January 2011

# Role Of Hyperglycemia, Hypoglycemia, and Glucose Transporter 4 on Brain Glucose Sensing, Counterregulation, and Neuronal Viability

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# ROLE OF HYPERGLYCEMIA, HYPOGLYCEMIA, AND GLUCOSE TRANSPORTER 4 ON BRAIN GLUCOSE SENSING, COUNTERREGULATION, AND NEURONAL VIABILITY

by

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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

May 2011

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

Role of Hyperglycemia, Hypoglycemia, and Glucose Transporter 4 on Brain Glucose Sensing, Counterregulation, and Neuronal Viability

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Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2011

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As glucose is the main fuel source for the brain and a major nutrient for peripheral tissues, the brain must sense and respond to changes in blood glucose in order to sustain its own nutritional requirements and maintain whole body energy homeostasis. Disruption of brain glucose sensing results in impaired glucose tolerance, a hallmark in the pathogenesis of diabetes, as well as increased risk of severe hypoglycemia as occurs with insulin therapy. Thus, understanding how the brain senses and responds to changes in glucose is particularly important to individuals with diabetes. Experiments in this thesis investigated (1) the role of neuronal glucose transporter 4 (GLUT4) in glucose sensing and the counterregulatory response to hypoglycemia, (2) the adaptive response of the brain to antecedent hypoglycemia, and (3) the role of hyperglycemia and the hexosamine biosynthetic pathway (HBP) in the hypothalamus on regulating energy homeostasis. Neuronal GLUT4 was found to play a crucial role in modulating peripheral insulin sensitivity and was necessary for eliciting a full counterregulatory response to hypoglycemia. Antecedent moderate hypoglycemia preconditioned and protected the brain from neuronal injury and cognitive dysfunction induced by an episode of severe

hypoglycemia. Finally, increased metabolism through the HBP in the hypothalamus decreased food intake and body weight and increased central and peripheral insulin sensitivity. These findings have important implications to individuals living with diabetes. Neuronal GLUT4 and the hypothalamic HBP both modulated whole body insulin sensitivity, and hence, both may be potential therapeutic targets to enhance insulin sensitivity, which would reduce the risk and improve management of diabetes. Further, patients on insulin therapy are at risk of experiencing hypoglycemia. Neuronal GLUT4 may be a therapeutic target for reducing the risk of hypoglycemia as the current findings identified its importance in the counterregulatory response to hypoglycemia. Finally, the finding that moderate hypoglycemia preconditions the brain may explain why insulin treated patients have no long-term cognitive impairments despite experiencing episodes of severe hypoglycemia. By investigating how the brain responses to both high and low blood sugar, this thesis identified critical aspects of brain glucose sensing/metabolism that modulate whole body energy and glucose homeostasis.

**ACKNOWLEDGMENTS**

To my mentor Simon Fisher for his guidance, wisdom, and advice throughout my thesis work. I am forever 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, Adam J. Bree, Kelly Diggs, Candance Reno, Julie Silverstein, Tariq Tanoli, Danielle Schwartz, Daniel Musikantow, Ioanna Georgopoulos, and Yasuko Suzuki for their incredible assistance and support.

To Dr. C. Zorumski, Dr. J. Gidday, Dr. P. Hruz, Dr. M. Mueckler, and Dr. K. Yamada, for their guidance and advice as members of my thesis committee.

To Ronaldo Perez for assistance with animal surgeries and cannulations.

To Dr. B.B. Kahn for her generous gift of the floxed GLUT4 mice. Thanks also to Dr. C.R. Kahn for providing the nestin-Cre mice.

To Dr. P. Cryer and Krishan Jethi for performing the catecholamine determinations.

To Dr. K Yamada and Nick Rensing for their help and expertise with Fluoro-Jade staining.

To Dr. P. Hruz for his guidance and assistance with the indinavir studies.

To Dr. D. Wozniak and Susan Maloney for their help with the animal behavioral testing.

To Dr. T. Coleman and Dr. C. Semenkovich for the use and assistance with the microscope and cryostat.

To everyone in the Medical Scientist Training Program Office and Molecular Cell Biology Program for their guidance.

Funding to conduct the experiments in this thesis was provided by the National Institute of Health (NIH) (DK073683), Washington University – Pfizer Biomedical Research Program, core grant support from the Washington University's Diabetes Research and Training Center (DK020579); the Clinical Nutrition Research Unit (DK056341). Juvenile Diabetes Research Foundation, and the NIH Neuroscience Blueprint Center Core Grant (NS057105) to Washington University.

Finally, I dedicate this thesis to my beloved family. To my wife Robyn, for her patience, support, and love. To my son Ethan, who provides me inspiration. To my parents Erasto and Judith Puente, who have sacrificed so such for me so I could pursue a higher education. To my siblings Jennifer, Neil, Emerson, Eva, and Erick, for their constant encouragement and support.

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## ABBREVIATIONS

| | |
|---|---|
| Akt | protein kinase B |
| ARC | arcuate nucleus of the hypothalamus |
| ANOVA | analysis of variance |
| CON | control |
| Cre | mice that express Cre recombinase under the nestin promoter |
| CRR | counterregulatory response to hypoglycemia |
| DCCT | Diabetes Control and Complications Trial |
| DG | deoxyglucose |
| DG-6P | deoxyglucose 6-phosphate |
| EEG | electroencephalogram |
| EUG | euglycemia |
| FJB+ | Fluoro-Jade B positive cells |
| GE | glucose excited |
| GFAT | glutamine:fructose-6-phosphate amidotransferase |
| GI | glucose inhibited |
| GLN | glucosamine |
| GLU | glucose |
| GLUT4 | glucose transporter 4 |
| GSIS | glucose stimulated insulin secretion |
| GTT | glucose tolerance test |
| HA | hypoglycemia unawareness |
| HAAF | hypoglycemia-associated autonomic failure |
| HBP | hexosamine biosynthetic pathway |
| H&E | hematoxylin and eosin |
| HFD | high fat diet |
| HGP | hepatic glucose production |
| ICV | intracerebroventricular |
| IDV | indinavir |
| IR | insulin receptor |
| IRAP | insulin related aminopeptidase |
| ITT | insulin tolerance test |
| Lox | mice with both GLUT4 alleles floxed |
| LTP | long-term potentiation |
| MBH | mediobasal hypothalamus |
| NE | norepinephrine |
| NG4KO | neuronal specific GLUT4 knock out |
| NIRKO | neuronal specific insulin receptor knock out |
| NPY | neuropeptide Y |
| O-GlcNAc | *O*-linked *N*-acetylglucosamine glycosylation |

| | |
|---|---|
| OGT | O-linked *N*-acetylglucosamine transferase |
| pAkt | phosphorylation Akt |
| PBS | phosphate buffered saline |
| POMC | proopiomelanocortin |
| PVN | paraventricular nucleus of the hypothalamus |
| $R_a$ | rate of glucose appearance |
| $R_d$ | rate of glucose disposal |
| RH | recurrent moderate hypoglycemia |
| SEM | standard error of the mean |
| SH60 | severe hypoglycemia of 60 min |
| SH90 | severe hypoglycemia of 90 min |
| UDP-GlcNAc | uridyl diphosphate-*N*-acetylglucosamine |
| VMH | ventromedial nucleus of the hypothalamus |
| WT | wild-type |

## THESIS INTRODUCTION

### Diabetes

Diabetes is a disease characterized by chronic elevated blood sugars. The elevated blood sugar is a consequence of either absolute or relative deficiency in insulin. Insulin, a major regulator of systemic glucose levels, is secreted from the pancreatic β-cell in response to a glucose load (e.g. ingestion of food). Insulin lowers blood glucose levels by stimulating glucose uptake into skeletal muscle and adipose tissue, inhibiting glucose release from the liver, and inhibiting glucagon secretion. In type 1 diabetes, pancreatic β-cells are destroyed leading to an absolute insulin deficiency. Type 2 diabetes is characterized by insulin resistance and relative insulin deficiency. In both cases, the abnormalities in insulin production and/or action result in elevated blood sugars. Chronic hyperglycemia is toxic to cells and leads to microvascular complications such as retinopathy, nephropathy, and neuropathy (1;2). Therefore, the management of both type 1 and type 2 diabetes includes insulin therapy aimed to reduce blood sugars towards normal to prevent the microvascular complications associated with diabetes. However, determining the precise amount of insulin needed to lower blood sugar to normal while avoiding hypoglycemia is difficult. Indeed, iatrogenic hypoglycemia is a common complication of insulin therapy and is the major barrier to the management of diabetes (3). Therefore, understanding how the body maintains glucose homeostasis may lead to improved therapies that reduce the risk and improve the management of diabetes.

Glucose is the main fuel source for the brain and provides the majority of the nutritional needs to peripheral tissues (4). Thus, blood glucose levels must be tightly controlled in order for the brain to sustain its own nutritional requirements as well as for

peripheral tissues to receive an appropriate amount of fuel. If glucose homeostasis is disrupted, the body may become exposed to the toxic effects of chronic elevated blood sugars, as occurs with diabetes. Central to maintaining glucose homeostasis is the ability of the brain to sense the amount of circulating glucose in the body and respond accordingly (5). Impaired brain glucose sensing may impair an individual's ability to sense and respond to severe hypoglycemia as occurs with tight glycemic control with insulin therapy, thus making them more susceptible to a loss of consciousness and potential neuronal injury (6). Thus, the aim of this thesis was to investigate the mechanisms in which brain senses glucose as well as the adaptive responses of the brain to both increased glucose levels as well as decreased glucose levels. Specifically, the experiments in this thesis investigated (1) the role of glucose transporter 4 (GLUT4) in glucosensing, (2) the effect of central hyperglycemia and the hexosamine biosynthetic pathway in feeding behavior, and (3) the adaptive response of the brain to recurrent, moderate hypoglycemia.

**Neuronal Glucose Sensing**

Several studies have demonstrated that the brain is exquisitely sensitive to changes in glucose, and brain glucose sensing is an essential component of feeding behavior, counterregulatory response, and peripheral glucose levels. First, feeding behavior is affected by brain glucose levels (7-9). Increasing central glucose levels by direct infusion of glucose into the brain reduces food intake and body weight (7;8). Central glucose deprivation by injection of 2-deoxy-D-glucose (a glucose analogue that is transported but not metabolized) into the third ventricle of the brain results in

hyperphagia and increased body weight (9). In addition to modulating feeding behavior, brain glucose sensing also modulates the counterregulatory response to hypoglycemia (10;11). In response to low blood sugar, the sympathetic nervous system is activated, epinephrine is released from the adrenal medulla, and glucagon is released from the α-cells of the pancreas to increase blood glucose levels. The brain is the critical glucose sensing organ that determines the counterregulatory response to hypoglycemia. If, under experimental conditions, brain glucose levels are maintained at normal levels despite peripheral hypoglycemia, the counterregulatory response is blocked (12). On the other hand, if the brain is deprived of glucose while the rest of the body is maintained at euglycemia, a robust counterregulatory response is produced (13). Finally, brain glucose can also affect peripheral glucose levels by modulating hepatic glucose production. Infusion of glucose into the brain lowers blood glucose by reducing gluconeogenesis and glycogenolysis (14). How the brain is able to sense these changes in ambient glucose has been an area of intense research.

Identification of "glucose responsive" neurons in the brain has been an important step forward in understanding how the brain senses changes in glucose (15;16). These distinct neuronal populations are expressed in several areas of the brain including the hypothalamus, an area important in the regulation of whole body energy metabolism (15;16). They are termed "glucose responsive" neurons because their membrane potential and activity responds to changes in glucose. Two types of glucose responsive neurons have been identified. First, glucose excited (GE) neurons are those neurons that depolarize and increase activity with increases in glucose levels (15;16). At low ambient glucose, GE neurons are quiescent. The other type of glucose responsive neurons is

glucose inhibited (GI) neurons which reduce electrical activity at high glucose levels and increase their activity when ambient glucose levels are low (15;16). The mechanism by which these glucosensing neurons respond to changes in glucose are only beginning to be understood. Glucokinase (GK) and $K_{ATP}$ channels have been shown to be important in the glucosensing ability of these cells (15;17-19). Disruption of glucose sensing in hypothalamic neurons by deletion of $K_{ATP}$ channels results in impaired whole body glucose tolerance (19). Several other proteins may also be important in glucose sensing, and several hormones can modulate neuronal glucose sensing.

Hormonal signals such as insulin can influence the glucose sensing properties of glucose responsive neurons. The presence of insulin modulates the electrical activity of glucose excited neurons in response to changing glucose levels (20). Further, the absence of insulin receptors in glucose excited neurons reduces the excitability of these neurons to increases in glucose (V. Routh, unpublished data). However, how insulin modulates glucose responsive neurons are unknown. Insulin has a plethora of potential targets from regulation of glucokinase, $K_{ATP}$ activity, as well as glucose transporter 4. The work in this thesis investigated the potential role of GLUT4 in modulating neuronal glucose sensing.

**Glucose Transporter 4 (GLUT4)**

Glucose transporter 4 (GLUT4) is a key protein involved in the regulation of glucose homeostasis. GLUT4, one of an expanding family of sugar transporter proteins (21), allows facilitative diffusion of hexoses, in particular glucose, across cell membranes. GLUT4 is unique in that it is mostly expressed in intracellular vesicles in an

unstimulated state. Then, in response to insulin and other stimuli, GLUT4 translocates to the plasma membrane, and subsequently, allows the transport of glucose into the cell (22). This phenomenon has been widely studied and described in peripheral tissues and cells such as skeletal muscle and adipocytes. Disruption of GLUT4, whether whole body deletion (23) or even disruption of one GLUT4 allele (24;25) results in impaired glucose tolerance, impaired insulin resistance, and an increased risk of diabetes. Tissue specific ablation of GLUT4 identified key roles of skeletal muscle GLUT4 (26;27) and adipocyte GLUT4 (28) in modulating glucose homeostasis. Interestingly, GLUT4 is also expressed in the central nervous system (29-32); however, whether brain GLUT4 also plays a role in regulating glucose homeostasis is unknown.

GLUT4 has been identified in specific areas of the brain, including the cortex, hippocampus, cerebellum, and importantly, the hypothalamus (29-32). It is primarily expressed in neuronal bodies and dendrites with little or no staining in non-neuronal cells such as glia (29;30). Interestingly, GLUT4 was demonstrated to be expressed not only in the plasma membrane but also in intracellular vesicles, similar to peripheral tissues (29;30). Further, up to 75% of glucose responsive neurons in the hypothalamus co-express GLUT4 and insulin receptor (15;16). These histological data suggest an interaction between insulin signaling and GLUT4 translocation in the brain, as occurs in peripheral tissues. Indeed, several studies found a correlation between the level of circulating insulin and GLUT4 translocation to the plasma membrane in the central nervous system (33). Specifically, in conditions of hyperinsulinemia, GLUT4 protein expression in the plasma membrane is significantly increased (33). In insulin-deficient states, GLUT4 expression in the plasma membrane is significantly reduced (34). Cell

culture studies have also demonstrated GLUT4 translocation to the plasma membrane in response to insulin stimulation (35). Thus, studies suggest insulin can increase GLUT4 translocation to the plasma membrane in neurons. However, the functional significance of brain GLUT4 has yet to be delineated.

Insulin action in the brain plays an important role in energy homeostasis and is critical for neuronal glucose sensing. Deletion of insulin receptor centrally results in peripheral insulin resistance, glucose intolerance, and increased susceptibility to diet induced obesity (36;37). Further, mice that lack the insulin receptor in the brain have impaired counterregulatory response to hypoglycemia (38), and recent data demonstrated that this impaired counterregulation is associated with a reduced ability of glucose-responsive neurons to respond to changes in glucose (39). If GLUT4 is also important in exerting insulin effects centrally, then neuronal GLUT4 would be critical in modulating energy homeostasis and neuronal glucose sensing as well. Thus, the experiments in this thesis investigated the role of GLUT4 in neuronal glucose sensing, energy homeostasis, and counterregulatory response to hypoglycemia.

**Hyperglycemia and the hexosamine biosynthetic pathway**

Dysregulated glucose homeostasis as occurs with diabetes lead to chronic elevated blood sugars. In addition to the toxicity of hyperglycemia to tissues, elevated blood sugar can further impair normal glucose homeostasis as part of a vicious cycle (40;41). Hyperglycemia results in peripheral insulin resistance, and as insulin is the major hormone in the regulation of glucose homeostasis, further disrupts the ability to maintain normal blood glucose levels. Hyperglycemia results in insulin resistance via

increased metabolism through the hexosamine biosynthetic pathway (HBP) (40;41). Of the glucose that is transported into the cell, 2-5% is metabolized through the HBP. The rate limiting enzyme in the HBP is glutamine:fructose-6-phosphate amidotransferase (GFAT), and the end product of this pathway is uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) (40;41) (Figure 18). UDP-GlcNAc can be attached to threonine and serine residues of proteins by *O*-linked β-*N*-acetylglucosamine transferase (OGT), termed *O*-linked glycosylation (40;41). Glucosamine, a molecule that directly enters the HBP, has also been shown to mimic hyperglycemia by increasing UDP-GlcNAc and *O*-linked glycosylation (40;41).

Several studies have linked increased UDP-GlcNAc and *O*-linked glycosylation to insulin resistance. Adipocytes and skeletal muscle cells exposed to either high concentrations of glucose or glucosamine results in impaired insulin-stimulated GLUT4 translocation and, consequently, reduced insulin stimulated glucose uptake (42-44). Interestingly, inhibition of the HBP and reduction of *O*-linked glycosylation by inhibiting GFAT prevented the insulin resistance caused by exposure to high concentrations of glucose (45). Further, increasing *O*-linked glycosylation by overexpression of OGT in adipocytes and skeletal muscle cells results in insulin resistance characterized by reduced insulin stimulated glucose uptake (46). Taken together, these studies indicate a major role of the HBP and protein glycosylation in the development of insulin resistance due to hyperglycemia.

Protein glycosylation is thought to disrupt insulin signaling by altering an array of signaling molecules as well as modulate transcription and translation (40). For example, protein glycosylation is thought to compete with insulin signaling enzymes for

phosphorylation sites on target proteins. Indeed, increased protein glycosylation reduces insulin-induced phosphorylation of protein kinase B (Akt) (47;48). These effects are well characterized in peripheral tissues (47;48). However, the effect of chronic hyperglycemia and metabolic flux through the HBP in the CNS has not been investigated. Indeed, excess nutrients by high fat feeding have been shown to disrupt neuronal glucose sensing (19).

The experiments in this thesis investigate whether excess glucose specifically in the CNS can affect neuronal glucose sensing and alter glucose homeostasis. Further, this thesis investigated whether the HBP in the hypothalamus is important in mediating the effects of central hyperglycemia on whole body energy homeostasis. If a similar phenomenon occurs in the CNS as it does in peripheral tissues, central glucosamine infusion into the hypothalamus and subsequent increases in protein glycosylation is hypothesized to induce neuronal insulin resistance. Central insulin resistance would be predicted to lead to hyperphagia, increased body weight, glucose intolerance, and reduced peripheral insulin sensitivity (36;37).

**Severe Hypoglycemia**

Intact neuronal glucose sensing is imperative for preventing large fluctuations in glucose, including severely low blood sugar levels. The ability to sense and respond to hypoglycemia is especially important for individuals who are on insulin therapy to control their diabetes. The Diabetes Control and Complications Trial (DCCT) demonstrated that intensive insulin therapy reduces the microvascular complications associated with diabetes, including retinopathy, neuropathy, and nephropathy (49).

However, intensive insulin therapy that aims to lower blood glucose levels towards normal also significantly increases the risk of hypoglycemia (50). Hypoglycemia is defined as a plasma glucose level less than 70 mg/dl, but varying degrees of severity of hypoglycemia exist (51). Mild hypoglycemia may be asymptomatic. Moderate hypoglycemia may cause hypoglycemic symptoms such as sweating, palpitations, and hunger and may acutely affect cognitive ability, leading to temporary stupor and confusion. Further declines in blood sugar may lead to severe hypoglycemia which is defined as an hypoglycemic event that requires assistance from another individual to administer carbohydrates or glucagon to increase blood sugar (51). Severe hypoglycemia can lead to seizures, coma, and death (4;52). Thus, discovering potential mechanisms to protect the brain from severe hypoglycemic injury is of great importance to individuals on insulin therapy who are at great risk of developing severe hypoglycemia.

Animal studies have unequivocally shown that severe hypoglycemia leads to neuronal injury and long-term impairments in memory. Severe hypoglycemia leads to significant neuronal damage in many areas in the brain including the cortex and hippocampus, areas particularly important in learning and memory (52). This brain injury leads to impaired spatial learning and memory even several weeks after the episode of severe hypoglycemia (53;54). Case studies in humans have shown severe hypoglycemia can lead to brain injury and encephalopathy (55). While many clinical studies also demonstrate a correlation between severe hypoglycemia and long term cognitive impairments (56-66) some studies do not (67-72). The discrepancy between studies could be attributed to many factors, but one variable in particular may be prior glycemic control (including hypoglycemia). The strongest risk factor for experiencing

severe hypoglycemia is the number of prior hypoglycemic events; that is, individuals who have experienced antecedent hypoglycemia are at greater risk for future hypoglycemia (49). The reason for this, as several studies have demonstrated, is that antecedent hypoglycemia impairs the body's counterregulatory response to subsequent hypoglycemia. This phenomenon is termed hypoglycemia-associated autonomic failure (HAAF). HAAF results from reduced sympathoadrenal responses leading to, first, defective glucose counterregulation (e.g. attenuated epinephrine response) and, second, hypoglycemic unawareness (HU), the reduction of neurogenic symptoms (sweating, palpitations, etc.) at a given level of hypoglycemia (3;73). Attenuated epinephrine responses increase the risk for severe hypoglycemia by 25-fold (74), and patients with HU have a six-fold increased risk for developing severe hypoglycemia (75).

This situation seems paradoxical in that the body seemingly maladapts to hypoglycemia by limiting its ability to defend against subsequent hypoglycemia. Several studies have indicated that a central nervous system (CNS) adaptation occurs after an episode of hypoglycemia. The brain adapts to episodic periods of lower levels of blood sugar and thus the glycemic threshold for initiating glucose counterregulation and neurogenic symptoms is shifted to lower glucose levels.

In other areas of brain research, the term preconditioning refers to the exposure to a sublethal stressful stimuli (e.g. ischemia) that will result in protection against larger doses of that same stressful stimuli (76). One of the first studies of preconditioning in animals found that rats exposed to brief anoxia had significantly increased survival following a subsequent exposure to prolonged anoxia (77). Further, hippocampal cell death after global ischemia is completely prevented when carotid blood flow is briefly

interrupted (resulting in mild ischemia) a few days before exposure to global ischemia (78). Clinical studies also support this phenomenon. Several studies have suggested that prior transient ischemic attacks precondition the brain and improve outcomes in people who experience stroke (79-81). The phenomena of preconditioning has also been described in other paradigms, including systemic hyperthermia, responses to lipopolysaccharide injections, and in response to various anesthetics (76). However, no study has investigated the possibility of recurrent hypoglycemia as a preconditioning stimulus. As stated earlier, antecedent hypoglycemia leads to adaptations within the brain that cause HAAF, presumably by allowing the brain to better tolerate and be less response to hypoglycemia (6;82). Intensively insulin treated people that experience severe hypoglycemia also have recurrent episodes of moderate hypoglycemia. Further, some studies suggest no long term cognitive impairments due to severe hypoglycemia in humans (67-72). Taken together, this thesis hypothesizes that recurrent moderate hypoglycemia can act to precondition the brain and protect it against neuronal injury and cognitive decline induced by severe hypoglycemia. Hypoglycemic preconditioning thus may explain why several clinical studies found no association between severe hypoglycemia and long-term cognitive dysfunction.

The ability of the brain to sense and respond to changes in blood glucose is critical to maintain glucose homeostasis. Disruption of the ability of the brain to sense and respond to changes in glucose occurs in pathological conditions of chronic hyperglycemia (diabetes) as well as hypoglycemia. Thus, the work of this thesis investigated the

mechanisms of brain glucose sensing and metabolism that are essential to glucose homeostasis and preserving neuronal viability.

# CHAPTER 1. GLUCOSE INTOLERANCE, REDUCED INSULIN SENSITIVITY, AND IMPAIRED COUNTERREGULATORY RESPONSE TO HYPOGLYCEMIA IN NEURONAL SPECIFIC GLUT4 KNOCK-OUT MICE

**ABSTRACT**

The specific role of the insulin-responsive glucose transporter 4 (GLUT4) in the brain has not been well characterized in spite of its unique distribution in key glucose sensing areas of the brain. GLUT4 was therefore selectively knocked-out in neurons using a Cre-Lox approach. Brain GLUT4 protein expression was reduced by 99% in NG4KO mice ($p<0.01$) compared to littermate controls. GLUT4 protein levels in the skeletal muscle, adipose tissue, and heart were unaffected by neuron-specific GLUT4 deletion (p=NS, ANOVA). Despite normal fed and fasting glycemia, NG4KO mice had significantly higher glucose levels during a glucose tolerance test (2g/kg) compared to littermates ($p<0.01$). NG4KO mice also had impaired insulin sensitivity as assessed by hyperinsulinemic (4 mU/kg/min) euglycemic (~110 mg/dl) clamps. NG4KO mice required a significantly lower glucose infusion rate to maintain euglycemia compared to littermate controls ($p<0.05$), and insulin-induced suppression of hepatic glucose production was impaired in NG4KO ($p<0.02$ vs controls). To assess the role of brain GLUT4 in glucose sensing, hyperinsulinemic (30 mU/kg/min) hypoglycemic (~33 mg/dl) clamps were performed in awake, cannulated, unrestrained mice. Epinephrine and glucagon responses to hypoglycemia in NG4KO mice were significantly reduced by 48% and 54%, respectively, while norepinephrine and corticosterone responses were normal. Additionally, c-fos activation in the hypothalamic paraventricular nucleus in response to hypoglycemia was significantly reduced in NG4KO compared to littermate controls ($p<0.01$). Thus, the impaired counterregulatory response to hypoglycemia, reduced insulin sensitivity, and impaired glucose tolerance in NG4KO mice indicate a critical role for brain GLUT4 in sensing and responding to changes in blood sugar.

## INTRODUCTION

The facilitative glucose transporter 4 (GLUT4) is the major glucose transporter in skeletal muscle and adipose tissue. In response to insulin stimulation, GLUT4 is translocated to the plasma membrane and facilitates glucose entry into the cell (83-85). Disruption of GLUT4 in either skeletal muscle or adipose tissue leads to impaired glucose tolerance and insulin resistance, two key features in the pathogenesis of diabetes (26;28). Recently, GLUT4 has been found to be expressed in the brain (30;31;86-88). However, the physiological role of GLUT4 in the brain has yet to be elucidated. GLUT4 is expressed predominantly in neuronal cells in discrete regions of the brain, including the hippocampus, cortex, and cerebellum. Of particular interest is that GLUT4 is also expressed in the hypothalamus, an area important in the regulation of whole body glucose and energy homeostasis (30;31;86-88). Further, GLUT4 is co-expressed with the insulin receptor in important glucose sensing areas of the brain including in glucose excited (GE) neurons in the mediobasal hypothalamus (15). Thus, central GLUT4 may play an important role in the neuronal glucose sensing and modulating whole body glucose homeostasis.

To determine the physiological roles of GLUT4 in the brain, GLUT4 was selectively knocked-out in neuronal tissue using a Cre-Lox approach and the effects on whole body glucose homeostasis were examined. Mice with the neuronal specific GLUT4 knock-out (NG4KO) were found to be glucose intolerant, have reduced insulin sensitivity, and impaired counterregulatory responses to hypoglycemia. Thus, these studies found a novel role for GLUT4 in regulating whole body energy homeostasis.

## MATERIAL AND METHODS

**Animals.** Mice that have the *GLUT4* gene floxed (Lox) (26;28) were crossed with transgenic mice that expressed Cre recombinase under the neuron specific promoter nestin (36;38;89). The resultant heterozygotes were crossed with each other to produce 4 experimental groups: wild-type (WT); mice that have both GLUT4 alleles floxed (Lox); nestin-Cre expressing mice (Cre); and knockout mice that express both nestin-Cre and have both GLUT4 alleles floxed (NG4KO). Mice were genotyped by PCR analysis of DNA extracted from tail tissue and using previously established primers and PCR conditions (26;28;36;38).

Mice were housed in a temperature and light controlled environment maintaining the animal's diurnal cycle (12hrs light, 12hrs dark) and fed a standard rodent chow or a high fat diet (60% calories from fat, Research Diets 12492; New Brunswick, NJ) ad libitum. Male Sprague-Dawley rats were individually housed, maintain on a 12 hour light/12 hour dark cycle, and fed standard rodent chow diet. 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.

**Western blot analysis.** Brain, skeletal muscle, heart, and white adipose tissue were homogenized in buffer containing 1% Igepal (Sigma, Saint Louis, MO), 0.5% sodium dodecyl sulfate (Sigma, Saint Louis, MO), 0.1 mM Phenylmethylsulfonyl fluoride (Sigma, Saint Louis, MO), 1X complete protease inhibitor (Sigma, Saint Louis, MO), 1 mM NaF (Sigma, Saint Louis, MO), and 1 mM $Na_3VO_4$ (Sigma, Saint Louis, MO) in phosphate buffered saline (pH=7.4), centrifuged at 14,000 rpm for 30 min, and

supernatant was collected. Protein samples were then separated on 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA), transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA), and were immunoblotted with antibodies against GLUT1 (1:1000, Chemicon, Temecula, CA), GLUT3 (1:2000, Chemicon, Temecula, CA), GLUT4 (1:1000, Chemicon, Temecula, CA), or against the loading control β-actin (1:6000; Sigma, St. Louis, MO). Horseradish peroxidase-conjugated secondary antibody (1:8000, Cell Signaling, Boston, MA) was used and antibody binding was detected by enhanced chemiluminescence ECL reagents (Perkin Elmer, Waltham, MA) on ISO-MAX films and quantified by ImageJ software analysis.

**Glucose and insulin tolerance tests.** Glucose tolerance tests (GTT) were accomplished by intraperitoneal injection of glucose (2 g glucose/kg body weight) after an overnight fast (15-17 hr). In addition to glucose sampling, tail vein blood samples (20 μl) were taken before glucose injection and at 15 minute intervals during the GTT to measure plasma insulin levels. Insulin tolerance tests (ITT) were performed in 5 hour fasted mice by intraperitoneal injection of human regular insulin (1 U insulin/kg body weight; Lilly, Indianapolis, Indiana). Tail vein blood glucose was measured using Ascensia Contour blood glucose monitors (Bayer, Tarrytown, NY).

**Glucose stimulated insulin secretion**. First phase glucose stimulated insulin secretion was assessed by an intraperitoneal injection of glucose (3 g glucose/kg body weight) in fasted (15-17 hr) mice. Tail vein blood glucose was measured and blood samples (20 μl) were collected for plasma insulin determinations immediately before injection and at 2, 5, 15, and 30 minutes after injection.

**High-Fat Feeding**. At 5-6 weeks of age, male NG4KO mice and their littermate controls were fed a high fat diet (60% calories from fat, Research Diets 12492; New Brunswick, NJ) for 11 weeks. Body weight was measured before the start of the high fat diet and weekly during the high fat feeding. Tail vein blood glucose was measured before the start of the high fat diet and at the end of the 11 week high fat feeding. Glucose tolerance and insulin tolerance tests were performed after 11 weeks of high fat feeding.

**Hyperinsulinemic-euglycemic clamp, glucose kinetics analysis, and brain glucose uptake.** Micro-renathane catheters (Braintree Scientific Inc., Braintree, Massachusetts, USA) were implanted in the jugular vein and femoral artery of anesthetized mice (Ketamine 87 mg/kg and Xylazine 2.6 mg/kg). Hyperinsulinemic-euglycemic clamp studies were conducted 5–8 days after surgery in awake, freely mobile mice after a 5-hour fast. Whole-body glucose flux was determined using a continuous infusion of [3-3H] glucose (PerkinElmer, Boston, Massachusetts, USA) at 0.05 μCi/min after an initial 5-μCi bolus. After a 90 minute basal period, one basal blood sample (25 μl) was taken for measurement of basal plasma insulin levels and three basal blood samples (15 μl) at 10 min intervals were taken for plasma [3H]glucose measurements. After basal sampling, a primed (40 mU/kg) continuous infusion (4 mU/kg/min) of human regular insulin (Humulin; Eli Lilly and Co.) in 0.1% BSA was co-administered with 50% glucose at variable rates to maintain blood glucose at approximately 110 mg/dl. Arterial blood glucose (~2 ul) was measured every 10 min.

Tissue-specific insulin-stimulated glucose transport was measured from a bolus (5 μCi) of 2-deoxy-D-[1-14C] glucose (2-[14C]DG; Amersham) administered 45 minutes

before the end of the clamp. For plasma 2-[14C]DG measurements, blood samples (10 µl) were taken immediately before 2-[14C]DG administration and at 0.5, 2, 3, 5, 10, 15, 20, 30, and 45 min following administration. For plasma [3H]glucose measurements, 20 min before the end of the clamp, blood samples (15 µl) were taken at 10 min intervals till the end of the clamp, and a 25 µl blood sample was taken at the end of the clamp to measure plasma insulin levels. Immediately after the conclusion of the clamp, animals were euthanized and skeletal muscle and adipose tissue rapidly were dissected and frozen in liquid nitrogen. Brains were rapidly harvested and quickly frozen in a dry ice and 2-methylbutane (Fisher, Saint Louis, MO) bath (-20$^{o}$ C). Plasma [3-3H] glucose and 2-[14C]DG concentrations were determined by a dual-channel scintillation counter (Tri-Carb 2800TR, PerkinElmer, Waltham, MA) after deproteinization and drying. Tissue 2-[14C]DG and 2-[14C]DG-6-phosphate (2-[14C]DG-6P) were separated by ion-exchange chromatography.

Whole-body glucose turnover was determined during the steady state from the ratio of the [3H] glucose infusion rate to the measured specific activity of plasma glucose (90). Hepatic glucose production (HGP) was determined by subtraction of the glucose infusion rate from the whole-body glucose turnover (90). Skeletal muscle and adipose tissue glucose uptake was calculated from tissue 2-[14C]DG-6P content normalized against the area under the plasma 2-[14C]DG decay curve (91).

For brain glucose uptake calculations, isotope concentrations in regions of interest were measured from 20µm thick coronal brain sections after exposure to autoradiograph film via optical densitometry. Precise identification of the areas of interest (paraventricular nucleus of the hypothalamus (PVN), arcuate nucleus (ARC),

ventromedial hypothalamus (VMH), hippocampus, cerebellum, and cortex) were accomplished by Nissl staining the radiolabeled sections and visualizing the desired areas (Figure 8). Mean local rates of glucose utilization were averaged from four matched sections from each region of interest then calculated according to Sokoloff's equation with rat rate constants, as mice rate constants have not been determined (92;93).

**Pharmacological inhibition of brain GLUT4 by indinavir (IDV).** Cannulas (Plastics One Inc, Roanoke, VA) were inserted in the third ventricle, and micro-renathane® (Braintree Scientific, Boston, MA) catheters was inserted into the left carotid artery and right jugular vein in anesthetized (Ketamine 87 mg/kg and Xylazine 2.6 mg/kg) nine week-old male Sprague Dawley rats. After one week of recovery, the animals were subjected to an hyperinsulinemic (20 mU/kg/min) hypoglycemic clamp (~45 mg/dl). Three hours prior to the start of and for the duration of the clamp, indinavir (10μg/min; Merck, White-house City, NJ) or vehicle (artificial cerebrospinal fluid, aCSF) was infused into the third ventricle. Indinavir is a pharmacologic agent that has been shown to inhibit GLUT4 activity and transport in cell culture and hippocampal brain slices (94-96). Blood samples were taken in the basal period and at 45, 60, and 90 min into the hyperinsulinemic hypoglycemic clamp.

**Hyperinsulinemic-hypoglycemic mice clamps and c-fos immunostaining.** Four month old mice anesthetized with ketamine/xylazine (87 and 13.4 mg/kg IP) were implanted with micro-renathane catheters (Braintree Scientific Inc., Braintree, MA) into both the right internal jugular vein and femoral artery. After a 5-7 day recovery period, 2 hour hyperinsulinemic (20mU/kg/min) hypoglycemic (30 mg/dl) clamps were performed in 5-hour fasted, freely mobile mice. A variable 12.5% glucose infusion was used to

carefully match blood glucose levels to 30 mg/dl. Arterial blood samples (~2 µl) were drawn at 10-min intervals to measure blood glucose (Ascenia Contour blood glucose monitor). Blood samples (110 µl) were taken in the basal period and at 50 and 90 min into the hyperinsulinemic hypoglycemic clamp to assess the counterregulatory response.

At the end of the 2 hr hypoglycemic clamp, mice anesthetized with isofluorane and intracardially 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 (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). 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). Four anatomically matched sections per animal were used to quantify c-fos immunostaining in the paraventricular nucleus of the hypothalamus (PVN). c-fos data was expressed as the average number of c-fos positive cells per section. An observer blinded to the mice genotype counted the number of c-fos positive cells in the PVN.

**Brain Glucose Uptake during a hyperinsulinemic-hypoglycemic clamp.** Awake, unrestrained, cannulated NG4KO and littermate Lox mice underwent a 2-hour hyperinsulinemic (80mU/kg/min) hypoglycemic (30mg/dL) clamp protocol. The experiments were similar in design to the euglycemic clamps noted above, except that the clamps were performed during hypoglycemia when glucose uptake into the brain may be rate limiting. At 45 minutes prior to the end of the clamp, a 5µCi bolus of

2[14C]deoxyglucose was administered intravenously and arterial blood samples (10 μl) were collected immediately before 2[14C] deoxyglucose injections and 0.5, 2, 3, 5, 10, 15, 20, 30, and 45 min following administration for analysis of arterial plasma glucose and plasma 2-[14C]DG levels. Isotope concentrations and brain glucose utilization in PVN, ARC, VMH, hippocampus, cerebellum, and cortex were measured as described above.

**Heat Stress.** Awake NG4KO and littermate control mice were exposed to an ambient temperature of 42°C for 60 minutes to induce heat stress. Blood samples were taken before and at the end of the heat stress period to measure epinephrine levels.

**Analytical measurements**. Blood glucose was measured by Ascensia Contour blood glucose monitors (Bayer HealthCare, LLC, Mishawaka, IN). Insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Crystal Chem, Chicago, Illinois). Catecholamine analysis was performed by a single isotope-derived (radioenzymatic) method (97). Radioimmunoassays were performed for glucagon (Linco Research, St. Charles, MO) and corticosterone (MP Biomedicals, Orangeburg, NY) measurements.

**Statistical analyses**. All data are presented as the mean ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$ and was determined by either Student's *t* test or analysis of variance (ANOVA), as indicated.

## RESULTS

### Verification of neuronal-specific GLUT4 deletion

Using the Cre-Lox system, neuronal specific GLUT4KO mice were created by crossing mice that express Cre under the nestin promoter with mice that have exon 10 of *GLUT4* flanked by loxP sites. Four experimental groups were generated: wild-type mice (WT); mice with both *GLUT4* genes floxed (Lox); mice that express Cre under the nestin promoter (Cre); and neuronal specific GLUT4 knockout mice (NG4KO) mice. To verify tissue specific deletion of GLUT4, GLUT4 protein concentration was measured by Western Blot analysis in the brain as well as the muscle, heart, and white adipose tissue. NG4KO mice had a greater than 99% reduction in brain GLUT4 protein levels relative to WT, Lox, and Cre mice (Figure 1). Importantly, GLUT4 content in the heart, muscle, and adipose tissue was present in NG4KO mice and expressed at similar levels to the littermate controls (Figure 1), verifying the specificity of the brain GLUT4 knock-out.

To determine if deletion of GLUT4 in the brain altered expression of other brain glucose transporters, GLUT1 and GLUT3 protein expression were measured by Western blot. No significant difference in whole brain GLUT1 and GLUT3 protein levels were observed between NG4KO and littermate controls (Figure 2).

### Effect of Brain GLUT4KO on energy and glucose homeostasis.

Body weight was measured weekly from 5 weeks to 12 weeks of age. No significant difference in body weight was observed between Cre mice and NG4KO mice in both males and females (Figure 3). Intriguingly, both Cre and NG4KO mice had significantly reduced body weights compared to WT and Lox mice (Figure 3). No

significant difference in body weight was observed between WT and Lox mice (Figure 3). Thus, the expression of Cre itself reduced body weight.

The reduction in body weight was partly attributable to the reduction in body length. Crown-rump body length was measured at 12 weeks of age and, consistent with the body weight, Cre and NG4KO were significantly shorter than WT and Lox mice (Figure 3). Brain mass was not significantly different between groups nor were fat pad mass (Figure 3).

*Neuronal GLUT4KO does not affect fasting or fed blood glucose levels, but results in fed hyperinsulinemia and glucose intolerance.*

To assess the effect of the brain GLUT4 KO on whole-body glucose homeostasis, fed and fasting glucose and insulin levels were measured and intraperitoneal glucose tolerance tests (IPGTT) and insulin tolerance tests (ITT) were performed in 12 week old mice on a normal chow diet. Fasting and random fed glucose levels were similar in all groups in both male and female mice. Further, in male mice, no difference in fasting or fed insulin levels were observed between groups (Figure 4A). Interestingly, female NG4KO mice had significantly higher fed plasma insulin levels compared to WT, Lox, and Cre littermates, though no difference was observed with fasted plasma insulin levels (Figure 4D).

The role of neuronal GLUT4 in handling and responding to changes in glucose was assessed by the administration of intraperitoneal glucose tolerance tests (IPGTT; 2 mg/kg) in overnight fasted mice. Interestingly, male NG4KO mice had significantly higher glucose excursions during the GTT than WT, Lox, and Cre littermates (Figure

5A). In female mice, glucose increased similarly in NG4KO and littermate controls (Figure 5B). To determine whether neuronal GLUT4 deletion also affected whole body insulin sensitivity, an insulin tolerance test (ITT, 1 U/kg) was performed in NG4KO and control mice. Despite the impaired glucose tolerance, male NG4KO mice had similar reduction in blood glucose in response to insulin compared to littermate controls (Figure 5). Further, no difference in insulin sensitivity as measured by the insulin tolerance test was observed between the groups in female mice (Figure 5).

The impaired glucose tolerance in male NG4KO was not attributed to altered glucose stimulated insulin secretion (GSIS). Plasma insulin levels were measured during the IPGTT, and no difference in first phase insulin secretion and no difference in plasma insulin release during the glucose tolerance test were observed between NG4KO and their littermate controls (Figure 6).

*NG4KO had reduced insulin sensitivity and hepatic insulin resistance*

Although blood glucose dropped similarly in all groups in response to insulin during the ITT, suggesting no difference in insulin sensitivity between NG4KO and their littermate controls, several variables can compound these results, such as differences in the counterregulatory response to hypoglycemia (discussed below). The gold standard for measuring insulin sensitivity and glucose kinetics is the use of the hyperinsulinemic euglycemic clamp. The effect of neuronal GLUT4 KO on whole body insulin sensitivity was directly assessed by 2-hour hyperinsulinemic-euglycemic clamp in NG4KO mice and their littermate controls. For statistical purposes, the three littermate control groups (WT, Lox, and Cre) were combined (CON) as there was no statistical differences between these control groups. Baseline blood glucose and blood glucose during the

clamp were not statistically different between the NG4KO and CON (Figure 7A). Interestingly, NG4KO mice required 22% lower glucose infusion rate (34 ± 3 versus 43 ± 2 mg/kg/min; $p < 0.02$) to maintain euglycemia compared to littermate controls, demonstrating whole-body insulin resistance (Figure 7B). The defect in whole-body insulin sensitivity in NG4KO mice was not due to changes in glucose disposal ($R_d$), because NG4KO mice had similar basal $R_d$ and similar insulin-stimulated increase in $R_d$ compared with controls (Figure 7C). Consistent with the similar $R_d$ values between groups, no difference in $^{14}$C-2-deoxyglucose determined muscle and fat-specific glucose uptake was observed between NG4KO and CON mice (Figure 7D and Figure 7E). However, the ability of insulin to suppress hepatic glucose production (HGP) was impaired by 44% in NG4KO compared to littermate controls (Figure 7F, $p < 0.02$).

To determine whether deletion of GLUT4 in the brain results in altered glucose uptake, brain glucose uptake was measured using $^{14}$C-2-deoxyglucose isotope determinations during the 2-hour hyperinsulinemic (4mU/kg/min) euglycemic clamp. Mean local rates of glucose uptake were calculated according to Sokoloff's equation (92). Deletion of neuronal GLUT4 did not result in any detectable changes in brain glucose uptake in select regions of the hypothalamus—the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC)—as well as in the hippocampus, cerebellum, and cortex (Figure 8).

**Neuronal GLUT4 deletion does not affect susceptibility to diet-induced obesity**

Because brain GLUT4 may have a role in modulating glucose sensing, the deletion of brain GLUT4 may result in altered ability to sense and/or respond to

alterations in nutrient levels within the hypothalamus. Impaired nutrient sensing is hypothesized to increase susceptibility to diet-induced obesity. To test whether NG4KO are at higher risk of developing diet-induced obesity, male NG4KO mice and their littermate controls were fed a high-fat diet (HFD, 60% kcal from fat) starting at 5 weeks of age. Body weight was measured weekly and random fed blood glucose was measured before the start of high fat feeding and at 11 weeks after. No difference in body weight change was observed between WT, Lox, Cre, and NG4KO mice on a HFD (Figure 9). Random fed glucose was similar between groups before and after the high fat diet (Figure 9). Further, blood glucose levels during a IPGTT (2 mg/kg) and ITT (1 U/kg) were not different between NG4KO and littermate control mice (Figure 9).

**Role of central GLUT4 on the counterregulatory response (CRR) to hypoglycemia**

*Pharmacological inhibition of central GLUT4 indinavir attenuates the counterregulatory response to hypoglycemia.*

To test whether brain GLUT4 is important in the counterregulatory response to hypoglycemia, indinavir, a GLUT4 inhibitor, or vehicle (artificial cerebrospinal fluid, aCSF) was infused into the third ventricle (10 µg/min) of chronically cannulated rats during a hyperinsulinemic (20 mU/kg/min) hypoglycemic clamp. Indinavir (IDV) is a pharmacologic agent that has been shown to inhibit GLUT4 activity and transport (94-96). Blood glucose was not different in the basal period or during the hyperinsulinemic clamp period (Figure 10A). Further, no difference in epinephrine, norepinephrine, glucagon, and corticosterone was observed in the basal period. Interestingly, during the clamp, the glucose infusion rate required to maintain glucose at ~50 mg/dl was 44%

higher in the IDV-treated rats compared to controls (Figure 10, P<0.05). The higher glucose infusion rates in IDV rats were associated with significantly attenuated counterregulatory response in these animals. Specifically, third ventricle infusion of IDV resulted in a 24% lower epinephrine response, a 22% lower norepinephrine response, and a 45% reduced glucagon response to hypoglycemia compared to controls (Figure 10).

*Genetic deletion of neuronal GLUT4 impairs the CRR to hypoglycemia*

A genetic approached was used to further elucidate the role of neuronal GLUT4 in the CRR to hypoglycemia. Hyperinsulinemic (20 mU/kg/min) hypoglycemic (~30 mg/dl) clamps were performed in chronically cannulated, freely mobile NG4KO mice and their littermate controls. Glucose levels during the basal period were similar and glycemia was carefully matched during the clamp period (Figure 11).

Basal epinephrine levels were similar in all groups (Figure 11B). During hypoglycemia, epinephrine levels rose similarly in the WT, Lox, and Cre mice (3133 ± 600; 2902 ± 560; 2633 ± 355 pg/ml, respectively; P=NS). However, epinephrine response in NG4KO was significantly lower (1575 ± 101 pg/ml) compared to the other three littermate controls ($P < 0.05$) (Figure 11B). Further, glucagon response to hypoglycemia was significantly attenuated in NG4KO mice (NG4KO: 299 ± 92 pg/ml and WT 650 ± 57; Lox 597 ± 74; Cre 651 ± 39 pg/ml, $p < 0.001$) (Figure 11C). Norepinephrine and corticosterone response to hypoglycemia were not significantly different between the 4 groups (Figure 11D and Figure 11E).

*Neuronal GLUT4KO results in reduced neuronal activation in the paraventricular nucleus in response to hypoglycemia.*

To determine whether the impaired counterregulatory response in the NG4KO mice was mediated by an impaired neuronal activation in response to hypoglycemia, c-fos activation in response to hypoglycemia was measured in the paraventricular nucleus (PVN) at the end of the 2 hour hyperinsulinemic hypoglycemic clamps. Despite matched levels of hypoglycemia, NG4KO mice had ~80% reduction in c-fos positive cells compared to littermate controls (KO 28 ± 17 versus WT 120 ± 20, Lox 138 ± 19, Cre 141 ± 20 c-fos positive cells, $P < 0.001$) (Figure 12).

*Brain glucose uptake during hypoglycemia*

Whether altered brain glucose uptake during hypoglycemia was associated with the impaired CRR to hypoglycemia, brain glucose uptake was measured using the $^{14}$C-2-deoxyglucose radioisotope determinations during hyperinsulinemic (80mU/kg/min) hypoglycemic (~30 mg/dl) clamps. No significant difference in brain glucose uptake was observed in NG4KO mice compared to Lox littermate controls in any of the regions measured (Figure 13).

*Impaired adrenomedullary response to stress is unique to hypoglycemia*

The reduced epinephrine response to hypoglycemia in NG4KO mice may be due to a generalized impairment in the adrenomedullary response to stress rather than a specific impairment in the ability to respond to low blood sugar. To test the adrenomedullary response to other types of stress, epinephrine levels were measured

from tail vein blood samples drawn from NG4KO mice and littermate controls that were subjected to 60 min of heat stress. No significant difference in epinephrine or norepinephrine levels during heat stress was observed between NG4KO and littermates (Figure 14).

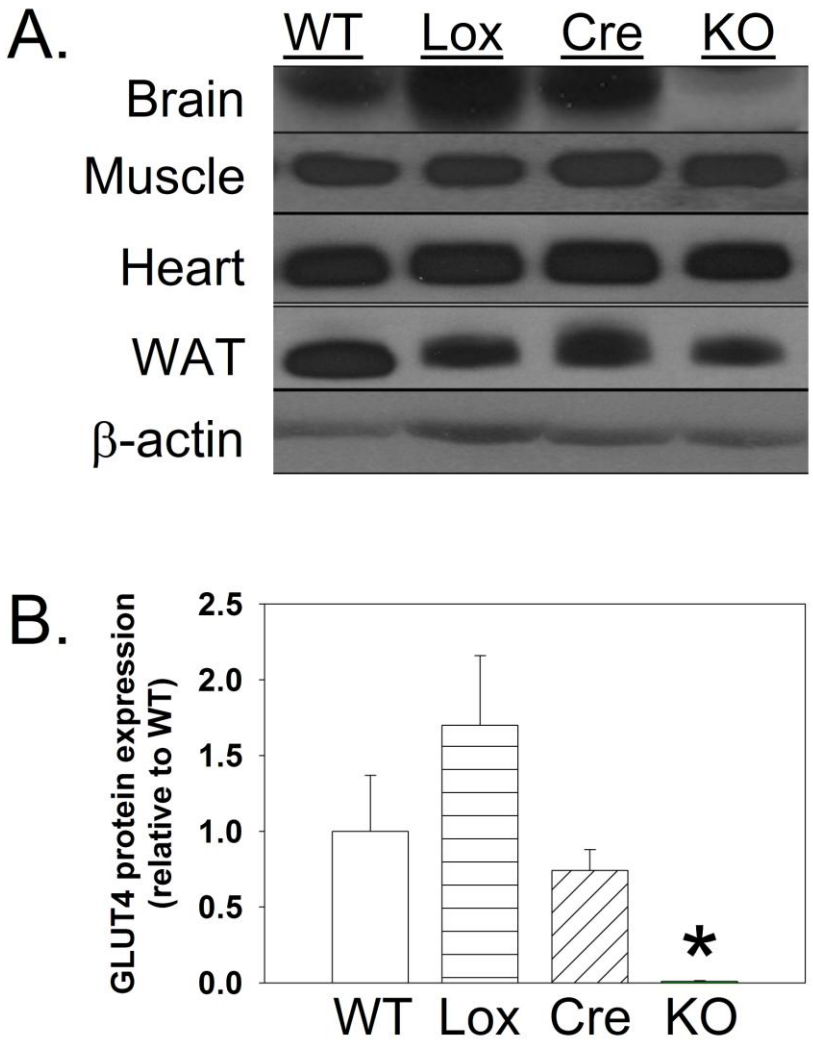


**Figure 1. Brain-specific deletion of GLUT4.** (A) Whole brain, gastrocnemius muscle, heart, and white adipose tissue (WAT) were harvested and homogenized for western blot analysis of GLUT4. GLUT4 was reduced in the brain of neuronal GLUT4KO mice (KO) compared to wild-type mice (WT), Lox expressing (Lox) and Cre expressing (Cre) mice. GLUT4 protein levels in muscle, heart, and WAT were similar between KO and littermate controls. (B) Quantification of brain GLUT4 protein content. KO (black bar, n=11) had a greater than 99% reduction in brain GLUT4 levels compared to WT (white bar, n=6), Lox (horizontal hash, n=6), and Cre (slanted hash, n=6). * $P < 0.001$ vs WT, Lox, and Cre mice. Data expressed as mean ± S.E.M.

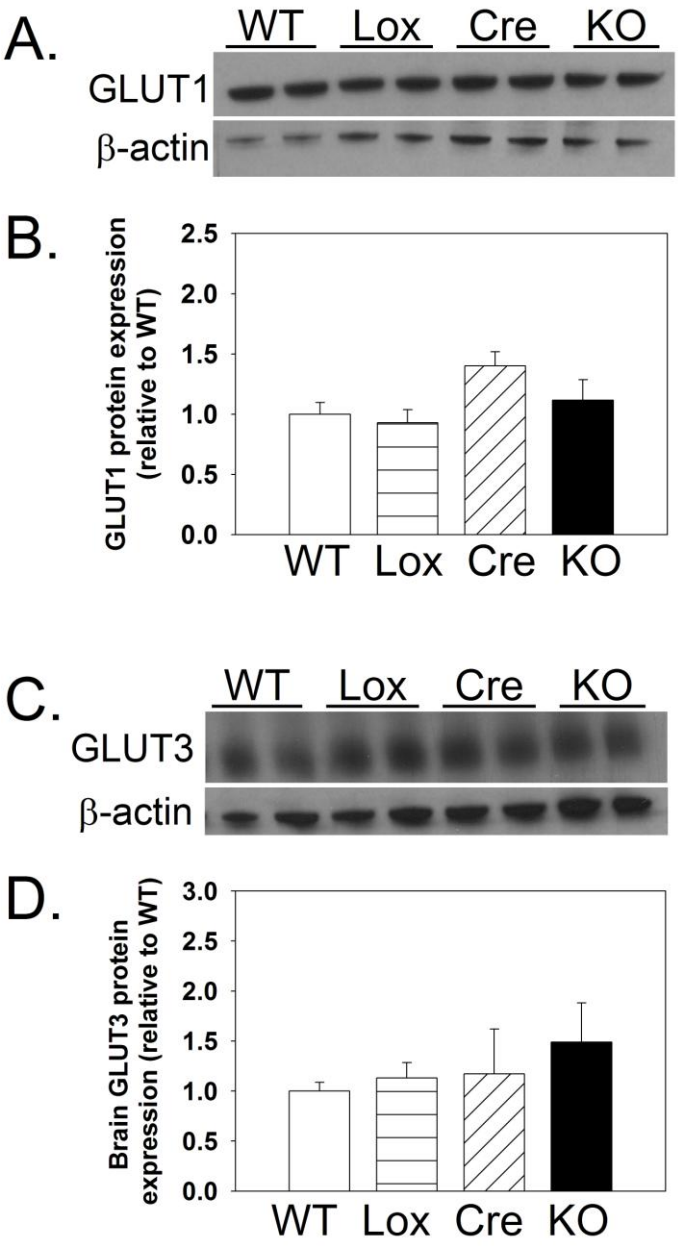


**Figure 2. Brain GLUT1 and GLUT3 protein expression was not altered in neuronal GLUT4 KO mice.** (A and B) Brain GLUT1 and (C and D) Brain GLUT3 protein expression as measured by Western blot analysis was not different in neuronal GLUT4KO mice (KO, black bar, n=5) versus wild-type (WT, white bar, n=4), Lox (horizontal hash, n=4), and Cre (slanted hash, n=4). Data expressed as mean ± S.E.M. n=4-6 per group.

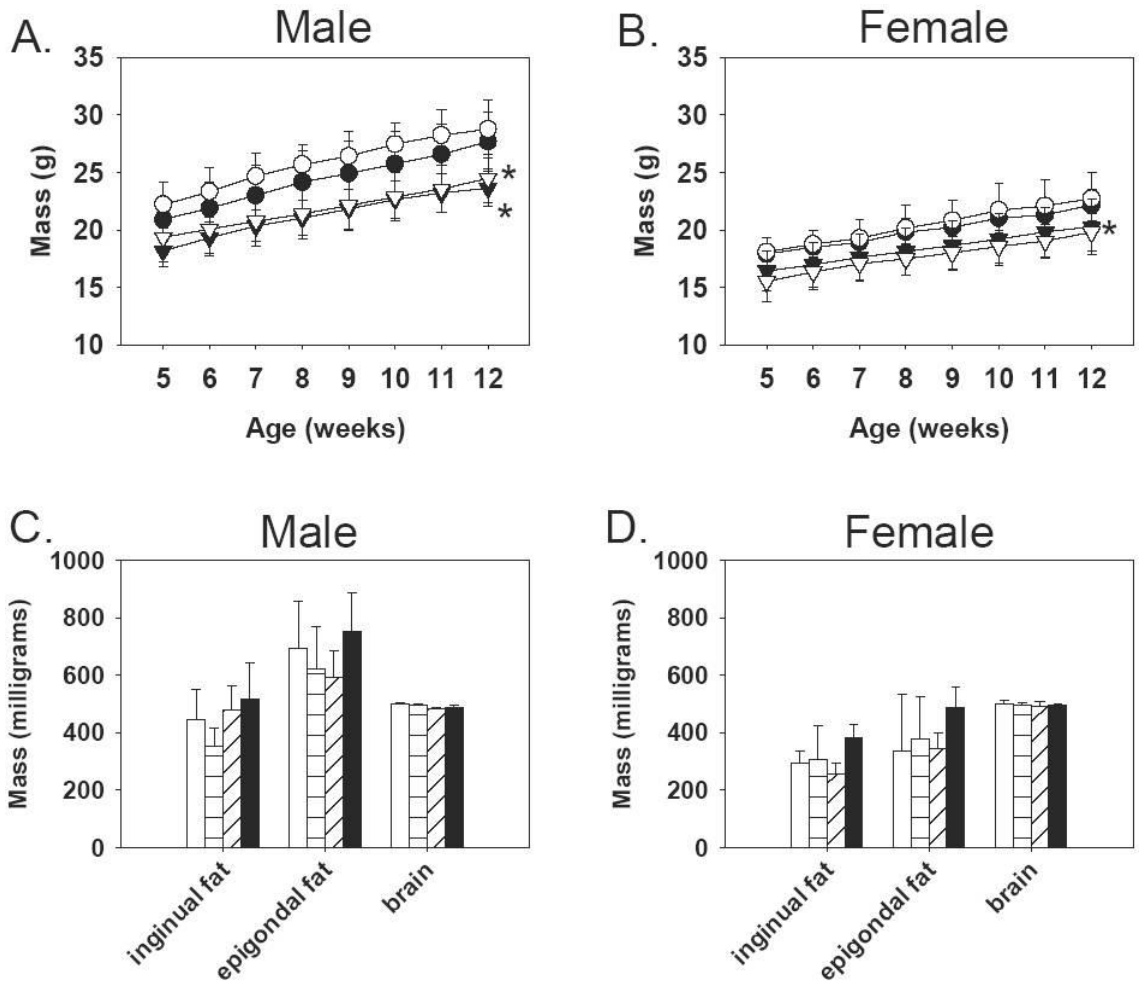


**Figure 3. Neuronal GLUT4 knock-out did not affect body weight.** (A and B) Body weight was similar between Cre (open triangle) mice and NG4KO (closed triangle) mice in both males (left panels) and females (right panels). Cre and NG4KO mice had slightly lower body mass compared to WT (open circle) and Lox (closed circle) mice. No difference in body weight was observed between WT and Lox mice. n=10-20 mice per group. (C and D) The reduction in body weight was attributable to smaller crown-rump length in Cre (slanted hash) and NG4KO (black bar) mice compared to wild type (open bar) and Lox (horizontal hash) mice. n=5-7 mice per group. (E and F) Brain mass, fat pad mass, and heart weight were not significantly different between groups. n=5-7 mice per group. * $P < 0.05$ vs. WT, Lox, and Cre. Data expressed as mean ± S.E.M.

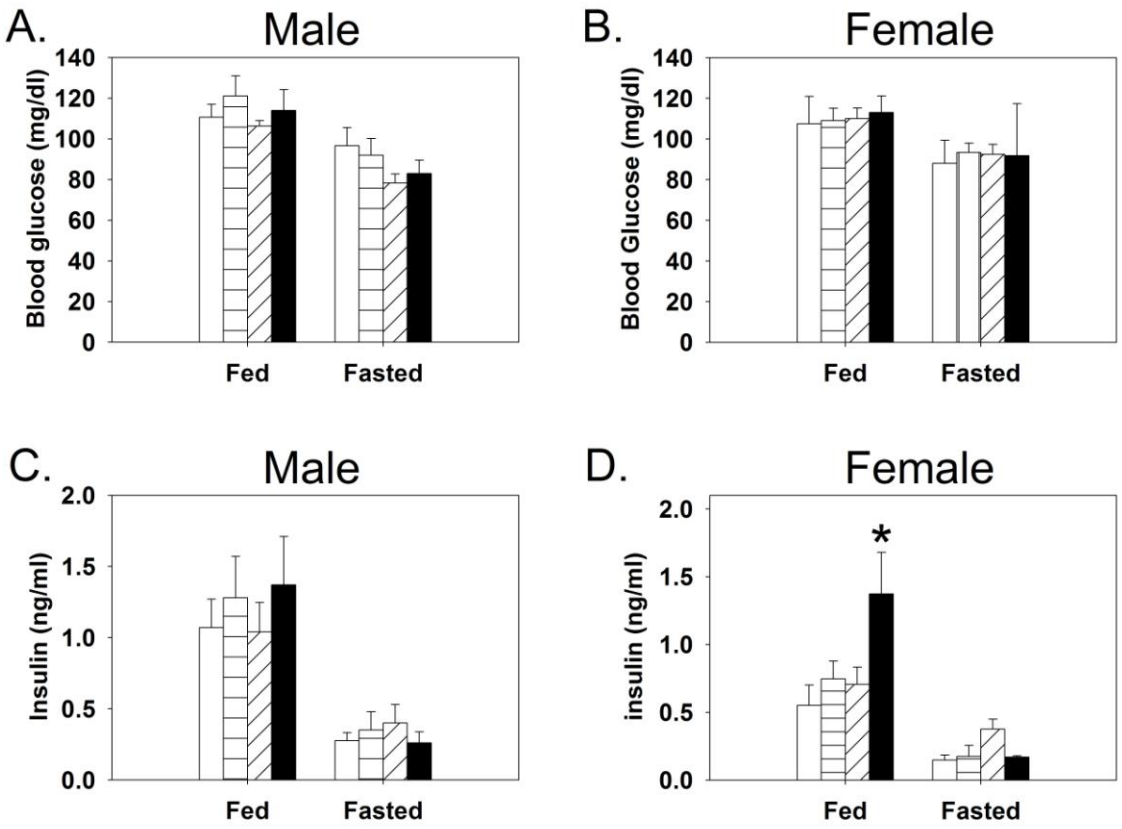


**Figure 4. Normal glucose but hyperinsulinemia in neuronal GLUT4 KO (NG4KO) mice.** (A and B) Male NG4KO (black bar) have similar fed and fasting blood glucose levels as well as similar fed and fasting plasma insulin levels compared to wild-type (open bar), Lox (horizontal hash), and Cre (slanted hashed). (C) Female NG4KO mice had similar fed and fasting blood glucose levels compared to littermate controls. (D) Female NG4KO mice had significantly higher plasma insulin levels in the fed state compared to littermate controls. No difference in fasted plasma insulin levels were observed. * $P < 0.05$ vs. WT, Lox, Cre. Data expressed as mean ± S.E.M. n=6-14 mice per group.

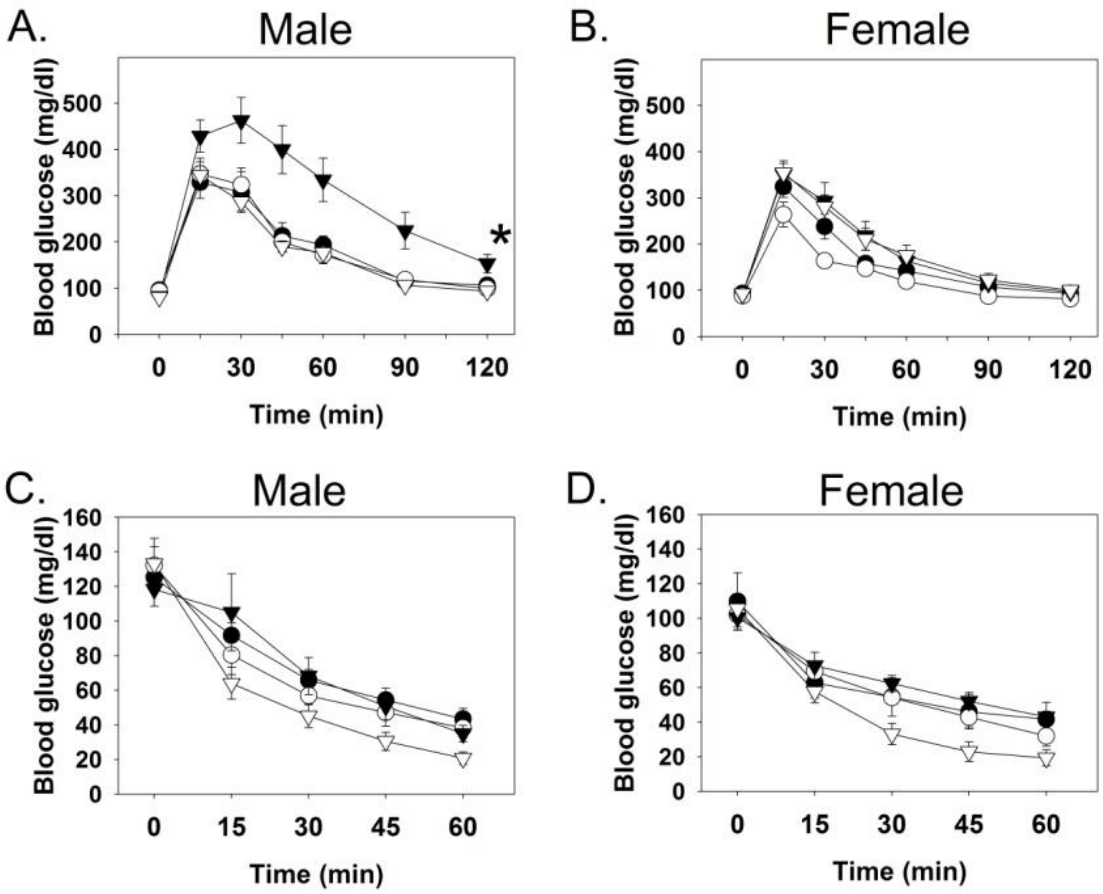


**Figure 5. Impaired glucose tolerance in NG4KO mice.** Intraperitoneal glucose tolerance tests (IPGTT, 2 mg/kg) were performed in 12 week old male and female mice. (A) Male NG4KO mice (closed triangles) had significantly higher excursions in blood glucose compared to WT (open circles), Lox (closed circles), and Cre (open triangles) mice. (B) No difference in blood glucose during the IPGTT was observed in female NG4KO mice compared to littermate controls. No difference in the blood glucose was observed between NG4KO mice and to littermate controls during an insulin tolerance test in either males (C) and females (D). * $P < 0.05$ vs. WT, Lox, Cre. Data expressed as mean ± S.E.M. n=7-17 mice per group.

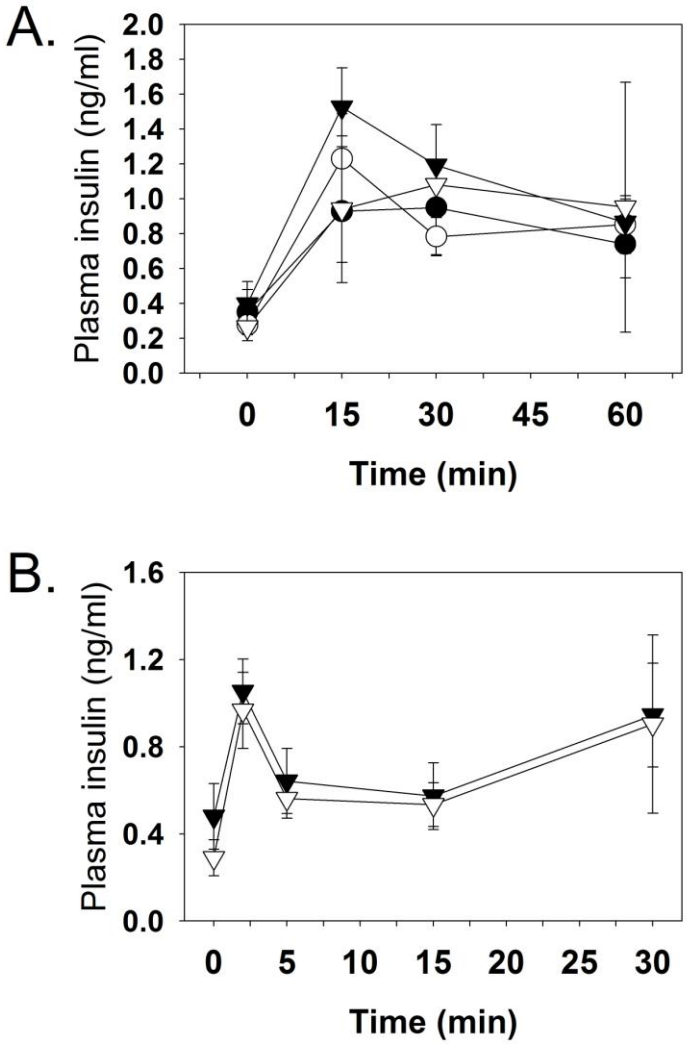


**Figure 6. Normal glucose stimulated insulin secretion in neuronal GLUT4 knock-out mice.** (A) Insulin secretion during a glucose tolerance test was similar between NG4KO (closed triangle, n=4), wild-type mice (open circle, n=9), and lox mice (closed circles, n=4) (p=NS). (B) In a separate study, first phase insulin secretion was measured during after an intraperitoneal injection of glucose in NG4KO (closed triangle, n=7) and Cre littermate controls (open triangle, n=8). First phase insulin secretion was not significantly different between groups. Data expressed as mean ± S.E.M.

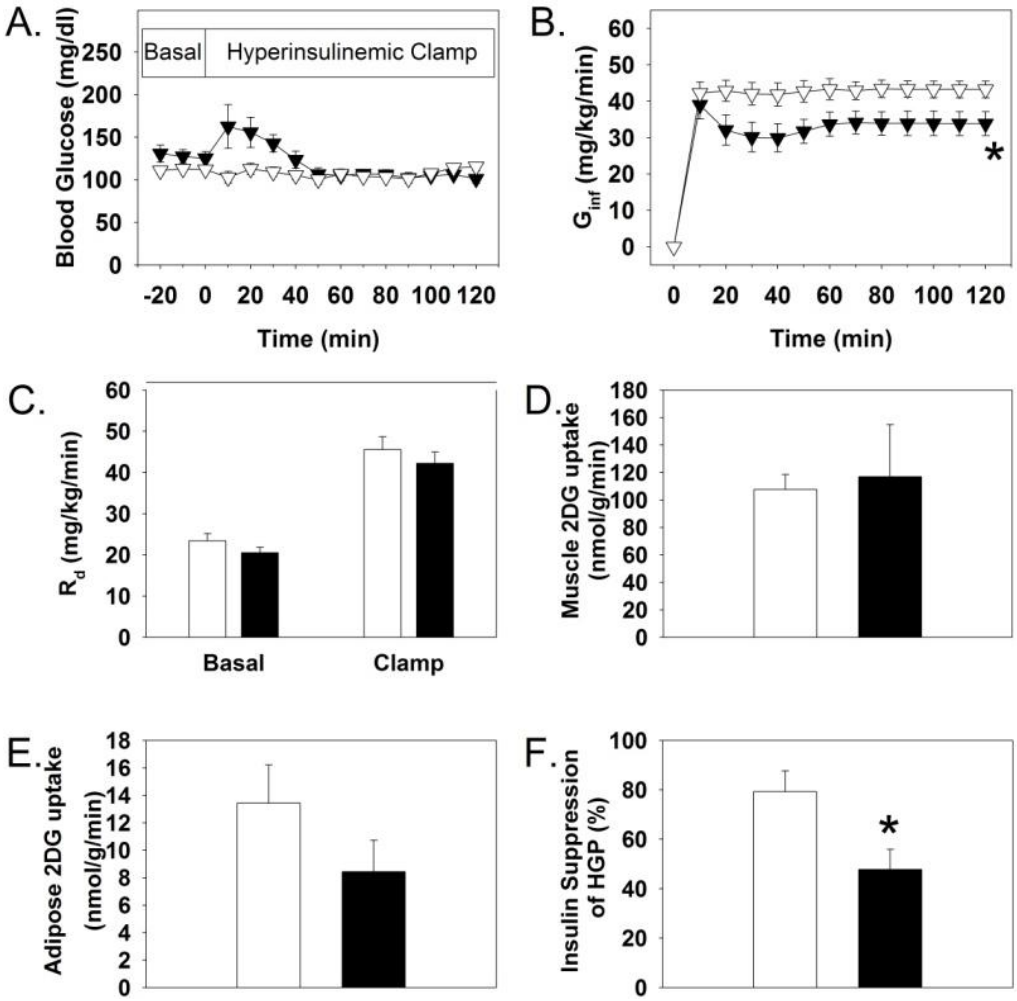


**Figure 7. Reduced insulin sensitivity and hepatic insulin resistance in NG4KO mice.** (A) Blood glucose during a hyperinsulinemic (4 U/kg/min) euglycemic clamp. No difference in blood glucose before or during the clamp was observed between NG4KO (closed triangles, n=12) and littermate controls (CON, open triangles, n=20). (B) Despite matched blood glucose levels, NG4KO mice (closed triangles, n=12) required a significantly lower glucose infusion rate to maintain euglycemia compared to CON (open triangles, n=20) indicating insulin resistance (* $P < 0.05$, NG4KO vs. CON). (C) Rate of whole body glucose disposal at baseline and during the hyperinsulinemic clamp was not significantly different between NG4KO (black bar, n=12) and CON (open bar, n=20). (D and E) Glucose uptake specifically in muscle (D) and adipose tissue (E) was similar between NG4KO (black bar, n=6) and littermate controls (white bar, n=11) during the hyperinsulinemic clamp. (F) Insulin failed to suppress hepatic glucose production in NG4KO (black bar) mice to the same degree as control mice (white bar, n=20) (48±8 and 79±8 % suppression, * $P < 0.02$) All data expressed as mean ± S.E.M.

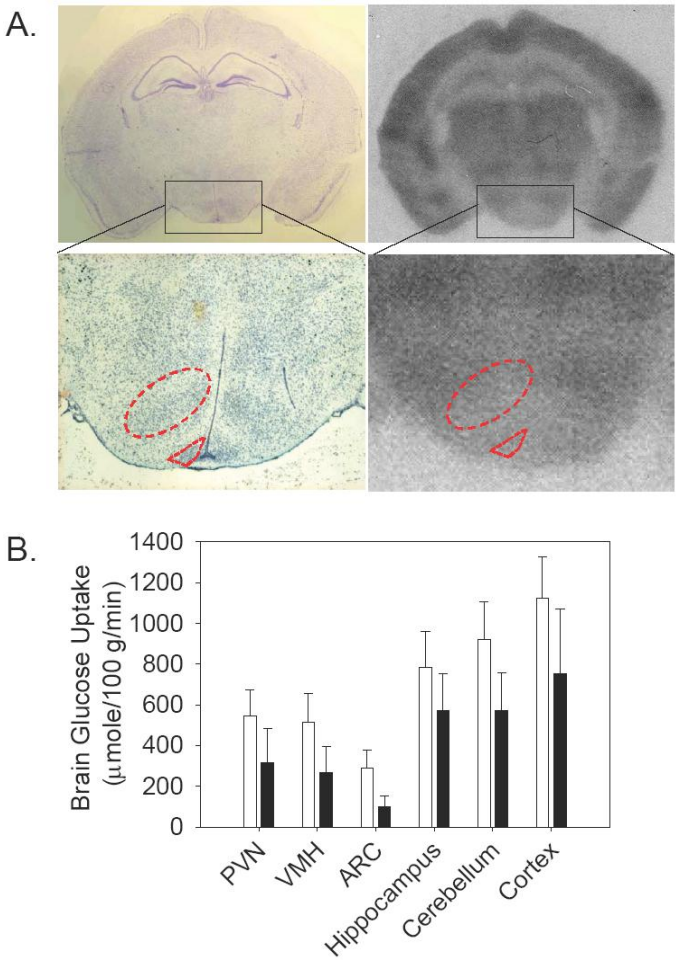


**Figure 8. Brain glucose uptake during a hyperinsulinemic hypoglycemic clamp in NG4KO mice.** Brain glucose uptake was measured using $^{14}$C-2-deoxyglucose autoradiography in mice that underwent a 2 hour hyperinsulinemic (4 mU/kg/min) euglycemic clamp. (A) Precise identification of the areas of interest were accomplished by Nissl staining the radiolabeled sections (left panels), visualizing the desired areas (i.e. ventromedial hypothalamus (VMH, red dashed oval outline) and arcuate nucleus (red dashed triangular outline), and measuring the density of the outlined areas of interest on the respective exposed autoradiographic films (right panels). (B) Quantification of brain glucose uptake during euglycemia using the Sokoloff's equation in NG4KO (black bar, n=6) and littermate control (white bar, n=11). Brain glucose uptake during the hyperinsulinemic euglycemic clamp was not significantly different in NG4KO mice compared to littermate controls in the areas measured: the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), arcuate nucleus (ARC), hippocampus, cerebellum, or cortex. Data expressed mean ± S.E.M.

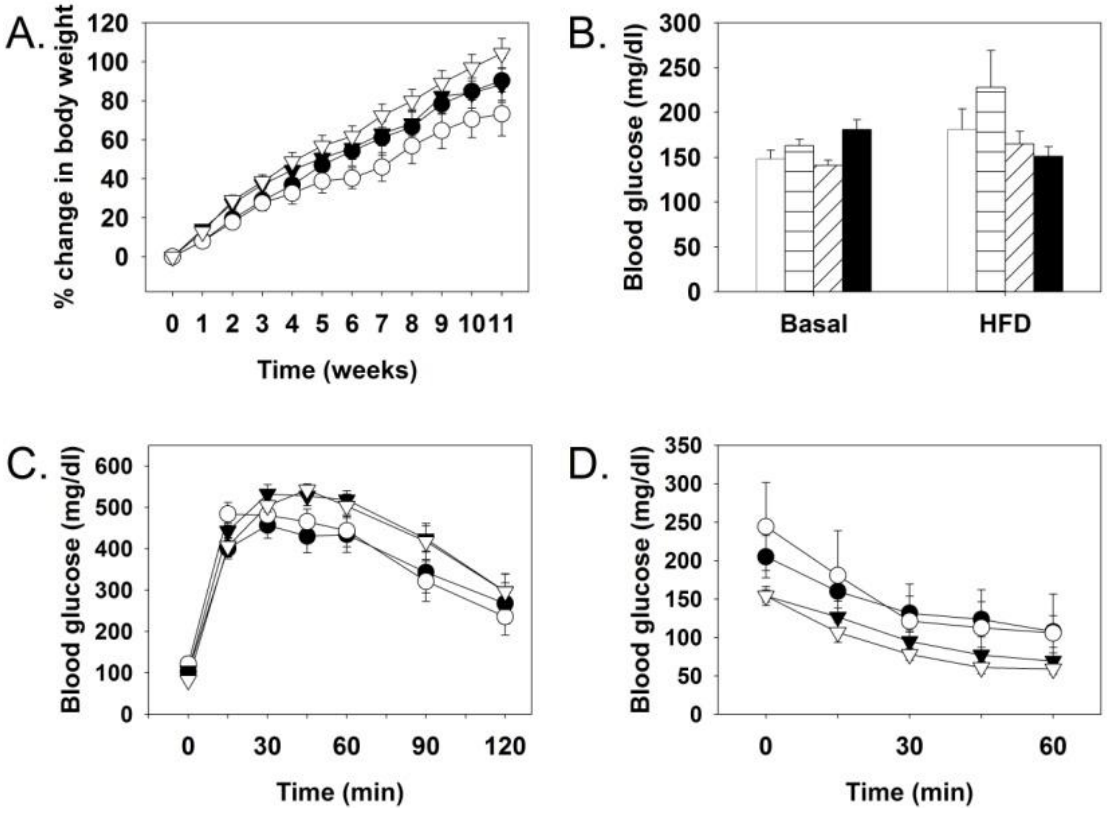


**Figure 9. Neuronal GLUT4KO did not affect susceptibility to diet-induced obesity.** Mice were fed a high fat diet (60% calories from fat, solid) at 5 weeks of age. (A) Body weight gain was similar between NG4KO mice (close triangles, n=14) and their littermate controls (WT, open circle, n=7; Lox, closed circle, n=9; Cre, open triangle, n=11). (B) Blood glucose before and during high fat feeding. No difference in basal blood sugar levels were observed between NG4KO (black bar) and WT (white bar), Lox (horizontal hash), and Cre (slanted hash). Further, blood glucose was not significantly different between groups after 11 weeks on a high fat diet. Glucose tolerance and insulin sensitivity as measured by a glucose tolerance test (C) and insulin tolerance test (D), respectively, were similar between NG4KO and littermate controls after 11 weeks on a high fat diet. Data expressed as mean ± S.E.M. n=7-14 mice per group.

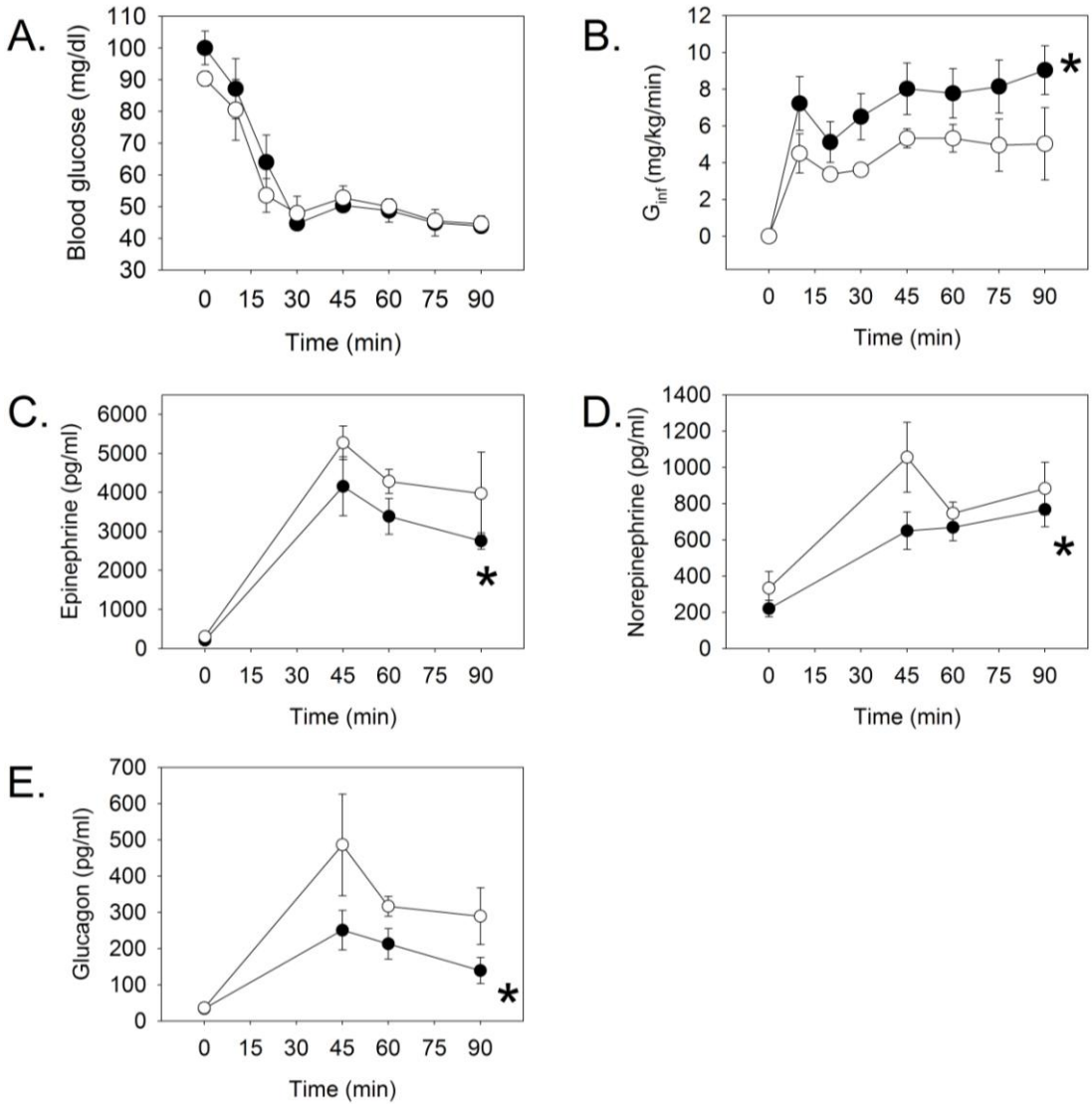


**Figure 10. ICV Infusion of an inhibitor of GLUT4 transport, indinavir, reduced the counterregulatory response (CRR) to hypoglycemia.** Indinavir (IDV, closed circle, n=4) or artificial cerebrospinal fluid (aCSF, open circle, n=9) was infused for 90 min before and for the duration of a 90 min hyperinsulinemic (20mU/kg/min) hypoglycemic (~50mg/dl) clamp in Sprague-Dawley rats. (A) Blood glucose was precisely matched between IDV and aCSF treated rats. (B) Despite matched blood glucose levels, IDV treated rats (closed circle) required a significantly higher glucose infusion rate than control rats (open circle). The higher glucose infusion rate was attributed to the attenuated CRR to hypoglycemia. Epinephrine (C), norepinephrine (D), and glucagon (E) response to hypoglycemia was significantly reduced in IDV rats versus controls (* $p<0.05$ by ANOVA). Data expressed mean ± S.E.M.

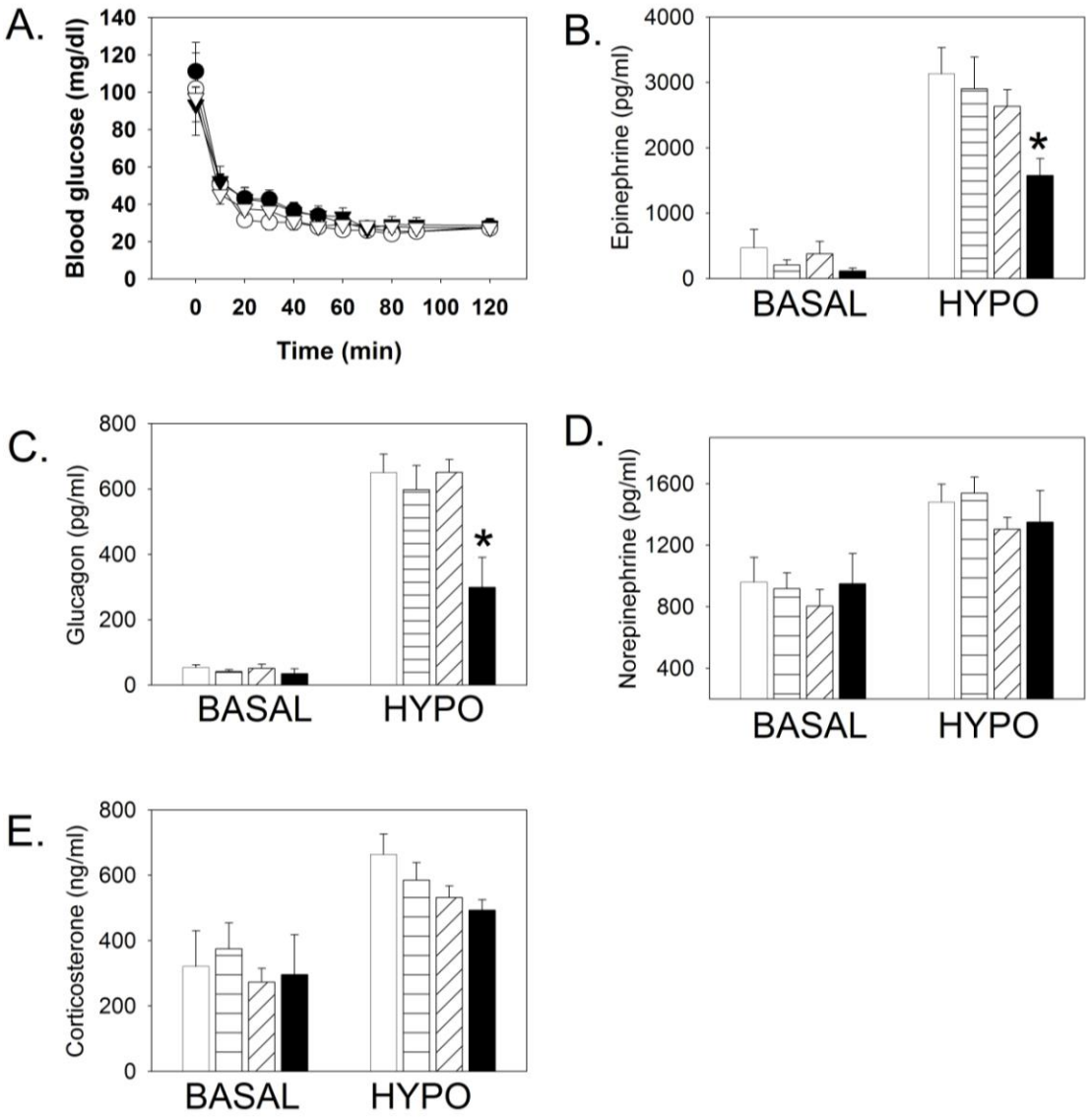


**Figure 11. Neuronal GLUT4KO mice had impaired counterregulatory response to hypoglycemia.** NG4KO and their littermate controls were subjected to hyperinsulinemic (20 mU/kg/min) hypoglycemic (~30 mg/dl) clamps. (A) Blood glucose was not different before or during the clamp between NG4KO (black bar, n=5), wild-type (white bar, n=5), Lox (horizontal hash, n=6), and Cre (diagonal hash, n=11). Epinephrine (B) and glucagon (C) responses to hypoglycemia were significantly reduced in NG4KO (black bar) mice compared to WT (closed bar), Lox (horizontal hash), and Cre (slanted hash). Norepinephrine (D) and corticosterone (E) levels during the hypoglycemic clamp were similar in all groups. * $P < 0.05$ versus WT, Lox, and Cre. Data expressed as mean ± S.E.M.

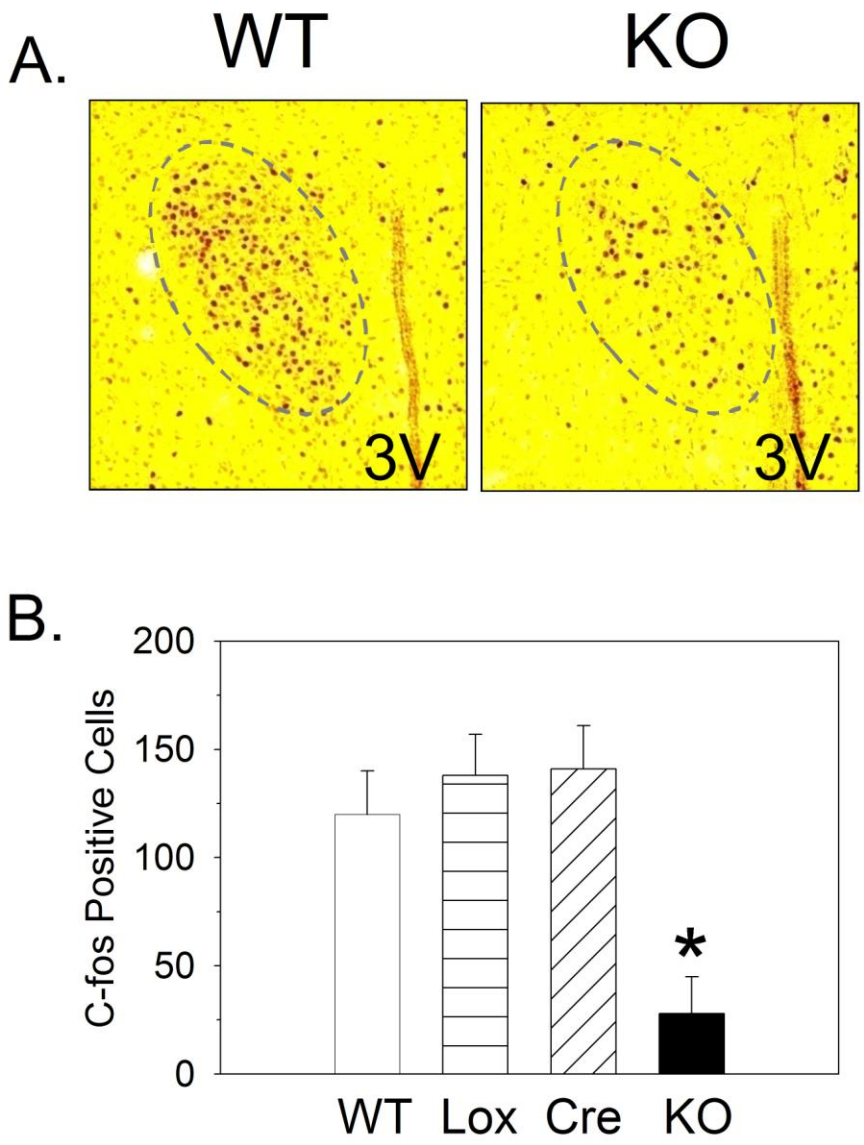


**Figure 12. Reduced neuronal activation in response to hypoglycemia in neuronal GLUT4KO mice.** Hypoglycemia was induced for 2 hours using a hyperinsulinemic (20 mU/kg/min) hypoglycemic (~30mg/dl) clamp in NG4KO mice and their littermate controls. After 2 hours, brains were harvested and processed for c-fos immunostaining. (A) Representative c-fos immunostaining of the hypothalamic paraventricular nucleus (PVN) in wild-type (WT) and NG4KO (KO) mice after 2 hours of hypoglycemia. (B) Quantification of c-fos immunostaining. The number of c-fos positive cells in the PVN was greatly reduced in NG4KO mice (black bar) compared to wild-type (open bar), Lox (horizontal hash), and Cre (slanted hash). * $P < 0.01$ versus WT, Lox, and Cre. Data expressed as mean ± S.E.M. n=4-8 per group.

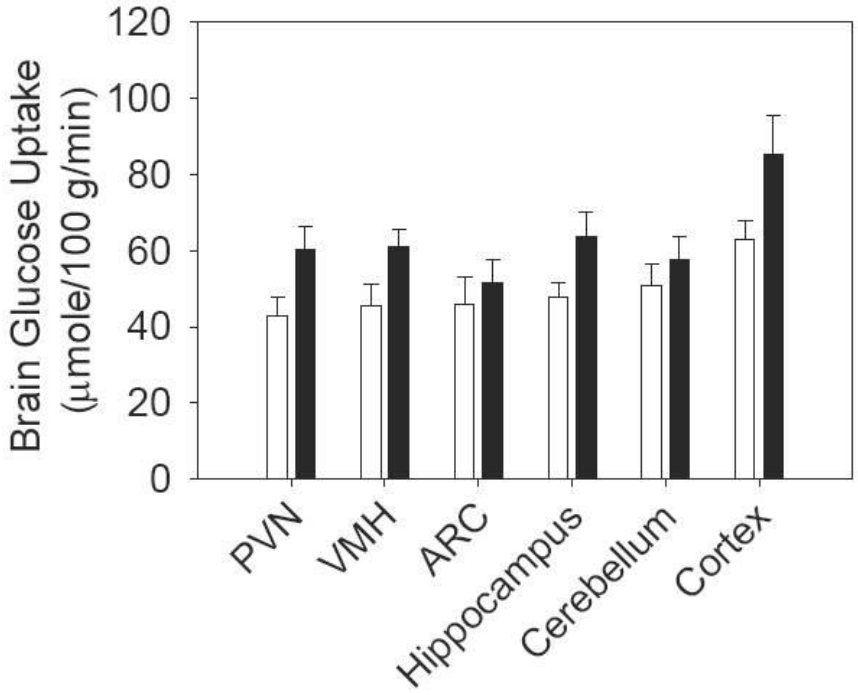


**Figure 13. Neuronal GLUT4 deletion does not alter brain glucose uptake during hypoglycemia.** Brain glucose uptake was measured using $^{14}$C-2-deoxyglucose autoradiography in mice that underwent a 2 hour hyperinsulinemic hypoglycemic clamp. Brain glucose uptake during hypoglycemia was quantified using the Sokoloff's equation in NG4KO (black bar, n=4) and Lox (horizontal hash, n=5). No difference in glucose uptake during hypoglycemia was observed between groups in either the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), arcuate nucleus (ARC), hippocampus, cerebellum, or cortex. Data expressed mean ± S.E.M.

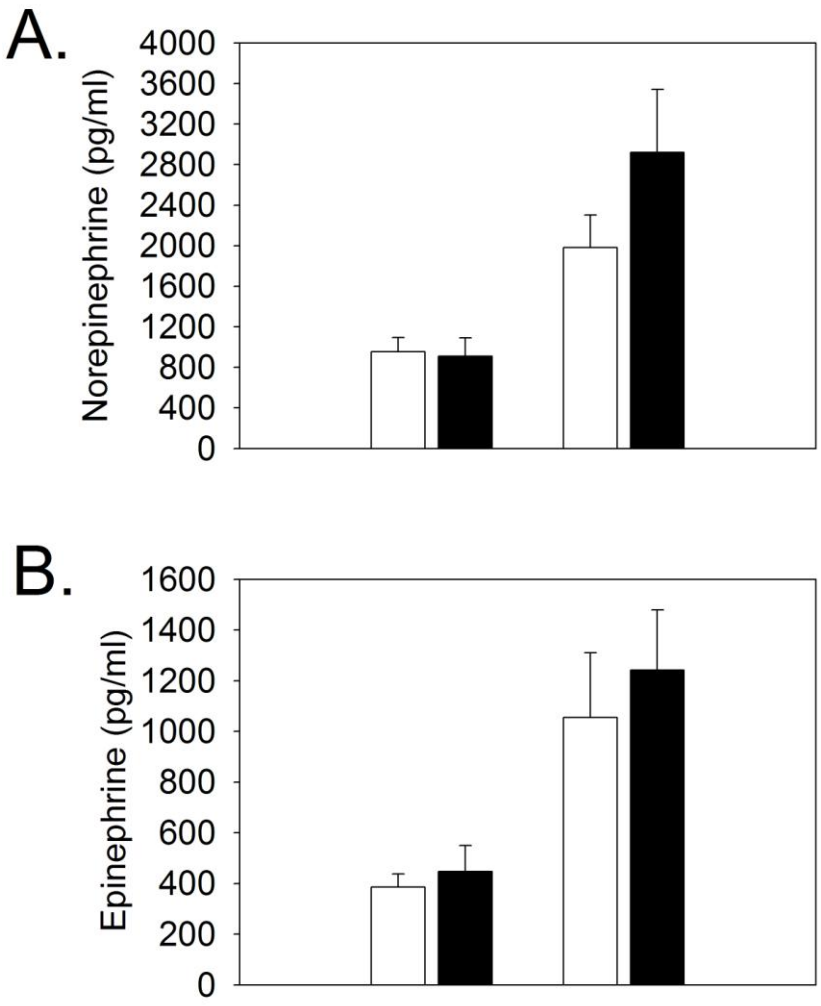


**Figure 14. Normal sympathoadrenal response to heat stress in neuronal specific GLUT4 knock-out mice.** To assess the whether the adrenomedullary response in NG4KO was also impaired to other forms of stress aside from hypoglycemia, NG4KO and littermate controls (CON) were subjected to 60 min of heat stress (ambient temperature of 42$^{o}$ C). In response to heat stress, NG4KO and CON mice had similar increases in norepinephrine (A) and epinephrine (B).

## DISCUSSION

Based on immunohistochemical studies that identified GLUT4 in discrete areas of the central nervous system including the hypothalamus (30;31;86-88), neuronal GLUT4 has long been speculated to play a key role in neuronal glucose sensing and in whole body energy homeostasis. However, the physiological roles of GLUT4 in the central nervous system have remained unknown. This current study demonstrated a novel role of neuronal GLUT4 in the modulation of whole body energy homeostasis as well as the counterregulatory response to hypoglycemia.

Tissue specific deletion of neuronal GLUT4 was accomplished by Cre-Lox technology. As expected, brain GLUT4 was specifically knocked out while GLUT4 protein expression was still normally expressed in peripheral tissues including the heart, adipose tissue, and skeletal muscle. As GLUT4 is the major glucose transporter for peripheral insulin stimulated glucose uptake, further evidence for the restriction of GLUT4 knock-out solely to the brain was that the rate of whole body glucose disposal was similar between NG4KO mice and littermate controls. Further, tissue specific glucose uptake in adipose tissue and skeletal muscle was normal in NG4KO mice.

An interesting finding in this study was the expression of nestin-Cre alone resulted in a mild reduction in body weight and crown-rump length, consistent with preliminary results from other labs (B.B. Lowell and B.B. Kahn, personal communication). As NG4KO and littermate Cre mice had no difference in body weight, neuronal GLUT4 deletion was concluded not to have an effect on overall body weight. Because of this effect of nestin-Cre expression, all experiments included WT, Lox, and Cre littermates as the 3 control groups to be compared to the NG4KO mice. Notably, Cre

mice had similar metabolic phenotypes to WT and Lox mice in all other metabolic parameters assessed such as blood glucose levels, insulin levels, glucose tolerance, insulin sensitivity, and counterregulatory responses. Thus, although nestin-Cre expression resulted in slightly decreased size, it did not affect any other metabolic phenotypes.

Fasting and fed glucose levels were similar in NG4KO mice and littermate controls, suggesting a lack of effect of neuronal GLUT4 on basal glucose homeostasis. Interestingly, female NG4KO mice had mild hyperinsulinemia in the fed state although no difference in fasted insulin levels were observed. This hyperinsulinemia may be indicative of mild insulin resistance, characteristic of a pre-diabetic state. When challenged with a glucose load during an intraperitoneal glucose tolerance test, male NG4KO had significantly higher blood glucose levels than littermate controls. NG4KO female mice did not demonstrate glucose intolerance. The gender specific difference was not surprising as other studies have observed phenotypes more pronounced in one sex than the other (36). For example, in neuronal specific insulin receptor knock-out mice (NIRKO), on a standard chow diet, female NIRKO mice had increased food intake and body weight compared to controls, while male NIRKO mice and controls had similar food intake and body weight. When an insulin tolerance test was performed, surprisingly, no difference in blood glucose was observed between male NG4KO and their littermate controls. The impaired glucose tolerance but seemingly normal insulin sensitivity was initially speculated to be attributed to a pancreatic β-cell secretory defect. However, glucose stimulated insulin secretion was measured and no difference in insulin

levels were seen between NG4KO and littermate controls after a glucose load. Thus, pancreatic β-cell function was intact and not affected by neuronal GLUT4 deletion.

An insulin tolerance test is an inexact measure of insulin sensitivity because several factors could confound interpretation of the results, including differences in counterregulatory responses. As discussed below, NG4KO had an attenuated CRR to hypoglycemia and this impairment may have masked any differences in insulin sensitivity present in NG4KO mice. To directly assess insulin sensitivity, the "gold standard" hyperinsulinemic-euglycemic clamps was performed in NG4KO mice and control mice. In the setting of carefully matched blood glucose levels, NG4KO mice required a significantly lower glucose infusion rate to maintain euglycemia than littermate controls, indicating insulin resistance. To better define the exact nature of the insulin resistance, glucose fluxes and glucose uptake into specific tissues were measured during the euglycemic clamp. Consistent with normal GLUT4 expression in peripheral tissues, NG4KO had a similar rate of glucose disposal ($R_d$) to littermate controls. Further, skeletal muscle and adipose tissue specific glucose uptake was not different between NG4KO and control mice. Interestingly, the ability of insulin to suppress hepatic glucose production (HGP) was significantly impaired in NG4KO mice. Thus, the insulin resistance in NG4KO mice is attributed to hepatic insulin resistance. This data is of particular interest as previous studies have shown that central insulin signaling is crucial for insulin's ability to fully suppress HGP (98;99). One possibility for the altered hepatic insulin sensitivity is that insulin action in the central nervous system requires central GLUT4 to suppress HGP. The absence of neuronal GLUT4 may prevent central

insulin from exerting its effects on HGP. More direct studies to understand this relationship between CNS GLUT4 and insulin are warranted.

As GLUT4 and insulin receptors are coexpressed in up to 75% of key glucose sensing neurons in the ventromedial hypothalamus (15), brain GLUT4 may have a particular role in the ability of neurons to sense and respond to changes in glucose levels. To test this possibility, the counterregulatory response to hypoglycemia (CRR) was assessed in animals that had central GLUT4 inhibited pharmacologically (by indinavir) or genetically (NG4KO mice). Indinavir has been shown to selectively inhibit glucose transport through GLUT4 in both cell culture and in brain slices (94-96). Both pharmacologic inhibition and genetic deletion of brain GLUT4 resulted in significantly attenuated CRR. Specifically, both approaches resulted in impaired epinephrine response as well as an attenuated glucagon response to hypoglycemia. Interestingly, consistent with the observation that brain GLUT4 is important in the counterregulatory response to hypoglycemia, streptozotocin diabetic rats that have reduced GLUT4 expression in the brain (34) also have an impaired epinephrine and glucagon responses to hypoglycemia (100;101). As with central insulin signaling, neuronal GLUT4 also has an important role in the CRR.

To determine whether this impaired CRR was unique to hypoglycemia or a result of a more global impairment in the sympathoadrenal response to stress in general, NG4KO mice and littermate controls were subjected to heat stress. The adrenomedullary response to heat stress were identical in NG4KO and littermate control mice, indicating that NG4KO have an intact sympathoadrenal response to stress and that the impaired CRR response in NG4KO is unique to hypoglycemic stress.

To determine whether the impaired CRR in the NG4KO mice was associated with impaired neuronal activation to hypoglycemia, c-fos activation was quantified in the PVN of mice that underwent a hyperinsulinemic hypoglycemic clamp (102;103). In response to hypoglycemia, c-fos expression increased predominantly in the PVN but not in the VMH, consistent with previous studies (104). Notably, PVN c-fos activation to hypoglycemia was significantly reduced in NG4KO mice compared to controls. The impaired hypoglycemia-induced impaired c-fos activation in NG4KO mice could represent reduced glucose sensing of PVN neurons. Alternatively, given that the PVN receives input from other glucose sensing neurons in other regions of the brain (i.e. the VMH), could represent an indirect reduction in afferent inputs from other brain regions. In either case, NG4KO had a profound impairment in c-fos activation, consistent with other models of impaired glucose sensing and impaired counterregulation (102;103).

To determine whether the impaired CRR and neuronal activation to hypoglycemia was associated with altered glucose uptake, brain glucose uptake was measured in NG4KO and littermate controls during a hyperinsulinemic hypoglycemic clamp. No difference in regional brain glucose uptake was observed between groups during the hypoglycemic clamp. Further, brain glucose uptake was also measured during a hyperinsulinemic euglycemic clamp. Again, no statistically significant difference in regional brain glucose uptake was observed between NG4KO and littermate control mice. To note, since brain GLUT4 expression is much lower than GLUT1 and GLUT3, it is likely that that majority of glucose uptake occurred through these glucose transporters, thus masking any subtle effect caused by GLUT4 deletion. Further, due to technical

limitations in the $^{14}C$ 2-deoxyglucose autoradiograph technique, we cannot rule out an effect of GLUT4 deletion on glucose uptake in individual glucose sensing neurons.

As these studies demonstrated a novel role of neuronal GLUT4 in energy homeostasis, whether deletion of neuronal GLUT4 would have an impact on the susceptibility to diet induced obesity was investigated. NG4KO mice and littermates were fed a high fat diet (HFD, 60% calories from fat) for 11 weeks. No difference in body weight change was observed between NG4KO and littermates, demonstrating that neuronal GLUT4 deletion and altered glucosensing did not increase the risk of diet induced obesity.

Several studies have suggested a relationship between insulin action in the CNS and GLUT4 (15;34;88;94). Specifically, as insulin stimulates GLUT4 translocation to the plasma membrane in the peripheral tissue, a similar phenomena may occur in the central nervous system (94). This current study does not directly address the role of GLUT4 in modulating central insulin effects. However, some similarities were observed with NG4KO mice and neuronal insulin receptor knock-out mice (NIRKO). Both NG4KO and NIRKO mice were glucose intolerant and insulin resistant (36). Further, both NG4KO mice and NIRKO mice had impaired epinephrine responses and impaired neuronal activation to hypoglycemia (38;39). Thus, central GLUT4 may play a role in exerting CNS insulin effects in regards to those roles. However, the metabolic phenotypes of NG4KO mice and NIRKO mice were not exactly the same. NIRKO mice had increased body weight and increased risk of diet induced obesity whereas NG4KO mice did not (36). NG4KO mice had an impaired glucagon response to hypoglycemia in addition to the impaired epinephrine response whereas NIRKO mice exhibited

impairment only in the epinephrine response (38). These observations are not surprising as mice with either muscle or adipose specific knock-out of GLUT4 (26;28) have drastically different phenotypes compared to mice with muscle or adipose specific knock-out of insulin receptor (105;106), respectively. GLUT4 and proteins involved in insulin signaling are co-expressed in many but not all areas of the brain. For example, there is a dissociation between GLUT4 expression and insulin related aminopeptidase (IRAP) expression in the cerebellum (94). Further, insulin has a plethora of actions independent of the stimulation of glucose uptake (107). Taken together, GLUT4 may be involved in exerting some but not all of central insulin effects.

# CHAPTER 2. PARADOXICALLY, HYPOTHALAMIC GLUCOSAMINE INFUSION REDUCES FOOD INTAKE AND LIMITS WEIGHT GAIN WHILE ENHANCING BRAIN INSULIN SENSITIVITY

**ABSTRACT**

In patients with uncontrolled diabetes, high blood sugar further worsens diabetes control by causing resistance to insulin action. Chronic hyperglycemia increases glucose flux through the hexosamine biosynthetic pathway (HBP) and impairs insulin signaling. Glucosamine, a molecule which mimics hyperglycemia by directly entering the HBP, has similarly been shown to induce insulin resistance in peripheral tissues. Knowing the importance of the brain in mediating systemic metabolism, chronic infusion of a high concentration of glucose or glucosamine into the mediobasal hypothalamus of the brain was hypothesized to induce hypothalamic insulin resistance and, consistent with other models of central insulin resistance, lead to increases in food intake and body weight. To test this hypothesis, glucose (GLU), glucosamine (GLN) or mannitol (MAN), an osmotic control, were each infused bilaterally into the mediobasal hypothalami of 9 week old, male, Sprague-Dawley rats for 3 weeks. Contrary to our original hypothesis, GLU-treated and GLN-treated rats weighed significantly less than their respective MAN-treated controls. Further, food intake was significantly reduced in GLN-treated rats compared to controls. The decrease in food intake occurred in the absence of changes in hypothalamic NPY or POMC expression. Interestingly, GLN-treated rats had increased central insulin sensitivity as ICV infusion of insulin resulted in a 2-fold greater phosphorylation of Akt in GLN-treated rats compared to MAN controls. This study demonstrates a novel nutrient sensing role for glucosamine and the hexosamine biosynthetic pathway in regulating brain insulin sensitivity, food intake, and body weight.

## INTRODUCTION

Obesity and caloric excess are major predisposing factors in the development of insulin resistance and Type 2 diabetes (108;109). Persistent overabundance of nutrients, such as glucose, can induce insulin resistance, a hallmark of Type 2 diabetes (44;110). The precise mechanisms of how excessive glucose leads to peripheral insulin resistance are incompletely understood. One mechanism is enhanced flux through the hexosamine biosynthetic pathway (HBP) and subsequent increase in protein glycosylation (40;111). Between 2-5% of glucose entering the cell enters the HBP (111), the end-product of which is uridyl diphosphate-N-acetylglucosamine (UDP-GlcNAc) (41;111) (Figure 18). UDP-GlcNAc can then be added to serine and threonine residues of proteins by O-linked N-acetylglucosamine transferase (OGT) (41;111) (Figure 18). The addition of UDP-GlcNAc, termed O-linked glycosylation (O-GlcNAc) is considered to be a measure of nutritional status (41;111;112) and excess O-GlcNAc leads to peripheral insulin resistance. At a cellular level, excess nutrients and increased O-GlcNAc negatively regulate nutrient import into the cell. In skeletal muscle and adipose tissue, incubation with high levels of glucose or glucosamine (a substrate that directly enters the HBP) increases O-GlcNAc and results in insulin-resistance and impaired insulin-stimulated glucose uptake (42;43;113).

In addition to systemic insulin resistance, perturbations of insulin signaling in the central nervous system have also been implicated in the pathogenesis of Type 2 diabetes (36;37). Animals with insulin receptors (IR) knocked-out in the brain were hyperphagic, glucose intolerant, and insulin resistant—hallmarks in the development of Type 2 diabetes (36;37). Insulin receptors are discretely expressed in particular areas of the

brain, including the mediobasal hypothalamus (MBH). The MBH is an important site for integrating hormonal and nutritional signals and modulating whole body energy homeostasis (114;115). Targeted disruption of IR in the MBH increased food intake and increased fat mass, similar to the whole brain IR knockout mice (36;37). Whether chronic exposure to hyperglycemia and increased O-GlcNAc in the mediobasal hypothalamus can induce hypothalamic insulin resistance and lead to hyperphagia has yet to be determined. In the current experiments, infusion of glucose or glucosamine (which directly enters the HBP and increases O-GlcNAc) in the MBH was hypothesized to induce hypothalamic insulin resistance, leading to increased food intake and body weight gain.

## METHODS AND PROCEDURES

**Animals care and surgical procedures**. Eight to nine week old male Sprague-Dawley rats (Charles River Laboratories) were individually housed in a temperature and light controlled environment maintaining the animal's diurnal cycle (12hrs light, 12hrs dark) and with an ad lib standard rat chow diet. During the experimental protocol, body weight and food intake was measured twice a week for 3 weeks. All studies were done in accordance with the Animal Studies Committee at the Washington University School of Medicine.

**Surgical procedures and hypothalamic infusions**. Animals were anesthetized with isofluorane and were implanted with microinjection cannulas. Cannulas (Plastics One Inc, Roanoke, VA) were targeted to the mediobasal hypothalamus bilaterally (2.6 mm posterior to the bregma, + 2.0 mm lateral to midline at a 9 degree angle, cannula length 10.6 mm) and attached to low flow rate osmotic pumps (Alzet model 2ML4, Durect, Cupertino, CA). In one set of studies, the osmotic pumps were filled with either glucose (Sigma-Aldrich) or the osmotic control mannitol (Sigma-Aldrich) for an infusion rate of 16.5 micromoles/hr. In another set of studies, osmotic pumps were filled with glucosamine (Sigma-Aldrich) or the osmotic control mannitol which were infused at a lower concentration (0.12 micromoles/hr) than the glucose infusions because glucosamine is a more potent activator of the HBP and pilot experiments determined that this dose of glucosamine achieved a physiologically relevant increase in protein glycosylation. Osmotic pumps were buried subcutaneously on the back of the animals. A third cannula to be used later for acute intracerebroventricular insulin or aCSF

injections was implanted into the 3rd ventricle (0.24 mm anterior to the bregma, on the midline, at an angle of 9 degrees, cannula length 10.5 mm).

**Short-term glucosamine infusion.** Cannulas were implanted into the 3rd ventricle of 8 week old Sprague-Dawley rats. After a week recovery, rats were fasted for 24 hours. One hour before the onset of the dark cycle, glucosamine or mannitol was slowly infused (0.1 µl/min) into the 3rd ventricle (2.8 micromoles/hr) and infusion continued for the duration of the experiment. Rats were given food at the onset of the dark cycle, and food intake was measured at 2 and 4 hours afterwards.

**Insulin Tolerance Test.** Animals were fasted overnight and given an intraperitoneal (i.p.) injection of 0.60 U/kg regular human insulin (Lilly). Tail vein glucose measurements were made immediately before injection and at 15 min intervals for 90 min after insulin administration.

**Central Insulin Sensitivity.** After 3 weeks of hypothalamic infusion of glucosamine or mannitol, insulin (15 mU) or aCSF was injected into the 3rd ventricle of overnight fasted rats. Twenty minutes later, brains were harvested and rapidly frozen in liquid nitrogen. Hypothalami were processed by western blot analysis to measure insulin mediated Akt phosphorylation.

**Western Blot.** Hypothalami were homogenized in buffer containing 1% Igepal (Sigma), 0.5% sodium dodecyl sulfate (Sigma), 0.1 mM Phenylmethylsulfonyl fluoride (Sigma), 1X complete protease inhibitor (Sigma), 1 mM NaF (Sigma), and 1 mM Na3VO4 (Sigma) in phosphate buffered saline (pH=7.4), centrifuged at 14,000 rpm for 30 min, and supernatant was collected. Protein samples (200 µg) were then separated on 12.5% Tris-HCl gel, transferred to nitrocellulose membrane (Bio-Rad), and were

immunoblotted with antibodies against phosphorylated Akt (1:400; Cell Signaling), total Akt (1:600; Cell Signaling), or beta-N-acetyl-D-glucosamine (O-GlcNAc) (1:1000; Covance).

**Brain stereotaxic punch biopsies of the arcuate nucleus and ventromedial hypothalamus.** After 3 weeks of bilateral hypothalamic infusion glucosamine or mannitol, brains were harvested and frozen in 2-methylbutane in dry ice at -20°C. Brain sections (500 μm) were cut and collected using a Leica CM1850 cryostat (Leica Microsystems Inc., Bannockburn, IL). According to a rat brain stereotaxic atlas (Paxinos and Watson), the arcuate nucleus (ARC) and ventromedial hypothalamus (VMH) were identified and punched out with a stainless steel needle. Punch biopsies were placed in Eppendorf tubes and stored at -80°C until real-time revere transcription polymerase chain reaction (RT-PCR) analysis.

**Real-time RT-PCR.** Punch biopsy total RNA was extracted with TriZOL (Invitrogen) and reversed transcribed to cDNA using Superscript (Invitrogen). Real-time PCR reactions were prepared using SYBR® Green PCR Master Mix (Applied Biosystems), and reactions were carried out with ABI Prism® 7000 Sequence Detection (Applied Biosystems). Primers for real-time PCR analysis are as follows: rat NPY (F 5'-GCCATGATGCTAGGTAACAAACG-3', R 5'-GTTTCATTTCCCATCACCACATG-3'); rat POMC (F 5'-CCAGGCAACGGAGATGAAC-3', R 5'-TCACTGGCCCTTCTTGTGC-3'); L32 (F 5'-TAAGCGAAACTGGCGGAAAC-3', R 5'-TCATTTTCTTCGCTGCGTAGC-3').

**Glucose, Insulin, and Leptin Measurements.** Tail vein blood samples were taken in overnight fasted rats one day prior to surgical implantation of cannulas and after

the 3 week infusion of hypothalamic infusions. Blood glucose was measured by glucose oxidase method (B-D logic Glucometer). Plasma insulin was measured by ultrasensitive rat insulin ELISA (Crystal Chem Inc., Downer's Grove, IL), and plasma leptin was measured by leptin ELISA assay (Crystal Chem Inc.).

**Statistical Analysis.** Data are represented as mean + S.E.M. Statistical analysis was performed using unpaired Student's t test or two-way ANOVA, as indicated. $P < 0.05$ was considered statistically significant.

## RESULTS

### Chronic hypothalamic infusion of glucose limited body weight gain.

To determine whether chronic hyperglycemia in the central nervous system would induce central insulin resistance and affect whole body energy homeostasis, glucose was infused bilaterally into the mediobasal hypothalamus for 3 weeks. Intriguingly, body weight was significantly lower in glucose-infused rats as compared to mannitol infused controls ($P<0.05$) (Figure 15A). No statistically significant difference in food intake was observed between groups (Figure 15B).

### Mediobasal glucosamine infusion increased hypothalamic protein glycosylation, reduced food intake, and limited body weight gain.

Through the hexosamine biosynthetic pathway, glucose and glucosamine can increase protein glycosylation, leading to insulin resistance in peripheral tissues (40;42;112). To assess the effect of enhanced flux through the HBP in the central nervous system, glucosamine or the osmotic control mannitol was infused into the mediobasal hypothalamus for 3 weeks and food intake and body weight were measured.

As with the hypothalamic glucose infusions, glucosamine treatment resulted in rats weighing significantly less than controls during the infusion period (Figure 16C). Further, glucosamine infusion resulted in significantly reduced food intake (Figure 16D). At the end of the 3 week infusion, hypothalami were harvested. As expected, hypothalamic protein glycosylation as measured by Western blot analysis using anti-O-GlcNAc antibody was increased ~2-fold in glucosamine infused rats compared to mannitol controls (Figure 16). Interestingly, the degree of hypothalamic protein glycosylation was inversely correlated with the change in body weight during the

infusion period (Figure 16C). Specifically, increased protein glycosylation correlated with reduced body weight gain. No significant difference was observed in fasting blood glucose, insulin, and leptin levels (Table 1).

**Central and peripheral insulin sensitivity is enhanced by glucosamine infusion.**

To test whether glucosamine could also affect insulin sensitivity in the central nervous system, at the end of the infusion period rats were fasted overnight, injected with intracerebroventricular (ICV) insulin (15 mU) or an equal volume of aCSF, and after 20 minutes hypothalami were harvested and analyzed for insulin-induced Akt phosphorylation (pAkt). Insulin induced Akt phosphorylation was significantly higher in glucosamine treated rats given ICV insulin compared to mannitol treated rats given ICV insulin (Figure 17).

Additionally, an insulin tolerance test was performed in rats at the end of the 3 week infusion period. An intraperitoneal injection of insulin (0.60 U/kg) was given and blood glucose was measured immediately before injection and at 15 minute intervals for 90 min. Glucosamine treated rats had significantly lower blood glucose during the insulin tolerance test compared to controls ($p < 0.05$ by ANOVA) (Figure 17C).

**Alteration in food intake and body weight were independent of changes in neuropeptide expression.**

To determine whether the effects on food intake and body weight elicited by glucosamine are dependent on changes in neuropeptide expression, punch biopsies of the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) were taken from glucosamine and mannitol treated rats. Real-time PCR analysis was performed to

measure neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) expression. No significant difference in NPY or POMC expression was observed between glucosamine and mannitol treated animals in either the VMH or ARC (Figure 17D and Figure 17E).

**Short-term glucosamine infusion does not alter food intake.**

In a separate study, to determine whether glucosamine can also acutely affect food intake, glucosamine or mannitol was continuously infused into the 3rd ventricle of 24 hour fasted rats 1 hour prior to the onset of the dark cycle till the end of the refeeding study. Rats were given food at the onset of the dark cycle and food intake was measured at 2 and 4 hours later. No significant difference in food intake was observed between groups (Figure 15E).

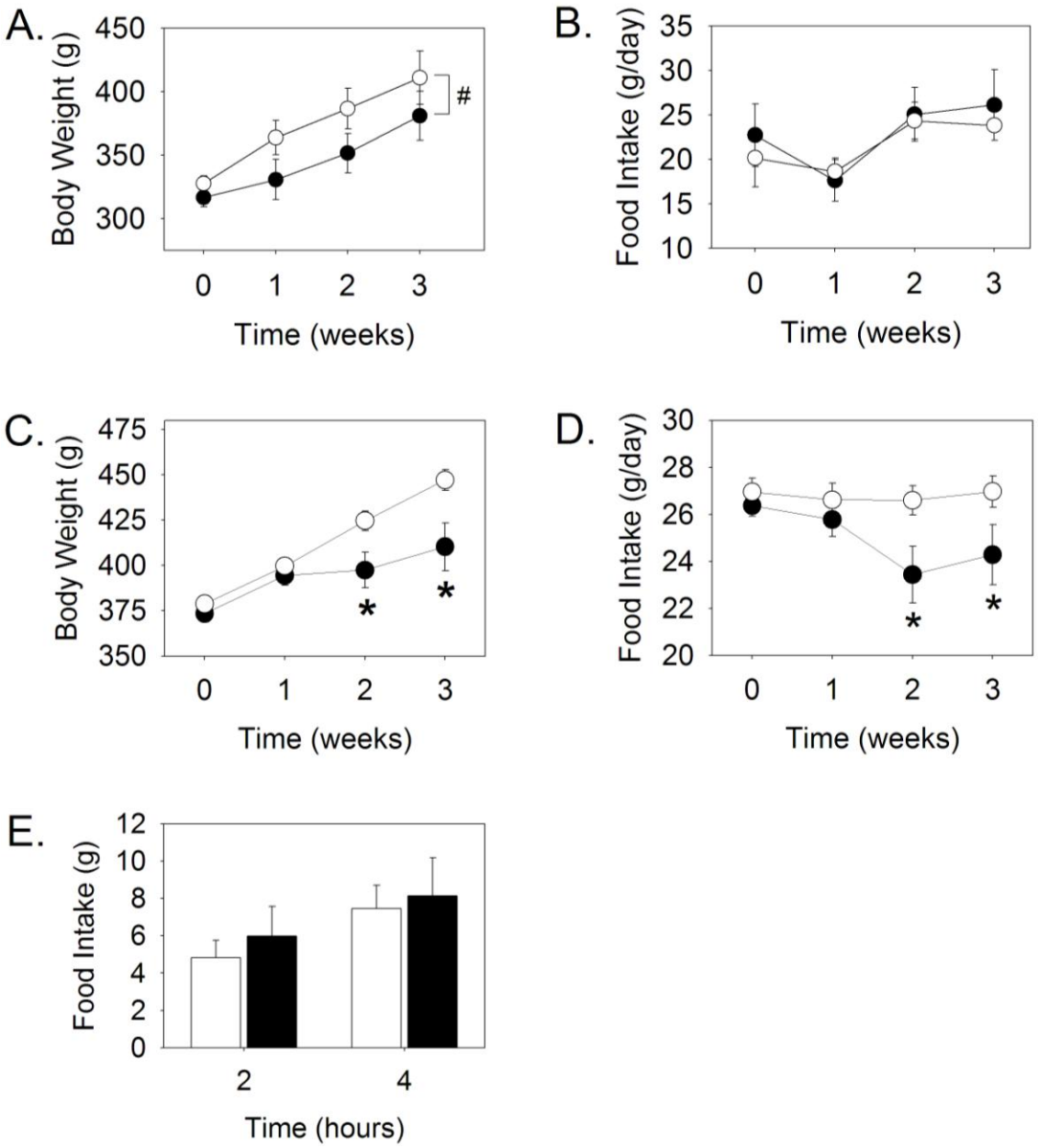


**Figure 15. Chronic hypothalamic glucose infusion reduced body weight while hypothalamic glucosamine infusion reduced body weight and food intake.** (A) Chronic hypothalamic glucose infusion (closed circles, n=9) significantly reduced the body weight gain compared to rats receiving hypothalamic mannitol (osmotic control) infusion (open circles, n=9). (B) Food intake was similar between glucose infused (closed circle) and mannitol infused (open circle) rats. (C) Consistent with hypothalamic glucose infusion, three weeks of glucosamine infusion (closed circles, n=22) resulted in lower body weight compared to mannitol controls (open circles, n=19). (D)

Hypothalamic glucosamine infusion (closed circles) also significantly decreased food intake compared to mannitol controls (open circles). (E) To test the acute effects of glucosamine infusion, glucosamine (black bars, n=6) or mannitol (white bars, n=7) was infused into the third ventricle of 24 hour fasted rats 1 hour prior to the onset of the dark cycle till the end of the refeeding study. Rats were then given food at the onset of the dark cycle. No significant difference in food intake was observed at 2 or 4 hours after the introduction of food. # $P < 0.05$ versus mannitol by ANOVA. * $P < 0.01$, compared to mannitol by ANOVA.

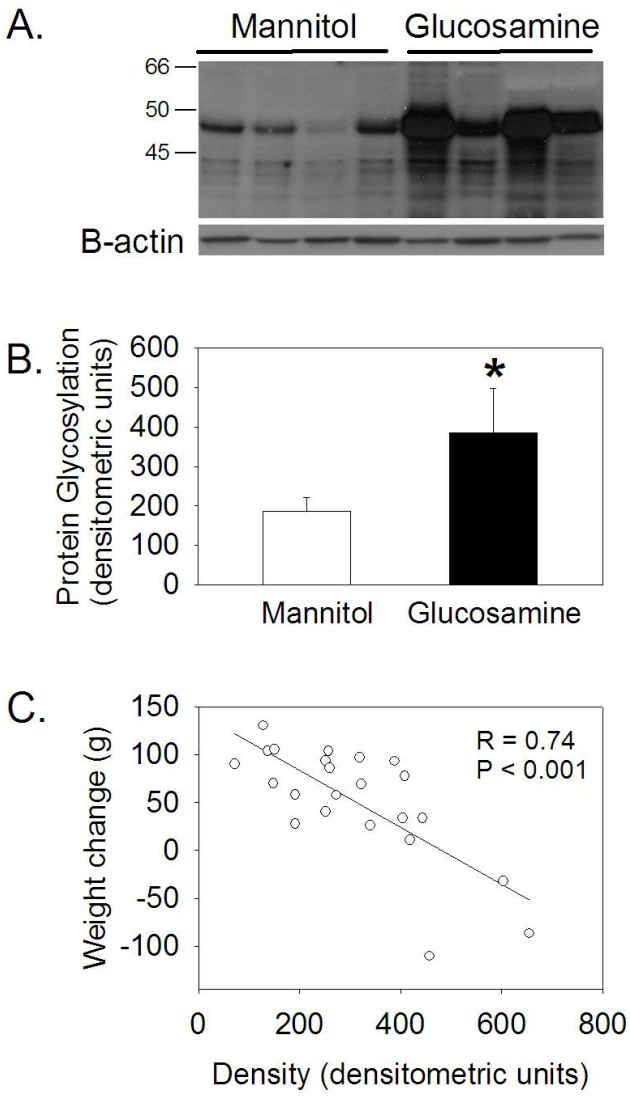


**Figure 16. Mediobasal hypothalamic glucosamine infusion increased hypothalamic protein glycosylation.** (A) Representative western blot analysis using anti-GlcNAc antibody. Overall protein glycosylation in the hypothalamus was increased after 3 weeks of glucosamine infusion compared to mannitol infused controls. Molecular marker weights are indicated with numbers on the left. (B) Quantification of western blots. Glucosamine infusion (black bar, n=14) increased hypothalamic glycosylation over 2-fold (385±112 vs 187±34 densitometric units) compared to mannitol controls (white bar, n=9). (C) Hypothalamic protein glycosylation inversely correlated with the change in body weight at the end of the 3 week infusions ($R=0.74$, $P < 0.001$; n=23). Increased hypothalamic protein glycosylation correlated with lower body weight change. * $P<0.05$, compared to mannitol.

| | Mannitol | | Glucosamine | |
|---|---|---|---|---|
| | *Basal* | *Post-infusion* | *Basal* | *Post-infusion* |
| **Glucose (mg/dl)** | 91 ±6 | 87 ±4 | 91±3 | 97±4 |
| **Insulin (pg/ml)** | 0.57± 0.31 | 0.59±0.16 | 0.34±0.08 | 0.36±0.16 |
| **Leptin (pg/ml)** | 1052±148 | 1764±350 | 800±127 | 1182±252 |

**Table 1. Metabolic Profile of mediobasal hypothalamic mannitol and glucosamine infused rats.** Glucosamine infusion did not affect fasting glucose (n=19-22 per group), insulin (n=7-10 per group) and leptin levels (n=7-10 per group) compared to mannitol-treated animals. Data represents ± S.E.M.

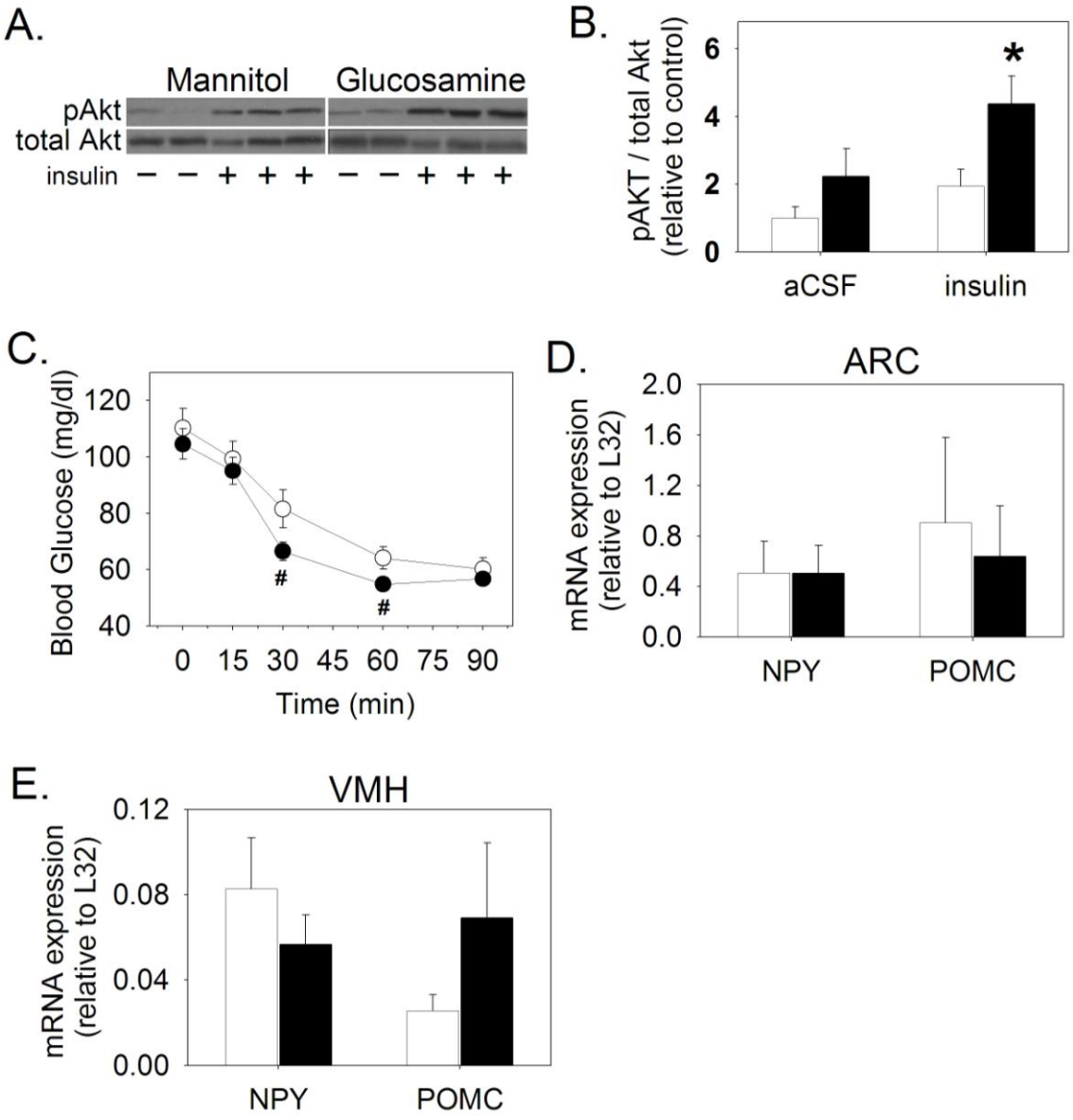


**Figure 17. Hypothalamic glucosamine infusion enhanced both central and peripheral insulin sensitivity.** (A) Representative Western blot of insulin stimulated phosphorylation of Akt (pAkt) in the hypothalamus. Insulin (15 mU) or aCSF was microinjected into the 3rd ventricle of overnight fasted rats. After 20 min, hypothalami were harvested and processed for western blot analysis. (B) Quantification of insulin-stimulated Akt phosphorylation in the hypothalamus. In response to 3rd ventricle insulin infusion, glucosamine treated rats (black bars, n=6) had significantly enhanced

phosphorylation of Akt (pAkt) compared to mannitol treated rats (white bar, n=4) while total Akt was unaffected. No significant difference in pAkt or total Akt levels was observed between glucosamine (black bars, n=7) or mannitol (white bars, n=4) treated rats receiving 3$^{rd}$ ventricle aCSF injection. (B) Insulin tolerance test (ITT) after 3 weeks of glucosamine (closed circles, n=8) or mannitol (open circles, n=8) infusion. Animals were injected intraperitoneally with 0.6 U/kg of insulin, and glucose was measured immediately before injection and at 15 min intervals. (D and E) There was no significant difference in NPY or POMC expression between glucosamine (black bar, n=9) and mannitol (white bar, n=8) treated rats in either the arcuate nucleus (D) or in the ventromedial hypothalamus (E), * $P < 0.05$ compared to mannitol + ICV insulin; $^{\#}P < 0.05$ vs. mannitol by ANOVA.

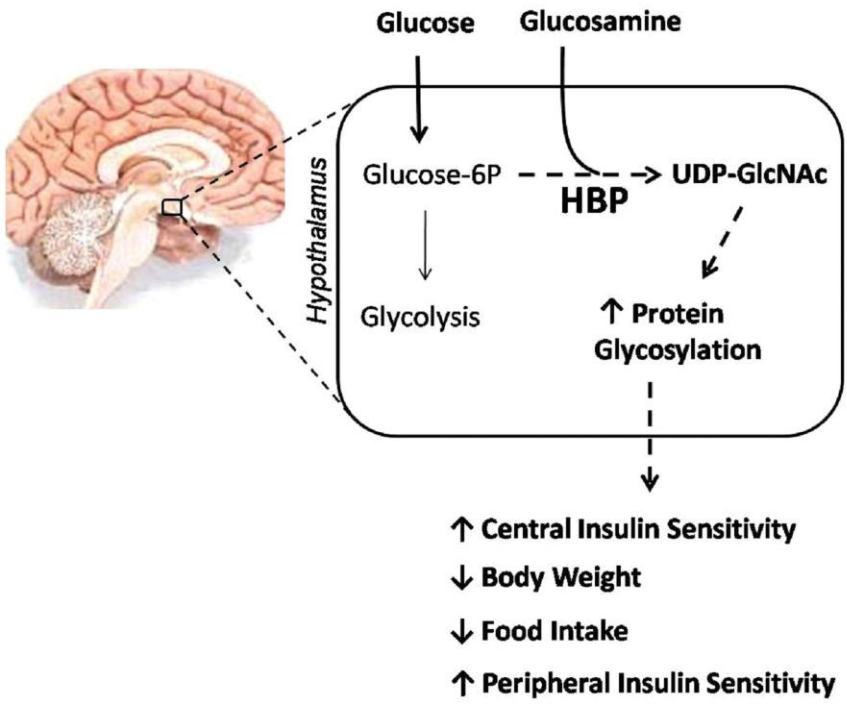


**Figure 18. Model of hypothalamic glucose and glucosamine infusion effects on whole body metabolism.** As glucose enters this cell in the hypothalamus, 2-5% of glucose flux enters the hexosamine biosynthetic pathway (HBP), ultimately leading to increased levels of uridyl diphosphate-*N*-acetylglucosamine (UDP-GlcNAc). Glucosamine directly enters the HBP and also increases UDP-GlcNAc levels. UDP-GlcNAc is then attached to proteins, increasing overall protein glycosylation. In this study, increased hypothalamic protein glycosylation led to increased hypothalamic insulin sensitivity, decreased food intake, decreased body weight, and enhanced peripheral insulin sensitivity.

## DISCUSSION

Hyperglycemia and increased flux through the hexosamine biosynthetic pathway induces insulin resistance in adipose tissue and skeletal muscle. In these studies, we tested the hypothesis that chronic infusion of glucose or glucosamine, a molecule that directly enters the HBP, into the hypothalamus would similarly induce central insulin resistance leading to obesity and hyperphagia. Contrary to our original hypothesis, hypothalamic glucose and glucosamine infusion reduced body weight while glucosamine infusion also reduced food intake and enhanced both central and peripheral insulin sensitivities. Our findings suggest a novel central nutrient sensing mechanism in which sugar flux through the hexosamine biosynthetic pathway can modulate food intake and body weight.

Contrary to our initial hypothesis, central glucose infusion resulted in rats weighing less than controls. Surprisingly, food intake was not altered despite a reduction in body weight. However, several studies have observed that changes in central nervous system (specifically within the hypothalamus) can result in alterations to whole body metabolism and body weight that may be independent of food intake (116-118). In a similar fashion, chronic glucose infusion into the mediobasal hypothalamus affected body weight without altering food intake.

One possible mechanism in which hypothalamic glucose infusion modulated body weight is increased flux through the hexosamine biosynthetic pathway (HBP). To determine whether the HBP in the hypothalamus could modulate whole body energy homeostasis, glucosamine, a molecule that directly enters the HBP pathway (Figure 18), was continuously infused into the mediobasal hypothalamus for three weeks. Interestingly, and consistent with the chronic high glucose infusion study, hypothalamic

glucosamine infusion also resulted in a reduction in body weight gain of a similar magnitude. Hypothalamic glucosamine infusion reduced food intake and reduced body weight as compared to control rats. Consistent with the reduced body weight, plasma leptin levels tended to be lower in glucosamine infused animals, although not statistically significant between groups. Systemic glucose and insulin levels were not different between treatment groups although the insulin tolerance test did demonstrate that the glucosamine treated rats were more insulin sensitive that controls (Figure 17C). Presumably, the reduced body weight in the glucosamine infused animals (Figure 15) accounted for the improvement in systemic insulin sensitivity, but it is possible that the enhanced hypothalamic insulin sensitivity (Figure 17A) may have directly contributed to the enhance peripheral insulin sensitivity (119).

Although antithetical to our tested hypothesis, these initially paradoxical findings support the notion that increased flux through the HBP acts as a nutrient sensor (41;111;112). Enhanced flux into the HBP is thought to signal nutrient excess (41;111;112). In peripheral tissues, increased HBP flux may lead to insulin resistance and thus limiting substrates (i.e. glucose) from entering the cell (41;112). In the same regard, increased metabolism through the HBP in the hypothalamus could signal nutrient surfeit and would lead to a suppression of food intake and, consequently, body weight. Indeed, previous studies have demonstrated that glucosamine can increase expression of satiety signals. Incubation of adipocytes with glucosamine resulted in increased expression of the anorexigenic hormone leptin (120). Further, the significant inverse correlation between the extent of hypothalamic protein glycosylation and body weight supports the evidence that increased HBP flux acts as a nutrient sensor. Indeed,

consistent with these findings are the observations that the degree of brain protein glycosylation changes with physiological changes in nutritional status, (e.g., fasting results in decreased brain glycosylation) (120;121). These data support the notion that protein glycosylation within the brain acts as a nutrient sensor, or acts to modify nutrient sensing in the hypothalamus, and thus modulates overall energy homeostasis.

Studies in pancreatic β-cells further support the concept that protein glycosylation via the HBP acts as a nutrient sensor. Increased protein glycosylation by transgenic overexpression of OGT leads to enhanced glucose-stimulated insulin secretion (46). Reduction of protein glycosylation by pharmacological inhibition of glutamine:fructose-6-phosphate amidotransferase (GFAT) or targeted disruption of OGT by siRNA resulted in reduced glucose-stimulated insulin secretion in isolated pancreatic β-cells (122;123). Thus, the HBP and protein glycosylation in the β-cell modulates glucose sensing and insulin secretion.

To determine whether glucosamine can acutely affect food intake, glucosamine or the osmotic control mannitol was infused into the third ventricle of 24 hour fasted rats. Glucosamine or mannitol was infused one hour prior to the onset of the dark cycle. Food was then provided at the onset of the dark cycle, and food intake was measured at 2 and 4 hours afterwards. No difference in food intake was observed between groups. This result is not surprising as many of the enzymes important in glycosylation, such as OGT, are highly expressed in the nucleus (40;41). Thus many of the effects of hypothalamic glucosamine infusion on whole body energy homeostasis may first depend on changes in gene expression and subsequent translation to protein synthesis instead of acutely modulating phosphorylation and signaling of various proteins and enzymes. This data is

consistent with the observation that a difference in body weight and food intake in glucosamine infused rats was delayed and not observed until the second week of hypothalamic infusions (Figure 15). Importantly, these results demonstrate that glucosamine does not act as a noxious stimulus to adversely affect short term food intake.

A possible mechanism for glucosamine's anorexigenic effects was its effect on central insulin signaling. Mediobasal hypothalamic infusion of glucosamine enhanced hypothalamic insulin sensitivity. Insulin has anorexigenic effects centrally (124-126), and the reduced food intake and body weight could be attributed to increased central insulin sensitivity. Intriguingly, expression of neuropeptide Y (NPY) and proopiomelanocortin (POMC), the major hypothalamic orexigenic and anorexigenic neuropeptides (115), were not affect by glucosamine infusion. Although the anorexigenic effect of hypothalamic insulin signal is thought to be mediated by reciprocal changes in hypothalamic NPY and POMC expression (127;128), the absence in changes in neuropeptide expression in these experiments point to the intriguing possibility that alternative mechanisms exist by which enhanced flux through HBP and/or enhanced insulin signaling act to reduce food intake and body weight

These data provide evidence of a novel nutrient sensing system in the hypothalamus. Enhanced metabolism through the HBP may increase O-GlcNAc, which in turn, leads to an activation of satiety signals (Figure 18). O-GlcNAc can interact with several signaling molecules and modulate gene transcription (40;111), though the exact targets of O-GlcNAc that lead to attenuation of food intake and body weight are still unknown and are currently being investigated. The HBP may become a potential

therapeutic target to modulate food intake and body weight and, hence, combat the ever-growing epidemic of obesity and diabetes.

# CHAPTER 3. RECURRENT, MODERATE HYPOGLYCEMIA AMELIORATES BRAIN DAMAGE AND COGNITIVE DYSFUNCTION INDUCED BY SEVERE HYPOGLYCEMIA

**ABSTRACT**

Although intensive glycemic control achieved with insulin therapy increases the incidence of both moderate and severe hypoglycemia, the extent to which severe hypoglycemia causes brain damage and cognitive impairments continues to be unresolved. It was hypothesized that recurrent moderate hypoglycemia might "precondition" the brain and protect the brain against damage caused by severe hypoglycemia. To test this hypothesis, nine-week old male Sprague-Dawley rats were subjected to either three consecutive days of recurrent, moderate (25-40 mg/dl) hypoglycemia (RH) or saline injections. On the fourth day, rats were subjected to a hyperinsulinemic (0.2 U/kg/min) severe hypoglycemic (~11 mg/dl) clamp for 60 or 90 minutes. Neuronal damage was subsequently assessed by H&E and Fluoro-Jade B staining. The functional significance of severe hypoglycemia induced brain damage was evaluated by motor and cognitive testing. The results demonstrated that following severe hypoglycemia, RH pretreated rats had 62-74% less brain cell death and significantly reduced deficits in cognitive performance. In summary, antecedent recurrent moderate hypoglycemia "preconditioned" the brain and markedly limited both the extent of severe hypoglycemia induced neuronal damage and cognitive impairment. In conclusion, changes brought about by recurrent moderate hypoglycemia can be viewed, paradoxically, as providing a beneficial adaptive response in that there is mitigation against severe hypoglycemia induced brain damage and cognitive dysfunction.

## INTRODUCTION

Hypoglycemia is the major obstacle in achieving tight glycemic control in people with diabetes (129). Intensive insulin therapy increases the risk of iatrogenic hypoglycemia (49). Episodes of both moderate and severe hypoglycemia have long-term clinical consequences. Recurrent moderate hypoglycemia induces a maladaptive response that limits symptoms of hypoglycemia (hypoglycemia unawareness) and limits the counterregulatory response to subsequent hypoglycemia (hypoglycemia associated autonomic failure – HAAF) and thus jeopardizes patient safety (82;129). By depriving the brain of glucose, more severe hypoglycemia has been shown to cause brain damage in animal studies. Severe hypoglycemia induced damage, especially in the hippocampus, leads to long-term impairments in learning and memory (54;130). However, studies examining the effect of severe hypoglycemia in humans have been conflicting. Severe hypoglycemia alters brain structure (64;131) and causes significant cognitive damage in many (56-66) but not all (67-72) studies. Reasons for the discrepancy between human and animal studies are unknown but a major contributing factor may be the extent of glycemic control (including recurrent hypoglycemia) prior to the episode of severe hypoglycemia.

In other models of brain damage, such as a stroke, it has been shown that brief, mild episodes of antecedent brain ischemia causes a beneficial adaptation that serves to protect the brain against a subsequent episode of more severe ischemia (a phenomena known as ischemic pre-conditioning) (76). In a similar fashion, it was hypothesized that antecedent, recurrent episodes of moderate hypoglycemia could protect the brain against damage caused by a subsequent episode of severe hypoglycemia.

To investigate this hypothesis, recurrent moderately hypoglycemic (25-40 mg/dl) rats and recurrent saline injected rats were subjected to hyperinsulinemic, severe hypoglycemic clamps (10-15 mg/dl). The rats were either sacrificed one week after the severe hypoglycemia to quantify brain damage or were evaluated by behavioral and cognitive tests 6-8 weeks after the episode of severe hypoglycemia. The results indicate that recurrent antecedent moderate hypoglycemia acts to "precondition" the brain and protect it against neurological damage and cognitive defects induced by a single episode of severe hypoglycemia.

## RESEARCH DESIGN AND METHODS

**Animals.** Nine week old male Sprague-Dawley rats (Charles River Laboratories) were individually housed in a temperature and light controlled environment maintaining the animal's diurnal cycle (12hrs light, 12hrs dark) and with an ad lib standard rat chow diet. All studies were done in accordance with the Animal Studies Committee at the Washington University School of Medicine.

**Implantation of arterial and venous catheters.** Rats were anesthetized with an intraperitoneal injection of Ketamine 87 mg/kg and Xylazine 2.6 mg/kg. A micro-renathane® (Braintree Scientific, Boston, MA) catheter was inserted into the left carotid artery and two catheters were implanted into the right jugular vein. To maintain patency, catheters were filled with a 40% polyvinylpyrrolidone (Sigma) in heparin (1000 USP U/ml) solution (Baxter Healthcare Corporation, Deerfield, IL).

**Recurrent Moderate Hypoglycemia**. One week after catheter implantation, non-fasted rats were injected with subcutaneous regular human insulin (RH) [6 U/kg on day 1; 5 U/kg on day 2; and 4 U/kg on day 3] (Lilly, Indianapolis, IN) while the control group (CON) were given injections of equal volume saline for three consecutive days. Food was withheld and tail vein blood samples were obtained hourly in both treatment groups. For insulin treated rats, blood glucose ranged between 25-40 mg/dl for three hours. Hypoglycemia was terminated after three hours by a subcutaneous injection of dextrose (Hospira, Lake Forest, IL), and the rats were then allowed free access to food.

**Hyperinsulinemic-Severe Hypoglycemia Clamp**. Animals were fasted overnight after the third day of injections. The following morning, vascular catheters were externalized for the hyperinsulinemic (0.2 U/kg/min) severe hypoglycemic clamp.

Rats were awake, unrestrained, and had free access to water. Arterial blood samples were obtained every 15 minutes to measure blood glucose using Ascensia Contour blood glucose monitors (Bayer HealthCare, LLC, Mishawaka, IN). Follow basal sampling, insulin was infused intravenously and glucose was co-infused. The target for severe hypoglycemia was glucose levels of 10-15 mg/dl, as this level of hypoglycemia was necessary to induce neuronal damage (130;132). Once the blood glucose dropped to below 15 mg/dl, the clock was re-set and severe hypoglycemia was maintained at 10-15 mg/dl by adjusting the rate of continuous intravenous glucose infusion. This technique allowed both groups to be precisely matched for duration and depth of hypoglycemia in unrestrained animals, without the confounding effects of anesthesia. Blood glucose was maintained below 15 mg/dl for either 60 minutes (CON-SH60, n=6; RH-SH60, n=10) or 90 minutes (CON-SH90, n=20; RH-SH90, n=18) (Figure 2A and 3A). To terminate hypoglycemia, insulin infusion was ceased and animals were given infusions of dextrose until they could maintain normal blood glucose values without dextrose infusion. Blood samples were taken during the basal period and 30 min into the severe hypoglycemia for epinephrine measurements, as determined by a single isotope derivative (radioenzymatic) method (97).

Episodes of seizure-like behavior were noted during the severe hypoglycemic clamps. Tonic-clonic seizure-like behavior was visually noted by characteristic brief (5-10 seconds) of neck extensions, tonic stretching, uncontrolled limb movements, and spontaneous spinning (132;133). The number of episodes of seizure-like behavior was quantified for each rat during the clamp period and was later correlated with histological and behavioral findings.

To provide additional groups of experimental control rats not exposed to severe hypoglycemia, two more groups of rats were made either recurrently hypoglycemic or given saline injections, as described above, and on the fourth day, instead of a severe hypoglycemic clamp, the rats underwent a 90 minute hyperinsulinemic (0.2 U/kg/min) euglycemic clamp with blood glucose maintained above 70 mg/dl (CON-EUG, n=9; RH-EUG, n=11). These two groups served as control rats treated in the same fashion except that they were not exposed to severe hypoglycemia.

Rats exposed to severe hypoglycemia and euglycemic controls were subsequently analyzed either for brain damage or subjected to tests of sensorimotor and cognitive function (Figure 19).

**Histology**. Rats exposed to severe hypoglycemia and euglycemic controls were anesthetized with isofluorane one week after their clamp and intracardially 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. Beginning at 2.8 mm posterior to the bregma, coronal cryostat sections (20 μm) were collected and mounted on superfrost coated slides (VWR, West Chester, PA). Four coronal sections, 120 μm apart, were then analyzed for neuronal damage by Fluoro-Jade B (Chemicon International, Inc., CA) and hematoxylin and eosin (H&E, Sigma, St. Louis, MO) staining, according to manufacturer's protocol. Fluoro-Jade B (FJB) is a well characterized stain for degenerating neurons (134). The number of fluorescent cells (Fluoro-Jade positive cells) was counted in both hemispheres of the cortex and of the hippocampal structures, CA1

and dentate gyrus. For each region of interest, data is expressed as the average number of Fluoro-Jade B positive (FJB+) cells per section. (CON-SH90, n=9; RH-SH90, n=8).

**Behavioral Testing.** Rats were given 6-8 weeks to recover from the hyperinsulinemic hypoglycemic (CON-SH90, n=11; RH-SH90, 9) and hyperinsulinemic euglycemic clamp (CON-EUG, n=7; RH-EUG, n=9), at which time they were moved to the behavioral testing facility and allowed one week to acclimate to the new environment. To determine if severe hypoglycemia produced long-term deficits in general activity level or sensorimotor function, (which could have confounded interpretation of cognitive test performance) the rats were evaluated on a 1-h locomotor activity test and on a battery of sensorimotor measures, as described below. Following these assessments, they were tested on the Morris water maze task to determine their spatial learning and memory capabilities.

***1-h Locomotor activity test and sensorimotor battery.*** General locomotor activity and exploratory behavior were evaluated over a 1-hr period using a computerized system (MotorMonitor, Kinder Scientific, LLC, Poway, CA) of photobeam pairs to separately quantify ambulations (whole body movements) and rearing frequency. To determine if severe hypoglycemia produced sensorimotor disturbances, a set of tests was conducted to measure balance, strength, coordination and initiation of movement using the ledge, platform, $90^{o}$ inclined screen, and walking initiation tests, as previously described (135).

***Water maze cognitive testing.*** Spatial learning and memory were assessed using the Morris water maze test utilizing general procedures similar to previously-published methods (135). A computerized tracking program (Polytrack, San Diego Instruments, San Diego, CA) was used to record the swim path lengths and time required to find the

platform. The cued condition involved conducting two sessions of three trials each (60-s maximum/trial) per day for two consecutive days where the rats were trained to swim to the submerged platform (1.5 cm below the surface) marked (cued) by a visible pole with 3 h intervening between sessions. The data were analyzed in blocks of three trials each for a total of four blocks. Three days later, the spatial learning capabilities of the rats were tested using the place condition in the water maze. This is a reference-memory based task where the rats were trained to learn the position of a submerged and non-visible (since pole is removed) platform which remained in the same location across all trials. The place trials protocol involved conducting two sessions of 3 trials each (60-s maximum) per day for five consecutive days using a 3-h interval between sessions. The data were analyzed in blocks of six trials each (two daily sessions) for a total of 5 blocks. On day 5, a probe trial was conducted 1 h after the last place trial which involved removing the platform from the pool and quantifying rats' search behaviors in the four pool quadrants for 30 s to evaluate retention of the platform location. Probe trial performance variables included: the number of times a rat passed directly over the platform location (platform crossings); the time spent in the target quadrant versus the time spent in each of the other pool quadrants (spatial bias), and average proximity (distance to the platform location sampled and averaged across 1-s epochs throughout the trial).

**Statistical Analysis**. All data are expressed as mean ± SEM, and were by either Student t-tests or analysis of variance (ANOVA) models. Quantification of brain damage and behavioral assessments were made by investigators blinded to treatment conditions. The behavioral data were analyzed by a two way ANOVA assessing variables of group

(severe hypoglycemia vs euglycemia) and treatment (recurrent hypoglycemia or saline injections). Where appropriate, ANOVA models also included one within-subjects (repeated measures) variable such as Blocks of Trials, and Huynh-Feldt correction was used for all within-subjects effects containing more than two levels. When multiple comparisons were conducted, Bonferroni correction was used to help maintain prescribed alpha levels (e.g., 0.05).

## RESULTS

**Recurrent hypoglycemia reduced cortical brain damage induced by 60 min of severe hypoglycemia.** To examine whether prior episodes of moderate hypoglycemia reduce susceptibility to severe hypoglycemia-induced brain damage, rats were treated with three days of insulin injections to induce moderate hypoglycemia (RH) or given control saline (CON) injections. On the fourth day, severe hypoglycemia was induced in awake, unrestrained animals in both treatment groups using a hyperinsulinemic severe hypoglycemic clamp. To verify that the recurrent moderate hypoglycemia recapitulated the syndrome of hypoglycemia associated autonomic failure (HAAF), epinephrine levels were measured before and during the hypoglycemic clamp. As expected, recurrently hypoglycemic rats exposed to 60 minute of severe hypoglycemia (RH-SH60) had a significantly impaired epinephrine response compared to control saline injected rats exposed to 60 minutes of severe hypoglycemia (CON-SH60) (2001$\pm$241 and 3487 $\pm$474 pg/ml, $p < 0.01$) (Figure 20B).

No significant difference in blood glucose was observed before, during, or after the 60 min severe hypoglycemic clamps between RH and CON rats (Figure 20A). During the 60 min of severe hypoglycemia, blood glucose was precisely maintained between 10-15 mg/dl for 60 min (RH-SH60:12.7 $\pm$ 0.9 and CON-SH60: 12.6 $\pm$ 0.5 mg/dl, p=NS). One hour after the clamp, blood glucose was not significantly different between groups (RH-SH60: 81 $\pm$19 and CON-SH60: 90 $\pm$15 mg/dl, p=NS) (Figure 20A).

Recurrent hypoglycemic treated rats had 64% less neuronal damage, as assessed by the number of Fluoro-Jade B positive (FJB+) cells, in the cortex than controls (173 $\pm$ 64 vs. 479 $\pm$ 170 cells, $p<0.05$) (Figure 20C and Figure 20D). It was noted that 60 min of

severe hypoglycemia did not induce significant damage in the hippocampus in either RH-SH60 or CON-SH60.

**Recurrent hypoglycemia attenuated cortical and hippocampal brain injury after 90 minutes of severe hypoglycemia.** To more consistently induce hypoglycemic brain damage in the hippocampus, the above experiments were repeated except that the duration of severe hypoglycemia was extended to 90 min. The average blood glucose during 90 min of severe hypoglycemia was 10.9±0.2 versus 11.0±0.3 mg/dl in the saline injected (CON-SH90) and recurrently hypoglycemic (RH-SH 90) rats, respectively (p=NS) (Figure 21A). As an additional set of experimental controls, euglycemic hyperinsulinemic clamps were also performed in recurrently hypoglycemic (RH-EUG) or saline injected control (CON-EUG) rats. Notably, in these euglycemic control groups, blood glucose was maintained on average at 76±5 and 84±6 mg/dl in the CON-EUG and RH-EUG, respectively (p=NS) (Figure 21A).

Again validating the model of HAAF, recurrent hypoglycemia resulted in the expected impairment of the epinephrine response to hypoglycemia (CON-SH90: 3175±516 and RH-SH90: 2077±426 pg/ml, $p<0.05$) (Figure 21B). Severe hypoglycemia of 90 min induced significant cellular damage in the cortex, as evidenced by the presence of pyknotic cells observed with H&E staining (Figure 22A) and the marked number of fluorescent cells with Fluoro-Jade B staining (Figure 22B). Interestingly, 90 min of severe hypoglycemia induced 6-fold greater cortical neuronal damage than with 60 min of severe hypoglycemia (Figure 23A and Figure 20D). Recurrent antecedent moderate hypoglycemia decreased cortical brain damage induced by 90 min of severe

hypoglycemia by 62% (RH-SH90: 1107 ± 428 and CON-SH90: 2918 ± 615 FJB+ cells, $p<0.05$). Unlike hypoglycemia of shorter duration, 90 min of severe hypoglycemia also induced hippocampal brain damage (Figure 22 and Figure 23). Recurrent antecedent hypoglycemia resulted in less hippocampal brain damage following 90 min of severe hypoglycemia compared to CON-SH90 (Figure 22 and Figure 23). Specifically, RH-SH90 had decreased FJB+ cells in the CA1 region by 74% (RH-SH90: 88±56 vs. CON-SH90: 334±91 cell, $p<0.05$) and by 67% in the dentate gyrus (RH-SH90:274±119 vs. CON-SH90: 833 ± 148, $p<0.05$) compared to CON-SH90 (Figure 23A).

Interestingly, recurrent hypoglycemia also reduced the episodes of seizure-like behavior observed during severe hypoglycemia (RH-SH90: 2.0±0.3 vs. CON-SH90: 3.4±0.3, $p<0.01$) (Figure 23B). There was a significant correlation between the number of episodes of seizure-like behavior and number of FJB+ cells ($R=0.572$, $p< 0.05$) (Figure 23C).

In the absence of severe hypoglycemia, virtually no Fluoro-Jade positive cells (Fluoro-Jade B staining) nor pyknotic cells (H&E) were observed in the cortex and hippocampus of either the euglycemic CON-EUG or RH-EUG group (Figure 22 and Figure 23). The lack of brain damaged cells indicated that the catheter implantation, recurrent moderate hypoglycemia, hyperinsulinemic clamp or glucose infusion *per se* does not cause significant brain damage.

**Preserved cognitive function in recurrently hypoglycemic rats.**

Based on the extensive severe hypoglycemia induced damage noted in the cortex, including the sensorimotor cortex, locomotor activity and sensorimotor tests were

performed on the rats to evaluate whether severe hypoglycemia induced any detectable motor or sensorimotor deficits. It was also important to assess the possible presence of sensorimotor deficits which could impede physical performance during the Morris maze testing and affect interpretation of spatial learning and memory results. General activity, as measured by total ambulations, was not found to differ among the groups in terms of either total activity summed over the entire session, the response to novelty of the test field (i.e. block 1), or in the habituation of activity over time (Figure 24A). Two way ANOVA indicated that as a group, the severe hypoglycemic rats (both CON-SH90 and RH-SH90) exhibited significantly ($p=0.02$) more rearing than the two groups of EUG rats (Figure 24B). Data from the walking initiation, ledge, platform, and $90^{o}$ incline tests were not significantly different between groups (Figure 24C-F).

During the cue (Figure 25A) and place (Figure 25C) trials, the CON-SH90 rats performed very poorly compared to the other three groups in spite of having normal swimming speeds (Figure 25B, D). The CON-SH90 group had significantly longer path lengths across the blocks of trials compared to the CON-EUG group ($P=0.0002$) documenting impaired performance as a result of the severe hypoglycemia. Other important comparisons showed that the RH-SH90 group had significantly shorter path lengths relative to the CON-SH90 group ($P=0.0025$), while no differences were observed between the RH-SH90 versus the CON-EUG group nor between the two EUG control groups. Analysis of the escape latency data (not shown) yielded essentially the same results.

During the place (spatial learning) trials, the CON-SH90 rats again showed significant performance deficits, with the degree of impairment appearing to be even

greater than that observed during the cued condition. Comparisons showed that the CON-SH90 group had significantly (P=0.0001) longer path lengths across the blocks of trials compared to the CON-EUG rats (Figure 25C). Importantly, in rats that experienced severe hypoglycemia, prior recurrent moderate hypoglycemia improved performance as evidenced by significantly ($p = 0.0006$) shorter path lengths in the RH-SH90 versus the CON-SH90 group (Figure 25C). Again, no differences were observed between the two euglycemic control groups. The same pattern of results was found for the escape latency (data not shown).

The results of the probe trial (Figure 26A-C) mirrored the results obtained during the cue and place trials showing impaired memory retention due to severe hypoglycemia and a "rescue" of this deficit with antecedent recurrent moderate hypoglycemia. During the probe trial, CON-SH90 rats made significantly fewer platform crossings relative to the CON-EUG controls (P=0.014) (Figure 26A). There were, however, no differences in platform crossings between the CON-SH90 and RH-SH90 rats. With regard to spatial bias and average proximity to previous platform location, CON-SH90 did show an impaired performance and RH-SH90 demonstrated a completely normal performance. In spatial bias analysis, pool quadrants times showed that the RH-SH90, CON-EUG, and RH-EUG groups all exhibited a spatial bias for the target quadrant whereby each group spent significantly more time in the target quadrant compared to the times spent in each of the other pool quadrants ($P < 0.0025$). Unlike the other groups, the CON-SH90 rats did not show a significant spatial bias for the target quadrant using these criteria (Figure 26B). The examination of average proximity to previous platform location, CON-SH90 group demonstrated an impaired performance with a significantly higher average

proximity scores compared to the CON-EUG controls (P = 0.014) (Figure 26C). Importantly, RH-SH90 group had significantly lower average proximity scores compared to the CON-SH90 group (p = 0.014) and performed similarly relative to the CON-EUG controls. In summary, during the probe trial, severe hypoglycemia (CON-SH90) significantly impaired all three tests of memory retention, and antecedent recurrent moderate hypoglycemia pretreatment (RH-SH90) significantly improved memory testing (indeed, completely prevented the severe hypoglycemia induced memory deficit) in 2 out of 3 tests.

Interestingly, the number of episodes of seizure-like behavior during severe hypoglycemia were positively correlated with place trial performance during Morris water maze testing (Figure 26D). Specifically, increases in the number of episodes of seizure-like behavior were associated with longer average path lengths during the place trials ($R=0.685$, $p<0.001$) (Figure 26D).

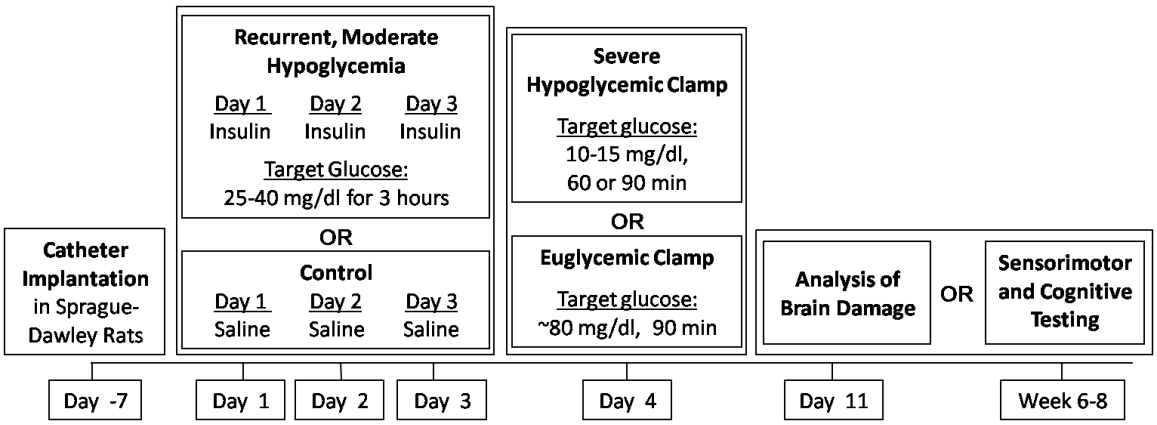


**Figure 19. Experimental Protocol for induction of recurrent moderate hypoglycemia, induction of severe hypoglycemia, and behavioral testing.** Arterial and venous catheters were implanted into 9 week-old Sprague Dawley rats. After one week of recovery, animals were either given an insulin injection for three consecutive days to induce moderate hypoglycemia (25-40 mg/dl) for three hours per day or they were given saline injections as a control. On the fourth day, rats underwent a severe hypoglycemic (10-15 mg/dl) hyperinsulinemic (0.2 U/kg/min) clamp for either 60 or 90 min, or alternatively, underwent a 90 min euglycemic (~80mg/dl) hyperinsulinemic (0.2 U/kg/min) clamp. Animals were either sacrificed one week later to assess neuronal damage by H&E and Fluoro-Jade B staining, or animals underwent sensorimotor and cognitive testing 6-8 weeks following the clamp.

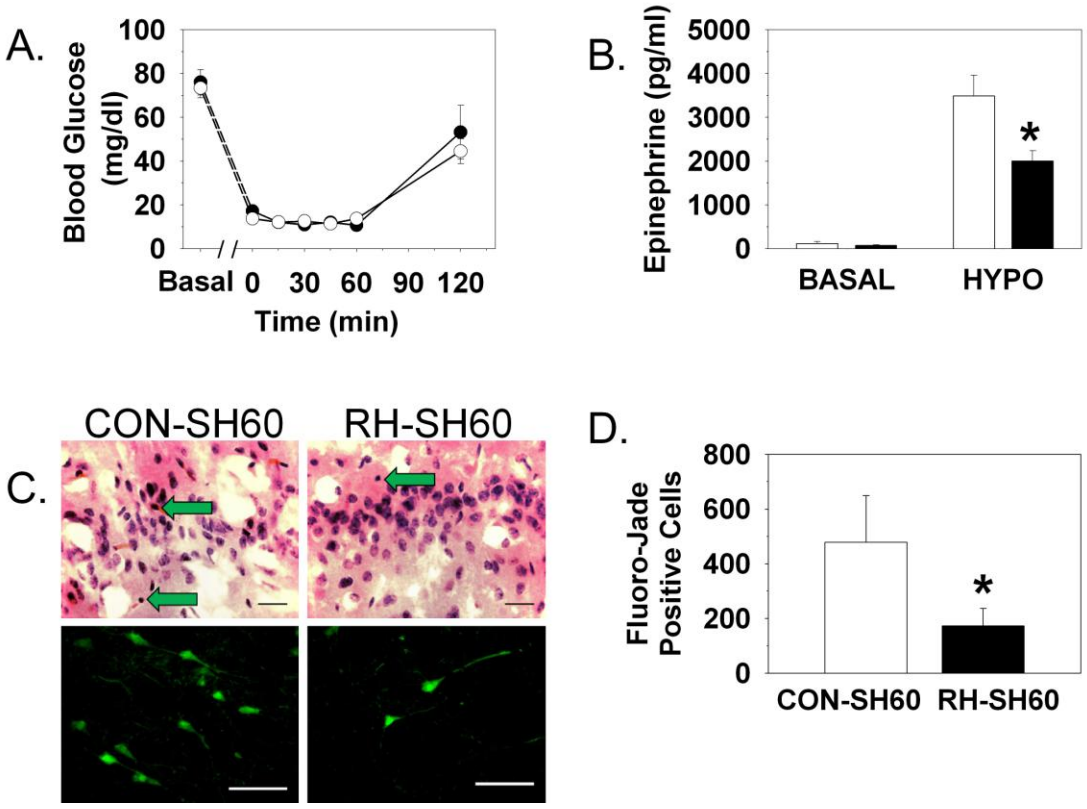


**Figure 20. Recurrent Hypoglycemia attenuates brain damage after 60 minutes of severe hypoglycemia.** (A) Blood glucose levels in rats subjected to a 60 minute severe hypoglycemic (SH) (10-15 mg/dl) hyperinsulinemic (0.2 U/kg/min) clamp. Blood glucose was not significantly different between saline-treated (CON-SH60, ○, n=6) and recurrently hypoglycemic rats (RH-SH60, ●, n=10) during 60 minutes of severe hypoglycemia. (B) Epinephrine levels were measured before the onset of severe hypoglycemia (basal) and 30 min into hypoglycemia. No difference was observed during the basal period, and as expected, RH-SH60 rats (black bar) had an attenuated epinephrine response to hypoglycemia compared to CON-SH60 rats (white bar) (* $p < 0.05$, by Student t-test). (C) Representative hematoxylin and eosin (H&E) and Fluoro-Jade B positive staining in the cortex of saline-treated (CON-SH60) and recurrently hypoglycemic (RH-SH60) rats one week following 60 min of severe hypoglycemia. Neuronal damage is indicated by pyknotic cells (H&E staining, green arrows) or with Fluoro-Jade B positive cells (green fluorescence). Scale bar = 100 μm (D) Quantification of Fluoro-Jade B staining in CON-SH60 (white bar, n=6) and in RH-SH60 (black bar, n=10). Following severe hypoglycemia, RH rats had significantly less degenerating cells in the cortex compared to CON rats (* $p < 0.05$, by Student t-test).

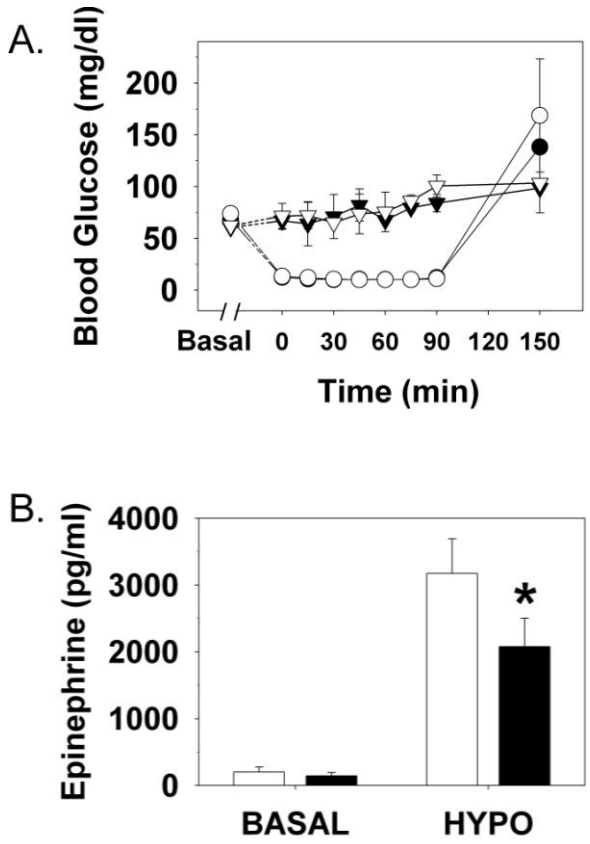


**Figure 21. Blood glucose and epinephrine response during 90 min of severe hypoglycemia.** (A) Blood glucose was not significantly different between saline-treated (CON-SH90, ○, n=9) and recurrently hypoglycemic rats (RH-SH90, ●, n=8) during 90 minutes of severe hypoglycemia. Hyperinsulinemic euglycemic (~80 mg/dl) clamps were also performed in saline treated (CON-EUG, ▽, n=9) and in recurrently hypoglycemic rats (RH-EUG, ▼, n=11). Blood glucoses were not significantly different between euglycemic groups. (B) Epinephrine levels were measured before the onset of severe hypoglycemia (basal) and 30 min into hypoglycemia. No difference was observed during the basal period, but RH-SH90 rats (black bar) had an attenuated epinephrine response to hypoglycemia compared to CON-SH90 (white bar) (* $p<0.05$).

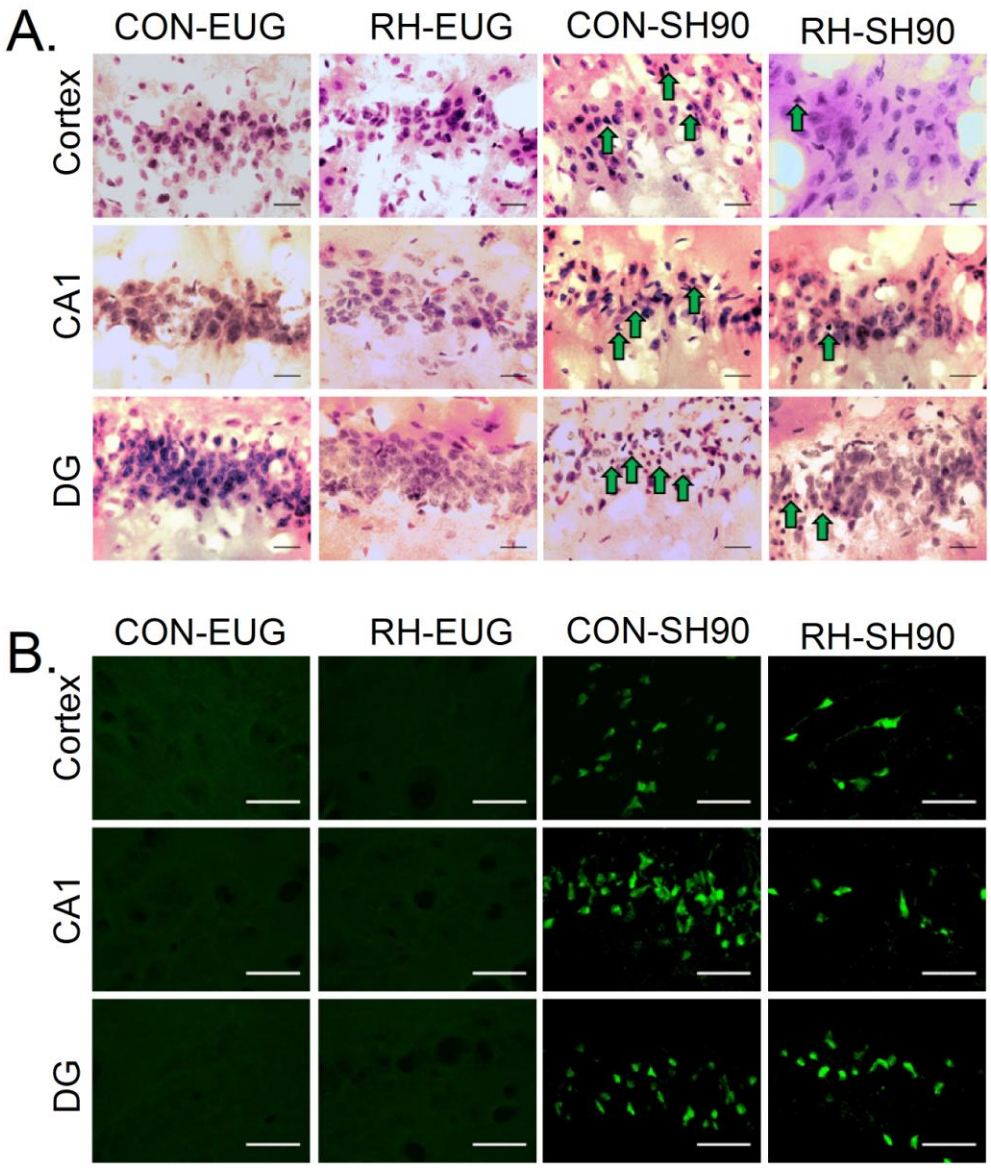


**Figure 22. Recurrent hypoglycemia limits brain cell death one week following 90 min of severe hypoglycemia.** (A) Representative hematoxylin and eosin staining of the cortex and hippocampal structures, CA1 and the dentate gyrus (DG), one week following 90 minute severe hypoglycemic clamps or euglycemic clamps in recurrently hypoglycemic (RH-SH90 and RH-EUG, respectively) and saline injected rats (CON-SH90 and CON-EUG). Rats that underwent severe hypoglycemia had damaged neurons characterized by pyknotic nuclei (green arrows) Scale bar = 100 μm (B) Fluoro-Jade B positive cells (FJB+, green fluorescence) in the cortex, hippocampal CA1 region and dentate gyrus (DG). Scale bar = 100 μm

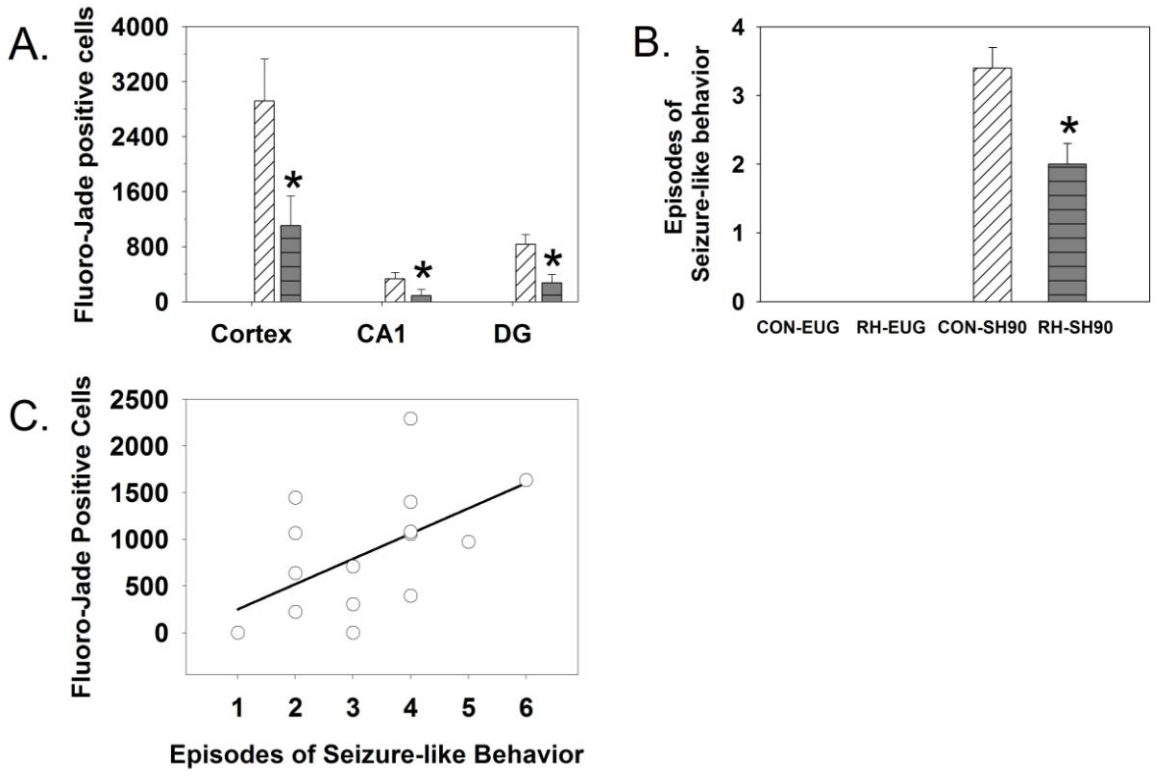


**Figure 23. Quantification of brain cell death following an episode of severe hypoglycemia and correlation to episodes of seizure-like behavior**. (A) Following 90 minutes of severe hypoglycemia, the markedly increased number of FJB+ cells in the cortex, CA1, and dentate gyrus observed in the CON-SH90 (▨) was significantly (*$p<0.05$) reduced by antecedent recurrent moderate hypoglycemia (RH-SH90, ▬). White and black bars representing Fluoro-Jade B in CON-EUG and RH-EUG groups are not visible in this figure as no appreciable brain damage was observed in euglycemic control rats. (B) Euglycemic rats (CON-EUG and RH-EUG) experienced no seizure-like behavior. Rats exposed to 90 min severe hypoglycemia exhibited seizure-like behavior, although RH-SH90 (▬) had significantly less seizure-like behavior than CON-SH90 (▨) ($p<0.01$). (C) In rats that experienced severe hypoglycemia (RH-SH90 and CON-SH90), seizure-like behaviors positively correlated with the amount of Fluoro-Jade B cells in the hippocampus ($R=0.572$, $P<0.05$).

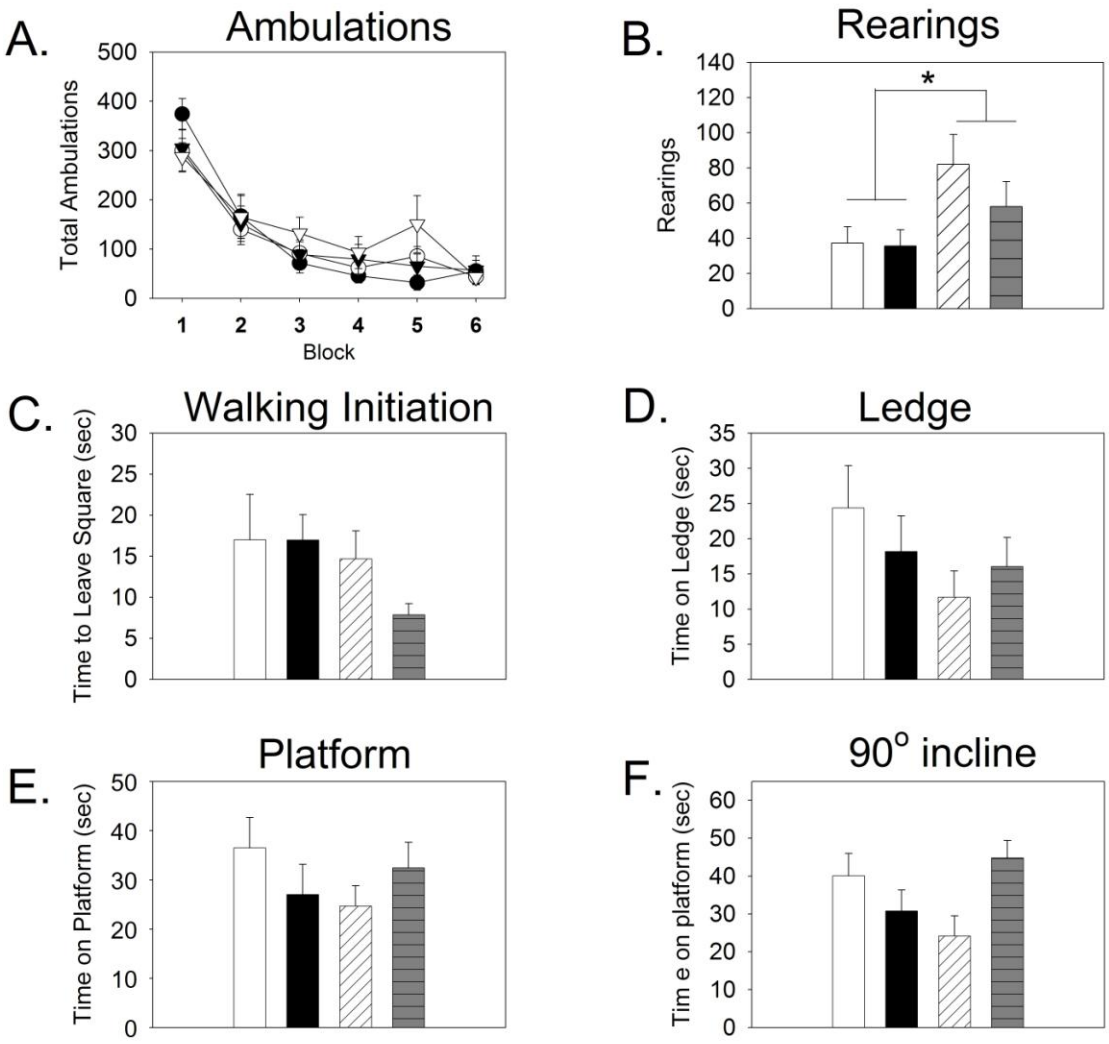


**Figure 24. Locomotor activity and sensorimotor function 6-8 weeks following severe hypoglycemia or euglycemic clamp.** (A) No significant differences were observed between groups in terms of ambulations (CON-EUG, ▽, n=7; RH-EUG, ▼, n=9; CON-SH90, ○, n=11; RH-SH90, ●, n=9). (B) The SH90 groups (CON-SH90, ▨, and RH-SH90, ▬) exhibited significantly ($p = 0.017$) more rearing than the EUG groups (CON-EUG, ▭ and RH-EUG, ▬). Sensorimotor function was also assessed by walking initiation task (C), ability to balance on a ledge (D), remain on a platform (E), or stay on a $90^{o}$ inclined screen (F). Analyses conducted on the data from these sensorimotor tests showed no significant differences between groups tested.

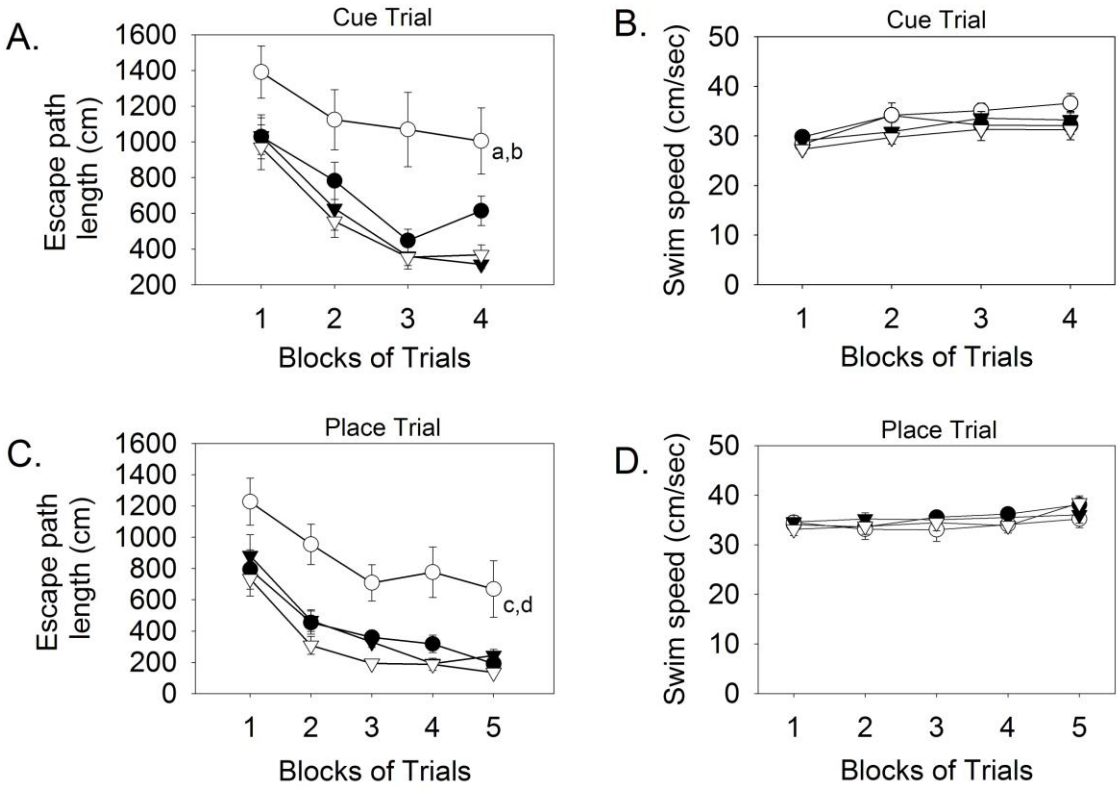


**Figure 25. Antecedent recurrent hypoglycemia mitigated cognitive dysfunction induced by severe hypoglycemia as assessed by the cue and place trials during Morris water maze testing.** Morris water maze testing was performed 6-8 weeks following severe hypoglycemic or euglycemic clamps. (A) During the cue trial, control rats exposed to 90 min of severe hypoglycemia (CON-SH90, ○, n=11) performed worse as evidenced by higher escape path lengths compared to euglycemic control (CON-EUG, ▽, n=7) ([a]p=0.002). Notably, rats exposed to recurrent moderate hypoglycemia before severe hypoglycemia (RH-SH90, ●, n=9) had shorter escape path lengths than CON-SH90 ([b]p=0.0025) and performed similarly to CON-EUG and RH-EUG (▼, n=9). (B) Swim speeds during the cue trials were similar between groups. (C) During the place trials, CON-SH90 had significantly higher escape path lengths compared to CON-EUG ([c]p=0.0001) and RH-SH90 ([d]p=0.0006). (D) In the place trials, swim speeds were similar between groups. [a] P<0.01 vs. CON-EUG, [b] P<0.01 vs. RH-EUG, [c] P <0.01 vs. RH-SH90. All statistical analyses by ANOVA.

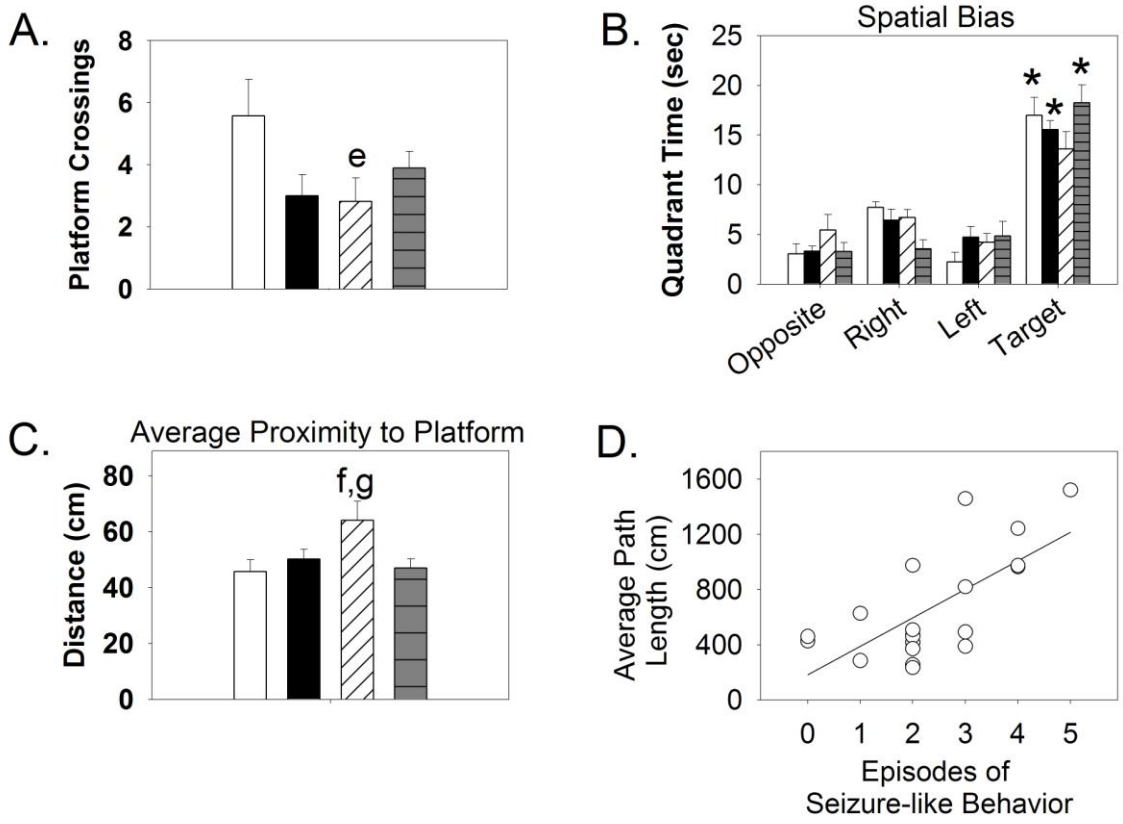


**Figure 26. Antecedent recurrent hypoglycemia limited cognitive impairments induced by severe hypoglycemia as assessed during the probe trials of Morris water maze testing.** (A) CON-SH90 (▨)had significantly less platform crossings than CON-EUG (▭) ($^{e}p=0.014$) . No significant difference was observed between CON-SH90 and RH-SH90 (▬) nor between CON-EUG and RH-EUG (■). (B) RH-SH90, CON-EUG, and RH-EUG had a spatial bias towards the target quadrant while CON-SH90 did not ($^{*}p<0.0025$). (C) During the probe trial, CON-SH90 rats showed an average proximity to the platform location that was significantly farther away than that of the CON-EUG ($^{f}p=0.014$). RH-SH90 rats swam significantly closer to the platform location than CON-SH90 ($^{g}p=0.014$), similar to euglycemic controls. (D) The number episodes of seizure-like behaviors observed during severe hypoglycemia 6-8 weeks prior positively correlated with average path length during the place trials ($R=0.685$, $P<0.001$, $n=20$).

## DISCUSSION

Since severe hypoglycemia affects 40% of insulin treated people with diabetes (136), concern regarding the hazardous potential for severe hypoglycemia to cause "brain damage" continues to be a very real barrier for patients to fully realize the benefits associated with intensive glycemic control (137). An area of intense controversy is the extent to which severe hypoglycemia causes neuronal damage and cognitive dysfunction. Animal models have unambiguously demonstrated that acute severe hypoglycemia (blood glucose <18 mg/dl) induces neuronal damage and subsequent deficits in learning and memory associated with severe hypoglycemia-induced neuronal damage (53;54;130). Due to the retrospective nature of clinical studies investigating severe hypoglycemia induced brain damage, a direct link between severe hypoglycemia and cognitive deficits has been less well established. Many studies have shown that episodes of severe hypoglycemia alters brain structure (64;131) and causes significant cognitive damage (56-66) yet other studies fail to show such an association (67-72). In the real world clinical setting, the extent of antecedent glycemic control (including recurrent hypoglycemia) could be a critical factor in explaining differences between animal and clinical studies and may partially explain the variable results from clinical trials. Patients with the highest incidence of severe hypoglycemia are those who maintain intensive glycemic control, and hence are likely to have had recurrent bouts of moderate hypoglycemia. In this study, it was observed that recurrent moderate hypoglycemia "preconditioned" the brain and protected it against brain damage and cognitive dysfunction induced by a subsequent episode of severe hypoglycemia.

In these experiments, severe hypoglycemic brain injury was consistently induced with hyperinsulinemic hypoglycemic clamps designed to carefully control the depth and duration of severe hypoglycemia. The dose of administered insulin was pharmacological by experimental design and was necessary to overcome the intact counterregulatory response present in non-diabetic rats and maintain glycemia in the 10-15 mg/dl range. The total insulin dose of 20-35 U/kg is consistent with doses used in other hypoglycemic rodent studies (54;138-142). The clamps were performed in awake animals in order to avoid the confounding effects of anesthesia (143-146). Profound neuronal damage was observed in rats that experienced an episode of severe hypoglycemia (blood glucose < 15 mg/dl). Severe hypoglycemia of 60 minute duration resulted in brain damage only in the cortex. 90 minutes of severe hypoglycemia resulted in 6-fold more cortical neuronal damage compared to 60 minutes of severe hypoglycemia. Further, 90 minutes of severe hypoglycemia resulted in significant damage in the hippocampus, an area important for learning and memory. These studies demonstrate that rats exposed to three days of recurrent, moderate hypoglycemia, had less severe hypoglycemic brain injury in both the cortex and hippocampus. Thus, as with ischemic preconditioning (76), hypoglycemic preconditioning attenuated brain damage by 62-74%. Although hypoglycemia-induced neuronal damage in the hypothalamus has been noted (147), other reports did not note damage to the hypothalamus (148), and in this study no hypoglycemia induced neuronal injury to the hypothalamus was observed.

It was important to assess whether the beneficial histopathological findings of reduced neuronal damage in recurrently, moderately hypoglycemic rats were associated with improved behavioral and cognitive performance. Consistent with other protocol

designs (53;54;140), histopathological outcomes were assessed one week following the hypoglycemic neuronal insult and cognitive studies were performed 6-8 weeks later in a separate group of similarly treated rats. This later assessment of cognitive damage has been shown to be a more useful measure of clinical outcome as well as a better functional index of neuroprotection because it allows for a more complete and integrated evaluation of ongoing damage and/or possible recovery (149). As compared to the extensive brain damage in rats exposed to severe hypoglycemia, it was hypothesized that the reduced neuronal damage in recurrently hypoglycemic rats would be associated with improved performance on several behavioral and cognitive indices.

The results of the 1-h locomotor activity test and series of sensorimotor measures suggested that the rats had no meaningful deficit in sensorimotor function resulting from severe hypoglycemia. These results imply that all groups were generally healthy and not suffering from residual malaise associated with the severe hypoglycemia. Interestingly, the rats subjected to the severe hypoglycemia reared significantly more often than the euglycemic controls which likely reflects possible changes in emotionality or information processing rather than sensorimotor disturbances and shows, importantly, that the severe hypoglycemia did not induce gross hindlimb dysfunction. Further, no differences were observed among the four groups in sensorimotor tests (Figure 24) or in swimming speeds during the cued and place trials (Figure 25). In summary, the data from the activity tests, sensorimotor measures, and swim speeds all indicate the rats exposed to the severe hypoglycemia were generally healthy at the beginning of behavioral testing and showed no signs of a sensorimotor impairment which could have affected interpretation of cognitive function testing as measured during in the Morris Water maze.

Cognitive testing results obtained during the water maze testing documented severe acquisition performance deficits in the CON-SH90 rats during the cued and place conditions, and that these impairments were prevented by the antecedent recurrent moderate hypoglycemic treatment. Specifically, analysis of the escape path length and latency data showed that the CON-SH90 rats were significantly impaired relative to the CON-EUG controls during both the cued and place trials, and that the RH-SH90 group performed significantly better than the CON-SH90 rats during both conditions.

There were three tests of memory performance evaluated with the probe trial (Figure 26). With regard to platform crossings, severe hypoglycemia induced a significant impairment in rats exposed to severe hypoglycemia. Recurrent hypoglycemia tended to improved performance but was not significant (RH-SH90 vs CON-SH90) indicating that the recurrent hypoglycemia was not able to completely reverse the retention deficits concerning the exact location of the platform. However, analysis of the spatial bias and average proximity data indicates that recurrent hypoglycemia did preserve the memory of a more generalized platform location. Specifically, the two euglycemic control groups each showed a significant spatial bias for the target quadrant that had contained the submerged platform while the CON-SH90 rats showed no such bias indicating a cognitive impairment in spatial bias induced by severe hypoglycemia. The RH-SH90 rats also showed a significant spatial bias for the target quadrant indicating that recurrent hypoglycemia treatment completely restored spatial bias. Assessment of average proximity again showed a cognitive impairment due to severe hypoglycemia as the CON-SH90 group had an average proximity that was farther away from the platform location than that of the CON-EUG and RH-SH90 rats. Since the average proximity

values for the RH-SH90 and CON-EUG groups were not different, recurrent hypoglycemia treatment fully reversed this cognitive deficit induced by severe hypoglycemia. These findings indicate that memory retention performance was impaired in the CON-SH90 rats relative to the CON-EUG controls on all probe trial variables, and recurrent hypoglycemia prevented impairments on 2/3 probe trials indices.

Since the CON-SH90 rats exhibited significant performance deficits during the cued trials, it is not possible to know whether the impaired place trials performance was due to compromised spatial learning and memory processing or to non-associative disturbances, or to both types of functional deficits. Reasons for the poor cued trials performance could include impairments in visual function, alterations in motivation, or a global decrement in generalized cognitive functioning which would encompass simple cued learning as well as more complex spatial learning and memory capabilities. Importantly, the profound cognitive defects induced by severe hypoglycemia in both cued learning deficits and compromised spatial learning and memory processing were prevented by antecedent recurrent moderate hypoglycemia.

Consistent with the notion that recurrent hypoglycemia induces an adaptive brain response is the observation that recurrent hypoglycemic rats had less seizure-like behavior during severe hypoglycemia (Figure 23), indicating the RH treated brain was better able to tolerate severe hypoglycemia. Consistent with previous studies from the laboratory (132), the degree of neuronal damage induced by severe hypoglycemia was correlated with the number of episodes of seizure-like behavior. Novel findings are now presented indicating that the number of episodes of seizure-like behavior observed during severe hypoglycemia was also correlated with cognitive performance. These

correlations, however, do not imply causality. In the setting of profound hypoglycemia, vulnerable brain regions may be susceptible to both damage and seizure activity. As in the real world setting, witnessed hypoglycemic seizures were defined clinically. In the absence of electroencephalogram (EEG) monitoring, the effect of subclinical seizures (i.e. seizures not associated with noticeable motor activity) on brain damage and cognition could not be assessed. Nonetheless, observable instances of seizure-like behavior correlated with the extent of neuronal damage and long-term cognitive function, and thus, can be used as a marker for neuronal injury and is prognostic of long-term cognitive outcomes. Indeed, clinical studies support these finding because more than severe hypoglycemia *per se*, the presence of hypoglycemic seizures correlate more closely with impaired cognitive function (59;65).

The amount and distribution of severe hypoglycemia induced neuronal damage was markedly different between the 60 minute and 90 minute clamp studies (Figure 20 and Figure 23). In spite of similar degrees of hypoglycemia (10-15 mg/dl) the extra 30 minutes of severe hypoglycemia induced a 6-fold increase in cortical brain damage and markedly increase hippocampal brain damage (which was minimal in the 60 minute clamp). These findings emphasize the importance of the duration of severe hypoglycemia, and not hypoglycemic nadir alone, as a critically important component in determining the extent of brain damage (53). Unlike noting the presences or absence of seizures, noting the duration of severe hypoglycemia is impractical to estimate clinically and, thus, unlike seizure activity would not be a clinically useful marker for neuronal damage. Interestingly though, the likely variability of severe hypoglycemia duration in the clinical situation could be another important factor contributing to the variable

amounts of cognitive impairments noted in the clinical literature. Given the variability in the extent of clinical symptoms associated with severe hypoglycemia, it is not surprising that there exists a marked variability in the reported neurological consequences of severe hypoglycemia in retrospective clinical reports.

Independent of episodes of severe hypoglycemia, previous studies have shown that recurrent moderate hypoglycemia can alter cognitive function. In one study, recurrent moderate hypoglycemia did not cause neuronal damage in the hippocampus (as confirmed in this study) but did impair hippocampal long-term potentiation (LTP), a cellular mechanism believed to be involved in learning and memory (150). The impaired LTP after recurrent hypoglycemia suggests that RH would lead to impaired cognitive function. Other studies showed an opposite effect in that antecedent hypoglycemia improved cognitive ability in rats tested in an euglycemic state (151;152). In the current study of euglycemic control rats that were not exposed to severe hypoglycemia, no detrimental or beneficial effect of recurrent hypoglycemia on cognitive ability was observed during the Morris water maze testing. This discrepancy between studies is likely due to the later point in time when cognition was tested. In the current study, cognitive ability was tested 6-8 weeks after the recurrent moderate hypoglycemia. LTP was measured a few days after recurrent hypoglycemia and normal LTP function may have recovered if LTP was measured at a later time point. Further, in studies that showed a beneficial cognitive effect of RH, cognition was measured a day following RH. Again, any changes brought about by RH alone in this study may have evanesced during a 6-8 week recovery period. Indeed, since 2-3 weeks of scrupulous avoidance of hypoglycemia negates the adaptive deficit in sympathoadrenal response associated with recurrent

hypoglycemia (153;154), any such adaptive effect of antecedent hypoglycemia on cognition would likely have dissipated after 6-8 weeks in euglycemic controls.

Although recurrent moderate hypoglycemia has repeatedly been shown to lead to maladaptive responses resulting in hypoglycemia unawareness and hypoglycemia associated autonomic failure (HAAF), the mechanism(s) by which recurrent hypoglycemia leads to these adaptations remain elusive. Similarly, the current experiments do not identify the mechanisms by which recurrent moderate hypoglycemia affords, [1] protection against hypoglycemia induced neuronal damage, [2] limitation to hypoglycemia induced neurocognitive dysfunction, and [3] increased thresholds for hypoglycemic seizures. Putative mechanisms for these beneficial adaptations could include glycogen supercompensation—increased brain glycogen content above pre-hypoglycemic levels (155-159). By keeping a higher level of stored fuel units, increased brain glycogen content has been shown to reduce hypoglycemic neuronal injury by maintaining brain electrical activity and forestalling EEG isoelectricity (4). Enhanced nutrient transport may also contribute to the neuroprotective effects of recurrent hypoglycemia (160;161) Monocarboxylate acid transport is increased during hypoglycemia in patients with well-controlled type I diabetes (160;161). Increase transport of monocarboxylate acids (e.g. lactate) could provide an alternative source of energy and maintain neuronal function (54). Several other possibilities could account for the neuroprotective effect such as alterations in brain metabolism or neuronal activity (155;162-165). Recurrent hypoglycemia has been shown to enhance the inhibitory neurotransmitter, GABA, which could reduce neuronal activity and limit excitotoxic damage (163). Further studies on the precise mechanisms of how recurrent

hypoglycemia exerts its neuroprotective effects to subsequent severe hypoglycemia are warranted.

These studies demonstrate that recurrent moderate hypoglycemia preconditions and protects the brain against severe hypoglycemia induced neuronal damage and its associated cognitive deficits. These intriguing findings suggest that recurrent bouts of moderate hypoglycemia that occur with intensive glycemic control might, paradoxically, render an individual more prone to, but less vulnerable to, an episode of severe hypoglycemia. If the current data indicating a neuroprotective preconditioning effect of recurrent moderate hypoglycemia were to be extrapolated to the clinical setting, it could explain the apparent divergent findings between animal and clinical studies and may also explain the seemingly incongruous clinical findings that intensively treated patients who experience recurrent moderate and severe hypoglycemia may be paradoxically protected from severe hypoglycemia induced brain damage and (fortunately) may not suffer from associated long-term cognitive damage (70;166).

## THESIS DISCUSSION

The major pathophysiological defects responsible for the development of type 2 diabetes have traditionally been attributed to insulin resistance in muscle and the liver and β-cell failure. However, recent research has found other organs that are involved in the pathogenesis of diabetes such as adipose tissue, the gastrointestinal tract, the pancreatic α-cell, and the kidneys (167). Of particular interest, the brain has also been implicated in contributing to the development of diabetes. The brain receives afferent neuronal and hormonal signals from the rest of the body and integrates these signals in order to elicit an appropriate response to maintain whole body energy homeostasis. Independent of these neuronal and hormonal signals, the brain can also directly sense nutrients, particularly glucose, and direct efferent signals to regulate glucose homeostasis. Disruption of the ability of the brain to sense glucose results in impaired glucose tolerance, a hallmark in the progression to diabetes (19). Additionally, impaired neuronal glucose sensing may also increase the risk of developing severe hypoglycemia, a common complication associated with tight glycemic control that occurs with intensive insulin therapy. As diabetes is characterized by disrupted energy homeostasis leading to hyperglycemia, glycemic management requires a careful balance between lowering glucose towards normal while avoiding hypoglycemia. Understanding the mechanisms in which the brain senses and responds to glucose to prevent hyperglycemia as well as hypoglycemia is of great importance to reducing the risk and improving the management of diabetes. The work of this thesis found important roles of neuronal GLUT4 and the hexosamine biosynthetic pathway (HBP) in the hypothalamus in the regulation of

peripheral insulin sensitivity and whole body energy homeostasis. Further, neuronal GLUT4 was found to be important in the counterregulatory response to hypoglycemia while antecedent hypoglycemia was found to precondition and protect the brain from severe hypoglycemia-induced neuronal damage.

**Insulin Signaling and Neuronal Glucose Sensing**

Insulin's role in the regulation of energy homeostasis has been extensively studied in peripheral tissues. Insulin inhibits hepatic glucose production and stimulates glucose uptake into skeletal muscle and adipose tissue in order to lower blood glucose. However, insulin has also been shown to act centrally to modulate brain glucose sensing and whole body energy homeostasis. One of the first studies demonstrating a role for insulin action in the brain revealed that infusion of insulin into the third cerebral ventricle of primates decreases food intake and body weight (126). Subsequent studies supported these findings and found more extensive functions of central insulin signaling. For example, infusion of insulin into the third cerebral ventricle suppresses hepatic glucose production (119). Studies involving genetic deletion of neuronal insulin receptors further underscore the importance of brain insulin action in the regulation of energy and glucose homeostasis. Knock-out of neuronal insulin receptors or knock down of hypothalamic insulin receptors results in hyperphagia, peripheral insulin resistance, and impaired glucose tolerance (36;37). Further, neuronal insulin signaling is required for eliciting a full counterregulatory response to hypoglycemia (38). Thus, identification of factors that modulate central insulin signaling may also be important in regulating energy and glucose homeostasis. GLUT4 is the major effector protein for insulin stimulated glucose

uptake. Deletion of GLUT4 in skeletal muscle or adipose tissue results in insulin resistance (26-28). Additionally, excess nutrients such as hyperglycemia have a negative impact on insulin signaling. Hyperglycemia reduces insulin stimulated glucose uptake, and this effect has been shown to be mediated by increased metabolism through the hexosamine biosynthetic pathway (42;48;113). As GLUT4 and hyperglycemia affect insulin signaling peripherally, this thesis observed important functions of GLUT4 and hyperglycemia in the central nervous system in modulating energy homeostasis.

*Brain Glucose Transporter 4*

To delineate the roles of GLUT4 in the brain, neuronal specific GLUT4 knockout mice (NG4KO) were created. Tissue specific deletion of neuronal GLUT4 did not affect body weight or fat pad mass. Further, neuronal GLUT4 did not have an effect on basal glucose homeostasis as fasting and fed glucose levels were similar in NG4KO mice compared to littermate controls. Interestingly, neuronal GLUT4 did have an important role in glucose homeostasis in response to a glucose load. Intraperitoneal glucose tolerance tests revealed impaired glucose tolerance in NG4KO mice compared to littermate controls. To directly assess insulin sensitivity, hyperinsulinemic-euglycemic clamps were performed. Neuronal GLUT4 deletion was found to result in whole body insulin resistance as NG4KO mice required a significantly lower glucose infusion rate to maintain euglycemia than littermate controls. The insulin resistance was not caused by changes in insulin stimulated glucose uptake in peripheral tissues because glucose uptake in skeletal muscle and adipose tissue were not different between NG4KO mice and their respective littermate controls. Instead, hepatic insulin resistance was responsible for the

lower glucose infusion rate and reduced insulin sensitivity seen in NG4KO mice. The ability of insulin to suppress hepatic glucose production (HGP) was significantly impaired in NG4KO mice. Interestingly, brain insulin signaling is also critical for insulin's ability to fully suppress HGP (98;99). It is feasible that the altered hepatic insulin sensitivity in NG4KO mice may be a result of the requirement for neuronal GLUT4 in exerting insulin's effects in the CNS. The absence of neuronal GLUT4 may prevent insulin action in the brain from exerting its effects on HGP. More direct studies to understand this relationship between central GLUT4 and insulin are warranted. Nonetheless, these studies found a novel role of brain GLUT4 in the regulation of insulin sensitivity, glucose tolerance, and hepatic glucose production.

*Hexosamine Biosynthetic Pathway and Hypothalamic Protein Glycosylation*

Nutrient excess in the setting of hyperglycemia increases flux through the hexosamine biosynthetic pathway leading to increased *O*-linked protein glycosylation. Increased protein glycosylation has been implicated as a mechanism by which hyperglycemia induces insulin resistance in adipose tissue and skeletal muscle. Whether these same processes also occur in the central nervous system to modulate central insulin sensitivity and, subsequently, energy homeostasis were investigated. The hypothesis that chronic infusion of glucose or glucosamine, a molecule that directly enters the HBP, into the hypothalamus would induce central insulin resistance leading to increased food intake and excessive body weight gain was tested.

Contrary to the original hypothesis, hypothalamic glucose infusion reduced body weight. Hypothalamic glucose concentrations may have not reached a level sufficient

enough to induce hypothalamic insulin resistance. As the brain is highly metabolically active, the infused glucose may have been simply utilized by the brain. Indeed, a previous study demonstrated a 7 day infusion of glucose into the third ventricle decreased body weight (8). In peripheral tissues, the hyperglycemia mimetic glucosamine is a more potent inducer of UDP-GlcNAc production (168) and, subsequently, insulin resistance (40;41;48). Glucosamine directly enters the hexosamine biosynthetic pathway (HBP) to increase protein glycosylation and consequently induces insulin resistance in peripheral tissues (40;41;48). Therefore, glucosamine was infused into the mediobasal hypothalamus and the effects on energy homeostasis were investigated.

As with hypothalamic glucose infusion, hypothalamic glucosamine infusion reduced body weight and additionally reduced food intake. Hypothalamic glucosamine infusion also resulted in the expected increase in overall *O*-linked protein glycosylation (Figure 16). Interestingly, the degree of hypothalamic protein glycosylation was significantly and inversely correlated to the degree of weight gain. That is, increased protein glycosylation correlated with lower the weight gain and vice versa (Figure 16). Further, the anorexigenic effects of hypothalamic glucosamine infusion could be attributed to its effects on central insulin signaling. Mediobasal hypothalamic infusion of glucosamine enhanced hypothalamic insulin sensitivity, and insulin is known to have anorexigenic effects centrally (124-126). Thus, the hexosamine biosynthetic pathway and the degree of protein glycosylation were important processes in the regulation of whole body energy homeostasis and insulin sensitivity centrally and peripherally.

Systemic glucose and insulin levels were not affected by hypothalamic glucosamine infusion. However, insulin tolerance tests did demonstrate that the

glucosamine treated rats were more insulin sensitive than controls (Figure 17). The reduced body weight in the glucosamine infused animals (Figure 15) could account for the improved peripheral insulin sensitivity, but the enhanced hypothalamic insulin sensitivity (Figure 17) may have also contributed to the improved peripheral insulin sensitivity (36;37).

These data provide evidence of a novel nutrient sensing system in the hypothalamus. Enhanced metabolism through the HBP may increase O-GlcNAc, which in turn, reduces food intake, limits body weight gain, and improves both central and peripheral insulin sensitivity. The HBP may become a potential therapeutic target to modulate food intake and body weight and, hence, combat the ever-growing epidemic of obesity and diabetes.

This thesis work demonstrated a role for both neuronal GLUT4 and hypothalamic HBP in modulating peripheral insulin sensitivity. As peripheral insulin resistance is a hallmark in the development of type 2 diabetes, both neuronal GLUT4 and the HBP may be potential therapeutic targets to combat type 2 diabetes.

**Hypoglycemia**

Intensive insulin therapy that lowers blood glucose towards normal reduces the long-term microvascular complications due to diabetes. However, insulin replacement is an imperfect process that often results in relative insulin excess and repeated episodes of hypoglycemia. Moderate hypoglycemia can acutely affect cognitive capabilities while more severe hypoglycemia can lead to seizures, coma, and even death. For patients who

manage their diabetes with insulin therapy, hypoglycemia is the major obstacle in achieving tight glycemic control. Thus, discovering mechanisms involved in preventing hypoglycemia as well as identifying ways to reduced brain injury due to severe hypoglycemia will have direct clinical impact for patients living with diabetes. This thesis work found a novel and important role of neuronal GLUT4 in producing a full counterregulatory response to hypoglycemia. Further, the studies of this thesis found a novel mechanism in neuroprotection, hypoglycemic preconditioning.

As brain insulin signaling is important in the counterregulatory response to hypoglycemia (CRR), the CRR was assessed NG4KO mice. Genetic deletion of central GLUT4 resulted in impaired epinephrine and glucagon responses to hypoglycemia. Further, pharmacologic inhibition of brain GLUT4 also reduced both the epinephrine and glucagon response to hypoglycemia. Interestingly, streptozotocin diabetic rats that have reduced GLUT4 expression in the brain (34) also have impaired epinephrine and glucagon responses to hypoglycemia (100;101). These findings suggest intact brain GLUT4 function is necessary for producing a full CRR and further support the notion that brain GLUT4 is important in the modulation of neuronal glucose sensing. Thus, brain GLUT4 may be a therapeutic target for the prevention of hypoglycemia.

If the counterregulatory response is inadequate to correct hypoglycemia, blood sugars may continue to fall leading to severe hypoglycemia. Severe hypoglycemia can cause brain injury and encephalopathy (4;55;169). Therefore, discovering ways to protect the brain from hypoglycemic brain injury is very important for patients who rely on insulin therapy and are at risk for experiencing severe hypoglycemia. One potential mechanism for preventing severe hypoglycemia induced brain damage is via

preconditioning. In other forms of stress such as ischemia, brief, mild episodes of ischemia will protect the brain from a subsequent, more severe ischemic episode. These adaptive and protective changes in the brain to a moderate stressful stimulus are termed preconditioning. This thesis work investigated whether moderate episodes of antecedent hypoglycemia may also precondition and protect the brain against a subsequent episode of more severe hypoglycemia.

These studies found that antecedent hypoglycemia was able to protect the brain from neuronal damage induced by a subsequent episode of severe hypoglycemia. Intriguingly, the number of seizure-like behaviors observed during the episode of severe hypoglycemia was also significantly reduced in rats that were pretreated with three days of antecedent, moderate hypoglycemia. These data suggest that antecedent, moderate hypoglycemia induces adaptive changes within the brain that allows the brain to protect and better tolerate low blood sugars. Further, these reductions in brain injury were associated with long-term preservation of cognitive function. Six weeks after the severe hypoglycemia, rats that were pretreated with three days of antecedent recurrent hypoglycemia had significantly better spatial learning ability and memory. Taken together, antecedent, moderate hypoglycemia did precondition the brain and protected it against severe hypoglycemia induced brain injury and cognitive dysfunction.

Several results from the investigations on hypoglycemic preconditioning have direct clinical implications and corollaries. First, the degree of neuronal damage and extent of cognitive impairments were strongly correlated with the number of episodes of seizure-like behaviors. Rats that experienced greater number of seizure-like behaviors had more neuronal injury. Further, rats that experienced more instances of seizure-like

behaviors during severe hypoglycemia also had more extensive long-term impairments in cognitive ability when spatial learning and memory were tested six weeks after the severe hypoglycemia. Thus, episodes of seizure-like behaviors during severe hypoglycemia may be markers of neuronal injury and may be prognostic of long-term cognitive outcomes. Indeed, clinical studies show that the presence of seizures during hypoglycemia correlate with impaired cognitive function (59;65).

Hypoglycemia is a major complication with insulin therapy. This thesis identified potential neuronal mechanisms that may prevent hypoglycemia as well as protect the brain against the deleterious effects of severe hypoglycemia. Specifically, neuronal GLUT4 is essential for eliciting a full epinephrine and glucagon response to hypoglycemia, and antecedent hypoglycemia can precondition the brain and protect it against the hypoglycemic brain injury.

**Summary**

Central to both the pathogenesis and treatment of diabetes is the regulation of glucose homeostasis. Dysfunction in glucose homeostasis such as insulin resistance and impaired glucose tolerance are the core pathophysiological processes involved in development of diabetes. The inability of the body to adequately prevent hypoglycemia during intensive glycemic control in patients living with diabetes is the major obstacle in the management of diabetes. The experiments in this thesis identified processes involved in brain glucose sensing/metabolism that are important in modulating central and peripheral insulin resistance, in preventing the occurrence of hypoglycemia, and in promoting neuronal viability. Particularly, brain GLUT4 and hypothalamic HBP both were found to

modulate peripheral insulin sensitivity, and hence, are potential therapeutic targets for improving insulin sensitivity. Further, brain GLUT4 was found to be a critical regulator of the counterregulatory response to hypoglycemia, thus is a potential therapeutic target for preventing the hypoglycemia that often occurs with insulin therapy. Finally, antecedent hypoglycemia preconditions and protects the brain against severe hypoglycemia-induced injury and cognitive function, a mechanism that may explain why individuals who experience both moderate and severe hypoglycemia may, fortunately, not suffer from long term cognitive dysfunction associated with severe hypoglycemia.

Reference List


1. 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

2. Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. *N.Engl.J.Med.* 342:381-389, 2000

3. Cryer,PE: Hypoglycaemia: the limiting factor in the glycaemic management of Type I and Type II diabetes. *Diabetologia* 45:937-948, 2002

4. Suh,SW, Hamby,AM, Swanson,RA: Hypoglycemia, brain energetics, and hypoglycemic neuronal death. *Glia* 55:1280-1286, 2007

5. Lam,CK, Chari,M, Lam,TK: CNS regulation of glucose homeostasis. *Physiology.(Bethesda.)* 24:159-170, 2009

6. Cryer,PE: Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes* 54:3592-3601, 2005

7. Berthoud,HR, Mogenson,GJ: Ingestive behavior after intracerebral and intracerebroventricular infusions of glucose and 2-deoxy-D-glucose. *Am.J.Physiol* 233:R127-R133, 1977

8. Davis,JD, Wirtshafter,D, Asin,KE, Brief,D: Sustained intracerebroventricular infusion of brain fuels reduces body weight and food intake in rats. *Science* 212:81-83, 1981

9. Miselis,RR, Epstein,AN: Feeding induced by intracerebroventricular 2-deoxy-D-glucose in the rat. *Am.J.Physiol* 229:1438-1447, 1975

10. Levin,BE, Dunn-Meynell,AA, Routh,VH: Brain glucose sensing and body energy homeostasis: role in obesity and diabetes. *Am.J Physiol* 276:R1223-R1231, 1999

11. 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

12. 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

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

14. Lam,TK, Gutierrez-Juarez,R, Pocai,A, Rossetti,L: Regulation of blood glucose by hypothalamic pyruvate metabolism. *Science* 309:943-947, 2005

15. 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

16. 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

17. 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

18. 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

19. 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

20. 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

21. 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

22. Chang,L, Chiang,SH, Saltiel,AR: Insulin signaling and the regulation of glucose transport. *Mol.Med.* 10:65-71, 2004

23. Katz,EB, Stenbit,AE, Hatton,K, DePinho,R, Charron,MJ: Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4. *Nature* 377:151-155, 1995

24. Stenbit,AE, Tsao,TS, Li,J, Burcelin,R, Geenen,DL, Factor,SM, Houseknecht,K, Katz,EB, Charron,MJ: GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. *Nat.Med.* 3:1096-1101, 1997

25. Rossetti,L, Stenbit,AE, Chen,W, Hu,M, Barzilai,N, Katz,EB, Charron,MJ: Peripheral but not hepatic insulin resistance in mice with one disrupted allele of the glucose transporter type 4 (GLUT4) gene. *J.Clin.Invest* 100:1831-1839, 1997

26. Zisman,A, Peroni,OD, Abel,ED, Michael,MD, Mauvais-Jarvis,F, Lowell,BB, Wojtaszewski,JF, Hirshman,MF, Virkamaki,A, Goodyear,LJ, Kahn,CR, Kahn,BB: Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat.Med.* 6:924-928, 2000

27. Kim,JK, Zisman,A, Fillmore,JJ, Peroni,OD, Kotani,K, Perret,P, Zong,H, Dong,J, Kahn,CR, Kahn,BB, Shulman,GI: Glucose toxicity and the development of diabetes in mice with muscle-specific inactivation of GLUT4. *J Clin.Invest* 108:153-160, 2001

28. Abel,ED, Peroni,O, Kim,JK, Kim,YB, Boss,O, Hadro,E, Minnemann,T, Shulman,GI, Kahn,BB: Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* 409:729-733, 2001

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

30. 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

31. Kobayashi,M, Nikami,H, Morimatsu,M, Saito,M: Expression and localization of insulin-regulatable glucose transporter (GLUT4) in rat brain. *Neurosci.Lett.* 213:103-106, 1996

32. Sankar,R, Thamotharan,S, Shin,D, Moley,KH, Devaskar,SU: Insulin-responsive glucose transporters-GLUT8 and GLUT4 are expressed in the developing mammalian brain. *Brain Res.Mol.Brain Res.* 107:157-165, 2002

33. Alquier,T, Leloup,C, Arnaud,E, Magnan,C, Penicaud,L: Altered Glut4 mRNA levels in specific brain areas of hyperglycemic-hyperinsulinemic rats. *Neurosci.Lett.* 308:75-78, 2001

34. 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

35. 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

36. 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

37. 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

38. 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

39. Diggs,K, Zhang,X, Puente,E, Daphna-Iken,D, Fisher,S: Brain insulin action regulates hypothalamic glucose sensing. *Diabetes* 57:A81, 2008

40. Copeland,RJ, Bullen,JW, Hart,GW: Cross-talk between GlcNAcylation and phosphorylation: roles in insulin resistance and glucose toxicity. *Am.J.Physiol Endocrinol.Metab* 295:E17-E28, 2008

41. Zachara,NE, Hart,GW: O-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. *Biochim.Biophys.Acta* 1673:13-28, 2004

42. Buse,MG: Hexosamines, insulin resistance, and the complications of diabetes: current status. *Am.J.Physiol Endocrinol.Metab* 290:E1-E8, 2006

43. Heart,E, Choi,WS, Sung,CK: Glucosamine-induced insulin resistance in 3T3-L1 adipocytes. *Am.J.Physiol Endocrinol.Metab* 278:E103-E112, 2000

44. Marshall,S, Bacote,V, Traxinger,RR: Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J.Biol.Chem.* 266:4706-4712, 1991

45. McClain,DA, Lubas,WA, Cooksey,RC, Hazel,M, Parker,GJ, Love,DC, Hanover,JA: Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. *Proc.Natl.Acad.Sci.U.S.A* 99:10695-10699, 2002

46. Cooksey,RC, McClain,DA: Transgenic mice overexpressing the rate-limiting enzyme for hexosamine synthesis in skeletal muscle or adipose tissue exhibit total body insulin resistance. *Ann.N.Y.Acad.Sci.* 967:102-111, 2002

47. Yang,X, Ongusaha,PP, Miles,PD, Havstad,JC, Zhang,F, So,WV, Kudlow,JE, Michell,RH, Olefsky,JM, Field,SJ, Evans,RM: Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. *Nature* 451:964-969, 2008

48. Vosseller,K, Wells,L, Lane,MD, Hart,GW: Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. *Proc.Natl.Acad.Sci.U.S.A* 99:5313-5318, 2002

49. Hypoglycemia in the Diabetes Control and Complications Trial. The Diabetes Control and Complications Trial Research Group. *Diabetes* 46:271-286, 1997

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

51. Defining and reporting hypoglycemia in diabetes: a report from the American Diabetes Association Workgroup on Hypoglycemia. *Diabetes Care* 28:1245-1249, 2005

52. Auer,RN: Hypoglycemic brain damage. *Forensic Sci.Int.* 146:105-110, 2004

53. Suh,SW, Aoyama,K, Chen,Y, Garnier,P, Matsumori,Y, Gum,E, Liu,J, Swanson,RA: Hypoglycemic neuronal death and cognitive impairment are prevented by poly(ADP-ribose) polymerase inhibitors administered after hypoglycemia. *J Neurosci.* 23:10681-10690, 2003

54. Suh,SW, Aoyama,K, Matsumori,Y, Liu,J, Swanson,RA: Pyruvate administered after severe hypoglycemia reduces neuronal death and cognitive impairment. *Diabetes* 54:1452-1458, 2005

55. Auer,RN, Hugh,J, Cosgrove,E, Curry,B: Neuropathologic findings in three cases of profound hypoglycemia. *Clin.Neuropathol.* 8:63-68, 1989

56. Bjorgaas,M, Gimse,R, Vik,T, Sand,T: Cognitive function in type 1 diabetic children with and without episodes of severe hypoglycaemia. *Acta Paediatr.* 86:148-153, 1997

57. Golden,MP, Ingersoll,GM, Brack,CJ, Russell,BA, Wright,JC, Huberty,TJ: Longitudinal relationship of asymptomatic hypoglycemia to cognitive function in IDDM. *Diabetes Care* 12:89-93, 1989

58. Hershey,T, Lillie,R, Sadler,M, White,NH: Severe hypoglycemia and long-term spatial memory in children with type 1 diabetes mellitus: a retrospective study. *J.Int.Neuropsychol.Soc.* 9:740-750, 2003

59. Kaufman,FR, Epport,K, Engilman,R, Halvorson,M: Neurocognitive functioning in children diagnosed with diabetes before age 10 years. *J.Diabetes Complications* 13:31-38, 1999

60. Langan,SJ, Deary,IJ, Hepburn,DA, Frier,BM: Cumulative cognitive impairment following recurrent severe hypoglycaemia in adult patients with insulin-treated diabetes mellitus. *Diabetologia* 34:337-344, 1991

61. Musen,G, Lyoo,IK, Sparks,CR, Weinger,K, Hwang,J, Ryan,CM, Jimerson,DC, Hennen,J, Renshaw,PF, Jacobson,AM: Effects of type 1 diabetes on gray matter density as measured by voxel-based morphometry. *Diabetes* 55:326-333, 2006

62. Northam,EA, Anderson,PJ, Werther,GA, Warne,GL, Andrewes,D: Predictors of change in the neuropsychological profiles of children with type 1 diabetes 2 years after disease onset. *Diabetes Care* 22:1438-1444, 1999

63. Northam,EA, Anderson,PJ, Jacobs,R, Hughes,M, Warne,GL, Werther,GA: Neuropsychological profiles of children with type 1 diabetes 6 years after disease onset. *Diabetes Care* 24:1541-1546, 2001

64. Northam,EA, Rankins,D, Lin,A, Wellard,RM, Pell,GS, Finch,SJ, Werther,GA, Cameron,FJ: Central nervous system function in youth with type 1 diabetes 12 years after disease onset. *Diabetes Care* 32:445-450, 2009

65. Rovet,JF, Ehrlich,RM: The effect of hypoglycemic seizures on cognitive function in children with diabetes: a 7-year prospective study. *J.Pediatr.* 134:503-506, 1999

66. Ryan,CM, Becker,DJ: Hypoglycemia in children with type 1 diabetes mellitus. Risk factors, cognitive function, and management. *Endocrinol.Metab Clin.North Am.* 28:883-900, 1999

67. Strudwick,SK, Carne,C, Gardiner,J, Foster,JK, Davis,EA, Jones,TW: Cognitive functioning in children with early onset type 1 diabetes and severe hypoglycemia. *J.Pediatr.* 147:680-685, 2005

68. Schoenle,EJ, Schoenle,D, Molinari,L, Largo,RH: Impaired intellectual development in children with Type I diabetes: association with HbA(1c), age at diagnosis and sex. *Diabetologia* 45:108-114, 2002

69. Austin,EJ, Deary,IJ: Effects of repeated hypoglycemia on cognitive function: a psychometrically validated reanalysis of the Diabetes Control and Complications Trial data. *Diabetes Care* 22:1273-1277, 1999

70. Jacobson,AM, Musen,G, Ryan,CM, Silvers,N, Cleary,P, Waberski,B, Burwood,A, Weinger,K, Bayless,M, Dahms,W, Harth,J: Long-term effect of diabetes and its treatment on cognitive function. *N.Engl.J.Med.* 356:1842-1852, 2007

71. Wysocki,T, Harris,MA, Mauras,N, Fox,L, Taylor,A, Jackson,SC, White,NH: Absence of adverse effects of severe hypoglycemia on cognitive function in school-aged children with diabetes over 18 months. *Diabetes Care* 26:1100-1105, 2003

72. Kramer,L, Fasching,P, Madl,C, Schneider,B, Damjancic,P, Waldhausl,W, Irsigler,K, Grimm,G: Previous episodes of hypoglycemic coma are not associated with permanent cognitive brain dysfunction in IDDM patients on intensive insulin treatment. *Diabetes* 47:1909-1914, 1998

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

74. White,NH, Skor,DA, Cryer,PE, Levandoski,LA, Bier,DM, Santiago,JV: Identification of type I diabetic patients at increased risk for hypoglycemia during intensive therapy. *N.Engl.J Med.* 308:485-491, 1983

75. Gold,AE, MacLeod,KM, Frier,BM: Frequency of severe hypoglycemia in patients with type I diabetes with impaired awareness of hypoglycemia. *Diabetes Care* 17:697-703, 1994

76. Gidday,JM: Cerebral preconditioning and ischaemic tolerance. *Nat.Rev.Neurosci.* 7:437-448, 2006

77. DAHL,NA, BALFOUR,WM: PROLONGED ANOXIC SURVIVAL DUE TO ANOXIA PRE-EXPOSURE: BRAIN ATP, LACTATE, AND PYRUVATE. *Am.J.Physiol* 207:452-456, 1964

78. Kitagawa,K, Matsumoto,M, Kuwabara,K, Tagaya,M, Ohtsuki,T, Hata,R, Ueda,H, Handa,N, Kimura,K, Kamada,T: 'Ischemic tolerance' phenomenon detected in various brain regions. *Brain Res.* 561:203-211, 1991

79. Malhotra,S, Savitz,SI, Ocava,L, Rosenbaum,DM: Ischemic preconditioning is mediated by erythropoietin through PI-3 kinase signaling in an animal model of transient ischemic attack. *J.Neurosci.Res.* 83:19-27, 2006

80. Moncayo,J, de Freitas,GR, Bogousslavsky,J, Altieri,M, Van Melle,G: Do transient ischemic attacks have a neuroprotective effect? *Neurology* 54:2089-2094, 2000

81. Wegener,S, Gottschalk,B, Jovanovic,V, Knab,R, Fiebach,JB, Schellinger,PD, Kucinski,T, Jungehulsing,GJ, Brunecker,P, Muller,B, Banasik,A, Amberger,N, Wernecke,KD, Siebler,M, Rother,J, Villringer,A, Weih,M: Transient ischemic attacks before ischemic stroke: preconditioning the human brain? A multicenter magnetic resonance imaging study. *Stroke* 35:616-621, 2004

82. Cryer,PE: Mechanisms of sympathoadrenal failure and hypoglycemia in diabetes. *J.Clin.Invest* 116:1470-1473, 2006

83. Cushman,SW, Wardzala,LJ: Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. *J.Biol.Chem.* 255:4758-4762, 1980

84. Huang,S, Czech,MP: The GLUT4 glucose transporter. *Cell Metab* 5:237-252, 2007

85. Suzuki,K, Kono,T: Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc.Natl.Acad.Sci.U.S.A* 77:2542-2545, 1980

86. Brant,AM, Jess,TJ, Milligan,G, Brown,CM, Gould,GW: Immunological analysis of glucose transporters expressed in different regions of the rat brain and central nervous system. *Biochem.Biophys.Res.Commun.* 192:1297-1302, 1993

87. Leloup,C, Arluison,M, Kassis,N, Lepetit,N, Cartier,N, Ferre,P, Penicaud,L: Discrete brain areas express the insulin-responsive glucose transporter GLUT4. *Brain Res.Mol.Brain Res.* 38:45-53, 1996

88. McEwen,BS, Reagan,LP: Glucose transporter expression in the central nervous system: relationship to synaptic function. *Eur.J.Pharmacol.* 490:13-24, 2004

89. Tronche,F, Kellendonk,C, Kretz,O, Gass,P, Anlag,K, Orban,PC, Bock,R, Klein,R, Schutz,G: Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat.Genet.* 23:99-103, 1999

90. 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

91. Norris,AW, Chen,L, Fisher,SJ, Szanto,I, Ristow,M, Jozsi,AC, Hirshman,MF, Rosen,ED, Goodyear,LJ, Gonzalez,FJ, Spiegelman,BM, Kahn,CR: Muscle-specific PPARgamma-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. *J Clin.Invest* 112:608-618, 2003

92. 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

93. Toyama,H, Ichise,M, Liow,JS, Modell,KJ, Vines,DC, Esaki,T, Cook,M, Seidel,J, Sokoloff,L, Green,MV, Innis,RB: Absolute quantification of regional cerebral glucose utilization in mice by 18F-FDG small animal PET scanning and 2-14C-DG autoradiography. *J.Nucl.Med.* 45:1398-1405, 2004

94. Fernando,RN, Albiston,AL, Chai,SY: The insulin-regulated aminopeptidase IRAP is colocalised with GLUT4 in the mouse hippocampus--potential role in modulation of glucose uptake in neurones? *Eur.J.Neurosci.* 28:588-598, 2008

95. Hertel,J, Struthers,H, Horj,CB, Hruz,PW: A structural basis for the acute effects of HIV protease inhibitors on GLUT4 intrinsic activity. *J.Biol.Chem.* 279:55147-55152, 2004

96. Murata,H, Hruz,PW, Mueckler,M: Indinavir inhibits the glucose transporter isoform Glut4 at physiologic concentrations. *AIDS* 16:859-863, 2002

97. 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

98. Okamoto,H, Obici,S, Accili,D, Rossetti,L: Restoration of liver insulin signaling in Insr knockout mice fails to normalize hepatic insulin action. *J.Clin.Invest* 115:1314-1322, 2005

99. Pocai,A, Lam,TK, Gutierrez-Juarez,R, Obici,S, Schwartz,GJ, Bryan,J, Aguilar-Bryan,L, Rossetti,L: Hypothalamic K(ATP) channels control hepatic glucose production. *Nature* 434:1026-1031, 2005

100. Chan,O, Inouye,K, Akirav,EM, Park,E, Riddell,MC, Matthews,SG, Vranic,M: Hyperglycemia does not increase basal hypothalamo-pituitary-adrenal activity in diabetes but it does impair the HPA response to insulin-induced hypoglycemia. *Am.J.Physiol Regul.Integr.Comp Physiol* 289:R235-R246, 2005

101. Shi,ZQ, Rastogi,KS, Lekas,M, Efendic,S, Drucker,DJ, Vranic,M: Glucagon response to hypoglycemia is improved by insulin-independent restoration of normoglycemia in diabetic rats. *Endocrinology* 137:3193-3199, 1996

102. 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

103. 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

104. 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

105. Bluher,M, Michael,MD, Peroni,OD, Ueki,K, Carter,N, Kahn,BB, Kahn,CR: Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Dev.Cell* 3:25-38, 2002

106. Bruning,JC, Michael,MD, Winnay,JN, Hayashi,T, Horsch,D, Accili,D, Goodyear,LJ, Kahn,CR: A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol.Cell* 2:559-569, 1998

107. Minokoshi,Y, Kahn,CR, Kahn,BB: Tissue-specific ablation of the GLUT4 glucose transporter or the insulin receptor challenges assumptions about insulin action and glucose homeostasis. *J.Biol.Chem.* 278:33609-33612, 2003

108. Lazar,MA: How obesity causes diabetes: not a tall tale. *Science* 307:373-375, 2005

109. Spiegelman,BM, Flier,JS: Obesity and the regulation of energy balance. *Cell* 104:531-543, 2001

110. Parker,GJ, Lund,KC, Taylor,RP, McClain,DA: Insulin resistance of glycogen synthase mediated by o-linked N-acetylglucosamine. *J.Biol.Chem.* 278:10022-10027, 2003

111. Slawson,C, Housley,MP, Hart,GW: O-GlcNAc cycling: how a single sugar post-translational modification is changing the way we think about signaling networks. *J.Cell Biochem.* 97:71-83, 2006

112. Rossetti,L: Perspective: Hexosamines and nutrient sensing. *Endocrinology* 141:1922-1925, 2000

113. Ross,SA, Chen,X, Hope,HR, Sun,S, McMahon,EG, Broschat,K, Gulve,EA: Development and comparison of two 3T3-L1 adipocyte models of insulin resistance: increased glucose flux vs glucosamine treatment. *Biochem.Biophys.Res.Commun.* 273:1033-1041, 2000

114. Porte,D, Jr., Baskin,DG, Schwartz,MW: Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. *Diabetes* 54:1264-1276, 2005

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

116. Cox,JE, Powley,TL: Intragastric pair feeding fails to prevent VMH obesity or hyperinsulinemia. *Am.J.Physiol* 240:E566-E572, 1981

117. Han,PW: Energy metabolism of tube-fed hypophysectomized rats bearing hypothalamic lesions. *Am.J.Physiol* 215:1343-1350, 1968

118. King,BM: The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. *Physiol Behav.* 87:221-244, 2006

119. Obici,S, Zhang,BB, Karkanias,G, Rossetti,L: Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat.Med.* 8:1376-1382, 2002

120. Wang,J, Liu,R, Hawkins,M, Barzilai,N, Rossetti,L: A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* 393:684-688, 1998

121. Liu,F, Iqbal,K, Grundke-Iqbal,I, Hart,GW, Gong,CX: O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. *Proc.Natl.Acad.Sci.U.S.A* 101:10804-10809, 2004

122. Akimoto,Y, Hart,GW, Wells,L, Vosseller,K, Yamamoto,K, Munetomo,E, Ohara-Imaizumi,M, Nishiwaki,C, Nagamatsu,S, Hirano,H, Kawakami,H: Elevation of the post-translational modification of proteins by O-linked N-acetylglucosamine leads to deterioration of the glucose-stimulated insulin secretion in the pancreas of diabetic Goto-Kakizaki rats. *Glycobiology* 17:127-140, 2007

123. Zraika,S, Dunlop,M, Proietto,J, Andrikopoulos,S: The hexosamine biosynthesis pathway regulates insulin secretion via protein glycosylation in mouse islets. *Arch.Biochem.Biophys.* 405:275-279, 2002

124. Brown,LM, Clegg,DJ, Benoit,SC, Woods,SC: Intraventricular insulin and leptin reduce food intake and body weight in C57BL/6J mice. *Physiol Behav.* 89:687-691, 2006

125. Clegg,DJ, Riedy,CA, Smith,KA, Benoit,SC, Woods,SC: Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes* 52:682-687, 2003

126. Woods,SC, Lotter,EC, McKay,LD, Porte,D, Jr.: Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature* 282:503-505, 1979

127. Benoit,SC, Air,EL, Coolen,LM, Strauss,R, Jackman,A, Clegg,DJ, Seeley,RJ, Woods,SC: The catabolic action of insulin in the brain is mediated by melanocortins. *J.Neurosci.* 22:9048-9052, 2002

128. 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

129. Cryer,PE: Diverse causes of hypoglycemia-associated autonomic failure in diabetes. *N.Engl.J.Med.* 350:2272-2279, 2004

130. Auer,RN: Hypoglycemic brain damage. *Metab Brain Dis.* 19:169-175, 2004

131. Perantie,DC, Wu,J, Koller,JM, Lim,A, Warren,SL, Black,KJ, Sadler,M, White,NH, Hershey,T: Regional brain volume differences associated with hyperglycemia and severe hypoglycemia in youth with type 1 diabetes. *Diabetes Care* 30:2331-2337, 2007

132. Bree,AJ, Puente,EC, Daphna-Iken,D, Fisher,SJ: Diabetes increases brain damage caused by severe hypoglycemia. *Am.J.Physiol Endocrinol.Metab* 297:E194-E201, 2009

133. Del Campo,M, Abdelmalik,PA, Wu,CP, Carlen,PL, Zhang,L: Seizure-like activity in the hypoglycemic rat: lack of correlation with the electroencephalogram of free-moving animals. *Epilepsy Res.* 83:243-248, 2009

134. Schmued,LC, Hopkins,KJ: Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res.* 874:123-130, 2000

135. Wong,M, Wozniak,DF, Yamada,KA: An animal model of generalized nonconvulsive status epilepticus: immediate characteristics and long-term effects. *Exp.Neurol.* 183:87-99, 2003

136. ter Braak,EW, Appelman,AM, van de,LM, Stolk,RP, van Haeften,TW, Erkelens,DW: Clinical characteristics of type 1 diabetic patients with and without severe hypoglycemia. *Diabetes Care* 23:1467-1471, 2000

137. Cox,DJ, Irvine,A, Gonder-Frederick,L, Nowacek,G, Butterfield,J: Fear of hypoglycemia: quantification, validation, and utilization. *Diabetes Care* 10:617-621, 1987

138. Butcher,SP, Sandberg,M, Hagberg,H, Hamberger,A: Cellular origins of endogenous amino acids released into the extracellular fluid of the rat striatum during severe insulin-induced hypoglycemia. *J.Neurochem.* 48:722-728, 1987

139. Nellgard,B, Wieloch,T: Cerebral protection by AMPA- and NMDA- receptor antagonists administered after severe insulin-induced hypoglycemia. *Exp.Brain Res.* 92:259-266, 1992

140. Suh,SW, Gum,ET, Hamby,AM, Chan,PH, Swanson,RA: Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. *J.Clin.Invest* 117:910-918, 2007

141. Telushkin,PK, Nozdrachev,AD, Potapov,PP, Medvedeva,NB, Stel'makh,AY: Glycolysis and oxidation enzyme activity in rat brain during insulin-induced hypoglycemia against the background of alloxan-induced diabetes mellitus. *Bull.Exp.Biol.Med.* 140:695-697, 2005

142. Wieloch,T, Engelsen,B, Westerberg,E, Auer,R: Lesions of the glutamatergic cortico-striatal projections in the rat ameliorate hypoglycemic brain damage in the striatum. *Neurosci.Lett.* 58:25-30, 1985

143. Alkire,MT, Pomfrett,CJ, Haier,RJ, Gianzero,MV, Chan,CM, Jacobsen,BP, Fallon,JH: Functional brain imaging during anesthesia in humans: effects of halothane on global and regional cerebral glucose metabolism. *Anesthesiology* 90:701-709, 1999

144. 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

145. Jeong,YB, Kim,JS, Jeong,SM, Park,JW, Choi,IC: Comparison of the effects of sevoflurane and propofol anaesthesia on regional cerebral glucose metabolism in humans using positron emission tomography. *J.Int.Med.Res.* 34:374-384, 2006

146. Nakao,Y, Itoh,Y, Kuang,TY, Cook,M, Jehle,J, Sokoloff,L: Effects of anesthesia on functional activation of cerebral blood flow and metabolism. *Proc.Natl.Acad.Sci.U.S.A* 98:7593-7598, 2001

147. Tkacs,NC, Pan,Y, Raghupathi,R, Dunn-Meynell,AA, Levin,BE: Cortical Fluoro-Jade staining and blunted adrenomedullary response to hypoglycemia after noncoma hypoglycemia in rats. *J.Cereb.Blood Flow Metab* 25:1645-1655, 2005

148. Tkacs,NC, Dunn-Meynell,AA, Levin,BE: Presumed apoptosis and reduced arcuate nucleus neuropeptide Y and pro-opiomelanocortin mRNA in non-coma hypoglycemia. *Diabetes* 49:820-826, 2000

149. Corbett,D, Nurse,S: The problem of assessing effective neuroprotection in experimental cerebral ischemia. *Prog.Neurobiol.* 54:531-548, 1998

150. Yamada,KA, Rensing,N, Izumi,Y, De Erausquin,GA, Gazit,V, Dorsey,DA, Herrera,DG: Repetitive hypoglycemia in young rats impairs hippocampal long-term potentiation. *Pediatr.Res.* 55:372-379, 2004

151. McNay,EC, Sherwin,RS: Effect of recurrent hypoglycemia on spatial cognition and cognitive metabolism in normal and diabetic rats. *Diabetes* 53:418-425, 2004

152. McNay,EC, Williamson,A, McCrimmon,RJ, Sherwin,RS: Cognitive and neural hippocampal effects of long-term moderate recurrent hypoglycemia. *Diabetes* 55:1088-1095, 2006

153. Dagogo-Jack,S, Rattarasarn,C, Cryer,PE: Reversal of hypoglycemia unawareness, but not defective glucose counterregulation, in IDDM. *Diabetes* 43:1426-1434, 1994

154. Fanelli,CG, Epifano,L, Rambotti,AM, Pampanelli,S, Di Vincenzo,A, Modarelli,F, Lepore,M, Annibale,B, Ciofetta,M, Bottini,P, .: Meticulous prevention of hypoglycemia normalizes the glycemic thresholds and magnitude of most of neuroendocrine responses to, symptoms of, and cognitive function during hypoglycemia in intensively treated patients with short-term IDDM. *Diabetes* 42:1683-1689, 1993

155. 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

156. Brown,AM, Sickmann,HM, Fosgerau,K, Lund,TM, Schousboe,A, Waagepetersen,HS, Ransom,BR: Astrocyte glycogen metabolism is required for neural activity during aglycemia or intense stimulation in mouse white matter. *J.Neurosci.Res.* 79:74-80, 2005

157. Brucklacher,RM, Vannucci,RC, Vannucci,SJ: Hypoxic preconditioning increases brain glycogen and delays energy depletion from hypoxia-ischemia in the immature rat. *Dev.Neurosci.* 24:411-417, 2002

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

159. Wender,R, Brown,AM, Fern,R, Swanson,RA, Farrell,K, Ransom,BR: Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. *J.Neurosci.* 20:6804-6810, 2000

160. Boyle,PJ, Kempers,SF, O'Connor,AM, Nagy,RJ: Brain glucose uptake and unawareness of hypoglycemia in patients with insulin-dependent diabetes mellitus. *N.Engl.J Med.* 333:1726-1731, 1995

161. Mason,GF, Petersen,KF, Levon,V, Rothman,DL, Shulman,GI: Increased brain monocarboxylic acid transport and utilization in Type 1 diabetes. *Diabetes* 55:929-34, 6 A.D.

162. Chan,O, Lawson,M, Zhu,W, Beverly,JL, Sherwin,RS: ATP-sensitive K(+) channels regulate the release of GABA in the ventromedial hypothalamus during hypoglycemia. *Diabetes* 56:1120-1126, 2007

163. 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

164. 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

165. 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

166. Amiel,SA: Hypoglycaemia in diabetes mellitus--protecting the brain. *Diabetologia* 40 Suppl 2:S62-S68, 1997

167. DeFronzo,RA: Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 58:773-795, 2009

168. Marshall,S, Nadeau,O, Yamasaki,K: Dynamic actions of glucose and glucosamine on hexosamine biosynthesis in isolated adipocytes: differential effects on glucosamine 6-phosphate, UDP-N-acetylglucosamine, and ATP levels. *J.Biol.Chem.* 279:35313-35319, 2004

169. Auer,RN, Kalimo,H, Olsson,Y, Siesjo,BK: The temporal evolution of hypoglycemic brain damage. II. Light- and electron-microscopic findings in the hippocampal gyrus and subiculum of the rat. *Acta Neuropathol.* 67:25-36, 1985