monitored every 10 min throughout the

protocol. Plasma samples were collected throughout the protocol for the determination of [3-^{3}H]-glucose specific activity as well as hormone levels.

Diet-Induced Model of Insulin-Resistance

Animals were fed a high fat (lard-oil enriched) diet (catalog no. 9389; Purina Mills Inc.), that was generated by supplementing the standard chow with 10% lard, for three days. Sprague Dawley rats on maintained on this diet for 3 days rapidly develop insulin resistance (124; 205); this permits an examination of CNS nutrient sensing during the acute-onset of this disease, paralleling the preceding evaluation of CNS lactate sensing during the early-onset of uncontrolled diabetes. The composition of the high fat diet is 45% carbohydrate, 22% protein, 33% fat (vs. 52, 31, and 17%, respectively, for the regular chow). A separate set of hyperinsulinemic (insulin dosage: 3 mU/kg.min)-euglycemic clamp experiment was performed in this high-fat fed model to evaluate whether high fat diet induce insulin resistance (see below). The *in vivo* infusion experiments lasted a total of 4.5 hr. A primed-continuous infusion of [3-^{3}H]-glucose (Perkin Elmer; 40 μCi bolus; 0.4 μCi/min thereafter) was initiated at 0 min. We infused i.c.v. vehicle (0.9% w/v NaCl), or i.c.v. 5 mM L-lactate from time 90 min and onwards till 270 min. A basal pancreatic insulin clamp was performed in the final 2hr (150 to 270 min) of the study: A continuous co-infusion of insulin (0.8 mU/kg·min) along with somatostatin (3 μg/kg·min) was administered, and a variable infusion of 25% glucose solution was administered as needed to clamp and maintain the plasma glucose concentration at levels similar to the basal state. Plasma samples were obtained at 10-min intervals for determination of [3-^{3}H]-glucose-specific activity as well as plasma insulin, glucagon and leptin levels.

Hyperinsulinemic-Euglycemic Pancreatic Clamp

A separate set of hyperinsulinemic-euglycemic clamp experiments was performed in a subset of rodents from both the regular chow-fed and the high-fat-fed model to evaluate whether the high-fat diet induces insulin resistance, as indicated by an impaired ability of hyperinsulinemia to suppress GP from the basal rate. These *in vivo* infusion experiments lasted a total of 3.5 h. A primed-continuous infusion of [3-^{3}H]-glucose (Perkin Elmer; 40 µCi bolus; 0.4 µCi/min thereafter) was initiated at 0 min and maintained throughout the study. At 90 min, a hyperinsulinemic-eugclyemic clamp was performed for the final 2hr (90 to 210 min) of the study, where insulin was infused at 3 mU·kg^{-1}·min^{-1} along with somatostatin (3 µg/kg·min). With this dose, plasma insulin concentration was raised from 0.71±0.15 ηg/mL (basal levels) to 1.981±0.28 ηg/mL (during the final 30 min of the clamp). A variable rate of 25% glucose was infused during the clamp to maintain plasma glucose concentration at levels similar to basal. Plasma samples were obtained at 10-min intervals for determination of [3-^{3}H]-glucose-specific activity as well as plasma insulin, glucagon and leptin levels.

Calculations

Statistical analysis was done by unpaired Student's *t* test. Data are presented as means ± SEM (standard error of the mean). The final 30 minutes of the experiments were averaged for the "i.c.v." time period and used for the calculation of plasma glucose, insulin and glucagon levels and GP rates presented in the Figures.

Results

First, we established an early-onset uncontrolled diabetic model with an i.v. injection of streptozotocin (STZ; 60 mg/kg); within 30 h of the STZ injection, the blood glucose level was elevated to at least 15 mM. After exposure to elevated glucose levels for 24-30 h, peripheral glucose metabolism was analyzed during a 3 h i.c.v. administration procedure (**Fig. 1b**). Via the use of tracer-dilution methodology, GP in i.c.v. saline-treated STZ rats was determined to be elevated to 22.7±1.3 mg/kg·min (**Fig. 2a**) – approximately twice the rate observed in normal rats 11.8±0.3 mg/kg·min) – resulting in a correspondingly elevated post-prandial plasma glucose level of 22.7±2.6 mM at the start of the i.c.v. infusion procedure. These elevations occurred in the presence of very low plasma insulin (**Fig. 2c**) but normal glucagon levels (**Fig. 2d**).

The effects of i.c.v. glucose and i.c.v. lactate at doses (2 and 5 mM, respectively, at a rate of 5 μL/h) that have been previously shown (103) to lower GP and plasma glucose level in normal rats were then evaluated in this uncontrolled diabetic model. While an acute elevation of central glucose failed to lower GP in diabetes (this impairment will be the focus of Study II), this was not the case with the i.c.v. administration of lactate. Interestingly, an acute elevation of central lactate resulted in a decrease in GP by ~6 mg/kg·min (**Fig. 2a**) which in turn led to a 4.3±0.8 mM drop (or 24.7±3.6 %) in plasma glucose (**Fig. 2b**). These data suggest that there is a loss of CNS glucose sensing in diabetes which lies upstream of lactate metabolism – this loss will be further examined later in Aim II of the project.

As STZ-induced uncontrolled diabetes is chiefly characterized by not only marked hyperglycemia but also hypoinsulinemia, the GP-lowering effects of central lactate were further evaluated in an experimentally-induced model of selective hypoinsulinemia. To generate this alternative model, normal rats were administered somatostatin for 4.5 h (**Fig. 3a**) at a dosage of 1 μg/kg·min, which resulted in a selective decrease (50%) in plasma

insulin levels (**Fig. 3d**); plasma glucagon levels (**Fig. 3e**) were unchanged. This resulted in an elevation of GP to 15.8±1.4 mg/kg·min and plasma glucose levels to 10.8±0.3 mM by 180-200 minutes into the procedure. By the end of the infusion procedure (**Fig. 3a**), GP and plasma glucose levels remained elevated in the hypoinsulinemic rodents receiving i.c.v. saline administration (15.6±1.1kg·min and 10.9±0.5 mM, respectively). In contrast, the administration of i.c.v. lactate prevented the rise in GP (10.2±2.1 mg/kg.min) (**Fig. 3c**) and plasma glucose levels (7.3±0.6 mM) (**Fig. 3b**) throughout the infusion protocol.

Together with the findings of the uncontrolled diabetic model, the above data indicate that basal insulin action is not required for central lactate metabolism to lower glucose levels by means of negatively regulating GP. Thus, it is highly likely that the activation of central lactate metabolism could bypass insulin resistance and lower GP.

To directly test this hypothesis, the metabolic effects of i.c.v. lactate were tested in an early-onset high fat diet-induced insulin resistant model (**Fig. 4**). In this highly established model (124; 205) rodents fed on a lard oil-enriched diet for merely three days rapidly increase their caloric consumption (**Fig. 4a**), develop hepatic insulin resistance (as determined by hyperinsulinemic-euglycemic clamps; **Fig. 4d**), and are hyperinsulinemic and hyperleptinemic (**Fig. 4b,c**) compared to rodents maintained on the standard chow. In this insulin resistant model, i.c.v. lactate administration lowered GP (from 13.2± 0.8 to 7.1± 0.4 kg·min) during the pancreatic basal insulin clamp procedure (**Fig. 5a**) – importantly, this metabolic effect of lactate i.c.v. was comparable to that in regular chow-fed rats (**Fig. 5a**). These data indicate that the acute elevation of central lactate and its subsequent metabolism was able to bypass insulin resistance in order to lower GP in the diet-induced insulin resistant model.

Taken together, we postulate that therapeutic strategy designed to activate hypothalamic lactate metabolism could bypass the diminished insulin effectiveness

observed in hypoinsulinemia and diet-induced insulin resistance and serve as one of the complimentary approaches to insulin injections to lower glucose levels in diabetes and obesity. The importance behind the results thus far of this Aim does not lie in the possibility of lactate administration as a therapeutic intervention; rather, these results pinpoint the central metabolic defect as lying upstream of lactate utilization, which facilitates the identification of molecular targets that can in turn be targeted in the design of novel therapeutics for use in diabetic and/or obese population.

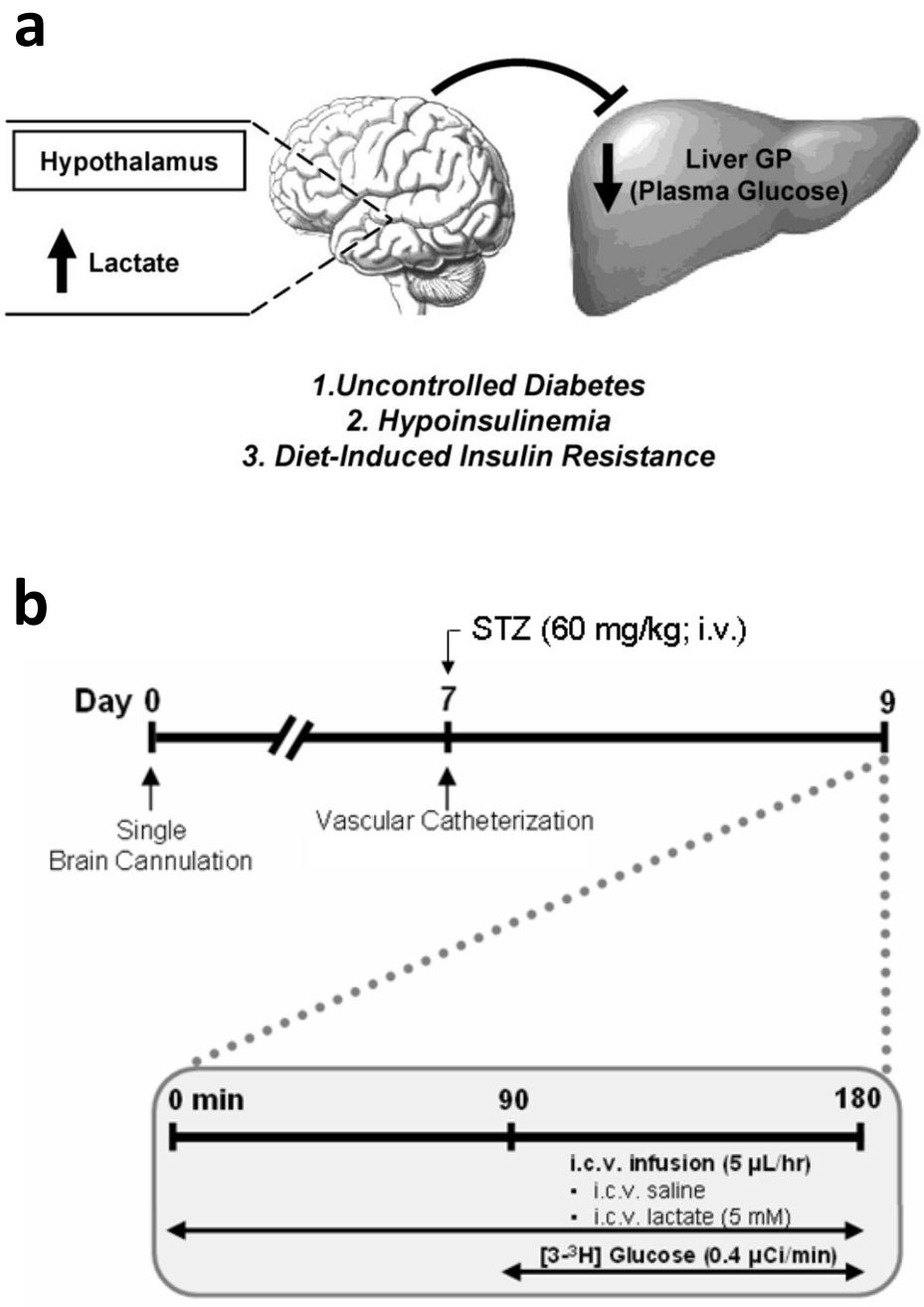

Figure 1. Schematic representation of Study 1 and experimental protocol for STZ-diabetic Rodents. (**a**) A schematic representation of the working hypothesis and study summary. Activation of hypothalamic lactate metabolism lowers GP and plasma glucose in the early-onset of uncontrolled diabetes, experimentally-induced selective hypoinsulinemia, and diet-induced insulin resistance. (**b**) Experimental protocol of i.c.v. infusion in normal and STZ-diabetic rats, whereby the effects of i.c.v. lactate (or vehicle saline) on *in vivo* glucose fluxes were determined using the tracer (^{3}H-glucose)-dilution methodology.

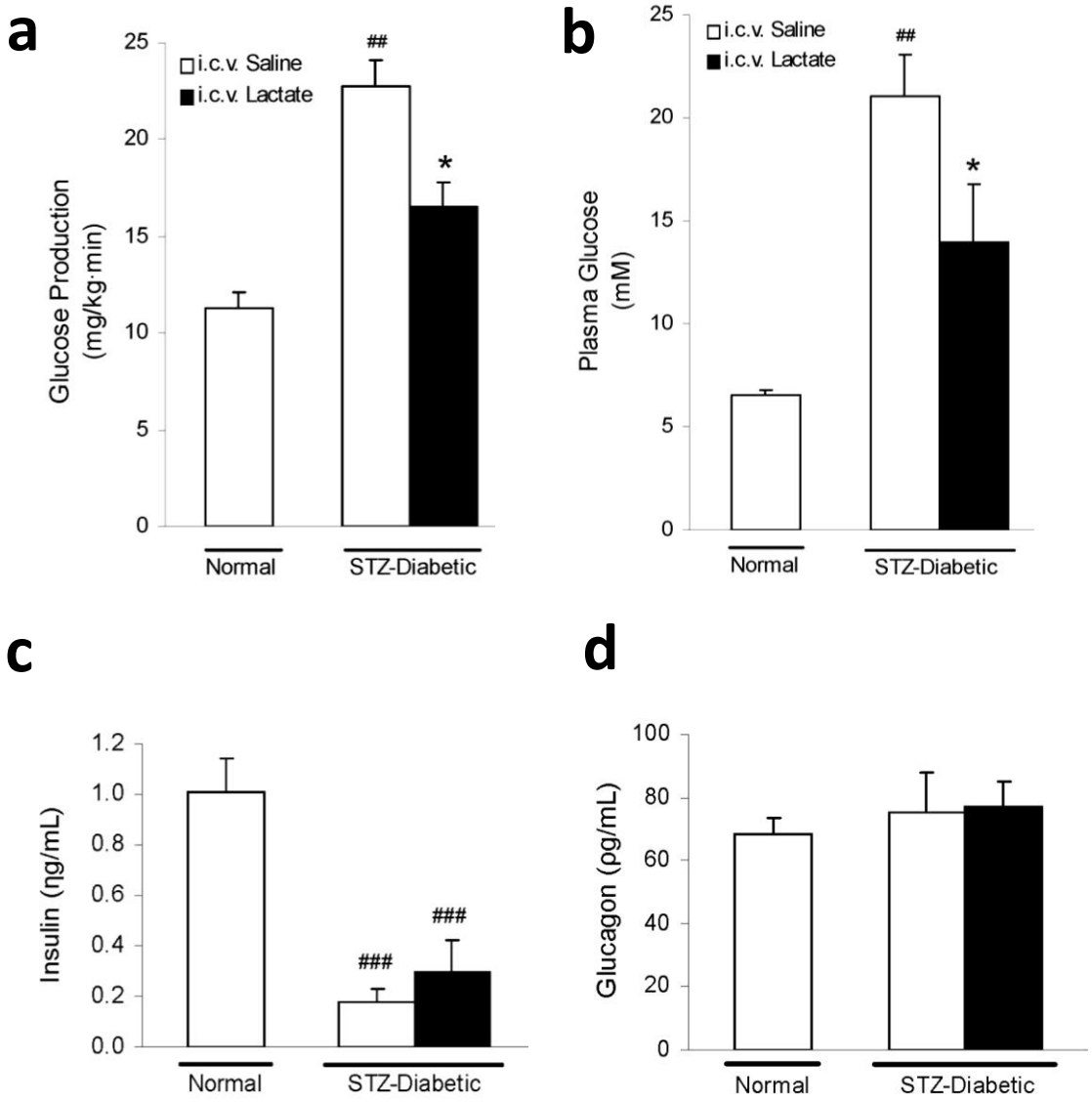

Figure 2. Activation of Central Lactate metabolism lowers Glucose Production and Plasma Glucose levels in Uncontrolled Diabetes. (**a**) STZ injection increased glucose production to ~22 mg/kg.min in i.c.v. saline ($^{\#\#}P$<0.01 vs. normal n=12) treated diabetic rodents (n=6). Administration of i.c.v. lactate lowered glucose production (*P<0.05 vs. i.c.v. saline-STZ) in uncontrolled diabetic rodents (n=6). (**b**) This suppression in glucose production led to a significant drop (~25%; *P<0.05) in plasma glucose levels by the end of the 3h i.c.v. lactate-infusion period vs. saline vehicle-infused diabetic rodents. (**c**) STZ injection lowered plasma insulin levels in i.c.v. saline- or lactate-treated STZ rats ($^{\#\#\#}P$<0.001 vs. normal). (**d**) Glucagon level was comparable in all groups.

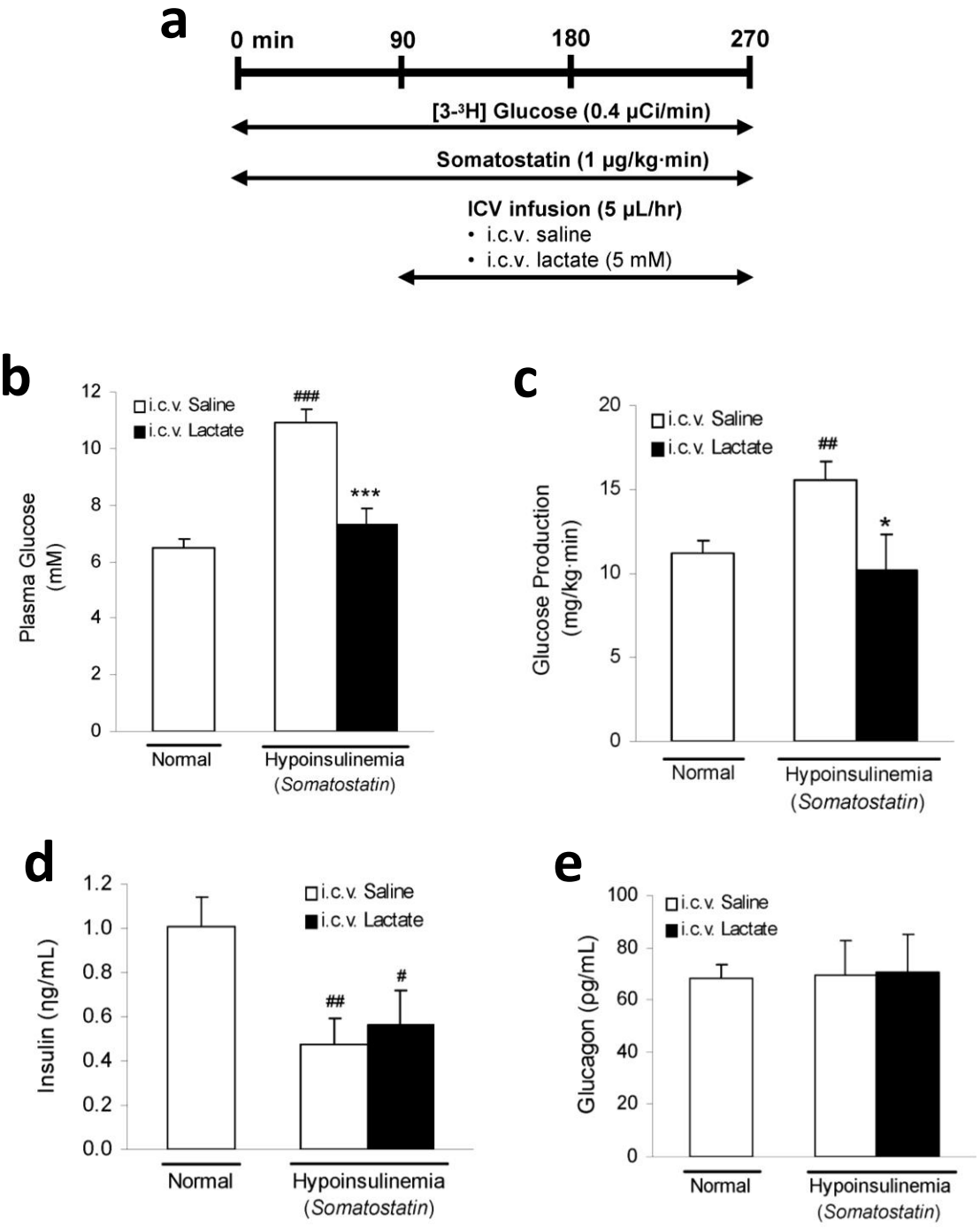

Figure 3. Activation of Central Lactate metabolism lowers Glucose Production and Plasma Glucose in experimentally-induced Hypoinsulinemia. (**a**) Experimental protocol of i.c.v. infusion in normal or hypoinsulinemic rats. Administration of i.v. somatostatin at the indicated dose with i.c.v. saline (n=6) increased (**b**) plasma glucose levels ($^{\#\#}P<0.01$ vs. i.c.v. saline-normal) and (**c**) GP ($^{\#}P<0.05$ vs. i.c.v. saline-normal), and lowered (**d**) insulin levels ($^{\#}P<0.05$ vs. i.c.v. saline-normal rats). (**e**) Administration of i.v. somatostatin with i.c.v. lactate (n=6) prevented the rise in (**b**) plasma glucose levels ($^{***}P<0.001$ vs. i.c.v. saline-hypoinsulinemic rats) and (**c**) glucose production ($^{*}P<0.05$ vs. i.c.v. saline-hypoinsulinemic rats) in the presence of low (**d**) insulin level ($^{\#}P<0.05$ vs. i.c.v. saline-normal rats). (**e**) Glucagon level was comparable in all groups.

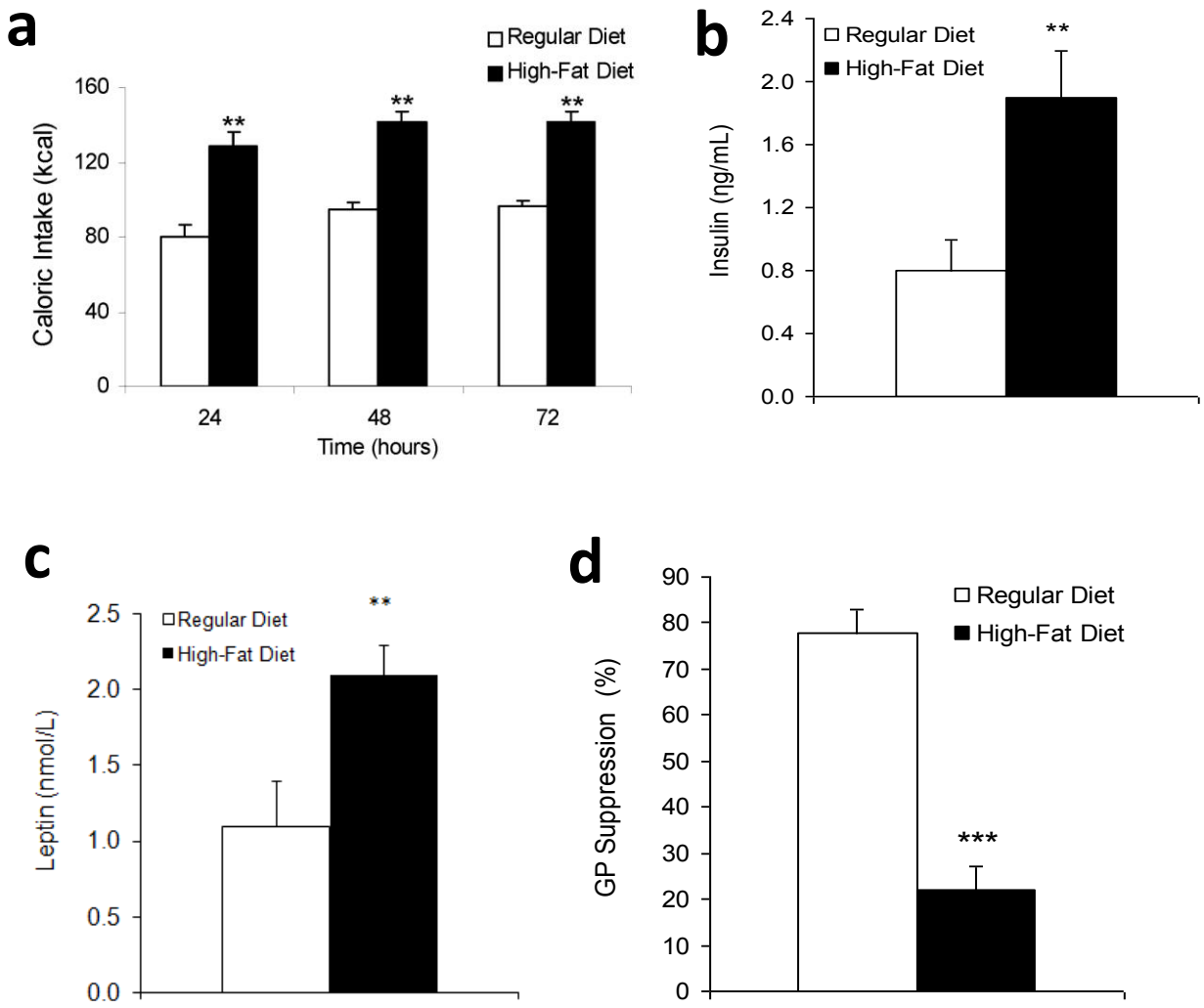

Figure 4. Metabolic characteristics of the high-fat diet-induced Insulin Resistant Rodents. (**a**) Over the course of the 3-day high-fat diet, Normal male SD rats rapidly develop a marked hypercaloric intake (n=5) vs. those on the regular chow diet (n=5), which ate ~85 kcal/day (***P*<0.01 vs. regular diet). By the end of the high-fat diet, the rodents are (**b**) hyperinsulinemic as well as (**c**) hyperleptinemic (***P*<0.01 vs. regular chow-fed rats). (**d**) A separate set of hyperinsulinemic-euglycemic clamp experiments were conducted (3 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin dosage) to evaluate whether this high-fat diet induced insulin resistance. By the final 30 min of the clamp, the ability of acute exogenous hyperinsulinemia to suppress hepatic glucose production was severely blunted in rodents on the high-fat diet (75±5% vs 23±3 %; ***P*<0.01 vs. regular chow-fed rats).

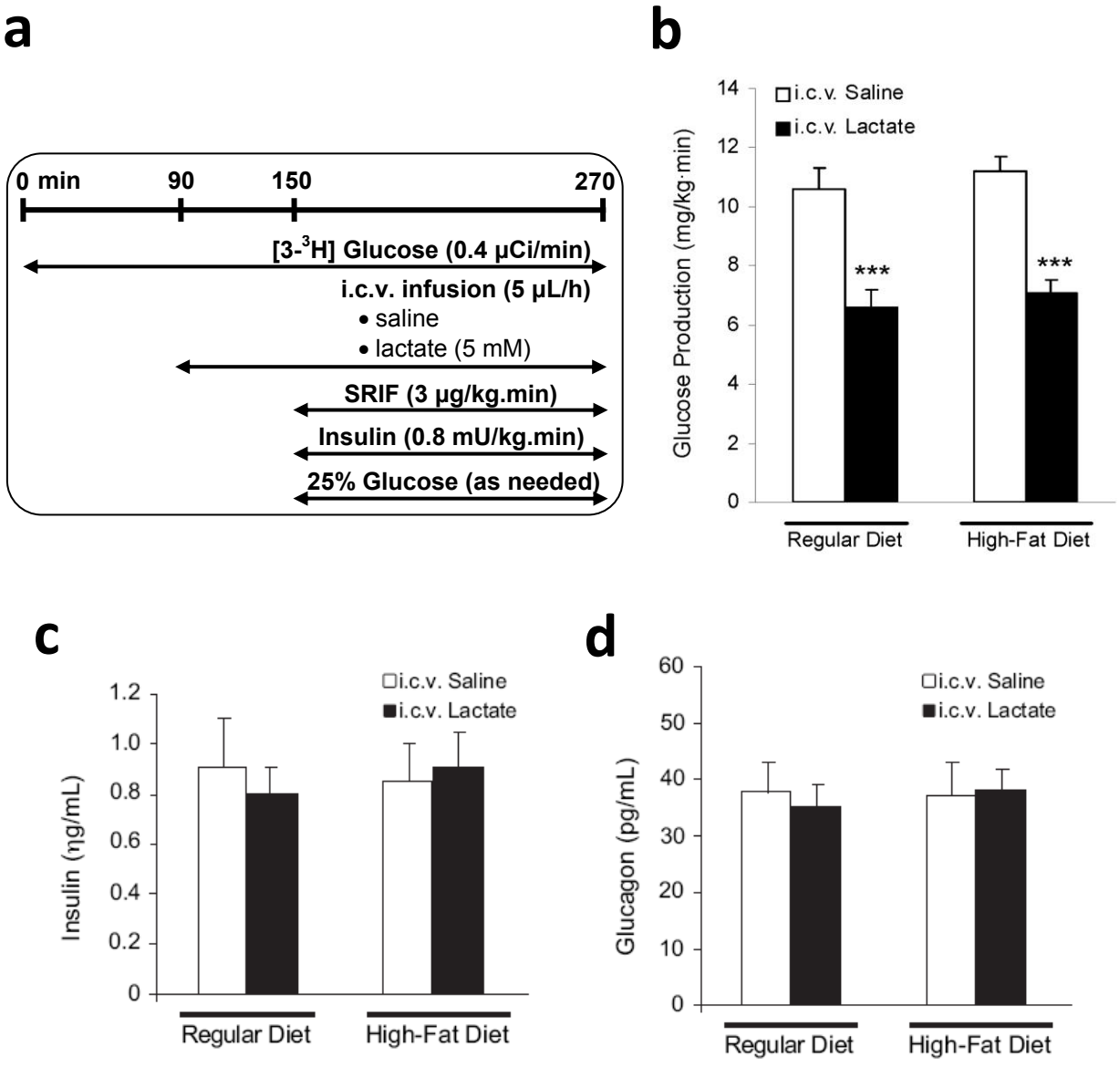

Figure 5. Activation of Central Lactate metabolism lowers Glucose Production in the high fat diet-induced Insulin Resistant Model. (**a**) The effects of central lactate (or vehicle control saline) on glucose production was evaluated using the tracer-dilution methodology in conjunction with the pancreatic (basal insulin) euglycemic clamp. (**b**) Administration of i.c.v. lactate (n=5) lowered glucose production in the high fat fed rats (***P<0.001 vs. i.c.v. saline-high fat diet, n=5) to the same extent as in the regular chow-fed rats (***P<0.001 vs. i.c.v. saline-regular diet, n=6 each). (**c**) Insulin levels were comparable in all groups during the clamp. Glucagon levels (**d**) were comparable in all groups during the clamp. Somatostatin, SRIF.

6

STUDY II: GLUCOTOXICITY AND CNS GLUCOSE SENSING

Abstract

Increases in hypothalamic glucose and its subsequent metabolism to lactate lower plasma glucose levels by inhibiting GP in normal rodents. We here begin to test the hypothesis that hypothalamic glucotoxicity impairs glucose sensing to lower GP in diabetes. First, peripheral glucose metabolism was analyzed in STZ-diabetic rodents with the use of a pancreatic (basal insulin) euglycemic clamp coupled to the tracer-dilution methodology, during a concurrent i.c.v. glucose (2 mM) infusion. The infusion of i.c.v. glucose lowered GP in normal rodents, confirming previous finding. However, this acute administration of central glucose, unlike lactate (Study 1), failed to lower GP in STZ-uncontrolled diabetes. To begin assessing whether glucotoxicity per se impairs hypothalamic glucose sensing, two models with selective hyperglycemia were tested. Hypothalamic glucose sensing was assessed in normal rats which had received (A) a 24 h exposure to increased circulating blood glucose levels (~20 mM) by means of direct i.v. glucose (37.5%) infusion or (B) a 24 h exposure to increased hypothalamic glucose levels via direct i.h. glucose (4 mM) administration or (C) vehicle control infusions. We found that i.h. glucose (2 mM) lowered GP in the rodents receiving 24 h i.h./i.v. saline-infusions but not the glucose-infusions, suggesting that whole-body as well as hypothalamic glucotoxicity are sufficient to inhibit CNS glucose sensing mechanisms. To determine whether glucotoxicity *per se* is responsible for the impaired hypothalamic glucose sensing in diabetes, STZ-diabetic rodents were treated with i.v. phlorizin, an inhibitor of renal glucose reabsorption, for 24 hours to normalize plasma glucose levels and prevent the hyperglycemic exposure. In these STZ+phlorizin rodents, lowering glucose levels *per se* in diabetes rescued the effect of i.h. glucose, suppressing GP (by ~40%). Together, these data suggest glucotoxicity as a means by which central/hypothalamic glucose effectiveness is disrupted to lower GP in uncontrolled diabetes.

Methods

Pancreatic (Basal Insulin) Euglycemic Clamps

We infused i.c.v./i.h. (as indicated) vehicle (0.9% w/v NaCl) or i.c.v/i.h. (as indicated) 2 mM glucose throughout the experiments. A primed-continuous infusion of [3-^{3}H]glucose (Perkin Elmer; 40 μCi bolus; 0.4 μCi/min thereafter) was initiated at 0 min and maintained through the study to assess glucose kinetics via the use of the tracer-dilution methodology. Ninety minutes after the basal period, a basal pancreatic insulin clamp was performed in the final 2hr (90 – 210 min) of the study: A continuous co-infusion of insulin (0.8 mU/kg·min) along with somatostatin (3 μg/kg·min) was administered, and a variable infusion of 25% glucose solution was administered as needed to clamp and maintain the plasma glucose concentration at levels similar to the basal state. Plasma samples were collected every 10 minutes for determination of [^{3}H]glucose specific activity as well as hormone levels (see **Table 1** and **Table 2** for insulin and glucagon levels, respectively, during the procedure across all Study 2 groups).

Early-onset Uncontrolled Diabetic Model

Uncontrolled diabetes was induced with a single intravenous (i.v.) injection of streptozotocin (STZ; Sigma, St. Louis, MO; 60 mg/kg), a potent diabetogenic agent that is cytolytic against pancreatic β–cells, dissolved in sterile saline at the end of the vascular surgery. The glycemic status in diabetic rats was monitored daily using a hand-held glucometer (Accu-Chek Compact Plus, Roche Diagnostics, Laval, QC); only rats that were sufficiently hyperglycemic (blood glucose >15 mM) were included in the diabetic studies. After 54 hr to 60 hr of post-STZ injections, the *in vivo* i.c.v. infusion experiments were carried out and lasted a total of 3.5 hr in rats that were limited to 20 g of standard chow the previous night (see above for clamp description).

Whole-Body Glucotoxic Model

To induce whole-body hyperglycemia and the resultant glucotoxicity, normal SD rats received a 24 hr infusion of i.v. glucose. A solution of 37.5% glucose was made and infused from a 60 cc syringe into the externalized i.v. catheter at 2 ml/hr for a total of 24 hr using a Harvard PHD 2000 syringe pump. To protect the exteriorized polyethylene catheter from being chewed by the rodent during the course of the infusion, the catheter was fed through a 12" stainless steel spring-coil tether (RT-12; Braintree Scientific, Braintree, MA) and was secured to the rodent in a saddle-style Velcro rodent jacket (RJ-M, Braintree Scientific). This infusion apparatus was out of reach to the rodent and still permitted the rodents to be mobile, unrestrained and have free access to food and water. As a control, normal rodents received a 24 hr i.v. infusion of vehicle saline (0.9% w/v NaCl). The next morning, after the 24 hr infusion period, a 1.0 mL blood sample was obtained from the intracarotid catheter for plasma glucose and hormone analyses – here referred to as the "pre-basal" time point. The i.v. glucose infusion was then terminated at this point, and the plasma glucose levels were allowed to normalize (this took approximately 1.5 hr) prior to the subsequent *in vivo* analyses via the pancreatic euglycemic clamp (see above), where the effects of i.h. glucose (2 mM) on glucose fluxes were assessed.

Hypothalamic Glucotoxic Model

To induce selective, hypothalamic hyperglycemia and the resultant glucotoxicity, normal SD rats received a 24 hr infusion of i.h. glucose. A solution of 4 mM glucose was made and infused from a 50 uL Hamilton glass syringe into the externalized bilateral i.h. catheter at 0.33 µl/hr for a total of 24 hr using a microdialysis pump. The overnight infusion apparatus was setup as described above for the whole-body glucotoxic, i.v. glucose-infused model. As a control, normal rodents received a 24 hr i.h. infusion of vehicle saline (0.9% w/v NaCl). The i.h. glucose infusion was terminated the next day after 24 hr, and, to keep the experimental

time course consistent with the whole-body glucotoxicity studies, the pancreatic euglycemic clamp was commenced 1.5 hr after this termination. During the pancreatic euglycemic clamp, the effects of i.h. glucose – at 2 mM – were evaluated.

Phlorizin-Treated Uncontrolled Diabetic Model

Uncontrolled diabetes was induced with a single intravenous (i.v.) injection of streptozotocin (STZ; Sigma, St. Louis, MO; 60 mg/kg), dissolved in sterile saline, at the end of the vascular surgery. The glycemic status in diabetic rats was monitored daily using a hand-held glucometer (Accu-Chek Compact Plus, Roche Diagnostics, Laval, QC); only rats that were sufficiently hyperglycemic (blood glucose >15 mM) were included in the diabetic studies. After ~24 hr of hyperglycemia, the STZ-diabetic rodents of this group were administered a continuous infusion of i.v. phlorizin.

Phlorizin is a naturally occurring product that is found in a number of fruit trees (55). The primarily pharmacological action of phlorizin is to produce renal glucosuria and block intestinal glucose absorption via the inhibition of the sodium/glucose cotransporters in the proximal renal tubule and the mucosa of the small intestine, respectively (55). This compound has previously been used experimentally to increase glucose clearance and lower plasma glucose levels.

To make the phlorizin solution, phlorizin dihydrate (Sigma-Aldrich, St. Louis, MO) was gradually dissolved in hot, near-boiling 20% propylene glycol (PG; Sigma) at a concentration of 5 mg/mL. The solution was freshly made for each treatment, and was allowed to cool to room temperature before being administered to the rodent. Phlorizin was given at a rate of 2.5 µl/min (12.5 µg/min) i.v. to continuously normalize glucose levels overnight and throughout the pancreatic clamp procedure outlined earlier, where the effects of 2 mM glucose were evaluated.

Calculations

Statistical analysis was done by unpaired Student's *t* test. Data are presented as means ± SEM (standard error of the mean). The presented "basal" values for glucose concentration and rates of GP and utilization are averages for the 60 through 90 minutes time points, while those for the "clamp" period are the averages for the final 30 minutes (180 – 210 min) of the procedure.

Results

In the presence of marked hypoinsulinemia (**Table 1**) and unchanged glucagon levels (**Table 2**) when compared to normal rodents, the basal GP was elevated to ~23 mg/kg·min in STZ-diabetic rodents (**Fig. 2a**). Despite the replacement of basal insulin levels during the clamp (**Fig. 1b**), an acute increase in central glucose was unable to suppress the rate of GP in diabetic rodents, as GP (18.2±3.2 mg/kg·min) remained elevated to a similar extent as the i.c.v. saline-infused STZ-diabetic rodents (18.8±2.4 mg/kg·min) during the clamp (**Fig. 2a**). When comparing the basal and clamp GP rates, the infusion of i.c.v. glucose was able to suppress GP by 53.6±10.5 % in normal rodents (**Fig. 2a**), thus requiring an exogenous glucose infusion rate (GIR) of 7.1±1.8 mg/kg·min during the clamp to maintain euglycemia (**Fig. 2b**). In line with previous studies (103), this central nutrient accumulation did not affect the rate of glucose disappearance (Rd) (**Fig. 2c**), thus confirming that this homeostatic effect is specific to the regulation of GP and not glucose utilization or clearance.

Thus, it is evident that the central glucose sensing mechanisms to lower GP are defective in uncontrolled diabetes. The molecular defect likely lies upstream of lactate uptake and metabolism, as data from the previous aim suggest that the central lactate sensing mechanism is preserved in diabetes. We then began to test the hypothesis that hypothalamic glucotoxicity (i.e. hyperglycemia *per se*) is responsible for this impairment of central glucose sensing to lower GP in diabetes

As outlined earlier, this concept of glucotoxicity in relation to hypothalamic nutrient (glucose) sensing was to be evaluated in multiple models. Acute STZ-induced uncontrolled diabetes is chiefly characterized by hyperglycemia and hypoinsulinemia, so to assess the effects of glucotoxicity on central glucose sensing it is necessary to evaluate models of selective hyperglycemia without any potential confounding effects of insulin deficiency on central glucose sensing – specifically hypothalamic glucose sensing from here on in.

First, we infused 37.5% glucose intravenously into normal rats at a dose to selectively raise plasma glucose levels. This continuous glucose infusion was then maintained for 24 hours (**Fig. 3a,** model 1) to mimic the hyperglycemic insult endured by the uncontrolled diabetic rodents. Immediately after the end of the 24 h period, the i.v. glucose-infused rodents had elevated glycemia similar to that of STZ-diabetic rodents (20.2±1.1 vs. 22.7±2.6 mM, **Fig. 3c**) and this glucose challenge had stimulated marked hyperinsulinemia (8.4±0.4 vs. 0.63±0.12 ηg/mL in normal rodents). There were no significant differences in glucagon levels (**Table 2**). Prior to the pancreatic clamp procedure, the glucose infusion was stopped and the plasma glucose levels were allowed to normalize, which occurred within 1-1.5 hours. Despite the normal plasma glucose levels, these rodents still had residual hyperinsulinemia at the start of the 210-min pancreatic clamp protocol (**Fig. 3b**). These 24 h i.v. glucose-infused normal rodents had an elevated basal GP ~18 mg/kg·min (**Fig. 4a**). Despite the infusion of i.h. glucose to increase hypothalamic glucose levels, these normoglycemic rodents did not undergo the suppression in GP (to 4.9±1.0 mg/kg·min) seen in their 24-hour vehicle-infused counterparts (**Fig. 4a**); rather, their clamp GP remained elevated at 14.6±1.9 mg/kg·min, similar to the 24-hour i.v. glucose-infused group receiving i.h. saline during the procedure (14.8±1.2 mg/kg·min). Thus, the whole-body glucotoxicity induced by infusing i.v. glucose prior to the clamp procedure was able to inhibit the ability of an acute hypothalamic glucose elevation to lower GP in normal rodents.

Although the aim of the i.v. glucose infusion was to induce whole-body hyperglycemia *per se,* these rodents were quite clearly hyperinsulinemic and likely insulin resistant as well. As such, it was important to evaluate hypothalamic glucose sensing in a true alternative model of glucotoxicity. In order to confine the effects of hyperglycemia even further, another group of rodents followed a similar experimental timeline, but instead received an intrahypothalamic (i.h.) as opposed to an intravenous infusion of glucose for 24 hours (at 4 mM) prior to undergoing the clamp protocol (**Fig. 3a,** model 2) to induce hypothalamic glucotoxicity. This was dose was selected on the basis of a dose-

response curve which showed that increasing plasma glucose levels to ~22 mM can achieve the same increase in hypothalamic glucose as a 4 mM central glucose infusate (102). Although this group did not display elevated plasma glucose levels like the i.v. glucose-infused rodents (**Fig. 3c**), the i.h. glucose infusion was still terminated 1.5 hours prior to the start of the pancreatic clamp protocol to keep the experimental timeline consistent (**Fig. 3b**). This hypothalamic glucose challenge did not significantly alter plasma insulin or glucagon levels versus normal rodents (**Table 2**). Although the hypothalamic glucotoxic rodents had similar a basal GP rate (**Fig. 4a**) as the normal rodents ("normal" group pools 24 h i.v. and i.h. saline-infused rodents, along with non-infused rodents), the clamp GP of hypothalamic glucotoxic rodents was at 8.9±1.0 mg/kg·min, thereby failing to display the strong suppression (57±10.5 %) seen in the normal group (**Fig. 4a**). Once again, there were no significant changes in glucose utilization/clearance rates between acute i.h. saline and i.h. glucose-infused rodents across all of the groups (**Fig. 4b**).

Although the whole-body and hypothalamic glucotoxicity models displayed normal plasma glucose levels, in both of those models hypothalamic glucose sensing to regulate GP was disrupted. Particularly with the use of the hypothalamic hyperglycemic model, where the 24 h glucose infusion induced selective glucotoxicity, we have demonstrated that hyperglycemia *per se* is sufficient to impair the acute effects of hypothalamic glucose-sensing to lower GP (**Fig. 3a**). To fully implicate glucotoxicity as a causative agent in this impairment, we investigated if the normalization of glycemia in uncontrolled diabetes could rescue hypothalamic glucose sensing to lower GP (**Fig. 5a**).

In order to do this, we looked into the use of phlorizin, an inhibitor of the renal sodium/glucose cotransporter that has previously been used experimentally to increase glucose clearance. However, our use of subcutaneous bolus treatments as per this study resulted in short-term (<3 h) normalization followed by a rebound of hyperglycemia (unpublished observations). So, a continuous infusion protocol was designed to establish

and maintain normoglycemia in diabetic rodents for 24 hours (**Fig. 6a,c**) when compared to STZ-diabetic rodents receiving the vehicle infusion of 20% propylene glycol (PG). To achieve this, rodents receiving STZ after the vascular catheterization were placed on the i.v. phlorizin treatment upon first observation of hyperglycemia (> 18 mM), and maintained normoglycemia up to and through the subsequent pancreatic clamp protocol one day later (**Fig. 5b**).

When compared to untreated STZ-diabetic rodents (pooling of STZ-diabetic rodents receiving the vehicle (VEH) infusion as well as non-infused rats), phlorizin-treated STZ-diabetic (STZ+Phlorizin) rodents were similarly hypoinsulinemic (**Fig. 6b**), but were normoglycemic (**Fig. 6c**) at the start of the 210 minute pancreatic clamp protocol. Untreated STZ-diabetic rodents receiving i.h. saline had a basal GP of 23.0±1.2 mg/kg·min, and during basal insulin replacement had a clamp GP of 19.0±2.2 mg/kg·min (**Fig. 7a**), representing a baseline suppression of 23.1±7.4 % (**Fig. 7b**). A similar story is seen with untreated STZ-diabetic rodents receiving i.h. glucose, as there was no additional suppression of the GP in a clamp setting like that seen in normal rodents (**Fig. 7a**). STZ + Phlorizin rats receiving an i.h. saline infusion had a basal GP of 13.3±0.9 mg/kg·min, which was suppressed by 24.4±4.4 % (**Fig. 7b**) to 9.8 mg/kg·min (**Fig. 7a**) during the basal insulin clamp. Unlike the case with untreated STZ-diabetic rodents, the infusion of i.h. glucose suppressed GP from 10.6±1.1 to 6.1±0.9 mg/kg·min (**Fig. 7a**) during the clamp, corresponding to a 48.3± 6.7 % decrease (**Fig. 7b**). The rates of glucose disappearance (**Fig. 7c**) during the clamp remain unchanged between i.h. saline and glucose treatments across all groups. Thus, the lowering of plasma glucose levels *per se* in diabetes plays a critical part in the restoration of central and/or hypothalamic glucose sensing to regulate GP (**Fig. 6a**).

Collectively, these multiple approaches of assessing the impairment and rescuing of hypothalamic glucose sensing in models of uncontrolled diabetes (with and without glycemic control) and whole-body or hypothalamic glucotoxicity demonstrate that the insult

of hyperglycemia *per se* is sufficient to impair the inhibitory effects of hypothalamic glucose on GP. The preservation of central lactate's, but loss of central glucose's, ability to regulate GP in diabetes is indicative of an impairment that lies upstream of lactate uptake and metabolism. Additional studies, which remain outside the overall scope of this thesis, are absolutely necessary to determine the glucotoxicity-induced cellular and/or molecular defect(s) that underlies the loss in hypothalamic glucose sensing to lower GP.

Table 1 – Plasma levels of Insulin (ηg/mL) during Study 2 Pancreatic Clamps

	Start	Basal	Clamp
Normal (n = 20)	0.67 ± 0.14	0.73 ± 0.07	0.66 ± 0.07
STZ-Diabetic (n = 16)	0.18 ± 0.04***	0.13 ± 0.05***	0.73 ± 0.06
24 hr i.v. glucose (n = 14)	2.41 ± 0.05#	1.42 ± 0.22#	0.70 ± 0.06
24 hr i.h. glucose (n = 11)	0.64 ± 0.07	0.74 ± 0.09	0.68 ± 0.10
STZ + Phlorizin (n = 15)	0.19 ± 0.05***	0.19 ± 0.05***	0.73 ± 0.09

Data are means ± SEM.

Start: t=0 min (of clamp). Basal: average of t=60-90 min. Clamp: average of t=180-210 min.

'Normal' pools 1) Normal (n=12), 2) 24h i.v. (n=4) and 3) 24 i.h. (n=4) saline-infused rodents.

'STZ-Diabetic' pools 1) STZ-Diabetic (n=11) and 2) STZ+PG (n=5).

***P < 0.001 vs. Normal, #P < 0.05 vs. Normal (Student's *t* test).

Table 2 – Plasma levels of Glucagon (ρg/mL) during Study 2 Pancreatic Clamps

	Start	Basal	Clamp
Normal (n = 20)	69.58 ± 7.70	66.60 ± 9.69	37.11 ± 5.95
STZ-Diabetic (n = 16)	68.19 ± 9.74	61.89 ± 5.33	42.13 ± 1.68
24 hr i.v. glucose (n = 14)	53.37 ± 6.30	56.35 ± 2.23	39.75 ± 1.53
24 hr i.h. glucose (n = 11)	60.09 ± 4.46	56.32 ± 2.53	41.75 ± 2.46
STZ + Phlorizin (n = 15)	68.41 ± 10.46	66.59 ± 9.69	36.73 ± 7.09

Data are means ± SEM.

Start: t=0 min (of clamp). Basal: average of t=60-90 min. Clamp: average of t=180-210 min.

'Normal' pools 1) Normal (n=12), 2) 24h i.v. (n=4) and 3) 24 i.h. (n=4) saline-infused rodents.

'STZ-Diabetic' pools 1) STZ-Diabetic (n=11) and 2) STZ+PG (n=5).

Start: t=0 min. Basal: average of t=60-90 min. Clamp: average of t=180-210 min.

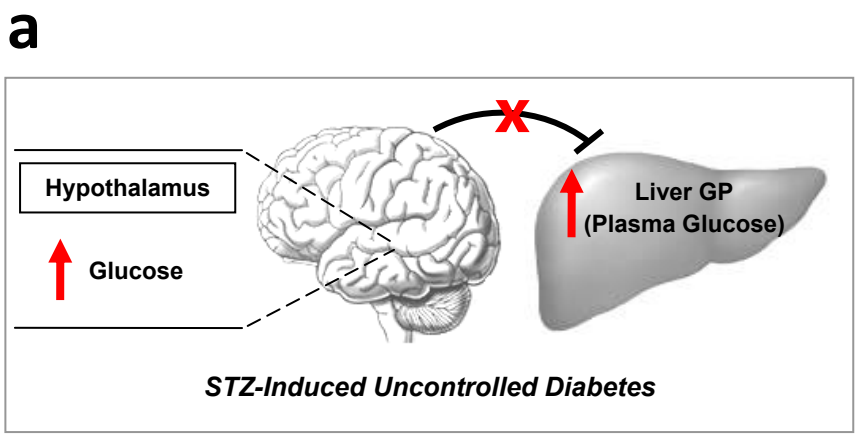

Figure 1. Schematic representation of working Hypothesis and Experimental Protocol. Schematic representation of the working hypothesis (**a**) for the glucotoxicity model experiments. Central glucose administration is not expected to lower glucose production in rodents with early-onset STZ-induced uncontrolled diabetes. (**b**) Experimental time course. The ability of central glucose to regulate whole-body glucose fluxes were evaluated in normal and STZ-Diabetic rats with the use of the pancreatic (basal insulin) clamp (shown on day 9).

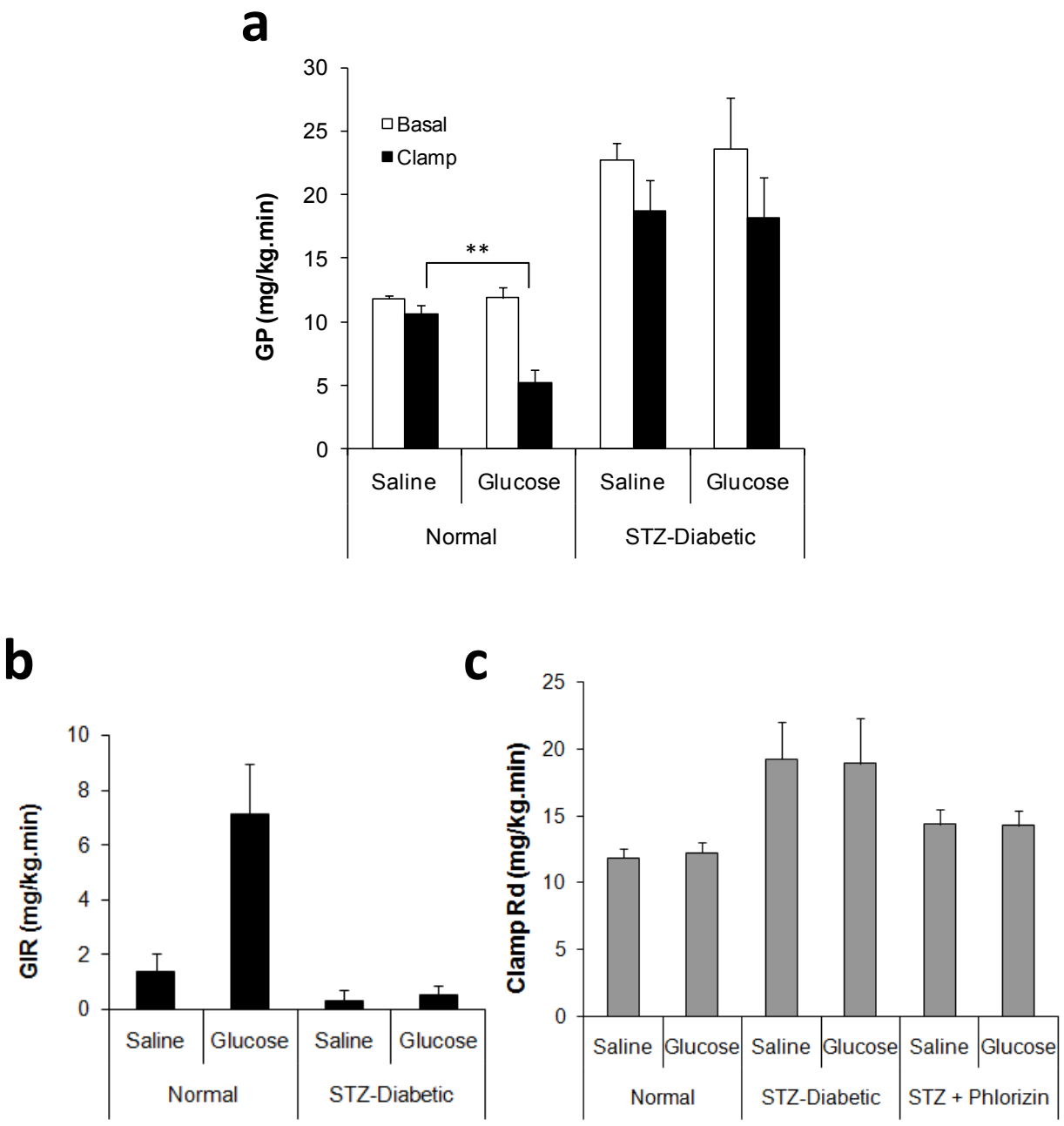

Figure 2. Acute elevation in Central Glucose is Unable to lower Glucose Production in Uncontrolled Diabetes. (**a**) STZ-Diabetic rats had a significantly elevated basal rate of glucose production (GP) - nearly double. In confirmation with previous studies, the infusion of i.c.v. glucose (2 mM) was able to reduce hepatic GP in normal rodents (n=6) during the clamp (**P<0.01 vs. i.c.v. saline (n=6)). However, no such suppression was observed in i.c.v. glucose-treated STZ-Diabetic rodents (n=5); values were similar to i.c.v. saline-treated STZ-Diabetic rodents (n=6). (**b**) The required glucose infusion rate (GIR) to maintain euglycemia during the clamp (**P<0.01 i.c.v. glucose-normal vs. i.c.v. saline-normal). The rate of glucose clearance (Rd) was not significantly different across the treatment groups for either normal or STZ-Diabetic rodents.

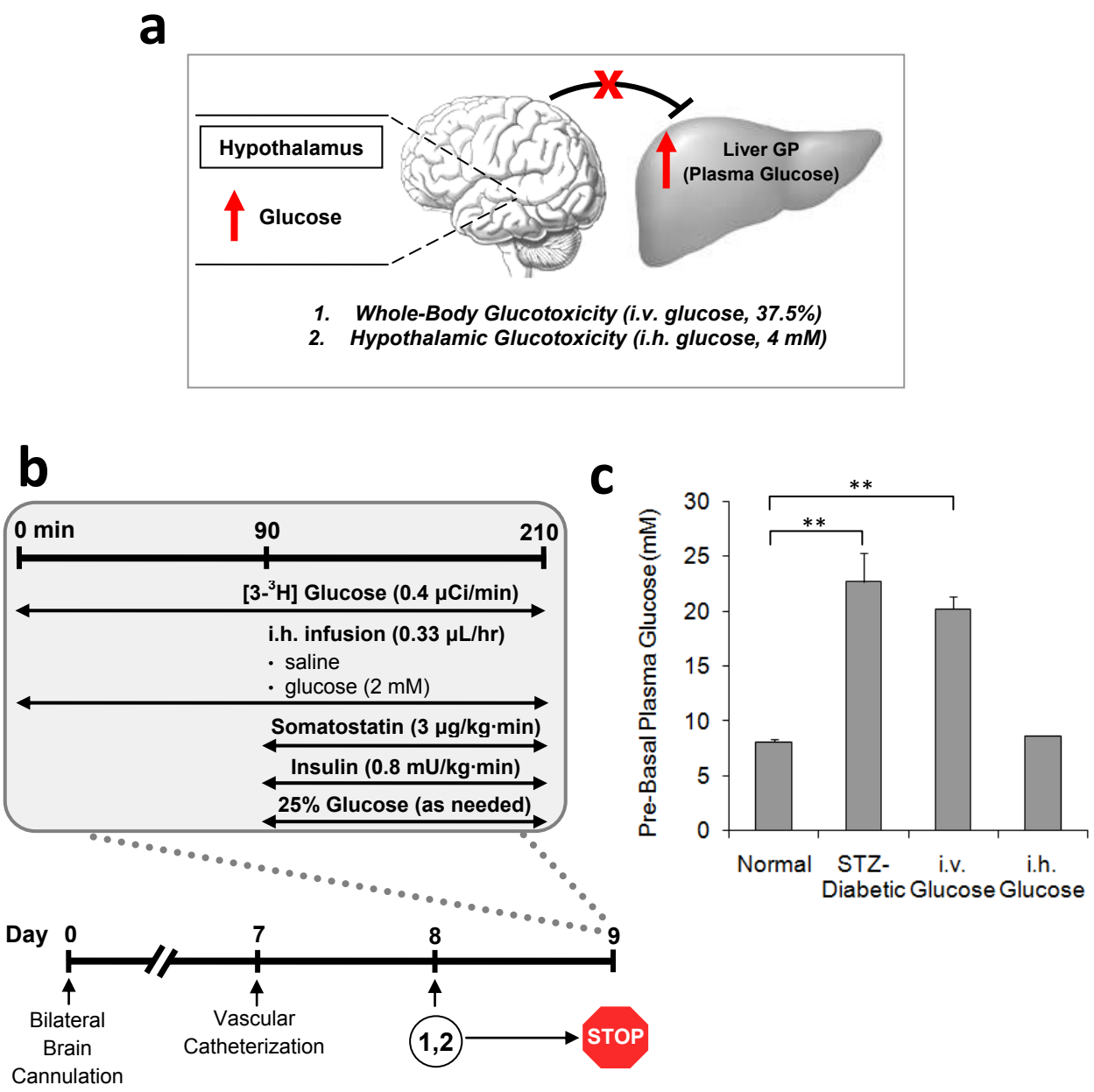

Figure 3. Working Hypothesis, Experimental Protocol and Glycemic Status for Glucotoxicity Models. (**a**) Schematic representation of the working hypothesis for the glucotoxicity model experiments. Hypothalamic glucose administration is not expected to lower glucose production in either the 1) whole-body or 2) hypothalamic glucotoxic models. (**b**) Experimental time course. Hypothalamic (i.h.) glucose-sensing was evaluated in normal, 24h i.v. (1) and i.h. glucose-infused (2) rodents with the use of the pancreatic (basal insulin) clamp. (**c**) Plasma glucose levels prior to clamp studies after 24h-infusion periods (normal i.e. saline-infused, n=4; i.v. glucose, n=11; i.h. glucose, n= 14). Values from earlier experiments done with STZ-Diabetic rodents are plotted for reference and comparison. **P<0.01 vs. normal rodents.

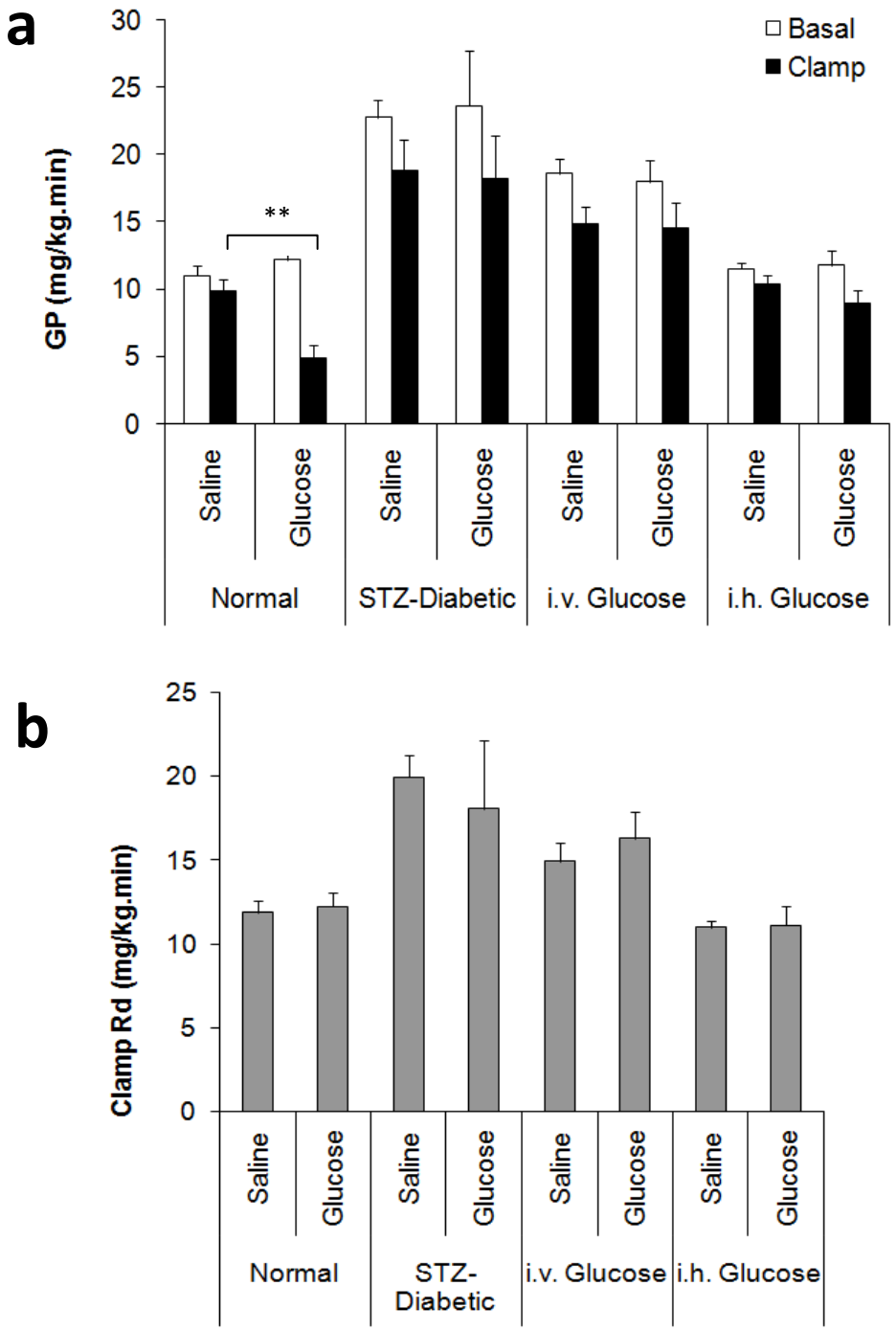

Figure 4. Glucose Fluxes in Hyperglycemic Models during i.h. Infusions. (**a**) An acute infusion of glucose directly into the hypothalamus (i.h.) was able to significantly lower glucose production in normal rodents (**P<0.01 vs. i.h. saline-normal; n=11 including n=4 24h saline-infused rodents). Pancreatic clamp analyses of normoglycemic rodents which received a 24h i.v glucose infusion revealed a defect in hypothalamic glucose sensing to lower glucose production, as the values for the i.h. glucose-treated group paralleled those of the i.h. vehicle saline-treated counterparts. A similar story is seen in the hypothalamic glucotoxic, 24h i.h. glucose-infused rodents. (**b**) The rate of whole body glucose disposal during the clamp (final 30 min) was consistent and insignificantly different across each of the groups, regardless of whether i.h. saline or glucose was received during the pancreatic clamp.

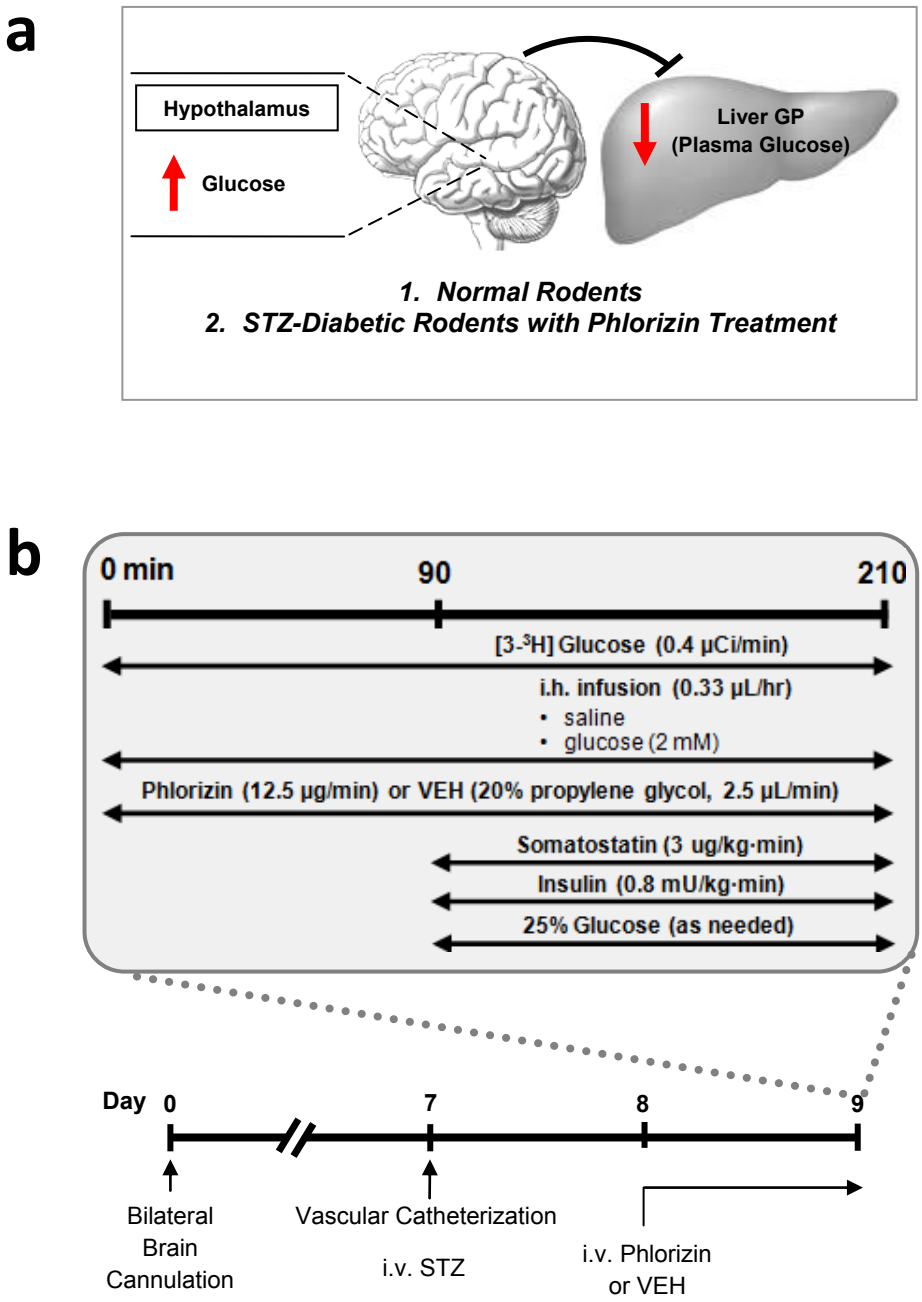

Figure 5. Schematic Representation of working Hypothesis and Gain-of-Function Experimental Protocol in Assessing Hypothalamic Glucose Sensing. (**a**) Schematic representation of the working hypothesis for the gain-of-function experiments. Hypothalamic glucose administration is anticipated to lower glucose production in STZ-Diabetic rodents which have had their plasma glucose levels selectively normalized. (**b**) Experimental time course. Hypothalamic (i.h.) glucose-sensing was evaluated with the use of the displayed pancreatic clamp in STZ-Diabetic rodents receiving a continuous i.v. phlorizin (or 20% propylene glycol, as vehicle control) administration that was initiated 24h prior to the clamp.

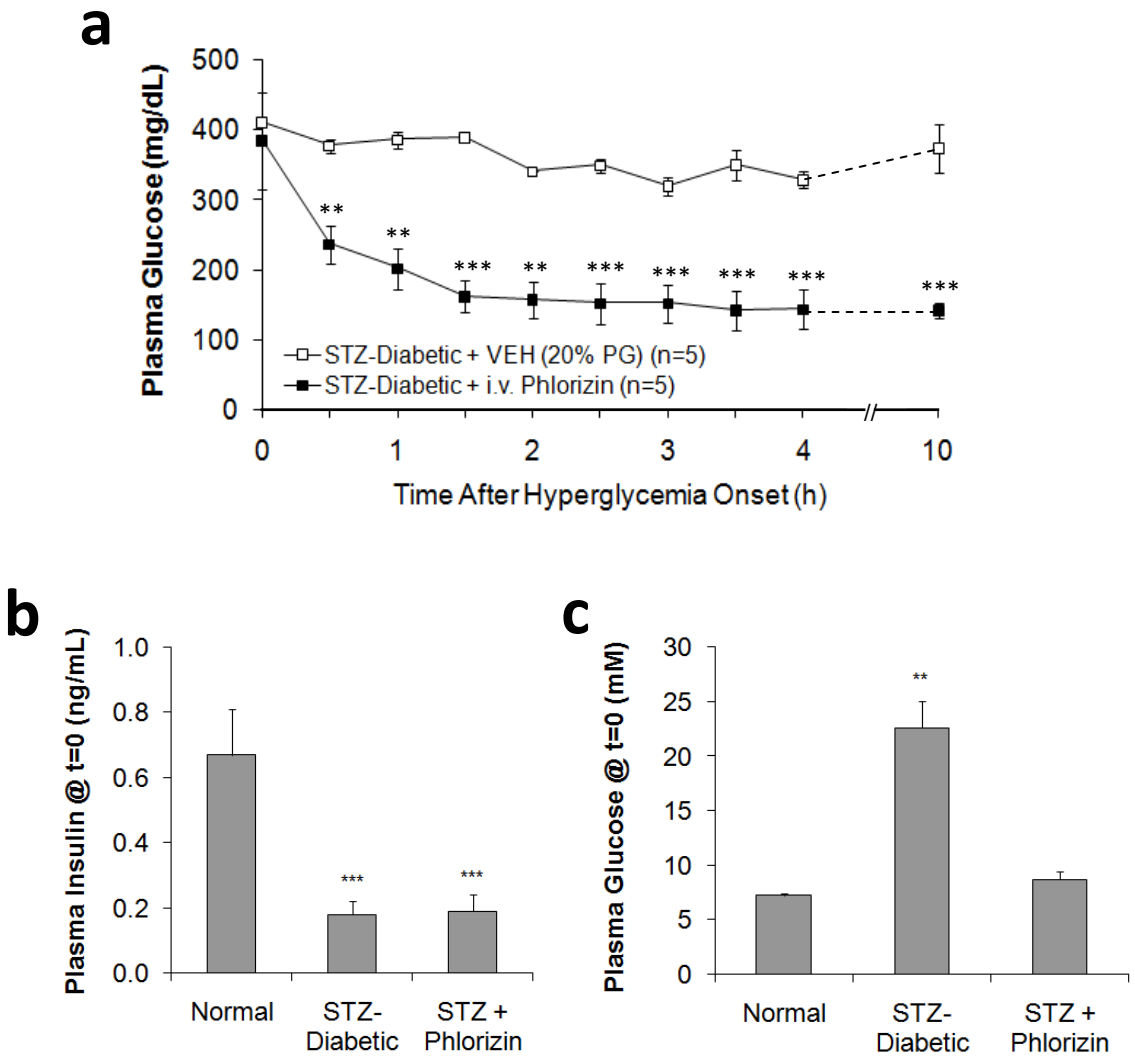

Figure 6. Effect of Continuous Phlorizin Administration on STZ-Diabetic Rodents. (**a**) The continuous intravenous (i.v.) infusion of phlorizin rapidly normalized and maintained plasma glucose levels for the duration of the infusion (**P<0.01, ***P<0.001 vs. 20% propylene glycol vehicle infusion). (**b**) STZ-diabetic rodents remained markedly hypoinsulinemic regardless of whether phlorizin was administered (***P<0.001 vs. Normal, n=12). (**c**) Starting plasma glucose levels prior to pancreatic clamp analyses where hypothalamic glucose sensing was evaluated. The continuous infusion of phlorizin successfully normalized plasma glucose levels in STZ-diabetic rodents (n=15), while untreated STZ-diabetic (n=15) remained markedly hyperglycemic (**P<0.01 vs. Normal). 'STZ-Diabetic' pools non-infused diabetic rodents (n=11) and PG-infused diabetic rodents (n=4).

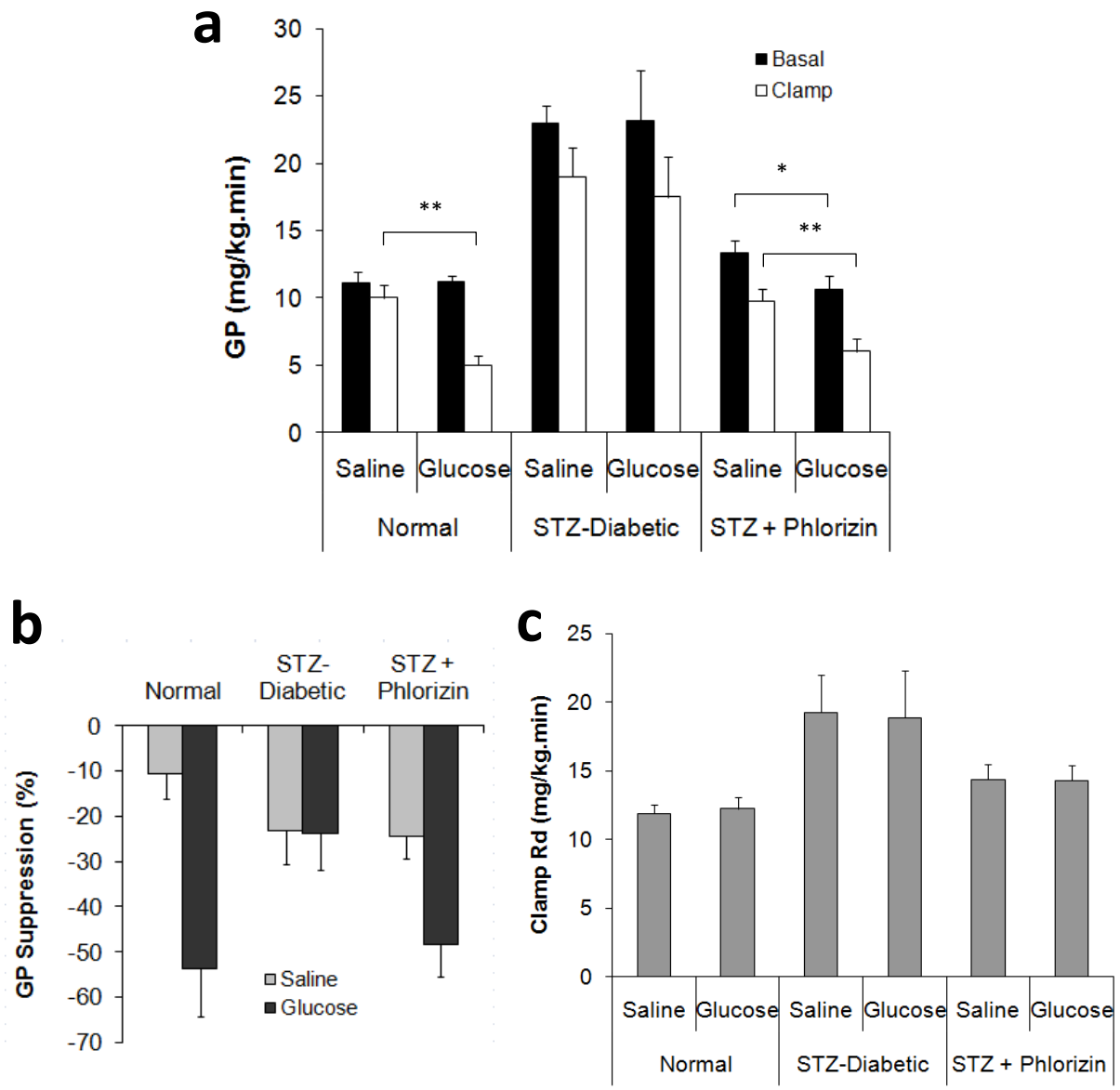

Figure 7. Insulin-Independent Normalization of Glycemia Rescues CNS Glucose Sensing in Diabetes. (**a**) Basal and clamp glucose production rates resulting from i.h. saline or glucose treatment during the pancreatic clamp analyses of normal, untreated STZ-diabetic and STZ+Phlorizin rodents. Acute i.h. glucose infusion in STZ+Phlorizin significantly lowered the rate of glucose production (GP) during the final 30 minutes of the clamp (**P>0.01 vs. i.h. saline-infused STZ+Phlorizin rodents). (**b**) The rate of glucose production suppression from the basal to clamp time periods. The ability of i.h. glucose to suppress GP is restored in phlorizin-treated STZ-Diabetic rodents (**P>0.01 vs. i.h. saline-infused STZ+Phlorizin rodents). (**c**) Across this study's groups, the rate of whole-body glucose disposal (Rd) remained unchanged regardless of the i.h. treatment.

7

GENERAL DISCUSSION

The primary function of the hypothalamus is to establish homeostasis, or the maintenance of key physiological parameters including thirst, hunger, body temperature and blood pressure. In recent history, this structure within the brain has been thoroughly demonstrated to be a vital mediator of the CNS' ability to directly sense nutrients (103; 136; 145) and hormones (40; 67; 80; 96; 97; 137) in the control of glucose homeostasis. Of particular relevance to the studies of this project, an acute increase in either hypothalamic glucose or lactate is able to trigger a regulatory neurocircuitry to inhibit hepatic glucose production in normal, healthy rodents (103). To date, the effectiveness of this carbohydrate-initiated glucoregulatory signal has yet to be adequately evaluated in experimental models of diabetes and/or obesity. In the project presented here, we have determined that central lactate retains its ability to trigger a GP-lowering effect in early-onset uncontrolled diabetes, but that glucose does not. Further, lactate is able to lower hepatic GP in settings of diminished insulin action: experimentally-induced hypoinsulinemia and diet-induced insulin resistance. Finally, lowering plasma glucose levels *per se* rescued the GP-lowering effect of hypothalamic glucose in diabetic rodents, demonstrating glucotoxicity as a primary physiological deterrant underlying the impaired CNS glucose sensing in diabetes.

The model of diabetes utilized in these studies was streptozotocin (STZ)-induced uncontrolled diabetes, which is a widely used experimental animal model. STZ, which is 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) and a derivative of glucosamine, is synthesized by the bacteria *Streptomycetes achromogenes* (187), and is a potent β-cell

cytotoxic agent that is also used in combating malignant pancreatic tumors in humans (167). After entering via the transporter GLUT2, this compound has a multi-faceted approach in causing the necrosis of the pancreatic cells, which overall results from DNA damage and dramatic ATP depletion. First, STZ is a nitrosurea and thus a potent agent that directly induces DNA damage via alkylation (187). Secondly, STZ metabolism leads to the formation of nitrous oxide (NO), which targets aconitase, a Krebs cycle enzyme (193). The subsequent limitation of ATP phosphorylation and production leads to an increase in its degradation products, including hypoxanthine which is a substrate for the superoxide anion-producing xanthine oxidase, the activity of which is inherently high in the β-cell (130). The production of hydrogen peroxide and hydroxyl radicals from superoxide generation then induce DNA damage (187). Thirdly, the damage to DNA resulting from alkylation and oxidant action leads to the activation of poly(ADP-ribosyl)ation catalyzed by poly (ADP-ribose) polymerase (PARP) in a nicotinamide adenine nucleotide (NAD+)-dependent manner: when DNA damage is less, PARP activity promotes DNA repair, but when the damage is massive, like that induced by substantial alkylation and oxidants, there is a rapid depletion of ATP (which is used to generate NAD+), which leads to a cellular energy crisis that ultimately results in β-cell death via necrosis (72). An extrapancreatic effect that is of particular relevance to this thesis is the ability of STZ to significantly reduce the activities of glycolytic enzymes, including HK, PFK, and PK, in the cerebral cortex and hippocampus (151).

A dose-response study which evaluated the metabolic effects of i.v. STZ bolus administrations demonstrated the highly specific nature of STZ on β-cells, and that the diabetogenic response could be fine-tuned using a relatively broad dosage range (87). When 65 mg/kg of STZ was administered intravenously to rodents, the diabetic state was established within 24 h: polyuria, glycosuria, and hyperglycemia (~18.3 mM) were evident, and there was a dramatic drop in pancreatic insulin content – only 5% of the intial amount remained. The metabolic readouts of the 55 mg/kg dose were very similar, and the 45

mg/kg dose led to less severe hyperglycemia (~15.6 mM) and pancreatic insulin loss (~80% reduction). Consistent with this, in our studies we observed that within 24 h, the male SD rats that were injected with 60 mg/kg had pronounced hyperglycemia (17.0±1.9 mM), which elevated further to ~21.6 mM after 48 h, when plasma insulin levels were reduced by >80%. Contributing to the hyperglycemia was an elevated rate of GP that was nearly double that of normal, non-diabetic rodents. Although chronic hyperglucagonemia is a contributor to hyperglycemia in diabetes (39), glucagon levels were unchanged in our STZ-diabetic vs. normal rodents. These results are in line with a previous study showing that the basal GP rate, in the post-absorptive state, was increased by 150% and that glucagon levels were similar to normal rodents up to 30 h post-STZ injection (65 mg/kg). However, after several days of STZ-diabetes, the plasma levels of glucagon as well as FFAs more than doubles (26). Although we did not, in our studies, measure the expression of glucoregulatory enzymes, this study by Burcelin *et al.* revealed that there was a dramatic increase in insulin-sensitive hepatic PEPCK and G6Pase activities after 12h of STZ-diabetes, along with marked depletion of hepatic glycogen stores (26). We anticipate that a similar enzymatic up-regulation occurred in our model, which is also early-onset but had been exposed to an additional day of hyperglycemia. This along with our results demonstrated that marked hypoinsulinemia is the major contributor of excessive GP in the early-onset of uncontrolled diabetes.

Study I

In 2005, the Luciano Rossetti's group revealed that an acute increase in central or hypothalamic lactate (at 5 mM) was able to lower hepatic glucose production by approximately 45%; this was done in normal rodents and in the setting of basal insulin levels (103). Here, despite the severe hypoinsulinemia and hyperglycemia, the acute i.c.v. administration of the same dose of lactate was still able to significantly lower GP in rodents with uncontrolled diabetes by more than 25% versus i.c.v saline-infused vehicle controls.

This enhancement of insulin sensitivity produced a drop in plasma glucose levels of more than 4 mM. To date, only one other group has evaluated the ability of hypothalamically-triggered mechanisms to regulate glucose homeostasis in uncontrolled diabetes. Insulin administered i.c.v. lowers GP (137) and hepatic insulin resistance develops in rodents with a reduction in hypothalamic insulin receptors (135). To further flesh out the importance of hypothalamic insulin action, Michael Schwartz' group tested the possibility that the successful treatment of uncontrolled diabetes requires intact central insulin-signaling mechanisms. Indeed, when a PI_3K-inhibitor (LY) was infused i.c.v. continuously, the blood-glucose lowering effect of daily subcutaneous insulin treatments was blunted by about one-third in rodents that had STZ-uncontrolled diabetes for two weeks (67). The i.c.v. infusion of LY alone in the absence of insulin injections had no worsening effect on blood glucose levels in these diabetic rodents whose insulin levels were 85% that of normal rodents (67), demonstrating that hypothalamic signaling cannot be triggered by circulating insulin at these diminished levels. This suggests that the i.c.v. lactate-induced lowering of glucose levels and GP observed in our studies was indeed the result of lactate *per se*, once again implying that central lactate signaling is functional in the absence of basal insulin levels.

To better assess this hypothesis, we evaluated the effects of i.c.v lactate in a rodent model of experimentally-induced hypoinsulinemia, that was previously used in dogs (33). In the dog study, Cherrington *et al.* infused somatostatin alone at 0.8 μg/kg/min for 4.5 h, which was able to suppress both plasma insulin and glucagon levels by >60% (33). As a result, the plasma glucose level and rate of GP in these conscious dogs had increased by ~130 and 150%, respectively, from the starting values (33). In our studies, Sprague-Dawley rats infused with somatostatin alone did indeed have a drop in plasma insulin levels by a similar amount, yet the glucagon levels remained unchanged from those seen in normal rodents, facilitating the evaluation of selective hypoinsulinemia on central lactate sensing. In this setting of insulin lack, the plasma glucose levels gradually rose during the 4.5 h

protocol to ~11 mM (increase of 160%), with a corresponding increase in GP by 150% (to 16 mg/kg/min) in rodents receiving a concurrent i.c.v. saline infusion. However, in rodents receiving a concurrent i.c.v. lactate infusion during the i.v. somatostatin infusion, the rise in plasma glucose level and GP was prevented. This along with the evaluation of central lactate in uncontrolled diabetes confirms that basal insulin signaling is not required for the effectiveness of i.c.v. lactate to trigger its' regulatory effect on hepatic glucose production.

The retained effectiveness of central lactate sensing during insulinopenia suggests that lactate could bypass insulin resistance to regulate glucose homeostasis. In keeping with the previous studies of this project thus far which have been acute models of insulin dysfunction, we chose to evaluate central lactate sensing in an established model of early-onset, diet-induced insulin resistance. Previous studies which have used this 3-day model of insulin resistance have reported a significant increase in caloric intake versus rodents on a regular chow diet (124; 205). Additionally, high fat-fed rodents had elevated plasma leptin and insulin levels, which is indirectly indicative of leptin and insulin resistance. To be sure, direct evaluation of resistance via blunted suppression of GP by hyperinsulinemic clamp and the failed anorectic action of exogenous leptin administration confirmed that these rodents were indeed leptin and insulin resistant (205). Similarly, in our study we have confirmed that the same strain of rats on a lard-oil enriched high-fat diet for 3 days rapidly develop a hypercaloric intake, hyperinsulinemia and hyperleptinemia. In this setting of insulin resistance, as determined by the results of a hyperinsulinemic clamp, the administration of i.c.v. lactate retained its' potent ability to lower hepatic GP, suppressing the rate by ~40% versus the i.c.v. saline-infused controls. This parallels the finding from a few years ago (102) indicating that i.c.v lactate retains its' effect to lower hepatic very low-density lipoprotein (VLDL) in the 3-day overfed model. Although the readout in this study is different, the hypothalamic control of hepatic GP and VLDL secretion are proposed to occur by the same pathway (hypothalamic glucose metabolism → activation of K_{ATP} channels → trigger brain-

liver circuitry via afferent vagus nerve). Given the demonstrated effectiveness of i.c.v. lactate in uncontrolled diabetes (characterized by severe hypoinsulinemia and hyperglycemia) as well as experimentally-induced hypoinsulinemia, the GP-lowering effect of central lactate in a model of insulin-resistance may not seem all that surprising. It should be noted, however, that the potent ability of hypothalamic insulin (137) and LCFA (136) to lower glucose production are blocked after high-fat feeding for merely 1 day or 3 days, respectively (124; 138). Clearly, further investigation is necessary to elucidate the mechanisms underlying this selective preservation in CNS sensing mechanisms in models of metabolic disease. Additionally, the effectiveness of central lactate was only evaluated in a single model of early-onset diet-induced insulin resistance the glucoregulatory effect of central lactate in more developed and pronounced stages of diet-induced insulin resistance/obesity needs to be assessed. After all, while the i.c.v. administration of leptin is effective in lowering GP in the early-onset of diet-induced insulin resistance, chronic overfeeding eventually leads to hypothalamic leptin resistance (57). As such, the "upper limit" of hypothalamic lactate sensing mechanisms needs to be determined.

Overall, the results of this Study reveal the criticality of preserving or enhancing the generation of lactate in the CNS, and hint at the importance of the earlier-described metabolic coupling of astrocytes and neurons (whereby neurons preferentially use the astrocyte-derived lactate to fuel their energetic demands). These results, along with the initial study which examined normal rodents (103) , are the first to take a closer look at hypothalamic lactate metabolism, but a few other notable studies have assessed the relevance of CNS lactate sensing in other areas of the brain involved in the regulation of glucose homeostasis. Specifically, the perfusion of the ventromedial hypothalamus (VMH) with lactate was sufficient to trick the region and severely blunt the counterregulatory hormone response to hypoglycemia, with a marked suppression of both glucagon and epinephrine release during a hypoglycemic clamp – a finding also seen when glucose was

perfused into the VMH (17). The caudal hindbrain has also been established as a sensor of local glucoprivation which subsequently activates a response to restore glycemia, and in an attempt to uncover the potential metabolic sensors involved, the role of lactate in particular was recently examined (146). Indeed, 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 (146). Conversely, an increase in caudal hindbrain lactate worsened insulin-induced peripheral hypoglycemia (146).

To further the therapeutic relevance of these results, the ability of central lactate sensing mechanisms to detect physiological increases in *circulating* lactate to regulate glucose homeostasis would have to be assessed in models of metabolic disease. This has not been covered in this project. However, our lab has recently established this potential in the normal, healthy setting. Although lactate is a gluconeogenic precursor, a study in humans revealed that an elevation in either alanine or lactate does not increase overall hepatic glucose output during a clamp despite more than doubling the rate of gluconeogenesis (84). To assess the role of centrally-mediated glucoregulatory mechanisms involved in counteracting this increase in gluconeogenesis (98), normal rodents received a continuous central infusion of oxamate during a concurrent i.v. infusion of lactate which raised plasma lactate ~2-fold (as seen in exercise). During this blocking of central lactate metabolism, the i.v. lactate infusion led to an increase in glucose (98), demonstrating that there is a central component to the restraining of hepatic glucose output during increased gluconeogenic flux. Future studies are necessary to determine if this lactate-sensing mechanism is intact in the early-onset of uncontrolled diabetes or diet-induced insulin resistance.

However, some caution must be exercised in completely embracing lactate-sensing mechanisms as a means to lower plasma glucose levels. As described earlier, hyperglycemia is a major risk factor for stroke. Once the stroke occurs, the resultant ischemia means that

the anaerobic metabolism from glucose to lactate (and thus lactic acid) is promoted; this "acidotoxicity" resulting from the low intracellular and extracellular pH can induce damage to neurons and glial cells, as well as the vasculature (116). As such it is important that there is adequate oxygen supply to ensure complete lactate oxidation to reduce the toxic effects of lactate accumulation. As well, lactate levels are chronically elevated in diabetic and obese individuals (111). Although this may be as an effect rather than a cause, given that lactate production by adipocytes is proportional to their volume (174), studies have shown that lactate can promote peripheral insulin resistance. Specifically, the chronic infusion of lactate (24 h) decreased glucose uptake in muscle, where there was also a corresponding decrease in GLUT4 expression (110). Even acutely (2.5 h), elevations in plasma lactate were able to reduce the glucose utilization in the heart and certain skeletal muscles (201). The latter study (201) did, however, confirm the earlier described finding that there was no effect of acute hyperlactatemia on total hepatic glucose output (during both basal and clamped settings), suggesting that the insulin resistance is localized extrahepatically.

Nevertheless, the results of our studies confirm the ability of central lactate to lower hepatic GP and flesh out the importance of central lactate metabolism in the regulation of glucose homeostasis in experimental models of uncontrolled diabetes, hypoinsulinemia, and diet-induced insulin resistance. The preservation of this nutrient-sensing pathway in the setting of metabolic dysregulation is intriguing, and given that central leptin action initially bypasses leptin resistance (155) but eventually diminishes after continued overfeeding (57), further follow-up experiments need to be performed to determine upper limit of central lactate's effectiveness.

Study II

In the same 2005 study that demonstrated the ability of central and hypothalamic lactate to lower GP in normal rodents, the administration of central/hypothalamic glucose

was also found to potently suppress hepatic GP and lower plasma glucose levels (103). The effects of central glucose and lactate were both inhibited when these metabolites were co-infused with oxamate, an inhibitor of the enzyme LDH, demonstrating the importance of glucose metabolism to trigger this brain-liver axis to lower hepatic GP (103). This finding of hypothalamic glucose sensing mechanisms regulating hepatic GP adds to the well-established phenomenon of "glucose effectiveness", which is the term coined to describe the process whereby acute increases in systemic glucose, independent of increases in insulin, are able to lower its own production from the liver and stimulate its clearance (1; 170; 192). In fact, when this acute systemic hyperglycemia is coupled to a concurrent inhibition of hypothalamic glucose metabolism, its suppressive effect on GP is blunted by nearly 40% (103), indicating that the hypothalamus is partly responsible for this action of glucose effectiveness.

To date, the extent of central glucose effectiveness to regulate glucose homeostasis, or more specifically suppress hepatic glucose output, has yet to be assessed in the diabetic setting. Given that glucose readily crosses the blood barrier, we can postulate that the hypothalamus is exposed to an elevated level of glucose in the setting of diabetic hyperglycemia. Indeed, in the landmark study by Silver and Erecinska (181), extracellular brain glucose levels, as determined by microdialysis in the intact rodent, were found to change in parallel with plasma glucose levels which ranged from hypoglycemia (via insulin bolus) through hyperglycemia (via glucose bolus). Although these brain glucose levels were measured in the cortex and not in the mediobasal hypothalamus, another more recent study which measured extracellular glucose in the ventromedial hypothalamus (VMH; which includes the arcuate nucleus as well as the ventromedial nucleus) during hypoglycemia and normoglycemia revealed consistent results (46). Additionally, by monitoring the brain extracellular fluid in BB rats which develop spontaneous poorly controlled diabetes, midbrain glucose levels were increased after a chronic (2 month) exposure to

hyperglycemia, and were nearly three times higher than those seen in normal rodents (83). No studies, to our knowledge, have measured the glucose levels in the interstitial space of the mediobasal hypothalamus during the acute onset of diabetes, however. Despite this lack of relevant literature, these studies suggest that it is likely that the diabetic hypothalamus is subjected to a proportionately elevated amount of glucose levels, especially considering that it lies immediately above the median eminence where the blood-brain barrier is considered to be incomplete (207). As such, we anticipated that the administration of exogenous glucose into the hypothalamus will fail to trigger the CNS glucose effectiveness to lower hepatic GP in uncontrolled diabetic rodents, and further hypothesize that sustained hyperglycemia *per se* (a.k.a. "glucotoxicity") will be a primary underlying variable that mediates this impairment.

We began testing this idea with the assessment of i.c.v. glucose administration, at the same dose (2 mM) that lowers plasma glucose levels and GP in normal rodents rodents (103), on *in vivo* glucose fluxes in rodents with an acute onset of STZ-induced uncontrolled diabetes, which was described earlier in greater detail. Central glucose effectiveness was evaluated during a pancreatic clamp where insulin levels were fixed at basal levels. In these severely hypoinsulinemic STZ-rodents, the basal GP was ~22 mg/kg/min regardless of whether i.c.v. saline or glucose was infused. Serving as a positive control, the infusion of i.c.v. glucose was found to lower GP during the clamp in normal rodents by ~50%, which is line with the previous study that first published this finding (103). However, the infusion of i.c.v. glucose at the same dose was completely ineffective at regulating glucose homeostasis in STZ-diabetic rodents, as the rate of GP during the clamp was unchanged from that measured during the i.c.v infusion of the vehicle control saline. As such, i.c.v. glucose was unable to further stimulate insulin sensitivity, even with the provision of basal insulin, and it appears as if the central glucose effectiveness to lower GP was indeed impaired in this setting acute-onset uncontrolled diabetes.

STZ-induced diabetes, within the first few days, is characterized primarily by hyperglycemia and hypoinsulinemia; hyperglucagonemia and dyslipidemia do not present until several days into the onset of this form of uncontrolled diabetes (26). Therefore, in order to more clearly test the effects of glucotoxicity on the impairment of central glucose sensing, alternative experimental models of selective hyperglycemia without any accompanying insulin deficiency are needed. As such, two additonal models of glucotoxicity were generated, whereby normal adult rodents were infused with intravenous (37.5%) or intrahypothalamic (4 mM) glucose for 24 h to induce whole-body or hypothalamic glucotoxicity, respectively. In a study published by Gordon Weir's group (106), normal Sprague Dawley rodents that were infused with a 35% glucose solution fpr 48 h were found to have impaired glucose-stimulated insulin secretion from the perfused pancreas. Twenty-four hours into the infusion, these hyperglycemic rodents have an elevation of plasma glucose levels to ~325 mg/dL with a ten-fold increase in plasma insulin over saline-infused rodents. Similarly, our studies were determined that the 37.5% glucose infusion after 24 h was able to increase glucose levels to ~360 mg/dL, with a nine-fold increase in plasma insulin levels over vehicle-infused controls. After plasma glucose levels normalized over the course of ~1.5h and during the basal period of the pancreatic clamp analyses, i.v. glucose-infused rodents still displayed hyperinsulinemia, which is indicative of an insulin-resistant state. Indicative of this is the elevated rate of glucose production seen in both the basal (~18 mg/kg/min) and clamped settings (~15 mg/kg/min), as compared to the range of 10-12 mg/kg/min seen in normal rodents in these settings.

Indeed, Rossetti and colleagues were the first to provide *in vivo* evidence that sustained hyperglycemia *per se* in an experimental rodent model leads to the development of insulin resistance. No such metabolic alterations (in insulinemia or glycemia) were displayed in 24 h i.h. glucose-infused rodents. It would not be reasonable to anticipate that the i.h. infusate *per se* (i.e. a leak into systemic circulation) would have effects on the whole

body. After all, the <8 uL of the 4 mM glucose solution that is infused over the 24h period is rather insignificant. However, neural signals initiated in the hypothalamus could have been altered by the nutrient overload, in turn causing peripheral metabolic dysfunction. Indeed, the pancreatic β–cell is heavily innervated (60), and the administration of epinephrine can impair insulin secretion in man during exogenous glucose and glucagon-stimulated hyperglycemic conditions (156). Epinephrine can also induce insulin resistance, as the co-infusion epinephrine with insulin severely blunted the effects of insulin on glucose utilization and the suppression of glucose production (49). Although whether the hypothalmus is involved in this exact process is not clear, it is well known that, as part of the "fight or flight" stress response, the hypothalamus stimulates the sympathetic nervous response which in turn activates the endocrine response, which in part involves the release of epinephrine and norepinephrine from the adrenal medulla in order to mobilize glucose from the liver. Further, the hypothalamic (VMH) administration of acetylcholine, which is one of the activators that increases the secretion of corticotropin-releasing hormone (CRH) (133) which marks the hypothalamic triggering of the stress response, promptly elevated plasma glucagon and glucose levels and suppressed insulin secretion (81). Thus, although 24 h i.h. glucose-infused (hypothalamic glucotoxic) rodents did not have any measurable alterations in insulin or glucose levels, it should be recognized that hypothalamic nuclei are additionally poised to regulate glucose homeostasis via altering insulin secretion.

When the effectiveness of hypothalamic glucose sensing was assessed in these 24 h-infused models, i.h. glucose (2 mM) lowered GP in normal, 24h-saline infused rodents. However, this acute administration of i.h. glucose was unable to do so in either of the 24 h glucose-infused rodents, despite their evident normoglycemia. Thus, the glucotoxic effects conferred by a prior hyperglycemic insult to the whole body or selectively in the hypothalamus were sufficient to nullify the glucoregulatory ability of hypothalamic glucose-sensing mechanisms. It has yet to be determined whether moderately longer normalization

periods would be sufficient on their own to restore hypothalamic glucose sensing – this is a simple future direction that can be easily completed, however.

Finally, in an attempt to create a model of rescue to further test the hypothesis that glucotoxicity, i.e. sustained hyperglycemia *per se*, plays a primary role in the impairment of hypothalamic glucose sensing in diabetes, the ability hypothalamic glucose sensing to lower GP was assessed in early-onset uncontrolled diabetic rodents in which plasma glucose were continuously normalized to prevent the exposure of hyperglycemia for 24 hours. For this, we sought the use of phlorizin, which is a common experimentally used agent that inhibits the sodium glucose-linked transporters (SGLTs) of the small intestine (SGLT1) and the renal tubule (SGLT2), and does not affect the facilitative GLUTs (55). Using phlorizin to normalize blood glucose in diabetic rodents, Rossetti *et al.* were the first to demonstrate that glucotoxicity is sufficient to impair insulin-stimulated glucose uptake (166). Further studies using phlorizin determined that hyperglycemia-induced disruptions in adipocyte glucose transport (89), and glucose- and arginine-stimulated insulin secretion (165).

In our studies, the continous i.v. infusion of phlorizin into diabetic rodents upon the first display of hyperglycemia (~1 day post-STZ) normalized glucose levels within 1.5 h and maintained normoglycemia without affecting insulin levels, as these STZ+Phlorizin rodents were still markedly hypoinsulinemic. This selective effect of normoglycemia with a preservation of hypoinsulinemia was also seen in phlorizin-treatic diabetic rodents which had their pancreatic tissues depleted via pancreatectomy (166). Accompanying this normalization of blood glucose was a lowered rate of basal glucose production, which was further lowered in the presence of i.h. glucose administration. During the pancreatic clamp, i.h. glucose-infused STZ+Phlorizin rodents had a rate of GP that was further suppressed by ~45%, mirroring the rate of suppression seen in hypothalamic glucose-infused non-diabetic rodents. STZ diabetic rodents receiving the phlorizin vehicle (20% propylene glycol)

throughout the protocol displayed no such normalization or suppression of GP. As well, glucose utilization rates within the i.h. treatment groups remained similar.

A closer look reveals that when compared to the i.h. saline treatment, the i.h. glucose treatment produces a greater additional suppression of GP in normal rodents than normoglycemic STZ-rodents. This may suggest that normalization of glycemia does not fully restore hypothalamic glucose effectiveness to regulate GP to its maximum potential, at least in the current experimental protocol which provides acute basal insulin replacement for only 2 hours. Although the absolute rate of suppression by i.h. glucose in normoglycemia STZ rodents matches that seen in normal rodents, there is a clear baseline effect in i.h. saline-infused normoglycemic rodents. This may have resulted from the process of attaining normoglycemia by phlorizin, as the removal of hyperglycemia may have improved insulin sensitivity since hyperglycemia *per se* has been shown to inhibit insulin secretion (106; 165) and reduce insulin sensitivity. This combined with the provision of basal insulin during the clamp (166) may have dropped the rate of GP even without any activation of hypothalamic sensing mechanisms. Thus, although glucotoxicity may not be the only physiological factor (e.g. altered insulin action/sensitivity) that causes the impairment in i.h. glucose administrations to regulate GP, it is quite clear that glycemia plays an important gatekeeper in hypothalamic glucose effectiveness.

The results of Study I and Study II overall indicate that CNS lactate-sensing mechanisms lowers glucose production in uncontrolled diabetes, but that CNS glucose-sensing mechanisms are completely impaired. This impairment appears to be mediated by hyperglycemia *per se*, as hypoinsulinemic STZ-rodents with normoglycemia had this GP-lowering circuitry restored. What could possibly be mediating this selective preservation of hypothalamic lactate-sensing mechanisms in diabetes? Purely based on the differential effects of central lactate and glucose, it is not illogical to postulate that the hyperglycemia-induced cellular defect lies upstream of lactate generation and utilization in the brain.

Although it can be argued that STZ is the causative agent itself altering brain glucose metabolism, since direct STZ administration into the lateral ventricle at subdiabetogenic doses significantly reduces the activities of glycolytic enzymes (including HK, PFK, and PK) in the cerebral cortex and hippocampus (151), our studies demonstrated that hypothalamic glucose sensing was also impaired in our normal rodents with sustained hyperglycemia *per se* in the whole body or selectively in the hypothalamus, achieved independently of STZ via glucose infusions.

One possibility is that, in the face of hyperglycemia, there is a compensatory decrease in the rate of glucose transport into brain cells. Indeed, the peripheral insulin resistance marked by a reduction in insulin-stimulated glucose utilization that is conferred by extended hyperglycemia (73; 214) is thought to be due in part to a decrease in glucose transport activity. Supporting this, a multitude of studies has determined that there is a significant decrease in the expression of GLUT4 in the insulin-sensitive muscle and adipose tissue in diabetes (10; 66; 88; 184). As far as the brain is concerned, the transport of glucose is primarily dependent on two glucose transporter proteins: GLUT1, which is expressed in the blood-brain barrier endothelium and in the underlying glial cells, and GLUT3, the predominant transporter in neurons (183). GLUT1, -3, and -4 are part of the high-affinity class I of glucose transporters, which have K_m values that are below the concentration of glucose seen in the blood (19). Due to this inherent ease of saturation, alterations in their cell surface expression significantly influence the rate of glucose entry into the cell.

Although there have been several previous studies assessing the effects of glycemic changes on brain glucose transporters and/or transport, the concept of CNS transport adaptation is controversial. The seminal paper in this area of research by Gjedde and Crone revealed that the chronic hyperglycemia in STZ-induced diabetes repressed blood-brain glucose transfer, and that this was not due to a decrease in blood flow to the brain (68).

The translation of this finding to our investigation direction is not seamless however, as the study employed the use of anesthetized rodents as well as a three-week diabetic model. A year later, another group using an acute rat model of STZ-diabetes similar to ours, in which glucose levels were elevated to ~22 mM after two days, demonstrated that chronic hyperglycemia suppressed the blood-brain transport of glucose (117). Interestingly, Ian Simpson and colleagues found (using *in situ* brain perfusion studies) that although there was an increase in glucose transport and a corresponding increase in GLUT1 protein and transcript in *hypo*glycemia, there were no changes in transporters or transport activity in STZ-induced *hyper*glycemia (182). Studies examining brain glucose utilization in humans are limited, but overall suggest that global brain glucose transport is not decreased in poorly controlled diabetes (58; 177). Also in contrast to the rodent findings, individuals with poorly controlled type 1 diabetes have been reported to have either unaltered (179) or even slightly *decreased* (20) brain glucose uptake when subjected to experimental hypoglycemia. It is important to note, however, that no studies to-date, to our knowledge, have specifically looked at hypothalamic glucose transport. The arcuate nucleus of the hypothalamus – which has emerged as the master nuclei for the regulation of energy and glucose homeostasis and is the focus of our studies – is situated at the base of the third ventricle and lies immediately above the median eminence where the blood-brain barrier is considered to be incomplete (207). Thus, it may be the case that the hypothalamus is poised to be subjected to various circulating nutrients to a greater degree, and as a result have unique and more acute adaptive mechanisms. As such it would be reasonable to assess the impact of our early-onset model of uncontrolled diabetes on hypothalamic glucose transporter expression.

Just downstream of the transport of glucose lays its phosphorylation which is mediated by hexokinases, the activity of which need to be intact to maintain glucose homeostasis. In the periphery, type 2 diabetic subjects have lower hexokinase II activity

and transcript levels in skeletal muscle (200), and the activity of GK, the rate-limiting enzyme of glucose metabolism in the β–cell and liver, is also diminished (30). Further, the ability of the normalization of glycemia *per se* in diabetic rodents to rescue the glucose effectiveness of acute hyperglycemia to lower GP correlated with an increased cytoplasmic localization of GK and decreased nuclear localization with its negative regulator, GKRP (64). With respect to the regulation of glucose homeostasis by the brain, Barry Levin's group (52; 92; 107; 108) has been at the forefront in the advancement of brain glucokinase (specifically, in the VMN) as a critical mediator of hypothalamic glucose sensing. Glucokinase has been found to be expressed in the majority of VMN glucosensing neurons (93), and its upregulation has been associated with the impairment of the counterregulatory response induced by recurrent hypoglycemia (52; 107). However, these above glycemia-correlated alterations in glucokinase activity may not translate perfectly to our situation of *hyper*glycemia-induced disruption hypothalamic glucose sensing because the high K_m of GK (~10 mM; (112)) make its hyperglycemia-induced down-regulations that are seen in the periphery, where such elevated glucose levels are easily seen in diabetes, unlikely in the brain where glucose levels range from 0.2 to 4.5 mM in response to variations in plasma glucose from ~3 to >15 mM (181).

In 2004, Ozcan *et al.* were the first to demonstrate the activation of ER stress as a means by which obesity progresses to insulin resistance and ultimately type 2 diabetes (142). In a follow-up study, they used orally active chemical chaperones to combat ER stress in diabetic and obese rodents and improve glycemia and insulin sensitivity (143). In the past year, the activation of the metabolic inflammation mediator IKKβ/NF-κB was shown to induce central leptin and insulin resistance via a diminishing of hormonal signalling (217). In the same study, IKKβ/NF-κB was found to be triggered by *hypothalamic* ER stress which in turn was caused by overnutrition mediated by a 6 h i.c.v. infusion of 20% glucose (217), and with this i.c.v. glucose infusion the hypothalamic glucose content was raised to 80% of the

levels seen in the diabetic and obese db/db mice (217). Although hypothalamic tissue lysates from our STZ-diabetic and 24 h i.v./i.h. glucose-infused have not been analysed for their glucose levels, these findings from Zhang *et al.* hint that the activation of ER stress mechanisms by overnutrition is a worthwhile mechanism to investigate as a hyperglycemia-induced cellular defect that impairs hypothalamic nutrient (specifically, glucose) sensing mechanisms.

In the meantime, there are some physiological assessments that can be done to further assess the impairment in CNS glucose sensing mechanisms to regulate hepatic GP by hyperglycemia *per se*, prior to tackling the potential cellular defects suggested above. In our studies, i.h. glucose was unable to inhibit GP in rodents receiving a prior insult of whole-body or hypothalamic glucose, even though the rodents were normoglycemic and the insult had been removed ~1.5 h prior to analyses. What if the rodents were tested after additional post-hyperglycemia recovery (e.g. one day)? In diabetic humans, an acute increase in plasma glucose levels sustains its' ability to lower hepatic GP provided the individuals were subjected to an intensive, 3-day normalization period (75), however, this is not the case after 1 day of glycemia normalization (120). This suggests that a slightly more prolonged normalization period may rescue the GP-suppressing action of central glucose effectiveness in 24 h glucose-infused rodents – the fact that alterations in glucose transport to the brain in response to changes in antecedent glycemia require ≥ 1 day (116) further strengthen this possibility. Another question to be addressed is whether the threshold to trigger hypothalamic glucose sensing mechanisms has been altered in the diabetic brain. Experimental rodent models of diabetes do have elevated hypothalamic glucose levels (217), so a simple test would be so see if acute i.h. administrations of glucose at a higher concentration (from 2 to 4 mM) could trigger the neurocircuitry to lower hepatic glucose production. Of course, the range of glucose concentrations would need to be tested within a physiological upper limit to increase the validity of this threshold assessment.

8

GENERAL CONCLUSION

Diabetes and obesity are generally perceived to be peripheral metabolic diseases. However, data from the remarkable surge in literature dedicated to the CNS control of nutrient and energy homeostasis suggest that the brain is an organ that is vital in the control of food intake, nutrient production, and fuel substrate oxidation, among other processes. Of particular note, the acute administration of lactate or glucose into the hypothalamus is able to trigger a brain-liver axis to potently curtail hepatic glucose production, independent of changes in glucoregulatory hormones including insulin and glucagon. These nutrient-sensing mechanisms had yet to be assessed in the setting of metabolic disease, and this void in the literature necessitated the conducting of the experiments that have been presented here in this thesis.

We have here determined that the acute administration of central lactate retains its ability to lower GP in multiple acute models of metabolic distress, including STZ-induced uncontrolled diabetes, experimentally-induced hypoinsulinemia, and diet-induced insulin resistance. Central glucose sensing mechanisms, however, could not be triggered in uncontrolled diabetic rodents. Further experimentation revealed that hypothalamic glucose administrations could not suppress GP in normoglycemic rodents that had received a prior 24 h infusion of i.v. or i.h. glucose to induce whole-body or hypothalamic glucotoxicity, respectively. However, by preventing the exposure to hyperglycemia via the administration of phlorizin to uncontrolled diabetic rodents, the effect of hypothalamic glucose to lower hepatic GP was rescued. Further work needs to be completed to 1) evaluate the potency of central lactate sensing in longer onset metabolic disease and 2) determine the glucotoxicity-

induced cellular or molecular defect that mediates the impairment of central glucose sensing in diabetes.

The studies completed here add to the growing literature that evaluates the effectiveness of hypothalamic sensing mechanisms in the normal and obese and/or diabetic setting. Dysfunctions in the ability of nutrient and hormones to trigger these hypothalamus-mediated regulatory effects of nutrient and energy homeostasis may be key players in the progression of insulin resistance and diabetes. Given the unfortunately enormous growth in diabetes around the world, a greater understanding of mechanisms that improve insulin sensitivity, curtail hepatic glucose production and lower plasma glucose levels is imperative. Such advancements will aid in the development of more effective therapeutics and help restrain this epidemic as a means to ultimately improve the health and well-being of those suffering from diabetes.

9

FUTURE DIRECTIONS

The results of this thesis led to the following questions that would be of interest to address with future experiments:

1) We here determined that central lactate lowers hepatic GP in various models of metabolic disease, including uncontrolled diabetes, experimentally-induced hypoinsulinemia, and diet-induced insulin resistance. All of these metabolic diseases, however, are of the early onset. Does central lactate sensing retain its effectiveness in longer established disease states? If not, when does it's "breaking point" occur? The effects of i.c.v. lactate administration on basal and clamped GP needs to be evaluated in rodents with at least 7 days of STZ-induced hyperglycemia and hypoinsulinemia, and high-fat diet-induced insulin resistance/obesity.

2) Our lab has recently determined that the inhibition of hypothalamic lactate sensing mechanisms leads to an increase in GP during the continuous i.v. infusion of the gluconeogenic substrate lactate. Is this hypothalamus-triggered effect of circulating lactate effective in uncontrolled diabetes? It would be of interest to see if this effect of circulating lactate on hepatic glucose output persists in the early-onset of STZ-induced diabetes, much like the effects of direct central lactate administrations do.

3) Direct hypothalamic administration of glucose fails to lower hepatic GP in normoglycemic rodents that were considered to have antecedent whole-body or hypothalamic glucotoxicity. Here, the effects of i.h. glucose on *in vivo* glucose fluxes were determined 1.5 h after the 24 h glucose insult was removed – how long does this glucose-induced

impairment last after discontinuation of the glucose infusion? The effect of i.h. glucose on hepatic GP should be assessed in whole-body and hypothalamic glucotoxic rodents after longer periods (e.g. one day) of normalization to determine the point at which hypothalamic glucose sensing is restored.

4) Interstitial brain glucose levels are markedly increased in the diabetic state. Sustained elevations in brain glucose may lead to the desensitization of hypothalamic nutrient sensing mechanisms that regulate GP and lower blood glucose levels. Do higher concentrations of glucose (e.g. 3-5 mM) administered hypothalamically trigger this glucoregulatory circuitry in diabetes? These experiments will allow us to ascertain whether the hyperglycemia increases the nutrient threshold required to activate hypothalamic glucose sensing mechanisms.

5) In another study, overnutrition via prolonged i.c.v. glucose infusion increase the glucose levels in hypothalamic tissue and activated ER stress, leading to the development of hypothalamic insulin and leptin resistance. What levels of glucose are achieved in the mediobasal wedge tissue after the 24 h glucose infusions? Are ER stress markers upregulated in the hypothalamus of the two glucotoxic models? Addressing these concerns will provide insight into whether hyperglycemia-induced ER stress mechanisms are inducing hypothalamic “glucose resistance” and impairing the ability of this nutrient to lower hepatic GP.

6) Although a controversial topic, the potential of altered hypothalamic glucose transport (into cells) in the presence of pronounced diabetes as a means to impair hypothalamic glucose sensing needs to be explored. Via immunoblotting or more ideally immunohistochemistry, the expression of hypothalamic glucose transporters should be determined – specifically that of GLUT1 and GLUT3, which are generally agreed upon to be the most responsible glucose transporters of the glial cells and neurons, respectively.

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