January 2010

Brain Insulin Action Regulates Hypothalamic Glucose Sensing and the Counterregulatory Response to Hypoglycemia

Kelly Diggs-Andrews
Washington University in St. Louis

WASHINGTON UNIVERSITY

Division of Biology and Biomedical Sciences

Molecular Cell Biology

Dissertation Examination Committee:

Simon J Fisher, Chair

Thomas Baranski

Paul Hruz

Steven Mennerick

Kelle Moley

Mark Sands

BRAIN INSULIN ACTION REGULATES HYPOTHALAMIC GLUCOSE SENSING AND THE COUNTERREGULATORY RESPONSE TO HYPOGLYCEMIA

by

Kelly Annette Diggs-Andrews

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2010

Saint Louis, MO

ABSTRACT OF THE DISSERTATION

Brain Insulin Action Regulates Hypothalamic Glucose Sensing and the Counterregulatory Response to Hypoglycemia

by

Kelly Annette Diggs-Andrews

Doctor of Philosophy in Biology and Biomedical Sciences

(Molecular Cell Biology)

Washington University in St. Louis, 2010

Dr. Simon J. Fisher, Chairperson

The brain is the primary organ that senses blood glucose levels and initiates a stress response when blood glucose levels are too low (hypoglycemia). Insulin-dependent people with Type 1 diabetes (T1DM) have an impaired ability to sense hypoglycemia and an impaired ability to activate this counterregulatory response (CRR) to hypoglycemia. As a result, T1DM are at a greater risk of experiencing insulin induced severe hypoglycemic episodes, which can result in seizures, brain damage, or even death. Since hypoglycemia is a major barrier that limits intensive blood glucose control, important research initiatives are needed to prevent or reduce the burden of hypoglycemia for people with Type 1 diabetes, specifically by defining the "mechanisms and modulators" of brain glucose sensing. The experiments in this thesis were designed to investigate the role and mechanism by which insulin may regulate brain glucose sensing. Recent evidence suggests that insulin acts in the brain to regulate glucose homeostasis, central

nervous system (CNS) glucose sensing, and the CRR to hypoglycemia, but the site and method of CNS insulin action are still unknown. This study 1) investigated whether insulin acts on hypothalamic neurons to regulate brain glucose sensing and 2) ascertained how insulin regulates glucose sensing by evaluating its effects on key glucose sensors and CNS glucose uptake. Taking advantage of a genetic mouse model that chronically lacks CNS insulin action (the neuronal insulin receptor knockout "NIRKO" mouse), this report assessed whether CNS insulin signaling regulates the brain's ability to detect and respond to hypoglycemia by analyzing glucose counterregulation and neuronal activation in response to hypoglycemia. Further, to clarify a mechanism of CNS insulin action, this study assessed whether insulin regulates key glucose sensors and/or CNS glucose uptake by examining the expression patterns of key glucose sensing proteins, including glucose transporters (GLUTs) and glucokinase (GK), and measuring regional brain glucose utilization. Understanding how the brain regulates the counterregulatory response to hypoglycemia is critical to devise therapies to combat severe hypoglycemia in diabetic patients. Overall, this thesis provides new insights into insulin's role in the brain to regulate CNS glucose sensing and the counterregulatory response to hypoglycemia.

ACKNOWLEDGMENTS

I would like to especially thank my advisor Dr. Simon J Fisher for his guidance and support throughout my thesis work. I am eternally grateful for the opportunity to pursue research in his laboratory and for his commitment and dedication to my research and education.

To all my fellow colleagues in the Fisher lab, especially Dorit Daphna-Iken, Xuezhao (Shanna) Zhang, Erwin Puente, Candace Reno, Julie Silverstein, Tariq Tanoli, Adam Bree for their incredible assistance and support.

To Dr. Moley, Dr. Hruz, Dr. Mennerick, Dr. Sands, and Dr. Baranski, for their guidance and advice as members of my thesis committee.

To R. Perez for his technical expertise with animal surgeries and cannulations.

Thanks also to Dr. C.R. Kahn who graciously supplied the NIRKO mice.

To Drs. V. Routh and Z. Song for performing the electrophysiological patch clamps.

To Drs. P. Cryer and K. Jethi for performing the catecholamine determinations.

To Dr. T. Coleman for his help with the microscope and cryostat.

To everyone in the Molecular Cell Biology Program for their guidance and support.

For funding to conduct research outlined in this thesis, I would like to acknowledge the Juvenile Diabetes Research Foundation, the National Institutes of Health (NIH) DK073683, Washington University's Diabetes Research and Training Center (DK020579) and Nutrition Obesity Research Center (P30DK056341). I would also like to acknowledge graduate student support from the National Institutes of Health (NIH) (F31DK084813), William H. Danforth Scholarship, and the Chancellor's Graduate Fellowship Program.

Finally, I dedicate this thesis to my most avid supporters—my family. To my husband Nicholas Andrews for his love, patience, and continuous encouragement. To my mother Deann Diggs and the loving memory of my grandmother Annette Taylor for their unwavering belief in my potential and genuine pride in my accomplishments. To my newest inspiration Miranda Nichole Andrews for providing additional motivation, commitment, and fervor to complete this journey.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
ACTH	adenocorticotrophin hormone
AMPK	adenosine monophosphate (AMP)-activated protein kinase
ANOVA	analysis of variance
ANS	autonomic nervous system
ARC	arcuate nucleus of the hypothalamus
BBB	blood-brain-barrier
BSA	bovine serum albumin
CDC	Center of Disease Control and Prevention
CFTR	cystic fibrosis transmembrane regulator
CNS	central nervous system
CON	control
Cre	mice that express Cre recombinase under the nestin promoter
CRH	corticotrophin releasing hormone
CRR	counterregulatory response to hypoglycemia
DCCT	Diabetes Control and Complications Trial
DG	deoxyglucose
DG-6P	deoxyglucose 6-phosphate
ELISA	enzyme-linked immunosorbent assay
G	glucose
GABA	gamma aminobutyric acid
GE	glucose excited
GI	glucose inhibited
GK	glucokinase
GLUT	facilitative glucose transporter
GSN	glucose sensing neuron
HGP	hepatic glucose production
HPA	hypothalamic-pituitary-adrenal
ICV	intracerebroventricular
IP	intraperitoneal
IR	insulin receptor or input resistance, as indicated
IV	intravenous
K_{ATP}	ATP-sensitive potassium channel
Lox	mice with both insulin receptor alleles floxed
MBH	mediobasal hypothalamus
MP	membrane potential
Na_3VO_4	sodium orthovanadate
NE	norepinephrine

NIRKO	neuronal specific insulin receptor knockout
NO	nitric oxide
NTS	nucleus of the solitary tract
PBS	phosphate buffered saline
PI3K	phosphatidylinositol-3-kinase
PMSF	phenylmethylsulfonyl fluoride
PVN	paraventricular nucleus of the hypothalamus
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VMH	ventromedial nucleus of the hypothalamus

THESIS INTRODUCTION

Diabetes

Glucose homeostatic mechanisms exist to maintain stable blood glucose levels. When mechanisms controlling whole-body glucose homeostasis are dysregulated, pathophysiologic consequences, including diabetes, can occur (1;2). Diabetes is a classification of metabolic diseases characterized by chronically elevated blood glucose levels (hyperglycemia). Diabetes develops predominately from impairments in the action and/or production of the key regulatory hormone, insulin. Insulin is an endocrine hormone secreted by the pancreas that stimulates glucose uptake into peripheral tissues and suppresses liver glucose production in order to reduce elevated blood glucose levels. For patients with type 1 diabetes (T1DM), autoimmune destruction of the insulin-producing pancreatic β-cells results in insulin deficiency. In type 2 diabetic patients (T2DM), insulin-responsive tissues (i.e. muscle and fat) become insensitive to the actions of endogenous insulin, causing insulin resistance. Overt diabetes develops in type 2 diabetes when pancreatic β-cells fail to secrete enough insulin to compensate for the degree of insulin resistance. For both type 1 and type 2 diabetic patients, the lack of appropriate insulin release and/or signaling results in chronically elevated blood glucose levels.

Due to hyperglycemia, patients with diabetes often suffer from a number of macro- and microvascular complications, including retinopathy, hypertension, renal failure, cardiovascular disease, etc., leading to increased morbidity/mortality and overall reducing

quality of life (3-5). Diabetes is a major health problem in the United States, with nearly 25 million citizens (10% of the total population) affected with diabetes (6). Diabetes is a chronic debilitating disease requiring years of treatment. As a result, diabetes causes an undue burden on the United States healthcare system and costs nearly $200 billion annually to treat cases of diabetes (6). This crisis is projected to worsen over the next 40 years as a recent Center for Disease Control and Prevention (CDC) study reported that the number of Americans afflicted with diabetes may triple by 2050 (7).

To limit major complications associated with diabetes, diabetic patients are often prescribed a drug regimen, along with lifestyle modifications, to help correct hyperglycemia and to improve insulin sensitivity. Insulin secretagogues and sensitizers, including sulfonylureas and thiazolidinediones, are commonly used oral medications that stimulate endogenous insulin release or enhance insulin action on peripheral tissues. All patients with type 1 and many patients with advanced type 2 diabetes require exogenous insulin therapy to correct glucose levels. An unfortunate consequence and major complication of insulin therapy is the increased risk of dropping glucose levels too low, causing hypoglycemia.

Hypoglycemia

As an obligate glucose consumer, the brain is critically dependent on glucose supply for normal function. Therefore, maintaining blood glucose levels within a tight physiological range is essential to achieve whole-body glucose homeostasis. For patients with diabetes, many of the glucose lowering drugs (including insulin) used to treat

hyperglycemia can reduce blood glucose levels below the physiological range and induce a state of hypoglycemia (low blood sugar). In response to hypoglycemia, the brain coordinates a hierarchical stress (counterregulatory) response to rapidly restore normal blood sugar levels (Figure 1). The normal counterregulatory response (CRR) initiates a primary systemic hormone response, involving a reduction in endogenous insulin secretion from the pancreatic β-cells and an increase in glucagon secretion from the pancreatic α-cells. The counterregulatory stress response also involves activation of critically important glucose sensing areas in the hypothalamus, which respond to hypoglycemia by activating sympathetic efferent signals to the adrenal medulla to rapidly release epinephrine (8-11). The hypothalamic release of corticotrophin releasing hormone (CRH) mediates release of adrenocorticotrophin hormone (ACTH) systemically to trigger cortisol release from the adrenal cortex, which may be an important counterregulatory response in the setting of more prolonged hypoglycemia. There is also the development of autonomic symptoms which are mediated by the release of catecholamines (norepinephrine and epinephrine). These symptoms include tremor, anxiety, and palpitations which occur as a result of norepinephrine and epinephrine being released from sympathetic postganglionic neurons and/or the adrenal medulla and sweating, hunger, and paresthesias which occur as a result of the release of acetylcholine from sympathetic postganglionic neurons. The awareness of these autonomic symptoms alert the hypoglycemic individual to take corrective action (i.e., consume sugar) to correct the hypoglycemic episode. When blood glucose levels drop below 50mg/dL, neuroglycopenic symptoms develop (impaired cognition, weakness, confusion, lethargy, coma and death).

Many patients with diabetes experience frequent episodes of severe, temporarily disabling hypoglycemia (12;13). Severe hypoglycemia is associated with several detrimental symptoms, including seizures, coma, brain damage, and even death (13). Hypoglycemia is caused by relative insulin excess and the condition is made worse in the setting of impaired counterregulation. There are many reasons why patients with both Type 1 and long-standing Type 2 diabetes have defective counterregulatory responses to hypoglycemia and are therefore susceptible to more frequent and more severe episodes of hypoglycemia. Several components of the counterregulatory response (specifically the suppression of endogenous insulin secretion and stimulation of glucagon and catecholamine release) are blunted or completely absent in diabetic patients (14-17). Accompanied by impaired counterregulation, intensive insulin therapy markedly increases the risk of severe hypoglycemia in type 1 diabetic patients (3). As a result, hypoglycemia stands as the rate-limiting step for tight glycemic management in diabetic patients (18). This barrier of hypoglycemia prevents people with diabetes from achieving the known benefits associated with intensive blood sugar control. To improve glycemic management of diabetic patients, a better understand of hypoglycemic counterregulation is needed. By understanding how the brain regulates the counterregulatory response to hypoglycemia and identifying key glucose sensing systems in the central nervous system, it will allow for the appropriate identification of clinical therapeutic strategies to combat severe hypoglycemia in patients with diabetes.

Insulin in the CNS

Once considered insulin-insensitive, insulin action in the brain has become an area of intense investigation. Insulin and insulin receptors have been localized throughout the central nervous system (CNS) (19-23) and act diversely to affect feeding, body weight, reproduction, and neurotransmission (24-31). More importantly, CNS insulin action has been implicated as a major contributor to the counterregulatory response to hypoglycemia (32;33). Insulin has been shown to cross the blood-brain barrier to directly activate the autonomic nervous system (34-38). Furthermore, in studies where insulin was selectively directed to the brains of hypoglycemic dogs, the counterregulatory response was greatly augmented (39;40). Conversely, genetic ablation of CNS insulin action (as seen in the NIRKO model) resulted in impaired counterregulation (41). Also, insulin treatment has been shown to increase the action potential frequency of glucose responsive neurons during hypoglycemia (42). These studies suggest that insulin operates within the CNS to regulate hypoglycemic counterregulation.

It is unclear how insulin modulates the counterregulatory response in the brain, but this thesis approaches deciphering the mechanism by analyzing similarities between mechanisms of insulin action in peripheral glucose sensors. Most interestingly, insulin receptors demonstrate overlapping expression with key glucose sensing proteins in key regions of the hypothalamus, specifically glucose transporters (GLUTs) and glucokinase (GK). As a result, it is postulated that brain insulin action may mediate its effects on glucose sensing via regulation of glucose transporters (GLUTs) and/or glucokinase (GK).

This thesis analyzes insulin's influence over these proteins in order to gain a better understanding of insulin's role in CNS glucose sensing.

Central Nervous System (CNS) Glucose Sensing

In order to detect slight fluctuations in glucose levels, the body is equipped with numerous glucose sensors located throughout the body, including the gut, portal vein, pancreas, and brain (43-47). Due to its high glucose demands, many studies have confirmed that the brain serves as the primary glucose sensor and the hypothalamus functions as the integrative center of glucose sensing (8-11). Within the hypothalamus, glucose sensing predominates in discrete regions, namely the ventromedial hypothalamus (VMH), arcuate nucleus (ARC), and paraventricular nucleus (PVN). Of which, the region most studied is the VMH. Several studies abolishing VMH function have demonstrated its role in CNS glucose sensing. Specifically, creating lesions or chemical destruction of the VMH deregulated peripheral glucose homeostasis (11;48-50).

While all neurons metabolize glucose as a fuel source, a set of critically important neurons within the VMH and brain stem sense and respond to changes in blood sugar. By coupling their neuronal activity (i.e., firing rate) in response to their metabolism of glucose, these specialized neurons have been classified as "glucose sensing" neurons and not merely as "glucose using" neurons. These glucose sensing neurons are categorized as either glucose-excited neurons (GE), which increase their firing rate when extracellular glucose concentrations are elevated or glucose-inhibited neurons (GI), which are activated by decreases in extracellular glucose concentration or by cellular glucoprivation

(50-52). Both neuronal populations are widely distributed throughout the brain, but display distinct enrichment in hypothalamic and hindbrain regions classically associated with neuroendocrine regulation, energy homeostasis, and nutrient metabolism.

The mechanism of CNS glucose sensing is not clearly defined but appears to be dependent on the metabolism of glucose within key glucose sensing neurons (GSN). Reports indicate that GSN located in the ventromedial hypothalamus (VMH) contain critical glucose sensors that mediate many of the counterregulatory responses to hypoglycemia (8-11). To better understand CNS glucosensing, researchers have drawn clues from more widely studied glucose sensing mechanisms, including the classic glucose sensing system—pancreatic β-cell (Figure 2). Glucose sensing in the β-cell is a dose-dependent process that joins elevations in glucose uptake/metabolism to insulin release. Analogous to the β-cell, GE neurons enhance their firing activities when extracellular glucose rises. Furthermore, the existences of key β-cell glucose sensing proteins, including glucose transporters (GLUTs), glucokinase (GK), and ATP-sensitive K^+ channels (K_{ATP}), have been reported in hypothalamic nuclei (44;52-54). Therefore, it is speculated that these proteins may have a broadened role to include CNS glucose sensing. The mechanism of glucose sensing in GI neurons is even less characterized. GI neurons contain many of the same glucose sensing proteins as GE neurons, including glucose transporters and glucokinase (55). However, the mechanism linking increases in glucose concentrations to suppression of neuronal firing may involve additional players, including regulation of the sodium-potassium channel activity and/or ATP-sensitive chloride channels, to facilitate glucose-mediated silencing (55-57). In addition to

regulating electrical activity, glucosensing neurons may also mediate their effects through nutrient sensors or the release of neurotransmitters in the brain, including GABA (inhibitory) and glutamate (excitatory) (58;59).

Glucose sensing neurons also play an important role in the detection of hypoglycemia and the initiation of the counterregulatory response; therefore changing any key glucose sensor may result in altered hypothalamic glucose sensing and impaired initiation of the counterregulatory response. As a consequence, these key glucose sensing proteins, among other hypothalamic nutrient sensors, may serve as therapeutic targets to combat hypoglycemia.

Facilitative Glucose Transporters (GLUTs)

Glucose transport is a fundamentally important process in energy metabolism. The body needs to maintain a relatively constant supply of blood glucose to sustain brain metabolism and to maintain transport of glucose to peripheral tissues for utilization and storage (60). Facilitative glucose transporters (GLUTs) support the passive influx of glucose into cells. Several GLUTs have been identified in the brain (61;62). Of these, GLUT1 and GLUT3 are the predominate isoforms expressed in the brain (63). Although found in lower quantities, GLUT2 is expressed in key glucose sensing regions (64;65). Similar to its role in β-cell glucosensing, mounting evidence suggests that GLUT2 may be involved in hypothalamic glucosensing (66;67). GLUT4, the insulin-responsive glucose transporter, is expressed along with the insulin receptor in many important brain regions involved in glucose sensing (68;69).

Glucokinase (GK)

Glucokinase (GK) is a hexokinase that regulates glucose phosphorylation and metabolism in the β-cell and liver (44;55). Similar to its peripheral functions, GK may also play a role in the hypothalamus to mediate glucose sensing (70). Demonstrated by both *in-situ* hybridization and RT-PCR, glucokinase is expressed in several hypothalamic regions (55;71). Further, modulating hypothalamic GK activity has been shown to regulate the counterregulatory response to hypoglycemia. Specifically, studies were performed to inhibit hypothalamic GK activity by injecting the glucokinase inhibitor alloxan or an adenovirus expressing a GK short hairpin RNA (to chronically reduce GK hypothalamic expression) into the ventricle that bathes the hypothalamus (intracerebroventricular—ICV). Results of these studies show that GK inhibition selectively enhanced the epinephrine response to hypoglycemia (71). Conversely, enhancing GK activity with a glucokinase activator (Compound A) markedly suppressed the counterregulatory response (71).

Ion channels: ATP-sensitive K^+ (K_{ATP}) and ATP-sensitive Cl^- channels

ATP-sensitive K^+ channels (K_{ATP}) may provide a link between glucose metabolism and electrical activity in glucose sensing neurons. Several K_{ATP} channel subunits (Kir6.2, SUR1, and SUR2) have been localized in key glucosensing regions of the hypothalamus (72;73). The involvement of the K_{ATP} channel in central glucose sensing and counterregulation has been also shown by several *in vivo* studies. Either ICV or direct VMH injection of the channel inhibitor glibenclamide blocks the counterregulatory response to acute hypoglycemia or anti-metabolite-induced glucopenia (74). K_{ATP}

channel inactivation (via Kir6.2 gene knockout) also leads to an impaired counterregulatory response to hypoglycemia. Further, K_{ATP} inactivity correlates to suppressed glucose-regulated firing activity of VMH neurons (75). In contrast, activation of ATP-sensitive K^+ channels in the VMH with channel opener diazoxide amplifies counterregulatory hormone responses to hypoglycemia in normal and recurrently hypoglycemic rats (76).

ATP-sensitive Cl^- channels are postulated to play an important role in the electrical activity of GI neurons. In response to decreased glucose levels, neuronal ATP levels are reduced, the Cl^- channel becomes inactivated, and the GI neuron becomes depolarized. This form of ATP-activated Cl^- conductance has been demonstrated in key glucose sensing systems, namely the pancreatic islet cells (77). Further, an example of these Cl^- channels, cystic fibrosis transmembrane regulator (CFTR), CFTR mRNA and protein has been localized in human and rat hypothalamus (78-80), suggesting a role of these channels in central glucose sensing mechanisms.

Other Glucose Sensing Players: AMPK and Neurotransmitters

AMP-activated protein kinase (AMPK) is an important nutrient sensor present in the brain. When nutrient supplies are low, there is an increase in cellular AMP, which leads to allosteric activation of AMPK. AMPK activity stimulates catabolic pathways and suppresses anabolic pathways to restore energy homeostasis. In the hypothalamus, AMPK activity is increased in response to insulin-induced hypoglycemia and reduced by peripheral or ICV glucose infusion (81;82). AMPK may also regulate the

counterregulatory response to hypoglycemia. Enhancing VMH-AMPK activity with an activator, AICAR, markedly increases endogenous glucose production during a hypoglycemic clamp and improved the counterregulatory response in animals with defective counterregulation (83;84). Conversely, blocking hypothalamic AMPK with compound C, expressing a dominant negative form of AMPK, or reducing hypothalamic AMPK expression via RNA interference strongly reduced counterregulation to insulin-induced hypoglycemia (83).

Two major classes of neurotransmitters in the brain, GABA (inhibitory) and glutamate (excitatory), have been postulated to play a role in CNS glucose sensing (58;59). Blocking GABA receptors in the VMH stimulates counterregulatory response to hypoglycemia (59;85) while blocking glutamate release in the VMH impairs the counterregulatory response to hypoglycemia (58). These studies suggest that neurotransmission in VMH neurons provide an unappreciated role to the CNS glucose sensing mechanism. Further, modulation of neurotransmission in the brain may act as an additional clinical target to prevent hypoglycemia.

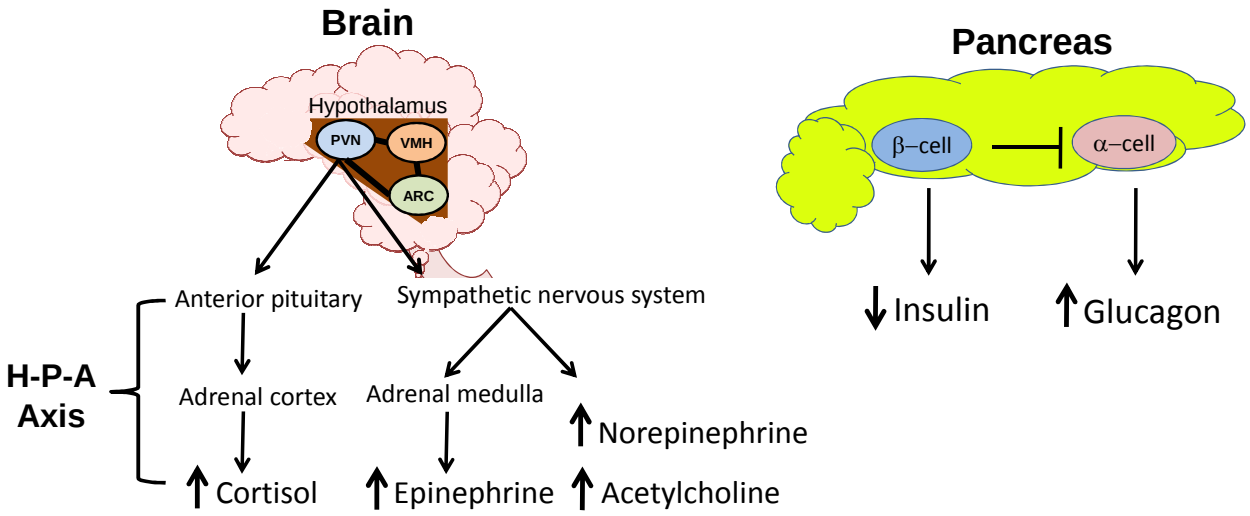

Figure 1. Glucose sensing and the physiological response to hypoglycemia.

In the normal response to hypoglycemia, the brain (esp. the hypothalamus) coordinates and triggers a stress response to restore euglycemia. This process includes stimulating the HPA axis and sympathetic nervous system to increase production of key counterregulatory hormones. Cortisol (via ACTH release) and epinephrine secretions arise from the adrenal cortex and medulla, respectively. The sympathetic nervous system also increases norepinephrine and acetylcholine release, to stimulate observable symptoms of hypoglycemia. At the level of the pancreas, endogenous insulin secretion from the β-cell is suppressed while glucagon release from the α-cell is elevated. This processes work in unison to normalize blood glucose levels.

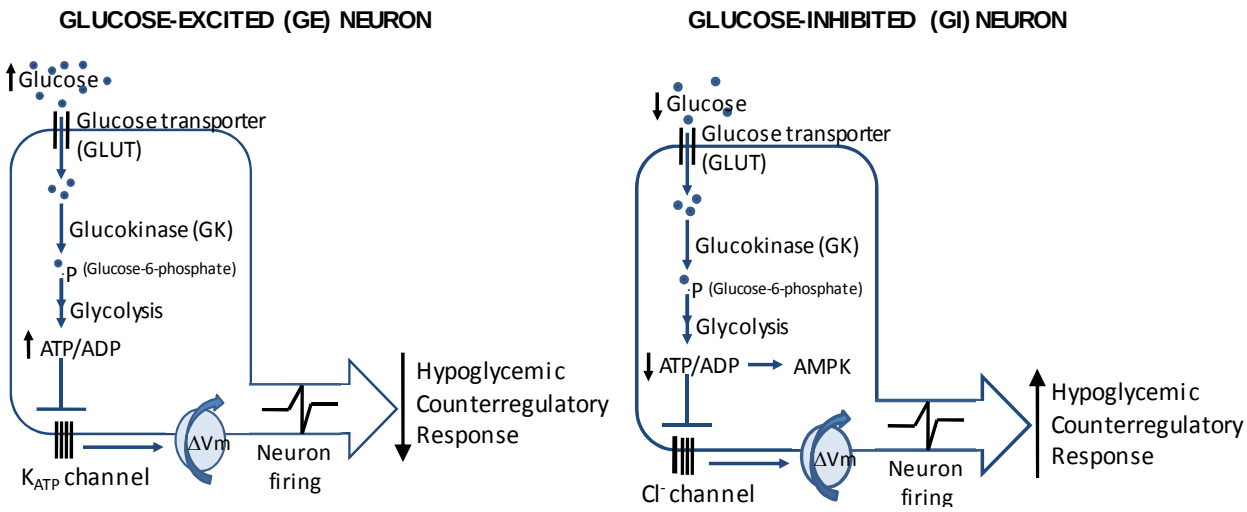

Figure 2. Proposed mechanism of glucose sensing in glucose sensing neurons.

During euglycemia or hyperglycemia, the proposed glucose-excited (GE) glucose sensing mechanism requires glucose uptake via glucose transporters (GLUTs), glucose phosphorylation by the rate-limiting enzyme glucokinase (GK), and subsequent metabolism of glucose to increase the intracellular ATP-to-ADP ratio. Following glucose metabolism, closure of ATP-sensitive K^+ channels, membrane depolarization, and neuronal firing occurs. When glucose levels fall, as seen during hypoglycemia, neuronal activity is decreased in GE neurons and glucose-inhibited (GI) neurons are activated. Similar to GE neurons, glucose sensing in GI is proposed to involve GLUTs, GK, and altered ATP-to-ADP ratio. Glucose sensing in the GI is speculated to also involve ATP-sensitive Cl^- channels and activation of the fuel sensor, AMPK, leading to initiation of the counterregulatory response to hypoglycemia. ΔVm: change in membrane potential, membrane depolarization.

CHAPTER 1. HYPOGLYCEMIC COUNTERREGULATION AND NEURONAL ACTIVATION IN A MODEL OF CHRONIC CENTRAL NERVOUS SYSTEM (CNS) INSULIN RESISTANCE

ABSTRACT

An impaired ability to sense and appropriately respond to insulin-induced hypoglycemia is a common and serious complication faced by insulin-treated diabetic patients. This study tests the hypothesis that insulin acts directly in the brain to regulate critical glucose sensing neurons in the hypothalamus to mediate the counterregulatory response to hypoglycemia. To delineate insulin actions in the brain, neuron-specific insulin receptor knockout (NIRKO) mice and littermate controls were subjected to graded hypoglycemic (100, 70, 50, and 30 mg/dL) hyperinsulinemic (20 $mU.kg^{-1}.min^{-1}$) clamps. Subsequently, counterregulatory responses and hypothalamic neuronal activation (with transcriptional marker *c-fos*) were measured. Additionally, electrophysiological activity of individual glucose-inhibited (GI) neurons was measured. NIRKO mice revealed a glycemia-dependent impairment in the sympathoadrenal response to hypoglycemia and demonstrated markedly reduced (70%) hypothalamic *c-fos* activation in response to hypoglycemia. Further, GI neurons in the ventromedial hypothalamus of NIRKO mice displayed significantly blunted glucose responsiveness to decreases in extracellular glucose from 2.5 to 0.5mM (membrane potential and input resistance responses were blunted 66% and 80%, respectively). We conclude that insulin acts in the brain to regulate the counterregulatory response to hypoglycemia by directly altering glucose-mediated activation of hypothalamic neurons and shifting the glycemic levels necessary to elicit a normal sympathoadrenal response to hypoglycemia.

INTRODUCTION

Intensive insulin therapy markedly increases the risk of severe hypoglycemia in people with type 1 (86) and type 2 (4) diabetes. Thus, hypoglycemia is the rate-limiting step for tight glycemic management in diabetic patients. In response to hypoglycemia, glucose sensors in the central and peripheral nervous system coordinate efferent autonomic responses resulting in the release of key counterregulatory hormones—glucagon, norepinephrine, epinephrine, and cortisol. This coordinated response stimulates hepatic glucose output and restricts glucose utilization in order to increase blood sugar levels. Patients with diabetes often have an impaired ability to sense and respond to hypoglycemia (12;13;87) because several components of the counterregulatory response have been shown to be either absent (i.e. fall in insulin, rise in glucagon) or markedly blunted (i.e. the sympathoadrenal response) (16;17).

While hypoglycemia is caused by absolute or relative insulin excess, the role of insulin in regulating the counterregulatory response is unclear. Studies have demonstrated that increased insulin levels may augment (32;88-90), diminish (91), or not change (33;92-94) the sympathoadrenal response to hypoglycemia. Given recent evidence indicating that insulin acts in the brain (28), some studies have investigated whether insulin's putative actions in regulating the counterregulatory response might be mediated via actions in the central nervous system. Again, conflicting reports suggest that insulin may act centrally to enhance (39-41), reduce (95;96) or not alter (97) the sympathoadrenal response to hypoglycemia. In this study, the neuronal specific insulin-receptor knockout (NIRKO) mouse model, which chronically lacks central nervous system (CNS) insulin signaling

(28;98), was used to investigate the role by which brain insulin action regulates central glucose sensing and the counterregulatory response to hypoglycemia.

MATERIALS AND METHODS

Animals. Mice homozygous for the floxed insulin receptor allele ($IR^{lox\text{-}lox}$) were bred with transgenic mice that express Cre recombinase cDNA from the rat nestin promoter to generate ($IR^{lox\text{-}lox}$:nestin-$Cre^{+/-}$) nervous system specific insulin receptor knockout (NIRKO) mice (28). Genotypes were determined by PCR of tail DNA. Unless otherwise indicated, 2-4 month old NIRKO ($IR^{lox\text{-}lox}$:nestin-$Cre^{+/-}$) and littermate control (Control, $IR^{lox\text{-}lox}$:nestin-$Cre^{-/-}$) mice were used for these experiments. All mice were housed on a 12-hour light/dark cycle and fed a standard rodent chow (Mouse Diet 9F, PMI Nutrition International, St. Louis, MO) *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Studies Committee of Washington University.

Hypoglycemic-hyperinsulinemic glucose clamps. Mice anesthetized with ketamine/xylazine (87 and 13.4 mg/kg IP) were implanted with catheters (MRE 025, Braintree Scientific Inc., Braintree, MA) into both the right internal jugular and the left carotid or femoral artery. After a 5-7 day recovery period, hyperinsulinemic ($20mU.kg^{-1}.min^{-1}$) hypoglycemic clamps were performed in 5-hour fasted, awake, unrestrained, NIRKO and control mice (n=6-9 per group). To create various degrees of hypoglycemic stress, arterial blood glucose (~2μL) was measured at ten-minute intervals as a continuous infusion (20 mU/kg/min) of human regular insulin (Humulin, Eli Lilly and Co.) in 0.1% BSA and 50% dextrose were administered. The rate of intravenous 50% dextrose infusion was variable and carefully adjusted to create equivalent levels of mild (70mg/dL), moderate (50mg/dL), and severe hypoglycemia (30mg/dL) as well as a

euglycemic (110mg/dL) control. HPLC-purified [3-^{3}H]-glucose tracer (NEN Life Science Products Inc., Boston, MA) was infused (10μCi bolus followed by 0.1μCi/min continuous infusion) for the assessment of hepatic glucose production (99). After a 90 minute basal period, three blood samples (10μL) at 10 minute intervals were taken for hepatic glucose production determination. Three blood samples were also taken during the last half hour of the clamp. An additional blood sample (130μL) was obtained for hormonal measurements (insulin, glucagon, epinephrine, norepinephrine, and corticosterone) during the basal period and at the end of the clamp. Whole-body glucose turnover was determined during the steady state from the ratio of the [^{3}H] glucose infusion rate to the measured specific activity of plasma glucose (99). Hepatic glucose production (HGP) was determined by subtraction of the glucose infusion rate from the whole-body glucose turnover (99).

Hypoglycemia-induced *c-fos* expression. Awake, 5-hour fasted NIRKO (n=4) and littermate controls (n=6) were given a single intraperitoneal (IP) injection of high dose insulin (3.0-3.5U/kg) to achieve a consistent and stable hypoglycemic insult (~30mg/dL) for 2 hours. Euglycemic controls (~110mg/dL, n=4 per group) were given an IP injection of saline. After a 2 hour duration of hypoglycemia (or euglycemia), mice anesthetized with isofluorane and transcardially perfused with 0.01 M PBS (Sigma, Saint Louis, MO) followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). The brains were immersed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose. Brain sections were then processed for *c-fos* immunostaining. Free-floating brain sections (20-30μm) containing the paraventricular nucleus (PVN) of the

hypothalamus were blocked with 30% normal goat serum diluted in 0.1% Triton-100/PBS and then incubated overnight at 4°C with *c-fos* antibody (1:2000, Ab-5, Calbiochem, San Diego, CA). Subsequently, the sections were mounted on slides and processed with biotinylated goat anti-rabbit immunoglobulin G (1:200) using the Elite ABC kit (Vector Laboratories, Burlingame, CA). As a negative control, alternative sections were incubated without primary antibodies. Anatomical landmarks were used to identify the paraventricular nucleus of the hypothalamus (100). Positively stained cells were counted by a blinded investigator. Four to six anatomically matched sections per mouse were quantified for statistical purposes.

Electrophysiological studies. Male 14-28 day old NIRKO and littermate control mice were anesthetized and transcardially perfused with ice-cold oxygenated perfusion. On the day of the experiment, mice were anesthetized and transcardially perfused with ice-cold oxygenated (95% O_2/5% CO_2) perfusion. Sections (350μm) through the hypothalamus were made on a vibratome (Vibroslice; Camden Instruments). The brain slices were maintained at 34°C in oxygenated high-Mg^{2+} low-Ca^{2+} artificial cerebrospinal fluid for 30 minutes and then transferred to normal oxygenated ACSF (2.4 mmol/l $CaCl_2$ and 1.3 mmol/l $MgCl_2$) for the remainder of the day. Viable neurons were visualized and studied under infrared differential-interference contrast microscopy using a Leica DMLS microscope equipped with a 40× long working-distance water-immersion objective. Current-clamp recordings (standard whole-cell recording configuration) from neurons in the VMH were made using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) as previously described (101;102). During recording, brain slices were perfused at

10mL/min with normal oxygenated ACSF. Borosilicate pipettes (1–3 MΩ; Sutter Instruments, Novato, CA) were filled with an intracellular solution containing (in mmol/l): 128 K-gluconate, 10 KCl, 4 KOH, 10 HEPES, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, and 2 Na_2ATP; pH 7.2. Osmolarity was adjusted to 290–300 mOsm with sucrose. Input resistance was calculated from the change in membrane potential in response to small 500-msec hyperpolarizing pulses (−10 to −20pA) given every 3 seconds. Briefly, hyperpolarizing current pulses varying from −10 to −120 pA were applied at 10- or 20-pA increments. At each increment, four pulses were applied. The duration of each pulse was 500 ms, and pulses were applied every 3 seconds. The membrane potential response during the last 5 ms of each pulse (when $dV/dt = 0$) was measured, and the average of the four pulses at each amplitude was calculated. The membrane potential response was measured only after the membrane response to altered extracellular glucose had stabilized, and this value was compared with controls that were measured immediately before changing extracellular glucose. Individual glucose inhibited (GI) neurons were identified as those neurons that increased their action potential frequency, membrane potential, and input resistance with decreases in extracellular glucose from 2.5 to 0.1mM.

Plasma Assays. Blood glucose was measured by a glucometer (Becton, Dickinson and Company, Franklin Lakes, NJ) while plasma glucose was assayed by the glucose oxidase method and a spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Radioimmunoassays were performed for glucagon (LINCO Research, Inc., St. Charles, MO) and corticosterone (ICN Biomedicals, Inc., Costa Mesa, CA). Insulin was assayed by enzyme-linked immunosorbent assay (ELISA) (Chrystal Chem. Inc., Downers Grove

IL). Plasma epinephrine and norepinephrine were measured with a single isotope derivative (radioenzymatic) method (103).

Statistics. All values are presented as the mean ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$, as determined by Student's *t* test.

RESULTS

Brain Insulin Action is Necessary for Full Sympathoadrenal Response to Hypoglycemia

To characterize the counterregulatory response to hypoglycemia in NIRKO mice, a series of hyperinsulinemic glucose clamps were performed. Blood glucose was clamped at 110, 70, 50 and 30mg/dL in control and NIRKO mice to induce various degrees of hypoglycemia (mild=70mg/dL, moderate=50mg/dL, and severe hypoglycemia=30mg/dL) or no hypoglycemia (euglycemic clamp=110mg/dL) (Figure 3). In response to insulin infusion, plasma insulin levels were similarly elevated in NIRKO and control mice (Figure 4). Severe hypoglycemia (30mg/dL) resulted in a 6-fold increase in glucagon levels and ~60% increase in corticosterone levels, but these increases were similar in both groups (Figures 4 and 5). Norepinephrine levels trended lower in NIRKO mice during moderate (50mg/dL) and severe (30mg/dL) hypoglycemia, but the difference did not reach significance (Figure 5).

In response to high dose insulin ($20mU.kg^{-1}.min^{-1}$), hepatic glucose production was completely inhibited during the hyperinsulinemic clamp at glycemic levels of 100, 70, and 50mg/dL. During severe hypoglycemia (30mg/dL), hepatic glucose production rose significantly; however, the rise in hepatic glucose production was significantly blunted in NIRKO mice (Figure 6). The epinephrine response was significantly impaired in NIRKO mice during moderate (50mg/dL) and severe (30mg/dL) hypoglycemia (Figure 7). The epinephrine response was highly correlated to glycemia levels in both control (R^2=0.76) and NIRKO (R^2=0.75) mice. Based on the shift in the hypoglycemia-epinephrine response curve (Figure 7-inset), the data was fitted to a four parameter logistic curve and

EC_{50} values were determine for control (46.5±3.6 mg/dL) and NIRKO (34.5±11.7mg/dL) mice.

Absent CNS Insulin Action Impairs Hypothalamic Neuronal Activation to Hypoglycemia

In order to assess the brain's response to hypoglycemia, *c-fos*-based functional mapping was used to demonstrate activated neurons and functional circuits that respond to hypoglycemic stress (104). Euglycemic (~110mg/dL) controls displayed low *c-fos* expression in the hypothalamus. In response to insulin-induced hypoglycemia (31.5±3.1mg/dL), both NIRKO and control mice markedly increased *c-fos* expression within the paraventricular nucleus (PVN) of the hypothalamus. However, NIRKO animals showed a 3-fold impairment in *c-fos* activation as compared to controls (Control: 99±16 vs. NIRKO: 31±5, $p<0.01$) (Figure 8).

Intact Neuronal Activation and Physiological Response to Extreme Glucose Deprivation

To determine whether NIRKO mice displayed an absolute impairment in their ability to respond to hypoglycemic insults, NIRKO mice were subjected to extreme glucose deprivation (blood glucose level below 30mg/dL) to evaluate their ability to fully stimulate a sympathoadrenal response and/or activate neurons in the PVN. Similar to the severe hypoglycemic insult, both NIRKO and control mice significantly stimulated neuronal activity as well as catecholamine responses to extreme glucose deprivation. However, when glucose levels were kept below 30mg/dL, NIRKO mice elevated *c-fos* expression and catecholamine levels similar to those seen in control mice (Figure 9).

Impaired Glucose Sensing in Individual Glucose-Inhibited Neurons

Whole-cell current clamp recordings were performed to evaluate the glucose sensitivity of individual glucose-inhibited (GI) neurons in the ventromedial hypothalamus (VMH) (Figure 10). As expected for VMH GI neurons bathed in sufficient 2.5mM glucose, action potentials in this basal state were absent in recordings from both control and NIRKO mice. There were also no group differences in membrane potential (MP) or input resistance (IR) in 2.5mM glucose (Control: MP -57±4 mV, IR 1209±272 MΩ; NIRKO: MP -59±3 mV, IR 1016±162 MΩ). Further, no group differences were observed in GI neurons in response to a maximal glucose decrease from 2.5 to 0.1mM. In contrast, GI neurons in NIRKO mice had a significantly impaired change in membrane potential and input resistance (66% and 80% impairment, respectively) in response to a glucose decrease from 2.5 to 0.5mM (Figure 11).

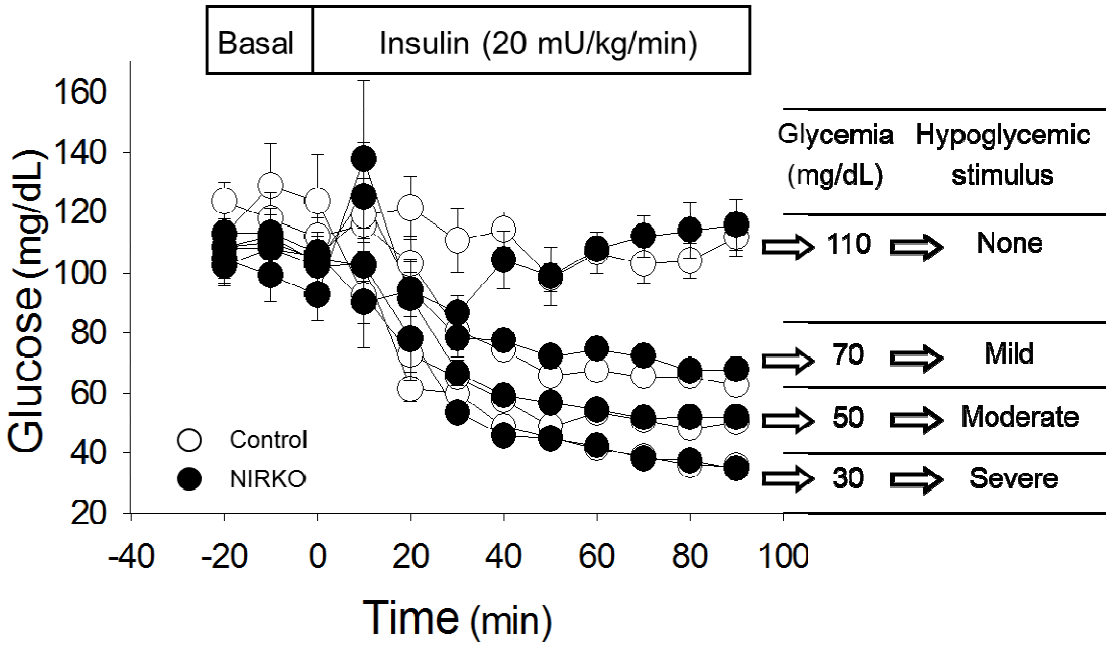

Figure 3. Glucose profile from hyperinsulinemic, graded hypoglycemic clamp protocol. Blood glucose levels are shown for NIRKO (closed circles) and control (open circles) mice (n=6-8 mice per group). After basal sampling, insulin was infused ($20mU.kg^{-1}.min^{-1}$) and blood glucose levels were measured at ten-minute intervals via arterial sampling. By adjusting the rate of intravenous glucose infusion, glucose levels were carefully lowered, then clamped at matched, pre-determined glycemic levels (110, 70, 50, and 30mg/dL) to create various degrees of hypoglycemic stress (none, mild, moderate, and severe, respectively).

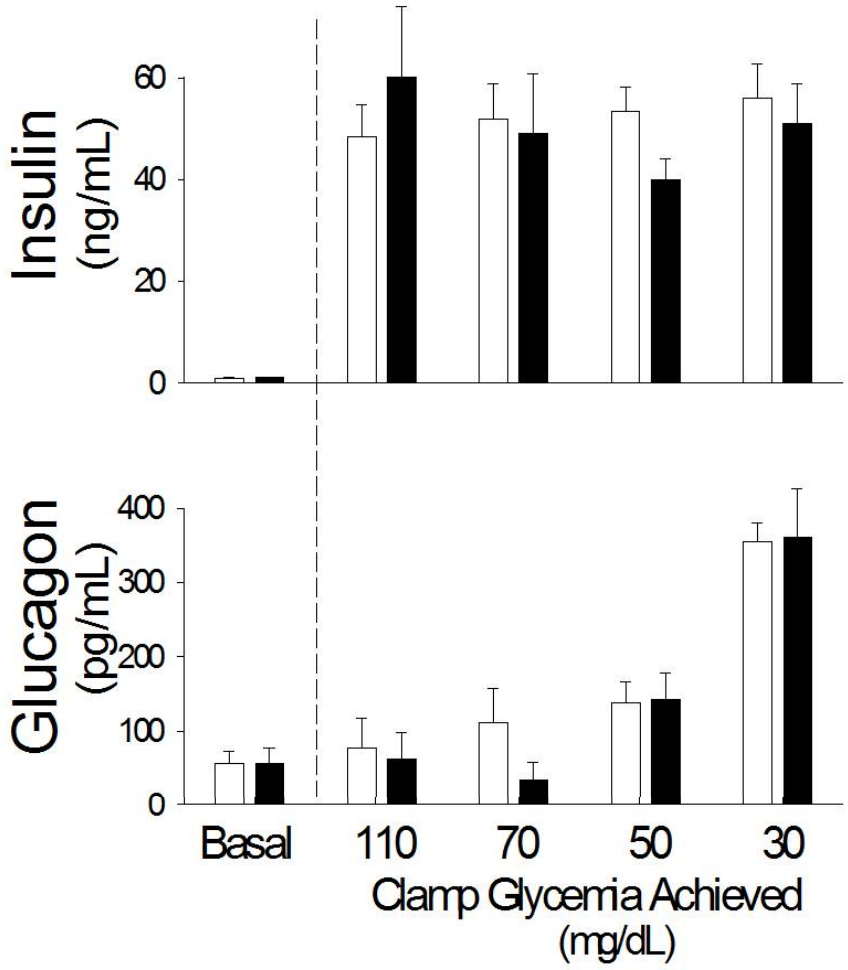

Figure 4. Pancreatic hormone levels during graded hyperinsulinemic glucose clamps. Results are shown for NIRKO (closed bars) and control (open bars) mice (n=6-8 mice per group). **Top:** By experimental design, insulin levels rose markedly, and similarly between groups during the hyperinsulinemic clamps. **Bottom:** Glucagon levels in both treatment groups rose significantly above basal levels ($p<0.05$) during moderate and severe hypoglycemia but similarly between treatment groups.

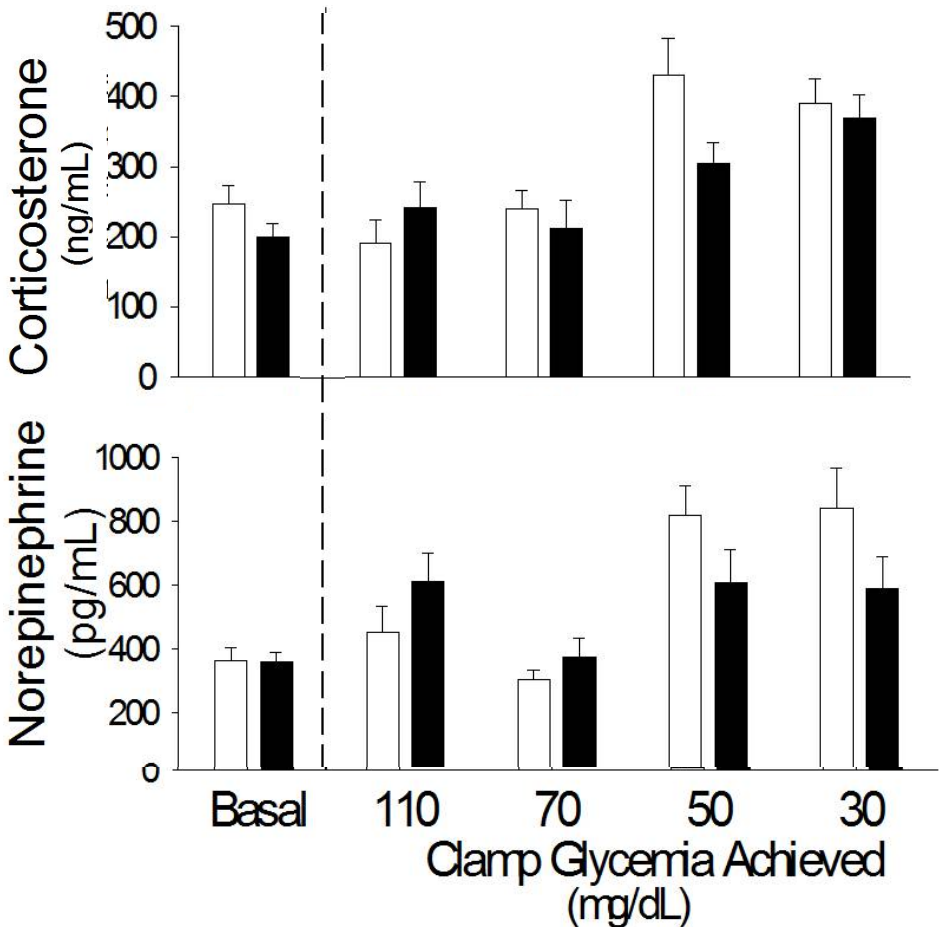

Figure 5. Corticosterone and norepinephrine levels during graded hyperinsulinemic glucose clamps. Results are shown for NIRKO (closed bars) and control (open bars) mice (n=6-8 mice per group). **Top:** Corticosterone levels in both treatment groups rose significantly above basal levels ($p<0.05$) during moderate and severe hypoglycemia but similarly between treatment groups. **Bottom:** Norepinephrine levels in both treatment groups rose significantly higher from the basal period during moderate and severe hypoglycemia but there was no difference between NIRKO and control responses.

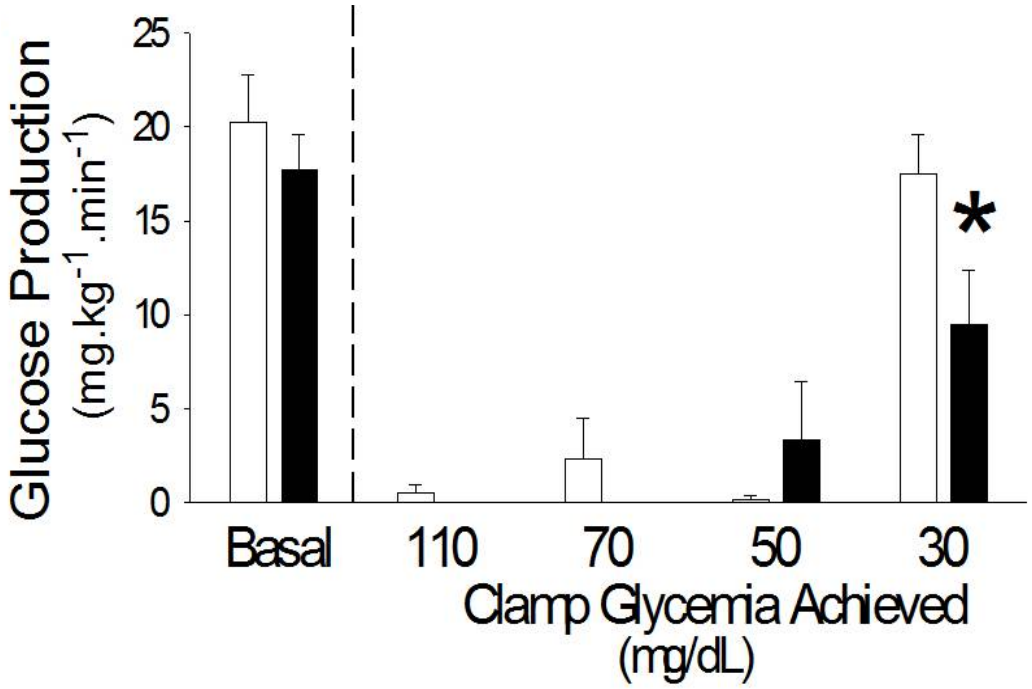

Figure 6. Hepatic glucose production during graded hyperinsulinemic glucose clamps. Results are shown for NIRKO (closed bars) and control (open bars) mice (n=6-8 mice per group). Hepatic glucose production (HGP) before and during the hyperinsulinemic clamps was determined by tracer dilution methodology. Hepatic glucose production (HGP), in the basal period prior to insulin infusion, was the same in control and NIRKO mice. During the hyperinsulinemic glucose clamps at mild and moderate hypoglycemia, HGP was suppressed. In spite of the hyperinsulinemia, during severe hypoglycemia (30mg/dL), HGP rose significantly, but remained lower in NIRKO as compared to control mice. (*$p<0.05$)

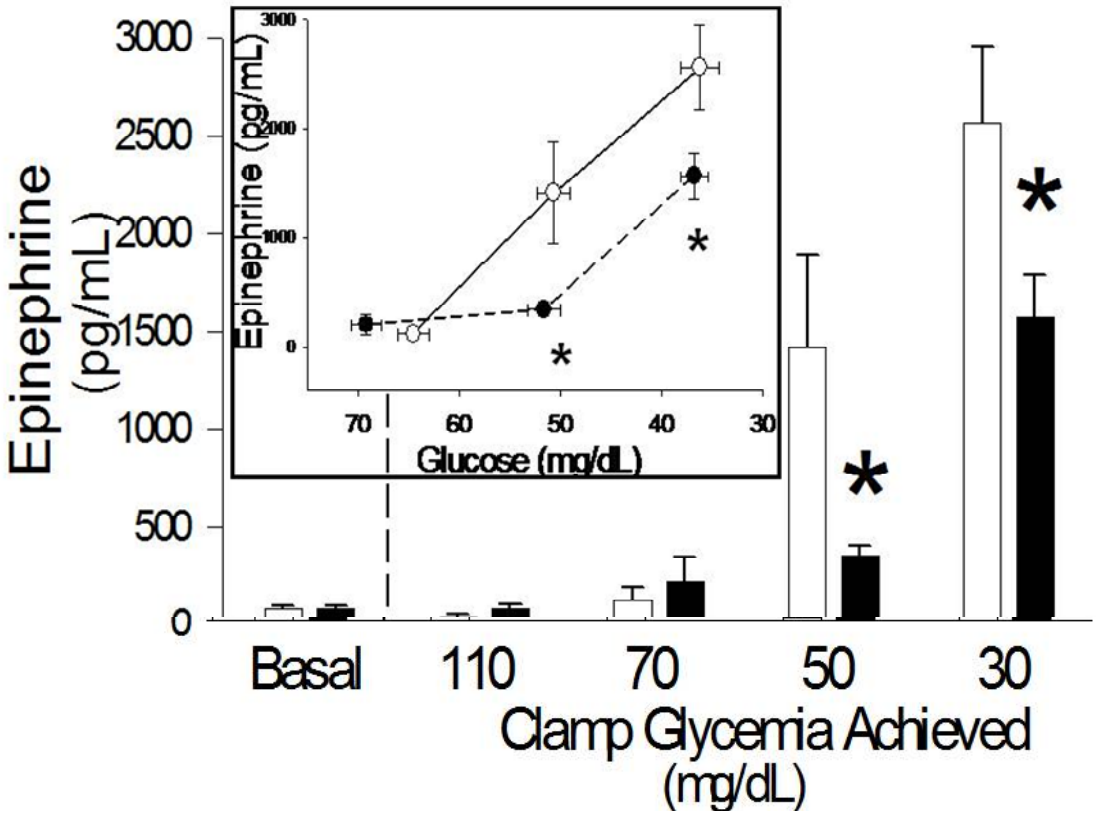

Figure 7. Adrenomedullary response in a series of hyperinsulinemic glucose clamps. Results are shown for NIRKO (closed bars) and control (open bars) mice (n=6-8 mice per group). The epinephrine response was significantly impaired in NIRKO mice during moderate (50mg/dL) and severe (30mg/dL) hypoglycemia. The inset picture demonstrates a shift in the hypoglycemia dose-response curve by the solid (controls) versus dashed (NIRKO) line. (*$p<0.05$ vs. control)

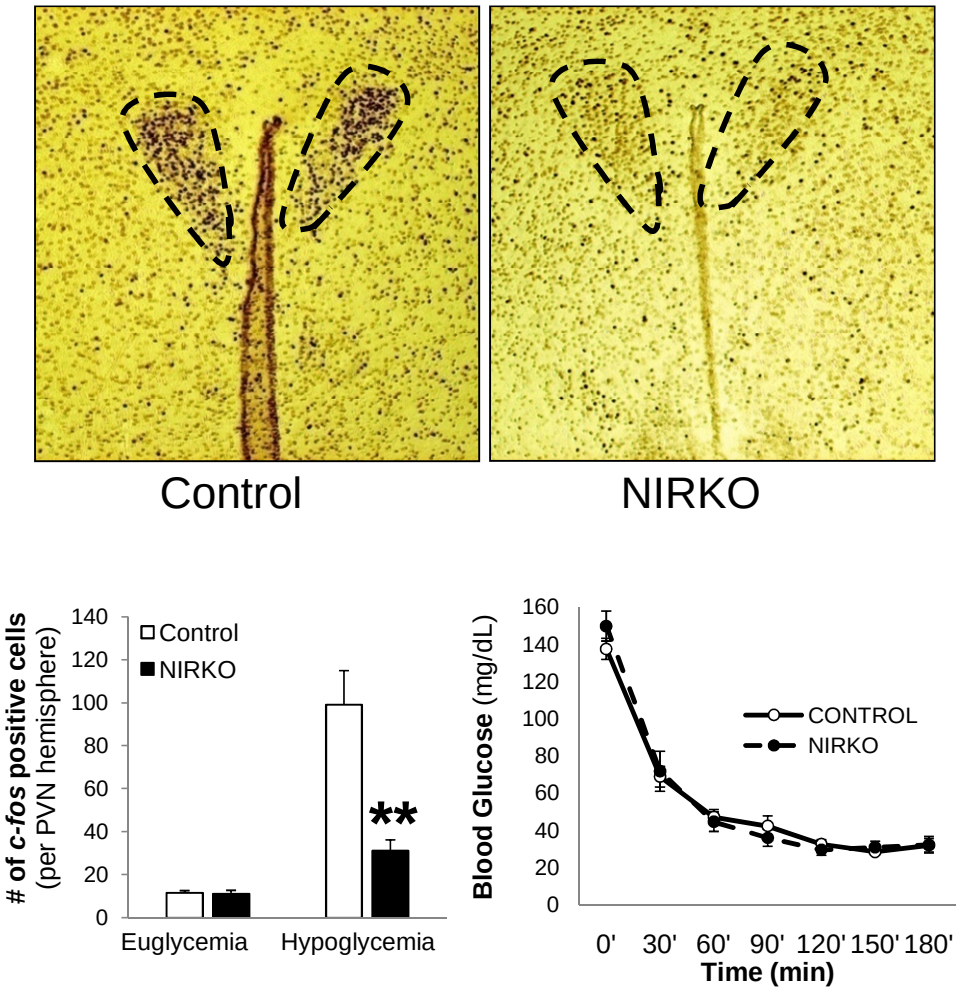

Figure 8. Blunted neuronal activation in response to hypoglycemia seen in NIRKO hypothalamus. **Top:** Representative images of matched hypothalamic sections highlighting *c-fos* staining in the paraventricular nucleus (PVN) in NIRKO (right image) and littermate controls (left image) after a 2 hour hypoglycemic insult. **Bottom:** Quantification of *c-fos* positive cells stimulated during euglycemia (n=4 mice per group) and matched hypoglycemia in NIRKO (n=4, closed bars) and littermate control (n=6, open bars) mice (left). Blood glucose levels are shown for NIRKO (closed circles) and control (open circles) mice during the hypoglycemic insult, illustrating a well-matched, stable hypoglycemic insult (right). (**p<0.01 vs. control)

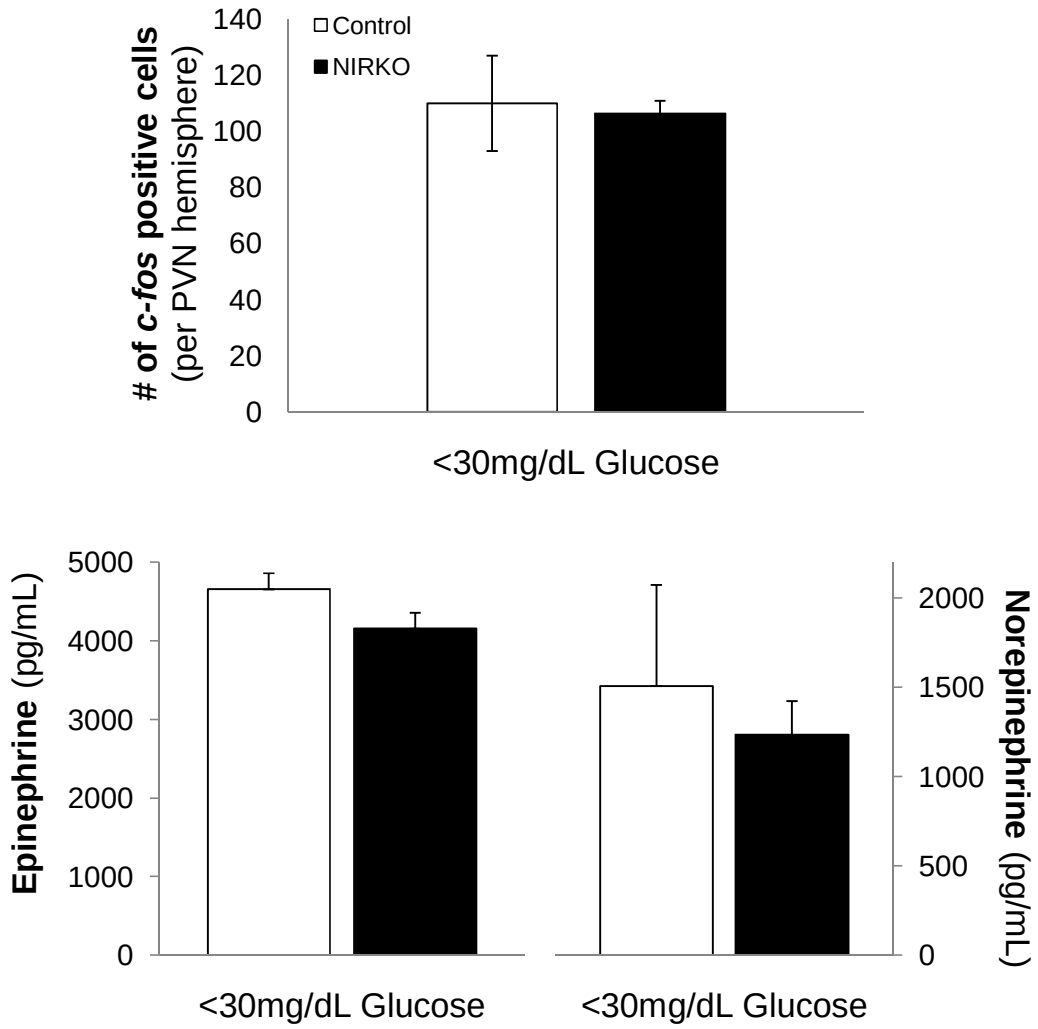

Figure 9. Intact neuronal activation and catecholamine response to extreme glucose deprivation. Results shown for NIRKO (closed bars) and littermate control (open bars) mice (n=3-6 per group). **Top:** Quantification of *c-fos* positive cells from a 2 hour hypoglycemic insult maintained below 30mg/dL glucose in NIRKO mice and littermate controls. **Bottom:** Catecholamines are robustly induced during extreme glucose deprivation. By maintaining blood glucose levels below 30mg/dL, NIRKO mice are able to stimulate both epinephrine (left) and norepinephrine (right) levels equally to control mice.

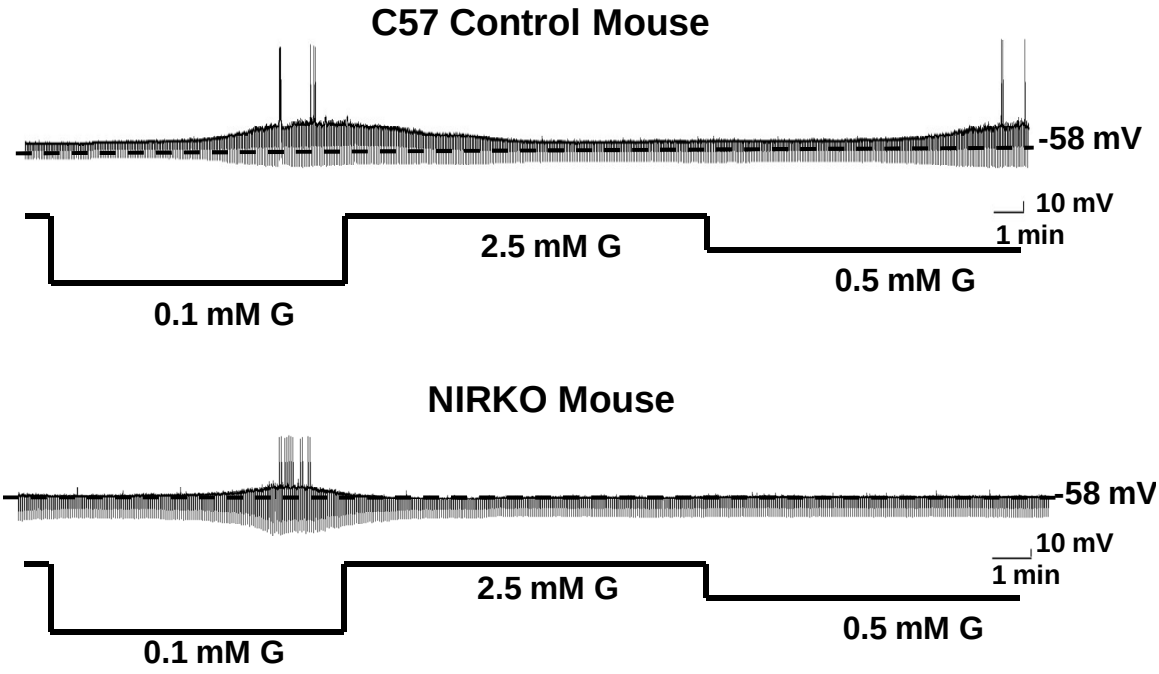

Figure 10. Individual glucose-inhibited neurons demonstrate impaired glucose responsiveness in NIRKO mice. Representative traces from standard whole-cell current clamp recordings of VMH glucose-inhibited (GI) neurons from a C57 control and NIRKO mouse in response to decreases in extracellular glucose (G) from 2.5 mM to 0.1 and 0.5mM. **Top:** In a control mouse, decreased G from 2.5 to 0.1 mM and 2.5 to 0.5 mM resulted in membrane depolarization, increased input resistance and the appearance of action potentials in this GI neuron. **Bottom:** In a GI neuron from a NIRKO mouse, decreased G from 2.5 to 0.1 mM resulted in membrane depolarization, increased input resistance and the appearance of action potentials identifying it as a GI neuron; however, this effect was not observed when G decreased from 2.5 to 0.5 mM. The resting membrane potential in 2.5 mM G is represented by the dashed line and is noted to the right of each trace. The upward deflections indicate action potentials and the downward deflections represent the membrane response to a constant current hyperpolarizing pulse.

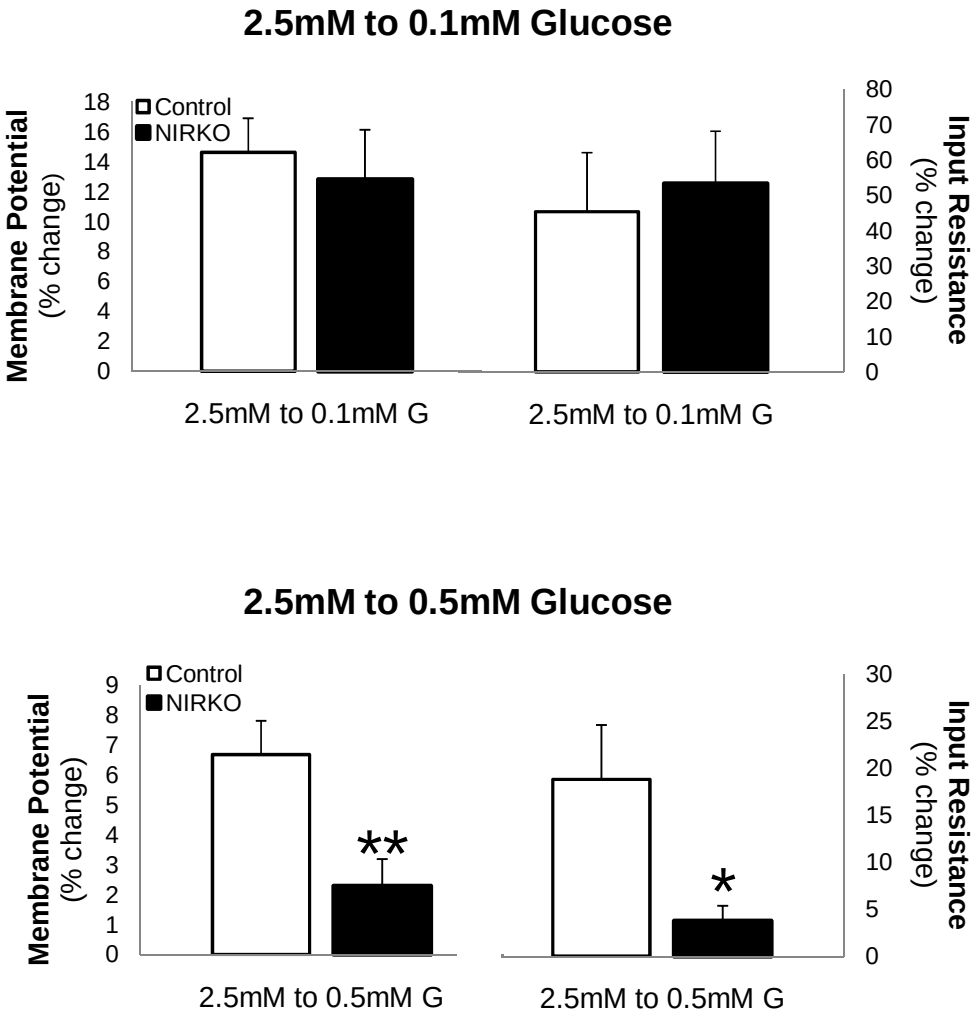

Figure 11. Changes in membrane potential and input resistance reduced in NIRKO glucose-inhibited neurons. **Top:** Graphs quantifying the percentage change of membrane potential (left) and input resistance (right) when identifying GI neurons in NIRKO (n=7, closed bars) and control (n=6, open bars) mice (decreases in extracellular glucose (G) from 2.5 to 0.1mM G). As noted, there is no difference between groups. **Bottom:** In response to lowering G from 2.5 to 0.5mM G, the percentage change in membrane potential (left) and input resistance (right) of glucose-inhibited neurons was significantly lower in NIRKO compared to littermate controls. (*$p<0.05$, **$p<0.01$)

DISCUSSION

Insulin's role in regulating the counterregulatory response to hypoglycemia is an area of active investigation. Insulin has been shown to increase (32;39-41;88-90), diminish (91;95), and not alter (33;92-94;97) the sympathoadrenal response to hypoglycemia. In this study, using a model of chronic brain insulin receptor deficiency, it was demonstrated that insulin action in the brain, 1) regulates the glucose sensitivity of glucose sensing neurons in the VMH, 2) regulates hypoglycemia-induced neuronal activation, and 3) modulates the sympathoadrenal response to hypoglycemia by altering the glycemic level required to elicit appropriate sympathoadrenal responses.

In these studies, a ~60% rise in corticosterone was observed in response to severe hypoglycemia in NIRKO and control mice (Figure 5). Although not well characterized in mice, this degree of hypothalamic-pituitary-adrenal (HPA) induced increment in corticosterone is consistent with other groups (105-109). Contrary to the stimulatory effect of insulin on the cortisol response to hypoglycemia observed in canine models (39;40), these studies in mice demonstrate that the absence of brain insulin action does not impair the HPA axis response to hypoglycemia.

Reports of insulin actions in the CNS in modulating the glucagon response to hypoglycemia are variable, with studies demonstrating insulin to increase (39;40), decrease (95), or not effect (41;97) the glucagon response to hypoglycemia. In the current studies, the pancreatic α-cell response to severe hypoglycemia showed a 6-fold increase in plasma glucagon levels that was not altered by the absence of CNS insulin

receptors in NIRKO mice. Interestingly, although catecholamines stimulate the α-cell, the impaired catecholamine response to hypoglycemia did not diminish the full glucagon response in NIRKO mice (Figure 4). These results indicate that factors other than central insulin action and systemic catecholamine responses (perhaps, local glycemia, intra-islet insulin/zinc, direct innervations, etc.) are more important mediators of the glucagon response to hypoglycemia.

The absence of brain insulin receptors resulted in a significantly impaired epinephrine response in NIRKO mice during moderate (50mg/dL) and severe (30mg/dL) hypoglycemia. However, the absence of brain insulin signaling did not result in total deficiency of hypoglycemic counterregulation. An epinephrine response of ~1500 pg/mL, which was achieved during moderate hypoglycemia (50mg/dL) in controls, was also elicited at a lower blood glucose level (30mg/dL) in NIRKO mice (Figure 7). Further, NIRKO mice were able to stimulate a comparable catecholamine response to controls when blood glucose levels were lowered well below 30mg/dL (Figure 9). Consistent with these findings, the shift in the hypoglycemia-epinephrine response curve (Figure 7-inset) indicates that in the absence of insulin signaling, NIRKO mice needed to reach lower glycemic levels to appropriately activate their adrenomedullary response. While insulin infusion suppressed hepatic glucose production during the clamps, only during severe hypoglycemia (30mg/dL) was the counterregulatory response of a sufficient magnitude to overcome the suppressive effects of insulin and significantly increase hepatic glucose production. In NIRKO mice, however, the counterregulatory-induced stimulation of hepatic glucose production was significantly blunted during severe

hypoglycemia (Figure 6), consistent with an impaired sympathoadrenal response. These findings indicate that chronic lack of CNS insulin action alters glucose sensing and/or responsiveness, leading to an impaired sympathoadrenal response and an impaired ability to defend against iatrogenic hypoglycemia.

Increased *c-fos* expression in the paraventricular nucleus (PVN) has been used as a marker of transcriptional activity in stress-related neural circuitry (104;110-112). Expression of *c-fos* was therefore measured to determine whether the impaired sympathoadrenal response in the NIRKO mice was related to impaired activation of hypothalamic sensing neurons (Figures 8 and 9). During hypoglycemia, increased *c-fos* expression was predominantly observed in the PVN and not seen in the VMH, consistent with other studies (113;114). Hypoglycemia-induced *c-fos* activation in the PVN may represent direct activation in response to hypoglycemia or indirect activation in response to afferent input from other areas containing glucose sensing neurons. Thus, the impaired *c-fos* activation in the PVN of NIRKO mice in response to severe hypoglycemia could represent reduced glucose sensing of PVN neurons; or given the abundance of insulin receptors in important VMH glucose sensing neurons, an indirect reduction in afferent inputs from glucose sensing neurons in the VMH. Whether this defect indicates impaired direct or indirect glucose sensing, the reduced *c-fos* activation in NIRKO mice was profound and consistent with other models of impaired glucose sensing and impaired counterregulation (112;115).

Whole cell current clamp recordings of spontaneous electrical activity were made in individual glucose-inhibited (GI) neurons to assess responses of individual glucose sensing neurons in the VMH. While a direct relationship between glucose sensing neurons and sympathoadrenal activation has yet to be definitively established, it is noteworthy that the ability of VMH GI neurons to sense a fall in ambient glucose levels is impaired under several conditions where the sympathoadrenal response to hypoglycemia is also impaired (i.e. rats treated with recurrent hypoglycemia or streptozotocin-induced diabetes) (102;116;117). In NIRKO mice, the observed impaired response of VMH GI neurons to reductions in glucose levels (Figures 10 and 11) is entirely consistent with the impaired neuronal (*c-fos*) activation (Figure 8) and the impaired sympathoadrenal activation during severe hypoglycemia (Figure 7). Further, the sympathoadrenal and *c-fos* findings along with the electrophysiological findings that NIRKO GI neurons respond normally to maximal glucose deprivation (blood glucose less than 30mg/dL and 0.1mM G), but show impaired responses at 0.5mM are consistent with a relative, not absolute, impairment in glucose sensing (Figures 9-11). These results indicate that insulin acts directly in the brain to regulate the glucose sensing ability of hypothalamic GI neurons that are critically important and functionally linked in mediating the sympathoadrenal response to hypoglycemia.

Of particular interest is that GI neurons of NIRKO mice have an impaired ability to respond to a fall in glucose even in the absence of insulin administration. Combining these *in vitro* findings to the *in vivo* findings suggest that it may not solely be a failure of insulin to acutely activate its receptor that leads to impaired glucose sensing and altered

neuronal responses; but rather, we propose that the chronic lack of insulin signaling in NIRKO mice causes long-term adaptations in gene transcription/transduction (i.e. altered expression of key hypothalamic glucose sensing proteins), leading to impaired glucose sensing. Alternatively, since neuronal nitric oxide (NO) production is required for GI neurons to sense decreased glucose (118;119) and insulin enhances NO production in VMH GI neurons (118), the chronic lack of insulin signaling in NIRKO mice may led to impaired glucose sensing by impairing nitric oxide production. It is entirely plausible that the chronic actions of insulin may be mechanistically very different from the acute actions of insulin in regulating neuronal glucose sensing and the counterregulatory response to hypoglycemia.

In summary, it is shown that the chronic lack of insulin receptor signaling in the central nervous system, 1) attenuates individual hypothalamic GI neuronal responses to low glucose, 2) impairs hypothalamic neuronal activation in response to hypoglycemia, and 3) reduces the sympathoadrenal response to hypoglycemia by shifting the glycemic level necessary to elicit appropriate sympathoadrenal responses. It is concluded that insulin acts directly in the brain to regulate both glucose sensing in hypothalamic neurons and the counterregulatory response to hypoglycemia. Since insulin-treated diabetic patients have an impaired ability to sense and appropriately respond to insulin-induced hypoglycemia, the mechanisms by which insulin regulates central nervous system glucose sensing need to be actively investigated as research scientists endeavor to supplant insulin-induced hypoglycemia as the rate-limiting factor in the glycemic management of diabetes.

CHAPTER 2. INSULIN-REGULATED GLUCOSE TRANSPORTER GLUT4 MAY MEDIATE THE UNIQUE RESPONSE TO HYPOGLYCEMIA SEEN IN MICE LACKING BRAIN INSULIN ACTION

ABSTRACT

Insulin acts in the brain to affect numerous physiological functions, including reproduction, neurotransmission, appetite, and glucose homeostasis. Recent reports in the CNS insulin receptor knockout (NIRKO) mouse also indicate a significant role of insulin action in the brain to regulate hypoglycemic counterregulation and glucose sensing. However, it is unclear whether these impaired responses in NIRKO mice are unique to hypoglycemia. Further, the mechanism of insulin action in the brain must still be clarified. In this study, experiments were conducted to determine whether the NIRKO model system exhibits a generalized (not hypoglycemia-specific) defect in the autonomic nervous system (ANS) mediated stress response. It was found that NIRKO mice displayed normal physiological responses to various generalized stressors (i.e. restraint, heat, and cellular glucopenia) comparable to littermate controls. To decipher a mechanism of CNS insulin action and glucose sensing, key glucose sensing proteins, including glucose transporters (GLUTs) and glucokinase (GK), were analyzed and measured. These glucose sensors were highly enriched in the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) of both NIRKO and control mice. GLUT1 and GLUT3 protein and glucokinase mRNA and protein expression levels were not significantly altered in NIRKO mice. Interestingly, expression of the insulin-responsive glucose transporter, GLUT4, within the ARC and VMH of NIRKO mice was found to be only 68% of control mice ($p<0.05$). In summary, lack of insulin signaling in the brain led to a hypoglycemia-specific impairment in the sympathoadrenal response to hypoglycemia noted in this model. Further, absent brain insulin action resulted in marked changes in glucose transporter expression, specifically GLUT4, which may account for the

previously noted glucose sensing impairment unique to this mouse model. Therefore, we conclude that insulin acts in the brain to regulate GLUT4 expression in critically important sites within the hypothalamus necessary for normal brain glucose sensing and the normal sympathoadrenal response to hypoglycemia.

INTRODUCTION

Insulin signaling is involved in many cellular processes, causing diverse effects on metabolism, longevity, and stress responses in various model systems (120). Insulin's actions also extend to the central nervous system where insulin can directly regulate food intake, body weight, and glucose homeostasis (24-31). As seen in the NIRKO model, chronic lack of brain insulin signaling results in an impaired sympathoadrenal response to hypoglycemia (41). Due to insulin's ability to diversely effect disparate processes as well as cross the blood-brain barrier to directly activate the autonomic nervous system (34-38), it is unclear whether the hypoglycemia impairment found in NIRKO mice is specific to altered glucose sensing or due to a generalized defect in its ability to respond to stressful insults.

Insulin's specific role in regulating CNS glucose sensing is ill-defined but growing evidence supports the hypothalamus (specifically the ventromedial hypothalamus) as the key glucose sensor that coordinates and triggers hypoglycemic counterregulation (8-11). Insulin receptors are highly expressed throughout the CNS, with specific enrichment in hypothalamic regions posited to be critical for regulating glucose homeostasis (19-23). As a result, insulin may play a predominate role in regulating CNS glucose sensing via glucose sensing neurons located in the ventromedial hypothalamus (VMH) (50;57;95;121;122). These glucose sensing neurons share metabolic similarities to other well-characterized glucose sensing cells (i.e. pancreatic β-cells), especially with regards to glucose transport and metabolism (44;52;53). Based on the expression of insulin receptors in the majority of glucosensing neurons in the VMH (68), it is postulated that

brain insulin action may mediate its effects on central glucose sensing by regulating expression of glucose transporters (GLUTs) and/or glucokinase (GK). Several members of the glucose transporter family are expressed throughout the CNS (123). GLUT1 and GLUT3 are the most abundant isoforms expressed in the brain (63;124-126), whose functions are thought to maintain basal brain glucose uptake. GLUT4, the insulin responsive glucose transporter, is co-expressed with the insulin receptor in several hypothalamic regions (68) and is down-regulated under diabetic conditions (127). Subsequent to glucose transport, it may be that the enzyme glucokinase, a key regulator of glucose phosphorylation and metabolism in the β-cell and liver, may mediate insulin's effects on glucose sensing in the hypothalamus (44;55). Similar to insulin receptors, glucokinase is highly expressed in hypothalamic neurons (44;55;65;66;70;128). Because insulin signaling regulates glucokinase expression and glucose sensing in liver and β-cells (129;130), it is thought that insulin signaling may similarly regulate glucokinase expression and glucose sensing in the hypothalamus. In this study, the NIRKO mouse model was utilized to assess insulin's role in modulating the stress response to low glucose relative to other stressors. Also, the potential mechanism of CNS insulin action was examined by evaluating alterations in key glucose sensing proteins that colocalize with insulin receptors, specifically GLUTs and GK.

MATERIALS AND METHODS

Animals. Mice homozygous for the floxed insulin receptor allele ($IR^{lox\text{-}lox}$) were bred with transgenic mice that express Cre recombinase cDNA from the rat nestin promoter to generate ($IR^{lox\text{-}lox}$:nestin-$Cre^{+/-}$) nervous system specific insulin receptor knockout (NIRKO) mice (28). Genotypes were determined by PCR of tail DNA. Unless otherwise indicated, 2-4 month old NIRKO and littermate control (Control, $IR^{lox\text{-}lox}$:nestin-$Cre^{-/-}$) mice were used for these experiments. All mice were housed on a 12-hour light/dark cycle and fed a standard rodent chow (Mouse Diet 9F, PMI Nutrition International, St. Louis, MO) *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Studies Committee of Washington University.

Brain Glucose Uptake. Briefly, awake, unrestrained, cannulated NIRKO and littermate control mice (n=6-9 per group) underwent a 2-hour hyperinsulinemic (40mU/kg/min) hypoglycemic (30mg/dL) clamp protocol. At 45 minutes prior to the end of the clamp, a 5μCi bolus of ^{14}C 2-deoxyglucose was rapidly infused intravenously and ten-timed (0, 0.5, 2, 3, 5, 10, 15, 20, 30, and 45 min) arterial blood samples (10μL) were collected for analysis of arterial plasma glucose and ^{14}C levels. Immediately after the conclusion of the clamp, animals were euthanized and brains were rapidly harvested and quickly frozen in a dry ice and 2-methylbutane (Fisher, Saint Louis, MO) bath (-20^{o} C). Plasma 2-^{14}C-DG concentrations were determined by a dual-channel scintillation counter (Tri-Carb 2800TR, PerkinElmer, Waltham, MA). Isotope concentrations in regions of interest, including the CA1, CA3, and dentate gyrus (DG) of the hippocampus, arcuate nucleus

(ARC), paraventricular nucleus (PVN), and ventromedial nucleus (VMH) of the hypothalamus, and nucleus of the solitary tract (NTS), were measured from 20μm thick coronal serial sections collected on heated coverslips at 60μm intervals. Following, sections were exposed to autoradiograph film along with precalibrated ^{14}C standards for 2-4 days and analyzed via optical densitometry (ARC 146E, American Radiolabeled Chemicals, Inc., St. Louis, MO). Regional glucose uptake was calculated according to Sokoloff's equation utilizing rat rate constants, as mice rate constants have not been established (131).

Restraint Stress. Awake, 5-hour fasted, control and NIRKO mice (n=6 per group) were placed in a mouse restrainer (Braintree Scientific, Braintree, MA) for 45 minutes to induce restraint stress. Cardiovascular parameters were obtained during the basal period and during the last 20 minutes of restraint stress using a tail-cuff system (Kent Scientific Corporation, Torrington, CT). Blood samples (50μL) were taken by previously implanted arterial cannula at the beginning and end of the restraint period to measure plasma epinephrine levels.

Heat Stress. Awake control and NIRKO mice (n=6 per group) were exposed to an ambient temperature of 42°C for 90 minutes to induce heat stress. Blood samples were taken at the end of the heat stress period to measure catecholamines (50μL). Subsequently, cryoprotected brains were analyzed for heat stress induced *c-fos* immunostaining, as described in the immunohistochemistry methods section.

Systemic 2-deoxyglucose induced glucopenia. In awake, 5-hour fasted, control and NIRKO mice, a single bolus of 2-deoxyglucose (600mg/kg IV, Sigma, St. Louis, MO) was given to induce glucopenic stress. Blood samples (100μL) were taken by a previously implanted arterial cannula at the beginning and end of the stress period to measure plasma catecholamines and corticosterone levels.

Central 2-deoxyglucose induced glucopenia. Two weeks prior to the study, the animals were anesthetized with ketamine/xylazine (87 and 13.4mg/kg IP), and microinjection cannula (Plastics One Inc., Roanoke, VA) were inserted into the third ventricle. For intracerebroventricular (ICV) injections, a solitary ICV cannula was inserted 1.6mm posterior to bregma, on the suture line, to a targeted depth of 5.5mm. After a 5-7 day recovery, mice were anesthetized isofluorane and were implanted with catheters (MRE 025, Braintree Scientific Inc., Braintree, MA) into the femoral artery. The animals were then allowed approximately 4-7 days to recover prior to the experiment. Simultaneously during the recovery period, mice were handled daily and the ICV cannula was tested to minimize stress the day of the experiment.

One the day of the experiment, awake, unrestrained animals were rested 2 hours following exteriorizing the vessel cannula. A basal blood sample (50μL) was taken at time 0 to measure basal catecholamine levels. Following, the animals were gently, yet quickly immobilized to give a single infusion of 2-deoxyglucose (1mg/mouse, Sigma, St. Louis, MO) or a control artificial cerebrospinal fluid (aCSF) solution and returned to their home cages. Blood glucose levels were monitored continuously throughout the

experiment. At 45 minutes after injection, another blood sample was taken to measure catecholamine levels. Animals were sacrificed at 120 minutes and the brains were cryoprotected for future *c-fos* analysis. Animals were excluded from analysis if the ICV cannula was not positioned properly as determined on posthumous examination by track formation and evidence of Evans blue dye (0.01%).

Immunohistochemistry. Mice anesthetized with isofluorane and transcardially perfused with 0.01 M PBS (Sigma, Saint Louis, MO) followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). The brains were immersed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose. Free-floating brain sections (20µm) were blocked with 30% normal goat serum diluted in 0.1% Triton-100/PBS and then incubated overnight at 4°C with *c-fos* antibody (1:2000, Ab-5, Calbiochem, San Diego, CA) or GLUT4 antibody (1:1000, generously gifted by Dr. M. Mueckler, Washington University in St. Louis). Subsequently, the sections were mounted on slides and processed with biotinylated goat anti-rabbit immunoglobulin G (1:200) using the Elite ABC kit (Vector Laboratories, Burlingame, CA). As a negative control, alternative sections were incubated without primary antibodies. Regions of interest were identified using anatomical landmarks to identify the paraventricular nucleus (PVN), arcuate nucleus (ARC), or ventromedial nucleus (VMH) of the hypothalamus (100). Positively stained cells were counted by a blinded investigator. Four to six anatomically matched sections per mouse were quantified for statistical purposes.

Western Blots. The medial basal hypothalamus, defined anatomically as posterior to the optic chiasm, anterior to the mammillary body, inferior to the thalamus, and ±1mm lateral to the midline was dissected and frozen for analysis. Samples were homogenized in buffer containing 1% Igepal (Sigma, Saint Louis, MO), 0.5% Sodium Dodecyl Sulfate (SDS) (Sigma, Saint Louis, MO), 0.1 mM Phenylmethylsulfonyl Fluoride (PMSF) (Sigma, Saint Louis, MO), 1X complete protease inhibitor (Sigma, Saint Louis, MO), 1 mM Sodium Fluoride (NaF) (Sigma, Saint Louis, MO), and 1 mM Sodium Orthovanadate (Na_3VO_4) (Sigma, Saint Louis, MO) in phosphate buffered saline (pH=7.4), centrifuged at 14,000 rpm for 30 minutes, and supernatant was collected. Homogenized hypothalamic protein extracts (20μg for GLUT1 and GLUT3; 100μg for GLUT4 and glucokinase) were fractionated by electrophoresis on a 10% Bis-Tris Criterion XT (Biorad, Hercules, CA) gel and subjected to transfer onto a nitrocellulose membrane (Biorad, Hercules, CA). Following, the membranes were immunoblotted against the following primary antibodies: GLUT1 (1:5000, Chemicon, Temecula, CA), GLUT3 (1:1000, Chemicon, Temecula, CA), GLUT4 (1:1000, kindly supplied by Dr. M. Mueckler, Washington University in St. Louis, St. Louis, MO), Glucokinase (1:1000, Calbiochem, San Diego, CA). The blots were developed using a horseradish peroxidase-conjugated secondary antibody (1:8000, Cell Signaling, Boston, MA). Primary antibody binding was detected by enhanced chemiluminescence ECL reagents (Perkin Elmer, Wellesley, MA) on ISO-MAX films and quantified by ImageQuant software analysis (Amersham Pharmacia, Piscataway, NJ). An antibody against β-actin (1:2000, Sigma, St. Louis, MO) served as a loading control.

Immunofluorescence. Mice anesthetized with isofluorane and transcardially perfused with 0.01 M PBS (Sigma, Saint Louis, MO) followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). The brains were immersed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose. Briefly, free-floating hypothalamic sections (20-30µm) throughout the VMH/ARC were taken from 1.46 to 1.82mm caudal to bregma, blocked, and incubated overnight at 4°C in the GLUT4 primary antibody (1:1000). Goat anti-rabbit Texas Red (1:200, Molecular Probes) was used as the secondary antibody. Subsequently, the sections were mounted on slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). As a negative control, alternative sections were incubated without primary antibodies. Regions of interest were identified using anatomical landmarks (100) and positively stained cells were counted by a blinded investigator. Four to six brain sections per mouse were quantified for statistical purposes.

RT-PCR. Sections (400µm) were taken from brain sections 1.46 to 1.86mm caudal to bregma. 0.5mm bilateral punch biopsies from the VMH and ARC (0.75mm from the piriform cortex) were collected from NIRKO mice and littermate controls (n=7-8 per group). The mRNA extracted with Trizol (Invitrogen Corporation, Carlsbad, CA) was subject to quantitative two-step RT-PCR reaction performed in triplicate in a fluorescent temperature cycler (GeneAmp 7700 Sequence Detector, Applied Biosystems) with glucokinase primers (glucokinase probe 5' -/56-FAM/ACC GCC AAT GTG AGG TCG GCA /3BHQ_1/-3'; glucokinase reverse 5'- AGC CGG TGC CCA CAA TC- 3'; and

glucokinase forward 5'- CCA CAA TGA TCT CCT GCT ACT ATG A-3'). The results were quantified after normalizing to ribosomal RNA L32mRNA.

Statistics. All values are presented as the mean ± standard error of the mean (SEM). Single comparisons between parameters were tested for statistical significance using a Student's *t* test. For multiple group comparisons, statistical significance was determined by analysis of variance (ANOVA) using repeated measures followed by a Bonferroni post-hoc test to assess differences between experimental groups. Statistical significance was set at $p<0.05$.

RESULTS

Absent CNS Insulin Signaling does not Influence Response to Restraint or Heat Stress

It is noted that NIRKO mice have an impaired response to hypoglycemia. Since the efferent response to stress involves common hypothalamic projections to spinal and medullary autonomic centers resulting in adrenomedullary activation (132), it was important to test the adrenomedullary response to other forms of stress in order to determine whether the impaired stress response in NIRKO mice was unique to hypoglycemia or part of a more generalized defect in the autonomic nervous system mediated stress (i.e., fight or flight) response unique to this knockout mouse. NIRKO and control mice were subjected to two, glycemia-independent, stressors—a mild stressor (restraint stress) and a more profound stressor (heat stress)—in order to evaluate sympathoadrenal activation in response to glycemia-independent stress. In response to milder restraint stress, plasma epinephrine levels were somewhat elevated in the basal state due to animal handling but rose similarly 2-fold in both littermate controls and NIRKO mice from this baseline during stress. The physiological increased heart rate to restraint stress was also similar in control and NIRKO mice (Figure 12). Heat stress induced a more pronounced catecholamine elevation than restraint stress (to levels within the range observed during hypoglycemia experiments), but the rise in both epinephrine and norepinephrine in response to heat stress was again not significantly different between groups (Figure 13).

To determine whether the impairment in neuronal activation seen in NIRKO mice was unique to hypoglycemia, *c-fos* expression was also assessed in response to heat stress.

Increased *c-fos* expression was noted in the paraventicular nucleus (PVN) in response to heat stress (Figure 14), to levels observed with severe hypoglycemia (Figure 8); however, in response to heat stress, there was no difference in *c-fos* expression between groups (Control: 123±4 vs. NIRKO: 129±10, p=NS).

Absent CNS Insulin Action does not Impair Physiological Responses to Systemic nor Central Cellular Glucopenia

To ascertain whether the impaired counterregulatory response seen in NIRKO mice is due solely to insulin-induced hypoglycemia, an alternate glucopenic stimulus was utilized. 2-deoxyglucose (2-DG) is a commonly used agent thought to pharmacologically simulate hypoglycemia in animals. 2-DG is a non-metabolized glucose analog that deprives cells of metabolizable glucose, thus inducing a glucoprivic signal that triggers a counterregulatory response even in the presence of normal systemic glucose concentrations. Other groups have demonstrated that administration of 2-DG can induce a profound counterregulatory response in animals, as well as induce neuronal activation within key glucose sensing regions of the hypothalamus (133;134). When given systemically, 2-deoxyglucose increased blood glucose levels in both NIRKO and control mice (Figure 15). There was also robust stimulation of epinephrine release, similar to levels induced by insulin-induced hypoglycemia, by NIRKO and control mice. However, the rise in epinephrine seen in NIRKO mice was not different from that of controls (Figure 16). Norepinephrine levels were not significantly elevated from baseline levels in either NIRKO mice or littermate controls in response to 2-DG (Figure 16). Basal corticosterone levels were lower in NIRKO mice and the incremental response (i.e. delta)

induced by 2-DG stress was significantly greater in NIRKO mice vs. control mice; however, peak corticosterone levels during 2-DG stress were comparable to those seen in control mice (Figure 15).

Subsequent experiments were performed to determine whether the counterregulation impairment seen in NIRKO mice was centrally-mediated. 2-DG was specifically administered to the 3rd ventricle of the hypothalamus to cause a localized glucoprivic insult. In response to central 2-DG, NIRKO and control mice showed a matched rise in blood glucose (Figure 17). Norepinephrine was not significantly elevated in either NIRKO or control mice in response to central 2-DG administration (Figure 17). The norepinephrine response seen in NIRKO mice was comparable to littermate controls. Similarly, epinephrine levels trended higher due to 2-DG stress in both control and NIRKO mice, but the rise was not significant (Control: p=0.09 vs. CSF, NIRKO: p=0.06 vs. CSF) (Figure 17).

Expression of Hypothalamic Glucose Sensors in Model of Abrogated Insulin Action

To assess whether CNS insulin action regulates key glucose sensors, including glucokinase (GK) and glucose transporters (GLUTs), in the brain, hypothalamic protein and mRNA expression was assessed. GK protein levels in the hypothalamus were comparable in control and NIRKO mice (Figure 18). GK mRNA expression was preferentially expressed in the VMH and arcuate nucleus, but there was no difference in expression levels between experimental groups (Figure 18), consistent with the glucokinase protein expression findings. Hypothalamic protein expression of GLUT1

and GLUT3 were 3-fold higher than hypothalamic protein expression of GLUT4. Relative to controls, GLUT1 protein levels in the hypothalamus were similar in NIRKO mice (Figure 19). Hypothalamic GLUT3 protein levels in NIRKO mice were slightly (80.5±9.8% of control), but not significantly ($p=0.08$) reduced (Figure 19). Interestingly, insulin-regulated GLUT4 protein levels were significantly reduced (68.5±5.5% of control, $p<0.05$) in the hypothalamus of NIRKO mice (Figure 19). When assessing GLUT4 regional localization, immunohistochemistry results demonstrated that GLUT4 protein was highly enriched in the ventromedial hypothalamus (VMH) and the arcuate nucleus (ARC) of control mice. In NIRKO mice, GLUT4 protein expression was markedly reduced in these regions (Figure 20).

Regional Brain Glucose Uptake during Hypoglycemia is not Altered in NIRKO Mice

Based on the impaired glucose sensing and reduced GLUT4 expression found in NIRKO mice, it was postulated that glucose uptake might be altered in critical glucose sensing areas of NIRKO mice. To measure regional specific brain glucose uptake, radiolabeled ^{14}C-2-deoxyglucose infusions during hyperinsulinemic-hypoglycemic clamps were used. In spite of reductions in glucose transporter expression in NIRKO mice, regional brain glucose uptake was not different between experimental groups within any areas assessed (Figure 21).

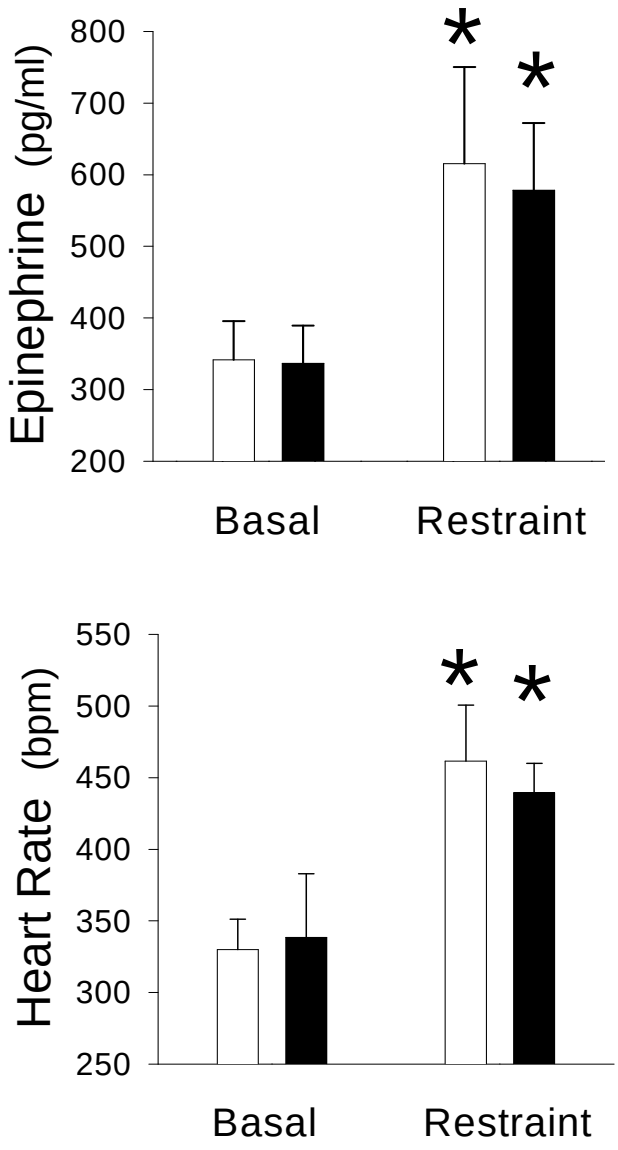

Figure 12. NIRKO mice have a normal physiological response to restraint stress. NIRKO (n=6, closed bars) and littermate control (n=6, open bars) mice were placed into a confining restraint device for 45 minutes. **A&B.** Plasma epinephrine levels (A) and heart rates (B) were elevated in response to restraint stress, but equally in control and NIRKO mice. (*$p<0.05$ vs. basal)

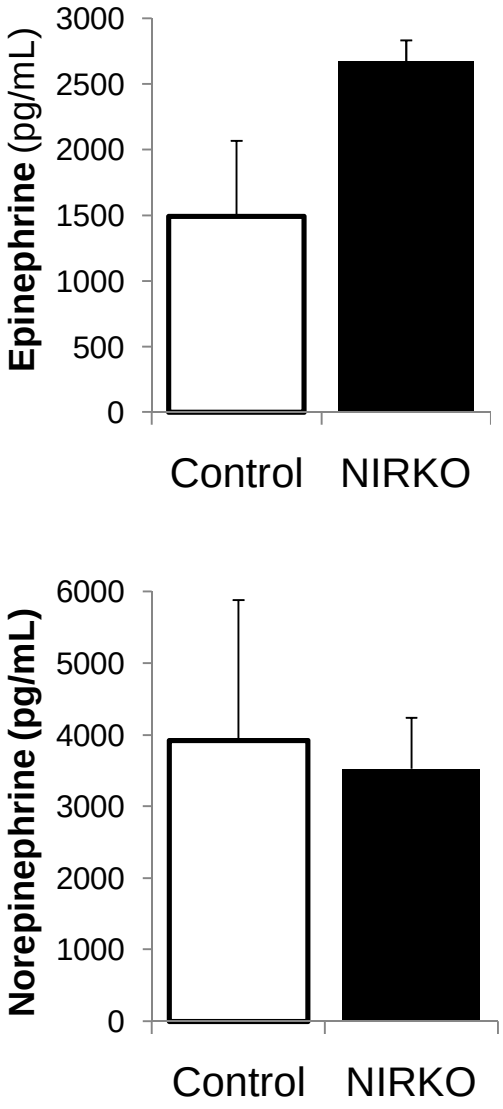

Figure 13. NIRKO mice display normal catecholamine responses to heat stress. NIRKO (n=6, closed bars) and littermate controls (n=6, open bars) were subjected to heat stress for 90 minutes. Plasma epinephrine (top) and norepinephrine (bottom) levels were significantly stimulated by heat stress; however, there was no difference between NIRKO and control mice.

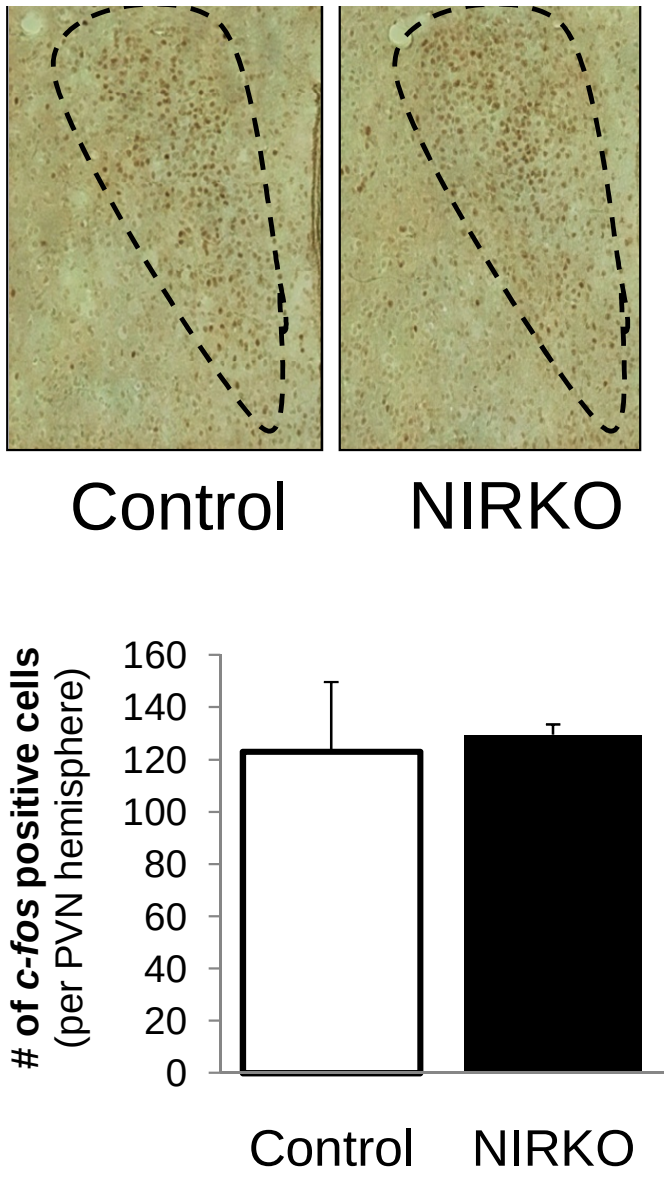

Figure 14. Normal neuronal activity in response to heat stress seen in NIRKO mice. NIRKO (n=6, closed bars) and littermate controls (n=6, open bars) were subjected to heat stress for 90 minutes. **Top:** Representative images of matched hypothalamic sections highlighting heat stress induced *c-fos* staining in the paraventricular nucleus (PVN). **Bottom:** Graph quantifying *c-fos* positive cells shows no difference between NIRKO and controls.

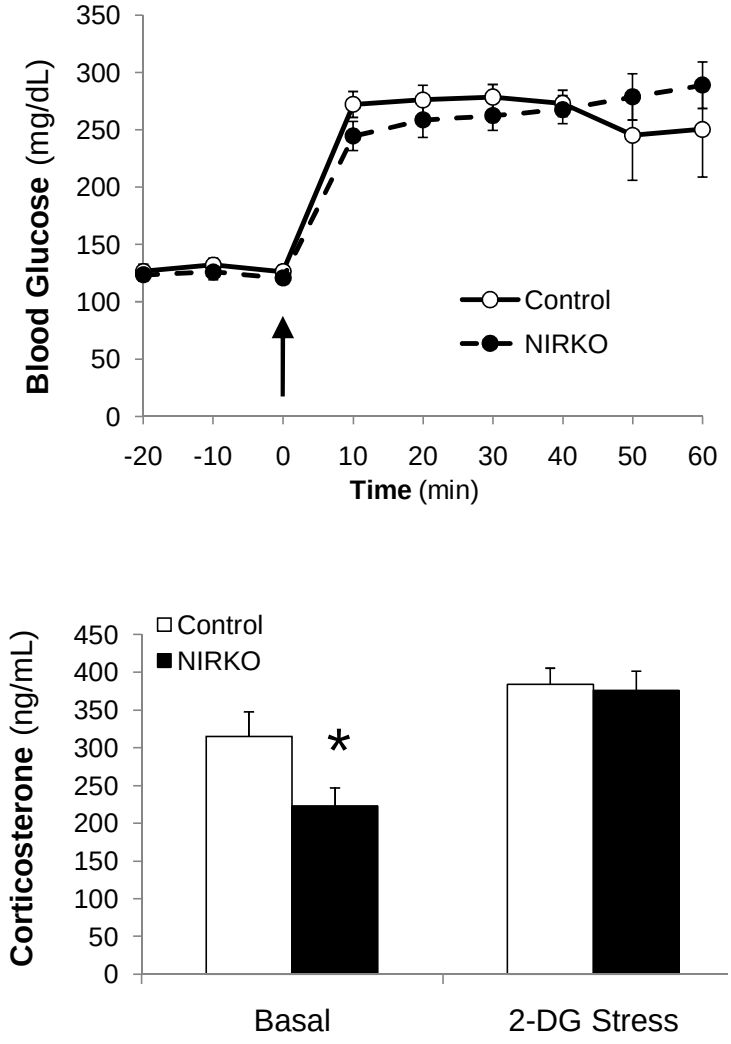

Figure 15. NIRKO mice show normal physiological response to systemic glucopenic stress. **Top:** Blood glucose levels were similarly elevated in NIRKO (closed circles) and control (open circles) mice following 2-DG administration (as indicated by arrow). **Bottom:** In NIRKO (closed bars) mice, basal corticosterone levels were slightly lower than controls (open bars). As a result, the rise (i.e. delta) in corticosterone seen in NIRKO mice is significant; however, relative to controls, 2-DG stress induced corticosterone levels were not different between NIRKO and littermate control mice. (*$p<0.05$ vs. control)

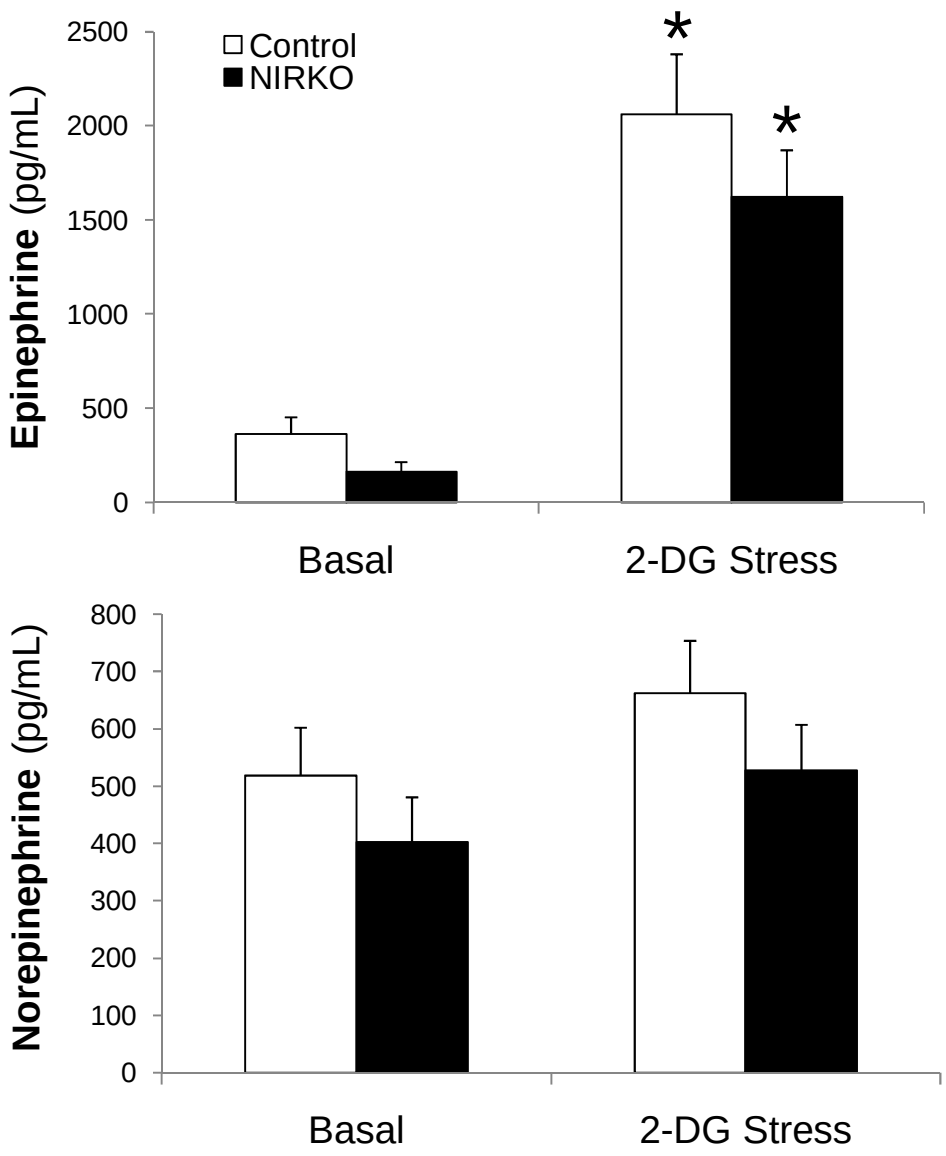

Figure 16. Catecholamine response to systemic glucopenic stress. **Top:** Epinephrine levels were significantly elevated in both NIRKO mice and littermate controls. **Bottom:** Norepinephrine levels were slightly, but equally elevated by 2-DG stress in both NIRKO and control mice. (*$p<0.05$ vs. control)

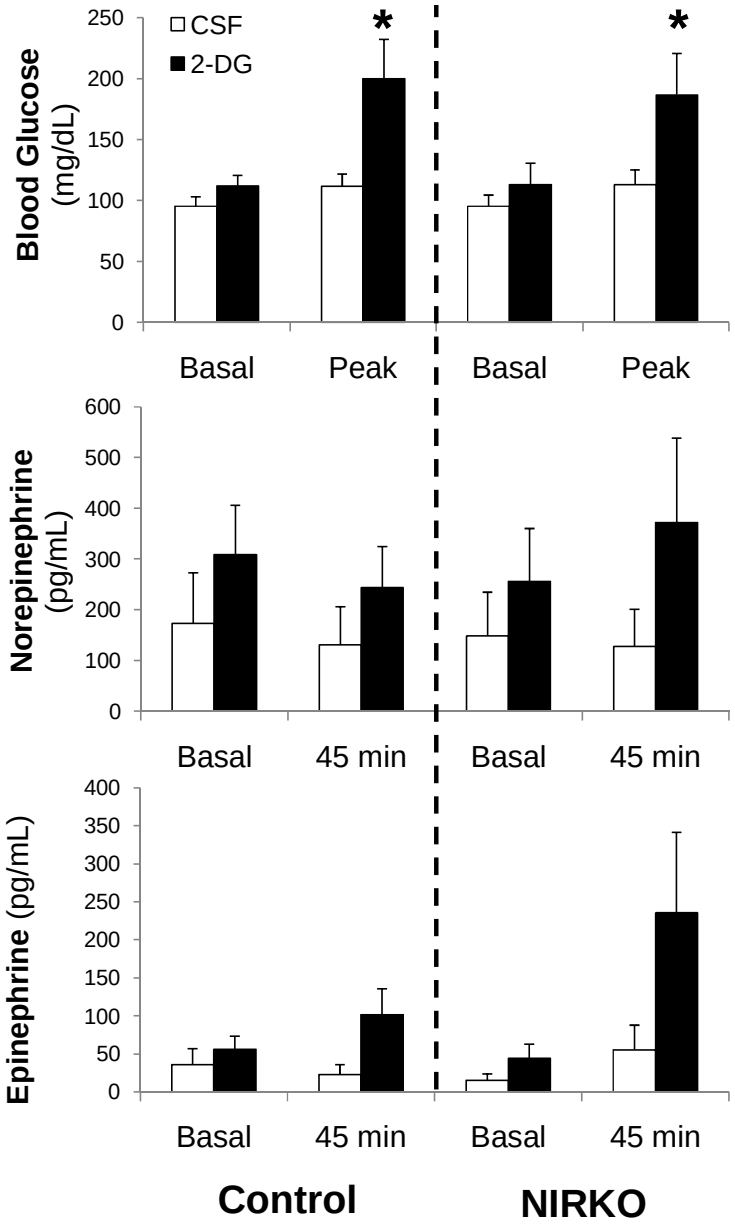

Figure 17. Central glucopenic stress induces modest physiological response in NIRKO mice. **Top:** Basal and peak blood glucose profile from NIRKO and control mice after 2-DG (closed bars) or aCSF (open bars) administration (n=4-7 mice per group). **Center:** Norepinephrine levels were similarly induced by 2-DG stress in NIRKO and littermate controls. **Bottom:** Epinephrine levels trended higher in response to 2-DG; however, these responses were comparable between groups. (*$p<0.05$ vs. CSF)

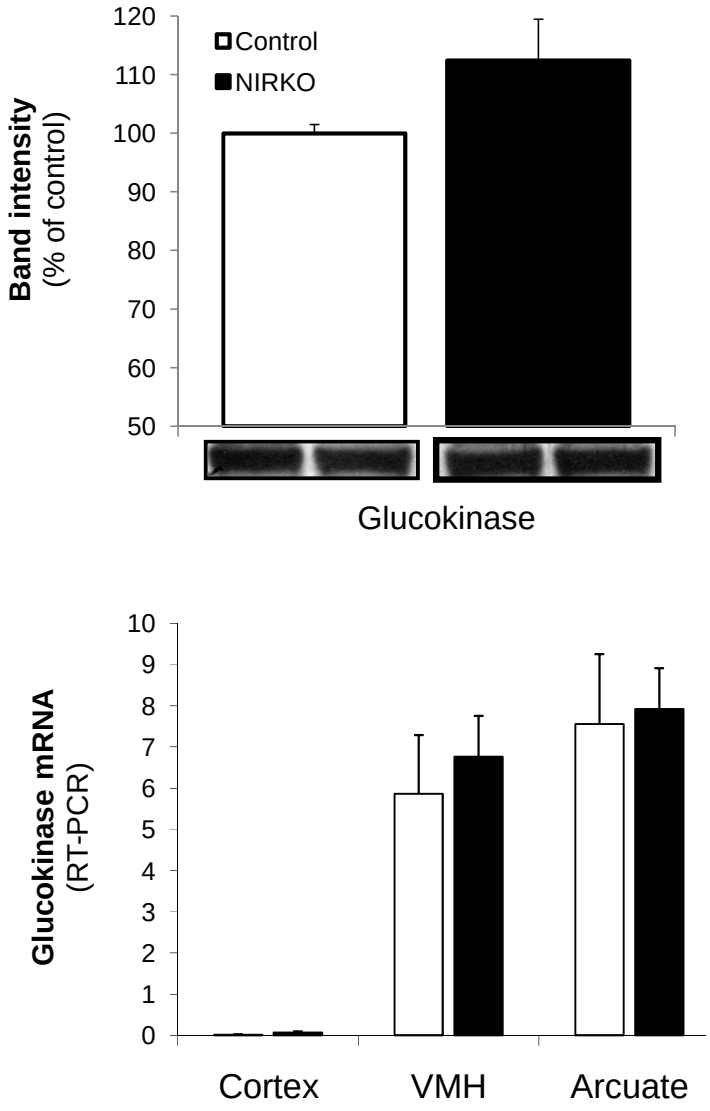

Figure 18. Glucokinase expression is not altered in NIRKO brains. **Top:** Western blots of whole hypothalamic extracts from NIRKO and littermate controls (n=5-6 mice per group). Graph quantifying protein expression (representative images below) is shown. **Bottom:** Although glucokinase mRNA was highly expressed in the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) as compared to the cortex, there was no difference between NIRKO (n=7, closed bars) and controls (n=8, open bars).

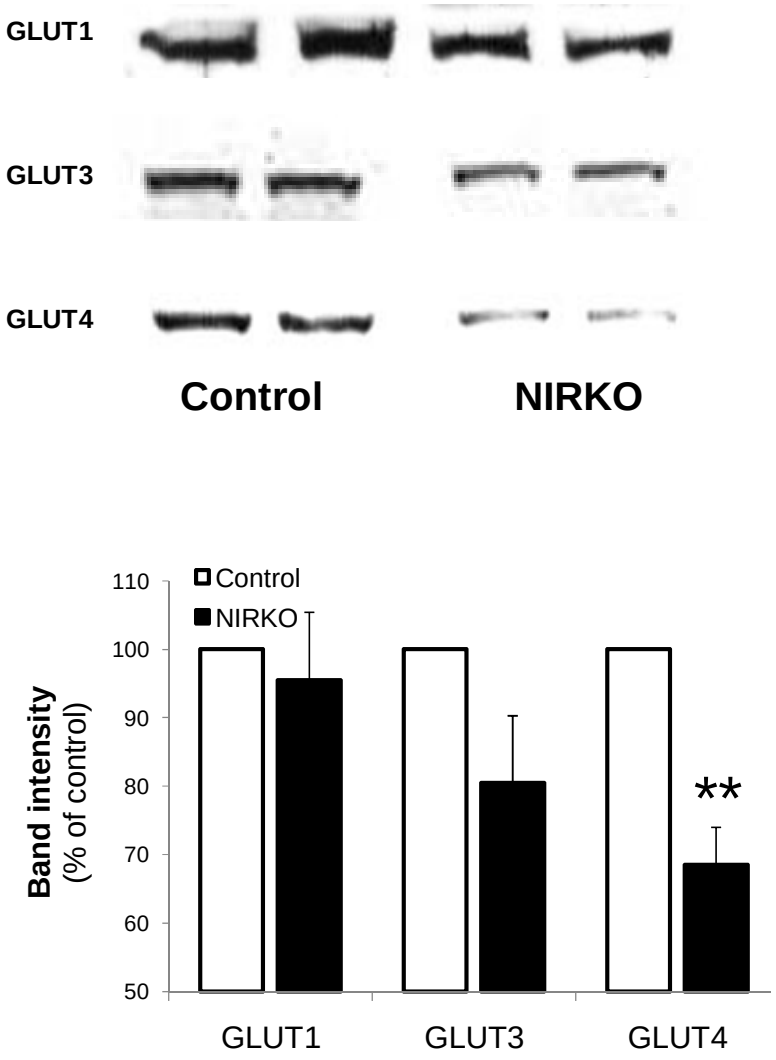

Figure 19. Insulin-regulated glucose transporter 4, not GLUT1 or GLUT3, is reduced in NIRKO brains. Western blots of whole hypothalamic extracts from NIRKO and littermate controls (n=5-6 mice per group) were performed. Representative images (top) and graph (bottom) quantifying protein expression of glucose transporters (GLUT1, GLUT3, and GLUT4) are shown. (**p<0.01 vs. control)

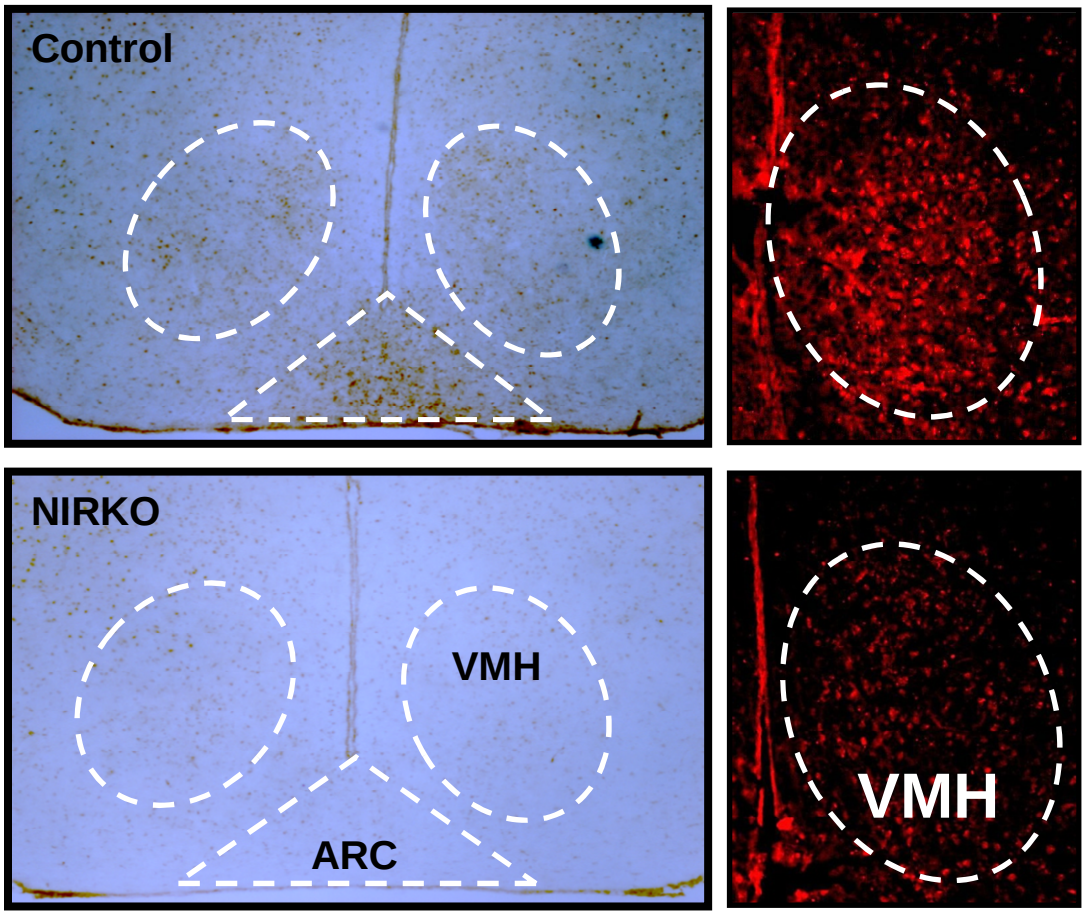

Figure 20. GLUT4 expression is specifically reduced in key glucose sensing regions of the hypothalamus in NIRKO mice. Regional localization of GLUT4 protein content, as determined by hypothalamic DAB staining (left) and immunofluorescence (right) of control (above) and NIRKO (below) mice, show enriched GLUT4 protein content in the ventromedial hypothalamus (VMH, circled) and arcuate nucleus (triangle) of control mice. GLUT4 protein content was 62±6% lower in NIRKO mice.

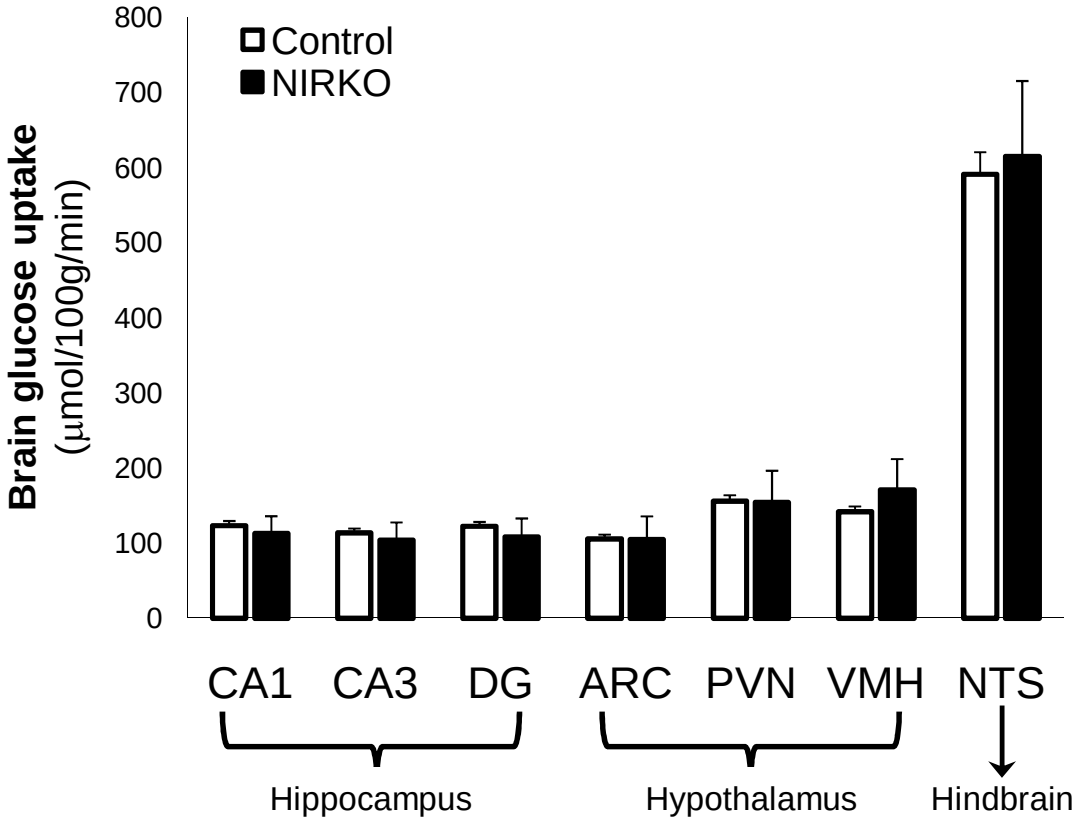

Figure 21. Glucose uptake during hypoglycemia is preserved in the NIRKO brain. Regional brain glucose uptake was quantified using ^{14}C 2-deoxyglucose during a hyperinsulinemic hypoglycemic (~30mg/dL) clamp. Results show that regional brain glucose uptake in all regions measured [hippocampus (CA1, CA3, DG), hypothalamus (VMH, ARC, PVN), and hindbrain (NTS)] are similar among NIRKO (n=6, closed bars) and control mice (n=9, open bars).

DISCUSSION

In this study, it was determined that lack of insulin receptor signaling in the central nervous system does not restrict the sympathoadrenal response to various glycemia-independent stressors, but specifically alters glucose sensing in response to insulin-induced hypoglycemia and alters the expression of a key glucose sensor, GLUT4, in the brain. These findings indicate that insulin acts in the brain to uniquely regulate CNS glucose sensing and the sympathoadrenal response to hypoglycemia.

Since the adrenomedullary response to hypoglycemia was not impaired during mild hypoglycemia in NIRKO mice, it was speculated that mild (restraint) stress, as noted by modest elevations in epinephrine levels (Figure 12), might not have been of sufficient magnitude to detect a differential response between control and NIRKO mice. However, by achieving comparable epinephrine levels during severe stress (heat) and severe hypoglycemia (30mg/dL) and finding a normal catecholamine response to heat stress in NIRKO mice, these findings indicate that absent CNS insulin signaling does not impair the normal adrenomedullary response even to severe non-hypoglycemic stress (Figures 9 and 13).

Further, in response to a non-hypoglycemic stressor, heat stress increased *c-fos* expression to a similar magnitude as observed during severe hypoglycemia (30mg/dL); however, no difference in heat-induced *c-fos* expression was noted between control and NIRKO mice (Figure 14). These results indicate that NIRKO mice have an intact neuronal circuitry for sensing and responding to non-hypoglycemic stress; therefore, the

impaired responses to hypoglycemia in the NIRKO mice appear to be unique to hypoglycemic stress and/or glucose sensing.

2-deoxyglucose (2-DG) is a commonly used agent as it is thought to serve as an insulin-independent, pharmacologically-induced hypoglycemia mimetic. Similar to glucose, 2-DG is transported into cells via facilitative glucose transporters and phosphorylated via hexokinases. However, because 2-DG lacks a hydroxyl group, it is not recognized by fructose isomerase and cannot be converted to fructose-6-phosphate, thus inhibiting its ability to undergo further glycolysis. Furthermore, 2-DG effectively titrates away key glycolytic machinery, especially hexokinases, needed for normal glucose metabolism, in effect, ceasing glycolysis and causing a cellular starvation signal even in the presence of glucose abundance.

Because 2-DG causes a cell to sense low glucose, it has been considered analogous to hypoglycemia for many experimental designs. Unfortunately, the inability to accurately equate a dose of 2-DG to a corresponding degree of hypoglycemia precludes our ability to directly compare these two stressors. In several studies, 2-DG has been used as a hypoglycemia surrogate in rat and mouse models of impaired counterregulation (58;75;135-139). Data from these model systems predominately observed impairments of the counterregulatory glucagon response. Of note, several studies (particularly mouse studies) failed to report if defects extended to other components of glucose counterregulation, namely catecholamine responses (58;75;137). In this study, we found 2-DG to stimulate the adrenomedullary response in NIRKO mice similarly to controls,

whether given systemically or centrally (Figures 16 and 17). As we observed a prominent adrenomedullary impairment resulting from insulin-induced hypoglycemia in the NIRKO model, these results suggest that 2-DG is not an equivalent stressor as insulin-induced hypoglycemia in our model. It is possible, due to the nature of the agent, 2-DG may have directly activated downstream glucose sensors to normalize the counterregulatory response in NIRKO mice. For example, the activity of nutrient sensor, AMPK, is increased by 30-50% in key hypothalamic regions of rats treated with 2-DG (137). Since AMPK is an important modulator of hypoglycemic counterregulation and its activity is associated with augmented counterregulatory responses (81;83;137;140), it is plausible to speculate that 2-DG restored the counterregulatory defect in NIRKO mice by targeting AMPK or other downstream glucose sensors. However, additional studies are required to address the specific mechanism of action.

Similar to its well-characterized actions in muscle and fat, insulin-mediated GLUT4 translocation has been demonstrated in neuronal cell lines (141), hippocampus (142), and hypothalamus (143). This translocation to the plasma membrane is decreased in the brains of insulin resistant rats (142;143). GLUT4 mediated glucose sensing has been speculated to be important at low glucose concentrations, where insulin-mediated glucose transport may act to supplement low intracellular glucose levels in hypothalamic glucose sensing neurons (57;122). Indeed, supporting a glucosensing role for insulin receptors and GLUT4 is their coexpression in up to 75% of glucose responsive neurons in the VMH (68). Further, neuronal GLUT4 has recently been shown to be an important mediator of hypoglycemic counterregulation and glucose sensing, as noted in neuronal

GLUT4 knockout mice (144). During hypoglycemia, when glucose transport becomes rate-limiting (145), it was speculated that decreased GLUT4 expression and/or deficient insulin action would result in reduced glucose uptake in critical glucose sensing regions of NIRKO mice. This study, however, noted equal regional brain glucose uptake during the hyperinsulinemic-hypoglycemic clamp (Figure 21), indicating that neither deficient insulin signaling nor the reduced GLUT4 levels altered glucose uptake in these brain areas. Since brain GLUT4 expression is much lower than other glucose transporters, it is likely that glucose uptake was primarily regulated by the (insulin-independent and) more abundant GLUT1 and GLUT3, thus masking any subtle effect caused by decreased GLUT4. While regional brain glucose uptake was not altered in NIRKO mice, an effect of insulin signaling and/or GLUT4 availability on mediating glucose uptake in individual glucose sensing neurons cannot be ruled out.

The enzyme glucokinase (GK) has also been implicated as a mediator of CNS glucose sensing. GK expression has been demonstrated in hypothalamic neurons by *in-situ* hybridization and RT-PCR (70). Further, studies have shown that modulation of hypothalamic GK activity can alter the counterregulatory response to hypoglycemia (55;71). Since insulin signaling regulates GK expression and glucose sensing in liver and β-cells (129;130;146), we postulated that insulin may also regulate GK expression in the brain. Similar to the β-cell (βIRKO) (129) and liver (LIRKO) (146) specific insulin receptor knockout models, we expected impaired hypoglycemic counterregulation and glucose sensing to correlate with decreased GK expression in NIRKO mice. Our studies, however, found no changes in GK expression (Figure 18). Although we cannot rule out

an effect of insulin on GK activity, based on normal hypothalamic mRNA and protein expression in NIRKO mice, insulin's effect on glucose sensing likely lies downstream of the glucose phosphorylation step.

In the absence of a change in hypothalamic glucose uptake or glucokinase expression, it may be that insulin acts on glucose sensing hypothalamic neurons (or glia) via downstream metabolic effectors that limit net glycogen breakdown (147), inhibit AMPK (82), increase glycolysis and change NADH levels (50;51), modify ATP/ADP ratio (52;121;148;149), or insulin may directly regulate ATP-activated Cl^- channel (101;119;150) or K_{ATP} channel activity (121;151;152) through actions of 3'-phospholipids (153;154).

In summary, it is shown that the chronic lack of insulin receptor signaling in the central nervous system decreases hypothalamic glucose transporter 4 expression, which may mediate insulin's effects on CNS glucose sensing. Further, the impaired sympathoadrenal response observed in NIRKO mice appears to be specific for glucose sensing, as the lack of CNS insulin signaling does not restrict neuronal activation or the adrenomedullary response to other generalized stressors. It is concluded that insulin acts directly in the brain to uniquely regulate CNS glucose sensing via GLUT4. By identifying GLUT4 as a key mediator of CNS insulin action, we are closer to uncovering the full mechanism by which insulin regulates central nervous system glucose sensing. This knowledge will allow us to identify key therapeutic targets to improve consequences associated with insulin-induced hypoglycemia in patients with diabetes.

THESIS DISCUSSION

Hypoglycemia continues to be a barrier in achieving glycemia control for patients with Type 1 and advanced Type 2 diabetes. Impaired brain glucose sensing limits the appropriate sympathoadrenal counterregulatory response and contributes to hypoglycemia unawareness, thus increasing the frequency and severity of hypoglycemia for patients with diabetes. Unraveling the biology of neuronal glucosensing, the experiments in this thesis demonstrated a key role of CNS insulin signaling in regulating hypoglycemic counterregulation and glucose sensing. Further, we have highlighted the role of a key component mediating insulin's central effects, GLUT4, which can serve as a likely therapeutic target for preventing hypoglycemia caused by intensive insulin therapy.

Insulin Therapy

Glycemic control is the primary goal of diabetes therapy. Unfortunately, achieving near-normal glucose levels is often complicated by high variability with traditional insulin treatments. Determining appropriate doses, timing, and routes of insulin delivery, conventional glucose management poses an enormous challenge in achieving and maintaining stable glucose levels. In light of evidence of insulin's role in the brain to regulate glucose homeostasis, prescribing the right insulin therapy now adds an additional level of complexity to glycemic management. With the development of novel insulin analogues (Glargine (Lantus), Detemir (Levemir), intra-nasal insulin, inhaled insulin, etc.), patients with diabetes are able to improve glycemic control while limiting additional risks associated with intensive therapy. However, these analogues have different pharmacodynamic properties that could enhance or restrict insulin transport

across the blood-brain-barrier (BBB) and thus alter the extent of insulin action in the CNS. Normally, circulating insulin crosses the BBB via a saturable transport mechanism (35). Yet, certain insulin analogues can cross the BBB more easily than human insulin, thus enabling it to enhance insulin signaling in targeted brain regions and modulate the counterregulatory response to hypoglycemia. For example, the long-acting acylated insulin analogue Determir may enhance CNS insulin signaling. Due to the lipophilic nature of Detemir, it may more easily cross the BBB relative to human insulin. In mice, detemir administration resulted in higher hypothalamic insulin signaling compared to human insulin (155). Further, detemir treatment is associated with lower incidences of hypoglycemia, less weight gain, and higher degrees of symptom awareness during hypoglycemia (156;157), indicative of enhanced CNS insulin signaling. Similarly, intra-nasal insulin can bypass the BBB, directly targeting brain tissue (158;159). Although it is a less common treatment, intra-nasal administration is associated with reduced bouts of hypoglycemia (160;161). Given the importance of aggressive insulin therapy in achieving and maintaining intensive blood glucose control, the extent to which insulin analogues act in the CNS is an important factor to consider as patients and healthcare providers assess the role of insulin action in the brain in mediating glucose sensing and/or the counterregulatory response to hypoglycemia.

CNS Insulin Action

Insulin and insulin signaling have been widely studied in classic “insulin-sensitive” peripheral tissues (i.e. fat, muscle, liver) for decades; yet, only recently has insulin action garnered appreciation for its role in the brain. Centrally, insulin signaling influences

divergent metabolic pathways. Of particular interest, brain insulin action has a novel role in regulating glucose homeostasis. Unlike its peripheral actions to suppress hepatic glucose outflow, CNS insulin action enhances glucose output under conditions of hypoglycemia. This dichotomous role of insulin action protects the body against overt glucose fluctuations and the associated consequences of glycemic dysregulation. In this study, we examined the role of CNS insulin action in regulating the counterregulatory response to hypoglycemia. The CNS-specific insulin receptor knockout (NIRKO) mouse was a powerful model system to conduct these experiments as it allowed us to isolate and clarify insulin's role in a targeted tissue. This study informed us of insulin's unique role in regulating the adrenomedullary response to hypoglycemia. It also highlighted its involvement in CNS glucose sensing. Further, it gave us clues as to the specific site(s) and mechanism of CNS insulin action.

In particular, this study recognized the ventromedial hypothalamus (VMH) as a potential site of CNS insulin action. Elegant studies performed by Borg and colleagues, among others, illustrated the role of the VMH in CNS glucose sensing and glucose homeostasis (11;48-50). With its targeted enrichment of insulin receptors and high population of glucosensing neurons, the VMH was postulated to be the most likely site of CNS insulin action. These studies revealed key impairments of glucose sensing in VMH neurons and a distinct reduction in the key glucose sensing protein GLUT4 of NIRKO mice. Overall, these results suggest the VMH serves as a site of insulin action; however, modifications observed in other brain regions suggest that the VMH is not the sole site of CNS insulin action.

The paraventricular nucleus (PVN) of the hypothalamus may also be a key site of insulin action. In this study, impaired *c-fos* activity was predominately observed in this region. The PVN is primarily thought of as an integrating center of neuronal inputs. Receiving input signals from the VMH and sending projections to autonomic centers, the PVN main purpose may be to serve as a second-level hypoglycemic detector that propagates neuronal responses initiated within the VMH. However, the PVN also contains a large subset of glucose sensing neurons and a demonstrated role in hypoglycemic counterregulation (114;162). As a consequence, the PVN may also act as a distinct, primary site of hypoglycemic detection and a key site of CNS insulin action. The pan-neural insulin receptor knockout of the NIRKO model established the importance of insulin action in the brain; however, additional studies are needed to clearly and definitively isolate specific nuclei as primary site(s) of insulin action. Some studies have created hypothalamic specific insulin receptor knockdown models (95;143;163) to pinpoint the CNS site of insulin action. Similar to NIRKO mice, targeted reductions in hypothalamic insulin receptor expression resulted in hyperphagia, weight gain, and insulin resistance in rats (95;143;163). These studies also noted a reduction of plasma membrane GLUT4 expression in the hypothalamus (143). Nevertheless, additional studies are required to unequivocally locate the specific site of CNS insulin action. Taking advantage of these approaches or generating nuclei- or cell type-specific insulin receptor knockout models via other transgenic, viral, or pharmacological approaches, the primary site(s) of CNS insulin action in regulating glucose sensing and the hypoglycemic counterregulatory response can be determined.

Insulin and Glucose Sensing Neurons

Although the focal point of CNS insulin signaling is yet to be defined, this report demonstrates a significant role of insulin in influencing a specialized neuronal population of glucose sensing neurons (GSN). Stimulated by fluxes in ambient glucose concentrations, it is believed that GSNs evolved to protect the brain against pathophysiologic glucose fluctuations, especially bouts of hypoglycemia (164). As a result, GSNs are considered the primary modulators of glucose sensing and glucose counterregulation in the brain. This thesis demonstrated impaired glucose responsiveness of hypothalamic glucose-inhibited (GI) neurons in NIRKO mice. As the primary function of GI neurons is believed to stimulate hypoglycemic counterregulation, this glucose sensing impairment correlated well with the impaired sympathoadrenal response to hypoglycemia noted in the NIRKO model. Similar to GI neurons, *in vitro* studies have also indicated a role for insulin to act directly on the glucose-excited (GE) neurons as well. Previous studies have shown insulin to increase action potential frequency of arcuate nucleus glucose-excited neurons under conditions of low glucose (42). Other studies have shown insulin to hyperpolarize VMH glucose-excited neurons by opening ATP-sensitive K^+ channels (72;121). Although GE neurons are presumably quiescent during hypoglycemia, these studies suggest that insulin may also regulate the glucose responsiveness of GE neurons, subsequently to regulate the counterregulatory response to hypoglycemia.

It is unclear which aspects of glucose counterregulation are affected by the different subsets of GSNs or if they play distinct roles in regulating the counterregulatory

response. However, emerging evidence suggest that GSNs may have distinct functions to coordinate the full counterregulatory response to hypoglycemia. Some studies attribute the sympathoadrenal response to GI neurons while the glucagon response to hypoglycemia may be primarily regulated by GE neurons (102;165;166). Due to the discrete localization of glucose sensing neurons in the VMH and technical challenges involving in isolating glucose sensing neurons, glucose responsiveness of GE neurons were not analyzed in our NIRKO mice. Since insulin can influence both GI and GE neurons, it is fair to speculate that, similar to GI neurons, some, if not all, parameters of glucose responsiveness would be impaired in NIRKO GE neurons. Alternatively, due to the intact glucagon response evident in the NIRKO model, glucose sensing in GE neurons may be preserved. In either case, evaluating glucose sensing in NIRKO GE neurons would further highlight the importance of CNS insulin action in central glucose sensing. It could also uncover the interplay between the two types of glucose sensing neurons and how they might communicate to regulate glucose sensing and hypoglycemic counterregulation.

CNS Insulin Action and Downstream Glucose Sensors

Insulin receptor signaling is an elaborate signaling cascade that coordinates the activation/regulation of a number of downstream mediators to facilitate insulin's effects on various metabolic processes. In peripheral glucose sensing, the mechanism of insulin's actions on various sensing proteins are well-defined. Whether central insulin signaling mimics peripheral action is unclear; however, identification of shared and novel CNS insulin action mediators draws us closer to defining the mechanism of CNS insulin

action. One major mediator of peripheral insulin action is the family of facilitative glucose transporters (GLUTs). Predominately, GLUT4 is a primary target of peripheral insulin action. This insulin-regulated transporter plays a key role in maintaining glucose homeostasis by enhancing insulin stimulated glucose uptake in peripheral tissues. Due to colocalization with insulin receptor in key glucosensing areas, it was speculated that, similar to peripheral actions, insulin may regulate brain GLUT4 (68), such that impaired GLUT4 activity and/or expression might contribute to defective CNS glucose sensing observed in the NIRKO mouse. Previous studies have shown that GLUT4 is localized to glucosensing regions of the hypothalamus and GLUT4 expression is down-regulated under diabetic conditions (127;142). In this thesis, immunohistochemistry results indicated that GLUT4 protein expression was markedly decreased (to approximately 30% of controls) in the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) of NIRKO mice. These results suggest that insulin acts in the brain to regulate glucose transporter expression in critical glucose sensing regions of the hypothalamus. Corroborating an important role of brain GLUT4, neuronal specific GLUT4 knockout mice also demonstrate impaired glucose sensing and hypoglycemic counterregulation (144).

Glucokinase (GK) has also been shown to be important sensing protein in glucosensing neurons (55;68;75;167) and may also mediate insulin's effects within the brain. In this study, there were no changes in GK expression levels; however, activity was not assessed in this study. Due to glucokinase's demonstrated role in regulating hypoglycemic counterregulation, it is worthwhile to assess insulin's direct effects on GK activity. Other

potential glucose sensors that may mediate insulin's central effects are ATP-sensitive ion channels and the fuel gauge AMPK. Similar to GLUTs and GK, mounting evidence highlights the emerging roles of both ion channels and AMPK in mediating hypothalamic glucose sensing (74-76;81-83;168). Spanswick and others found that insulin can regulate GE neuronal responsiveness via K_{ATP} activation (121;122). In the periphery, the effects on insulin on AMPK activity have been variable. In the heart, insulin inhibits AMPK (169;170) while reduced insulin signaling correlated to decreased AMPK activity in trophoblast stem cells (171). There is also some evidence that insulin may regulate hypothalamic AMPK activity (82); however, the nature of insulin's effects on hypothalamic AMPK activity must be further clarified. By evaluating insulin's effects on these downstream glucose sensors in further detail, a clear description of insulin's mechanism of CNS action will develop.

Summary

On a quest to achieve near-normoglycemia, hypoglycemia and impaired glucoregulation are serious challenges faced by insulin-treated diabetic patients. Insulin effects extend beyond classic insulin-sensitive tissues to include major influences in the brain to regulate glucose homeostasis, central nervous system (CNS) glucose sensing, and the counterregulatory response to hypoglycemia. The experiments in this thesis investigated insulin's role in regulating hypothalamic glucose sensing and the counterregulatory response to hypoglycemia. Utilizing a genetic mouse model which chronically lacks CNS insulin action (NIRKO mouse), this report demonstrated CNS insulin action to regulate the glucose sensitivity of glucose sensing neurons in the VMH. This report also showed CNS insulin action to uniquely modulate hypothalamic neuronal activation and

the sympathoadrenal response to hypoglycemic stress, not other stressors. Finally, it was found that insulin action in the brain regulates GLUT4 expression, which may operate as the primary mediator of CNS insulin action. Results of this study provided novel insights into insulin's role in regulating CNS glucose sensing and the counterregulatory response to hypoglycemia. This insight can aid in the overall understanding as to how the brain regulates the counterregulatory response to hypoglycemia in order to devise therapies to combat severe hypoglycemia.

REFERENCES

1. Olson,AL, Pessin,JE: Structure, function, and regulation of the mammalian facilitative glucose transporter gene family. *Annu.Rev.Nutr.* 16:235-256, 1996

2. Watson,RT, Kanzaki,M, Pessin,JE: Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. *Endocr.Rev.* 25:177-204, 2004

3. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N.Engl.J.Med.* 329:977-986, 1993

4. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 352:837-853, 1998

5. Nathan,DM, Cleary,PA, Backlund,JY, Genuth,SM, Lachin,JM, Orchard,TJ, Raskin,P, Zinman,B: Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N.Engl.J.Med.* 353:2643-2653, 2005

6. National Institute of Diabetes and Digestive and Kidney Diseases. National Diabetes Statistics, 2007 fact sheet. 2008. U.S. Department of Health and Human Services, National Institutes of Health.

7. Boyle,JP, Thompson,TJ, Gregg,EW, Barker,LE, Williamson,DF: Projection of the year 2050 burden of diabetes in the US adult population: dynamic modeling of incidence, mortality, and prediabetes prevalence. *Popul.Health Metr.* 8:29, 2010

8. Borg,MA, Borg,WP, Tamborlane,WV, Brines,ML, Shulman,GI, Sherwin,RS: Chronic hypoglycemia and diabetes impair counterregulation induced by localized 2-deoxy-glucose perfusion of the ventromedial hypothalamus in rats. *Diabetes* 48:584-587, 1999

9. Borg,MA, Sherwin,RS, Borg,WP, Tamborlane,WV, Shulman,GI: Local ventromedial hypothalamus glucose perfusion blocks counterregulation during systemic hypoglycemia in awake rats. *J.Clin.Invest* 99:361-365, 1997

10. Borg,WP, Sherwin,RS, During,MJ, Borg,MA, Shulman,GI: Local ventromedial hypothalamus glucopenia triggers counterregulatory hormone release. *Diabetes* 44:180-184, 1995

11. Borg,WP, During,MJ, Sherwin,RS, Borg,MA, Brines,ML, Shulman,GI: Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia. *J.Clin.Invest* 93:1677-1682, 1994

12. Laing,SP, Swerdlow,AJ, Slater,SD, Botha,JL, Burden,AC, Waugh,NR, Smith,AW, Hill,RD, Bingley,PJ, Patterson,CC, Qiao,Z, Keen,H: The British Diabetic Association Cohort Study, II: cause-specific mortality in patients with insulin-treated diabetes mellitus. *Diabet.Med.* 16:466-471, 1999

13. Jones,TW, Davis,EA: Hypoglycemia in children with type 1 diabetes: current issues and controversies. *Pediatr.Diabetes* 4:143-150, 2003

14. Gerich,JE, Langlois,M, Noacco,C, Karam,JH, Forsham,PH: Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. *Science* 182:171-173, 1973

15. Bolli,G, De Feo,P, Compagnucci,P, Cartechini,MG, Angeletti,G, Santeusanio,F, Brunetti,P, Gerich,JE: Abnormal glucose counterregulation in insulin-dependent diabetes mellitus. Interaction of anti-insulin antibodies and impaired glucagon and epinephrine secretion. *Diabetes* 32:134-141, 1983

16. Hirsch,BR, Shamoon,H: Defective epinephrine and growth hormone responses in type I diabetes are stimulus specific. *Diabetes* 36:20-26, 1987

17. Dagogo-Jack,SE, Craft,S, Cryer,PE: Hypoglycemia-associated autonomic failure in insulin-dependent diabetes mellitus. Recent antecedent hypoglycemia reduces autonomic responses to, symptoms of, and defense against subsequent hypoglycemia. *J.Clin.Invest* 91:819-828, 1993

18. Cryer,PE: The barrier of hypoglycemia in diabetes. *Diabetes* 57:3169-3176, 2008

19. Havrankova,J, Mate,R, Belanger,R, D'Amour,P, Ste-Marie,LG, Petit,JL: [Non-insulin-dependent diabetes in obesity (type IIB): study of insulin secretion, insulin receptors and response to low-calorie diet]. *Union Med.Can.* 116:337-341, 1987

20. Werther,GA, Hogg,A, Oldfield,BJ, McKinley,MJ, Figdor,R, Allen,AM, Mendelsohn,FA: Localization and characterization of insulin receptors in rat brain and pituitary gland using in vitro autoradiography and computerized densitometry. *Endocrinology* 121:1562-1570, 1987

21. Marks,JL, Porte,D, Jr., Stahl,WL, Baskin,DG: Localization of insulin receptor mRNA in rat brain by in situ hybridization. *Endocrinology* 127:3234-3236, 1990

22. Hopkins,DF, Williams,G: Insulin receptors are widely distributed in human brain and bind human and porcine insulin with equal affinity. *Diabet.Med.* 14:1044-1050, 1997

23. Freychet,P: Insulin receptors and insulin actions in the nervous system. *Diabetes Metab Res Rev.* 16:390-392, 2000

24. Baskin,DG, Figlewicz,LD, Seeley,RJ, Woods,SC, Porte,D, Jr., Schwartz,MW: Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res.* 848:114-123, 1999

25. Schwartz,MW, Figlewicz,DP, Baskin,DG, Woods,SC, Porte,D, Jr.: Insulin in the brain: a hormonal regulator of energy balance. *Endocr.Rev.* 13:387-414, 1992

26. Jonas,EA, Knox,RJ, Smith,TC, Wayne,NL, Connor,JA, Kaczmarek,LK: Regulation by insulin of a unique neuronal Ca2+ pool and of neuropeptide secretion. *Nature* 385:343-346, 1997

27. Rossetti,L, Massillon,D, Barzilai,N, Vuguin,P, Chen,W, Hawkins,M, Wu,J, Wang,J: Short term effects of leptin on hepatic gluconeogenesis and in vivo insulin action. *J Biol.Chem.* 272:27758-27763, 1997

28. Bruning,JC, Gautam,D, Burks,DJ, Gillette,J, Schubert,M, Orban,PC, Klein,R, Krone,W, Muller-Wieland,D, Kahn,CR: Role of brain insulin receptor in control of body weight and reproduction. *Science* 289:2122-2125, 2000

29. Pliquett,RU, Fuhrer,D, Falk,S, Zysset,S, von Cramon,DY, Stumvoll,M: The effects of insulin on the central nervous system--focus on appetite regulation. *Horm.Metab Res.* 38:442-446, 2006

30. Plum,L, Belgardt,BF, Bruning,JC: Central insulin action in energy and glucose homeostasis. *J.Clin.Invest* 116:1761-1766, 2006

31. Morton,GJ, Cummings,DE, Baskin,DG, Barsh,GS, Schwartz,MW: Central nervous system control of food intake and body weight. *Nature* 443:289-295, 2006

32. Davis,MR, Mellman,M, Shamoon,H: Physiologic hyperinsulinemia enhances counterregulatory hormone responses to hypoglycemia in IDDM. *J.Clin.Endocrinol.Metab* 76:1383-1385, 1993

33. Davis,SN, Goldstein,RE, Price,L, Jacobs,J, Cherrington,AD: The effects of insulin on the counterregulatory response to equivalent hypoglycemia in patients with insulin-dependent diabetes mellitus. *J.Clin.Endocrinol.Metab* 77:1300-1307, 1993

34. Schwartz,MW, Bergman,RN, Kahn,SE, Taborsky,GJ, Jr., Fisher,LD, Sipols,AJ, Woods,SC, Steil,GM, Porte,D, Jr.: Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. *J Clin.Invest* 88:1272-1281, 1991

35. Baura,GD, Foster,DM, Porte,D, Jr., Kahn,SE, Bergman,RN, Cobelli,C, Schwartz,MW: Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *J.Clin.Invest* 92:1824-1830, 1993

36. Morgan,DA, Balon,TW, Ginsberg,BH, Mark,AL: Nonuniform regional sympathetic nerve responses to hyperinsulinemia in rats. *Am.J Physiol* 264:R423-R427, 1993

37. Schultz-Klarr,S, Wright-Richey,J, Dunbar,JC: Plasma glucose, insulin and cardiovascular responses after intravenous intracerebroventricular injections of

insulin, 2-deoxyglucose and glucose in rats. *Diabetes Res.Clin.Pract.* 26:81-89, 1994

38. Demuro,G, Obici,S: Central nervous system and control of endogenous glucose production. *Curr.Diab.Rep.* 6:188-193, 2006

39. Davis,SN, Colburn,C, Dobbins,R, Nadeau,S, Neal,D, Williams,P, Cherrington,AD: Evidence that the brain of the conscious dog is insulin sensitive. *J.Clin.Invest* 95:593-602, 1995

40. Davis,SN, Dunham,B, Walmsley,K, Shavers,C, Neal,D, Williams,P, Cherrington,AD: Brain of the conscious dog is sensitive to physiological changes in circulating insulin. *Am.J.Physiol* 272:E567-E575, 1997

41. Fisher,SJ, Bruning,JC, Lannon,S, Kahn,CR: Insulin signaling in the central nervous system is critical for the normal sympathoadrenal response to hypoglycemia. *Diabetes* 54:1447-1451, 2005

42. Wang,R, Liu,X, Hentges,ST, Dunn-Meynell,AA, Levin,BE, Wang,W, Routh,VH: The regulation of glucose-excited neurons in the hypothalamic arcuate nucleus by glucose and feeding-relevant peptides. *Diabetes* 53:1959-1965, 2004

43. Thorens,B: Mechanisms of glucose sensing and multiplicity of glucose sensors. *Ann.Endocrinol.(Paris)* 65:9-12, 2004

44. Yang,XJ, Kow,LM, Funabashi,T, Mobbs,CV: Hypothalamic glucose sensor: similarities to and differences from pancreatic beta-cell mechanisms. *Diabetes* 48:1763-1772, 1999

45. Donovan,CM, Hamilton-Wessler,M, Halter,JB, Bergman,RN: Primacy of liver glucosensors in the sympathetic response to progressive hypoglycemia. *Proc.Natl.Acad.Sci.U.S.A* 91:2863-2867, 1994

46. Oomura,Y, Ono,T, Ooyama,H, Wayner,MJ: Glucose and osmosensitive neurones of the rat hypothalamus. *Nature* 222:282-284, 1969

47. Bingham,EM, Hopkins,D, Smith,D, Pernet,A, Hallett,W, Reed,L, Marsden,PK, Amiel,SA: The role of insulin in human brain glucose metabolism: an 18fluoro-deoxyglucose positron emission tomography study. *Diabetes* 51:3384-3390, 2002

48. Lynch,JP, Wojnar,MM, Lang,CH: Ventromedial hypothalamic lesions impair glucoregulation in response to endotoxin. *Am.J.Physiol* 272:R1525-R1531, 1997

49. Fugo,KR, Lawson,MA, Lee,BJ: Ibotenic acid lesions reduce noradrenergic activation in ventromedial hypothalamus during hypoglycemia. *Brain Res.* 1111:105-110, 2006

50. Yang,XJ, Kow,LM, Pfaff,DW, Mobbs,CV: Metabolic pathways that mediate inhibition of hypothalamic neurons by glucose. *Diabetes* 53:67-73, 2004

51. Mobbs,CV, Kow,LM, Yang,XJ: Brain glucose-sensing mechanisms: ubiquitous silencing by aglycemia vs. hypothalamic neuroendocrine responses. *Am.J.Physiol Endocrinol.Metab* 281:E649-E654, 2001

52. Schuit,FC, Huypens,P, Heimberg,H, Pipeleers,DG: Glucose sensing in pancreatic beta-cells: a model for the study of other glucose-regulated cells in gut, pancreas, and hypothalamus. *Diabetes* 50:1-11, 2001

53. Penicaud,L, Leloup,C, Lorsignol,A, Alquier,T, Guillod,E: Brain glucose sensing mechanism and glucose homeostasis. *Curr.Opin.Clin.Nutr.Metab Care* 5:539-543, 2002

54. McCrimmon,R: The mechanisms that underlie glucose sensing during hypoglycaemia in diabetes. *Diabet.Med.* 25:513-522, 2008

55. Kang,L, Dunn-Meynell,AA, Routh,VH, Gaspers,LD, Nagata,Y, Nishimura,T, Eiki,J, Zhang,BB, Levin,BE: Glucokinase is a critical regulator of ventromedial hypothalamic neuronal glucosensing. *Diabetes* 55:412-420, 2006

56. Song,Z, Levin,BE, McArdle,JJ, Bakhos,N, Routh,VH: Convergence of pre- and postsynaptic influences on glucosensing neurons in the ventromedial hypothalamic nucleus. *Diabetes* 50:2673-2681, 2001

57. Levin,BE, Routh,VH, Kang,L, Sanders,NM, Dunn-Meynell,AA: Neuronal glucosensing: what do we know after 50 years? *Diabetes* 53:2521-2528, 2004

58. Tong,Q, Ye,C, McCrimmon,RJ, Dhillon,H, Choi,B, Kramer,MD, Yu,J, Yang,Z, Christiansen,LM, Lee,CE, Choi,CS, Zigman,JM, Shulman,GI, Sherwin,RS, Elmquist,JK, Lowell,BB: Synaptic glutamate release by ventromedial hypothalamic neurons is part of the neurocircuitry that prevents hypoglycemia. *Cell Metab* 5:383-393, 2007

59. Chan,O, Zhu,W, Ding,Y, McCrimmon,RJ, Sherwin,RS: Blockade of GABA(A) receptors in the ventromedial hypothalamus further stimulates glucagon and sympathoadrenal but not the hypothalamo-pituitary-adrenal response to hypoglycemia. *Diabetes* 55:1080-1087, 2006

60. Brown,GK: Glucose transporters: structure, function and consequences of deficiency. *J.Inherit.Metab Dis.* 23:237-246, 2000

61. Choeiri,C, Staines,W, Messier,C: Immunohistochemical localization and quantification of glucose transporters in the mouse brain. *Neuroscience* 111:19-34, 2002

62. Dwyer,DS, Vannucci,SJ, Simpson,IA: Expression, regulation, and functional role of glucose transporters (GLUTs) in brain. *Int.Rev.Neurobiol.* 51:159-188, 2002

63. Gerhart,DZ, Leino,RL, Borson,ND, Taylor,WE, Gronlund,KM, McCall,AL, Drewes,LR: Localization of glucose transporter GLUT 3 in brain: comparison of rodent and dog using species-specific carboxyl-terminal antisera. *Neuroscience* 66:237-246, 1995

64. Leloup,C, Arluison,M, Lepetit,N, Cartier,N, Marfaing-Jallat,P, Ferre,P, Penicaud,L: Glucose transporter 2 (GLUT 2): expression in specific brain nuclei. *Brain Res.* 638:221-226, 1994

65. Roncero,I, Alvarez,E, Chowen,JA, Sanz,C, Rabano,A, Vazquez,P, Blazquez,E: Expression of glucose transporter isoform GLUT-2 and glucokinase genes in human brain. *J Neurochem.* 88:1203-1210, 2004

66. Zhou,J, Roane,DS, Xi,X, Bogacka,I, Li,B, Ryan,DH, Martin,RJ: Short-term food restriction and refeeding alter expression of genes likely involved in brain glucosensing. *Exp.Biol.Med.(Maywood.)* 228:943-950, 2003

67. Garcia,ML, Millan,C, Balmaceda-Aguilera,C, Castro,T, Pastor,P, Montecinos,H, Reinicke,K, Zuniga,F, Vera,JC, Onate,SA, Nualart,F: Hypothalamic ependymal-

glial cells express the glucose transporter GLUT2, a protein involved in glucose sensing. *J.Neurochem.* 86:709-724, 2003

68. Kang,L, Routh,VH, Kuzhikandathil,EV, Gaspers,LD, Levin,BE: Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. *Diabetes* 53:549-559, 2004

69. El Messari,S, Leloup,C, Quignon,M, Brisorgueil,MJ, Penicaud,L, Arluison,M: Immunocytochemical localization of the insulin-responsive glucose transporter 4 (Glut4) in the rat central nervous system. *J.Comp Neurol.* 399:492-512, 1998

70. Lynch,RM, Tompkins,LS, Brooks,HL, Dunn-Meynell,AA, Levin,BE: Localization of glucokinase gene expression in the rat brain. *Diabetes* 49:693-700, 2000

71. Levin,BE, Becker,TC, Eiki,J, Zhang,BB, Dunn-Meynell,AA: Ventromedial hypothalamic glucokinase is an important mediator of the counterregulatory response to insulin-induced hypoglycemia. *Diabetes* 57:1371-1379, 2008

72. Ashford,ML, Boden,PR, Treherne,JM: Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K+ channels. *Pflugers Arch.* 415:479-483, 1990

73. Dunn-Meynell,AA, Rawson,NE, Levin,BE: Distribution and phenotype of neurons containing the ATP-sensitive K+ channel in rat brain. *Brain Res.* 814:41-54, 1998

74. Evans,ML, McCrimmon,RJ, Flanagan,DE, Keshavarz,T, Fan,X, McNay,EC, Jacob,RJ, Sherwin,RS: Hypothalamic ATP-sensitive K + channels play a key role

in sensing hypoglycemia and triggering counterregulatory epinephrine and glucagon responses. *Diabetes* 53:2542-2551, 2004

75. Miki,T, Liss,B, Minami,K, Shiuchi,T, Saraya,A, Kashima,Y, Horiuchi,M, Ashcroft,F, Minokoshi,Y, Roeper,J, Seino,S: ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. *Nat.Neurosci.* 4:507-512, 2001

76. McCrimmon,RJ, Evans,ML, Fan,X, McNay,EC, Chan,O, Ding,Y, Zhu,W, Gram,DX, Sherwin,RS: Activation of ATP-sensitive K+ channels in the ventromedial hypothalamus amplifies counterregulatory hormone responses to hypoglycemia in normal and recurrently hypoglycemic rats. *Diabetes* 54:3169-3174, 2005

77. Kinard,TA, Satin,LS: An ATP-sensitive Cl- channel current that is activated by cell swelling, cAMP, and glyburide in insulin-secreting cells. *Diabetes* 44:1461-1466, 1995

78. Sheppard,DN, Robinson,KA: Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane conductance regulator Cl- channels expressed in a murine cell line. *J.Physiol* 503 (Pt 2):333-346, 1997

79. Mulberg,AE, Wiedner,EB, Bao,X, Marshall,J, Jefferson,DM, Altschuler,SM: Cystic fibrosis transmembrane conductance regulator protein expression in brain. *Neuroreport* 5:1684-1688, 1994

80. Mulberg,AE, Weyler,RT, Altschuler,SM, Hyde,TM: Cystic fibrosis transmembrane conductance regulator expression in human hypothalamus. *Neuroreport* 9:141-144, 1998

81. Han,SM, Namkoong,C, Jang,PG, Park,IS, Hong,SW, Katakami,H, Chun,S, Kim,SW, Park,JY, Lee,KU, Kim,MS: Hypothalamic AMP-activated protein kinase mediates counter-regulatory responses to hypoglycaemia in rats. *Diabetologia* 48:2170-2178, 2005

82. Minokoshi,Y, Alquier,T, Furukawa,N, Kim,YB, Lee,A, Xue,B, Mu,J, Foufelle,F, Ferre,P, Birnbaum,MJ, Stuck,BJ, Kahn,BB: AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* 428:569-574, 2004

83. McCrimmon,RJ, Fan,X, Cheng,H, McNay,E, Chan,O, Shaw,M, Ding,Y, Zhu,W, Sherwin,RS: Activation of AMP-Activated Protein Kinase Within the Ventromedial Hypothalamus Amplifies Counterregulatory Hormone Responses in Rats With Defective Counterregulation. *Diabetes* 55:1755-1760, 2006

84. McCrimmon,RJ, Fan,X, Ding,Y, Zhu,W, Jacob,RJ, Sherwin,RS: Potential role for AMP-activated protein kinase in hypoglycemia sensing in the ventromedial hypothalamus. *Diabetes* 53:1953-1958, 2004

85. Chan,O, Cheng,H, Herzog,R, Czyzyk,D, Zhu,W, Wang,A, McCrimmon,RJ, Seashore,MR, Sherwin,RS: Increased GABAergic tone in the ventromedial hypothalamus contributes to suppression of counterregulatory responses after antecedent hypoglycemia. *Diabetes* 57:1363-1370, 2008

86. The DCCT Research Group: Epidemiology of severe hypoglycemia in the diabetes control and complications trial. *Am.J Med.* 90:450-459, 1991

87. Cryer,PE, Davis,SN, Shamoon,H: Hypoglycemia in diabetes. *Diabetes Care* 26:1902-1912, 2003

88. Davis,SN, Shavers,C, Collins,L, Cherrington,AD, Price,L, Hedstrom,C: Effects of physiological hyperinsulinemia on counterregulatory response to prolonged hypoglycemia in normal humans. *Am.J.Physiol* 267:E402-E410, 1994

89. Davis,SN, Goldstein,RE, Jacobs,J, Price,L, Wolfe,R, Cherrington,AD: The effects of differing insulin levels on the hormonal and metabolic response to equivalent hypoglycemia in normal humans. *Diabetes* 42:263-272, 1993

90. Lingenfelser,T, Overkamp,D, Renn,W, Buettner,U, Kimmerle,K, Schmalfuss,A, Jakober,B: Insulin-associated modulation of neuroendocrine counterregulation, hypoglycemia perception, and cerebral function in insulin-dependent diabetes mellitus: evidence for an intrinsic effect of insulin on the central nervous system. *J Clin.Endocrinol.Metab* 81:1197-1205, 1996

91. Diamond,MP, Hallarman,L, Starick-Zych,K, Jones,TW, Connolly-Howard,M, Tamborlane,WV, Sherwin,RS: Suppression of counterregulatory hormone response to hypoglycemia by insulin per se. *J Clin.Endocrinol.Metab* 72:1388-1390, 1991

92. Liu,D, Moberg,E, Kollind,M, Lins,PE, Adamson,U: A high concentration of circulating insulin suppresses the glucagon response to hypoglycemia in normal man. *J Clin.Endocrinol.Metab* 73:1123-1128, 1991

93. Mellman,MJ, Davis,MR, Shamoon,H: Effect of physiological hyperinsulinemia on counterregulatory hormone responses during hypoglycemia in humans. *J Clin.Endocrinol.Metab* 75:1293-1297, 1992

94. Kerr,D, Reza,M, Smith,N, Leatherdale,BA: Importance of insulin in subjective, cognitive, and hormonal responses to hypoglycemia in patients with IDDM. *Diabetes* 40:1057-1062, 1991

95. Paranjape,SA, Chan,O, Zhu,W, Horblitt,AM, McNay,EC, Cresswell,JA, Bogan,JS, McCrimmon,RJ, Sherwin,RS: Influence of insulin in the ventromedial hypothalamus on pancreatic glucagon secretion in vivo. *Diabetes* 59:1521-1527, 2010

96. Paranjape,S, Chan,O, Zhu,W, Wang,A, Fan,X, McCrimmon,R, Sherwin,R: Insulin signaling in the ventromedial hypothalamus (VMH) regulates glucagon secretion during eu- and hypoglycemia. *Diabetes* 57:A39, 2008

97. Ishihara,KK, Haywood,SC, Daphna-Iken,D, Puente,EC, Fisher,SJ: Brain insulin infusion does not augment the counterregulatory response to hypoglycemia or glucoprivation. *Metabolism* 2009

98. Schubert,M, Gautam,D, Surjo,D, Ueki,K, Baudler,S, Schubert,D, Kondo,T, Alber,J, Galldiks,N, Kustermann,E, Arndt,S, Jacobs,AH, Krone,W, Kahn,CR, Bruning,JC: Role for neuronal insulin resistance in neurodegenerative diseases. *Proc.Natl.Acad.Sci.U.S.A* 101:3100-3105, 2004

99. Fisher,SJ, Kahn,CR: Insulin signaling is required for insulin's direct and indirect action on hepatic glucose production. *J Clin.Invest* 111:463-468, 2003

100. Paxinos G, Franklin KBJ: The mouse brain in stereotaxic coordinates. London, Academic Press, 2001,

101. Song,Z, Routh,VH: Differential effects of glucose and lactate on glucosensing neurons in the ventromedial hypothalamic nucleus. *Diabetes* 54:15-22, 2005

102. Song,Z, Routh,VH: Recurrent hypoglycemia reduces the glucose sensitivity of glucose-inhibited neurons in the ventromedial hypothalamus nucleus. *Am.J.Physiol Regul.Integr.Comp Physiol* 291:R1283-R1287, 2006

103. Shah,SD, Clutter,WE, Cryer,PE: External and internal standards in the single-isotope derivative (radioenzymatic) measurement of plasma norepinephrine and epinephrine. *J.Lab Clin.Med.* 106:624-629, 1985

104. Kovacs,KJ: c-Fos as a transcription factor: a stressful (re)view from a functional map. *Neurochem.Int.* 33:287-297, 1998

105. Bolli,GB, Fanelli,CG: Physiology of glucose counterregulation to hypoglycemia. *Endocrinol.Metab Clin.North Am.* 28:467-93, v, 1999

106. Jones,TW, Borg,WP, Borg,MA, Boulware,SD, McCarthy,G, Silver,D, Tamborlane,WV, Sherwin,RS: Resistance to neuroglycopenia: an adaptive response during intensive insulin treatment of diabetes. *J Clin.Endocrinol.Metab* 82:1713-1718, 1997

107. Semakula,C, Damberg,G, Kendall,D, Seaquist,ER: The use of the hypoglycaemic clamp in the assessment of pituitary function. *Clin.Endocrinol.(Oxf)* 51:709-714, 1999

108. Inouye,K, Shum,K, Chan,O, Mathoo,J, Matthews,SG, Vranic,M: Effects of recurrent hyperinsulinemia with and without hypoglycemia on counterregulation in diabetic rats. *Am.J Physiol Endocrinol.Metab* 282:E1369-E1379, 2002

109. Chan,O, Chan,S, Inouye,K, Shum,K, Matthews,SG, Vranic,M: Diabetes impairs hypothalamo-pituitary-adrenal (HPA) responses to hypoglycemia, and insulin treatment normalizes HPA but not epinephrine responses. *Diabetes* 51:1681-1689, 2002

110. Tsay,HJ, Li,HY, Lin,CH, Yang,YL, Yeh,JY, Lin,MT: Heatstroke induces c-fos expression in the rat hypothalamus. *Neurosci.Lett.* 262:41-44, 1999

111. Harikai,N, Tomogane,K, Sugawara,T, Tashiro,S: Differences in hypothalamic Fos expressions between two heat stress conditions in conscious mice. *Brain Res.Bull.* 61:617-626, 2003

112. Paranjape,SA, Briski,KP: Recurrent insulin-induced hypoglycemia causes site-specific patterns of habituation or amplification of CNS neuronal genomic activation. *Neuroscience* 130:957-970, 2005

113. Niimi,M, Sato,M, Tamaki,M, Wada,Y, Takahara,J, Kawanishi,K: Induction of Fos protein in the rat hypothalamus elicited by insulin-induced hypoglycemia. *Neurosci.Res.* 23:361-364, 1995

114. Evans,SB, Wilkinson,CW, Bentson,K, Gronbeck,P, Zavosh,A, Figlewicz,DP: PVN activation is suppressed by repeated hypoglycemia but not antecedent corticosterone in the rat. *Am.J Physiol Regul.Integr.Comp Physiol* 281:R1426-R1436, 2001

115. Kale,AY, Paranjape,SA, Briski,KP: I.c.v. administration of the nonsteroidal glucocorticoid receptor antagonist, CP-472555, prevents exacerbated hypoglycemia during repeated insulin administration. *Neuroscience* 140:555-565, 2006

116. Powell,AM, Sherwin,RS, Shulman,GI: Impaired hormonal responses to hypoglycemia in spontaneously diabetic and recurrently hypoglycemic rats. Reversibility and stimulus specificity of the deficits. *J.Clin.Invest* 92:2667-2674, 1993

117. Canabal,DD, Potian,JG, Duran,RG, McArdle,JJ, Routh,VH: Hyperglycemia impairs glucose and insulin regulation of nitric oxide production in glucose-

inhibited neurons in the ventromedial hypothalamus. *Am.J.Physiol Regul.Integr.Comp Physiol* 293:R592-R600, 2007

118. Canabal,DD, Song,Z, Potian,JG, Beuve,A, McArdle,JJ, Routh,VH: Glucose, insulin, and leptin signaling pathways modulate nitric oxide synthesis in glucose-inhibited neurons in the ventromedial hypothalamus. *Am.J.Physiol Regul.Integr.Comp Physiol* 292:R1418-R1428, 2007

119. Murphy,BA, Fakira,KA, Song,Z, Beuve,A, Routh,VH: AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons. *Am.J.Physiol Cell Physiol* 297:C750-C758, 2009

120. McColl,G, Rogers,AN, Alavez,S, Hubbard,AE, Melov,S, Link,CD, Bush,AI, Kapahi,P, Lithgow,GJ: Insulin-like signaling determines survival during stress via posttranscriptional mechanisms in C. elegans. *Cell Metab* 12:260-272, 2010

121. Spanswick,D, Smith,MA, Mirshamsi,S, Routh,VH, Ashford,ML: Insulin activates ATP-sensitive K+ channels in hypothalamic neurons of lean, but not obese rats. *Nat.Neurosci.* 3:757-758, 2000

122. Cotero,VE, Routh,VH: Insulin blunts the response of glucose-excited (GE) neurons in the ventrolateral-ventromedial hypothalamic nucleus (VL-VMN) to decreased glucose. *Am.J.Physiol Endocrinol.Metab* 2009

123. Joost,HG, Bell,GI, Best,JD, Birnbaum,MJ, Charron,MJ, Chen,YT, Doege,H, James,DE, Lodish,HF, Moley,KH, Moley,JF, Mueckler,M, Rogers,S, Schurmann,A, Seino,S, Thorens,B: Nomenclature of the GLUT/SLC2A family of sugar/polyol transport facilitators. *Am.J.Physiol Endocrinol.Metab* 282:E974-E976, 2002

124. Kalaria,RN, Gravina,SA, Schmidley,JW, Perry,G, Harik,SI: The glucose transporter of the human brain and blood-brain barrier. *Ann.Neurol.* 24:757-764, 1988

125. Vannucci,SJ, Clark,RR, Koehler-Stec,E, Li,K, Smith,CB, Davies,P, Maher,F, Simpson,IA: Glucose transporter expression in brain: relationship to cerebral glucose utilization. *Dev.Neurosci.* 20:369-379, 1998

126. Uehara,Y, Nipper,V, McCall,AL: Chronic insulin hypoglycemia induces GLUT-3 protein in rat brain neurons. *Am.J Physiol* 272:E716-E719, 1997

127. Vannucci,SJ, Koehler-Stec,EM, Li,K, Reynolds,TH, Clark,R, Simpson,IA: GLUT4 glucose transporter expression in rodent brain: effect of diabetes. *Brain Res.* 797:1-11, 1998

128. Dunn-Meynell,AA, Routh,VH, Kang,L, Gaspers,L, Levin,BE: Glucokinase is the likely mediator of glucosensing in both glucose-excited and glucose-inhibited central neurons. *Diabetes* 51:2056-2065, 2002

129. Otani,K, Kulkarni,RN, Baldwin,AC, Krutzfeldt,J, Ueki,K, Stoffel,M, Kahn,CR, Polonsky,KS: Reduced {beta}-cell mass and altered glucose sensing impairs insulin secretory function in mice with pancreatic {beta}-cell knockout of the insulin receptor. *Am.J.Physiol Endocrinol.Metab* 2003

130. Matschinsky,FM: Regulation of pancreatic beta-cell glucokinase: from basics to therapeutics. *Diabetes* 51 Suppl 3:S394-S404, 2002

131. Sokoloff,L, Reivich,M, Kennedy,C, Des Rosiers,MH, Patlak,CS, Pettigrew,KD, Sakurada,O, Shinohara,M: The [14C]deoxyglucose method for the measurement

of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J.Neurochem.* 28:897-916, 1977

132. Heymsfield,SB, Greenberg,AS, Fujioka,K, Dixon,RM, Kushner,R, Hunt,T, Lubina,JA, Patane,J, Self,B, Hunt,P, McCamish,M: Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA* 282:1568-1575, 1999

133. Briski,KP, Brandt,JA: Oxytocin and vasopressin neurones in principal and accessory hypothalamic magnocellular structures express Fos-immunoreactivity in response to acute glucose deprivation. *J.Neuroendocrinol.* 12:409-414, 2000

134. Hoffman,GE, Smith,MS, Verbalis,JG: c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Front Neuroendocrinol.* 14:173-213, 1993

135. Briski,KP, Sylvester,PW: Hypothalamic orexin-A-immunpositive neurons express Fos in response to central glucopenia. *Neuroreport* 12:531-534, 2001

136. Schmidt,S, Richter,M, Montag,D, Sartorius,T, Gawlik,V, Hennige,AM, Scherneck,S, Himmelbauer,H, Lutz,SZ, Augustin,R, Kluge,R, Ruth,P, Joost,HG, Schurmann,A: Neuronal functions, feeding behavior, and energy balance in Slc2a3+/- mice. *Am.J.Physiol Endocrinol.Metab* 295:E1084-E1094, 2008

137. Alquier,T, Kawashima,J, Tsuji,Y, Kahn,BB: Role of hypothalamic adenosine 5'-monophosphate-activated protein kinase in the impaired counterregulatory response induced by repetitive neuroglucopenia. *Endocrinology* 148:1367-1375, 2007

138. Sanders,NM, Ritter,S: Repeated 2-deoxy-D-glucose-induced glucoprivation attenuates Fos expression and glucoregulatory responses during subsequent glucoprivation. *Diabetes* 49:1865-1874, 2000

139. Karlsson,S, Scheurink,AJ, Ahren,B: Gender difference in the glucagon response to glucopenic stress in mice. *Am.J.Physiol Regul.Integr.Comp Physiol* 282:R281-R288, 2002

140. Mountjoy,PD, Bailey,SJ, Rutter,GA: Inhibition by glucose or leptin of hypothalamic neurons expressing neuropeptide Y requires changes in AMP-activated protein kinase activity. *Diabetologia* 50:168-177, 2007

141. Benomar,Y, Naour,N, Aubourg,A, Bailleux,V, Gertler,A, Djiane,J, Guerre-Millo,M, Taouis,M: Insulin and leptin induce Glut4 plasma membrane translocation and glucose uptake in a human neuronal cell line by a phosphatidylinositol 3-kinase- dependent mechanism. *Endocrinology* 147:2550-2556, 2006

142. Piroli,GG, Grillo,CA, Reznikov,LR, Adams,S, McEwen,BS, Charron,MJ, Reagan,LP: Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus. *Neuroendocrinology* 85:71-80, 2007

143. Grillo,CA, Tamashiro,KL, Piroli,GG, Melhorn,S, Gass,JT, Newsom,RJ, Reznikov,LR, Smith,A, Wilson,SP, Sakai,RR, Reagan,LP: Lentivirus-mediated downregulation of hypothalamic insulin receptor expression. *Physiol Behav.* 92:691-701, 2007

144. Puente,E, Daphna-Iken,D, Bree,A, Suzuki,Y, Georgopoulos,I, Kahn,BB, Fisher,S: Impaired counterregulatory response to hypoglycemia and impaired glucose

tolerance in brain glucose transporter 4 (GLUT4) knockout mice. *Diabetes* 58:A13, 2009

145. Suda,S, Shinohara,M, Miyaoka,M, Lucignani,G, Kennedy,C, Sokoloff,L: The lumped constant of the deoxyglucose method in hypoglycemia: effects of moderate hypoglycemia on local cerebral glucose utilization in the rat. *J.Cereb.Blood Flow Metab* 10:499-509, 1990

146. Michael,MD, Kulkarni,RN, Postic,C, Previs,SF, Shulman,GI, Magnuson,MA, Kahn,CR: Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol.Cell* 6:87-97, 2000

147. Choi,IY, Seaquist,ER, Gruetter,R: Effect of hypoglycemia on brain glycogen metabolism in vivo. *J.Neurosci.Res.* 72:25-32, 2003

148. Schwartz,MW, Woods,SC, Porte,D, Jr., Seeley,RJ, Baskin,DG: Central nervous system control of food intake. *Nature* 404:661-671, 2000

149. Kennedy,HJ, Pouli,AE, Ainscow,EK, Jouaville,LS, Rizzuto,R, Rutter,GA: Glucose generates sub-plasma membrane ATP microdomains in single islet beta-cells. Potential role for strategically located mitochondria. *J Biol.Chem.* 274:13281-13291, 1999

150. Benani,A, Troy,S, Carmona,MC, Fioramonti,X, Lorsignol,A, Leloup,C, Casteilla,L, Penicaud,L: Role for mitochondrial reactive oxygen species in brain lipid sensing: redox regulation of food intake. *Diabetes* 56:152-160, 2007

151. O'Malley,D, Shanley,LJ, Harvey,J: Insulin inhibits rat hippocampal neurones via activation of ATP-sensitive K+ and large conductance Ca2+-activated K+ channels. *Neuropharmacology* 44:855-863, 2003

152. Belgardt,BF, Okamura,T, Bruning,JC: Hormone and glucose signalling in POMC and AgRP neurons. *J.Physiol* 587:5305-5314, 2009

153. Shyng,SL, Barbieri,A, Gumusboga,A, Cukras,C, Pike,L, Davis,JN, Stahl,PD, Nichols,CG: Modulation of nucleotide sensitivity of ATP-sensitive potassium channels by phosphatidylinositol-4-phosphate 5-kinase. *Proc.Natl.Acad.Sci.U.S.A* 97:937-941, 2000

154. Shyng,SL, Nichols,CG: Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science* 282:1138-1141, 1998

155. Hennige,AM, Sartorius,T, Tschritter,O, Preissl,H, Fritsche,A, Ruth,P, Haring,HU: Tissue selectivity of insulin detemir action in vivo. *Diabetologia* 49:1274-1282, 2006

156. Rossetti,P, Porcellati,F, Ricci,NB, Candeloro,P, Cioli,P, Bolli,GB, Fanelli,CG: Different brain responses to hypoglycemia induced by equipotent doses of the long-acting insulin analog detemir and human regular insulin in humans. *Diabetes* 57:746-756, 2008

157. Tschritter,O, Schafer,SA, Klett,J, Pfafflin,A, Haring,HU, Hennige,AM, Fritsche,A: Insulin detemir causes increased symptom awareness during hypoglycaemia compared to human insulin. *Diabetes Obes.Metab* 11:1017-1026, 2009

158. Kern,W, Born,J, Schreiber,H, Fehm,HL: Central nervous system effects of intranasally administered insulin during euglycemia in men. *Diabetes* 48:557-563, 1999

159. Hanson,LR, Frey,WH: Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. *BMC.Neurosci.* 9 Suppl 3:S5, 2008

160. Brandt G, Sileno A, Cohen AS, Souza S, Spann B, Costantino HR, Seiberling M, Quay S: Intranasal Insulin: Phase 2 Glucose Tolerance Study in Type 2 Diabetics (Abstract). *Diabetes (Abstract)*, 2008

161. Francis,G, Martinez,J, Liu,W, Nguyen,T, Ayer,A, Fine,J, Zochodne,D, Hanson,LR, Frey,WH, Toth,C: Intranasal insulin ameliorates experimental diabetic neuropathy. *Diabetes* 58:934-945, 2009

162. Evans,SB, Wilkinson,CW, Gronbeck,P, Bennett,JL, Taborsky,GJ, Jr., Figlewicz,DP: Inactivation of the PVN during hypoglycemia partially simulates hypoglycemia-associated autonomic failure. *Am.J Physiol Regul.Integr.Comp Physiol* 284:R57-R65, 2003

163. Obici,S, Feng,Z, Karkanias,G, Baskin,DG, Rossetti,L: Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nat.Neurosci.* 5:566-572, 2002

164. Routh,VH: Glucose Sensing Neurons in the Ventromedial Hypothalamus. *Sensors* 10:9002-9025, 2010

165. Fioramonti,X, Marsollier,N, Song,Z, Fakira,KA, Patel,RM, Brown,S, Duparc,T, Pica-Mendez,A, Sanders,NM, Knauf,C, Valet,P, McCrimmon,RJ, Beuve,A, Magnan,C, Routh,VH: Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. *Diabetes* 59:519-528, 2010

166. Fioramonti,X, Song,Z, Vazirani,RP, Beuve,A, Routh,VH: Hypothalamic NO in hypoglycemia detection and counter-regulation: A two edged sword. *Antioxid.Redox.Signal.* 2010

167. Parton,LE, Ye,CP, Coppari,R, Enriori,PJ, Choi,B, Zhang,CY, Xu,C, Vianna,CR, Balthasar,N, Lee,CE, Elmquist,JK, Cowley,MA, Lowell,BB: Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. *Nature* 449:228-232, 2007

168. McCrimmon,RJ, Fan,X, Evans,ML, McNay,E, Chan,O, Ding,Y, Sherwin,RS: VMH K-ATP channels play a key role in sensing hypoglycemia and triggering counterregulatory hormonal responses. *Diabetes* 53 (Suppl. 2):A42, 2004

169. Kovacic,S, Soltys,CL, Barr,AJ, Shiojima,I, Walsh,K, Dyck,JR: Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart. *J.Biol.Chem.* 278:39422-39427, 2003

170. Horman,S, Vertommen,D, Heath,R, Neumann,D, Mouton,V, Woods,A, Schlattner,U, Wallimann,T, Carling,D, Hue,L, Rider,MH: Insulin antagonizes ischemia-induced Thr172 phosphorylation of AMP-activated protein kinase alpha-subunits in heart via hierarchical phosphorylation of Ser485/491. *J.Biol.Chem.* 281:5335-5340, 2006

171. Louden,E, Chi,MM, Moley,KH: Crosstalk between the AMP-activated kinase and insulin signaling pathways rescues murine blastocyst cells from insulin resistance. *Reproduction.* 136:335-344, 2008