ABSTRACT

Title of Document:	THE EFFECTS OF INSULIN-INDUCED MODERATE HYPOGLYCEMIA ON HIPPOCAMPAL PLASTICITY
	Joseph Frankel, Kelley Gunther, Lex Matthews, Siddarth Plakkot, Eileen Ser, Melanie Shapiro, Peter Weng, Danielle Wilkin, Linhan Xu
Directed by:	Dr. Erica Glasper, Ph.D Department of Psychology, Program in Neuroscience and Cognitive Science

Self-regulation of blood glucose in diabetics via insulin administration introduces the risk of hypoglycemia. Previous studies have shown hypoglycemia damages the dentate gyrus, an area of the hippocampus associated with anxiety- and depressive-like behavior. To date, only depressive-like behaviors have been observed following moderate hypoglycemia. This study sought to examine whether acute moderate hypoglycemia induces both behaviors due to high clinical comorbidity. One episode of moderate hypoglycemia was induced in a male Sprague-Dawley rat. Twenty-four hours later, hippocampal function was evaluated via the elevated plus maze and the forced swim test to assess anxiety-like and depressive-like behavior. Results, though not statistically significant, suggested that acute moderate hypoglycemia may increase anxiety- and depressive-like behavior. These findings may elucidate hypoglycemia-related behavioral changes.

THE EFFECTS OF INSULIN-INDUCED MODERATE HYPOGLYCEMIA ON
HIPPOCAMPAL PLASTICITY

by

Team PANCREAS
Preventing Adverse Neurological Consequences of Reduced Episodic Amounts of Sugar

Joseph Frankel
Kelley Gunther
Lex Matthews
Siddarth Plakkot
Eileen Ser
Melanie Shapiro
Peter Weng
Danielle Wilkin
Linhan Xu

Thesis submitted in partial fulfillment of the requirements of the Gemstone Program
University of Maryland
2015

Advisory Committee:

    Dr. Erica Glasper
    Dr. Justicia Opoku-Edusei
    Dr. Leslie Pick
    Dr. Matthew Roesch
    Dr. David Yager
    Dr. Richard Yi

Acknowledgements

Table of Contents

List of Figures

List of Abbreviations

ANLSH – Astrocyte-Neuron-Lactate Shuttle Hypothesis
BGL – Blood Glucose Level
BrdU – Bromodeoxyuridine
CORT - Corticosterone
DSM-5 – Diagnostic and Statistics Manual of Mental Disorders 5
IP – Intraperitoneal
MCT – Monocarboxylate Transporters
MRI – Magnetic Resonance Imaging
NAD(+) – Nicotinamide Adenine Dinucleotide
NADH – Nicotinamide Adenine Dinucleotide
PARP-1 – Poly(ADP-ribose) Polymerase-1
PET – Positron Emission Tomography
T1D – Type 1 Diabetes

Introduction

Prevalence & Consequences

Type 1 diabetes (T1D) is a chronic autoimmune disorder characterized by destruction of the insulin-producing pancreatic beta cells (Like & Rossini, 1976; Nakhooda et al., 1977; Makino et al., 1980). A lack of insulin, a hormone produced by beta cells in the pancreas, leads to elevated blood sugar levels, better known as hyperglycemia (Nakhooda et al., 1977). Although the direct cause of T1D is yet unknown, research suggests that genetic susceptibility is polygenic, involving mutations in a number of genes involved in immunological tolerance, which may allow initiation of the autoimmune response. Additionally, environmental factors including exposure to the sun, ingestion of milk products, and infection with certain microbes have also been suggested to play a role in diabetes pathogenesis (Bluestone et al., 2010).

The incidence of T1D is on the rise, with an estimated annual increase of 3%, affecting approximately three million children and adults in the U.S. (Juvenile Diabetes Research Foundation, 2014b). Patients with T1D must exercise great care in regulating their blood glucose levels and be constantly vigilant against potentially deadly complications including, but not limited to, kidney failure, heart attack, and stroke (Krolewski et al., 1987; McGovern et al., 2013). Diet modifications and medication are often used to help mediate diabetes, but insulin therapy is usually required (Turner et al., 1999). Self-administration of insulin, via multiple daily injections or subcutaneous insulin infusion, reduces, but cannot completely eliminate, the risk of developing extensive complications (JDRF, 2014). Type 1 diabetics have a target glucose level to maintain through glucose testing plus subsequent insulin injections and are advised to stay within a

range low enough to avoid complications linked to hyperglycemia, such as diabetic ketoacidosis (buildup of ketones) but high enough to reduce the risks associated with hypoglycemia, or low blood glucose (Davis et al., 1998).

Management of otherwise unregulated blood glucose levels can lead to unintended consequences when glucose levels drop outside of the range that is considered euglycemic, 80 mg/dL. Adverse neurological effects have been attributed to glucose levels below the euglycemic threshold, with significant damage occurring as a consequence of severe episodes of hypoglycemia (Vriesendorp et al., 2006). A number of studies have focused on the effects of severe hypoglycemic episodes on the adult brain and discovered that these episodes can cause lasting detriments to brain structure and function (Kalimo & Olsson, 1980; Fujioka et al., 1997; Asvold et al., 2010). Severe hypoglycemia causes lesions in many areas of the brain including the basal ganglia, cerebral cortex, and hippocampus (Kalimo & Olsson, 1980; Fujioka et al., 1997). It has been shown that participants with severe childhood hypoglycemic episodes performed worse as adults on cognitive tests in domains such as memory, psychomotor efficiency, problem solving, and spatial function compared to participants who did not experience severe hypoglycemic episodes (Asvold et al., 2010).

Hypoglycemic episodes can also result in short-term effects such as fatigue, formal thought disorders, behavioral changes, emotional instability, seizures, and even coma (Towler et al., 1993). Improper insulin dosage leading to hypoglycemia now accounts for a greater number of T1D-related hospital admissions than hyperglycemia (Lipska et al., 2014) and incidences of malglycemia, or blood glucose levels outside of the euglycemic range, are quite common. Modern insulin therapy has evolved to

drastically increase the lifespan of type 1 diabetics, but still relies upon human management and is therefore prone to error.

Hypoglycemia in a Clinical Context

Conventional and intensive insulin therapies are two major approaches in the management of T1D. Conventional therapy involves a more restrictive diet as well as one or two daily insulin injections to supplement the lack of endogenous insulin, with blood glucose monitored sparsely (Dhingra et al., 2009). In intensive therapy, diabetics use three or more daily injections to aim for the euglycemic threshold, 80 mg/dL. This method requires regular blood glucose monitoring throughout the day (Dhingra et al., 2009). With the use of intensive insulin therapy, the risk of hypoglycemia is greater due to overshooting target insulin dosage. The use of insulin comes with a variety of inherent risks that T1D patients must assess. The choice of conventional insulin therapy versus intensive insulin therapy is heavily debated. Although intensive control has been shown to reduce the risk of many long-term complications of T1D including neuropathy, retinopathy, and nephropathy, patients are placed at higher risk for more frequent and severe hypoglycemic episodes (Dhingra et al., 2009).

Proper brain function is dependent upon an adequate supply of glucose. The brain depends upon facilitated diffusion of glucose across the blood-brain barrier. Generally, there is a positive correlation between glucose levels in the blood throughout the body and blood glucose levels in the brain (Gruetter et al., 1998). Changes in blood glucose levels in the brain resulting from insulin injections affect neurons, though the neurons themselves are insulin-independent (reference?). The release of insulin in the blood during hyperglycemia results in glucose moving into the body's cells to sustain them.

However, neurons do not experience the uptake of glucose in the presence of insulin because they are not dependent on it. Thus, during hypoglycemia, the brain becomes nearly devoid of glucose, causing functional brain failure and providing an opportunity for permanent neuronal damage even after euglycemia is restored (Suh et al., 2005a). With this, significant neuronal damage typically accompanies severe hypoglycemia, <36 mg/dL (Puente et al., 2010). Furthermore, structural damage can even be seen after cases of moderate hypoglycemia, usually manifesting as a consequence of oxidative stress (Puente et al., 2010; Won et al., 2012b).

Structural brain damage occurring as a consequence of hypoglycemia can induce behavioral deficits associated with the areas damaged. The hippocampus is a subcortical structure in the brain that is particularly susceptible to hypoglycemic insult (Auer et al., 1984). Therefore a number of cognitive functions that the hippocampus mediates can experience deficits in light of such damage, such as spatial memory (Moser et al., 1995; Bannerman et al., 2003), anxiety (Bannerman et al., 2003; Trivedi & Coover, 2004), and depression (Seminowicz et al., 2004). Although many studies have focused on hippocampally-mediated cognitive functions (Puente et al., 2010; Won et al., 2012b), few have studied hippocampally-controlled mood-related behaviors in the context of hypoglycemia.

Anxiety, Depression, and Adult Neurogenesis

As previously stated, anxiety and depression are among behaviors mediated in part by the hippocampus (Seminowicz et al., 2004). The hippocampus is a structure within the cerebral cortex that is divided into a variety of subsections including the dentate gyrus (Ramon y Cajal, 1901). The dentate gyrus has been shown to mediate

depressive-like behavior, suggesting that this part of the hippocampus could be responsible for findings that suggest the hippocampus is responsible for regulating mood (Jacobs et al., 2000). Thus, the dentate gyrus will serve as a representative area of the hippocampus for the purpose of this study.

Moreover, the dentate gyrus has been shown to display a high rate of adult neurogenesis, the birth of new neurons. The Neurogenesis Theory of Depression postulates that a decrease in adult neurogenesis can be a correlate of depression (Snyder et al., 2011b). It is hypothesized that hypoglycemia alters adult neurogenesis via a reduction in brain glucose that ultimately elicits a stress response. Stress hormones, such as corticosterone, are released in attempts to ameliorate the havoc wreaked by the sudden deficit of a necessary fuel (Diggs-Andrews et al., 2010). Research suggests that elevated levels of corticosterone can hinder adult neurogenesis in the hippocampus (Gould et al., 1992; Montaron et al., 2006). This decline in adult neurogenesis has been not only linked to an increase in depressive-like behavior, but also to an increase in anxiety-like behavior (Jacobs et al., 2000; Revest et al., 2009; Snyder et al., 2011b). Anxiety is also mediated by the hippocampus (Jacobs et al., 2000; Revest et al., 2009), so it stands to reason that both anxiety- and depressive-like behavior could be similarly affected by a decrease in adult neurogenesis. Given the high comorbidity of depression and anxiety (Regier et al., 1998), measuring the levels of anxiety- and depressive-like behavior, as well as the survival of newly-proliferated cells, in rats who have sustained a single episode of moderate hypoglycemia will lend insight into how an acute episode of moderate hypoglycemia affects adult neurogenesis, and what, if any, behaviors may be altered as a result of this physiological change.

Research Questions, Hypothesis, and Objectives

How does acute moderate hypoglycemia affect anxiety- and depressive-like behavior? How does acute moderate hypoglycemia affect the survival of newly born cells in the hippocampus?

We hypothesized that a single episode of moderate hypoglycemia would increase both anxiety- and depressive-like behavior in male Sprague-Dawley rats as compared to rats that simply received a control injection of saline. To quantitatively measure rodent anxiety- and depressive-like behavior, we used standardized behavioral tests: the Elevated Plus Maze test and the Forced Swim task. The elevated plus maze is used to assess anxiety-like behavior of rodents (Walf & Frye, 2007), while the forced swim test is a validated method to assess depressive-like behavior of rodents (Porsolt et al., 1979). We expected that there would be a decrease in new cell survival in the dentate gyrus of the hippocampus in rats receiving an episode of acute moderate hypoglycemia as compared to those that remained euglycemic for the duration of the experiment.

This study specifically investigated the neural and behavioral effects of hypoglycemia, as opposed to those of hyperglycemia, in order to further understand the complications that arise when using insulin therapy. Although insulin therapy has been shown to reduce instances of hyperglycemia and its negative consequences on the human body, hypoglycemia remains a major negative consequence of insulin therapy (Dhingra et al., 2009; DCCT et al., 1993; Lager et al., 1986). Previous research has found that both single and multiple episodes of severe hypoglycemia can cause brain damage and resulting behavioral changes (Kalimo & Olsson, 1980; Fujioka et al., 1997; Asvold et al., 2010). Additionally, multiple episodes of moderate hypoglycemia can lead to detrimental

effects to the brain (Won, 2012b; Puente, 2010; Moore, 2010). However, the effect of a single/acute moderate episode of hypoglycemia has not been thoroughly investigated. Park et al. 2012 is one of very few studies suggesting that an acute episode of moderate hypoglycemia can result in behavioral change even after euglycemia is restored. Thus, this study seeks to replicate such findings and further investigate the effects of an acute episode of moderate hypoglycemia on the brain and resultant behavioral changes.

The survival of newly born cells in the hippocampus was quantified using Bromodeoxyuridine (BrdU), a DNA synthesis marker for newly birthed cells. Utilizing a peroxidase labeled antibody against the BrdU, new cells within the hippocampus can be quantified (Czeh, Michaelis, & Fuchs, 2001). In order to test our hypotheses, we pursued the following objectives: 1) determine the impact of acute moderate hypoglycemia on anxiety- and depressive-like behavior in male Sprague-Dawley rats, and 2) determine the effects of acute moderate hypoglycemia on the survival of newly born cells in the dentate gyrus of the hippocampus.

Experimental Approach

In order to investigate the behavioral and cellular changes following an acute moderate hypoglycemic episode, we used male non-diabetic Sprague-Dawley rats as the animal model. This breed of albino rat was an optimal choice due to its docility and ease of handling, as compared to other rat breeds (Ulrich & Azrin, 1962). We utilized a non-diabetic model over a diabetic model in order to have complete control over glycemic variability. In diabetic rats, in which the pancreatic beta cells are destroyed, it is possible that the animal will experience episodes of hyperglycemia prior to experimentation, which would confound our data. In non-diabetic rats, however, it is much more likely that

7

the rat will naturally maintain euglycemia, and the only fluctuation would be the induced hypoglycemic episode via insulin injections. While this allowed us to isolate a hypoglycemic event and examine its effects, it limits our ability to extrapolate our research findings to diabetic humans, as type 1 diabetics will likely experience more than one hypoglycemic episode throughout their lifetime.

Male Sprague Dawley rats were randomly assigned to two groups for behavioral analysis. The control group was given an equivalent number of saline injections as the experimental group was given insulin and dextrose injections, so as to remove injection stress as a confounding variable. The experimental group underwent a single episode of moderate hypoglycemia. Each group also received an injection of BrdU, for later quantification of newly proliferated cells in the dentate gyrus of the hippocampus. Twenty-four hours after injection of BrdU, both groups underwent behavioral testing to compare the impact of a hypoglycemic episode on hippocampal function. Immediately following the behavioral tests, rats were humanely euthanized and their brains were perfused for immunohistochemical processing and analysis.

Our research builds upon a foundation of studies on brain structure and function in response to hypoglycemia, from the molecular to the macroscopic level. Current research shows that moderate hypoglycemia increases depressive-like behavior in a rodent model (Park et al., 2012), but does not fully explore how this trauma might impact anxiety-like behavior. Research also suggests that depressive-like behavior may be mediated by a decrease in adult neurogenesis in the dentate gyrus (Jacobs et al., 2000), providing a possible mechanism by which hypoglycemia-related changes in mood could

occur. This study seeks to link behavioral changes caused by an acute moderate episode of hypoglycemia with subsequent structural damage to the brain.

Literature Review

The Structure and Function of Hippocampus and Dentate Gyrus

The limbic system consists of a complex network of structures in the brainstem, midbrain, and cortex. The medial temporal lobe of the brain houses multiple important structures to the limbic system, such as the hippocampus, amygdala, and the rhinal cortex. Limbic system functions include emotion, learning, memory, olfaction, and homeostasis.

The hippocampus is a small, comma-shaped structure located within the medial temporal lobe and is conventionally divided into various subsections – the dentate gyrus and the CA1, CA2, CA3, and CA4 subfields (Ramon y Cajal, 1901). The hippocampus, particularly the posterior, or dorsal, hippocampus, is responsible for a wide variety of crucial cognitive functions and has been extensively studied in the context of spatial learning and memory development (Moser et al., 1995). The anterior, or ventral, hippocampus is responsible for emotional regulation and the neuroendocrine regulation of the stress axis (Kjelstrup et al., 2002; Bannerman et al., 2004) (Figure 1).
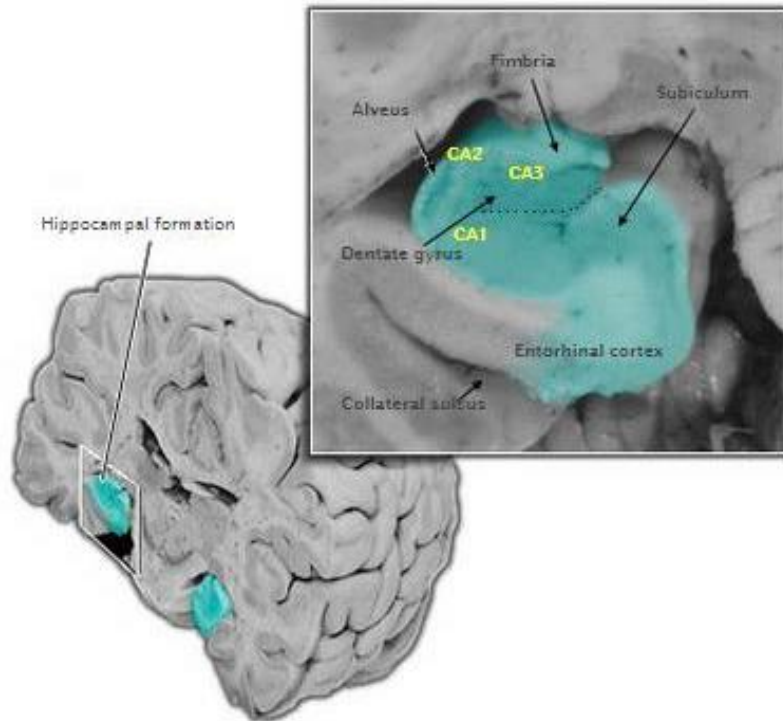
Figure 1. Brain section showing CA1, CA2, and CA3 areas of the hippocampus (Purves et al., 2011).

Rodent studies have provided evidence for the two-fold function of the hippocampus delineated by spatial region. Lesions to the ventral region of the hippocampus, including the dentate gyrus, resulted in reduced levels of anxiety-like behavior, demonstrating emotional dysregulation, while spatial learning remained unchanged. This was evidenced by a higher level of activity in a light-dark box task and a shorter latency in eating in hyponeophasia assessments, both of which quantify anxiety-like behavior. This suggests that the ventral hippocampus is related to emotional regulation. Furthermore, this study shows that ablation of this area will lead to emotional dysregulation by affecting anxiety-related behaviors (Bannerman et al., 2003).

Additionally, another study by Trivedi and Coover (2004) also demonstrated that ventral hippocampus lesions reduce anxiety-like behavior, but dorsal hippocampus lesions do not. Rats with both types of hippocampal lesions were tested with the elevated T-maze, another measure for anxiety-like behavior similar to the elevated plus maze. Rats with ventral hippocampal lesions had a lower latency to leave the closed arms of the maze, indicated reduced anxiety-like behavior. However, rats with dorsal hippocampal lesions did not show this decrease in time to exit the closed arms and enter the open arms, further supporting the ventral hippocampus' role in preferentially controlling emotional regulation (Trivedi & Coover, 2004).

Conversely, a study ablating the dorsal region of the hippocampus validated the same notion. A study by Bannerman et al. (2003) found that dorsal lesioning of the hippocampus hindered spatial memory on both a rewarded T-maze and a water maze task. The complete lesioning of the hippocampus produced similar deficits, as well as an absence of anxiety-like behavior when tested using social interaction, the plus maze, and hyponeophasia. These findings differentiate the functions of the dorsal region of the hippocampus and those of the hippocampus collectively (Bannerman et al., 2003).

The Role of the Hippocampus and Dentate Gyrus in Depression and Anxiety

As the ventral hippocampus mediates emotional regulation and the stress axis response, it has been shown to be clinically relevant by also mediating depression and depressive-like behavior (Seminowicz et al., 2004). The Diagnostic and Statistics Manual of Mental Disorders 5 (DSM-5) is the American Psychiatric Association's current guide for classifying the criteria in mental illness diagnoses. The DSM-5 defines depression as a disorder characterized by qualities such as feelings of low mood, helplessness, and

11

decreased interest in previously enjoyable activities (American Psychiatric Association, 2013).

Human studies have elucidated the relevant brain structures underlying depression. Positron emission tomography (PET) has revealed connections between the hippocampus, anterior cingulate gyrus, lateral prefrontal cortex, and anterior thalamus, among other brain structures. These limbic system pathways are active in the brains of patients who are responsive to drug treatments for major depressive disorder. The presence of the hippocampus in these pathways suggest that the hippocampus plays a role in mediating depressive-like symptoms (Seminowicz et al., 2004). Additionally, multiple studies analyzing the morphology of the hippocampus in human subjects, typically using magnetic resonance imaging (MRI), note that decreased hippocampal volume is correlated with the presence of depression (Bremner et al., 2000; Frodl et al., 2006; Kronmüller et al., 2008; MacMaster et al., 2014), while other studies have observed no such correlation (Rusch et al., 2001; Keller et al., 2008).

Hypoglycemia and the Hippocampus

The hippocampus is a region of the brain that is highly vulnerable to damage, indicating that the hippocampus is also susceptible to complications stemming from hypoglycemia (Auer et al., 1984). Severe hypoglycemia has been shown to induce the most damage to areas of the hippocampus including the dentate gyrus and CA1 region (Auer et al., 1984). This neuronal damage, that can be brought about as a result of severe hypoglycemia, usually leads to necrosis, morphological changes to cells that culminate in lysis and cellular death (Suh et al., 2005b). In addition to neuronal necrosis, severe hypoglycemia leads to transient increases in dentate gyrus adult neurogenesis that is

thought to replace dead neurons (Suh et al., 2005b). After this spike in in adult

neurogenesis, adult neurogenesis decreases below that found in the normal, non-

hypoglycemic brain (Suh et al., 2005b). Impaired proliferation and augmented necrosis

may act in combination, resulting in a net loss in the number of dentate gyrus neurons.

During moderate hypoglycemia, cell death rarely occurs in the dentate gyrus

(Tkacs et al., 2005). Injury to neurons post-moderate hypoglycemic insult manifests

instead as oxidative damage to dendrites. This less severe type of damage is a result of

mitochondrial production of reactive oxygen species (Won et al., 2012b). A recent study

has shown that one episode of moderate hypoglycemia is not enough to induce oxidative

damage as suggested by staining of 4-hydroxy-2-nonenal, a product of lipid peroxidation,

overlapped with staining of MAP2, a dendritic marker. However, five consecutive daily

episodes of moderate hypoglycemia in both diabetic and non-diabetic rats lead to a

significant increase in oxidative damage in CA1 dendrites (Won et al., 2012b). All of the

preceding forms of structural damage to neurons, including necrosis, oxidative damage,

and loss of proliferation, may be responsible for functional changes in behavior and

cognition that have been discussed above.

Adult Neurogenesis and Depression

The hippocampus, and in particular, the dentate gyrus, is one of the few structures

in the adult rodent brain that display high rates of adult neurogenesis, or cell birth in

adulthood. Neurogenesis is the generation of new neural stem cells, which can then

differentiate into neurons or glial cells (Altman & Das, 1965). In addition to the

hippocampus' presence in pathways for depression, evidence suggests that decreased

adult neurogenesis in the hippocampus, particularly the dentate gyrus, may mediate

depression and depressive-like behavior (Snyder et al., 2011a). Increased stress leads to a myriad of physiological responses, including increased glucocorticoid release. Increased glucocorticoid levels can impair adult neurogenesis, highlighting the connection between stress and emotional dysregulation (Gould et al., 1992).The release of stress hormones can be adaptive, but an excess in these levels may also be dangerous (personal communication, Justicia Opoku-Edusei).

A growing body of experimental evidence suggests that neurogenesis may play a role in depression. One study used tianeptine, an antidepressant, to prevent stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation (Czéh et al., 2001). After treatment with tianeptine and injection of BrdU following exposure to stressful conditions, tree shrews demonstrated greater cell birth in the dentate gyrus as compared to the control group exposed to stressful conditions without the antidepressant treatment.

Other studies also confirm the role of impaired birth of new cells in depressive-like behavior. A study by Santarelli et al. (2003) demonstrates that adult neurogenesis in the hippocampus is necessary for the behavioral effects of antidepressants to work to their fullest extent. This study showed that blocking adult neurogenesis upregulated by antidepressants, like fluoxetine and haloperidol, decreases their behavioral effects, as compared to a control group that receives them without impairing adult neurogenesis. When adult mice given antidepressant treatment underwent radiation to block neurogenesis, they demonstrated higher levels of anxiety than antidepressant-treated mice without eliminated neurogenesis, where the anxiety-like behavior should have been moderated by the antidepressants. This is further evidence confirming adult

neurogenesis' role in mediating the effects of antidepressant medication (Santarelli et al., 2003).

Adult Neurogenesis and Anxiety

A disorder that tends to be comorbid with depression is anxiety (Regier et al., 1998). According to the DSM-5, anxiety is a disorder characterized by an excessive state of fear and its behavior responses, where the fearful response is not appropriate for the context (American Psychiatric Association, 2013). Anxiety accounts for approximately 30% of psychiatric cases and is a broad term that encompasses generalized anxiety disorder, obsessive-compulsive disorder, and posttraumatic stress disorder (Kessler et al., 2010). Due to the high comorbidity of anxiety and depression, impairment of adult neurogenesis in the hippocampus not only mediates depression, but potentially anxiety-like behavior.

Research has suggested that increased adult neurogenesis, which can come as a result of episodes of hypoglycemia, can underlie instances of anxiety-like behavior. Revest et al. (2009) conducted a study to investigate the link between decreased adult neurogenesis and elevated anxiety-like behavior. They demonstrated that impairing adult neurogenesis increases the presence of anxiety-related behaviors in a mouse model. A pro-apoptotic drug suppressed neurogenesis in the mouse hippocampus. Mice with impaired neurogenesis displayed an increase in anxiety-like behavior, as assessed with by performance in the elevated plus maze. Additionally, these rats exposed to benzodiazepine, an anxiolytic drug, demonstrated fewer anxiety-related behaviors. The results of these behavioral tests and pharmacological manipulations suggest a strong connection between anxiety and adult neurogenesis in the hippocampus (Revest et al.,

2009). The table below illustrates the suggested hypothesis regarding the comorbidity of anxiety and depression and their inverse relation to neurogenesis.

Moderate and Severe Hypoglycemia Impair Hippocampal Function

Moderate and severe hypoglycemia are complications of managing T1D that impact hippocampal function. Severe hypoglycemia has been associated with many functional deficits including impaired recognition memory in the Novel Object Test and diminished spatial memory in the Morris Water Maze, likely due to resultant neuronal death in the dentate gyrus (Puente et al., 2010). Moderate hypoglycemia-related neuronal injury has also been correlated with damaged cognition. Evidence of impairments in spatial learning have been noted using the hippocampally-dependent Barnes maze task, although this decreased functionality has been shown to exist in diabetic, rather than non-diabetic, rats undergoing recurrent moderate hypoglycemia (Won et al., 2012b). In contrast to the functional results reported by Won et al., many other studies have found moderate hypoglycemia to induce demonstrable changes in behavior in non-diabetic rats (Park et al., 2008; Moore et al., 2010; Park et al., 2012).

In non-diabetic rats, recurrent moderate hypoglycemia has been associated with an escalation of fear-related behaviors associated with anxious tendencies (Moore et al., 2010). Non-recurrent hypoglycemia has also been shown to induce similar behavioral effects. A significant increase in depressive-like behavior has been found in non-diabetic mice twenty-four hours following a single forty-five minute episode of moderate hypoglycemia, as demonstrated by immobility in the Forced Swim Task (Park et al., 2008). In a prior study by Park et al. (2008b), a forty-five minute induction of moderate hypoglycemia also caused an increase in social withdrawal less than twenty-four hours after the episode. These studies collectively indicate that a single moderate episode of hypoglycemia is enough to induce functional impairment to the hippocampus in non-diabetic animals.

Park et al. imply that social withdrawal is closely linked with the presence of depressive- and anxiety-like behavior, although the authors have not published any data to suggest that they have performed experiments with any standardized behavioral tests for anxiety (Park et al., 2008). Thus, we hypothesize that an episode of moderate hypoglycemia would not only increase depressive-like behavior as evidenced by the Forced Swim Test, but also increase anxiety-like behavior as evidenced by the Elevated Plus Maze.

Conclusion

Given the research about hippocampal structure and function, adult neurogenesis, and moderate hypoglycemia, behavioral deficits seen after one episode of moderate hypoglycemia could be attributed to a structural change other than neuronal death or oxidative damage (Won et al., 2012b). The mechanism(s) behind depression and anxiety

can be increasingly associated with decreased adult neurogenesis (Thomas & Peterson, 2003). Incidentally, the survival of newly birthed cells has yet to be studied in the context of moderate hypoglycemia. We predict that moderate hypoglycemia increases depression- and anxiety-like behavior via inhibition of adult neurogenesis in the dentate gyrus. A description of our experimental design including an experimental timeline and protocols will follow this chapter.

Methodology

The following methodology is for the experimental protocol that was performed. However, a different protocol was proposed and attempted prior to this. See Appendix D for the methodology of that protocol.

Research Design

Given the existing knowledge of the connection between hypoglycemia, or low blood sugar levels, and brain injury as elucidated in the Literature Review, high-constraint laboratory practices, using an animal model are the most appropriate method for this research. A connection has been established between increased depressive-like behavior and moderate hypoglycemic episodes (Park et al., 2012). There has also been a connection established between increased anxiety and diabetes (Won et al., 2012), and there is a high rate of comorbidity between depression and anxiety (Aina & Susman, 2006). The mechanism by which moderate hypoglycemia causes behavioral changes after a hypoglycemic episode has not fully been explored. The adult neurogenesis theory of depression states that reduced adult neurogenesis in the dentate gyrus may lead to

depressive-like behavior, providing a possible pathway by which hypoglycemia may act to cause these behavioral changes (Santarelli, 2003).

Animals

Twenty adult, male Sprague-Dawley rats with a weight of 225-250 grams ordered from Taconic Biosciences, Inc. was used in this experiment. The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and rat care methods followed the guidelines in the *Guide for the Care and Use of Laboratory Animals, 8th Edition* (Council, 2011). Males were used rather than females, as the estrous cycle in female rats influences hormone levels that may affect behavioral test performance (Marcondes et al., 2001). The rats were subjected to a 12/12-hour light-dark schedule to maintain their natural diurnal cycle, where experiments were conducted during the animal's dark cycle (during the human daylight hours) (Council, 2011). At this time, activity levels were high as the animal's feeding patterns are intrinsically nocturnal (Henning & Gisel, 1980). While aspects of rat physiology have circadian rhythms, all experiments were performed at the same time of day to eliminate any bias due to differences in glucose metabolism (Cayen et al., 1972).

Before beginning the experiment, the rats were given at least a five-day acclimation period (Lawlor, 2002) following their arrival to the vivarium. At the end of the study, animals were euthanized using an intraperitoneal (IP) injection of ketamine and xylazine followed by transcardial perfusion and then decapitation, consistent with the American Veterinary Medical Association's Guidelines on Euthanasia.

Induction of Hypoglycemia

Subcutaneous injections of insulin were used to induce hypoglycemia. Although catheters and IP injections have widely been used in similar studies, subcutaneous injections were most effective in slow delivery of insulin into the bloodstream without rapidly reducing the experimental rats' blood glucose levels (BGLs). This was in accordance with the Puente et al., 2010 protocol for induction of moderate hypoglycemia.

Baseline BGL for all rats were noted and food was removed to eliminate contributing factors that could potentially increase BGL. Using the mass of each rat, all control and experimental rats were given 0.004 U/g of 1:10 diluted insulin or saline, respectively. The BGL of rats from both groups was measured every fifteen minutes, using blood obtained by tail-vein extraction (See Appendix C). When the experimental rat's BGL reached the target range of 30-45(+/- 5) mg/dL, a timer was set for one hour. BGL was then measured every fifteen minutes during the hour-long episode to ensure that the experimental subject's BGL remained in the target range. The control group's BGL was also tested to limit any confounding stress caused by the tail vein extraction. If rats in the experimental group did not reach target levels within one hour after first insulin injection and BGL was not dropping, then an insulin correction was performed subcutaneously (See Appendix B). If BGL was below 50 mg/dL, a dextrose correction was performed intraperitoneally (See Appendix B). If a dextrose correction was performed on the experimental rats using an IP injection, a corresponding saline IP injection was performed on the control rats. In alignment with the Puente et al. (2010) hypoglycemia induction protocol, dextrose, as opposed to its isomer glucose, was used to correct unsafe drops in BGLs.

If an insulin correction was performed on the experimental rat, a control rat received a saline injection of equal volume. Hypoglycemia was terminated using a 1 mL IP injection of dextrose after the experimental group rats remained within the target range for one hour. A corresponding sham termination using 1 mL saline was done on the control rats. The BGL was then monitored again every fifteen minutes for up to three hours until the rats returned to euglycemia (BGL above 100 mg/dL for one hour). An additional 1 mL of dextrose was given if glucose levels of the animal did not rise or dipped below 100 mg/dL. All rats were returned to the colony after reaching euglycemia.

Behavior

While control rats (n=8) experienced a behavioral test after no hypoglycemic episode, the experimental adult male Sprague-Dawley rats (n=8) underwent behavioral tests after a hypoglycemic episode (BGL of 30-45 mg/dL). Two extra subjects were included in each group to account for the possibility of early termination of Sprague-Dawley rats due to lack of BGL control. The elevated plus maze and forced swim test were examined for analyzing anxiety and depression, respectively.

Elevated Plus Maze

The elevated plus maze is a behavioral task used to measure anxiety related tendencies associated with the hippocampus in rodents (Walf & Frye, 2007). The elevated plus maze consisted of four arms (55 cm long x 11.5 cm wide) in the shape of a plus sign. Forty-five cm high walls surrounded two of the arms, while the other two arms were open. The maze was elevated 75 cm from the floor.
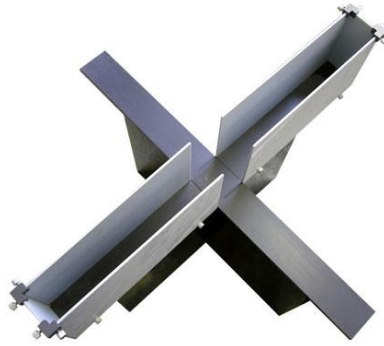
Figure 2: Illustration of Elevated Plus Maze with closed and open arms (PanLab)

For this behavioral task, rats were placed into the center of the maze facing an open arm (Walf & Frye, 2007). The behavior of the rats on the maze was recorded for 5 minutes using EthoVision XT 8.5 (Noldus Information Technology Inc., Leesburg, Virginia) video tracking software. If the rat fell off the maze, it was immediately picked up and placed back onto an open arm to complete the task (Walf & Frye, 2007). Anxiety-like behavior was assessed for both groups by comparing the amount of time spent in the open and closed arms, overall distance moved in the maze, and number of head dips (Walf & Frye, 2007). Increased time spent on the open arm indicated less anxious behavior, while more time spent on the closed arm indicated more anxious behavior (Walf & Frye, 2007). An open arm entry was defined when all four paws of the rat are in the open arm. It is noted as a head dip when the rat nose point is looking over the open arm edge. Greater tendency to head dip is indicative of less anxious behavior. Greater overall distance spent on the elevated plus maze is indicative of higher anxiety. Latency - or the time required for the initiation of a behavior (movement in elevated plus maze) - was also measured, with a greater latency period indicative of less anxious behavior

(personal communication, Molly Hyer). The elevated plus maze has been validated behaviorally and hormonally as a model of anxiety. Animals confined to the closed arm exhibit increased fear-related behaviors and elevated corticosterone levels as compared to animals confined to the open arm (Pellow et al., 1985).

Forced Swim Task

The forced swim task is a behavioral measure used to identify depressive-like behavior in rodents (Porsolt, 1979). For this test, rats were placed in a clear cylindrical container (diameter: 30 cm; height: 43 cm) containing 20 cm of room temperature water for 5 minutes. Motion of the rats was tracked using EthoVision XT 8.5 video tracking software. A number of immobility parameters were monitored for the duration of the test. Immobility was indicative of depressive-like behavior (Park et al., 2012). We measured latency to immobility, bouts of immobility, and percentage of total time spent immobile. A high latency period and/or high frequency of immobility were indicative of depressive-like behavior (personal communication, Molly Hyer). Immobility during the forced swim task has been shown to decrease with the administration of antidepressants in a number of labs, which validates this test as a model for depression in rats (Przegalinski et al., 1980; Borsini et al., 1981).

Mobility   **Immobility**
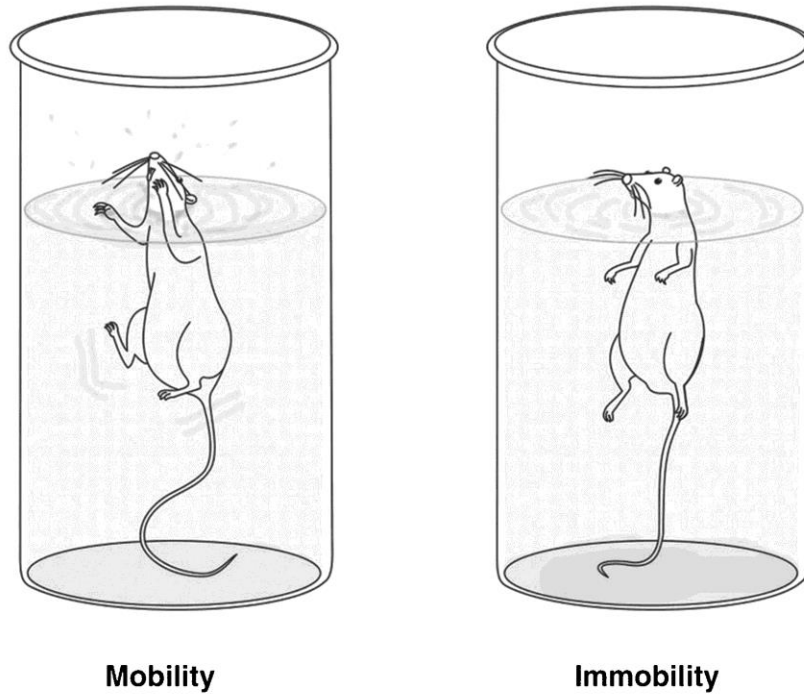
Figure 3: Illustration of Forced Swim Task showing a rat in the mobile and immobile phases (Abelaira et al., 2013)

Statistical Analysis

      All statistics were performed using GraphPad 6.0 (GraphPad Software Inc., San Diego, California). Results for the Elevated Plus Maze were compiled based upon time spent on open and closed arms, latency of behavior, overall distance moved, and frequency of visits to the head dip region. For the Forced Swim Test, results were obtained based upon differences between the control (saline induced) and experimental (insulin induced) group in the time spent in the immobile phase, latency for immobility, and bouts of immobility. Mean differences were deemed significant if $p < 0.05$. Statistical significance was evaluated using ANOVA and Student's t-test.

Results

Insulin lowers blood glucose levels

Pilot experiments were conducted to determine the appropriate dose of insulin to use in our thesis experiments. Four pilot experiments were performed.

The first pilot experiment used a single injection of 0.005 U/g insulin. An injection of 0.005 U/g insulin resulted in significantly lower BGLs when compared to rats administered saline. A significant difference between rats administered insulin and dextrose was observed on BGLs after 45 minutes ($F (3, 20) = 10.47$, $p = 0.0425$; (Figure 4). Post hoc analysis revealed that BGLs were significantly different between saline and insulin treated rats at 60, 120, and 180 minutes post injection; however, rats given insulin were unable to return to euglycemia.

A subsequent pilot experiment utilized a single injection of 0.004 U/g insulin. On four subsequent days, insulin significantly lowered BGLs when compared to baseline by an average of 73.00 mg/dL, 45 minutes post-injection 82.75 mg/dL, 90 minutes post-injection ($F = (3, 35) = 11.31$, $p = 0.0396$, Figure 5). Additionally, the rat was able to return to euglycemia following dextrose injection during the first three days of insulin injections. By day four, the rat was not able to return to euglycemia.

Pilot experiments three and four then tested the effectiveness of a single day of insulin injection of 0.004 U/g against rats given equal saline injections. The third and fourth pilot provided usable data, which served as the start of our thesis project.

The effectiveness of bolus injections of dextrose after 0.004 U/kg injection of insulin

Use of a single 1 mL dextrose injection to terminate hypoglycemia was rarely successful. Most rats required additional dextrose injections in order for the rat to

maintain euglycemia after termination of hypoglycemia. In five out of the seven instances where BGLs fell below 15 mg/dL, it was extremely difficult for the rat to maintain euglycemia despite repeated administration of dextrose. If the glucometer registered a "LO" reading, meaning BGL fell below 15 mg/dL, the rats' BGLs would fluctuate between euglycemia and hypoglycemia after the administration of dextrose, as well as following the administration of subsequent dextrose corrections. Rats that were unable to sustain euglycemia died or were euthanized (Figure 6). Because of these repeated fluctuations in and out of hypoglycemia, the behavioral data could not be collected from these rats, as they never returned to sustained levels of euglycemia. Rats subjected to the protocol used in the second through fourth pilot experiments, but whose BGLs never dipped below 30 mg/dL required only one or two additional dextrose injections before they were able to self-sustain euglycemia (data not shown).

Elevated Plus Maze measurement of anxiety-like behavior

When placed in the elevated plus maze, the control rat spent approximately 60% of the duration of the test in the closed arms, as compared to the experimental rat, which was in the closed arm 85% of the time (see Figure 7a). The experimental rat never entered the open arms (Figure 7b). The control rat was in the head dip region 18 times as compared to the experimental rat that was in the head dip region 8 times (Figure 7a). Additionally, the total distance moved throughout the maze was approximately the same for the control and experimental rats. Therefore, the rats exhibited similar activity levels (see Figure 7c).

Forced Swim Test assessment of depressive-like behavior

During the forced swim test, the control rat spent approximately 18 seconds immobile, as compared to the experimental rat, which spent 30 seconds immobile (Figure 8a). The control rat spent approximately 5.5% of the time in the immobile phase as compared to the experimental rat, which spent approximately 9.3% of the time in the immobile phase (Figure 8b). The control rat exhibited 49 bouts of immobility, as compared to the experimental rat, which exhibited 101 bouts of immobility (Figure 8c). It took 138.51 seconds for the control rat to first become immobile, versus 191.39 seconds for the experimental rat (Figure 8d).

Discussion

Summary of Goals

The objectives of this research project were twofold. We first sought to understand the effects of one moderate hypoglycemic episode on anxiety- and depressive-like behavior in Sprague-Dawley rats. Next, we attempted to find a basis for the behavioral results by assessing cell survival in the dentate gyrus. Unfortunately, due to time constraints, only the behavioral assessments could be performed. Furthermore, due to problems with inducing hypoglycemia, only two rats successfully underwent the intended behavioral experiment. A sample size of $N = 1$ is not enough to declare our results statistically significant, which ultimately hampered our ability to extrapolate the data collected in this project.

Behavioral Tests

In order to assess the behavioral effects of one moderate episode of hypoglycemia, two tests were administered: the Elevated Plus Maze and the Forced Swim Test.

Elevated Plus Maze

During the time that the rats were allowed to move throughout the Elevated Plus Maze, the insulin-treated rat dipped its head over the side of the maze much less than the saline-treated rat, as evidenced by nose point tracking (Figure 7a). Head dip movement is considered exploratory, since the animal is leaving the protection of the arm walls to peer over the side (Walf & Frye, 2007). Thus, a decrease in head dips in the insulin-treated rat is indicative of increased anxiety-like behavior as compared to the saline-treated control. Distance moved throughout the maze during the entirety of the testing period did not appear different between the insulin-treated and saline-treated rat (Figure 7c), confirming that the decrease in head dips seen in the insulin-treated rat was not simply a result of decreased mobility. This test would need to be repeated with additional rats to determine whether the decrease in head dips was significant.

The insulin-treated rat spent more time in the closed arms and less time in the center than the saline-treated rat (Figure 7b). Additionally, the insulin-treated rat never entered the open arms of the maze, while the saline-treated rat occasionally did (Figure 7b). More time spent in the closed arms than the open arms is considered indicative of anxiety-like behavior, as the closed arms offer protection to the animal (Walf & Frye, 2007). Thus, the insulin-treated animal expressed increased anxiety-like behavior in

28

comparison to the saline-treated rat. However, these experiments would need to be repeated with more rats in order to increase confidence in our results.

A past study on the behavioral effects of a single episode of hypoglycemia utilized the Barnes Maze Test, a different test that measures anxiety-like tendencies (Won et al., 2012b). Although the test is commonly used to evaluate spatial learning and memory, escape from the maze into the open center of the open field test box in which the maze is placed can suggest a decrease in anxiety-like behavior. After one episode of moderate hypoglycemia and a subsequent six-week waiting period, subjects were tested on the Barnes Maze. No significant difference was seen between the hypoglycemia and sham hypoglycemia groups in total time or distance spent in the center of the open field (Won et al., 2012b). However, the six week waiting period may have allowed for behavioral recovery, considering that the depressive-like behaviors that were confirmed after a single episode of moderate hypoglycemia disappeared 48 hours after the episode was terminated (Park et al., 2008). There is a lack of literature assessing anxiety directly with the Elevated Plus Maze after insulin-induced moderate hypoglycemia. The effects of a single episode of moderate hypoglycemia on performance 24 hours later in the Barnes Maze Test and the Elevated Plus Maze should be further evaluated.

Forced Swim Task

The insulin-treated group spent more time exhibiting immobility as compared to the saline-treated group over the cumulative duration of the Forced Swim Task (Figure 8a,b). Immobility is considered to be indicative of a higher level of depressive-like behavior (Bogdanova et al., 2013). Although it took longer for the insulin-treated rat to begin showing signs of immobility as compared the saline-treated rat (Figure 8d), the

29

insulin-treated rat exhibited over twice the number of bouts of immobility (Figure 8c). A previous study in mice found a single episode of moderate hypoglycemia to significantly increase depressive-like behavior, similarly evidenced by increased immobility in the Forced Swim Task (Park et al., 2008). In order to provide more convincing evidence in agreement with Park et al., it would be necessary to repeat the Forced Swim Task with more rats. While our data cannot be considered significant at the moment, the fact that results are in agreement with previously reported literature appears uncontroversial.

Limitations

If given the opportunity to repeat this study with modifications, there are a number of changes that would have led to a more successful experiment and a statistically significant conclusion.

Issues Inducing Hypoglycemia and Controlling Blood Glucose Levels

Consistent with previous methodology, subcutaneous insulin injections lowered blood glucose levels. A significant drop in blood glucose levels was consistently noted by 60 minutes following each insulin injection (Figure 4), as corroborated by an older hypoglycemic preconditioning study (personal communication, Simon Fisher). Upon changing our protocol to include more blood glucose measurements in order to prevent rats from dropping into severe hypoglycemia, a significant drop in blood glucose levels was noted by 45 minutes (Figure 5), in accordance with previous literature inducing moderate hypoglycemia (Park et al., 2008; Park et al., 2012).

Although other researchers such as Puente et al. had success in utilizing clamps to maintain the delicate balance of a low blood glucose level in rodent subjects (Puente et al., 2010), we were unable to use this method of insulin administration. We determined

that the catheterization of rats for a hypoglycemic clamp was outside of the team budget, and the utilization of the clamp was not within the realm of team expertise. We therefore modeled our experimental design off of previous studies, which were able to induce hypoglycemia utilizing subcutaneous and intraperitoneal injections of insulin and dextrose (Herzog et al., 2008; Jiang et al., 2009; Choi et al., 2013). Despite basing our experimental procedure off these studies, we were seldom successful in rendering our rats moderately hypoglycemic for the desired time allotment. As evident in Figure 6, it was difficult to successfully drop and maintain a lower blood glucose level, which often led to their seizing and death. Our protocol called for a .004 U/g subcutaneous injection of insulin in order to lower blood glucose levels to 30-60 mg/dL. However, the dosage commonly lowered the blood glucose levels further than 30 mg/dL, resulting in a severe hypoglycemic episode. This, in turn, rendered the rats unfit for our study. This outcome was puzzling, considering similar studies injected .006-.01 U/g of insulin in order to achieve blood glucose levels of 30-60 mg/dL (McNay & Sherwin, 2004; Herzog et al., 2008; Jiang et al., 2009).

Issues Utilizing Dextrose as a Means to Restore Euglycemia

Intraperitoneal doses of dextrose to correct for dangerously low blood glucose levels were ineffective in reestablishing euglycemia. If rats dropped below 30 mg/dL into severe hypoglycemia or had a glucometer reading of "LO," indicating a BGL lower than 15 mg/dL, then dextrose was administered to restore euglycemia. Unfortunately, even when the rats were given the correct dosage of dextrose, the rats later fluctuated between hypoglycemia and hyperglycemia, which hampered their ability to return to euglycemia (Figure 6).

Such findings have many implications for future directions for a protocol to induce hypoglycemia and successfully restore euglycemia. The inconsistent results that we found regarding the efficacy of 1 mL dextrose at restoring euglycemia may be explained by the personal variation in intraperitoneal injection technique. It is also possible that a 1 mL dose of dextrose to terminate hypoglycemia may be more effective if administered by a different method. Other studies investigating the effects of hypoglycemia have injected dextrose intravenously or with an oral gavage (Suh et al., 2005b; Dave et al., 2011). Utilization of a variety of means of dextrose administration may lead to better control of BGL and return to euglycemic levels.

Stress as a Confounding Variable

Stress hormones have the capability to impact blood glucose levels, which may have also affected our success in inducing and maintaining a consistent level of moderate hypoglycemia in each individual rat. Additionally, a rat that was not calm was difficult to manage, which led to unfavorable injection conditions. The restraint system appeared to initially be a stressful experience for the rat subjects, with stereotyped stress responses elicited even upon first attempts to place the animals in the restrainer. Specific examples of high-level stress include: vocalizations, hyperventilation, excessive fecal production, porphyrin secretion from the eyes and nose, seizures, and, ultimately, death. Attempts to precondition the rats to the restrainer for five minutes on five consecutive days prior to injection proved unsuccessful. Even after the preconditioning protocol was carried through, the rats still exhibited a near-identical stress response, indicating that preconditioning to the restrainer was ineffective and the stress response still had the capability to confound experimental conditions.

Developing a more consistent and better-controlled insulin/dextrose administration system to drop and maintain the blood glucose level of rats and ensure their survival would have lent to a larger sample size of rats. Only one rat that received the insulin injections survived, as well as only one rat in the control condition. While these small sample sizes were a result of high mortality rates rather than an experimental choice, this means that the future implementation of a tighter control mechanism for blood glucose, such as the aforementioned clamp, would lead to significantly higher survival rates and therefore a higher n-value. Only one rat in each group was not enough to sufficiently account for experimental variability, and a larger sample size would have ameliorated this issue and perhaps lent statistically significant results.

Clinical Implications

Our research suggests that moderate hypoglycemic events may lead to increased depressive and anxiety-like behavior upon return to euglycemia. Although a clinical study has implied that severe hypoglycemia can cause increased depression and anxiety up to 1.5 days after the episode, it is not known whether moderate hypoglycemia could induce the same behavioral changes in humans (Strachan et al., 2000). However, this lack of clinical research is not specific to anxiety and depressive-like behavior. Extremely few clinical studies have examined behavioral changes after moderate episodes of hypoglycemia, perhaps due to patients and doctors being more concerned about severe hypoglycemia.

Interestingly, an abundance of research in T1D patients has detailed the anxiety and depression resulting from fear of developing hypoglycemia (Barnard et al., 2010; Solli et al., 2010; Böhme et al., 2013). This is equivalent to suggesting that assessing

anxiety- and depressive-like behavior in humans post-moderate hypoglycemic episode might be difficult because pre-existing anxiety and depression would confound behavioral changes noticed after hypoglycemia. On the other hand, it is possible that the existence of anxiety and depression before development of hypoglycemia could have a synergistic effect, leading to even more elevated levels of depression and anxiety post-hypoglycemia. Since clinical studies have also shown that increased glycemic variability, which can include episodes of both hyperglycemia and hypoglycemia, also increases anxiety and depression, determining the effect of solely hypoglycemia on behaviors in humans would be extremely difficult to control (Penckofer et al., 2012).

The limitations presented above discourage application of our results to any drastic clinical recommendations. Although it is known that the use of intensive insulin therapy, as opposed to conventional insulin therapy, increases the risk of hypoglycemia, the risks presented here do not pose a convincing case to avoid intensive therapy. Complications posed by frequent hyperglycemia, such as neuropathy, retinopathy, and nephropathy, can be more easily avoided with intensive therapy (Dhingra et al., 2009). The benefits of intensive therapy clearly outweigh the potential risks. As such, our study suggests that doctors should make their patients using intensive insulin therapy aware of the potential for increased anxiety and depression post-hypoglycemia and perhaps suggest that they inform their friends and families of the potential for these mood changes. An understanding of hypoglycemia-related behavioral changes could serve to increase empathy in those that interact with and are caretakers for diabetic individuals.

Future Directions

  With modifications in experimental design to allow for the replication of this experiment, there are a number of items that could be added to allow for a larger contribution to the scientific community. Primary is pursuing the immunohistochemistry aspect of the experiment, which was left incomplete at the culmination of the project for the issue of practicality. The proposed BrdU staining would offer insight into what neurological changes may have been behind any noted behavioral changes.

  We speculate that moderate hypoglycemia would decrease cell survival in the dentate gyrus, in accordance with the Neurogenesis Theory of Depression (Snyder et al., 2011b). Although severe hypoglycemia is known to increase cell proliferation in the dentate gyrus at as little as 4 days after the episode (Suh et al., 2005b), severe hypoglycemia induces neuronal death, while moderate hypoglycemia does not (Puente et al., 2010; Won et al., 2012b). Moderate hypoglycemia would lack the purpose of inducing cells to proliferate without the motive of replacing missing neurons. Whether moderate hypoglycemia would cause cell survival to decrease, however, is not entirely clear.

Figure Legends

Figure 4. Blood glucose levels (BGLs) over time following insulin injections. BGLs were measured in rats given a saline (n=4) or 0.005 U/g insulin (n=8) injection at time 0. Rats were monitored for 120 additional minutes. A significant difference was observed between groups at time 60, 120, 180. Data represent Mean ± SEM.* indicates $p < 0.05$.

Figure 5. Average blood glucose levels (BGLs), over 4 days, for a single rat administered 0.004 U/g insulin. A statistically significant difference in BGLs was observed between time 0 and times 45 and 90 minutes post injection. Hypoglycemia was terminated with a 1.0 mL dextrose injection between time 90 and 135, resulting in a significant difference in BGLs between time 45 and 135. Data represent Mean ± SEM.* indicates $p < 0.05$

Figure 6. Fate of rats whose blood glucose levels (BGLs) dropped below 15 mg/dL following injection of 0.004 U/g insulin. Five of seven rats whose blood glucose levels reached 15 mg/dL or lower died or were not able to return to euglycemia.

Figure 7. Elevated plus maze performance 24 hours following induction of hypoglycemia. (a) The insulin rat (n=1) entered the head dip region fewer times as compared to the saline rat (n=1) during the elevated plus maze test. Less time spent in the head dip region indicates higher anxiety-like behavior, as the rat is exhibiting less exploratory behavior. (b) Cumulative duration the saline (n=1) and insulin (n=1) rat spent

in different arms of the elevated plus maze. The insulin rat spent approximately 45 more seconds in the closed arms, less time in the center, and did not enter the open arms, as compared to the saline rat. More time spent in the closed arms, and less time spent in the center and open arms, is indicative of higher anxiety-like behavior. (c) Distance traveled throughout the elevated plus maze was approximately the same between saline (n=1) and insulin (n=1) rat. The differences in location of the rats are not due to mobility changes, suggesting that the difference is due to exploration or anxiety.

Figure 8. Forced swim test performance 24 hours following induction of hypoglycemia. The hypoglycemic rat spent more time floating (a), spent a greater percentage time floating (b), and performed more bouts of immobility (c) in the FST, compared to the euglycemic rats. Increased floating, or immobility, demonstrates depressive-like behavior. However, the latency to the first bout of immobility was longer for the hypoglycemic rat compared to the euglycemic rat. (d) A shorter latency to become immobile would demonstrate a greater propensity for depressive-like behavior.
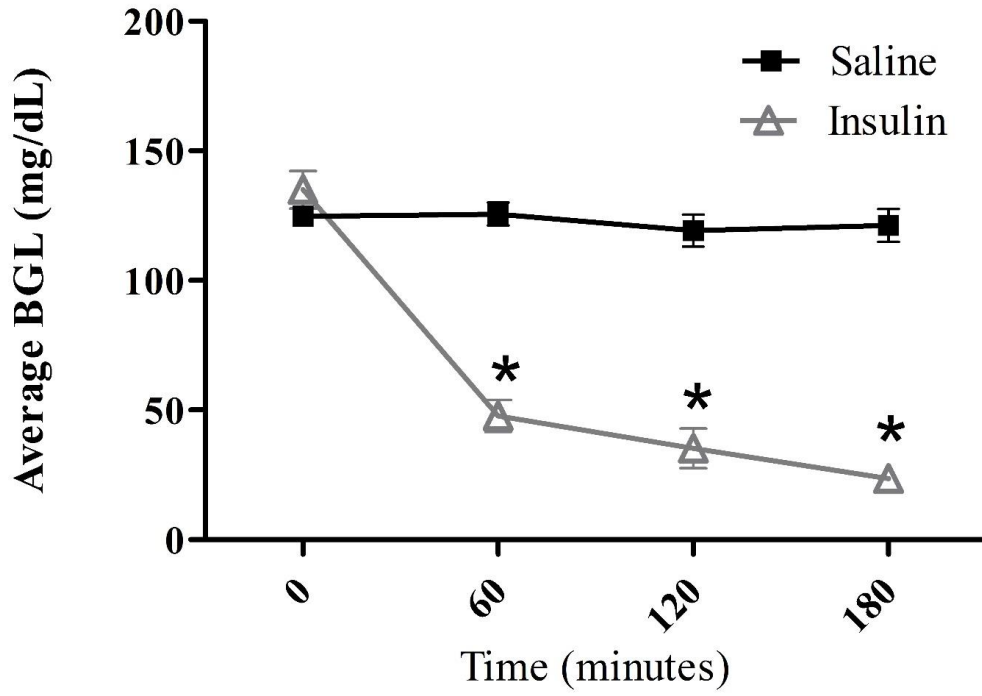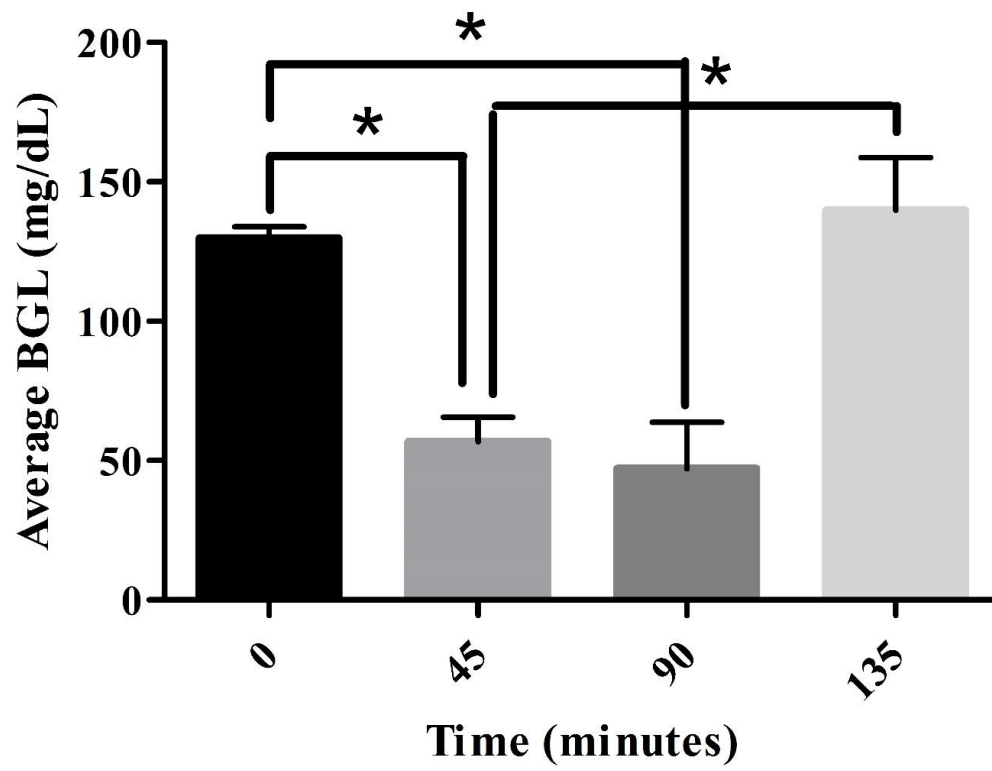
Experimental Figures
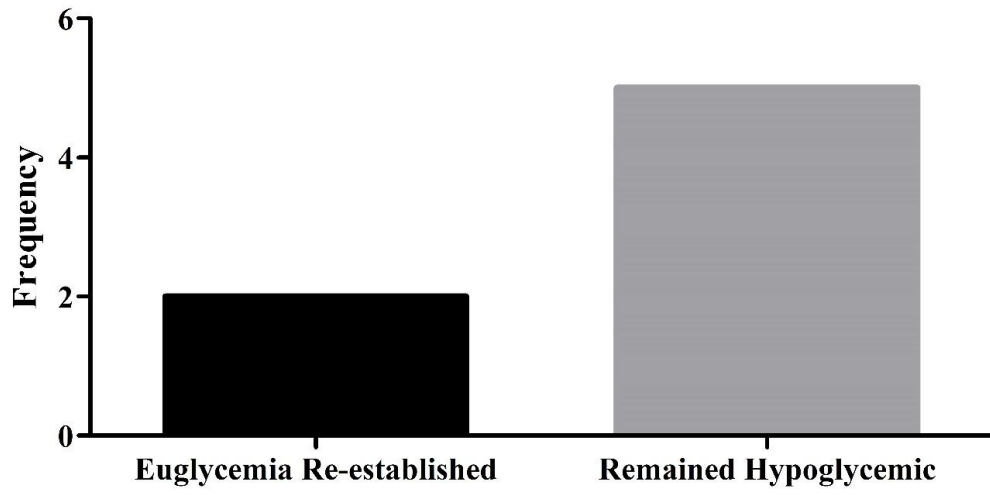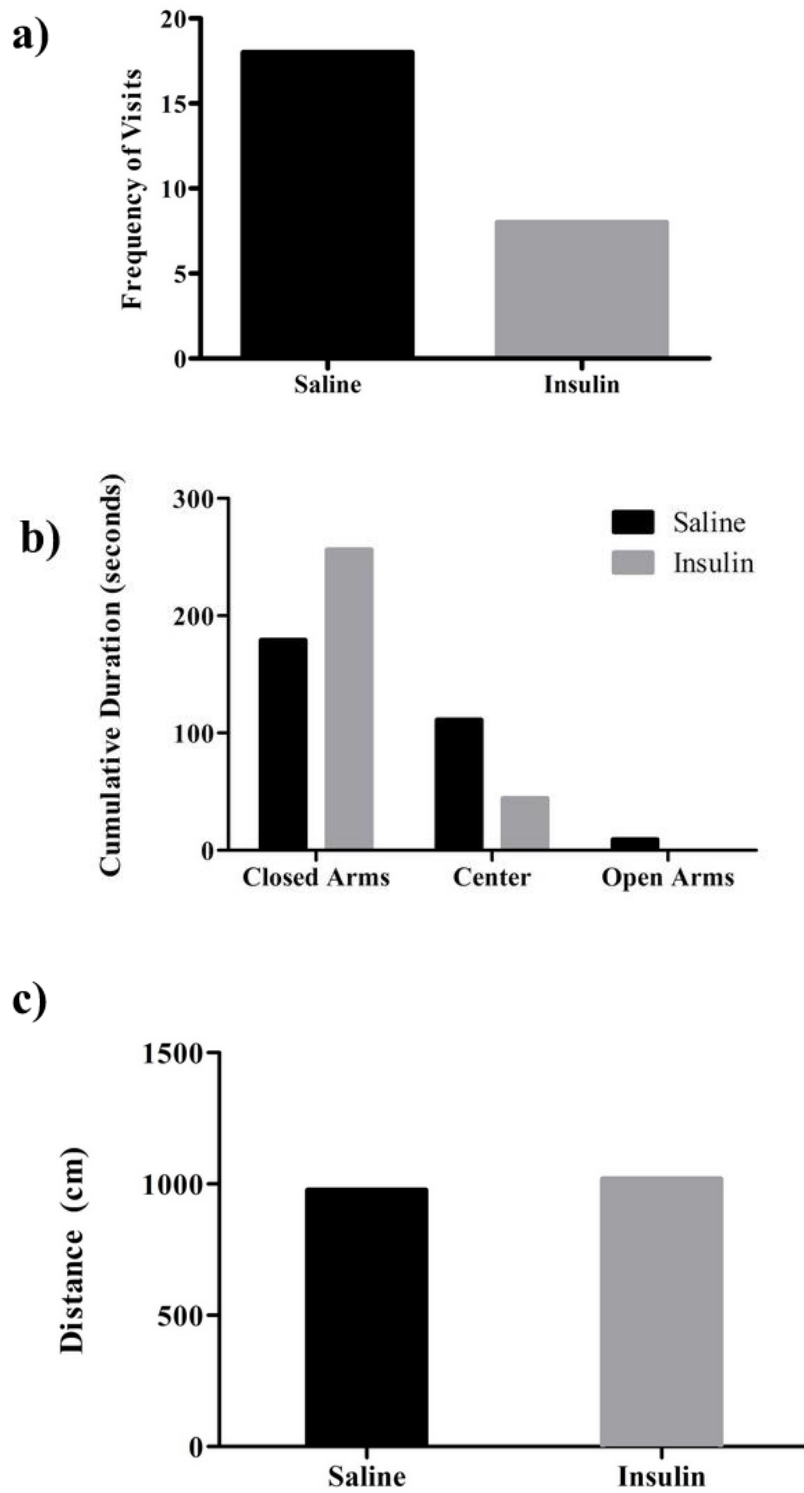
Figure 4

Figure 5

Figure 6
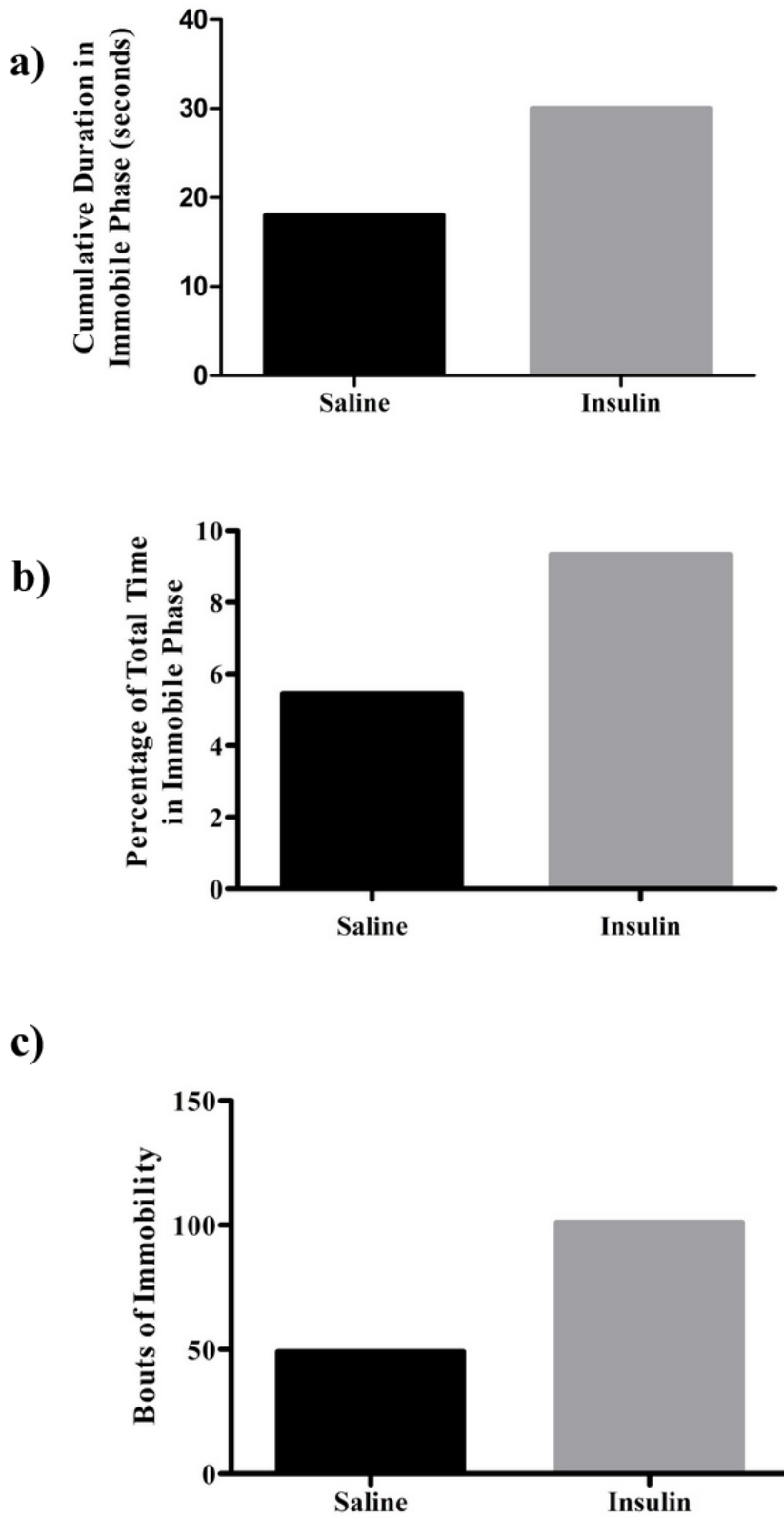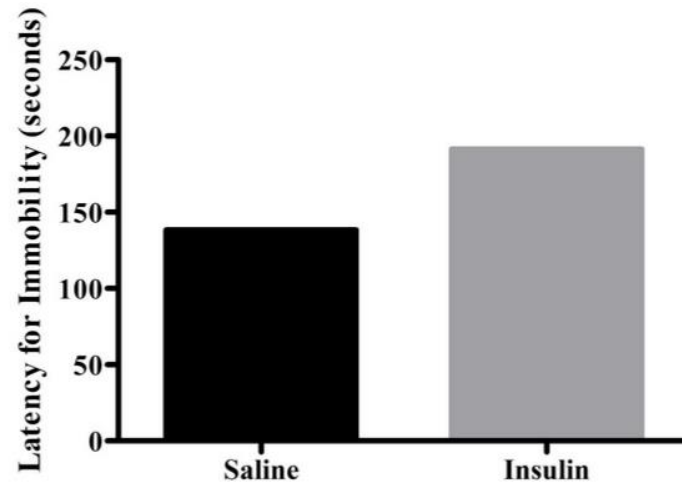
Figure 7

a)

b)

c)

Figure 8

a)

b)

c)

d)

Appendix A: Literature Review Related to Prior Experimental Design

Lactate Utilization as a Mechanism of Preconditioning

When the brain is deprived of glucose, it becomes capable of taking up and utilizing alternate fuels such as lactate and pyruvate (Suh et al., 2005a; Zhou et al., 2012). Pyruvate is the end product of glycolysis and is utilized in the Krebs cycle and eventually oxidative phosphorylation. Traditionally, it was viewed that under anaerobic conditions, pyruvate would be converted into lactate via lactate dehydrogenase in order to restore the number of nicotinamide adenine dinucleotide (NAD(+)) oxidizing agents involved in generating ATP from glucose (Brooks et al., 1999). However, it has now been recognized that glycolytic processes generate a substantial amount of lactate in circulation even under aerobic conditions (Brooks, 1986). Studies have also shown that, in addition to glucose, the brain can oxidize lactate as a source of energy (De Feyter et al., 2013).

A potential role for lactate could be the preservation of neurological activity during high stress. Recent studies have shown that hippocampal neuronal death related to hypoglycemia may not occur exclusively during periods of low blood glucose. Instead, a significant portion of damage is initiated during the glucose reperfusion stage due to oxidative stress (Suh et al., 2007a). As glucose re-enters the bloodstream, neuronal cells may be unable to metabolize it due to the activation of poly(ADP-ribose) polymerase-1 (PARP-1) (Suh et al., 2003). PARP-1 is an enzyme that consumes cytosolic NAD(+) (Burzio et al., 1979). As glycolysis requires NAD(+) for breakdown of glucose, a scarcity of NAD(+) prevents neurons from obtaining metabolic sustenance even with sufficient glucose in the bloodstream (Burzio et al., 1979). In response to activated PARP-1, alternative metabolites such as pyruvate and lactate may be utilized in order to bypass the glycolytic mechanism requiring NAD(+) (Suh et al., 2005a; Won et al., 2012a).

Pyruvate, the glycolytic end product, has been thoroughly studied, as it is an important regulatory molecule involved in the transition from anaerobic (glycolysis) to aerobic (Krebs Cycle and oxidative phosphorylation) respiration. Reperfusion of glucose combined with pyruvate has been shown to reduce hypoglycemia-related neuronal damage by 70-90% relative to glucose reperfusion alone (Suh et al., 2005a). Reduction of pyruvate by the oxidation of Nicotinamide Adenine Dinucleotide (NADH) results in the production of lactate, a molecule whose concentration is ten times greater than that of pyruvate during basal circulation levels (Henderson et al., 2004). Lactate supplementation of glucose following hypoglycemia has also been proven to reduce hypoglycemia-related neuronal death in the hippocampus relative to glucose by 80% (Won et al., 2012a). As a result, lactate may be more readily available for neuronal metabolism during hypoglycemia.

Hypoglycemia increases the levels of a number of glucocorticoids, a class of stress hormones, including corticosterone (CORT) (Diggs-Andrews et al., 2010). CORT is known to decrease the activity of a variety of glycolytic enzymes necessary for conversion of glucose to pyruvate, including hexokinase, phosphofructokinase, pyruvate kinase, and glyceraldehyde-3-phosphodehydrogenase (Hoyer and Lannert, 2008). However, CORT upregulates the activity of lactate dehydrogenase, which catalyzes the reduction of pyruvate to lactate, by three to four times (Hoyer and Lannert, 2008). Because CORT inhibits glycolysis, an essential step in respiration, it negatively affects the oxidation of glucose during glucose reperfusion. This encourages the use of alternate fuel sources when CORT is present. Since CORT is induced by moderate hypoglycemic episodes and promotes lactate synthesis, it is possible that the resulting lactate production

may prevent neuronal cell death if a future severe hypoglycemic episode were to occur (Diggs-Andrews et al., 2010). Although both lactate and pyruvate have been studied as potential treatments for post-hypoglycemic neuronal damage, it is unclear as to whether preconditioning might cause these energy sources to be favored over glucose (Suh et al., 2005a; Zhou et al., 2012). Thus, it seems plausible that the mechanism behind the neuronal protection provided by preconditioning severe hypoglycemic episodes with multiple moderate episodes of hypoglycemia might result from the brain's acquired preference of lactate usage over glucose.

When De Feyter et al. (2013) discovered that brain lactate concentrations during hypoglycemia are five times higher in diabetic patients compared to normal control subjects, it was one of the first studies that hypothesized that the recurrent exposure to hypoglycemic episodes potentially causes elevated lactate levels in the brain. Additionally, these elevated lactate levels were correlated with a preservation of neuronal metabolism during glucose deprivation (De Feyter et al., 2013). However, the cause for elevated levels of lactate in the brain is unclear. It has been suggested that the brain may be increasing lactate uptake from circulation, oxidizing lactate during glucose deficiency, or decreasing the export of lactate through monocarboxylate transporters (MCTs) (De Feyter et al., 2013). In regards to the increased lactate levels, a recent study by Herzog et al. (2013) identified that rodent brain metabolism is also altered after recurrent hypoglycemia. In particular, the study identifies that although lactate concentration is increased, it does not add to the brain's oxidative capacity and is not largely consumed. The study proposed a model, which demonstrated that the metabolism of glucose in the rodent brain is maintained through increased lactate levels. Taken together, this research

suggests that increased lactate in the brain is modulating and regulating neuronal metabolic activity and possibly glucose metabolism, but is not necessarily directly consumed (Herzog et al., 2013).

Lactate Transporters and Hypoglycemia

Neurons are metabolically supported by astrocytes in the brain. During hypoglycemia, astrocytes transport lactate to neurons via the astrocyte-neuron-lactate shuttle hypothesis (ANLSH), allowing for lactate metabolism and subsequent ATP production (Figure 9) (Pellerin and Magistretti, 1994; Suh et al., 2007b). This holds true when limited glucose is available for use; during euglycemia, glucose is the primary substrate utilized by neurons (Pfeuffer et al., 2000). Transport of lactate from astrocytes to neurons is facilitated by MCTs specifically, MCT1 and MCT2 (Garcia et al., 1994; Garcia et al., 1995). MCT1 and MCT2 are expressed in high concentration in the dentate gyrus, specifically by granule cells, which endure most of the hypoglycemic necrosis that occurs in the hippocampus (Auer et al., 1985). This indicates that the expression of these transporters may be upregulated in order to move lactate into neurons most prone to hypoglycemic death.

Both MCT1 and MCT2 are responsible for lactate transport in the hippocampus; however, astrocytes express MCT1 much more strongly than MCT2 and neurons express MCT2 much more strongly than MCT1 (Debernardi et al., 2003). It is important to note that MCT1 and MCT2 have different affinities for lactate, as measured by K_m values. K_m, the Michaelis-Menten constant, is the concentration of substrate at which a transporter is operating at half its maximum velocity. Therefore, a lower K_m value indicates a higher affinity for the substrate. MCT1 has a lactate K_m value of 3-5 mM and MCT2 has a K_m

value of 0.7 mM, so MCT2 has a much higher affinity for lactate than MCT1 (Halestrap and Price, 1999). Since the basal blood lactate level is 0.80 ± 0.27 mM, MCT2 transporters operate relatively efficiently during euglycemia, while MCT1 transporters are not very active (Abi-Saab et al., 2002). However, it is expected that blood lactate levels will rise following a hypoglycemic episode to 1.36 ± 0.76 mM, allowing MCT1 transporters to begin to become saturated (Abi-Saab et al., 2002). Thus, both MCT1 and MCT2 transporter expression should be studied to obtain an accurate representation of net lactate transport in the brain under conditions of euglycemia interrupted by hypoglycemia.

In addition to MCT1 and MCT2, MCT4 is also found in astrocytic tissue (Figure 10). However, its K_m value for lactate is 28 mM, which indicates that MCT4 has a much lower affinity for lactate than both MCT1 and MCT2, and probably does not contribute much to net lactate transport (Halestrap and Price, 1999). Thus, it seems somewhat unlikely that MCT4 would be significantly upregulated as a consequence of repetitive hypoglycemia as compared to the other two transporters. Additionally, it is important to note that MCT 1 and MCT2 on neurons import ketone bodies in addition to lactate (Figure 10). Thus, an increase in MCT1 and MCT2 protein expression may indicate increased import of both lactate and/or ketone bodies, without the possibility of differentiating between augmented import of one or the other (Halestrap and Price, 1999). However, increased levels of MCT1 expression could only be attributed to increased lactate export, since MCT1 is associated with astrocytes as opposed to neurons (Figure 10). In the context of the ANLSH hypothesis, increased levels of both dentate gyrus-

associated MCT1 and MCT2 expression would likely indicate an increase in lactate transport (Halestrap and Price, 1999).

It is known that severe hypoglycemia upregulates neuronal MCT2, implicating that use of the ANLSH pathway is increased (Vavaiya and Briski, 2008a, b). However, this has yet to be implicated in the context of preconditioning. Furthermore, studies have yet to elucidate whether hypoglycemia induces the overexpression of astrocytic MCT1 or MCT2. We hypothesize that in rats preconditioned with repeated moderate hypoglycemic events, neuroprotection may result from an increased ability to import lactate to neurons, due to an overexpression of MCT2 in neurons and MCT1 in astrocytes.

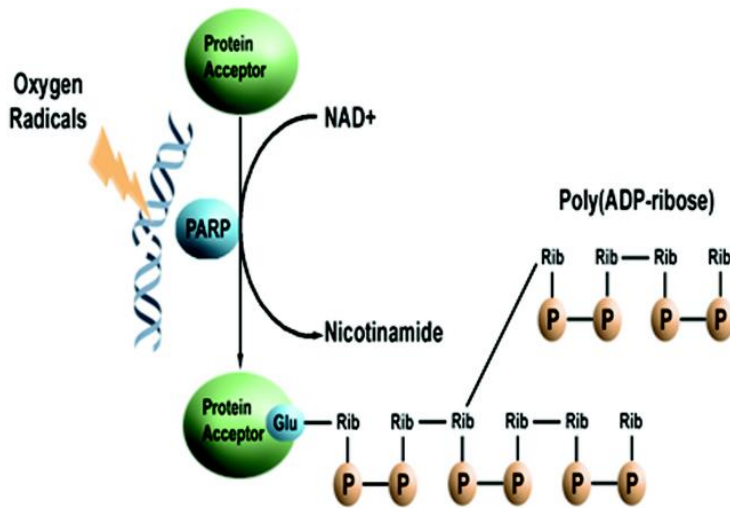## Figures for Literature Review & Appendix A

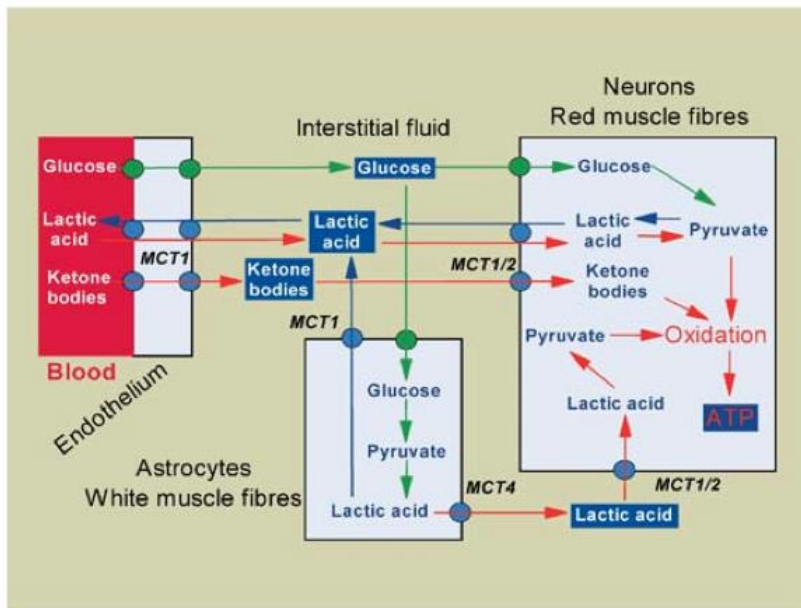Figure 9. PARP-1 and NAD+ interaction (Giannone et al., 2011)

Figure 10. Cellular distribution of MCT1, 2, and 4 and pathway of lactate transport (Halestrap and Meredith, 2004).

Appendix B: Inducing Hypoglycemia Subprotocols

Insulin Dosages and Corrections

   Rats in the experimental group were given a subcutaneous injection of regular human insulin diluted 1:10 by saline. The dosage administered was calculated by multiplying 4 U of insulin by the mass of the animal in kilograms, and converting the resulting value into milliliters using the insulin's concentration (100 U).

   If rats in the experimental group did not reach target levels within one hour after receiving an insulin injection and their blood glucose level was not dropping, then an insulin correction was performed subcutaneously. This correction was determined by multiplying the originally calculated insulin dosage by the current blood glucose level minus the target (30 mg/dL), divided by 120 mg/dL, the amount of blood glucose points that the original dosage was theoretically supposed to drop blood sugar levels by. If any rat received an insulin correction, the matched control animal received a corresponding amount of saline subcutaneously to control for the confounding variable of stress. The calculations for insulin dosages and corrections were developed by our team from a protocol in Puente, 2010.

Dextrose Corrections

   After insulin administration, if the blood glucose level of any rat dropped below 50 mg/dL, a 50% dextrose correction was performed intraperitoneally, so as to prevent the animal from dropping into severe hypoglycemia. The matched control animal received a corresponding amount of saline injected intraperitoneally to control for the confounding variable of stress. The correction amounts in the table below were

determined by monitoring blood glucose rise after administration of 1 mL dextrose in

pilot animals.

BGL	Amount of Dextrose
43-45 mg/dL	0.05 mL
40-42 mg/dL	0.1 mL
38-39 mg/dL	0.15 mL
35-37 mg/dL	0.2 mL
33-34 mg/dL	0.25 mL
30-32 mg/dL	0.3 mL
27-28 mg/dL	0.35 mL ****
25-26 mg/dL	0.4 mL ****
23-24 mg/dL	0.45 mL ****
20-22 mg/dL	0.5 mL ****

**** If any animals dropped to this blood glucose level, they were considered to have reached the threshold for severe hypoglycemia, and therefore, were terminated before the conclusion of the study.

Appendix C: Blood Glucose Measurement Subprotocols

Tail Vein Blood Withdrawal

Prior to blood withdrawal, the tail of the rat was submerged in a bowl of warm (40∘C) water to cause vasodilation, or enlargement, of veins (Jones, 2012). Next, the rats were placed in a 554-BSRR rodent restrainer from PLAS-LABS, INC. The tail was swabbed with 70% isopropyl alcohol to increase the visibility of the vein (Jones, 2012). The middle third of the tail was examined and either one of the two lateral veins was located (Jones, 2012). The tail was restrained while the vein was covered with the non-dominant hand (Jones, 2012). Next, with the bevel of the needle facing upward and the needle parallel to the vein, the needle was inserted into the visible tail vein (Jones, 2012). It was confirmed that the needle was inserted into the vein by applying negative pressure to the plunger (Jones, 2012). If the needle was correctly inserted into the vein, then a small amount of blood appeared in the hub of the needle once pressure was applied (Jones, 2012). The vein occlusion was released and either the needle was removed or the plunger of the syringe was slowly pulled upward to remove a blood sample from the vein (Jones, 2012). Then, the needle was removed from the vein and moderate pressure was applied with a cotton swab to stop the bleeding (Jones, 2012). The animal was removed from the restrainer and placed back into its home cage, where its movements were observed for 5-10 minutes to ensure that the bleeding fully stopped (Jones, 2012).

Habituation to Restrainer

To remove the confounding effects of stress (Girotti M1, 2006) and ease tail vein blood withdrawal process, habituation of the rats to the restrainer was necessary. Five days prior to beginning experimentation, both the experimental and control rats were

removed from the colony and taken to the lab, where they would be placed into the restrainer individually. This occurred at the same time of day when induction of hypoglycemia eventually took place once the experimentation actually begun. Each subject was held inside the restrainer for approximately five minutes and then placed back into the cage. This habituation process continued for five additional days. This process was used to control stress that the animals experienced when placed into the restrainer during the start of the experiment.

Appendix D. Changes to Methodology

An explicit, step-by-step protocol for the induction of moderation hypoglycemia was not found during literature review, meaning a protocol had to be developed based on existing data and consultation with experts. As we became increasingly aware of the limitations imposed by our protocol for induction of hypoglycemia, our research questions were redefined, and the protocol was remodeled to suit new experimental demands.

Experimental Design	Changes to Research Questions	Adaptations to Protocol
See Appendix D1	N/A	- Dilution of insulin to a 1:10 ratio with saline, after personal communication with Puente - Determination that severe hypoglycemia, as induced by accidental 10-fold insulin concentration, was unfeasible due to seizures

See Appendix D2	• Behavioral tests changed to Elevated Plus Maze and Forced Swim Task due to accessibility of EPM equipment and literature review suggesting that moderate hypoglycemia does not affect spatial reasoning, respectively • Oxidative dendritic damage assessed because neuronal death sparse after moderate hypoglycemia	• Research question no longer involved preconditioning (no severe episode, 5 recurrent moderate episodes as modeled by Won et al, 2012) • Assessed different insulin dosages (3 U/kg, 4 U/kg, and 5 U/kg) from Puente's recurrent hypoglycemia protocol until deciding on 4 U/kg dosage • Switched to subcutaneous insulin injections rather than intraperitoneal, personal communication with Puente • Blood glucose testing every 30 minutes as described in Won et al, 2012
See Appendix D3	N/A	• Five days of recurrent moderate hypoglycemia shortened to three days due to unpredictability of blood glucose readings • Blood glucose readings increased to every fifteen minutes to aid in decisions to administer dextrose corrections • Implementation of precautionary dextrose corrections, by team design

See Experimental Design in Main Text of Chapter 3	• Cell proliferation assessed rather than dendritic oxidative damage because single episode of hypoglycemia does not cause oxidative damage, but does affect depressive-like behavior	• Three days of recurrent moderate hypoglycemia shortened to one day due to unpredictability of blood glucose returning to euglycemia • Habituation to restrainer performed due to high animal stress

Appendix D1: First Iteration

Experiment I

        Twenty male Sprague-Dawley (S-D) rats were randomly assigned into two groups for behavioral testing. The control group (N=10) did not undergo preconditioning and the experimental group (N=10) was exposed to hypoglycemic preconditioning.

        Upon their arrival, the S-D rats received five days of rest to become acclimated to their new habitat. After this rest period, the rats in the experimental group were administered appropriate amounts of insulin to induce moderate hypoglycemia for 3 hours once daily for 3 days to serve as the preconditioning treatment. Saline administration to the control group limited the confounding effect from stress induced by a substance administration to the rats. On day four, both the preconditioned and control groups received a bolus of insulin to induce a severe hypoglycemic episode for 1.5 hours. Behavioral tests occurred approximately 5 weeks after the severe hypoglycemic episode for both the control and experimental groups (Puente et al., 2010). On day 39, the subjects underwent the novel object recognition test to assess temporal memory. After a day of rest, day 41 consisted of the light-dark box test to measure anxiety-linked

tendencies. Following another day of rest, the probe test for the Morris water maze was performed on day 43. Training for the Morris water maze occurred on days 44 through day 51, followed by the probe test on day 53 to test spatial memory. Euthanasia occurred after all behavioral tests were performed.

Experiment II

Ten male S-D rats were randomly assigned into two groups to assess neuronal viability. The control group (N=5) did not undergo preconditioning; the experimental group (N=5) did receive preconditioning. A week after the severe hypoglycemic episode, all rats were sacrificed in order to perform Fluoro-Jade B staining (Puente et al., 2010).

Upon their arrival, the S-D rats received five days of rest to become acclimated to their new habitat. During the first 3 days of the experiment, the rats in the experimental group were administered appropriate amounts of insulin to induce moderate hypoglycemia for 3 hours once daily for 3 days to serve as preconditioning. Saline administration to the control group limited the confounding effect that results from stress induced by a substance administration to the rats. On day four, both groups received a bolus of insulin to induce a severe episode of hypoglycemia for 1.5 hours in order to induce hippocampal damage (Puente et al., 2010), and euglycemia was restored on day 5. Then, the rats were allowed to rest until day eleven, when they were perfused for post-mortem biochemical testing of hippocampal tissue. After euthanasia, each rat brain underwent subsequent immunohistological processing and quantification of neuronal damage in the dentate gyrus associated with the hypoglycemic episodes via a Fluoro Jade B stain.

Experiment III

        Thirty male S-D rats were randomly assigned into two groups to assess MCT1 and MCT2 transporter protein expression. The control group (N=15) did not undergo moderate episodes of hypoglycemia; the experimental group (N=15) received one to three bouts of moderate hypoglycemia. Five S-D rats from the experimental group and five S-D rats from the control group were sacrificed after every moderate hypoglycemic episode in order to perform double immunofluorescence staining of MCT1 and MCT2.

        Upon their arrival, the S-D rats received five days of rest to become acclimated to their new habitat. After this rest period, the rats in the experimental group were administered appropriate amounts of insulin to induce moderate hypoglycemia once daily for 3 hours to serve as preconditioning. Saline administration to the control group limited the confounding effect that results from stress induced by a substance administration to the rats. After each moderate episode (or saline injection), 5 rats from the experimental group and 5 rats from the control group were euthanized 2 hours after euglycemia was achieved for post-mortem testing of hippocampal tissue (Vavaiya and Briski, 2008). Lactate transporter expression was quantified with immunohistochemistry (IHC) to measure protein expression of the MCT1 and MCT2 transporters.

Novel Objection Recognition

        Rats completed a temporal order task that has been proven to involve the hippocampus, particularly the dentate gyrus (Jessberger et al., 2009). During the first sample phase of the experiment, subjects were given four minutes to explore two identical objects. One hour later, the second sample phase proceeded with subjects given four minutes to explore two identical objects differing from the first set. This was

followed by a delay of three hours. The test trial setup involved a copy of one of the objects from sample one and one from sample two, positioned in similar spacing as the objects in the exposure phases were placed. Rats were given three minutes to explore the two different objects and time exploring each object were recorded. Software detected exploration by measuring the distance between the nose of the rat and the object. The rats were considered to be exploring an object if their nose was less than two centimeters away from the object (Personal communication, Dr. Erica Glasper). If hippocampal function was unimpaired, subjects would spend more time exploring the object from sample 1, the less recently presented object, than the object from sample 2, the more recently presented (Barker and Warburton, 2011).

Light/Dark Box

The light/dark box test is a method of examining the behavior of rodents when exposed to stressors, consisting of light and an unfamiliar environment. Anxiety has been shown to be an indicator of overall hippocampal function, particularly within the dentate gyrus (Nolen-Hoeksema, 2011). The rodents succumbed to either their neophobia, fear of the unfamiliar, or their natural predisposition to explore new surroundings. Rats were placed in a box with two sections, one that was illuminated and the other that was darkened, with a small hole for travelling between the two sections. Subjects were first placed in the light side of the box. They had five minutes to explore and their movements between the sections were tracked via a camera. Rodents who spent more time in the light side, or who made more transitions between the two sections had lower levels of anxiety-like behavior. Higher anxiety levels indicated hippocampal damage (Bourin and Hascoet, 2003).

59

Morris Water Maze

The Morris water maze is one of the most common and reliable tests for assessing spatial learning and therefore dentate gyrus function (Vorhees and Williams, 2006). The setup for the test consisted of a circular vessel filled halfway with opaque water (Vorhees and Williams, 2006). This opacity was achieved by adding a small amount of tempera paint to the water (Neigh et al., 2004). The clouded water obfuscated a submerged platform that the rat sought in efforts to escape the pool. The pool utilized was divided into four quadrants (Vorhees and Williams, 2006). To begin, each rat was placed in the water individually and its motions were tracked to determine which quadrant it naturally gravitated toward. Next, the platform was placed in the quadrant in which, on average, the rats naturally spent the least amount of time. After a week of training to this location, the rat was exposed to the probe test and assessed on its spatial memory to reach the hidden platform (Personal communication, Dr. Erica Glasper). The motions of each rat were monitored via a camera device and tracking software, and the directness of their path was indicative of their capability of spatial learning (Vorhees and Williams, 2006). The less time that it took the rat to reach the platform and the fewer crosses into other quadrants, the better their spatial learning and the higher their hippocampal function (Morris, 1984).

Fluoro-Jade B

Necrotic neurons can be stained and visualized by light and electron microscopy; however, fluorescence microscopy has become the method of choice for quantifying neuronal death. Fluoro-Jade B is a widely-used marker and has a high affinity for degenerating neurons (Schmued and Hopkins, 2000), and is thus suitable for our experimentation.

Following the severe hypoglycemic episode and a week of rest, rats in the FJB group were anesthetized and later perfused. The brains were then post fixed in paraformaldehyde followed by sucrose (Schmued and Hopkins, 2000). Coronal sections were cut into 40 μm thick pieces using a Vibratome slicing instrument in order maximize Fluoro-Jade B contrast (Matzen et al., 2008; Morris et al., 2010). These sections were then placed on a freezing sliding microtome and immersed in 0.1 M neutral phosphate buffer (Matzen et al., 2008; Morris et al., 2010). The sections were then mounted on 2% gelatin coated slides and air dried on a slide warmer at 50 ºC (Morris et al., 2010). Slides were then washed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 minutes (Morris et al., 2010). Slides were washed again for 2 minutes in 70% alcohol and distilled water, and subsequently transferred to a solution of 0.06% potassium permanganate for 10 minutes (Schmued and Hopkins, 2000; Morris et al., 2010). Slides were finally rinsed in distilled water for 2 minutes (Morris et al., 2010).

The Fluoro-Jade B stock solution was prepared using a mixture of dye powder, distilled water, and acetic acid, with a resulting dye concentration of 0.001% (Morris et al., 2010). The slides remained in the staining solution in order for the dye to set, then rinsed in distilled water and dried (Matzen et al., 2008). Non-aqueous and non-fluorescent material were used to cover the slides for analysis via epifluorescence microscopy (Matzen et al., 2008; Morris et al., 2010). The microscope emitted a blue-violet 420-490 nm light in order to excite the fluorescence, causing the degrading neurons to glow in contrast to the background (Schmued and Hopkins, 2000). The more that the sample fluoresced, the more neuronal death occurred.

MCT Immunohistochemistry (IHC) Assay

Double immunofluorescence staining techniques were used to quantify protein expression of MCT1 and MCT2 lactate transporters. Immunofluorescent staining required additional sections of perfused hippocampal tissue, approximately 40 μm thick, to be prepared from brains postfixed using paraformaldehyde in phosphate buffer solution (PBS) and sucrose. Sections of samples were prepared on microscope slides in the same manner as for Fluoro Jade B staining.

Samples were stained with MCT1 and MCT2 primary antibodies overnight, binding to MCT1 and MCT2 antigens in the sample. This was followed by washing the samples with PBS and staining with secondary fluorochromes of differing colors for one hour that each specifically adhered to one of the two primary antibodies (Vavaiya and Briski, 2008). Amount of fluorescence was examined via epifluorescence microscopy to determine if MCT1 and MCT2 up-regulation occurred in preconditioned animals. This involved specifically examining dentate gyrus tissue within each hippocampal slice and measuring intensity of the fluorescence from each dye used.

Appendix D2: Second Iteration

Experimental Design

Thirty male S-D rats were randomly assigned into two groups to assess MCT1 and MCT2 transporter protein expression and twenty male S-D rats were randomly assigned into two groups to assess behavioral changes and dendritic oxidative damage following five recurrent episodes of moderate hypoglycemia. The MCT control group (N=15) did not undergo moderate episodes of hypoglycemia; the MCT experimental group (N=15) received one to five bouts of moderate hypoglycemia. Five S-D rats from

the experimental group and five S-D rats from the control group were sacrificed after the first, third, and fifth moderate hypoglycemic episodes in order to perform double immunofluorescence staining of MCT1 and MCT2.

Upon their arrival, the S-D rats received five days of rest to become acclimated to their new habitat. After this rest period, the rats in the first experimental group were administered appropriate amounts of insulin to induce moderate hypoglycemia once daily for one hour. The control group was given saline injections in order to limit the confounding variable of stress due to substance administration to the rats. After each moderate episode (or saline injection), 5 rats from the first experimental group and 5 rats from the first control group were euthanized 2 hours after euglycemia was achieved for post-mortem testing of hippocampal tissue (Vavaiya and Briski, 2008). MCT transporter expression was quantified with immunohistochemistry (IHC) to measure protein expression of the MCT1 and MCT2 transporters. As the amount of moderate hypoglycemic episodes increased, we expected to see greater protein expression of MCT1 and MCT2.

Twenty additional rats were randomly assigned into a second control group (N=10) and a second experimental group (N=10). The experimental rats also underwent five days of moderate hypoglycemia while the controls received sham hypoglycemia, followed by the Elevated Plus Maze and Forced Swim Task on day six. They were then sacrificed and their brains stained with anti-MAP2 and anti-4HNE in order to assess dendritic oxidative damage (Won et al., 2012).

The Elevated Plus Maze and Forced Swim Task were used with the expectation that recurrent moderate hypoglycemia would increase anxiety and depressive-like

behavior. IHC was also used to measure the amount of dendritic oxidative damage seen in the DG (See *Assessing dendritic oxidative damage)*. As the amount of moderate hypoglycemic episodes increased, we expected to see greater amounts of damage in the hippocampus.

Assessing dendritic oxidative damage protocol

Sections of the brain were fixed with a 1:1 methanol/acetone solution and then incubated in a buffer consisting of 0.01% PBS, 0.2% Triton-X and 0.1% BSA for 30 minutes, at 4 degrees C to block and permeabilize the cells. They were then incubated for 24 hours with a 1:1,000 dilution of anti-MAP2 antibodies to stain the neurons and dendrites. Following this, the samples were incubated with a secondary IgG that was conjugated with a fluorescent dye and diluted in buffer. The sections were also stained with an antibody against 4-Hydroxy-2-nonenal (4HNE) and a different color secondary fluorophore. The production of 4HNE is indicative of oxidative damage in the cell (Suh et al., 2007).

Appendix D3: Third Iteration

Experimental Design

Thirty male S-D rats were randomly assigned into two groups to assess MCT1 and MCT2 transporter protein expression. The control group (N=15) did not undergo moderate episodes of hypoglycemia; the experimental group (N=15) received one to three bouts of moderate hypoglycemia. Five S-D rats from the experimental group and five S-D rats from the control group were sacrificed after every moderate hypoglycemic episode in order to perform double immunofluorescence staining of MCT1 and MCT2.

Upon their arrival, the S-D rats received five days of rest to become acclimated to their new habitat. After this rest period, the rats in the experimental group were administered appropriate amounts of insulin to induce moderate hypoglycemia once daily for one hour. Saline administration to the control group limited the confounding effect that results from stress induced by a substance administration to the rats. After each moderate episode (or saline injection), 5 rats from the experimental group and 5 rats from the control group were euthanized 2 hours after euglycemia was achieved for post-mortem testing of hippocampal tissue (Vavaiya and Briski, 2008). MCT transporter expression was quantified with immunohistochemistry to measure protein expression of the MCT1 and MCT2 transporters. As the amount of moderate hypoglycemic episodes increased, we expected to see greater protein expression of MCT1 and MCT2.

An additional twenty rats were randomly assigned into a second control group (N=10) and a second experimental group (N=10). The experimental rats underwent three days of moderate hypoglycemia while the controls received sham hypoglycemia, followed by the Elevated Plus Maze and Forced Swim Task on day four. After perfusion, brain samples were subjected to IHC in order to measure the amount of dendritic oxidative damage (See *Assessing dendritic oxidative damage*).

Citations

Abi-Saab WM, Maggs DG, Jones T, Jacob R, Srihari V, Thompson J, Kerr D, Leone P, Krystal JH, Spencer DD, During MJ, Sherwin RS (2002) Striking differences in glucose and lactate levels between brain extracellular fluid and plasma in conscious human subjects: effects of hyperglycemia and hypoglycemia. J Cereb Blood Flow Metab 22:271-279.

Abelaira HM, Réus GZ, Quevedo J (2013) Animal models as tools to study the pathophysiology of depression. Rev Bras Psiquiatr 35 Suppl 2:S112-120.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

American Psychiatric Association (2013) Diagnostic and Statistics Manual of Mental Disorders 5. In: (Association AP, ed): doi:10.1176/appi.books.9780890425596.dsm05.

Asvold BO, Sand T, Hestad K, Bjørgaas MR (2010) Cognitive function in type 1 diabetic adults with early exposure to severe hypoglycemia: a 16-year follow-up study. Diabetes Care 33:1945-1947.

Auer R, Kalimo H, Olsson Y, Wieloch T (1985) The dentate gyrus in hypoglycemia: pathology implicating excitotoxin-mediated neuronal necrosis. Acta Neuropathol 67:279-288.

Auer RN, Wieloch T, Olsson Y, Siesjö BK (1984) The distribution of hypoglycemic brain damage. Acta Neuropathol 64:177-191.

Bannerman DM, Grubb M, Deacon RM, Yee BK, Feldon J, Rawlins JN (2003) Ventral hippocampal lesions affect anxiety but not spatial learning. Behav Brain Res 139:197-213.

Bannerman DM, Rawlins JNP, McHugh SB, Deacon RMJ, Yee BK, Bast T, Zhang WN, Pothuizen HHJ, Feldon J (2004) Regional dissociations within the hippocampus - memory and anxiety. Neuroscience and Biobehavioral Reviews 28:273-283.

Barnard K, Thomas S, Royle P, Noyes K, Waugh N (2010) Fear of hypoglycaemia in parents of young children with type 1 diabetes: a systematic review. BMC Pediatr 10:50.

Bluestone JA, Herold K, Eisenbarth G (2010) Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 464:1293-1300.

Bogdanova OV, Kanekar S, D'Anci KE, Renshaw PF (2013) Factors influencing behavior in the forced swim test. Physiol Behav 118:227-239.

Borsini F, Bendotti C, Velkov V, Rech R, Samanin R (1981) Immobility test: effects of 5-hydroxytryptaminergic drugs and role of catecholamines in the activity of some antidepressants. JPHP Journal of Pharmacy and Pharmacology 33:33-37.

Bremner JD, Narayan M, Anderson ER, Staib LH, Miller HL, Charney DS (2000) Hippocampal volume reduction in major depression. Am J Psychiatry 157:115-118.

Brooks GA (1986) Lactate production under fully aerobic conditions: the lactate shuttle during rest and exercise. Fed Proc 45:2924-2929.

Brooks GA, Dubouchaud H, Brown M, Sicurello JP, Butz CE (1999) Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. Proc Natl Acad Sci U S A 96:1129-1134.

Burzio LO, Riquelme PT, Koide SS (1979) ADP ribosylation of rat liver nucleosomal core histones. J Biol Chem 254:3029-3037.

Böhme P, Bertin E, Cosson E, Chevalier N, group G (2013) Fear of hypoglycaemia in patients with type 1 diabetes: do patients and diabetologists feel the same way? Diabetes Metab 39:63-70.

Cayen GaK (1972) Effect of diurnal rhythm and food withdrawal on serum lipid levels in the rat. 28:502-503.

Choi BY, Kim JH, Kim HJ, Yoo JH, Song HK, Sohn M, Won SJ, Suh SW (2013) Pyruvate administration reduces recurrent/moderate hypoglycemia-induced cortical neuron death in diabetic rats. PLoS One 8:e81523.

Council NR (2011) Guide for the Care and Use of Laboratory Animals. In, 8th Edition, p 246. Washington, D.C.: The National Academies Press.

Czéh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, Bartolomucci A, Fuchs E (2001) Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. Proc Natl Acad Sci U S A 98:12796-12801.

Dave KR, Tamariz J, Desai KM, Brand FJ, Liu A, Saul I, Bhattacharya SK, Pileggi A (2011) Recurrent hypoglycemia exacerbates cerebral ischemic damage in streptozotocin-induced diabetic rats. Stroke 42:1404-1411.

Davis EA, Keating B, Byrne GC, Russell M, Jones TW (1998) Impact of improved glycaemic control on rates of hypoglycaemia in insulin dependent diabetes mellitus. Arch Dis Child 78:111-115.

De Feyter HM, Mason GF, Shulman GI, Rothman DL, Petersen KF (2013) Increased brain lactate concentrations without increased lactate oxidation during hypoglycemia in type 1 diabetic individuals. Diabetes 62:3075-3080.

Debernardi R, Pierre K, Lengacher S, Magistretti PJ, Pellerin L (2003) Cell-specific expression pattern of monocarboxylate transporters in astrocytes and neurons observed in different mouse brain cortical cell cultures. J Neurosci Res 73:141-155.

Dhingra V, Crampton L, Cook D, Finfer S, Bellomo R, Chittock D (2009) Intensive versus conventional glucose control in critically ill patients. New England Journal of Medicine 360:1283-1297.

Diggs-Andrews KA, Zhang X, Song Z, Daphna-Iken D, Routh VH, Fisher SJ (2010) Brain insulin action regulates hypothalamic glucose sensing and the counterregulatory response to hypoglycemia. Diabetes 59:2271-2280.

Fanselow MS, Dong HW (2010) Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65:7-19.

Frodl T, Schaub A, Banac S, Charypar M, Jäger M, Kümmler P, Bottlender R, Zetzsche T, Born C, Leinsinger G, Reiser M, Möller HJ, Meisenzahl EM (2006) Reduced hippocampal volume correlates with executive dysfunctioning in major depression. J Psychiatry Neurosci 31:316-323.

Fujioka M, Okuchi K, Hiramatsu KI, Sakaki T, Sakaguchi S, Ishii Y (1997) Specific changes in human brain after hypoglycemic injury. Stroke 28:584-587.

Garcia CK, Brown MS, Pathak RK, Goldstein JL (1995) cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1. J Biol Chem 270:1843-1849.

Garcia CK, Goldstein JL, Pathak RK, Anderson RG, Brown MS (1994) Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. Cell 76:865-873.

Giannone PJ, Alcamo AA, Schanbacher BL, Nankervis CA, Besner GE, Bauer JA (2011) Poly(ADP-ribose) polymerase-1: a novel therapeutic target in necrotizing enterocolitis. Pediatr Res 70:67-71.

Girotti M1 PT, Gaylord RI, Rubin BA, Herman JP, Spencer RL. (2006) Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. Neuroscience 4:1067-1081.

Gould E, Cameron H, Woodley C, McEwen B, Daniels D (1992) Adrenal hormones suppress cell division in the adult rat dentate gyrus. The Journal of Neuroscience 12:3642-3650.

Gruetter R, Ugurbil K, Seaquist ER (1998) Steady-state cerebral glucose concentrations and transport in the human brain. J Neurochem 70:397-408.

Halestrap AP, Price NT (1999) The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem J 343 Pt 2:281-299.

Halestrap AP, Meredith D (2004) The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 447:619-628.

Henderson GC, Horning MA, Lehman SL, Wolfel EE, Bergman BC, Brooks GA (2004) Pyruvate shuttling during rest and exercise before and after endurance training in men. J Appl Physiol (1985) 97:317-325.

Henning SJ, Gisel EG (1980) Nocturnal feeding behavior in the neonatal rat. 25 603-605.

Herzog RI, Chan O, Yu S, Dziura J, McNay EC, Sherwin RS (2008) Effect of acute and recurrent hypoglycemia on changes in brain glycogen concentration. Endocrinology 149:1499-1504.

Herzog RI, Jiang L, Herman P, Zhao C, Sanganahalli BG, Mason GF, Hyder F, Rothman DL, Sherwin RS, Behar KL (2013) Lactate preserves neuronal metabolism and function following antecedent recurrent hypoglycemia. J Clin Invest 123:1988-1998.

Hoyer S, Lannert H (2008) Long-term effects of corticosterone on behavior, oxidative and energy metabolism of parietotemporal cerebral cortex and hippocampus of rats: comparison to intracerebroventricular streptozotocin. Journal of Neural Transmission 115:1241-1249.

Jacobs BL, van Praag H, Gage FH (2000) Adult brain neurogenesis and psychiatry: a novel theory of depression. Mol Psychiatry 5:262-269.

JDRF (2014) Insulin Delivery Methods. In. Juvenile Diabetes Research Foundation.

Jiang L, Herzog RI, Mason GF, de Graaf R, Rothman D, Sherwin RS, Behar KL (2009) Recurrent antecedent hypoglycemia alters neuronal oxidative metabolism in vivo. Diabetes 58.

Jones K (2012) Tail Vein Injections in the Mouse and Rat SOP. In. UBC Animal Care Guidelines.

Kalimo H, Olsson Y (1980) Effects of severe hypoglycemia on the human brain. Neuropathological case reports. Acta Neurol Scand 62:345-356.

Keller J, Shen L, Gomez RG, Garrett A, Solvason HB, Reiss A, Schatzberg AF (2008) Hippocampal and amygdalar volumes in psychotic and nonpsychotic unipolar depression. Am J Psychiatry 165:872-880.

Kessler R, Birnbaum H, Demler O, Falloon I, Gagnon E, Guyer M (2010) The prevalence and correlates of nonaffective psychosis inthe National Comorbidity Survey Replication (NCS-R). Biological Psychiatry 58:668-676.

Kjelstrup KG, Tuvnes FA, Steffenach HA, Murison R, Moser EI, Moser MB (2002) Reduced fear expression after lesions of the ventral hippocampus. Proc Natl Acad Sci U S A 99:10825-10830.

Krolewski AS, Kosinski EJ, Warram JH, Leland OS, Busick EJ, Asmal AC, Rand LI, Christlieb AR, Bradley RF, Kahn CR (1987) Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. Am J Cardiol 59:750-755.

Kronmüller KT, Pantel J, Köhler S, Victor D, Giesel F, Magnotta VA, Mundt C, Essig M, Schröder J (2008) Hippocampal volume and 2-year outcome in depression. Br J Psychiatry 192:472-473.

Lager I, Attvall S, Blohmé G, Smith U (1986) Altered recognition of hypoglycaemic symptoms in type I diabetes during intensified control with continuous subcutaneous insulin infusion. Diabet Med 3:322-325.

Lawlor M (2002) Comfortable quarters for rats in research institutions. In: Comfortable Quarters for Laboratory Animals, 9th Edition (Reinhardt V, Reinhardt A, eds), pp 26-32. Washington, D.C.: Animal Welfare Institute.

Like AA, Rossini AA (1976) Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. Science 193:415-417.

Lipska KJ, Ross JS, Wang Y, Inzucchi SE, Minges K, Karter AJ, Huang ES, Desai MM, Gill TM, Krumholz HM (2014) National trends in US hospital admissions for hyperglycemia and hypoglycemia among Medicare beneficiaries, 1999 to 2011. JAMA Intern Med 174:1116-1124.

MacMaster FP, Carrey N, Langevin LM, Jaworska N, Crawford S (2014) Disorder-specific volumetric brain difference in adolescent major depressive disorder and bipolar depression. Brain Imaging Behav 8:119-127.

Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y (1980) Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 29:1-13.

Marcondes FK, Miguel KJ, Melo LL, Spadari-Bratfisch RC (2001) Estrous cycle influences the response of female rats in the elevated plus-maze test. Physiology & Behavior 74:435-440.

McGovern AP, Rusholme B, Jones S, van Vlyman JN, Liyanage H, Gallagher H, Tomson CR, Khunti K, Harris K, de Lusignan S (2013) Association of chronic kidney disease (CKD) and failure to monitor renal function with adverse outcomes in people with diabetes: A primary care cohort study. BMC Nephrol 14:198.

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

Montaron MF, Drapeau E, Dupret D, Kitchener P, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2006) Lifelong corticosterone level determines age-related decline in neurogenesis and memory. Neurobiol Aging 27:645-654.

Moore H, Craft TK, Grimaldi LM, Babic B, Brunelli SA, Vannucci SJ (2010) Moderate recurrent hypoglycemia during early development leads to persistent changes in affective behavior in the rat. Brain Behav Immun 24:839-849.

Moser MB, Moser EI, Forrest E, Andersen P, Morris RG (1995) Spatial learning with a minislab in the dorsal hippocampus. Proc Natl Acad Sci U S A 92:9697-9701.

Nakhooda AF, Like AA, Chappel CI, Murray FT, Marliss EB (1977) The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. Diabetes 26:100-112.

Park M, Yoo S, Choe B, Dantzer R, Freund G (2012) Acute hypoglycemia causes depressive-like behaviors in mice. Metabolism 61:229-236.

Park MJ, Guest CB, Barnes MB, Martin J, Ahmad U, York JM, Freund GG (2008) Blocking of beta-2 adrenergic receptors hastens recovery from hypoglycemia-associated social withdrawal. Psychoneuroendocrinology 33:1411-1418.

Pellerin L, Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A 91:10625-10629.

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. Journal of Neuroscience Methods 14:149-167.

Penckofer S, Quinn L, Byrn M, Ferrans C, Miller M, Strange P (2012) Does glycemic variability impact mood and quality of life? Diabetes Technol Ther 14:303-310.

Pfeuffer J, Tkác I, Gruetter R (2000) Extracellular-intracellular distribution of glucose and lactate in the rat brain assessed noninvasively by diffusion-weighted 1H nuclear magnetic resonance spectroscopy in vivo. J Cereb Blood Flow Metab 20:736-746.

Porsolt RD, Bertin A, Blavet N, Deniel M, Jalfre M (1979) Immobility induced by forced swimming in rats: effects of agents which modify central catecholamine and serotonin activity. Eur J Pharmacol 57:201-210.

Przegalinski E, Bigajska K, Siwanowicz J (1980) Psychopharmacological profile of dexamisole. Polish journal of pharmacology and pharmacy 32.

Puente EC, Silverstein J, Bree AJ, Musikantow DR, Wozniak DF, Maloney S, Daphna-Iken D, Fisher SJ (2010) Recurrent moderate hypoglycemia ameliorates brain damage and cognitive dysfunction induced by severe hypoglycemia. Diabetes 59:1055-1062.

Purves D, Augustine GJ, Fitzpatrick D, Hall WC, LaMantia S, White LE (2011) Neuroscience, 5 Edition: Sinauer Associates, Inc.

Ramon y Cajal S (1901) Estudios sobre la corteza cerebral humana. Trab Inst Cajal Invest Biol 1:1-227.

Regier DA, Rae DS, Narrow WE, Kaelber CT, Schatzberg AF (1998) Prevalence of anxiety disorders and their comorbidity with mood and addictive disorders. Br J Psychiatry Suppl:24-28.

Revest JM, Dupret D, Koehl M, Funk-Reiter C, Grosjean N, Piazza PV, Abrous DN (2009) Adult hippocampal neurogenesis is involved in anxiety-related behaviors. Mol Psychiatry 14:959-967.

Rusch BD, Abercrombie HC, Oakes TR, Schaefer SM, Davidson RJ (2001) Hippocampal morphometry in depressed patients and control subjects: relations to anxiety symptoms. Biol Psychiatry 50:960-964.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

Seminowicz DA, Mayberg HS, McIntosh AR, Goldapple K, Kennedy S, Segal Z, Rafi-Tari S (2004) Limbic-frontal circuitry in major depression: a path modeling metanalysis. Neuroimage 22:409-418.

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron Ha (2011a) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour Nature 476:458-461.

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011b) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature 476:458-461.

Solli O, Stavem K, Kristiansen IS (2010) Health-related quality of life in diabetes: The associations of complications with EQ-5D scores. Health Qual Life Outcomes 8:18.

Strachan MW, Deary IJ, Ewing FM, Frier BM (2000) Recovery of cognitive function and mood after severe hypoglycemia in adults with insulin-treated diabetes. Diabetes Care 23:305-312.

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

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

Suh SW, Bergher JP, Anderson CM, Treadway JL, Fosgerau K, A. SR (2007b) Astrocyte glycogen sustains neuronal activity during hypoglycemia: studies with the glycogen phosphorylase inhibitor CP-316,819 ([R-R*,S*]-5-chloro-N-[2-hydroxy-3-(methoxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-indole-2-carboxamide). Journal of Pharmacology and Experimental Therapeutics 321:45-50.

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

Suh SW, Fan Y, Hong SM, Liu Z, Matsumori Y, Weinstein PR, Swanson RA, Liu J (2005b) Hypoglycemia induces transient neurogenesis and subsequent progenitor cell loss in the rat hippocampus. Diabetes 54:500-509.

The Diabetes Control and Complications Trial Research Group (DCCT) (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes melitus. N Engl J Med 329: 977-86

Thomas RM, Peterson DA (2003) A neurogenic theory of depression gains momentum. Mol Interv 3:441-444.

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

Towler DA, Havlin CE, Craft S, Cryer P (1993) Mechanism of awareness of hypoglycemia. Perception of neurogenic (predominantly cholinergic) rather than neuroglycopenic symptoms. Diabetes 42:1791-1798.

Trivedi MA, Coover GD (2004) Lesions of the ventral hippocampus, but not the dorsal hippocampus, impair conditioned fear expression and inhibitory avoidance on the elevated T-maze. Neurobiol Learn Mem 81:172-184.

Turner RC, Cull CA, Frighi V, Holman RR (1999) Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Group. JAMA 281:2005-2012.

Ulrich RE, Azrin NH (1962) Reflexive fighting in response to aversive stimulation. J Exp Anal Behav 5:511-520.

Vavaiya KV, Briski KP (2008a) Effects of caudal fourth ventricular lactate infusion on hypoglycemia-associated MCT2, GLUT3, GLUT4, GCK, and sulfonylurea receptor-1 gene expression in the ovariectomized female rat LHA and VMH: impact of estradiol. J Mol Neurosci 34:121-129.

Vavaiya KV, Briski KP (2008b) Effects of caudal hindbrain lactate infusion on insulin-induced hypoglycemia and neuronal substrate transporter glucokinase and sulfonylurea receptor-1 gene expression in the ovariectomized female rat dorsal vagal complex: Impact of estradiol. J Neurosci Res 86:694-701.

Vriesendorp TM, DeVries JH, van Santen S, Moeniralam HS, de Jonge E, Roos YB, Schultz MJ, Rosendaal FR, Hoekstra JB (2006) Evaluation of short-term consequences of hypoglycemia in an intensive care unit. Crit Care Med 34:2714-2718.

Walf AA, Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc 2:322-328.

Won SJ, Jang BG, Yoo BH, Sohn M, Lee MW, Choi BY, Kim JH, Song HK, Suh SW (2012a) Prevention of acute/severe hypoglycemia-induced neuron death by lactate administration. J Cereb Blood Flow Metab 32:1086-1096.

Won SJ, Yoo BH, Kauppinen TM, Choi BY, Kim JH, Jang BG, Lee MW, Sohn M, Liu J, Swanson RA, Suh SW (2012b) Recurrent/moderate hypoglycemia induces hippocampal dendritic injury, microglial activation, and cognitive impairment in diabetic rats. Journal of Neuroinflammation 9:182.

Zhou D, Qian J, Chang H, Xi B, Sun RP (2012) Pyruvate administered to newborn rats with insulin-induced hypoglycemic brain injury reduces neuronal death and cognitive impairment. European Journal of Pediatrics 171:103-109.