Glucosensing, Hypoglycemia, and the Brain

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Glucosensing, Hypoglycemia, and the Brain

by

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List of Abbreviations

ACTH- adrenocorticotropic hormone
ARC- arcuate nucleus
BG4KO- brain glucose transporter 4 knockout
CRR- counterregulatory response
Db/db- diabetic mouse
ECG- electrocardiogram
EEG- electroencephalogram
EGP- endogenous glucose production
GE- glucose excited neuron
GI- glucose inhibited neuron
GLUT- glucose transporter
HAAF- hypoglycemia associated autonomic failure
HA1c- hemoglobin A1c
HYP- hypothalamus
ICV- intracerebroventricular
IR- insulin receptor
NIRKO- neuronal insulin receptor knockout
NTS- nucleus tractus solitarius
Ob/ob- obese mouse
PVC- premature ventricular contraction
PVN- paraventricular nucleus
QTc- QT interval corrected for heart rate
VMH- ventromedial hypothalamus

VMN- ventromedial nucleus
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Glucose homeostasis can be peripherally and centrally mediated. Within the periphery, GLUT4 in muscle and adipose tissue is required for normal glucose homeostasis and disruption of GLUT4 translocation in these tissues leads to glucose intolerance and insulin resistance, hallmarks of Type 2 diabetes. Within the brain, specialized glucose sensing neurons regulate whole body glucose homeostasis. Unlike GLUT4’s role in the periphery, brain GLUT4 regulation of glucose tolerance is not well defined. Understanding how brain glucose sensing, mediated by GLUT4, regulates glucose tolerance and insulin sensitivity will aid in the development of treatment strategies to protect against the pathogenesis of diabetes. In addition, the brain regulates hypoglycemic counterregulation. Hypoglycemia is a major limitation to the treatment of diabetes. Severe hypoglycemia can lead to brain damage, cognitive dysfunction, and death. Understanding how to prevent the complications of severe hypoglycemia is critical to helping patients that are at risk. This thesis investigated 1) the role of brain GLUT4 in mediating peripheral insulin sensitivity, brain glucose sensing, and the counterregulatory response to hypoglycemia, 2) the effect of chronic insulin therapy in
protecting the diabetic brain against neuronal damage and cognitive dysfunction due to severe hypoglycemia, and 3) the mechanisms by which severe hypoglycemia leads to sudden death. Brain GLUT4 was shown to be critical for normal glucose homeostasis and the counterregulatory response to hypoglycemia. This indicates that therapeutic targeting strategies can aim to enhance brain GLUT4 function to improve glucose homeostasis in the setting of diabetes. Chronic insulin therapy in diabetic rats decreased the extent of brain damage due to severe hypoglycemia. This indicates that insulin therapy in diabetes decreases susceptibility to severe hypoglycemia-induced brain damage. The mechanisms of severe hypoglycemia induced sudden death are shown for the first time to be regulated by sympathetic mediated fatal cardiac arrhythmias. This thesis reveals the importance of the brain in the regulation of glucose homeostasis and the counterregulatory response to hypoglycemia and reveals paradoxical evidence that inhibition of the counterregulatory response acts to protect against severe hypoglycemia-induced mortality.
Chapter 1: Thesis Introduction
Diabetes

Diabetes is a growing epidemic with more than 25 million people in the US alone diagnosed. Diabetes is defined by an elevated blood glucose resulting from autoimmune destruction of pancreatic beta cells leading to insulin deficiency (Type 1 diabetes) or a combination of insulin resistance and beta cell failure (Type 2 diabetes). Chronic hyperglycemia in diabetes can result in both macrovascular (heart disease, stroke) and microvascular (neuropathy, nephropathy, retinopathy) complications. A major contributor to glucose intolerance and insulin resistance in models of Type 2 diabetes is impaired glucose transporter 4 (GLUT4) expression and/or translocation to the plasma membrane for insulin stimulated glucose uptake. Insulin acts to lower blood glucose levels by both suppressing endogenous glucose production as well as increasing peripheral GLUT4-dependent glucose uptake. Impaired insulin action leads to inefficient glucose uptake into muscle and adipose tissue as well as altered regulation of hepatic glucose production resulting in hyperglycemia.

Because of its potent effects to lower blood glucose, insulin is used in the treatment of diabetes to control blood glucose levels. However, insulin treatment leads to increased risk of iatrogenic hypoglycemia. Hypoglycemia prevalence in insulin treated diabetic patients is as high as 2.1 times per 24 hours. To function properly, the brain is constantly dependent on circulating glucose. Thus when severe, hypoglycemia can result in seizures, brain damage, cognitive decline, coma, and even death. These are all major concerns for insulin treated diabetic patients and their families. Determining the mechanisms of how hypoglycemia-induced brain damage, cognitive decline, and sudden death are regulated is critically important in order to find treatments.
to protect patients who are at risk for hypoglycemia. This thesis will specifically address
1) the role of brain glucose transporter 4 in glucose homeostasis, 2) the role of antecedent blood glucose control in reducing severe hypoglycemia induced brain damage and cognitive dysfunction, and 3) the mechanisms by which severe hypoglycemia can cause sudden death.

**Glucose homeostasis**

Regulation of glucose homeostasis is a tightly controlled process involving cross-talk between liver, skeletal muscle, adipose tissue, and the brain to maintain blood glucose in a normal physiologic range. Insulin’s actions in the periphery to lower blood glucose levels have been studied extensively but its central actions have just begun to be elucidated. The brain has an important role in energy homeostasis due to its requirements for a constant glucose supply. Thus, studying the brain’s role in sensing and responding to changes in glucose will aid in development of better strategies to treat or prevent diabetes.

**Anatomy of brain glucose sensing**

Although all brain cells utilize glucose, only a few specialized neurons in the brain truly sense and respond to a reduced glucose supply. Rodent studies indicate that glucose is sensed in brain regions known to be important in metabolism and energy homeostasis, particularly in the hypothalamus, where glucose sensing neurons are located\(^\text{11}\). Most studies to date indicate that it is the hypothalamus that principally initiates (or at least coordinates) the counterregulatory response (CRR) to hypoglycemia
by stimulating hormone secretion in the pituitary gland, pancreas, and adrenal glands resulting in a coordinated response (Figure 1). Clinical studies confirm the importance of the hypothalamus as a critical glucose sensing area because blood flow to the hypothalamus increases significantly during hypoglycemia, even before counterregulatory hormones rise\textsuperscript{12}. Within the hypothalamus, key regions that respond to changes in circulating glucose levels are the ventromedial hypothalamus (VMH) which contains the ventromedial nucleus (VMN) and the arcuate nucleus (ARC)\textsuperscript{13}. Studies show that glucose infusion into the VMH in the setting of peripheral hypoglycemia blunts the epinephrine counterregulatory response to hypoglycemia\textsuperscript{14,15}. These studies indicate that decreases in glucose are detected by the VMH and are required to fully activate the sympathoadrenal response to hypoglycemia.

The VMH contains glucose excitatory (GE, increases in glucose increase neuronal activity) and glucose inhibited (GI, increases in glucose decrease neuronal activity) neurons\textsuperscript{16}. Together, the actions of these neurons results in a coordinated process of trying to maintain plasma glucose levels within a normal physiological range. It is thought that in response to hypoglycemia, concerted activation of GI neurons and suppression of GE neurons, as an initiating part of a neural network, result in a coordinated efferent process that activates the sympathoadrenal response. This balance is disrupted in conditions such as diabetes\textsuperscript{17}.

**Brain glucose uptake**

The brain contains different isoforms of glucose transporters (GLUT) responsible for neuronal and astrocyte glucose uptake. The ubiquitous GLUT1 and the neural
specific GLUT3 are the primary isoforms present in the brain that are responsible for glucose transport across the blood brain barrier and into the brain. Based on discrete regional expression, the potential contribution of other glucose transporters has been investigated. A role for GLUT2 in mediating CNS glucose sensing has been proposed. In addition, because of its important role in peripheral glucose tolerance, the insulin-sensitive brain GLUT4 has been hypothesized to play a role in glucose and energy homeostasis.

**Glucose transporter 4**

GLUT4 is an insulin sensitive facilitative glucose transporter primarily expressed in adipose tissue, skeletal muscle, and heart with a lower expression in the brain. GLUT4 is expressed in various regions of the brain, including the hypothalamus (VMH, PVN, ARC), cerebellum, hippocampus, and cortex, regions important in metabolism, learning, and memory. Translocation of intracellular GLUT4 to the plasma membrane in adipose tissue and skeletal muscle is important in maintaining glucose homeostasis. Altered GLUT4 translocation in adipose tissue and muscle leads to glucose intolerance and insulin resistance, both characteristics of Type 2 diabetes. It has been hypothesized that brain GLUT4 works in a parallel insulin-dependent manner to that in the periphery. Similar to its known actions in peripheral tissues, GLUT4 translocation occurs in neuronal cell lines, hippocampus, and hypothalamus in response to insulin. In addition, studies have shown that GLUT4 expression in the brain is altered in response to insulin levels. Diabetic mice (db/db, hyperinsulinemia) have increased GLUT4 protein expression while streptozotocin induced diabetic rats (hypoinsulinemia)
have decreased GLUT4 protein expression in the cerebellum\textsuperscript{25}. In addition, ob/ob mice (insulin resistant) have increased GLUT4 expression in the hypothalamus\textsuperscript{25}. Therefore, regulation of GLUT4 protein expression in different regions of the brain may be dependent on plasma insulin levels or insulin sensitivity, indicating a potential pathogenic role for brain GLUT4 in the development of diabetes.

In addition to insulin regulation of brain GLUT4 protein expression and translocation, co-localization of GLUT4 and insulin receptor (IR) within the brain indicates an important relationship in the regulation of metabolism. Within the VMH, up to 75\% of the glucose sensing neurons co-express GLUT4 and IR\textsuperscript{16}. Insulin receptors are also highly expressed in the paraventricular nucleus (PVN) and arcuate nucleus (ARC) of the hypothalamus\textsuperscript{26} suggesting an important role for brain insulin action in metabolism. A recent report from our laboratory has shown the importance of insulin action in the brain in mediating both glucose sensing and the counterregulatory response to hypoglycemia. Neuronal insulin receptor knockout (NIRKO) mice have impaired hypoglycemic counterregulation, characterized by a blunted epinephrine and norepinephrine response\textsuperscript{27}. Consistent with insulin exerting its effect primarily in the hypothalamus, NIRKO mice have impaired glucose sensing in VMH GI neurons and impaired activation of PVN neurons in response to hypoglycemia\textsuperscript{27}. In addition, insulin induced suppression of hepatic glucose production is impaired in NIRKO mice, confirming earlier reports of central insulin regulation of hepatic glucose production\textsuperscript{28}. Together, these studies implicate hypothalamic insulin signaling as an important regulator of glucose sensing to control hepatic glucose production and the normal counterregulatory response to hypoglycemia. Interestingly, NIRKO mice have a 68\%
reduction in hypothalamic GLUT4 protein expression indicating that insulin’s actions in metabolic homeostasis may be mediated in part by brain GLUT4.

Because peripheral GLUT4 is important in the etiology of type 2 diabetes and brain GLUT4 is co-localized with the insulin receptor and its protein expression may be controlled by insulin, a brain GLUT4 KO (BG4KO) mouse was generated to study the importance of brain GLUT4 in glucose homeostasis and hypoglycemic counterregulation and is the focus of chapter 2.

Hypoglycemia

The treatment of diabetes remains suboptimal due to the barrier of iatrogenic hypoglycemia. Insulin treated diabetic patients experience an average of two symptomatic hypoglycemic episodes per week while severe hypoglycemia requiring assistance is experienced approximately once per year. Since the brain is continuously dependent on glucose for metabolism, robust counterregulatory mechanisms exist to rapidly increase blood glucose levels in order to protect the brain from hypoglycemia induced neuronal dysfunction and injury. Both severe and even more moderate episodes of hypoglycemia can be harmful. Recurrent episodes of moderate hypoglycemia can lead to a decreased awareness of hypoglycemia and decreased counterregulation, a phenomenon known as hypoglycemia associated autonomic failure (HAAF). HAAF can lead to an increased risk of more severe episodes of hypoglycemia. Severe hypoglycemia can alter brain structure, cause brain damage, cognitive dysfunction and sudden death. The counterregulatory response (CRR) to hypoglycemia has an important role in preventing the fall in glucose
to reduce the risk of the damaging effects of severe hypoglycemia, but, paradoxically, this same response can be harmful, leading to increased susceptibility to the damaging effects of severe hypoglycemia.

The counterregulatory response to hypoglycemia

In the setting of absolute or relative hyperinsulinemia, the CRR is normally initiated when glucose levels fall below 80 mg/dl. The CRR to hypoglycemia normally includes suppression of endogenous insulin secretion and increased secretion of catecholamines (epinephrine, norepinephrine), glucagon, cortisol, and growth hormone, which together act to increase plasma glucose levels by stimulating hepatic glucose production and limiting glucose utilization in peripheral tissues (Figure 1).

Sympathetic activation

In response to hypoglycemia, patients with Type 1 diabetes and advanced Type 2 diabetes are not able to suppress circulating (exogenous) insulin levels nor increase glucagon secretion\textsuperscript{32}. Thus, in the absence of appropriate insulin and glucagon responses, patients with diabetes rely extensively on the sympathoadrenal system as their primary counterregulatory defense against hypoglycemia\textsuperscript{33}. Adrenergic activation leads to the release of norepinephrine at nerve terminals located throughout the periphery. Within the adrenal gland, adrenergic stimulation leads to epinephrine secretion. Activation of the adrenergic system reverses falling glucose levels by increasing glucose production, reducing peripheral glucose utilization, and eliciting symptoms of hypoglycemia.
Catecholamines

As systemic blood glucose levels fall, glucose levels within the brain also decrease. A fall in glucose levels within the VMH is associated with increased norepinephrine release in the VMH and the initiation of the counterregulatory response to hypoglycemia\textsuperscript{34,35}. Adrenergic receptors within the brain, particularly the VMH, are important mediators that trigger the sympathoadrenal response to hypoglycemia\textsuperscript{36}. However, recurrent episodes of hypoglycemia can lead to a blunted counterregulatory response to subsequent hypoglycemia as well as hypoglycemia unawareness, characteristics of HAAF. A recent study by Ramanathan et al.\textsuperscript{37} showed that intravenous infusion of adrenergic blockers (phentolamine and propranolol) on day 1 of hypoglycemia prevented the induction of counterregulatory failure in the subsequent response on day 2 of hypoglycemia. This study implicates that HAAF is induced by antecedent sympathoadrenal responses to hypoglycemia. Taken together, extending these findings to their potential pharmacologic and therapeutic implications indicates that paradoxically blocking the action of catecholamines within the CNS may protect against subsequent hypoglycemic bouts by limiting the development of HAAF, but unfortunately, blocking the action of catecholamines in the periphery would tend to increase the severity of acute hypoglycemia. Future research is needed to develop CNS specific adrenergic blockers that alter brain mediated counterregulatory responses without disrupting the peripheral sympathoadrenal response.
In addition to an impaired sympathoadrenal response to hypoglycemia, insulin deficient diabetes also results in loss of the glucagon counterregulatory response to hypoglycemia. The mechanisms that regulate glucagon secretion during hypoglycemia are unclear. Recent work suggests multiple mechanisms of regulation of glucagon secretion including fall in glucose levels, intra-islet crosstalk, and autonomic stimulation.

In response to hypoglycemia the counterregulatory glucagon response may be mediated, as least in part, via direct action on pancreatic α cells. In addition, other mechanisms of intra-islet control of glucagon secretion during hypoglycemia exist including α cell signaling, indirect β cell to α cell signaling, and δ cell secretory products. In particular, δ cell somatostatin secretion can stimulate glucagon secretion during hypoglycemia. Somatostatin within the pancreas and in circulation is elevated in diabetes and may contribute to the defective glucagon response during hypoglycemia. A recent study showed that the glucagon response to hypoglycemia in diabetic rats can be restored by somatostatin receptor type 2 antagonism accomplished by infusion of a selective somatostatin receptor type 2 antagonist during hypoglycemia in streptozotocin-induced diabetic rats. Pancreatic glucagon was elevated in diabetic rats suggesting that there is a glucagon secretory defect in diabetic rats during severe hypoglycemia rather than a glucagon synthesis defect. These studies suggest that elevated somatostatin in diabetes is one mechanism in which the glucagon response is lost during hypoglycemia.
Autonomic inputs of the pancreas include actions from both the parasympathetic and sympathetic nervous systems. Parasympathetic activation includes secretion of acetylcholine (AcH), which bind to receptors directly on the α and β cells\textsuperscript{48}. The sympathetic nervous system releases norepinephrine\textsuperscript{48}. Norepinephrine acts on β2 adrenergic receptors present on pancreatic α cells to stimulate glucagon secretion\textsuperscript{49}. In addition, the sympatoadrenal system results in release of AcH in adrenal medulla cells to release epinephrine which acts on the pancreas to stimulate glucagon secretion\textsuperscript{50}. The redundant mechanisms involved in the regulation of glucagon secretion makes the defects in diabetes more interesting in that many mechanisms have to fail in order get impaired glucagon secretion. Determining which factors are necessary and sufficient for glucagon secretion can potentially help patients at risk for recurrent hypoglycemia (impaired epinephrine responses) better combat hypoglycemic bouts in order to prevent severe hypoglycemia.

**Severe hypoglycemia**

Defects in the brain’s ability to sense changes in glucose levels can lead to impairments in the hypoglycemia induced counterregulatory response, which in a viscous cycle can lead to more severe episodes of hypoglycemia characterized by brain damage, cognitive impairment, and even death. Understanding the mechanisms that lead to severe hypoglycemia-induced brain damage and sudden death is important to help patients who are at risk for hypoglycemia.
Hypoglycemia and brain damage

Acute severe hypoglycemia induces neuronal damage in the cortex and hippocampus. Deficits in learning and memory have been shown to be a direct consequence of severe hypoglycemia-induced hippocampal neuronal damage. However, clinical studies investigating severe hypoglycemia effects on cognition have been variable, with some demonstrating and others not demonstrating cognitive problems. In our laboratory, a rat model of recurrent hypoglycemia revealed that brain damage and cognitive defects induced by severe hypoglycemia could be protected by exposure to recurrent moderate hypoglycemia demonstrating a functional relationship between brain damage and long-term cognitive function. In addition, it has been reported in our lab that diabetes per se increases the extent of severe hypoglycemia induced neuronal damage, but neither the effects on long term cognitive function nor the effects of insulin treatment were studied. Consistent with the notion that insulin deficiency worsens outcomes following brain insults, insulin has been shown to protect cells from death in both in vitro and in vivo studies. It was hypothesized that restoration of near normal glucose levels with chronic insulin therapy in diabetic rats protects against severe hypoglycemia-induced brain damage and cognitive dysfunction and is the focus of chapter 3.

Hypoglycemia and Mortality

Although fortunately rare, severe hypoglycemia can be lethal. The sudden unexplained death of patients with Type 1 diabetes has been noted by Tattersall and Gill with death 10-fold higher in young people with Type 1 diabetes.
The “dead in bed syndrome” identifies otherwise healthy young individuals with Type 1 diabetes that were found dead in bed with no clear cause of death\(^{75,77,79}\). Recent case reports consistently suggest that severe hypoglycemia due to excess insulin administration contributed to their death\(^{70-74,76,77,80}\). Given the rise of the number of cases of the dead in bed syndrome, research is needed in order to understand the mechanisms by which severe hypoglycemia can be lethal.

Central nervous system glucopenia is thought to be the major contributor to severe hypoglycemia-induced mortality. The brain cannot function properly without a continuous supply of glucose from the blood. During hypoglycemia, lack of brain glucose can lead to neuronal damage\(^91\) and seizures\(^{82,83}\) both of which may increase susceptibility to mortality. For instance, studies of sudden death in epileptic patients showed that seizures triggered an autonomic response leading to fatal cardiac arrhythmias\(^{84}\). In our model of insulin induced severe hypoglycemia, lack of brain glucose leads to seizures\(^{60,61}\); however, whether or not seizures correlate with increased risk of fatal cardiac arrhythmias in the setting of severe hypoglycemia is unknown.

Acute onset cardiac arrhythmias are speculated to be the primary cause of severe hypoglycemia-induced sudden death. Clinical studies using electrocardiograms (ECG) have found that cardiac arrhythmias associated with QTc prolongation occur during moderate hypoglycemia in both healthy and diabetic patients\(^{85-90}\). QTc prolongation represents dispersion of ventricle depolarization and repolarization and can lead to increased risk of fatal cardiac arrhythmias\(^{91-94}\). However, due to the difficulty of studying the complications of severe hypoglycemia in a clinical setting, studies are
unable to confirm that fatal cardiac arrhythmias result from severe hypoglycemia in the human population.

In addition to central nervous system counterregulation to hypoglycemia, systemic regulation of hypoglycemia can lead to cardiac arrhythmias. It is well established that enhanced catecholamine levels can cause ventricular arrhythmias. In addition, patients exposed to moderate levels of hypoglycemia (~45 mg/dl) experienced cardiac arrhythmias associated with QTc prolongation that could be rescued with potassium or beta blocker infusion. In the setting of insulin-induced hypoglycemia, both the insulin induced decrease in potassium levels and the counterregulatory induced increase in catecholamine levels have been speculated to contribute to fatal cardiac arrhythmias. This thesis tests the hypothesis that the prevention of hypokalemia and/or adrenergic receptor blockade protects against arrhythmias and cardiorespiratory arrest during severe hypoglycemia. It also examines the extent to which cardiac arrhythmias are centrally mediated. Whether central or systemic, possible mechanisms by which fatal cardiac arrhythmias occur during severe hypoglycemia are increased catecholamine surge, hypokalemia, seizures, and/or hypoxemia that occurs with apneic episodes. Exploration of these potential mediators of sudden death due to severe hypoglycemia is the focus of chapter 4.

Summary

The brain has recently been shown to be an important regulator of whole body glucose homeostasis, but how the brain plays a role in the pathogenesis of diabetes is not well defined. Brain-mediated metabolic regulation has implications for the
development of drug targeting strategies that can target the brain without adverse effects on peripheral metabolism. In addition, diabetes treatment strategies are currently limited due to the concern of iatrogenic hypoglycemia. Therefore, this thesis aims to address how brain glucose sensing controls peripheral metabolism and how the complications of severe hypoglycemia induced brain damage and mortality can be prevented.
Figure 1. The counterregulatory response to hypoglycemia. Hypoglycemia is first sensed in various brain regions including the hypothalamus (HYP) and brain stem. Low glucose in these brain regions stimulates the autonomic nervous system to release norepinephrine and acetylcholine at postganglionic nerve terminals. A principal counterregulatory response is the secretion of glucagon which may be stimulated by various mechanisms including independent α-cell glucose sensing, autonomic innervation, epinephrine stimulation, and a decrement of intra-islet insulin secretion. Via autonomic stimulation, epinephrine is released from the adrenal medulla. Not shown is the hypothalamic-pituitary-adrenal axis by which the release of adrenocorticotropic hormone (ACTH) from the pituitary stimulates cortisol release from the adrenal cortex. The net effect of the autonomic nervous system and counterregulatory hormones is to act at the level of the liver to increase hepatic gluconeogenesis and glycogenolysis while action at muscle and adipose tissue (fat) decrease peripheral glucose utilization.
Chapter 2. Glucose Intolerance, Insulin Resistance, and Impaired Hypoglycemic Counterregulation in Brain GLUT4 Knockout Mice
Abstract

The role of insulin-responsive glucose transporter 4 (GLUT4) in the central nervous system has not been well characterized. To assess its importance, a selective knock-out of brain GLUT4 (BG4KO) was generated by crossing Nestin-Cre mice with GLUT4-floxed mice. BG4KO mice had a 99% reduction in brain GLUT4. Despite normal fed and fasting glycemia, BG4KO mice were glucose intolerant, demonstrated hepatic insulin resistance, and had reduced brain glucose uptake during a hyperinsulinemic clamp. In response to hypoglycemia, BG4KO mice had impaired glucose sensing noted by impaired epinephrine and glucagon responses and impaired c-fos activation in the hypothalamic paraventricular nucleus. Moreover, \textit{in-vitro} glucose sensing of glucose inhibitory neurons from the ventromedial hypothalamus was impaired in BG4KO mice. In summary, glucose intolerance, insulin resistance, and impaired glucose sensing in BG4KO mice indicate a critical role for brain GLUT4 in sensing and responding to changes in blood glucose.
Introduction

The facilitative glucose transporter 4 (GLUT4) is the major glucose transporter in skeletal muscle, heart, and adipose tissue. In response to insulin stimulation, GLUT4 is translocated to the plasma membrane to facilitate glucose entry into the cell\textsuperscript{101,102}. Disruption of GLUT4 in either skeletal muscle or adipose tissue leads to impaired glucose tolerance and insulin resistance, two prominent features associated with the pathogenesis of diabetes\textsuperscript{1,2}. GLUT4 is also expressed in the brain\textsuperscript{21}, although the physiological role of GLUT4 in the brain has yet to be elucidated. The level of brain GLUT4 protein expression appears to be dependent on insulin levels, as the diabetic hyperinsulinemic \textit{db/db} mouse has increased GLUT4 expression while the insulin deficient streptozotocin diabetic rodent has reduced brain GLUT4 levels\textsuperscript{103}. Interestingly, neuronal insulin receptor knockout (NIRKO) mice have a 68\% reduction in hypothalamic GLUT4 protein\textsuperscript{27}. Together, this suggests a potential important role of insulin responsive GLUT4 in the brain.

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\textsuperscript{21}. Interestingly, GLUT4 is co-expressed with the insulin receptor in glucose sensing neurons\textsuperscript{16}. Whether dependent or independent of brain insulin action, GLUT4 is strategically located to play an important role in the neuronal glucose sensing and modulating whole body glucose homeostasis.

To determine the physiological role of GLUT4 in the brain, GLUT4 was selectively knocked-out in the brain using a Cre-Lox approach and the effects on whole
body glucose homeostasis were examined. Mice with brain specific GLUT4 knock-out (BG4KO) were found to be glucose intolerant, have reduced insulin sensitivity, impaired counterregulatory responses to hypoglycemia and impaired hypothalamic glucose sensing in glucose inhibited (GI) neurons. Thus, these studies identify a novel role for brain GLUT4 in regulating whole body energy homeostasis.

These studies are part of co-authored work with a previous graduate student, Erwin Puente. There was equal contribution to the writing and execution of the experiments. I helped write and fully revise the text for this section. I maintained the mouse colony. The experiments which I conducted were the hyperinsulinemic, euglycemic clamps and all assays associated with them including glucose turnover and glucose uptake in brain, adipose, and skeletal muscle. I performed all experiments for western blots of hypothalamic and liver pAkt and Insulin receptor. I performed the hyperinsulinemic, hypoglycemic clamps for the whole body GLUT4 knockout mice and also did the c-fos assays for that experiment. I also set up the collaboration with V Routh for the electrophysiology studies conducted in her lab. I helped with many of the other experiments described in this chapter.

**Methods**

**Animals.** To create brain specific GLUT4 KO mice, GLUT4 floxed mice$^{1,2,104}$ were crossed with transgenic mice that expressed Cre recombinase under the neuron specific promoter nestin$^{105-107}$. Male mice of the C57Bl6 background were used at 2-4 month of age in all studies unless otherwise specified. The 4 experimental groups were: wild-type mice (GLUT4$^{+/+}$;nestin-Cre$^{-/-}$); mice that have both GLUT4 alleles
floxed \( (\text{GLUT4}^{\text{lox-lox}}, \text{nestin-Cre}^{+/+}) \); mice expressing Cre under the nestin promoter \( (\text{GLUT4}^{+/+}, \text{nestin-Cre}^{+/+}) \); and brain specific GLUT4 knockout mice \( (\text{GLUT4}^{\text{lox-lox}}, \text{nestin-Cre}^{+/+}) \). For whole body GLUT4 knockout studies, Zona pellucida Cre mice were crossed with GLUT4 lox mice and generously provided by B. Kahn. Mice were genotyped by PCR analysis of DNA extracted from tail tissue using previously established primers and PCR conditions\(^1,2,105,106\).

Mice and male Sprague-Dawley rats 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 ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Studies Committee of Washington University.

**Western blot analysis.** Brain, skeletal muscle, heart, and white adipose tissue were homogenized, centrifuged, and 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)\(^27\). Hypothalamus and liver were immunoblotted with antibodies against pAKT (1:500, Cell Signaling, Danvers, MA), tAKT (1:500, Cell Signaling, Danvers, MA) Insulin Receptor β (anti-CD220, 1:500 BD Biosciences, San Jose, CA) and β-actin from 5 hour fasted mice 30 minutes (hypothalamus) or 15 minutes (liver) after intraperitoneal injection of human regular insulin (3U/kg body weight) or saline injection. 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). Tail vein blood samples (20 μl) were taken before 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 (Bayer HealthCare, LLC, Mishawaka, IN) blood glucose monitors.

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

**Hyperinsulinemic-euglycemic clamp, glucose kinetics analysis, and brain glucose uptake.** Hyperinsulinemic (4 mU.kg\(^{-1}\).min\(^{-1}\))-euglycemic (~110 mg/dl) clamp studies were performed as previously described\(^{108}\). 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, samples were taken for basal plasma insulin levels and plasma [3H]-glucose measurements. A primed (40 mU/kg) continuous infusion (4 mU.kg⁻¹.min⁻¹) 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.

Tissue-specific insulin-stimulated glucose uptake was measured with 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 were taken immediately before 2-[¹⁴C]DG administration and at 0.5, 2, 3, 5, 10, 15, 20, 30, and 45 min following administration. Blood samples were taken at the end of the clamp for plasma [3H]glucose and plasma insulin measurements. Immediately following the clamp, mice were euthanized and skeletal muscle and adipose tissue were rapidly dissected and frozen in liquid nitrogen. Brains were harvested and quickly frozen in a dry ice and 2-methylbutane (Fisher, Saint Louis, MO) bath (-20°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 at steady state from the ratio of the [³H] glucose infusion rate to the measured specific activity of plasma glucose. Hepatic glucose production (HGP) was determined by subtraction of the glucose infusion rate from the whole-body glucose turnover. 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.

For brain glucose uptake calculations, isotope concentrations in regions of interest were measured from 20μm thick coronal serial 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 desired areas (figure 4). Mean local rates of glucose utilization were calculated according to Sokoloff’s equation with rat rate constants, as mice rate constants have not been calculated.

Hyperinsulinemic-hypoglycemic mice clamps and c-fos immunostaining. Hyperinsulinemic (20 mU.kg⁻¹.min⁻¹) hypoglycemic (~30 mg/dl) clamps were performed as previously described. Four month old mice underwent 2 hour hyperinsulinemic (20 mU.kg⁻¹.min⁻¹) hypoglycemic (30 mg/dl) clamps as previously described. A variable 12.5% glucose infusion was used to carefully match blood glucose levels to 30 mg/dl. Blood samples were taken in the basal period and at the end of the clamp for hormone assays.
At the end of the 2 hr hypoglycemic clamp, mice were 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. Four anatomically matched sections per animal were used to quantify c-fos immunostaining in the paraventricular nucleus of the hypothalamus (PVN).

To test brain glucose sensing in a different model of GLUT4 knockout mice, hyperinsulinemic (120mU.kg\(^{-1}\).min\(^{-1}\)) hypoglycemic (~35 mg/dl) clamps were performed in 5 week old whole body GLUT4 knockout mice created by crossing floxed GLUT4 mice with zona pellucida Cre expressing mice. The resultant KO mice lacked GLUT4 in all tissues, including the brain. During the hypoglycemic clamp, variable 50% glucose was infused to match blood glucose levels, as described above.

**Heat Stress.** Awake BG4KO and littermate control mice were exposed to an ambient temperature of 42°C for 60 minutes to induce heat stress. Arterial blood samples were taken from an indwelling cannula before and at the end of the heat stress period to measure epinephrine levels.

**Analytical measurments.** Blood glucose was measured by Ascensia Contour blood glucose monitors (Bayer) and plasma glucose was assayed by the glucose oxidase method and a spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Insulin levels were measured by enzyme-linked immunosorbsent assay (ELISA) (Crystal Chem,
Chicago, Illinois). Catecholamine analysis was performed by a single isotope-derived (radioenzymatic) method\textsuperscript{112}. Radioimmunoassays were performed for glucagon (Linco Research, St. Charles, MO) and corticosterone (MP Biomedicals, Orangeburg, NY) measurements.

**Pharmacological inhibition of brain GLUT4 by indinavir (IDV).** Following recovery from intracerebroventricular cannula (Plastics One Inc, Roanoke, VA) and vascular implantation surgery, nine week-old male Sprague Dawley rats were subjected to a hyperinsulinemic (20 mU.kg\(^{-1}\).min\(^{-1}\)) hypoglycemic clamp (~45 mg/dl) as previously described\textsuperscript{113}. 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\textsuperscript{114-116}. Arterial blood samples were taken in the basal period and at 45, 60, and 90 min into the hyperinsulinemic clamp.

**Electrophysiological studies.** BG4KO and nestin-Cre positive littermate control mice (28-42 day old) were anesthetized and brains sectioned as previously described\textsuperscript{27}. VMH GI neurons from both control and BG4KO mice were identified as reversibly depolarized and increased their action potential frequency and input resistance in response to a glucose decrease from 2.5 to 0.1mM. Input resistance was calculated by dividing the membrane voltage change (mV) by the current (pA). The effects of glucose and insulin (5nM) on action potential frequency, membrane potential and input resistance were
evaluated in current-clamp recordings (standard whole-cell recording configuration) from neurons in the VMH as previously described\textsuperscript{27,117,118}. Current-voltage relationships were determined in the voltage-clamp recording mode using 10mV voltage steps from -50 to -150mV from a holding potential of -60mV.

**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 brain-specific GLUT4 deletion**

Using the Cre-Lox system, brain specific GLUT4KO mice were created by crossing mice that express Cre driven by the nestin promoter with mice that have exon 10 of \textit{GLUT4} flanked by loxP\textsuperscript{1,2,104}. Four experimental groups were chosen for phenotypic analysis: wild-type mice (WT); mice that have both GLUT4 alleles floxed (Lox); mice expressing Cre under the nestin promoter (Cre); and brain specific GLUT4 knockout mice (BG4KO) that are homozygous GLUT 4 floxed and express Nestin Cre. 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 adipose tissue. BG4KO mice had a greater than 99% reduction in brain GLUT4 protein levels relative to WT, Lox, and Cre mice (Figure 2). Importantly, GLUT4 content in the heart, muscle, and adipose tissue of BG4KO mice was expressed at similar levels to the littermate controls (Figure 2), verifying brain specific GLUT4 knock-out.
To determine if deletion of brain GLUT4 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 BG4KO and littermate controls (Figure 3A,B). Furthermore, to confirm brain insulin signaling was unaltered by brain GLUT4 deletion, insulin receptor and pAKT protein were measured in the hypothalamus after a bolus IP insulin injection (3U/kg). There were no significant differences in insulin receptor or insulin stimulated pAKT protein expression between the groups (Figure 3C).

Effect of Brain GLUT4KO on energy and glucose homeostasis.

Body weight was measured weekly from 5 to 12 weeks of age. No significant difference in body weight was observed between Cre and BG4KO mice (Figure 4A). Intriguingly, both Cre and BG4KO mice had significantly reduced body weights compared to WT and Lox mice (Figure 4A). No significant difference in body weight was observed between WT and Lox mice (Figure 4). Thus, the expression of Cre itself reduced body weight consistent with a previous report\textsuperscript{119}.

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 body weight, Cre and BG4KO were significantly shorter than WT and Lox mice (Figure 4B). Brain mass and perigonadal fat pad mass were not significantly different between groups (Figure 4C).
Brain GLUT4KO does not affect fasting or fed blood glucose levels, but results in glucose intolerance.

To assess the effect of the brain GLUT4KO on whole-body glucose homeostasis, fed and fasting glucose and insulin levels were measured in 12 week old mice on a normal chow diet. Fasting and random fed glucose levels were similar in all groups (Figure 5A). Further, no difference in fasting or fed insulin levels were observed between groups (Figure 5B).

To determine the role of brain GLUT4 in handling and responding to changes in glucose, dynamic tests were performed in overnight fasted mice. In response to an intraperitoneal glucose tolerance test (IPGTT; 2 mg/kg) BG4KO mice had significantly higher glucose excursions during the GTT than WT, Lox, and Cre littermates (Figure 5C). To determine whether brain GLUT4 deletion also affected whole body insulin sensitivity, insulin tolerance tests (ITT, 1 U/kg) were performed in BG4KO and control mice. Despite the impaired glucose tolerance, BG4KO mice had similar reduction in blood glucose in response to insulin compared to littermate controls (Figure 5D). To determine whether the impaired glucose tolerance in BG4KO mice was attributed to altered glucose stimulated insulin secretion (GSIS), plasma insulin levels were measured during the IPGTT. No difference in plasma insulin levels during the glucose tolerance test (Figure 5E) and no difference in first phase insulin secretion (Figure 5F) were observed between BG4KO mice and controls.
BG4KO have reduced insulin sensitivity and hepatic insulin resistance

The effect of brain GLUT4KO on whole body insulin sensitivity was directly assessed by hyperinsulinemic-euglycemic clamps in BG4KO mice and controls. For statistical analyses, littermate control WT, Lox, and Cre mice were combined into one control group (CON) since no statistically significant differences were observed between these groups during the euglycemic clamp (e.g. glucose infusion rate, glucose kinetics, hepatic glucose production). Blood glucose levels during the baseline period and during the last hour of the clamp were not statistically different between BG4KO and CON (Figure 6A). Basal and clamp insulin were also not significantly different between the groups (data not shown). Interestingly, BG4KO mice required 22% lower glucose infusion rate (34±3 versus 43±2 mg/kg/min; p<0.02) to maintain euglycemia compared to controls, demonstrating whole-body insulin resistance (Figure 6B). Since BG4KO mice had similar basal glucose disposal (R_d) and similar insulin-stimulated increase in R_d as compared to CON mice, the defect in whole-body insulin sensitivity was not due to changes in glucose disposal (R_d) (Figure 7A). Consistent with similar R_d values between groups, no difference in tissue-specific glucose uptake in muscle or adipose tissue was observed between BG4KO and CON mice (Figure 7B, C). However, the ability of insulin to suppress endogenous glucose production (EGP) was only 48% in BG4KO as compared to 79% in CON mice (Figure 8A, B, p<0.02). To test if insulin resistance is due to impaired hepatic insulin signaling, phospho AKT (pAKT) and total AKT (tAKT) protein expression were analyzed by Western blot following IP insulin. In
response to insulin (3U/kg), hepatic pAKT protein expression increased similarly in BG4KO and control mice (Figure 8C, D).

To determine whether deletion of GLUT4 in the brain results in altered glucose uptake, brain glucose uptake was measured using a radioisotope glucose uptake assay during a 2-hour hyperinsulinemic euglycemic clamp. Mean local rates of glucose uptake were calculated according to Sokoloff’s equation\textsuperscript{110,111}. Brain glucose uptake was lower in BG4KO mice compared to control mice in select regions of the brain including the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) (p<0.05 t-test) with a trend for decreased uptake in the paraventricular nucleus (PVN), hippocampus, cerebellum, nucleus tractus solitarius (NTS), and cortex (Figure 9A, B).

Role of central GLUT4 on the counterregulatory response to hypoglycemia

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

To test whether brain GLUT4 is important in the counterregulatory response to hypoglycemia, indinavir (IDV), 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 hypoglycemic (~45 mg/dl) clamp. IDV is a pharmacologic agent that has been shown to inhibit GLUT4 activity and glucose transport\textsuperscript{114-116}. 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 ~45 mg/dl was 44% higher in the
IDV-treated rats compared to controls (Figure 10B, 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 10C,D,E).

**Genetic deletion of brain GLUT4 impairs the counterregulatory response to hypoglycemia**

A genetic approach was used to further elucidate the role of brain GLUT4 in the counterregulatory response to hypoglycemia. Hyperinsulinemic hypoglycemic (~30 mg/dl) clamps were performed in chronically cannulated, freely mobile BG4KO mice and their littermate controls. Basal glucose levels were similar and glycemia was carefully matched during the clamp period (Figure 11A). Insulin levels were also similar in the basal and clamp conditions (Figure 11B). Despite matched glucose levels, BG4KO mice required a lower glucose infusion rate to maintain ~30 mg/dl compared to Cre controls (315±162 vs 611±125 AUC; p < 0.05 ANOVA). However, glucose infusion rate was not statistically significantly different to WT and flox controls (data not shown). Basal epinephrine levels were similar in all groups (Figure 11C). 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, the epinephrine response in BG4KO was significantly lower (1575±101 pg/ml) compared to littermate controls (P<0.05) (Figure 11C). Further, glucagon response to hypoglycemia was significantly attenuated in BG4KO mice (BG4KO 299±92 pg/ml; WT 650±57; Lox 597±74; Cre 651±39 pg/ml, p
< 0.001) (Figure 11D). Norepinephrine and corticosterone responses to hypoglycemia were not significantly different between the 4 groups (Figure 11E, F).

**Impaired adrenomedullary response to stress is unique to hypoglycemia**

To test if the reduced epinephrine response to hypoglycemia in BG4KO mice was due to a generalized impairment in the adrenomedullary response to stress, epinephrine levels were measured in BG4KO and control mice subjected to 60 min of heat (42°C) stress. There was a significant increase in epinephrine and norepinephrine levels induced by heat stress but the increment was similar in both groups of mice (Figure 12A, B).

**Brain GLUT4KO reduces hypoglycemic-induced neuronal activation in the paraventricular nucleus**

To determine whether the impaired counterregulatory response in the BG4KO mice was associated with impaired hypothalamic neuronal activation, c-fos activation in response to hypoglycemia was measured in the hypothalamus following the hyperinsulinemic hypoglycemic clamps. Despite matched levels and duration of hypoglycemia, BG4KO mice had ~80% reduction in paraventricular nucleus (PVN) c-fos positive cells compared to controls (BG4KO 28 ± 17 versus WT 120 ± 20, Lox 138 ± 19, Cre 141 ± 20 c-fos positive cells, p < 0.001) (Figures 13A, B).

To further test the role of brain GLUT4 glucose sensing in response to hypoglycemia, whole body (zona-pellucida Cre driven) GLUT4 knockout mice underwent hyperinsulinemic (120mU.kg⁻¹.min⁻¹) hypoglycemic (~35 mg/dl) clamps.
Decreased neuronal activation during hypoglycemia was confirmed in these whole body GLUT4 knockout mouse model. Whole body GLUT4 knockout mice that underwent 2 hours of hypoglycemia had reduced number of c-fos positive cells in the PVN (G4KO 67.6±18 vs WT 142.1±28; p<0.05) and VMH (G4KO 22.6±9 vs WT 53.1±4; p <0.05; Figure 13C, D).

Brain GLUT4KO causes impaired neuronal glucose sensing

In order to test whether the observed impaired counterregulatory response to insulin-induced hypoglycemia in BG4KO mice is a result of impaired brain glucose sensing or impaired insulin action, changes in membrane potential and input resistance of individual glucose inhibited (GI) neurons of the ventromedial hypothalamus (VMH) were measured using whole-cell current clamp recordings in brain slices both in the presence and absence of insulin. As expected for VMH GI neurons bathed in sufficient 2.5mM glucose, action potential frequency in this basal state was low in recordings from control mice (0.43 +/- 0.12 Hz; N=14). Although, baseline action potential frequency tended to be higher in recordings of VMH GI neurons from BG4KO mice (0.75+/0.22; N=16) it did not reach statistical significance due to interneuron variation. On the other hand, VMH GI neurons from BG4KO mice exhibited significantly higher baseline input resistance (BG4KO: 1465+/-90MΩ, N = 16; Control: 1042+/-77 MΩ, N=14; P<0.01) and a depolarized membrane potential compared to GI neurons from control mice (BG4KO: -56+/-1mV; Control: -60+/-1mV; P<0.01). Increased excitability of VMH GI neurons in the absence of Glut4 is consistent with reduced glucose uptake.
The glucose sensitivity of VMH GI neurons from control and BG4KO mice is illustrated by the whole cell current clamp recordings in figures 14A and 14B, respectively. No group differences were observed in GI neurons in response to a maximal glucose decrease from 2.5 to 0.1mM. In contrast, GI neurons from BG4KO mice had a significantly (p<0.0001) impaired change in membrane potential and input resistance (62% and 71% impairment, respectively) in response to a glucose decrease from 2.5 to 0.5mM (Figures 14A-D) indicating BG4KO mice have impaired glucose sensing.

To determine whether the effects of insulin on VMH GI neurons are mediated by GLUT4, we evaluated the effects of 5nM insulin on the electrical properties of VMH GI neurons from BG4KO and control mice. Interestingly, insulin reversed the excitatory effects of 0.1mM glucose by decreasing input resistance and hyperpolarizing the membrane potential of VMH GI neurons to a similar extent in both genotypes (BG4KO % change: membrane potential -8+/−0.4, input resistance -14+/−0.7, n=7; control %change: membrane potential -9+/−0.6, input resistance -16+/−1, n = 8; P=NS between genotypes) indicating insulin’s actions on these neurons is independent of GLUT4. Particularly interesting was that the current-voltage relationships obtained in voltage clamp indicate that the reversal potential for the effects of insulin and glucose were significantly (p<0.05) different, with the effect of insulin reversing near the K⁺ equilibrium potential (-94+/−4mV, n =4; Kₑq -99mV) and the effect of glucose reversing near the Cl⁻ equilibrium potential (-71+/−6, n=5; Clₑq -60mV, figure 14E).
Figure 2. 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 markedly reduced in the brain of BG4KO mice (BG4KO) 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 BG4KO and control mice. (B) Quantification of brain GLUT4 protein content. BG4KO (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 3. Brain GLUT1, GLUT3, pAKT, and insulin receptor protein expression was not altered in neuronal GLUT4 KO mice. (A) Brain GLUT1 and (B) brain GLUT3 protein expression as measured by Western blot analysis was not different in brain 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). C) Hypothalamic phospho-AKT (pAKT) and insulin receptor (IR β) protein expression were not different between BG4KO (gel lanes 3 and 4, black bar) and control mice (Cre, gel lanes 1 and 2, white bar) 30 minutes after an IP insulin (3U/kg) injection. Data expressed as mean ± S.E.M. n=4-5 per group.
Figure 4. Brain GLUT4 knock-out did not affect body weight. (A) Body weight was similar between Cre (open triangle) mice and BG4KO (closed triangle) mice. Cre and BG4KO 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. (B) The reduction in body weight was attributable to smaller crown-rump length in Cre (slanted hash) and BG4KO (black bar) mice compared to wild type (open bar) and Lox (horizontal hash) mice. (C) 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 5. Normal blood glucose but impaired glucose tolerance in BG4KO mice.
(A) Male BG4KO (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). (B) BG4KO mice had similar fed and fasting blood insulin levels compared to controls. (C) Intraperitoneal glucose tolerance tests (IPGTT, 2 mg/kg) were performed in 12 week old male mice. BG4KO mice (closed
triangles) had significantly higher excursions in blood glucose compared to WT (open circles), Lox (closed circles), and Cre (open triangles) mice where indicated. (D) No difference in the blood glucose was observed between BG4KO and control mice during an insulin tolerance test. (E) Insulin secretion during a glucose tolerance test was similar between BG4KO (closed triangle, n=4), wild-type (open circle, n=9), lox (closed circle, n=4) and Cre mice (open triangle, n=4) (p=NS). (F) In a separate study, first phase insulin secretion was measured during an intraperitoneal injection of glucose in BG4KO (closed triangle, n=7) and Cre controls (open triangle, n=8). First phase insulin secretion was not significantly different between groups. *p < 0.05 vs. WT, Lox, Cre.

Data expressed as mean ± S.E.M. n=6-17 mice per group.
Figure 6. Euglycemic clamp glucose and glucose infusion rate. (A) Blood glucose during a hyperinsulinemic euglycemic clamp. No difference in blood glucose before or during the last hour of the clamp was observed between BG4KO (closed triangles) and control mice (CON, open triangles). (B) Despite matched blood glucose levels, BG4KO mice (closed triangles) required a significantly lower glucose infusion rate to maintain euglycemia compared to CON (open triangles) indicating insulin resistance (*p < 0.05, BG4KO vs. CON, all timepoints). Data expressed as mean ± S.E.M. n= 12-20 mice per group. CON= WT, Flox, and Cre mice.
Figure 7. Normal glucose disposal in BG4KO mice during a euglycemic clamp. (A) Rate of whole body glucose disposal at baseline and during the last 30 minutes of the hyperinsulinemic clamp was not significantly different between BG4KO (black bar) and CON (open bar). (B and C) $^{14}$C-2deoxyglucose determined glucose uptake specifically in muscle (B) and adipose tissue (C) was similar between BG4KO (black bar, n=7) and CON (white bar, n=17) during the hyperinsulinemic clamp. Data expressed as mean ± S.E.M. n= 12-20 mice per group. CON= WT, Flox, and Cre mice. All data expressed as mean ± S.E.M.
Figure 8. Hepatic insulin resistance in BG4KO mice during a euglycemic clamp.

(A) Insulin suppressed endogenous glucose production (EGP) in BG4KO (black bar) mice to a lesser degree as compared to CON mice (white bar) (BG4KO 12.3±2.3 vs controls 5.1±2.3 mg/kg/min, *p < 0.04, ANOVA) B) Percent suppression of endogenous glucose production was reduced in BG4KO mice compared to controls (BG4KO 48±8% vs controls 79±8% suppression, *p < 0.02) during the last hour of the hyperinsulinemic clamp. C) Hepatic expression of phospho-AKT (pAKT), total AKT (tAKT) and β-actin as shown by western blot after an IP bolus insulin (3U/kg) or saline injection. D) Quantification of hepatic pAKT shows that in both control (CON) and BG4KO mice hepatic pAKT increases in response to insulin (black bar) equally as compared to saline (white bar) in both groups (n=3 per group). Data expressed as mean ± S.E.M. n= 12-20 mice per group. CON= WT, Flox, and Cre mice. All data expressed as mean ± S.E.M.
Figure 9. Reduced brain glucose uptake in BG4KO mice. Brain glucose uptake was measured using $^{14}$C-2-deoxyglucose autoradiography in mice that underwent a hyperinsulinemic euglycemic clamp. (A) Precise identification of the areas of interest were accomplished by Nissl staining (left panels) and identifying 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 exposed autoradiographic films (right panels). (B) Quantification of brain glucose uptake during euglycemia using Sokoloff’s equation in BG4KO (black bar, n=5) and control mice (white bar, n=5). Brain glucose uptake during the hyperinsulinemic euglycemic clamp was significantly reduced in BG4KO mice compared to control mice in ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) (*p < 0.05, t-test). Data expressed mean ± S.E.M.
Figure 10. Intracerebroventricular (ICV) infusion of an inhibitor of GLUT4 transport, indinavir, reduced the counterregulatory response 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 hypoglycemic (~45 mg/dl) clamp in Sprague-Dawley rats. (A) Blood glucose was precisely matched between rats treated with ICV infusions of either IDV or aCSF. (B) Despite matched blood glucose levels, IDV treated rats (closed circle) required a significantly higher glucose infusion rate than control rats (open circle) (*p < 0.01, t-test of all timepoints). The higher glucose infusion rate was attributed to the attenuated counterregulatory response to hypoglycemia due to the reduced GLUT4-mediated glucose sensing. Epinephrine (C), norepinephrine (D), and glucagon (E) response to hypoglycemia was significantly reduced in IDV rats versus controls (* p<0.05 by ANOVA for all hypoglycemic timepoints for epinephrine and norepinephrine, AUC for glucagon). Data expressed mean ± S.E.M.
Figure 11. BG4KO mice have impaired counterregulatory response to hypoglycemia. BG4KO and control mice were subjected to hyperinsulinemic hypoglycemic (~30 mg/dl) clamps. (A) Blood glucose was not different before or during the clamp between BG4KO (closed triangle, n=5), wild-type (open circle n=5), Lox (closed circle, n=6), and Cre (open triangle, n=11). B) Insulin levels at basal and during the hypoglycemic clamp were not different between the groups. Wild type (open bar), Lox (horizontal hash), Cre (slanted hash) and BG4KO (black bar). (C and D) Epinephrine (C) and glucagon (D) responses to hypoglycemia were significantly reduced in BG4KO (black bar) mice compared to WT (open bar), Lox (horizontal hash), and Cre (slanted hash). Norepinephrine (E) and corticosterone (F) 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. n= 4-11 per group.
Figure 12. Normal sympathoadrenal response to heat stress in brain GLUT4 knock-out mice. Heat stress was induced to determine if the impaired counterregulatory response in BG4KO mice was specific for hypoglycemia. Norepinephrine (A) and epinephrine (B) levels increased during heat stress but there was no difference between the control (white bar) or BG4KO mice (black bar). Data expressed as mean ± S.E.M.
Figure 13. Brain specific and whole body GLUT4 KO mice have impaired hypothalamic neuronal activation during hypoglycemia. A and B) Nestin-Cre GLUT4 KO mice (A) Representative c-fos immunostaining of the hypothalamic paraventricular nucleus (PVN) in wild-type (WT) and BG4KO (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 BG4KO mice (black bar) compared to wild-type (open bar), Lox (horizontal hash), and Cre (slanted hash). *p < 0.05 versus WT, Lox, and Cre. n= 4-11 per group. C and D) Zona-Pellucida Cre GLUT4 KO mice (Whole body GLUT4 KO) (C) Immunohistochemistry of brain sections from WT (left) and G4KO (right) mice after 2 hours of a hypoglycemic clamp. C-fos positive staining is shown in the paraventricular nucleus (PVN) and ventromedial hypothalamus (VMH) of WT and G4KO mice. (D) Quantification of c-fos positive cells represented as total number per
mouse. G4KO mice (black bar) had significantly less $c$-$fos$ positive cells in the PVN and VMH compared to WT mice (white bar). PVN: $68\pm18$ vs. $142\pm28$ (*$p<0.04$). VMH: $23\pm9$ vs. $53\pm4$ (*$p<0.02$). n=6/group. Data presented as mean±sem. 3V= third ventricle
Figure 14. Impaired glucose sensing in VMH GI neurons from BG4KO mice. Whole cell current clamp traces of VMH glucose-inhibited (GI) neurons in brain slices from nestin-cre control (A) and BG4KO (B) mice. The upward deflections represent action potentials, the resting membrane potential is given to the left of each trace, and the downward deflections are the voltage response to a constant current pulse. As compared to GI neurons from controls (white bars), GI neurons from BG4KO mice (black bars) had markedly impaired changes in membrane potential (C) and input resistance (D) when glucose levels were changed from 2.5 mM to 0.5 mM. E) Current-voltage relationship for a VMH GI neuron from control mice. The reversal potential for the effect of glucose (2.5 vs 0.1 mM glucose, upward dotted arrow) is close the theoretical Cl- equilibrium potential ($E_{Cl} \approx -60$ mV) suggesting that glucose activates a chloride channel. In contrast the reversal potential for the effect of insulin (0.1mM
glucose vs 0.1mM glucose + 5 nM insulin, downward dashed arrow) is near the theoretical K+ equilibrium potential (EK ~-99mV) suggesting that insulin activates a potassium channel. *p < 0.001 versus control, data expressed as mean ± S.E.M. n=6-9 per group. Performed in collaboration with Dr. Vanessa Routh, New Jersey Medical School.
**Discussion**

Based on immunohistochemical studies that identified GLUT4 in discrete areas of the central nervous system including the hypothalamus, brain GLUT4 has been suggested to have a role in neuronal glucose sensing and whole body energy homeostasis. However, the physiological roles of central GLUT4 have remained unknown. This current study demonstrated a novel role of brain GLUT4 in the modulation of whole body glucose homeostasis, brain glucose sensing, and the counterregulatory response to hypoglycemia.

Tissue specific deletion of brain GLUT4 was accomplished by Cre-Lox technology. As expected, knockout of GLUT4 in the brain was virtually complete, while GLUT4 protein expression was still expressed at normal levels in heart, adipose tissue, and skeletal muscle. Further evidence for unaltered peripheral expression of GLUT4 was noted by similar rates of whole body, adipose, and skeletal muscle glucose uptake in BG4KO and control mice during a hyperinsulinemic euglycemic clamp.

An interesting finding in this study was the expression of nestin-Cre alone resulted in a slight reduction in body weight and crown-rump length, similar to previous studies, which may be attributed to Nestin-Cre’s mild hypopituitarism. Since both BG4KO and nestin-Cre mice express Cre, the lack of difference in body weight between these two Cre groups indicate that deletion of brain GLUT4 did not have an effect on overall body weight. In consideration of this effect of nestin-Cre expression, all experiments were compared to Cre littermates as a control group. Notably though, WT, lox, and Cre mice had similar metabolic phenotypes with regard to 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 a smaller size, it did not affect any other measured metabolic phenotype parameters. Importantly, a pharmacologic approach using indinavir to inhibit brain GLUT4 glucose transport during hypoglycemia produced similar results as the genetic approach indicating brain GLUT4 is important in brain glucose sensing. Consistent with a critical role for GLUT4 in mediating brain glucose sensing, the whole body G4KO mice, recently generated in the Kahn laboratory, also showed impaired neuronal activation in response to hypoglycemia.

Fasting and fed glucose levels were similar in BG4KO and control mice, suggesting little contribution of brain GLUT4 in regulating basal glucose homeostasis. When challenged with a glucose load during an IP glucose tolerance test, BG4KO mice had significantly higher blood glucose excursion than control mice indicating glucose intolerance. Consistent findings of glucose intolerance have recently been reported in a distinct GLUT4 neuron ablation mouse model (Ren and Accili, Diabetes Suppl.1, A6, 2011). Dynamic insulin tolerance tests, showed no difference in blood glucose lowering effect of insulin between BG4KO and control mice. The impaired glucose tolerance, but seemingly normal insulin sensitivity, could be attributed to diminished insulin secretion. However, glucose-stimulated insulin secretion was measured and no difference in insulin levels was seen between BG4KO and control mice after a glucose challenge. Thus, pancreatic β-cell responses to glucose remained unaffected by brain GLUT4 deletion.

An insulin tolerance test is an imprecise measure of insulin sensitivity because with falling blood glucose levels, differential counterregulatory responses to
hypoglycemia (specifically an attenuated counterregulatory response) may mask the
degree of insulin resistance. To directly assess insulin sensitivity, hyperinsulinemic-
euglycemic clamps were performed in BG4KO and control mice. BG4KO mice required
a significantly lower glucose infusion rate to maintain euglycemia than 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,
BG4KO mice had a similar rate of glucose disposal as control mice. Further, glucose
uptake in skeletal muscle and adipose tissue was not different between BG4KO and
control mice. The insulin resistance in BG4KO mice can be attributed to hepatic insulin
resistance as noted by the impaired ability of insulin to suppress endogenous glucose
production. Since insulin stimulated AKT phosphorylation in the liver was found to be
normal in BG4KO mice, hepatic insulin resistance in BG4KO mice seems to be
independent of direct insulin signaling in the liver and is likely mediated via indirect
mechanisms. Potential indirect roles of altered suppression of plasma free fatty acid
levels in indirectly regulating EGP\textsuperscript{122} could not be determined as free fatty acids were
not measured in this current study. Recent studies have demonstrated a role for central
insulin signaling as an indirect mechanism by which insulin acts to suppress EGP,
possibly via efferent vagal and sympathetic actions\textsuperscript{123,124}. The absence of brain GLUT4
in BG4KO mice may have limited the central actions of insulin to indirectly suppress
EGP; although this is not due to proximal defects in hypothalamic insulin signaling as
this study demonstrates hypothalamic insulin signaling is preserved in BG4KO mice,
both \textit{in vivo} and \textit{in vitro}. One explanation for hepatic insulin resistance in BG4KO mice
may be that chronic adaptations in BG4KO mice led to altered neuronal development and plasticity altering the neuronal circuitry involved in glucose homeostasis\textsuperscript{125}. Alternatively, reduced centrally mediated insulin inhibition of glucagon secretion may lead to a failure to suppress hepatic glucose production compared to controls\textsuperscript{126}. Although glucagon was not measured during the euglycemic clamp, impaired regulation of glucagon secretion in the BG4KO mice was noted in the hypoglycemia studies. Regardless of the mechanism, the results indicate that insulin acts to regulate hepatic glucose production, at least in part, through brain GLUT4.

Several studies have suggested a relationship between central insulin action and GLUT4\textsuperscript{16,21,127}. When experimental conditions of hyperinsulinemia were studied that could maximize the potential difference in brain glucose uptake between BG4KO and control mice, brain glucose uptake into regions that normally express relatively high levels of GLUT4 was reduced in BG4KO mice. The reduced brain glucose uptake in BG4KO mice was expected to be relatively small because GLUT4 is normally expressed at a low level and only expressed in a fraction of all neurons\textsuperscript{16}. Although recent studies have shown insulin-stimulated GLUT4 translocation occurs in neurons\textsuperscript{22,114,128} the current in vivo techniques are not of sufficient detail to resolve individual neuron glucose uptake. Further in vitro studies from neurons of BG4KO mice are needed to assess the role of GLUT4 neuron specific glucose uptake.

As GLUT4 and insulin receptors are co-expressed in up to 75\% of glucose sensing neurons in the VMH\textsuperscript{16}, brain GLUT4 may have an important role in the ability of neurons to sense and respond to changes in glucose. To test this possibility, the counterregulatory response to hypoglycemia was assessed in rodents that had central
GLUT4 inhibited pharmacologically (by indinavir) or genetically (BG4KO mice). Indinavir has been shown to selectively inhibit glucose transport through GLUT4 in both cell culture and in brain slices\(^{114-116}\). Both pharmacological inhibition as well as genetic deletion of central GLUT4 resulted in impaired epinephrine response as well as an attenuated glucagon response to hypoglycemia. Acute pharmacological inhibition of CNS GLUT4 was associated with a blunted counterregulatory response, demonstrated by an appropriately elevated glucose infusion rate during the hypoglycemic clamp. However, the paradoxically low glucose infusion rates in the BG4KO mice were likely due to their insulin resistance. Interestingly, since streptozotocin diabetic rats have reduced brain GLUT4 expression\(^{103}\) and impaired epinephrine and glucagon responses to hypoglycemia\(^{129}\), it may be that the impaired counterregulatory response to hypoglycemia associated with insulin deficient diabetes is mediated, at least in part, by decreased brain GLUT4.

To determine whether this impaired counterregulatory response was unique to hypoglycemia or a result of a more global impairment in the sympathoadrenal response to general stress, BG4KO and control mice were subjected to heat stress. The adrenomedullary responses to heat stress were identical in BG4KO and control mice indicating that BG4KO mice have an intact sympathoadrenal response to stress and that the impaired counterregulatory response in BG4KO is unique to hypoglycemic stress.

To determine whether the impaired counterregulatory response to hypoglycemia in the BG4KO mice was associated with impaired neuronal activation, c-fos staining was quantified in hypothalamic PVN of mice that underwent a hyperinsulinemic
hypoglycemic clamp\textsuperscript{130}. In response to hypoglycemia, \textit{c-fos} expression increased predominantly in the PVN, consistent with previous studies\textsuperscript{131}. Notably, PVN \textit{c-fos} activation to hypoglycemia was significantly reduced in BG4KO mice compared to controls. This finding of impaired \textit{c-fos} activation in the PVN in response to hypoglycemia was independently confirmed in another whole body (including the brain) GLUT4 knockout model in which there was impaired neuronal activation in the PVN and VMH in response to hypoglycemia. The impaired hypoglycemia-induced \textit{c-fos} activation in the brains of both of these GLUT4 knockout mouse models could represent reduced glucose sensing of PVN neurons or, given that the PVN receives input from other glucose sensing neurons in other regions of the brain, could represent an indirect reduction in afferent inputs from other brain regions. In either case, the brains of GLUT4 knockout mice have a profound impairment in \textit{c-fos} activation, consistent with other models of impaired glucose sensing and impaired counterregulation\textsuperscript{130}. To more specifically identify the role of brain GLUT4 in glucose sensing within ventromedial hypothalamic neurons, electrophysiological studies were performed. GI neurons from the VMH of BG4KO mice have an impaired response to decreasing glucose concentrations as measured by whole-cell clamp recordings thus supporting the findings that BG4KO mice have impaired hypothalamic glucose sensing.

The simultaneous attainment of hyperinsulinemia and hypoglycemia with the hyperinsulinemic clamp studies do not allow us to determine whether reduced brain glucose sensing/uptake in the BG4KO mice is due to the absence of the acutely induced insulin-mediated neuronal GLUT4 translocation or reduced glucose transport due to the chronic absence of GLUT4, however our electrophysiology data support the
latter. First, in the absence of insulin, the finding of enhanced baseline excitability of VMH GI neurons from BG4KO mice is consistent with reduced intracellular glucose. Secondly, the addition of insulin equally inhibited VMH GI neurons from control and BG4KO mice. Finally, the current-voltage relationships indicate that insulin did not lead to activation of a Cl⁻ channel as would be expected if insulin increased glucose uptake. Interestingly, while insulin has been shown to inhibit hypothalamic AMPK, this is unlikely to explain insulin’s effects on GI neurons because AMPK inhibition stimulates GI neurons by closing a Cl⁻ channel. Rather, the inhibitory effect of insulin on VMH GI neurons resulted from activation of a K⁺ channel. This insulin sensitive K⁺ channel is possibly the ATP-sensitive K⁺ channel since insulin has been reported to activate this channel in other hypothalamic glucose sensing neurons. These data thus suggest that insulin inhibits GI neurons through a Glut4/glucose independent activation of a K⁺ channel.

It has previously been reported that neuronal insulin receptor knock-out (NIRKO) mice have a reduced content of brain GLUT4, and similar to the current BG4KO mice, NIRKO mice also demonstrate 1) glucose intolerance, 2) mild insulin resistance, 3) impaired epinephrine responses to hypoglycemia, 4) impaired neuronal activation to hypoglycemia, and 5) impaired glucose sensing in GI neurons of the VMH. The similarities in the phenotype of NIRKO and BG4KO mice suggest that many of the actions of insulin in the brain are mediated by GLUT4. Paradoxically though, the current study demonstrates that hypothalamic insulin signaling was unaltered by GLUT4 deletion and acute electrophysiological responses to insulin administration are independent of GLUT4. Collectively, it is hypothesized that the chronic lack of insulin
signaling or GLUT4 in the brains of NIRKO or BG4KO mice, respectively, that leads to the observed impairments in neuronal glucose sensing appears to be mechanistically distinct from the acute actions of insulin and/or GLUT4 translocation in regulating neuronal glucose sensing and the counterregulatory response to hypoglycemia.

As GLUT4 is expressed in diverse regions of the brain, BG4KO mice will be a useful tool in elucidating other potential roles of brain GLUT4 in cognition, learning and memory, and balance and coordination. In conclusion, central nervous system GLUT4 plays an important role in regulating whole body glucose homeostasis, brain glucose sensing, and the counterregulatory response to hypoglycemia.
Chapter 3. Antecedent Glycemic Control Reduces Severe Hypoglycemia-Induced Neuronal Damage in Diabetic Rats
Abstract

Brain damage due to severe hypoglycemia occurs in insulin treated people with diabetes. This study tests the hypothesis that chronic insulin therapy that normalizes elevated blood glucose in diabetic rats would be neuroprotective against brain damage induced by an acute episode of severe hypoglycemia. Male Sprague-Dawley rats were split into 3 groups, 1) control, non-diabetic, 2) STZ-diabetic, and 3) insulin treated STZ-diabetic. After 3 weeks of chronic treatment, all rats underwent acute hyperinsulinemic/severe hypoglycemic (10-15 mg/dl) clamps for 1 hour. Rats were subsequently analyzed for brain damage and cognitive function. Severe hypoglycemia-induced 15-fold more neuronal damage in diabetic rats as compared to non-diabetic rats. Chronic insulin treatment of diabetic rats, that nearly normalized glucose levels, markedly reduced neuronal damage induced by severe hypoglycemia. Fortunately, no cognitive defects associated with the hypoglycemia-induced brain damage were observed in any group. In conclusion, antecedent blood glucose control represents a major modifiable therapeutic intervention that can afford diabetic subjects neuroprotection against severe hypoglycemia-induced brain damage.
Introduction

Severe hypoglycemia is a side effect of insulin therapy affecting ~40% of insulin treated diabetics\textsuperscript{29}. Insulin treated diabetic patients experience an average of two symptomatic hypoglycemic episodes per week while severe hypoglycemia is experienced approximately once a year\textsuperscript{29}. By depriving the brain of glucose, severe hypoglycemia can lead to brain damage. The cortex and hippocampus are especially sensitive to the brain damaging effects of severe hypoglycemia\textsuperscript{61}. Deficits in learning and memory have been shown to be a direct consequence of severe hypoglycemia-induced hippocampal neuronal damage\textsuperscript{51,53,54}. Clinical research has shown that severe hypoglycemia induces cognitive deficits in some\textsuperscript{7,8,55,56} but not all studies\textsuperscript{57-59}. Animal studies have shown that rats exposed to severe hypoglycemia perform worse in a Morris water maze\textsuperscript{60} indicating that brain damage is functionally related to poorer cognitive performance. Since uncontrolled diabetes magnifies the extent of severe hypoglycemia-induced brain damage\textsuperscript{61}, it was hypothesized that chronic hyperglycemia associated with chronic insulin deficiency induces a mal-adaptive process that predisposes the brain to more extensive hypoglycemia-induced damage. Insulin has been shown to protect against ischemia-induced neuronal death both in vitro\textsuperscript{64} and in vivo\textsuperscript{67}, but the potential neuroprotective effects of long-term insulin therapy for diabetic animals have yet to be examined in response to severe hypoglycemia induced brain damage.

The aim of this study was therefore to determine if chronic insulin therapy resulting in the near normalization of glycemia could protect diabetic rats from hippocampal and cortical brain damage induced by a single episode of severe
hypoglycemia. It was also determined if this neuroprotective effect would also safeguard against hypoglycemia-induced deficits in learning and memory.

**Methods**

**Animals and Surgery.** Nine-week-old male Sprague-Dawley rats (Charles River Laboratories) were housed individually in temperature- and light-controlled environments fed ad libitum. All studies were done in accordance with and approved by the Animal Studies Committee at Washington University School of Medicine. In rats destined to undergo severe hypoglycemia, indwelling catheters were implanted under anesthesia in the left carotid artery and right jugular vein, as previously described. In control rats not destined to undergo hypoglycemia, the left carotid artery and right jugular vein were ligated under anesthesia.

**Induction of diabetes.** Three days post-surgery, rats received intraperitoneal injections of either streptozotocin (STZ; 65 mg/kg, n = 37; Sigma) to induce diabetes or vehicle 0.1 mM sodium citrate (Fisher Scientific) buffer for non-diabetic controls (CON, n = 18) (Figure 15). Some rats were injected a second time with STZ to ensure random glucose >300 mg/dl (Ascensia Contour BG monitors, Bayer HealthCare, Mishawaka, IN). (Figure 16A)

**Insulin treatment.** Eighteen of the diabetic rats were treated with subcutaneous insulin pellets (STZ+Ins; Linplant, ~2U/day, Lin Shin, Toronto, ON, Canada) to achieve blood glucose levels <250 mg/dl. If these rats were noted to have tail vein blood glucose >250
mg/dl, insulin (Lantus, 0-2.5U/day; sanofi-aventis, Bridgewater, NJ) was injected subcutaneously once daily. If blood glucose values were below 50 mg/dl for 3 or more days, rats were excluded from the study.

**Hyperinsulinemic hypoglycemic clamp.** Three weeks after STZ or vehicle injections, hyperinsulinemic (0.2 U·kg\(^{-1}\)·min\(^{-1}\)) hypoglycemic (10-15 mg/dl) clamps were performed in non-diabetic CON, uncontrolled STZ-diabetic, and chronically insulin treated STZ rats, as previously described\(^60,61\). At the start of insulin (Humulin R; Eli Lilly, Indianapolis, IN) infusion, all clamped rats received intravenous glucose (50% dextrose; Hospira, Lake Forest, IL) to match the rate of glucose decline observed in diabetic rats. All groups were precisely matched for duration (1 hour) and depth of hypoglycemia (10–15 mg/dl). After 1 hour of severe hypoglycemia, insulin infusion was discontinued, and glucose was given to end hypoglycemia. Rats subjected to severe hypoglycemia were examined either for brain damage or behavioral testing (Figure 15).

**Fluoro-Jade B and hematoxylin-eosin staining.** One week after the severe hypoglycemic clamps, anesthetized rats were intracardially perfused with 0.01 mol/l PBS (Sigma) followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA)\(^60,61\). Brains were immersed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose. Beginning at 2.8 mm posterior to the bregma, four coronal cryostat sections (20 \(\mu\)m), 120 \(\mu\)m apart, were analyzed for neuronal damage by Fluoro-Jade B (Chemicon International) and hematoxylin-eosin (H-E) (Sigma) staining. Fluoro-Jade B is a well-characterized stain for degenerating neurons and was
performed as described by Schmued and Hopkins\textsuperscript{137}. Fluorescent cells (Fluoro-Jade–positive cells) were quantified in both hemispheres of the cortex and of the hippocampal structures CA1 and dentate gyrus. For each region of interest, data are expressed as the average number of Fluoro-Jade B positive (FJB+) cells per section.

**Behavioral testing.** Consistent with other protocol designs\textsuperscript{52,54,138}, histopathological outcomes were assessed 1 week following the hypoglycemic neuronal insult, while cognitive studies were performed 6–8 weeks later in a separate group of similarly treated rats. This later assessment of cognitive function is a more useful measure of clinical outcome and a better functional index of neuroprotection because it allows for a complete and integrated evaluation of ongoing damage and possible recovery\textsuperscript{139}. After a 6- to 8-week recovery from the severe hypoglycemic clamp, CON (n=12), STZ (n=11), and STZ+Ins (n=12) rats, as well as a fourth group of sham operated negative control diabetic rats not subjected to hypoglycemia (STZ No Hypoglycemia, STZ-NH, n=10) were evaluated on a 1-hour locomotor activity test, a battery of sensorimotor measures, and on the Morris water maze (MWM) task\textsuperscript{60}.

**One-hour locomotor activity test and sensorimotor battery.** General locomotor activity and exploratory behavior were evaluated for 1 h in an open field (41 x 41 x 38.5 cm high) constructed of Pleixglas and containing computerized photobeam instrumentation consisting of a 16 x 16 matrix of photocell pairs (MotorMonitor; Kinder Scientific) to quantify ambulations (whole body movements) and vertical rearing frequency. As previously described\textsuperscript{140}, the ledge, platform, 90°-inclined screen, and
walking initiation tests were conducted to measure balance, strength, coordination, and initiation of movement.

**Cognitive testing in the Morris water maze:** Spatial learning and memory were assessed for CON (n=12), STZ (n=11), STZ+Ins (n=12), and STZ-NH (n=10) groups using the Morris water maze test similarly to previously published methods\(^{140}\). Cued (visible platform, variable location) and place (submerged, hidden platform, constant location) trials were conducted to train rats to navigate to a submerged platform (1.5 cm below the surface of opaque water) in a round pool (118 cm diameter). A computerized tracking system (ANY-maze, Stoelting Co., Wood Dale, IL) was used to quantify escape path length, latency, and swimming speeds which served as dependent variables. Rats first received cued trials to determine if non-associative dysfunctions (sensorimotor or visual disturbances or alterations in motivation) were likely to affect performance in subsequent place trials. The cued condition involved conducting two sessions of three trials each (60-s maximum/trial) per day for two consecutive days during which the rats were trained to swim to the submerged platform 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 hidden (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/trial) 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 days 3 and 5, a probe trial was conducted 1 h after the last place trial which involved removing the platform from the pool and quantifying the 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; and spatial bias which involved comparing the time spent in the target quadrant with time spent in each of the other quadrants.

Statistics. All data are represented as means ± sem. Data were analyzed by one-way or repeated measures analysis of variance (ANOVA and rmANOVA, respectively). The spatial learning data were analyzed using an ANOVA model containing one between-subjects variable (Treatment: CON; STZ; STZ+Ins; and STZ-NH) and one within-subjects (repeated measures) variable such as Blocks of Trials. For rmANOVAs, the Huynh-Feldt correction was used for all within-subjects effects containing more than two levels to help protect against violations of sphericity/compound symmetry assumptions underlying the rmANOVA model. In addition, Bonferroni correction was used to help maintain prescribed alpha levels (e.g., 0.05) when multiple comparisons were conducted.

Results
Chronic insulin treatment nearly normalizes glucose level of diabetic rats. Rats that received streptozotocin displayed elevated blood glucose levels two days post injection and remained diabetic throughout the experiment compared to non-diabetic
controls (CON 100±1 mg/dl vs. STZ 484±10 mg/dl; Figure 16A). Insulin treatment reduced blood glucose levels to near normal (STZ+Ins 160±11 mg/dl). Sham operated diabetic rats that did not undergo severe hypoglycemia (STZ-NH) had similar glucose levels as the STZ group (STZ-NH 467±12 mg/dl). Prior to randomization, body weight did not differ between groups (CON: 302±10, STZ: 308±5; STZ+Ins: 302±7; STZ-NH: 310±9g; Figure 16B). On the day of the severe hypoglycemic clamp, the non-diabetic and insulin-treated diabetic rats had an increase in body weight to 408±37 and 387±14g, respectively, but 3 weeks of uncontrolled diabetes in the STZ and STZ-NH rats resulted in a failure to increase body weight (329±22 and 339±10g, respectively, p=NS compared to pretreatment; Figure 16B).

**Hyperinsulinemic-hypoglycemic clamp.** All rats experienced the same degree of severe hypoglycemia (CON 12±0.3 mg/dl, STZ 13±0.7 mg/dl, STZ+Ins 12±0.3 mg/dl; Figure 17A). During severe hypoglycemia, animals lost their righting reflex, displayed seizure-like activity, and became minimally responsive. After one hour of severe hypoglycemia, glucose reperfusion was started so that animals reached euglycemia within 30 minutes (CON 159±48 mg/dl, STZ 181±31 mg/dl, STZ+Ins 172±48 mg/dl). Almost immediately animals regained consciousness and their righting reflex and seizure-like activity ceased. The animals were allowed to recover for 4 hours and then returned to their cages. The following day blood glucose levels were back to their respective values (CON 101±6 mg/dl, STZ 418±49 mg/dl, STZ+Ins 82±13 mg/dl).

Overall mortality associated with the episode of severe hypoglycemia was 29%. Control rats had a mortality of 22% while STZ and STZ+Ins rats tended to have
increased mortality to 36% and 42%, respectively, but this did not reach significance (p<0.25, Fisher exact test, Freeman Halton extension; Figure 17B).

**Chronic insulin treatment decreases severe hypoglycemia-induced brain damage.** One week following the acute severe hypoglycemic episode, rats were sacrificed and brains sectioned for neuronal damage using the marker Flouro-jade B which stains for degenerating neurons. In spite of equal depth and duration of hypoglycemia, diabetic rats had markedly increased (15-fold) brain damage in the hippocampus and cortex compared to non-diabetic rats (Figure 18A,B). In particular in the hippocampus, the CA1, CA3, and dentate gyrus regions had a 5-fold, 15-fold, and 45-fold increase in damage, respectively. Chronic antecedent insulin treatment reduced neuronal damage in diabetic rats (STZ+INS) in all regions to levels not significantly different from that of non-diabetic rats (Figure 18A,B).

**Severe hypoglycemia does not disrupt behavior.** Severe hypoglycemia did not significantly impair locomotor or sensorimotor functions (Figure 19A,B) when tested 6-8 weeks post-treatment (severe hypoglycemic clamp). An rmANOVA did not reveal any significant effects involving treatment on total ambulations (whole body movements) measured during the 1-hour locomotor activity test (Figure 19A) nor were any differences observed in vertical rearing frequency (not shown). Similarly, no performance differences between treatment groups were observed with regard to any measure on the battery of sensorimotor tests. For example, although the CON group tended to perform not as well as the diabetic groups in terms of being able to remain on
a small elevated platform, a one-way ANOVA showed no significant differences among
groups on this sensorimotor test (Figure 19B) nor on other sensorimotor measures of
balance (ledge), strength (90° inclined screen) or initiation of movement (walking
initiation) out of a small delineated space (not shown).

Interestingly, we also found no general performance differences between
treatment groups on the learning and memory indices quantified during testing in the
Morris water maze. Specifically, no effects involving treatment were found following
rmANOVAs conducted on the escape path length (Figure 19C), latency, or swimming
speed data (not shown) during the cued (visible platform location) trials. These results
suggest that there were no visual, sensorimotor, or motivational disturbances in any of
the groups that would compromise swimming performance and thus confound
interpretation of the subsequent place trials data. With regard to the acquisition (spatial
learning) performance of the treatment groups during the place trials (Figure 19D), an
rmANOVA conducted on the path length data yielded a significant Treatment by Blocks
of Trials interaction, \(F_{12,64} = 2.54; p=0.011\). Subsequent comparisons showed that this
was mostly due to increased path length in the STZ NH group versus the STZ-Ins
\(p=0.036\) or the CON \(p=0.037\) groups during the first block of trials only, but not
thereafter. A significant Treatment by Blocks of Trials interaction \(F_{12,64} = 2.32; p=0.016\)
was also found for escape latency (not shown), although only the comparison between
the STZ NH versus STZ+Ins groups were significantly different \(p=0.017\). Note that
although the comparisons cited here for block 1 revealed certain large \(p<0.05\)
differences between treatment groups, none of the “\(P\)” values were less than the
Bonferroni corrected level \(p=0.05/4= 0.0125\). Thus, it is appropriate to conclude that
the performance of the treatment groups did not differ at any time with regard to path length or latency during acquisition (place trials). Consistent with the general lack of differences between treatment groups during the place trials were comparable performance levels observed among groups during the probe trials. For example, one-way ANOVAs revealed non-significant treatment effects with regard to time in the target quadrant during probe trial 1 (mid-acquisition) (Figure 19F) and platform crossings (not shown). In addition, all groups demonstrated significant spatial bias for the target quadrant that had contained the hidden platform as evidenced by each group spending significantly more time in the target quadrant compared to times spent in each of the other quadrants (p<0.01). Similar results were found for the same variables during the second probe trial conducted 1 hour after the last place trial on day 5 (not shown).
Figure 15. Experimental protocol for analysis of brain damage and cognitive function after severe hypoglycemia. After recovery from arterial and venous cannulation, rats were injected with streptozotocin (STZ) or vehicle (Con, n=18). Three days later insulin treatment was started in a subset of the STZ diabetic rats (STZ+Ins, n=15) by implanting an insulin pellet and starting insulin injections as needed to maintain blood glucose <250 mg/dl. About 3 weeks later, hyperinsulinemic (0.2 U·kg$^{-1}$·min$^{-1}$) severe hypoglycemic (10-15 mg/dl) clamps were performed in all rats except a group of diabetic rats that were not subjected to the severe hypoglycemic clamp (STZ-NH). After the episode of severe hypoglycemia, either neuronal damage was assessed by Flouro jade B and H&E staining one week later, or rats were studied 6-8 weeks later with sensorimotor and cognitive testing. Brain damage study: CON (n=6), STZ (n=6), STZ+Ins (n=6); Cognitive testing study: CON (n=12), STZ (n=13), STZ+Ins (n=12), STZ-NH (n=10)
Figure 16. Glucose levels and body weight during the brain damage and cognitive testing protocols. A) Blood glucose levels throughout the experiment. The non-diabetic (CON, closed circle) rats had glucose levels of ~100 mg/dl. After STZ injection, STZ diabetic rats (open circle) and STZ diabetic rats no hypoglycemia(NH, open triangle) had glucose levels of >450 mg/dl. The diabetic rats chronically treated with insulin (STZ+Ins, closed triangle) group had markedly improved blood glucose levels (160 ± 11 mg/dl) throughout the experimental timeline. HYPO= day of severe hypoglycemic clamp B) The non-diabetic (CON) and insulin treated diabetic (STZ+Ins) groups increased their body weight over time while the STZ and STZ-NH groups did not increase their body weight. Note: Time axis not to scale. Wk= week; n= 10-18 per group. Data shown as mean ± SEM.
Figure 17. Severe hypoglycemic clamp glucose and mortality. A) Glucose levels during the hyperinsulinemic/hypoglycemic clamp. Insulin infusion decreased glucose levels over a 4 hour period in the CON (n=18), STZ (n=19), and STZ+Ins (n=18) groups until glucose reached 15 mg/dl. Glucose was clamped between 10-15 mg/dl for 1 hour and then hypoglycemia was terminated with increased glucose infusion throughout a 4 hour recovery period. B) Mortality associated with severe hypoglycemia was 22% in CON, 36% in STZ, and 42% in STZ+Ins treated rats but the difference was not significant (p<0.25). Note: Time axis not to scale.
Figure 18. Severe hypoglycemia induced brain damage. A) Representative Fluoro-Jade B (top 3 rows) and hematoxylin and eosin (H&E –bottom 3 rows) staining of the cortex and hippocampal structures, CA1, CA3 and the dentate gyrus (DG), one week following an episode of severe hypoglycemia in non-diabetic (CON), uncontrolled STZ-diabetic (STZ), and chronically insulin treated STZ (STZ+Ins) rats. Neuronal damage is indicated with Fluoro-Jade B positive cells (green fluorescence) or by pyknotic cells (H&E staining, black arrows). Blue= dapi, nuclear stain. B) Brain damage was markedly elevated in the cortex and hippocampus in STZ (white bar) rats compared to controls (black bar). STZ+Ins (diagonal hash bar) reduced this damage in both regions. (*p<0.05 ANOVA n=6 per group). Data shown as mean ± SEM.
Figure 19. Severe hypoglycemia does not produce impairment in behavioral and cognitive functions. A) There were no significant effects involving treatment (severe hypoglycemia) on general activity as measured by total ambulations (whole body movements) during a 1-h locomotor activity test. B) Although the CON group tended to perform not as well as the diabetic groups in terms of being able to remain on a small elevated platform, there was no significant differences among groups on this sensorimotor test. C) Groups did not differ in escape path length during the cued (visible platform location) trials. D) A rmANOVA conducted on the path length data during the place (spatial learning) trials yielded a significant Group (Treatment) by Blocks of Trials interaction, (*p=0.011). This was mostly due to increased path length in the STZ NH group versus the STZ-Ins (†p=0.036) or the CON (††p=0.0369) groups during the first block of trials only, but not thereafter. E) No differences among groups were observed in swimming speeds during the place trials. F) Groups performed similarly in terms of time in the target quadrant during probe trial 1 (mid-acquisition). In addition, all groups demonstrated significant spatial bias for the target quadrant that had contained the
hidden platform as evidenced by each group spending significantly more time in the target quadrant compared to times spent in each of the other quadrants (*p<0.01). TGT= target quadrant, LFT= left quadrant, OPP= opposite quadrant, RGT= right quadrant; Performed in collaboration with Dr. David Wozniak, Washington University in St. Louis.
Discussion

Studies investigating the potential neuroprotective role of insulin often do not differentiate the acute versus chronic effects of insulin therapy. The present findings show that while acute insulin-induced hypoglycemia can cause brain damage, chronic insulin treatment in diabetic rats to restore normoglycemia is markedly neuroprotective and significantly reduces the extent of severe hypoglycemia-induced brain damage.

It was hypothesized that the uncontrolled diabetic rats that displayed markedly elevated hypoglycemia-induced brain damage would perform much worse than other rats tested, and that the marked neuroprotective effects of chronic insulin therapy would mitigate against demonstrable deficits in cognitive performance. As with clinical studies, animal studies of hypoglycemia and cognitive performances are variable. However, it was somewhat surprising that Morris maze testing was unable to demonstrate differences in cognitive performances between the groups. Our previous Morris maze studies in normal rats\textsuperscript{60} showed rapid improvements in escape path lengths and latencies in sequential trial tests, very similar to the currently tested groups of rats, suggesting that all of the current groups of rats performed equally well with no detectable cognitive deficit. It is suspected that the extent of brain damage achieved in the current studies did not reach a high enough threshold to cause demonstrable deficits in learning/memory performances as determined by Morris maze testing. Previous experiments of longer duration of hypoglycemia that cause 3-6 fold more brain damage than observed in the current study indeed do result in demonstrable deficits in Morris maze testing\textsuperscript{60}. Although considered, the relatively high mortality in the current series of rats precluded hypoglycemia experiments of longer duration. Interestingly, a
dissociation between severe hypoglycemia-induced brain damage and detectable cognitive deficit is consistent with clinical studies in which severe hypoglycemia alters brain structure\(^4\) yet detectable cognitive deficits are seen in some\(^4,8\) but not all\(^59\) studies.

Consistent with a previous report from our lab revealing that diabetic rats are more susceptible to severe hypoglycemia-induced death\(^141\), mortality due to severe hypoglycemia tended to be increased in both the uncontrolled diabetic (STZ) and insulin-treated diabetic (STZ+Ins) rats compared to controls. However, since the difference was not significant, future research is focusing on how diabetes per se might increase risk of severe hypoglycemia-induced death. While the cause of death in the current experiments is unknown, it is hypothesized that severe hypoglycemia induces fatal cardiac arrhythmias which is further discussed in chapter 4.

Hypoglycemia has been shown to induce neuronal cell death via a cascade of molecular events, including increased release of glutamate, calcium, zinc, and reactive oxygen species, leading to cell death\(^142\). The mechanism by which hypoglycemia-induced neuronal damage was markedly augmented in diabetic rats was not directly assessed in these studies although many potential mechanisms exist. 1) The greater net drop in glycemia experienced in the diabetic rats (i.e. from 484 to 13 mg/dl) may have exacerbated brain damage. 2) The decreased number of brain glucose transporters\(^103\) and altered glucose transport and metabolism\(^143\) may have led to the increased susceptibility to brain damage in the diabetic rats. 3) An up-regulation of glutamate leading to calcium overload may have enhanced neuronal death in diabetic rats\(^144\). 4) STZ-diabetic rats show a down regulation of cortical NMDA receptors\(^145\), which may be particularly important given the role of excess glutamate in mediating
hypoglycemia-induced neuronal damage. 5) Following the hypoglycemic episode, the effects of chronic hyperglycemia may have been toxic to vulnerable neurons resulting in increased brain damage.

In the absence of severe hypoglycemia, uncontrolled diabetes (induced by streptozotocin) does not result in brain damage as shown by a previous report. Therefore, in the current studies, diabetic rats not subjected to severe hypoglycemia (STZ-NH) were not analyzed for brain damage.

The mechanism by which chronic insulin therapy afforded neuroprotection against brain damage cannot be precisely determined in this model. It was hypothesized that chronic insulin deficiency may predispose the diabetic brain to greater hypoglycemia-induced damage. Studies in ischemic brain injury models are consistent with the notion that insulin directly acts in the brain to afford neuroprotection.

Alternatively, since the down regulation of brain glucose transporters by chronic hyperglycemia may predispose the brain to profound neuroglycopenia in response to acute severe hypoglycemia, it may be that the correction of chronic hyperglycemia alone could be neuroprotective. Another possible mechanism for the neuroprotective effect of chronic insulin therapy could be related to the preconditioning effect of recurrent hypoglycemia. Although rats that displayed recurrent hypoglycemia were specifically excluded from the study, we cannot rule out the possibility that in an attempt to achieve near normalization of blood glucose levels, some of the insulin treated diabetic rats might have had episodes of undetected antecedent hypoglycemia which could have preconditioned the brain and protected it against the subsequent severe hypoglycemic episode. Regardless of the mechanism, the effect was profound. The
10-15 fold reduction in hypoglycemia-induced brain damage indicates that antecedent blood glucose achieved with chronic insulin therapy is a major beneficial therapeutic intervention that provided the diabetic rats significant neuroprotection.

For patients and clinicians, the choice to pursue an intensive insulin regime as a therapeutic approach to realize the established long-term microvascular benefits associated with tight glycemic control, has to be weighed against the increased risk of severe hypoglycemia and associated adverse outcomes including brain damage. In this study, chronic insulin treatment in diabetic rats that lead to a near normalization of glucose levels, markedly reduced the extent of brain damage induced by severe hypoglycemia. These intriguing findings indicate that chronic insulin therapy that improved glycemic control, paradoxically, rendered the diabetic rats more prone to, but less vulnerable to, an episode of severe hypoglycemia. In conclusion, antecedent blood glucose control represents a major modifiable therapeutic intervention that can afford diabetic subjects neuroprotection against a subsequent episode of severe hypoglycemia-induced brain damage.
Chapter 4. Sudden Deaths due to Lethal Cardiac Arrhythmias during Severe Hypoglycemia are Mediated by Sympathoadrenal Activation
Abstract

For people with insulin treated diabetes, severe hypoglycemia can be lethal. Potential mechanisms by which hypoglycemia can lead to sudden death are not well understood. To study how severe hypoglycemia can be fatal, 1-3 hour hyperinsulinemic, severe hypoglycemic (10-15 mg/dl) clamps were performed in Sprague-Dawley rats with simultaneous continuous electrocardiogram recording. With a goal towards reducing severe hypoglycemia induced mortality, the hypotheses tested were: 1) antecedent glycemic control impacts ability to survive an episode of severe hypoglycemia, 2) by limiting hypokalemia, potassium supplementation could limit cardiac arrhythmic deaths, 3) by preventing central neuroglycopenia, brain glucose infusion could prevent hypoglycemia associated arrhythmic deaths, and 4) by limiting the effects of sympathoadrenal activation during hypoglycemia, adrenergic blockers could provide a survival advantage by preventing lethal cardiac arrhythmias. Mortality rates associated with severe hypoglycemia were worsened by diabetes, but recurrent antecedent hypoglycemia markedly improved survival. Potassium supplementation trended to reduce mortality in non-diabetic and diabetic rats. Severe hypoglycemia caused numerous cardiac arrhythmias including premature ventricular contractions, 1st, 2nd, and 3rd degree heart block, and ventricular tachycardia. Intracerebroventricular glucose infusion reduced cardiac arrhythmias and mortality due to severe hypoglycemia. Beta adrenergic blockade reduced cardiac arrhythmias and completely abrogated deaths due to severe hypoglycemia. In conclusion, severe hypoglycemia induced sudden death was preceded by fatal cardiac arrhythmias. Mortality was entirely prevented by blocking the effective sympathoadrenal response. It is concluded that
brain neuroglycopenia and the marked catecholaminergic sympathoadrenal counterregulatory response are the primary mediators of fatal cardiac arrhythmias induced by severe hypoglycemia.
**Introduction**

The rate of sudden death in young people with Type 1 diabetes is 10-fold higher than age matched controls\(^7\). Sudden nocturnal deaths account for up to 27\% of all unexplained deaths in people with Type 1 diabetes\(^7\,75,77\). The aptly named “dead in bed syndrome” identifies otherwise healthy young individuals with Type 1 diabetes that were found dead in bed with no clear cause of death\(^69-71,73-78\). It has been speculated that their deaths were due to excess insulin administration and resultant severe hypoglycemia\(^70-74,76,77,80\). However, the mechanisms by which severe hypoglycemia can be lethal is unclear.

The brain requires a constant supply of glucose to function normally. During hypoglycemia, lack of glucose supply to neurons can lead to confusion, brain damage\(^81\), seizures\(^82,83\) and if hypoglycemia is low enough for long enough, it may be fatal. Indeed, 6-10\% of deaths in people with Type 1 diabetes are directly attributable to hypoglycemia\(^59,148,149\). It has been hypothesized that acute onset cardiac arrhythmias contribute to severe hypoglycemia-induced sudden death. Clinical studies using electrocardiograms (ECG) have found that cardiac arrhythmias associated with QTc prolongation occur during moderate hypoglycemia\(^85-89\). Prolongation of the QTc interval can lead to increased risk of fatal cardiac arrhythmias\(^91-93\). It remains undetermined to what extent severe hypoglycemia induced cardiac arrhythmias are mediated by central neuroglycopenia versus systemic factors affecting cardiac tissue.

In the setting of insulin-induced hypoglycemia, both the insulin-induced decrement in potassium levels and the counterregulatory-induced increase in catecholamine levels have been speculated to contribute to arrhythmias\(^88,97,98\). It has
been shown previously that potassium or beta blockers can reduce cardiac arrhythmias in patients during moderate hypoglycemia (~45 mg/dl)\textsuperscript{88}. It is well established that increased catecholamine levels can lead to ventricular arrhythmias\textsuperscript{95,96}. However, it remains to be determined whether the prevention of hypokalemia and/or blocking the actions of the catecholamines protect against lethal arrhythmias and cardiorespiratory arrest during the setting of severe hypoglycemia.

In order to determine preventable causes of death during severe hypoglycemia, protocols described below describe how experimental rats were monitored for cardiac arrhythmias and mortality during an episode of severe hypoglycemia during which time putative protective agents were administered.

**Methods**

**Animals.** Nine-week-old male Sprague-Dawley rats (Charles River Laboratories) were housed individually in a temperature- and light-controlled environment fed ad libitum with standard rat chow diet and water. All studies were done in accordance with and approved by the Animal Studies Committee at Washington University School of Medicine.

**Implantation of arterial and venous catheters and ECG leads.** Nine week old male, Sprague Dawley rats were anesthetized with an intraperitoneal injection of Ketamine 87 mg/kg and Xylazine 2.6 mg/kg. A micro-renathane\textsuperscript{®} (Braintree Scientific, Boston, MA) catheter was inserted into the left carotid artery for sampling access and two catheters were implanted into the right jugular vein for infusions. To maintain patency, at the time
of implantation catheters were filled with a 40% polyvinylpyrrolidone (Sigma) in heparin (1000 USP U/ml) solution (Baxter Healthcare Corporation, Deerfield, IL). In some studies, at the time of surgery one electrocardiogram (ECG) wire lead was placed in the right supraclavicular fossa and another wire lead was placed exterior to the lower left rib cage, in the mid axillary line, to approximate clinical lead II position. ECG wire leads were secured and sutured in place to the underlying muscle. A reference lead was placed subcutaneously over the back. The free ends of the three wire leads and vessel catheters were buried subcutaneously on the animal's back for easy retrieval on the day of the clamp. Rats were allowed to recover for 4-10 days. All rats had restored their pre-operative weight before being subjected to the hypoglycemic clamp.

**Hyperinsulinemic-severe hypoglycemia clamp.** After an overnight fast, vascular catheters and ECG leads were externalized under brief isoflorane anesthesia. Animals were allowed to rest for 1.5 to 2 hours before basal samples were taken. Awake, unrestrained rats were subjected to hyperinsulinemic (0.2 U · kg⁻¹ · min⁻¹) severe hypoglycemic (10-15mg/dl) clamps for 3 hours unless otherwise noted. Rats had free access to water. Insulin (Humulin R; Eli Lilly, Indianapolis, IN) and glucose (50% dextrose; Hospira, Lake Forest, IL) were co-infused intravenously to lower blood glucose to 10-15 mg/dl over a period of 2 to 2 ½ hours. During the first hour of insulin infusion, glucose levels were maintained at ~70 mg/dl in order to assess the effects of hyperinsulinemia on the electrocardiogram, in the absence of profound hypoglycemia. Arterial blood samples were obtained every 15 minutes to measure blood glucose (Ascensia Contour blood glucose monitors, Bayer HealthCare, LLC, Mishawaka, IN). All
Experimental groups were precisely matched for duration and depth (10-15 mg/dl) of hypoglycemia. Mortality due to severe hypoglycemia was determined by cessation of breathing.

Blood samples were taken during the basal period and at various times throughout the severe hypoglycemic clamp for epinephrine, norepinephrine, glucagon, and insulin measurements. Insulin was measured by ELISA (Crystal Chem, Downers Grove, IL). Glucagon was measured by RIA (Millipore, Billerica, MA). Plasma epinephrine and norepinephrine levels were determined by a single isotope derivative (radioenzymatic) method\textsuperscript{112}. Blood pressure (Noninvasive BP monitor, Columbus instruments, Columbus, OH) was measured by tail cuff in the basal period and every 15 minutes during severe hypoglycemia in select studies.

**Arterial blood gas and electrolytes.** Blood samples were taken at various timepoints throughout the clamp for analysis of potassium, oxygen, carbon dioxide, calcium, and pH on an ABG machine (pHOx Plus C, Nova Biomedical, Waltham, MA). Respiration was calculated as breaths per minute by visibly counting breaths.

**Seizures.** 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) neck extensions, tonic stretching, uncontrolled limb movements, and spontaneous spinning\textsuperscript{61,150}. The incidence of seizure-like behavior was quantified for each rat during the clamp period. A subset of rats also had electroencephalogram
(EEG) recording (XLTEK Video EEG System, Oakville, Ontario) to analyze brain waves before, during, and after hypoglycemia-induced seizures.

Study 1: Overall mortality analysis in control, diabetic and recurrently hypoglycemic experiments. Control (n=123), diabetic (n=95), and recurrent hypoglycemic (n=27) rats that underwent 1 hour of severe hypoglycemia were analyzed for mortality.

Diabetes. Approximately two weeks before the severe hypoglycemic clamp, diabetes was induced by intraperitoneal injection of streptozotocin (STZ; 65 mg/kg; Sigma) as previously described. Control non-diabetic rats received vehicle injections of 0.1 mM sodium citrate (Fisher Scientific) buffer. Some rats were injected with a second dose of STZ to ensure random glucose >300 mg/dl (Ascensia Contour BG monitors, Bayer HealthCare, Mishawaka, IN).

Recurrent hypoglycemia. For three consecutive days before the severe hypoglycemic clamp, moderate hypoglycemia (25-40 mg/dl) was recurrently induced in non-diabetic rats with injections of subcutaneous regular human insulin (Lilly, Indianapolis, IN; 6 U/kg on day 1; 5 U/kg on day 2; and 4 U/kg on day 3) as previously described. Control rats received saline injections. Severe hypoglycemic clamps were performed on the 4th day.

Study 2: Potassium supplementation. Potassium (KCl, 0.375 mEq/ml, Hospira, Lake Forest, IL) was infused with saline at 4 μl/min. Non-diabetic rats either received (n=12) or didn’t receive (n=15) potassium and diabetic rats either received (n=28) or didn’t receive potassium (n=45) throughout a hyperinsulinemic/severe hypoglycemic clamp, as described above.
Study 3: Cardiac arrhythmias in control experiments

ECG studies. To explore the possibility of arrhythmias as a possible cause of death, control non-diabetic rats (n=6) underwent severe hypoglycemic clamps for 90 minutes as described above, with continuous ECG recording (ADI Powerlab 26T, Colorado Springs, CO). ECG analyses included heart rate, QTc length, and other arrhythmias (ADI LabChart Pro 7). QTc was calculated using Bazett’s formula\(^{151}\). Cardiac arrhythmias were analyzed for incidence and frequency. For frequency analysis, arrhythmias were counted individually and divided by the amount of time spent in severe hypoglycemia (either 180 or minutes or time of death).

Study 4: ICV glucose infusion. Using a digital stereotactic neurosurgery apparatus and under continuous 1% isofluorane anesthesia, intracerebroventricular (ICV) cannulas (internal cannula 31g, outer guide cannula 24g, 9.5mm depth; Plastics One Inc, Roanoke, VA) were placed on the skull in the midline -2.8 mm posterior to bregma. Screws were affixed to the bare skull and glue was applied (SNAP, Parkell Inc, Edgewood, NY) to fasten the cannula in place. Rats were allowed to recover 1 week post ICV surgery before implantation of vascular catheters and ECG leads as described above. On the day of the clamp, the ICV cap was removed and a cannula primed with either 2mM D-glucose (n=9) (Sigma, St. Louis, MO) or 2mM D-mannitol (n=9) (Sigma) was placed in through the guide cannula. Glucose or mannitol (as a control) were infused at 0.1 \(\mu\)l/min via the internal ICV cannula at the start, and throughout, the hyperinsulinemic/severe hypoglycemic clamp, as described above. Correct ICV cannula
placement in the third ventricle was confirmed in all rats studied by post mortem staining with Evan’s blue dye.

**Study 5: Adrenergic blockade.** On the day of the clamp, after a 2 hour basal period, $\alpha$ (prazosin, Sigma, $\alpha_1$ receptor blocker, 1 mg/ml dissolved in sterile distilled water and infused at 1 $\mu$l/min) and $\beta$ (propranolol, Sigma, $\beta_1/\beta_2$ receptor blocker, 1 mg/ml infused at 2.5 $\mu$l/min) blockers were infused intravenously either concurrently (n=13) or individually (n=5-6) starting 30 minutes prior to insulin infusion and continued throughout the severe hypoglycemic clamp, as described above. Saline was infused in controls (n=12). The drug doses chosen were based on previous studies describing the use of adrenergic blockers in rats\textsuperscript{98} and by pilot studies.

**Statistical analyses.** All data are represented as mean±sem. Analysis of variance (one- and two-way where appropriate), repeated measures ANOVA, and student’s t-test were used to determine significance. Holm-Sidak’s and Dunn’s method were used for post-hoc analyses. Fisher exact test with the Freeman-Halton extension was used to determine significance for mortality and arrhythmia incidence. Significance was determined by $p<0.05$. 
**Results**

**Study 1: Overall mortality analysis in control, diabetic and recurrently hypoglycemic experiments**

In order to understand how diabetes and recurrent hypoglycemia might affect the ability to survive an episode of severe hypoglycemia, rats were placed into 3 groups: 1) control, non-diabetic, 2) uncontrolled diabetic (streptozotocin), and 3) recurrent moderate hypoglycemia (3 days of glucose 25-40 mg/dl for 3 hours). Basal morning glucose levels for control non-diabetic rats were 88±5 mg/dl. Uncontrolled STZ-diabetic rats had a fasted basal glucose of 348±37 mg/dl. Rats that underwent recurrent hypoglycemia had a basal glucose of 74±2 mg/dl. All three groups of rats underwent a hyperinsulinemic/severe hypoglycemic clamp for 1 hour. During severe hypoglycemia, mean glucose levels were similar for control (11.5±0.21mg/dl), diabetic (12.1±0.24mg/dl), and recurrent hypoglycemia (11.9±0.41mg/dl) rats. The epinephrine response to hypoglycemia was normal in diabetic rats, but epinephrine was blunted in recurrent hypoglycemia rats (2001±241 vs 3487±474 pg/ml in controls\(^60\)). All rats lost their righting reflex, went into a coma, and experienced brief seizure like activity. Analysis of severe hypoglycemia induced mortality rates was found to be 21% in control, non-diabetic rats and increased to 36% in diabetic rats (p<0.05; Figure 20A). Interestingly, rats that had been previously treated with the recurrent moderate hypoglycemia protocol had markedly less mortality (only 4%) during their severe hypoglycemic clamp (p<0.05; Figure 20A). Differences in death rates were hypothesized to be due to hypokalemia, neuroglycopenia, and/or the sympathoadrenal
response, each of which could have increased susceptibility to cardiac arrhythmias and are each individually tested in the subsequent experiments.

**Study 2: Potassium supplementation**

In order to determine if cause of death due to severe hypoglycemia resulted from hypokalemia, potassium was supplemented in non-diabetic and diabetic rats during a 1 hour severe hypoglycemic clamp. Morning basal glucose levels were similar for rats that either received or did not receive potassium supplementation. Control rats had basal glucose levels of 127±7 mg/dl and 116±12 mg/dl, respectively. Basal glucose levels for diabetic rats were 479±77 mg/dl and 394±54 mg/dl, respectively. Mean glucose levels during severe hypoglycemia were not different among the non-diabetic rats that did (12.6±1.18mg/dl) or did not (12.5±0.76mg/dl) receive potassium or the diabetic rats that did (12.1±0.84mg/dl) or did not (13.4±0.39 mg/dl) receive potassium. Potassium levels decreased during severe hypoglycemia in both non-diabetic rats (from 4.2±0.15 to 2.9±0.06 mmol/l) and in diabetic rats (from 4.0±0.11 to 2.9±0.23 mmol/l) that did not receive potassium (Figure 20B). With potassium supplementation, potassium levels were maintained during severe hypoglycemia compared to basal in non-diabetic (4.4±0.26 to 3.9±0.38mmol/l) and diabetic (4.5±0.25 to 3.8±0.47mmol/l) rats (Figure 20B). Mortality associated with severe hypoglycemia trended to be reduced with potassium supplementation compared to rats that did not receive potassium supplementation in both non-diabetic (from 33% to 8%) and diabetic (from 44% to 25%) rats, but this did not reach significance (p<0.08; Figure 20C).
Study 3: Cardiac arrhythmias in control experiments

We observed that sudden death in the setting of severe hypoglycemia seemed to be related to electrolyte (i.e. potassium) concentration. Since hypokalemia can be pro-arrhythmic, in order to determine if severe hypoglycemia induced mortality is mediated by cardiac arrhythmias, control, non-diabetic rats (n=6) were subjected to hyperinsulinemic/severe hypoglycemic clamps with continuous ECG recording. Glucose levels were gradually decreased and glucose was held between 10-15 mg/dl for 90 minutes (Figure 21A). With this longer duration of severe hypoglycemia, all the rats died (Figure 21B).

Following insulin administration and during the initial period of moderate hypoglycemia, heart beats per minute (bpm) remained normal as compared to the basal state (Figure 21C). It was not until severe hypoglycemia that heart rate increased. Additionally, during severe hypoglycemia, multiple cardiac arrhythmias were noted, discussed below. Heart rate was variable toward the end of severe hypoglycemia due to the few survivors towards the end of the clamp and due to the multiple types of cardiac arrhythmias that tended to, in some cases, reduce the heart rate while at other times increase the heart rate. Periods of tachycardia (>400 bpm) as well as bradycardia (<300 bpm) were noted with increasing frequency with the duration of severe hypoglycemia.

Widening of the QTc complex as noted on the ECG rhythm, represents dispersion of ventricle depolarization and repolarization and is known to be pro-arrhythmic. Marked QTc prolongation was observed shortly after insulin infusion when glucose levels were only in the moderate hypoglycemia range (47±6 mg/dl) (Figure 21D). As compared to basal QTc of 122±2ms, peak QTc during severe hypoglycemia
was 172±8ms, indicating a 40% increased ventricular depolarization and repolarization phase (p<0.001).

Epinephrine and norepinephrine increased during severe hypoglycemia compared to the basal period (p < 0.001; Figure 21E,F; Table 1). Plasma epinephrine peaks around the start of severe hypoglycemia and decreases as severe hypoglycemia prolongs indicating possible adrenal failure. Plasma norepinephrine gradually increases during severe hypoglycemia.

In the setting of hypoglycemia, QTc prolongation, hypokalemia, and elevated catecholamines can increase risk of cardiac arrhythmias. Cardiac arrhythmias did not occur during early moderate hypoglycemia. It was only following 15 minutes of severe hypoglycemia did premature ventricular contractions (PVCs) and narrow complex second degree heart block (Mobitz II) started to be noted as the initial cardiac arrhythmias (Figure 22). Frequency of PVCs and 2nd degree heart block were 1.4±0.7 and 2.1±0.8 per minute, respectively (Figure 23A). In addition, other cardiac arrhythmias typically seen during severe hypoglycemia were premature atrial contractions (PACs) and 1st degree heart block (Figure 22). Occasionally, brief (<10 seconds) runs of ventricular fibrillation and non-sustained ventricular tachycardia were noted, but these rhythms were not noted to precede sudden death (Figure 23B). During severe hypoglycemia, a consistent pattern of sequential progression of arrhythmias that preceded cardiorespiratory arrest was carefully noted. With prolonged hypoglycemia, more frequent and longer duration second degree heart block as well as higher grade second degree heart block (ie, atrial:ventricular conduction ratio of 4:1) were noted. Following second degree heart block, the most commonly noted terminal arrhythmias
were third degree heart block (complete atroventricular dissociation), characterized by a terminal bradycardic ventricular rhythm (Figure 22).

Arterial blood gas and electrolytes. Oxygen and carbon dioxide levels remained normal throughout the experiment until just prior to death, where oxygen levels decreased and carbon dioxide levels increased (Figure 23C, Table 1).

Potassium levels decreased during severe hypoglycemia compared to the basal period (p< 0.05, Table 1). In the moribund phase, potassium levels increased. Following a similar pattern, pH levels also remained normal throughout the clamp and only decreased just prior to death (p<0.05, Table 1). Calcium and bicarbonate levels did not change during severe hypoglycemia (Table 1).

Blood pressure. Blood pressure increased from 105±14/60±2 during the basal period to 177±16/93±9 mmHg during severe hypoglycemia (Figure 23D). Blood pressure remained elevated until immediately prior to death when it suddenly decreased. Before becoming immeasurable, the last recorded blood pressure was 77±10/59±10 mmHg.

Seizures. Seizure-like activity was noted in all rats that underwent hyperinsulinemic/severe hypoglycemic clamps. Seizure-like activity was quantified by counting the number of characteristic, brief (<5 sec), myoclonic convulsions. A subset of rats also had simultaneous EEG recording during the clamp. During severe hypoglycemia, EEG tracings showed characteristic high-amplitude slow waves and EEG isoelectricity coincided with a non-responsive comatose state (Figure 24). Also during severe hypoglycemia, high frequency high amplitude spikes of increased electrical activity were noted during seizures. Given that all severely hypoglycemic rats
had seizures, yet only a fraction of severe hypoglycemic rats died, it was not surprising that no correlation was found between seizure activity and mortality.

**Study 4: ICV glucose infusion**

In order to understand and to isolate the extent to which neuroglycopenia during hypoglycemia mediates sudden death, glucose was infused into the 3rd ventricle of the brain during the severe hypoglycemic clamps to prevent brain neuroglycopenia. Compared to controls, ICV glucose infusion reduced mortality due to severe hypoglycemia from 86% to 33% (p < 0.05; Figure 25A). Systemic blood glucose was similar during severe hypoglycemia in ICV mannitol (MAN, n=9) and ICV glucose (GLU, n=9) infused rats (11.7±0.1 vs 12.2±0.2 mg/dl, respectively; p = 0.13; Figure 25B; Table 2). Despite matched glucose levels, ICV glucose infused rats required a higher peripheral glucose infusion rate (5.4±0.56 mg/kg/min; p < 0.005) during severe hypoglycemia compared to MAN (4.1±0.41 mg/kg/min; Figure 25C). Insulin levels were comparable between both groups during basal and severe hypoglycemia (Table 2).

**ECG analyses.** During severe hypoglycemia, heart rate was significantly lower in the GLU group compared to the MAN group (p<0.05, Figure 25D). Mean heart rate increased in the ICV mannitol group from 373±4 in the basal to 414±9 BPM during severe hypoglycemia (p < 0.01) while ICV glucose infusion prevented this rise in heart rate (basal:357±4 vs. severe hypoglycemia:357±8 BPM). Mortality incidence was associated with duration of sinus tachycardia (heart rates greater than 400 BPM; Figure 26A); however, ICV glucose infused rats had decreased mortality compared to ICV mannitol infused rats despite more than 30 minutes of tachycardia.
QTc increased during severe hypoglycemia compared to basal in both MAN and GLU groups with no significant differences between the groups (p = 0.14; Figure 25E). Greater QTc length was associated with an increased mortality risk (Figure 26B). However, mortality rate was lower in ICV glucose infused rats compared to ICV mannitol infused rats despite QTc prolongation.

Cardiac arrhythmias were absent in 25% of the ICV glucose infused rats. Frequencies of PVCs and second degree heart block were nonsignificantly reduced with ICV glucose infusion compared to ICV mannitol infusion (p<0.07 and p<0.06, respectively; Figure 26C,D). Incidence of 3rd degree heart block was decreased in the GLU group compared to the MAN group (p<0.05; Figure 26E). Third degree heart block was observed just before death in all rats that died in both groups. Rats that lived did not experience third degree heart block suggesting that third degree heart block was the terminal fatal arrhythmia associated with severe hypoglycemia. Other life threatening arrhythmias such as ventricular tachycardia leading to ventricular fibrillation was witnessed in 33% of the MAN group and 0% of the GLU group but this difference was not significant (Figure 26F).

Epinephrine increased in both the MAN and GLU groups during severe hypoglycemia to 1926±320 and 2155±448 pg/ml, respectively, with no difference between the groups (Table 2). Norepinephrine increased in the MAN group during severe hypoglycemia (1441±646 pg/ml) but this response was attenuated in the GLU group (464±48 pg/ml) (p< 0.05; Figure 25F, Table 2). Interestingly, just prior to death, norepinephrine was significantly elevated in ICV glucose infused rats (p<0.001 vs severe hypoglycemia; Table 2).
**Arterial blood gas and electrolytes.** Potassium levels significantly decreased during severe hypoglycemia in both the MAN and GLU groups compared to basal (Table 2). Potassium during severe hypoglycemia was significantly lower in the GLU group (p<0.05).

Oxygen and carbon dioxide levels did not change during severe hypoglycemia (Table 2). Just before respiratory arrest in MAN and GLU rats that died, oxygen decreased to 35±10 and 18±17%, respectively, and carbon dioxide increased to 47±3 and 53±5 mmHg, respectively (Table 2).

Mean respiration during severe hypoglycemia for the MAN and GLU groups was not different (85±4.4 and 78±4 breaths per minute, respectively; Table 2) but ICV glucose infused rats had lower respiration during severe hypoglycemia compared to basal. Respiration decreased just prior to death in the MAN and GLU groups, respectively (p <0.01 vs basal; Table 2).

Seizure occurrence was 100% in the MAN group but only 78% in the GLU group; however, this was not statistically significantly different (data not shown).

**Study 5: Adrenergic blockade**

In order to test the hypothesis that the sympathoadrenal response increases risk of fatal cardiac arrhythmias during severe hypoglycemia, rats had adrenergic receptors blockers infused during a 3 hour hyperinsulinemic/severe hypoglycemic clamp. Mortality associated with severe hypoglycemia was 33% in control rats (n=12) while both combined α and β blocker infusion (α/β, n=13) and β blocker infusion alone (n=5)
completely prevented death ($p < 0.029$; Figure 27A). $\alpha$ blocker infusion alone had no effect on mortality (50%; n=6).

Glucose levels during severe hypoglycemia were equally matched in control, combined $\alpha/\beta$ blocker, $\alpha$ blocker alone, and $\beta$ blocker alone infused rats (11.7±0.2, 12.3±0.3, 11.6±0.5, and 12.4±0.5 mg/dl, respectively; Figure 27B). Consistent with a blunted effective counterregulatory response, glucose infusion rate was increased during severe hypoglycemia in $\alpha/\beta$ blocker infused rats (4.87±0.24mg/kg/min; $p < 0.01$; Figure 27C) compared to controls (3.3±0.41mg/kg/min). Glucose infusion rates during severe hypoglycemia were nonsignificantly increased in $\beta$ blocker infused rats (4.5±0.65 mg/kg/min) and nonsignificantly decreased in $\alpha$ blocker infused rats (2.1±0.61 mg/kg/min) compared to controls ($p = 0.12$; Figure 27C). Insulin levels were not significantly different between the groups (Figure 28A).

**ECG analyses.** Heart rate was variable throughout the clamp. During severe hypoglycemia mean heart rate was 331±5, 225±5, 339±9, and 198±4 BPM in CON, $\alpha/\beta$ blocker, $\alpha$ blocker, and $\beta$ blocker infused rats, respectively (Figure 27D). $\alpha/\beta$ and $\beta$ blocker infused rats had significantly lower heart rates during severe hypoglycemia ($p<0.05$, ANOVA). While soon after the start of insulin infusion and glucose lowering the $\alpha$ blocker infused rats had a higher heart rate compared to controls, during severe hypoglycemia heart rate was similar to controls. The lower heart rate in the $\alpha/\beta$ blocker and $\beta$ blocker infused rats indicates that the effects of beta blockade were working to prevent the rise in heart rate seen in control rats. Additionally, increased heart rate in $\alpha$ blocker infused rats indicates that the alpha blockade treatment was effective. In the control and $\alpha$ blocker groups, sinus tachycardia (heart rate greater than 400 BPM) was
experienced in all of the rats that died with more than 30 minutes of tachycardia resulting in 100% mortality (Figure 28B). Treatment with β blocker, and combined α/β blocker completely abrogated the hypoglycemia induced tachycardia and mortality. Therefore, sinus tachycardia is a predictor of mortality in these experiments and prevention of tachycardia was associated with a mortality benefit.

From baseline levels, there was significant QTc prolongation during severe hypoglycemia in control (105±6 to 163±2ms), α blocker (129±3 to 210±5ms), and β blocker (117±3 to 164±2 ms) infused rats (p < 0.001) but QTc prolongation was blunted in α/β blocker (99±2 to 134±3ms; p < 0.001) infused rats compared to controls (Figure 27E). QTc prolongation was associated with an increased risk of mortality in the control and α blocker rats (Figure 28C). In control and α blockade rats, markedly increased QTc prolongation lengths of more than 200ms resulted in 33% and 67% mortality, respectively. Interestingly, the β blocker infused rats had increased QTc prolongation very similar to control rats (Figure 27E) yet had a 100% survival rate indicating that QTc prolongation alone may not be a direct mediator of lethality associated with severe hypoglycemia, but rather may be a harbinger of impending mortality. QTc prolongation appears to be a marker of severe hypoglycemia, but is not in itself sufficient to trigger the more lethal types of cardiac rhythms.

While the control and α blocker infused rats experienced PACs, PVCs, and 1st, 2nd, and 3rd degree heart block, the α/β blocker and β blocker infused rats mostly only experienced a few PACs, PVCs and 2nd degree heart block but with less frequency. PVCs occurred in 25% of the rats with α/β blocker infusion but only during or after a visible seizure whereas heart block occurred in only 8% of the rats (p<0.001). Single α
and β blocker infused rats had reduced arrhythmias overall compared to controls. Frequency of PVCs was reduced in combined α/β blocker and β blocker infused rats compared to controls (p<0.001; Figure 29A). Although α blocker infused rats trended to have lower frequency of PVCs, there was no significant difference compared to controls. Frequency of 2nd degree heart block was significantly reduced in combined α/β blocker (0.0004±0.0004 per minute; p< 0.001) and trended to be reduced in α blocker (0.08±0.06 per minute), and β blocker (0.17±0.18 per minute) infused rats compared to controls (1.1±0.43 per minute; Figure 29B). Third degree heart block was prevented in α/β blocker and β blocker infused rats but was only significantly different with α/β blocker infusion (p< 0.024; Figure 29C). Rats with α blocker infusion experienced similar incidence of 3rd degree heart block as controls. These results indicate that although β blocker infusion is sufficient to prevent death, both α and β adrenoreceptors need to be blocked to nearly completely prevent arrhythmias.

Epinephrine and norepinephrine significantly increased during severe hypoglycemia compared to basal in control, α/β blocker, α blocker, and β blocker infused rats (Figure 29D,E); however, while measurable in the peripheral circulation, the actions of epinephrine and norepinephrine were dually blocked with combined α/β blockade or partially blocked with single α or β blockade. Epinephrine peaked during the first 30 minutes of severe hypoglycemia and was significantly higher in β blocker infused rats (5610±599pg/ml) compared to controls (2603±353pg/ml; p<0.004) and α/β blocker infused rats (3097±396; p<0.011; Figure 29D). Epinephrine levels in α blocker infused rats (3335±716 pg/ml) were not different from controls. Norepinephrine was significantly
increased in the α blocker group (2720±435 pg/ml; p<0.001) compared to the control (667±164 pg/ml), α/β blocker (591±69 pg/ml), and β blocker groups (1006±312 pg/ml; Figure 29E).

Glucagon increased during severe hypoglycemia compared to basal in control, α/β blocker, and α blocker infused rats with no significant difference in β blocker infused rats. Glucagon was significantly higher in α blocker infused rats compared to all three groups (p<0.001 for AUC; Table 3). Elevated glucagon is consistent with the elevated norepinephrine in the α blocker group which may be acting on beta adrenergic receptors on pancreatic alpha cells to stimulate glucagon secretion at a higher rate compared to controls.

**Arterial blood gas and electrolytes.** Mean potassium levels decreased in all groups during severe hypoglycemia although potassium was significantly higher in β blocker infused rats (p <0.01, Table 3). The similar degree of low potassium between the control, α/β blocker, and α blocker infused rats indicates that in the setting of severe hypoglycemia, hypokalemia alone is not sufficient to cause cardiac arrhythmias because α/β blocker infusion prevents arrhythmias in the presence of hypokalemia.

Oxygen saturation and carbon dioxide levels were normal for all groups throughout the clamp (Table 3). Control rats had a mean respiration during severe hypoglycemia of 76±3 breaths/min (Table 3). Respiration decreased in the α/β blocker and β blocker infused rats to 51±2 and 54±2 breaths/min, respectively (p < 0.001), while α blocker infused rats had similar respiration (65±8 breaths/min) compared to controls. Decreased respiration was consistent with higher carbon dioxide levels in α/β blocker and β blocker infused rats versus controls (p < 0.001; Table 3). Respiration decreased
in the control and α blocker infused rats just prior to death with a concomitant decrease in oxygen and increase in carbon dioxide levels (Table 3).

Seizure like activity was witnessed in 100% of the control, α blocker, and β blocker infused rats but only 71% of the α/β blocker infused rats (data not shown). However, this difference was not significant and no correlations were found between seizure-like activity and mortality.

**Blood pressure.** Mean systolic blood pressure during severe hypoglycemia for control, α/β blocker, α blocker, and β blocker infused rats was 133±14, 140±10, 129±8, and 112±4 mmHg with β blocker infusion resulting in a significantly lower blood pressure (p<0.001; Figure 28D). Mean diastolic blood pressure during severe hypoglycemia was 91±16, 90±9, 92±8, and 77±4 mmHg, respectively, with a significantly lower blood pressure in β blocker infused rats (p<0.001; Figure 28E). Most importantly blood pressure did not suddenly increase or decrease prior to death in any of the groups indicating that blood pressure is maintained throughout severe hypoglycemia.
Figure 20. Severe hypoglycemia induced mortality in diabetic, non-diabetic, recurrently hypoglycemic rats, and the effects of potassium supplementation (studies 1 and 2). A) Analysis of mortality associated with severe hypoglycemia revealed 21% mortality in non-diabetic control rats (n=123). Mortality was increased in diabetic rats to 36% (n=95) and decreased to 4% (n=27) in rats that underwent recurrent hypoglycemia prior to severe hypoglycemia (*p<0.05 Fisher exact test, Freeman-Halton extension). B) Potassium levels in rats that had no potassium supplementation (No K+, non-diabetic: white bar, diabetic: diagonal slash) versus rats with potassium supplementation (K+, non-diabetic: black bar, diabetic: horizontal slash) both in the basal state and during severe hypoglycemia. Rats without supplementation had potassium levels significantly decrease during severe hypoglycemia while rats with supplementation maintained normal potassium during severe hypoglycemia. Potassium levels in nondiabetic rats without potassium (white bar), nondiabetic rats with potassium
(diagonal slash), diabetic rats without potassium (black bar), diabetic rats with potassium (horizontal slash). C) Potassium supplementation in nondiabetic and diabetic rats trended to reduce severe hypoglycemia-induced mortality from 33 to 8% (n=12-15) and 44 to 25% (n=28-45), respectively, but this did not reach significance. Data expressed as mean+sem.
Figure 21. Severe hypoglycemic clamp in control experiments (study 3). A) Glucose levels of control, nondiabetic rats during a severe hypoglycemic clamp. With insulin infusion and careful glucose infusion, plasma glucose levels are decreased over a 2 ½ hour period to 15 mg/dl (time 0). Severe hypoglycemia is held for 1 ½ hours or until death. B) Mortality associated with severe hypoglycemia started as early as 25 minutes into severe hypoglycemia with 50% mortality at 50 minutes into severe hypoglycemia. All rats had died 90 minutes into severe hypoglycemia. C) Heart rate during the clamp remains normal until just before severe hypoglycemia when glucose levels reach ~35 mg/dl at which time heart rate increases. At the onset of severe hypoglycemia, heart rate decreases. Heart increases again immediately prior to death. The slower heart rates that occur towards the end of the clamp can be attributed to
multiple cardiac arrhythmias associated with bradycardia. D) QTc prolongation starts to occur during insulin infusion when glucose levels are moderately hypoglycemic (~35 mg/dl) and persists into severe hypoglycemia. Mean QTc during severe hypoglycemia increased to 158±5 ms compared to 122±2 ms in the basal period (p<0.01). E) Epinephrine levels increased from 495±60 in the basal period and peak at 3034±383 pg/ml at the onset of severe hypoglycemia, but decline as severe hypoglycemia persists (p<0.001). F) Norepinephrine levels increased from 197±20 in the basal period and start to increase at the onset of severe hypoglycemia and continue to rise throughout severe hypoglycemia, peaking at 748±116 pg/ml (p<0.001). n=6; Data expressed as mean±sem.
Figure 22. ECG changes in response to severe hypoglycemia (study 3). Electrocardiogram (ECG) recordings from one representative control rat from basal (euglycemia) through severe hypoglycemia induced death. The order of the graphs is a typical representation of what occurs in all rats during severe hypoglycemia and just prior to death. At the top of the left column, the ECG shows a rat in normal sinus rhythm during euglycemic conditions. During moderate hypoglycemia, cardiac arrhythmias are rarely witnessed. At the start of severe hypoglycemia, heart rate tends to return to basal levels followed by QTc prolongation and the initiation of several types of cardiac arrhythmias. Cardiac arrhythmias that occur during severe hypoglycemia are premature atrial contractions (PAC noted by arrow), premature ventricular contractions (PVC noted by arrow), nonsustained ventricular tachycardia (v-tach), 1st degree heart block (PR
prolongation noted by arrow), and occasional 2\textsuperscript{nd} degree heart block (absent QRS complex noted by arrow). With severe hypoglycemia of increased duration, frequency of 2\textsuperscript{nd} degree heart block increases followed by 3\textsuperscript{rd} degree heart block. At the time cardiorespiratory arrest, heart rate is markedly decreased.
Figure 23. Arrhythmias, blood gas, and blood pressure in response to severe hypoglycemia in control experiments (study 3). A) Frequency of both PVCs and 2\textsuperscript{nd} degree heart block occur at a rate of 1.4±0.74 and 2±0.78 per minute, respectively, during severe hypoglycemia. B) 3\textsuperscript{rd} degree heart block occurred in 100% of the rats and v-tach occurred in 75% of the rats. C) Oxygen (open diamond, left axis) and carbon dioxide (black square, right axis) levels remain normal during severe hypoglycemia (97% O2 and 32 mmHg CO2). During apnea and just prior to death, oxygen levels decrease (38±6%) and carbon dioxide levels increase (53±3 mmHg). D) Blood pressure increased at the start of severe hypoglycemia and remained elevated throughout severe hypoglycemia. Blood pressure decreased as the rats died. n=6. Data expressed as mean±sem.
Figure 24. Electroencephalogram (EEG) recordings in response to hypoglycemia in a control rat during a hyperinsulinemic/severe hypoglycemia clamp (study 3). The progressive series of EEG recordings initially shows basal brain activity during the euglycemic state followed by slow, high amplitude brain waves, characteristic of severe hypoglycemia. Isoelectric activity on the EEG corresponded to rats in a comatose state. The sudden onset of chaotic, high amplitude EEG voltages of short duration (~5 seconds) corresponded to visible tonic-clonic seizure-like behavior.
Figure 25. Severe hypoglycemic clamp with ICV glucose infusion (study 4). A) Mortality due to severe hypoglycemia was significantly reduced from 89% in ICV mannitol to 33% in ICV glucose infused rats (*p<0.05 Fisher exact test, Freeman-Halton extension). B) Blood glucose levels were evenly matched between the ICV mannitol (white circle) and ICV glucose (black circle) infused rats. Once blood glucose levels reached 15 mg/dl, severe hypoglycemia was started (time 0). Severe hypoglycemia was held for a duration of 3 hours. C) Mean glucose infusion rates during severe
hypoglycemia were significantly elevated in ICV glucose (black bar) infused rats (5.4±0.6 mg/kg/min) compared to ICV mannitol (white bar) infused rats (4.2±0.41 mg/kg/min). *p<0.01 ttest. D) Heart rate remained normal compared to basal in both groups until 45 minutes into severe hypoglycemia when ICV mannitol infused rats had elevated heart rate. ICV glucose infused rats had lower heart rates throughout severe hypoglycemia compared to ICV mannitol infused rats. Mean heart during severe hypoglycemia was 414±9 BPM and 357±8 in ICV mannitol and ICV glucose infused rats, respectively (*p < 0.05 ttest). E) The QTc length was increased after the start of insulin infusion during moderate hypoglycemia and reached statistical significance at 30 minutes into insulin infusion. During severe hypoglycemia, QTc length was not different between ICV mannitol and ICV glucose infused rats with mean QTc values of 180±2 and 187±3 ms, respectively. F) Norepinephrine levels were increased during severe hypoglycemia in ICV mannitol infused rats compared to the basal state, but this response was blunted in ICV glucose infused rats. *p<0.05, ttest. n=9/group. Data expressed as mean±sem.
Figure 26. Mortality and arrhythmia incidence in ICV glucose infusion (study 4). A) Mortality incidence was associated with duration of sinus tachycardia (heart rates greater than 400 bpm). Tachycardia lasting <15 minutes resulted in no mortality. Tachycardia lasting 16-30 minutes resulted in 17% and 20% death in the ICV mannitol and ICV glucose infused rats, respectively. Tachycardia lasting longer than 30 minutes led to 80% death in ICV mannitol infused rats but only 50% death in ICV glucose infused rats. B) The incidence of mortality increased with increased duration of ventricular depolarization/repolarization dispersion as noted by greater QTc prolongation. Basal QTc duration was 122±2 ms but increased in response to hypoglycemia. Increased QTc lengths of 150-179 ms did not increase mortality. Increased QTc lengths of 180-200ms was associated with 14 and 13% mortality in ICV.
mannitol and ICV glucose infused rats, respectively. QTc lengths of more than 200 ms was associated with 83% mortality in ICV mannitol infused rats but only 40% in ICV glucose infused rats. C) During severe hypoglycemia, the frequency of premature ventricular contractions (PVCs) was nonsignificantly reduced from 2.2±1.2 per minute in ICV mannitol to 0.54±0.32 per minute in ICV glucose infused rats (p = 0.07). D) During severe hypoglycemia, the frequency of 2nd degree heart block was 2.6±0.66 and 1.1±0.45 per minute for ICV mannitol and ICV glucose infused rats (p=NS). E) During severe hypoglycemia, the incidence of 3rd degree heart block was 89% and 38% for the ICV mannitol and ICV glucose infused rats (*p<0.05). F) Ventricular tachycardia (v-tach) during severe hypoglycemia occurred in 38% of the ICV mannitol infused rats but none of the ICV glucose infused rats experienced v-tach (p=NS). white circle/bar= ICV mannitol; black circle/bar= ICV glucose. *p<0.05, ttest. n=9/group. Data expressed as mean±sem.
Figure 27. Severe hypoglycemic clamp with adrenergic blockade (study 5). A) Control rats had a mortality rate of 33% (n=12) in response to severe hypoglycemia. Alpha blockade had no effect on mortality (50%; n=6). Death was prevented in both $\alpha/\beta$ (n=13) and $\beta$ blocker (n=5) infused rats (*p<0.029, Fisher exact test, Freeman-Halton extension). B) Glucose levels were evenly matched throughout the clamp. Glucose was clamped between 10-15 mg/dl for 3 hours. C) Mean glucose infusion rate during severe hypoglycemia. $\alpha/\beta$ blockade significantly increased glucose infusion rate. Alpha blockade and beta blockade alone did not differ compared to controls but $\alpha$ blocker rats
had lower glucose infusion rate compared to $\alpha/\beta$ blocker and $\beta$ blocker infused rats (p<0.05, ANOVA). D) Heart rate was consistently lower in $\alpha/\beta$ and $\beta$ blockade rats throughout the clamp compared to control and $\alpha$ blockade rats (*p<0.001 for individual timepoints, ANOVA). E) QTc length was significantly increased in all groups during severe hypoglycemia (p<0.001, ANOVA). Combined $\alpha/\beta$ blockade significantly blunted the QTc prolongation compared to controls (*p<0.001 for mean QTc during severe hypoglycemia, ANOVA). White circle= control; black circle= $\alpha/\beta$ (a/b) blocker; white triangle= $\alpha$ (a) blocker; black triangle= $\beta$ (b) blocker. Data expressed as mean±sem.
Figure 28. Insulin levels, mortality incidence and blood pressure levels during severe hypoglycemia with or without adrenergic blockade (study 5). A) Insulin levels were not different among the groups in the basal or severe hypoglycemia periods. B) Incidence of severe hypoglycemia induced death was increased with duration of sinus tachycardia (defined as >400BPM). Tachycardia did not develop in α/β or β blocker rats. Alpha blockade rats all died with less than 15 minutes of tachycardia...
whereas control rats had 100% mortality with more than 30 minutes of tachycardia. C) Incidence of mortality increased with QTc length. During severe hypoglycemia, rats that had a QTc prolongation of 150-179ms all survived, whereas in rats where hypoglycemia increased QTc lengths to 180-200ms there was an associated increase in mortality to 25% in control rats. In control and alpha blockade rats, QTc prolongation of more than 200ms resulted in 33% and 67% mortality, respectively. D and E) Systolic (D) and diastolic (E) blood pressure (BP) during severe hypoglycemia were lower in β blocker infused rats compared to controls (*p<0.001 Two-way ANOVA). White bar= control; black bar= α/β (a/b) blocker; diagonal slash= α (a) blocker; horizontal slash= β (b) blocker. n=5-13. Data expressed as mean±sem.
Figure 29. Severe hypoglycemia induced arrhythmias and catecholamines levels during adrenergic blockade (study 5). A) As compared to control rats, PVC frequency during severe hypoglycemia was significantly decreased in $\alpha/\beta$ and $\beta$ blockade rats (*$p < 0.001$, ANOVA) with a trend for decreased frequency in $\alpha$ blockade rats. B) Second degree heart block frequency was significantly reduced in $\alpha/\beta$ blockade rats with only 1 rat experiencing 2$^{\text{nd}}$ degree heart block. Alpha and beta blockade alone trended to reduce frequency of 2$^{\text{nd}}$ degree heart block but this was not significantly different
compared to controls. C) Incidence of 3rd degree heart block was 40% in control rats and 16% in α blockade rats. No 3rd degree heart block occurred in α/β or β blockade rats. (*p<0.02 Fisher exact test, Freeman-Halton extension). D) Epinephrine levels increased significantly in all groups during severe hypoglycemia. The beta blockade group had significantly higher epinephrine levels compared to all other groups. (*p<0.01 ANOVA) E) Norepinephrine levels increased significantly in all groups during severe hypoglycemia. Norepinephrine levels in the alpha blockade group were significantly higher compared to all 3 other groups. (*p<0.001 ANOVA). White bar= control; black bar= α/β (a/b) blocker; diagonal slash= α (a) blocker; horizontal slash= β (b) blocker. n=5-13. Data expressed as mean±sem.
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Table 1. Hormones and arterial blood gas and electrolytes in control experiments (study 3). Epinephrine and norepinephrine increased during severe hypoglycemia. Oxygen and carbon dioxide levels remain normal during severe hypoglycemia compared to basal. Just prior to death, oxygen levels decrease and carbon dioxide levels increase. Potassium levels slowly decline during insulin infusion with significantly lower potassium levels during severe hypoglycemia. Just before death, potassium levels increase. pH levels remain normal throughout severe hypoglycemia but just prior to death. Calcium and bicarbonate levels were not significantly different throughout the clamp. *p<0.05 vs basal. n=6. Data represented as mean±sem.
**Table 2. Glucose, hormones, and arterial blood gas and electrolytes for ICV glucose study (study 4).** Blood glucose levels were evenly matched during severe hypoglycemia in both ICV mannitol (MAN) and ICV glucose (GLU) groups. Insulin increased to a similar extent in the severe hypoglycemic clamp in both groups. Epinephrine and norepinephrine increased during severe hypoglycemia to the same extent in both groups. Just prior to death, norepinephrine increased in both treatment groups. Potassium decreased to a similar extent in both groups during severe hypoglycemia. Blood oxygen and carbon dioxide remained normal during severe hypoglycemia compared to the basal state. Blood oxygen levels decreased, and carbon dioxide levels increased, in rats that died in both groups immediately prior to death.

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<td></td>
<td>±27</td>
<td>±17</td>
<td>±646</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>4.66</td>
<td>4.43</td>
<td>3.58a</td>
</tr>
<tr>
<td></td>
<td>±0.13</td>
<td>±0.13</td>
<td>±0.14</td>
</tr>
<tr>
<td>Oxygen (SO2%)</td>
<td>97</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>±0.23</td>
<td>±0.29</td>
<td>±1.8</td>
</tr>
<tr>
<td>Carbon Dioxide (mmHg)</td>
<td>29</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>±0.49</td>
<td>±0.65</td>
<td>±1.3</td>
</tr>
<tr>
<td>Respiration (breaths/min)</td>
<td>94</td>
<td>95</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>±3</td>
<td>±4</td>
<td>±4</td>
</tr>
</tbody>
</table>

Blood glucose levels were evenly matched during severe hypoglycemia in both ICV mannitol (MAN) and ICV glucose (GLU) groups. Insulin increased to a similar extent in the severe hypoglycemic clamp in both groups. Epinephrine and norepinephrine increased during severe hypoglycemia to the same extent in both groups. Just prior to death, norepinephrine increased in both treatment groups. Potassium decreased to a similar extent in both groups during severe hypoglycemia. Blood oxygen and carbon dioxide remained normal during severe hypoglycemia compared to the basal state. Blood oxygen levels decreased, and carbon dioxide levels increased, in rats that died in both groups immediately prior to death.
Respiration remained normal in control rats during severe hypoglycemia but ICV glucose infused rats had lower respiration compared to the basal state. Prior to death, respiration significantly decreased in both groups. \(^a_p<0.05\) vs basal, \(^b_p<0.05\) vs control, ttest. \(n=9\)/group. Data represented as mean±sem.
### Table 3. Glucagon, arterial blood gas and electrolytes for adrenergic blockade study (study 5).

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>(\alpha/\beta) blockers</th>
<th>(\alpha) blocker</th>
<th>(\beta) blocker</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucagon</strong> (AUC)</td>
<td>3733</td>
<td>4137</td>
<td>10096(^a)</td>
<td>4559</td>
</tr>
<tr>
<td>Basal</td>
<td>+1342</td>
<td>+751</td>
<td>+1192</td>
<td>+483</td>
</tr>
<tr>
<td><strong>Potassium</strong> (mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>4.4</td>
<td>4.0</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>+0.14</td>
<td>+0.11</td>
<td>+0.09</td>
<td>+0.14</td>
</tr>
<tr>
<td>Severe Hypoglycemia</td>
<td>3.12(^a)</td>
<td>2.84(^a)</td>
<td>3.01(^a)</td>
<td>3.74(^{ab})</td>
</tr>
<tr>
<td></td>
<td>+0.18</td>
<td>+0.08</td>
<td>+0.21</td>
<td>+0.05</td>
</tr>
<tr>
<td>Pre-Death</td>
<td>3.6</td>
<td>N/A</td>
<td>4.2</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>+0.48</td>
<td></td>
<td>+0.21</td>
<td></td>
</tr>
<tr>
<td><strong>Oxygen</strong> (SO2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>96</td>
<td>97</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>+1.9</td>
<td>+0.28</td>
<td>+0.3</td>
<td>+0.32</td>
</tr>
<tr>
<td>Severe Hypoglycemia</td>
<td>97</td>
<td>98</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>+0.34</td>
<td>+0.15</td>
<td>+0.36</td>
<td>+0.14</td>
</tr>
<tr>
<td>Pre-Death</td>
<td>26(^a)</td>
<td>N/A</td>
<td>35(^a)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>+12</td>
<td></td>
<td>+0.28</td>
<td></td>
</tr>
<tr>
<td><strong>Carbon Dioxide</strong> (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>32</td>
<td>33</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>+1.7</td>
<td>+0.69</td>
<td>+0.87</td>
<td>+0.91</td>
</tr>
<tr>
<td>Severe Hypoglycemia</td>
<td>32</td>
<td>38(^{ab})</td>
<td>36</td>
<td>42(^{ab})</td>
</tr>
<tr>
<td></td>
<td>+1.4</td>
<td>+0.97</td>
<td>+2.2</td>
<td>+2.2</td>
</tr>
<tr>
<td>Pre-Death</td>
<td>48(^a)</td>
<td>N/A</td>
<td>63(^a)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>+2.5</td>
<td></td>
<td>+11</td>
<td></td>
</tr>
<tr>
<td><strong>Respiration</strong> (breaths/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>86</td>
<td>88</td>
<td>96</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>+4</td>
<td>+4</td>
<td>+8</td>
<td>+4</td>
</tr>
<tr>
<td>Severe Hypoglycemia</td>
<td>76</td>
<td>51(^{ab})</td>
<td>66(^a)</td>
<td>54(^{ab})</td>
</tr>
<tr>
<td></td>
<td>+3</td>
<td>+2</td>
<td>+8</td>
<td>+2</td>
</tr>
<tr>
<td>Pre-Death</td>
<td>28(^a)</td>
<td>N/A</td>
<td>8</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>+8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Glucagon area under the curve (AUC) was greater in alpha blockade rats and similar between combined alpha/beta and beta blockade rats compared to controls. Potassium levels decreased in all groups during severe hypoglycemia. Beta
blockade resulted in higher potassium levels during severe hypoglycemia compared to controls. Oxygen saturation remained normal in all groups throughout severe hypoglycemia. Prior to death in control and alpha blocker infused rats, oxygen decreased compared to basal. Carbon dioxide was higher during severe hypoglycemia in \( \alpha/\beta \), \( \alpha \), and \( \beta \) blocker infused rats compared to basal and control. Just prior to death in control and \( \alpha \) blockade rats, carbon dioxide levels increased. Respiration was significantly decreased during severe hypoglycemia in \( \alpha/\beta \), \( \alpha \), and \( \beta \) blocker infused rats compared to basal. Respiration was lower during severe hypoglycemia in \( \alpha/\beta \) and \( \beta \) blocker infused rats compared to controls. Respiration decreased just prior to death in control \( \alpha \) blocker infused rats. \(^{a}p<0.05\) vs basal, \(^{b}p<0.05\) vs control. n=5-13; Data represented as mean+sem.
Discussion

Mortality due to iatrogenic insulin administration represents a major concern for insulin treated diabetic patients and their families. Determining the mechanisms by which hypoglycemia induces sudden death is critically important in order to find treatments to protect patients who are at risk. Animal studies are needed to investigate the causes of mortality associated with severe hypoglycemia. In the current study, diabetes was found to exacerbate, while recurrent hypoglycemia was found to attenuate, severe hypoglycemia-induced sudden death. The cause of death in these preliminary rat studies was unknown. Cardiac arrhythmias have been shown to occur during moderate hypoglycemia in humans\textsuperscript{88} and fatal cardiac arrhythmias are speculated to mediate the “dead in bed syndrome” but no studies to date have shown this as it is difficult to investigate in the clinical setting. Using a rat model, we show for the first time that fatal cardiac arrhythmias occur as a result of severe hypoglycemia and such arrhythmias can be reduced by ICV glucose infusion and prevented by beta adrenergic blockade. These results indicate that brain neuroglycopenia and the sympathoadrenal response mediate fatal cardiac arrhythmias that occur during severe hypoglycemia (figure 30).

Diabetes increased mortality due to severe hypoglycemia although the mechanism(s) involved remains unclear. Uncontrolled diabetes is hypothesized to increase risk of fatal cardiac arrhythmias. Diabetes has been shown in animal models to alter the myocardium composition\textsuperscript{152} and lead to cardiac mechanical defects such as defects in left ventricular function and velocity ejection\textsuperscript{153}. Aside from chronic maladaptations associated with insulin deficiency, it may be that the greater absolute
decrement in blood glucose in diabetic (eg, from 348 to 12 mg/dl) versus control rats (from 88 to 12 mg/dl) may have contributed to overall increased mortality by inducing a greater net insult going from high to low glucose levels. Although STZ diabetic rats have been shown to be at higher risk for arrhythmias\textsuperscript{154}, in the absence of ECG data from these preliminary experiments, the cause of increased mortality rates in diabetic rats remains unknown.

Recurrent hypoglycemia significantly reduced severe hypoglycemia-induced mortality. These findings are consistent with a pre-conditioning effect of recurrent hypoglycemia to limit severe hypoglycemia induced seizures, brain damage and cognitive dysfunction\textsuperscript{60}. It is speculated that the blunted catecholamine response that occurred with recurrent hypoglycemia may be the mechanism by which recurrently hypoglycemic rats were protected from death. Interestingly, the blunted catecholamine response in the recurrently hypoglycemic rats apparently rendered them more prone to, but paradoxically less vulnerable to, an otherwise lethal episode of severe hypoglycemia. This putative mechanism is supported by the beta blockade treatment study that noted reduction of arrhythmias and prevention of death. In the absence of ECG recordings though, the extent to which cardiac arrhythmias were reduced by antecedent recurrent hypoglycemia is unknown.

As compared to human ECG recordings, rat ECGs are characterized by shorter interval durations, more rapid heart rate, absent Q waves, and absence of an isoelectric ST segment\textsuperscript{155}. Supporting our assertion that the reported ECG changes truly reflect ECG responses in humans, similar to the presented rate and rhythm disturbances noted by ECG monitoring in the current rat study, clinical ECG observations during
hypoglycemia include QTc prolongation\textsuperscript{87-89,156}, sinus bradycardia\textsuperscript{85,156}, and multifocal ventricular ectopies\textsuperscript{85}. Furthermore, rats respond to the effects of anti-arrhythmic agents such as adrenoreceptor blockers similar to humans as seen previously in a rat model in which combined alpha and beta adrenergic receptor blockade reduced sinus tachycardia and ST elevation induced by immobilization stress\textsuperscript{98}. Taken together, the rat ECG changes presented in these studies closely resemble those observed in humans during hypoglycemia, indicating that beta blockers have clinical significance to prevent severe hypoglycemia-induced cardiac arrhythmias and sudden death.

The mechanisms regulating severe hypoglycemia-induced sudden death are thought to be multifactorial; however, in these experiments a timeline of events leading to sudden death (Figure 31) can be summarized as follows: 1) In the setting of hyperinsulinemia, mild hypokalemia and mild QTc prolongation manifest first, even during euglycemic conditions. 2) As glucose levels are further lowered, potassium levels continue to drop even further and QTc prolongation increases reaching its maximum during severe hypoglycemia. Rarely, minor cardiac arrhythmias, such as PACs, are witnessed during more moderate levels of hypoglycemia (20-40 mg/dl) but were never witnessed during euglycemic conditions. 3) At the onset of severe hypoglycemia, epinephrine levels peak. 4) Cardiac arrhythmias develop shortly after, including PVCs, 1\textsuperscript{st} and 2\textsuperscript{nd} degree heart block, ventricular tachycardia, and atrial fibrillation. 5) Norepinephrine levels peak near 1 hour of severe hypoglycemia. 6) Shortly thereafter, sinus tachycardia occurs, and if not spontaneously corrected is very predictive of ensuing death. 7) In rats that die, sinus tachycardia is followed by increased frequency of PVCs and high grade 2\textsuperscript{nd} degree heart block. 8) If sinus tachycardia is not
spontaneously reversed, 3rd degree heart block is most often seen as the terminal cardiac rhythm that is associated with profound bradycardia followed by the rapid sequential onset of reduced cardiac output (noted by hypotension), respiratory depression, hypoxemia, hypercarbia, and acidosis. Sinus tachycardia and 3rd degree heart block were the strongest predictors of sudden death due to hypoglycemia. The sensitivity and specificity of sinus tachycardia (heart rate > 400 bpm) as a predictor of mortality was 88% and 74%, respectively, while 3rd degree heart block was 89% and 95%, respectively. Other predictors of mortality in the current experiments were QTc prolongation and increased frequency of 2nd degree heart block. The sensitivity and specificity of QTc prolongation (>200ms) as a predictor of mortality was 81% and 60%, respectively, while the sensitivity and specificity of 2nd degree heart block was 94% and 53%, respectively.

Moderate hypoglycemia has been previously shown to lead to QTc prolongation, which is known to be proarrhythmic. In the present studies, severe hypoglycemia induced a significant increase in QTc length indicating that hypoglycemia induces a type of acquired long-QTc syndrome. QTc lengthening was associated with increased risk of mortality. With adrenergic blockade, blunting the rise in QTc during hypoglycemia may have contributed to the reduced incidence of arrhythmias and ultimately the prevention of death. However, hypoglycemia induced QTc prolongation in beta blocker and ICV glucose infused rats were similar to their respective controls indicating that QTc prolongation per se does not lead to cardiac arrhythmias and death. Since providing the brain with glucose reduced mortality but did not reduce QTc prolongation, it is concluded that systemic factors such as low glucose, low potassium, and elevated
epinephrine levels that affect ventricular dispersion, and not neuroglycoenia per se, that regulate QT length. Taken together, these studies indicate that QTc prolongation is a marker of severe hypoglycemia and is predictive of ensuing cardiac arrhythmias, but is not sufficient to cause cardiac arrhythmias.

A role for potassium in regulation of cardiac arrhythmias has been noted. Hypokalemia can trigger cardiac arrhythmias during moderate hypoglycemia in humans. In addition, potassium supplementation in the current studies trended to reduce mortality in non-diabetic and diabetic rats indicating that hypokalemia increases risk of death due to severe hypoglycemia. However, because the mortality differences were not significant, it is still undetermined to what extent hypokalemia regulates severe hypoglycemia induced sudden death.

Blood potassium levels are known to be regulated by insulin, adrenergic activation and pH, each of which affects potassium entry into cells. 1) Insulin is known to lower plasma potassium levels by increasing potassium entry into cells. Insulin was shown to lower blood potassium levels during euglycemic conditions during the clamps but hypokalemia did not cause cardiac arrhythmias in this euglycemic setting indicating that low glucose levels are required in addition to hypokalemia to cause arrhythmias. 2) Beta and alpha adrenergic receptor stimulation are known to increase and decrease potassium entry into the cell thus resulting in decreased and increased potassium in the blood, respectively. In the current studies, beta blockade led to significantly higher blood potassium levels whereas alpha blockade led to a nonsignificant decrease in potassium levels during the clamp. Interestingly, combined alpha/beta blockade led to similar blood potassium levels as controls. This indicates that
although potassium levels were higher in the beta blockade group, this is not necessarily why mortality was prevented in this group because combined alpha/beta blockade resulted in potassium levels similar to controls yet all rats in this group lived. 3) Finally, acidosis is known to impair potassium entry into the cell thus leading to elevated plasma potassium. pH levels did not change during the clamps until just before death when acidosis occurred. When acidosis occurred it was also noted that hyperkalemia was present. However, hyperkalemia is not considered a cause of death but rather a result of acidosis. Taken together, the results from these studies indicate that the level of hypokalemia achieved in the setting of insulin induced hypoglycemia was not sufficient, or not of sufficient magnitude, to cause lethal cardiac arrhythmias.

Cardiac arrhythmias can also result from changes in respiration. In the current studies, the respiratory rate decreased slightly as the rats entered a severe hypoglycemia-induced coma, but the respiratory rate declined to a similar extent in all groups, although sometimes lower with beta blockade. Since there was no difference in the respiratory rate and since oxygen and carbon dioxide levels did not change during the clamps, it can be concluded that respiratory arrest developed only after cardiac arrest. Therefore, defects in respiration or respiratory drive, including brief post-ictal apneic episodes, are not primary contributors to severe hypoglycemia-induced sudden death but rather occur following a terminal cardiac arrhythmia.

Another potential contributor to cardiac arrhythmias is marked changes in blood pressure. During severe hypoglycemia, decreased cardiac output and resultant hypotension could lead to decreased blood flow to the brain and/or heart thereby increasing risk for fatal cardiac arrhythmias. However, based on the current studies,
blood pressure was maintained throughout the period of severe hypoglycemia and was therefore not thought to contribute to severe hypoglycemia induced mortality. In the control experiments from study 3, blood pressure was elevated during severe hypoglycemia and only dropped following a terminal bradycardic arrhythmia. It is surmised that hypotension did not contribute to cardiac death, but rather was a marker of sudden cardiac failure.

In addition to the above mentioned peripheral effects of insulin-induced severe hypoglycemia, central effects on hypoglycemic counterregulation are also important. In particular, the hypothalamus plays a critical role in regulating the hypoglycemic counterregulatory response. Low glucose in the ventromedial hypothalamus (VMH) is required for local release of norepinephrine\textsuperscript{15} which leads to increased adrenergic signaling and epinephrine release from the adrenal gland. Since glucose infusion into the VMH blunts the counterregulatory response to hypoglycemia in a dose dependent manner\textsuperscript{158} and brain centers are known to control heart contractility and respiration\textsuperscript{159}, it was hypothesized that if the brain has an adequate glucose supply during a hyperinsulinemic/severe hypoglycemic clamp, then cardiac arrhythmias and mortality would be reduced. Consistent with previous studies using a low dose of ICV glucose infusion\textsuperscript{158}, the ICV glucose dose infused in the current study was not enough to blunt the epinephrine response; however, the norepinephrine response was blunted. Interestingly, the blunted norepinephrine response during ICV glucose infusion may have contributed to reduced mortality. Although epinephrine remained elevated, heart rate in the ICV glucose infused rats was normal possibly due to the blunted norepinephrine response and/or an increased vagal tone. Reduced mortality rate with
ICV glucose infusion reveals that the brain plays an important role in maintaining normal heart rate and reducing cardiac arrhythmias during severe hypoglycemia even in the presence of elevated epinephrine. It can be concluded from the current studies that brain neuroglycopenia contributes to severe hypoglycemia-induced cardiac arrhythmias and mortality. Further research should focus on how cellular changes in brain glucose sensing and efferent pathways that occur during severe hypoglycemia may contribute to sudden death.

Hypoglycemic seizures were also noted to occur in most rats. However, with the exception of the recurrent hypoglycemia protocol, even though the incidence of seizures tended to be reduced in some studies, there was no correlation found between number of visible seizure-like activity and survival benefit. Consistent with other studies demonstrating survival following hypoglycemia induced seizures, these current studies indicate that seizures are not a primary contributor to severe hypoglycemia-induced mortality but rather serve as a marker of severe hypoglycemia.

Whether central or systemic, the rise in catecholamines during severe hypoglycemia was hypothesized to increase risk of cardiac arrhythmias and sudden death. By blocking the effects of catecholamines (epinephrine and norepinephrine) with simultaneous α/β or single β adrenergic receptor blockers during a hyperinsulinemic, severe hypoglycemic clamp, glucose infusion rate increased, heart rate decreased, and cardiac arrhythmias were reduced. The major finding of these studies is that combined alpha/beta and single beta adrenergic blockade prevented death. These studies indicate that enhanced catecholamine signaling through beta adrenergic receptors mediates fatal cardiac arrhythmias in the setting of severe hypoglycemia.
Beta blockade (propranolol) is known to reduce the clearance of epinephrine and norepinephrine. This correlates with the observed increased epinephrine in the beta blockade group and the trend for an increased norepinephrine\(^{160}\). Similarly, alpha blockade (phentolamine) has been shown previously to double plasma norepinephrine levels\(^{160}\), which in the present study norepinephrine was increased 2.8 fold with prazosin.

Although adrenergic receptors located within the brain have been shown to be involved in mediating the counterregulatory response\(^ {36}\), the current study cannot distinguish whether the adrenergic blocker effect was mediated via central or peripheral actions. However, regardless of their site of action, their effect was profound. It is speculated that the beta-adrenergic norepinephrine response, may be the lethal arrhythmogenic provocateur, based on the following observations: 1) epinephrine levels peaked early, at the onset of severe hypoglycemia, then declined before the onset of serious arrhythmias; whereas norepinephrine levels continued to rise during severe hypoglycemia and peaked immediately before the time of arrhythmogenic death, 2) the reduced rates of hypoglycemia-induced arrhythmias and deaths with ICV glucose infusion was associated with a blunted norepinephrine response and not a blunted epinephrine response, and 3) beta, not alpha adrenergic blockade, markedly reduced arrhythmias and completely prevented death, even in the presence of high epinephrine levels. Taken together, it is proposed that it was the hypoglycemia-induced increased norepinephrine response, acting primarily via beta adrenergic receptors, that mediated the severe hypoglycemia-induced fatal cardiac arrhythmias.
Cardiac specific beta blockers (ie, β1 blockers), that do not adversely limit the counterregulatory response to hypoglycemia, have been shown to prevent QTc prolongation associated with moderate hypoglycemia\textsuperscript{88}. In the current study, non-selective beta-blockade did not prevent QTc prolongation, but more importantly, reduced the incidence of life-threatening arrhythmias and overall mortality associated with severe hypoglycemia. Future research is needed to investigate potential life-saving effects of adrenergic β1 and/or β2 receptor blockade in the setting of hypoglycemia.

\textbf{Summary}

Under the conditions studied, diabetes worsens, while recurrent antecedent hypoglycemia protects against severe hypoglycemia-induced mortality. Thus, the odds of surviving an episode of severe hypoglycemia are dependent on antecedent blood sugar control. Seizures are markers of severe hypoglycemia, but are not sufficient to cause death. Under the conditions studied, severe hypoglycemia-induced sudden death is caused by fatal cardiac arrhythmias. Deaths due to severe hypoglycemia were mediated by brain neuroglycopenia and the counterregulatory sympathoadrenal (adrenergic) response that lead to fatal cardiac arrhythmias (3\textsuperscript{rd} degree heart block with a slow ventricular response) causing low cardiac output (hypotension) followed by respiratory arrest. Implications of the current studies could lead to improvement in treatment strategies that aim to reduce mortality of individuals at high risk for insulin-induced hypoglycemia.
Figure 30. Proposed mechanism of sudden cardiac death due to insulin-induced severe hypoglycemia. Hypoglycemia is detected at the level of the brain. Through autonomic innervation, local release of norepinephrine at nerve terminals within the heart may lead to fatal cardiac arrhythmias. Autonomic innervation of the adrenal gland results in epinephrine release. Both circulating epinephrine in addition to circulating norepinephrine act at the level of the heart to increase risk of fatal cardiac arrhythmias. Within the blood, hypokalemia is also thought to contribute to hypoglycemia induced cardiac arrhythmias. Both these direct and indirect actions on the heart lead to QTc prolongation, but QTc prolongation is not thought to be a major contributor to mortality. Hypoglycemia induced sudden cardiac death results from enhanced adrenergic signaling at the level of the heart that leads to sinus tachycardia. Tachycardia is followed by 3rd degree heart block that culminates into a fatal bradycardic rhythm causing cardiorespiratory failure and sudden death. Red= circulating glucose, electrolytes, and hormones; blue= autonomic innervation
Figure 31. Timeline of events leading to severe hypoglycemic sudden death. During hyperinsulinemic/euglycemic conditions when glucose is ~79 mg/dl, hypokalemia occurs and QTc prolongation begins. During moderate levels of hypoglycemia (~40 mg/dl), PACs occur occasionally. At the start of severe hypoglycemia (15 mg/dl), epinephrine peaks. As severe hypoglycemia prolongs, hypokalemia worsens, QTc prolongation peaks, and cardiac arrhythmias develop (PVCs and 1st and 2nd degree heart block). Norepinephrine peaks around 1 hour into severe hypoglycemia and is followed by tachycardia. If tachycardia is followed by a return to normal sinus rhythm, the rat survives. If tachycardia is followed by an increase in the frequency of PVCs and 2nd degree heart block, then 3rd degree heart block associated with bradycardia will manifest and lead to sudden cardiac death. PAC= premature atrial contraction, PVC= premature ventricular contraction
Chapter 5: Thesis Discussion
Regulation of glucose homeostasis involves coordination between liver, muscle, adipose tissue, and brain. The major understanding of the pathogenesis of insulin resistance characterized by Type 2 diabetes stems from research on skeletal muscle and adipose tissue in which a reduced ability to translocate GLUT4 to the plasma membrane in these tissues characterizes glucose intolerance and insulin resistance\textsuperscript{1,2}. In recent years, the role of the brain in regulating overall energy homeostasis has been emphasized. In particular, the hypothalamus consists of integrated neural networks with both efferent and afferent pathways leading to and from the periphery, respectively, to control whole body glucose homeostasis. Therefore, studying the role of brain glucose sensing in the settings of both euglycemic and hypoglycemic conditions is important to understanding the etiology of normal glucose homeostasis and altered glucose homeostasis as occurs in conditions such as insulin resistance in diabetes.

**Glucose transporter 4**

GLUT4 is an insulin responsive glucose transporter that is required in muscle and/or adipose for normal glucose homeostasis. To emphasize the important role for GLUT4 in mediating glucose homeostasis, GLUT4 knockout in skeletal muscle and adipose tissue leads to glucose intolerance and insulin resistance, hallmarks of Type 2 diabetes\textsuperscript{1,2}. GLUT4 is also present in the brain with differential regional expression in hypothalamus, hippocampus, cortex, cerebellum, and other regions\textsuperscript{21}. Since the hypothalamus is an important neural center that mediates many metabolic regulatory pathways, understanding GLUT4 action in this region is integral to understanding normal glucose homeostasis.
Insulin action in the brain is known to control hepatic glucose production and the counterregulatory response to hypoglycemia\textsuperscript{27,106,124}. Many glucose sensing neurons located within the hypothalamus contain both insulin receptor and GLUT4\textsuperscript{16}. In addition, neuronal insulin receptor knockout (NIRKO) mice which exhibit impaired suppression of hepatic glucose production and impaired hypoglycemic regulation also have reduced hypothalamic GLUT4 protein expression\textsuperscript{27,106} indicating that decreased brain GLUT4 may be contributing to the observed phenotype of NIRKO mice. However, brain GLUT4’s role in glucose homeostasis has not been clearly defined. As an insulin sensitive glucose transporter, a role in glucose uptake in the brain would need to be one of metabolic importance to be initiated only when insulin is present. Studies presented in this thesis have revealed the importance of brain GLUT4 in mediating whole body glucose homeostasis. Brain GLUT4 knockout mice exhibit glucose intolerance, hepatic insulin resistance, regional specific reduced brain glucose uptake, impaired hypoglycemic counterregulation, and impaired glucose sensing in VMH glucose inhibited neurons. These novel findings will help guide therapeutic strategies aimed at treating and preventing glucose intolerance and insulin resistance. Future research will need to focus on understanding how differential regional expression of the various glucose transporters regulate glucose metabolism and the counterregulatory response to hypoglycemia.

**Hypoglycemia**

One of the major limitations in the treatment of diabetes is hypoglycemia. Well controlled diabetes is essential for patients to prevent micro- and macrovascular
complications associated with hyperglycemia; but avoidance of hypoglycemia can be difficult. Severe hypoglycemia is clinically defined as a low blood sugar level during which time the patient is unable, by themselves, to correct their low blood sugar (ie, consume carbohydrates) and therefore require external assistance (from a family member/friend or emergency services) in order to return glucose levels to normal. In the lab, we define severe hypoglycemia as a glucose level of 10-15 mg/dl in order to maintain consistency in all studies. Clinical case reports have indicated that severe hypoglycemia can lead to brain damage, seizures, coma, and death, all of which are observed in our rat model of insulin-induced severe hypoglycemia.

Brain damage due to hypoglycemia is disconcerting. Understanding how brain damage is caused is essential in order to prevent hypoglycemia mediated brain damage from occurring in diabetic patients at risk. In this thesis it was demonstrated that brain damage can be affected by antecedent glucose control. Uncontrolled diabetes exacerbates while insulin treated diabetes, that nearly normalizes blood glucose levels, reduces severe hypoglycemia induced brain damage. Interestingly, no effects on cognitive function were observed in this study. This indicates that although the duration of hypoglycemia was enough to cause some brain damage, the extent of brain damage was not of sufficient magnitude to translate into demonstrable cognitive deficits.

In addition to the brain damaging effects of severe hypoglycemia, perhaps an even scarier reality is that severe hypoglycemia can be lethal. The ‘dead in bed’ syndrome refers to instances where otherwise healthy young individuals with Type 1 diabetes are found dead in their beds in the morning with no clear cause of death upon autopsy. Severe hypoglycemia has often been hypothesized to be the cause of death
In this syndrome. A recent case report where a patient was found dead in their bed and had been wearing a continuous glucose monitor revealed that severe hypoglycemia occurred overnight just prior to death. This was the first case report demonstrating severe hypoglycemia associated with the dead in bed syndrome. However, how exactly severe hypoglycemia leads to sudden death in humans is unknown. Ethical limitations restrict the study of severe hypoglycemia in the human population. It has been hypothesized that fatal cardiac arrhythmias result from severe hypoglycemia but to this end has never been shown in clinical practice.

In this current thesis, it is shown for the first time in a rat model that severe hypoglycemia leads to cardiac arrhythmias which lead to respiratory arrest. These fatal cardiac arrhythmias can be reduced by providing the brain with glucose thus indicating that neuroglycopenia plays an important role in mediating cardiac arrhythmogenic deaths. Similarly, fatal cardiac arrhythmias were prevented by blocking beta adrenoreceptors indicating that the marked rise in epinephrine and norepinephrine likely mediate cardiac arrhythmogenic deaths. These findings have direct clinical implications in that, paradoxically, blocking the actions of the catecholamines during severe hypoglycemia actually prevents death. These findings are consistent with the recurrent antecedent hypoglycemia observations. Rats exposed to recurrent moderate hypoglycemia for 3 days followed by a hyperinsulinemic/severe hypoglycemic (10-15 mg/dl) clamp for 1 hour the following day had blunted sympathoadrenal responses which was also associated with reduced mortality due to severe hypoglycemia. Thus, consistent with the adrenoreceptor blockade study, blunted epinephrine response of rats exposed to recurrent hypoglycemia was found to significantly improve overall
survival. Interestingly, in human studies, beta blockade prevented the QTc prolongation that normally occurs during moderate hypoglycemia. Similarly in this thesis, beta-adrenergic receptor blockade during severe hypoglycemia prevented both cardiac arrhythmias and death. Beta blockers are already in use in heart failure patients and improve their survival rates. Taken together, fatal cardiac arrhythmias that occur in the setting of severe hypoglycemia can be prevented with beta adrenergic blockade, and future research should consider the use of beta blockade as a potential treatment in diabetic patients at risk for severe hypoglycemic cardiac arrest.

In addition to the catecholamine surge, hypokalemia is known to be proarrhythmic. Therefore, one of the goals to prevent severe hypoglycemia-induced mortality in our rat model was to supplement potassium during the hyperinsulinemic/severe hypoglycemic clamp. Severe hypoglycemia-induced mortality trended to be reduced in both non-diabetic and diabetic rats that received potassium supplementation but neither reached significance. Hypokalemia results from the activation of sodium/potassium ATPases by insulin and, in the subsequent ECG studies, hypokalemia occurred even prior to severe hypoglycemia. However, hypokalemia is present at equal levels in several of the studies reported in this thesis, including the beta blocker studies, indicating that hypokalemia itself is not sufficient to cause sudden death during severe hypoglycemia. However, it remains undetermined whether or not potassium infusion during episodes of hypoglycemia will prevent cardiac arrhythmias.

A major question remains that if we can prevent sudden death due to severe hypoglycemia, what will be the consequences of surviving such a severe episode?
Brain damage and cognitive defects as a result of severe hypoglycemia can tremendously decrease quality of life. Therefore, future studies need to be aimed at reducing severe hypoglycemia-induced brain damage and mortality simultaneously in order to increase quality of life in patients at risk for the damaging effects of severe hypoglycemia.

**Summary**

Understanding the pathogenesis of diabetes is crucial in order to reduce the rise in diabetes prevalence. The complications of diabetes and diabetes treatments are also important to study due to the very high incidence of diabetes in the world. The mechanisms of the etiology of glucose intolerance and insulin resistance are well defined in the periphery. However, the brain has an important role in glucose homeostasis and has recently gained much attention. In addition, complications of diabetes treatment remain a major concern for diabetic patients and their families. This thesis reveals how brain centers control glucose tolerance and insulin sensitivity, and how brain damage and mortality due to severe hypoglycemia can be prevented. First, it was shown that brain GLUT4 is important in regulating hepatic glucose production and the counterregulatory response to hypoglycemia in that knockout of brain GLUT4 led to glucose intolerance, hepatic insulin resistance, and impaired hypoglycemic glucose sensing. Second, antecedent blood glucose control regulates the extent of brain damage induced by severe hypoglycemia in that insulin treatment that nearly normalized blood glucose levels in diabetic rats reduced severe hypoglycemia-induced brain damage compared to uncontrolled diabetic rats. Third, severe hypoglycemia
induced mortality is mediated by fatal cardiac arrhythmias that can be reduced by brain glucose infusion and prevented by beta adrenergic blockade indicating that brain neuroglycopenia and enhanced catecholamine signaling are major contributing factors to severe hypoglycemia-induced mortality.
References


