OBERLIN II, DOUGLAS J., Ph.D. Neither Recurrent Hypoglycemia nor Chronic Aerobic Training Alter the Content of MCTs in the Ventromedial Hypothalamus (2016) Directed by Dr. Laurie Wideman 144pp.

Many individuals with diabetes use medications or exercise to control blood glucose concentrations, which can lead to episodes of hypoglycemia. Although chronic hyperglycemia leads to many diabetic complications, hypoglycemia is an acute threat to the health of individuals, and can lead to myocardial ischemia and arrhythmias, as well as increasing inflammation, oxidative stress, and thrombotic and fibrinolytic processes. Either antecedent exercise or antecedent hypoglycemia lead to a blunted counter-regulatory response to a subsequent hypoglycemia episode. Acute exercise has been shown to increase monocarboxylate transport proteins (MCTs) in the ventromedial hypothalamus (VMH) of the brain, which is involved in regulating the counter-regulatory response to restore euglycemia. The MCTs shuttle lactate in and out of cells, however when is lactate infused into the VMH has been shown to interfere with the counter-regulatory response. Additionally, antecedent recurrent hypoglycemia has been shown to increase lactate transport in the brain. Therefore, the current studies investigated what effect exercise training or recurrent antecedent hypoglycemia had on MCT proteins in the VMH. Adult male Sprague-Dawley rats were used for both studies, randomized to receive either 6-7 weeks of aerobic training, sedentary behavior, 3 days of insulin induced hypoglycemia, or 3 days of saline injection. The increases in cytochrome c oxidase activity among the aerobically trained group showed that training adaptations occurred, however, there were no significant differences in MCT proteins within the VMH between the trained versus sedentary rats. While each of the 3 days of

hypoglycemia or saline injection showed differences in 30 minute post-injection glucose concentrations, no significant differences in MCTs were observed in the VMH between the 2 groups on day 4.

NEITHER RECURRENT HYPOGLYCEMIA NOR CHRONIC AEROBIC TRAINING ALTER THE CONTENT OF MCTS IN THE VENTROMEDIAL HYPOTHALAMUS

by

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A Dissertation Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
2016

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APPROVAL PAGE

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ACKNOWLEDGEMENTS

I would like to thank Dr. Wideman, Dr. Beverly, Dr. Starnes, Dr. Morrison, and Dr. Goldfarb for their help and guidance throughout my Ph.D. and my dissertation project. I would also like to thank the other graduate students and staff who helped me complete this project, including: Anthony Boccine, Peter Christopher, Paula Cooney, Mariel Fecych, Mary Martinez, Coleman Murray, Charles Park, Vince Porcelli, and Lauren Vervaecke.

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LIST OF ABBREVIATIONS

4CIN……...…..α-cyano-4-hydroxyCINnamate

Akt…….....….protein kinase B

AMPA……. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMP……..….Adenosine MonoPhosphate

AMPK……...…AMP activated protein Kinase

ATP…….Adenosine TriPhosphate

ANLS…….Astrocyte to Neuron Lactate Shuttle

cAMP……..…cyclic Adenosine MonoPhosphate

CFTR…….……Cystic Fibrosis Transmembrane conductance Regulator

CNS…….Central Nervous System

CREB……..cAMP Response Element Binding protein

CRTC2……. CREB Regulated Transcription Coactivator 2

DM…….Diabetes Mellitus

DTT…….…. DiThioThreitol

ECL……...… Enhanced ChemiLuminescence

fMRI…….. functional Magnetic Resonance Imaging

FOXO1…… FOrkhead boX protein O1

G6Pc…….…..Glucose 6 Phosphatase catalytic

GABA…….....… Gamma-AminoButyric Acid

GAPDH……..…..GlycerAldehyde 3 Phosphate DeHydrogenase

GE neurons……..…Glucose Excited neurons

GI neurons……..….Glucose Inhibited neurons

GluR……..…….Glutamate Receptor

GLUT……..…..GLUcose Transport protein

GSK……..…..Glycogen Synthase Kinase

HAAF……...Hypoglycemia Associated Autonomic Failure

HPLC……....High Performance Liquid Chromatography

IDDM…….….Insulin Dependent Diabetes Mellitus

K_{ATP}……..ATP sensitive potassium channel

MCT…….....MonoCarboxylate Transport protein

PDK……. Phosphoinositide Dependent protein Kinase-1

PEPCK……..PhophoEnolPyruvate CarboxyKinase

PET……..Positron Emission Tomography

PI3K……..…PhosphoInositol 3 Kinase

PIP2……..… PhosphatidylInositol 4,5-bisPhosphate

PIP3…….….. PhosphatidylInositol (3,4,5)-trisPhosphate

PKA……Protein Kinase A

PVDF…… PolyVinyliDene Fluoride

SIK…….…. Salt-Induced Kinases

T1D …….Type 1 Diabetes Mellitus

T2D……...….Type 2 Diabetes Mellitus

TBST…….…..Tris Buffered Saline with Tween*20

TNFα……..….Tumor Necrosis Factor Alpha

VMH……...VentroMedial Hypothalamus

CHAPTER I

INTRODUCTION

Specific Aims

The regulation of blood glucose concentrations is required for the maintenance of life and for the overall health of an individual. The ability of the brain to sense the level of glucose within the body is paramount to survival. Glucose sensing neurons are found throughout the brain, including in the hindbrain and hypothalamus, specifically the ventromedial hypothalamus (VMH). Blood glucose levels above (5.5 mmol/l) or below (3.9 mmol/l) the normal range (3.9 – 5.5 mmol/l), initiates a plethora of responses that when functioning properly, result in the re-establishment of homeostasis in blood glucose concentrations. While blood glucose concentrations fluctuate throughout the day (~3 – 10 mmol/l), the ability of the body to respond to these fluctuations and normalize glucose concentrations is considered a fundamental physiological process.

The hallmark of diabetes mellitus, either type 1 (T1D) or type 2 (T2D), is the inability to maintain blood glucose in the normal healthy range. Over the past 60 years, the number of individuals with diagnosed diabetes in the United States has risen from 1.5 million to about 21 million people, however the number, including those undiagnosed, may be as high as 29.1 million [1,2]. Euglycemia is the goal of many diabetic treatments, however addressing the rises and falls in glucose throughout the day can be difficult.

Exercise is commonly recommended for individuals with diabetes (particularly T2D) to help manage hyperglycemia. However, when exercise is combined with medications that help lower blood glucose, it can lead to hypoglycemia, a more immediate medical risk. In addition, many individuals with both T1D and T2D use exogenous insulin to regulate their blood glucose. Insulin, while effective at reducing blood glucose concentrations, must be carefully dosed to avoid hypoglycemia.

Hyperglycemia results in increased vascular dysfunction which contributes to retinopathy, neuropathy, and nephropathy often observed in diabetic patients, but it also poses long term health risks that may lead to increased risk of cardiovascular disease and cancer [3-10]. These adverse long-term health consequences of hyperglycemia are the impetus for recommendations related to stringent blood glucose control within diabetic individuals by way of lifestyle modifications (exercise and/or diet), various oral medications (i.e. sulphonylureas or glucophage) or with exogenous insulin. Unfortunately, intensive treatment focused on avoidance of hyperglycemia, particularly with insulin and sulphonylureas often leads to increased rates of hypoglycemic events[11,12]. Hypoglycemia is a more urgent, acute threat to the health of individuals with diabetes. Hypoglycemic episodes can lead to myocardial ischemia and arrhythmias, as well as increasing inflammation, oxidative stress, and thrombotic and fibrinolytic processes [11,12]. Therefore, while long-term regulation of hyperglycemia is important, recurring incidents of hypoglycemia can pose a more immediate health risk.

The dangers of hypoglycemia are compounded by the effect that an antecedent episode of hypoglycemia can have on a subsequent occurrence. It has been shown that

individuals who are exposed to repeated bouts of hypoglycemia have a blunted counter-regulatory response to restore euglycemia[13]. This cycle of hypoglycemia leading to inhibited responses to subsequent hypoglycemia, as well as reduced awareness of hypoglycemia, is often called hypoglycemia associated autonomic failure (HAAF). The prevalence of autonomic failure among individuals with diabetes has been estimated around 25% of insulin treated individuals with diabetes[14]. However, studies that examine standard treatment compared to more intense treatment (earlier and/or greater insulin and oral anti-hyperglycemic medications) of diabetes show that more intense treatment often leads to increased risk of hypoglycemia, which may lead to HAAF [11]. The long-term consequences appear to be a depressed counter-regulatory response to hypoglycemia and a decreased sensation of hypoglycemic symptoms. This puts individuals with insulin dependent diabetes mellitus (IDDM) at an even greater risk when they are trying to maintain tight control over their blood glucose concentrations, regardless of whether they originally developed T1D or T2D [15,16].

Interestingly, the HAAF response to hypoglycemia has also been observed when an incident of hypoglycemia follows an exercise session [17-19]. The blunted counter-regulatory response observed in autonomic failure has also been observed up to 24 hours after acute exercise. Although those who have an antecedent exercise induced HAAF response and those who have antecedent hypoglycemia induced HAAF response display common symptoms, it is unclear if the underlying mechanism in the HAAF response after antecedent exercise is the same as the mechanisms resulting from antecedent recurrent insulin induced hypoglycemia. Recent studies have shown that there may also

be exercise-induced adaptations in the brain area associated with glucose sensing [20,21]. A recent study by Takimoto et al. showed that a single bout of exercise in rats was sufficient to increase monocarboxylate transport proteins (MCTs) in several brain areas, including the hypothalamus. This is consistent with observations of acute MCT up-regulation in rat muscle after exercise [22]. These MCTs transport lactate (as well as other monocarboxylates) into, out of, or between cells within the brain and other tissues, and may be related to changes in lactate concentrations in the ventromedial hypothalamus, a region known to be involved with regulation of blood glucose concentrations. Other studies have shown that antecedent recurrent hypoglycemia can lead to increased lactate transport in the brain [23]. While it is unknown if these two adaptations have similar mechanisms, microdialysis infusion of lactate has been shown to suppress the counter-regulatory response to insulin induced hypoglycemia, similar to what is seen with HAAF [24]. This has also been shown with more physiological infusion of lactate into the VMH [25]. In either case, there is decreased sympathetic output, and reduced glucagon and catecholamines, despite hypoglycemia [24,25]. Additionally, MCT inhibition (in the ventromedial hypothalamus (VMH)) was able to rescue the counter-regulatory response, suggesting that lactate may play some role in HAAF [24].

Therefore, the overall objective of the current study was to investigate what effect exercise training or recurrent hypoglycemia has on MCT protein content within the VMH . Our approach will be to compare the MCTs in; 1) exercise trained rats or sedentary rats and 2) rats exposed to antecedent recurrent hypoglycemia or euglycemia. Our central hypothesis is that aerobic training as well as recurrent hypoglycemia will

increase MCT proteins within the VMHs of trained or recurrently hypoglycemic animals. Our rationale for investigating the changes in MCTs is to: 1) attempt to find if this is part of a unifying mechanism for antecedent exercise and antecedent recurrent hypoglycemia induced HAAF, 2) increase information related to two common treatments for individuals with diabetes, and 3) identify potential targets for management of HAAF to allow for tight control of blood glucose concentrations in individuals with IDDM.

Specific Aim 1) Determine if there is a cumulative effect of exercise training on MCT protein content in the VMH by comparing 6-7 weeks of exercise training to sedentary rats (part of another ongoing study).

Hypothesis 1; We hypothesize that the amount of MCT2 and MCT4 will be greater in rats trained 6-7 weeks compared to sedentary rats.

Rationale; studies have shown that, in addition to acute treadmill running, 3 weeks of aerobic training on a treadmill increased MCT content in skeletal muscle [26,27]. In addition, treadmill running has been shown to increase MCTs in many brain areas [20,21].

Specific Aim 2a) Determine if 3 days of insulin induced hypoglycemia leads to increases in MCT 1, 2, or 4 in the ventromedial hypothalamus compared to control rats.

Hypothesis 2a; It was-expected that elevated levels of MCT 2 and 4 protein in the VMH would occur to recurrently hypoglycemic rats to a greater extend compared to the control rats.

Rationale; MCTs have been reported to be approximately doubled in diabetic rats which also show signs of HAAF compared to control rats [20]. We also expected this increase because 1) lactate can be used to sustain neurons under glucoprivic conditions,

2) lactate has been shown to blunt the counter-regulatory response when injected, and 3) because antecedent recurrent hypoglycemia has been shown to lead to increased lactate transport in the brain as well as HAAF [23,24,28]. Therefore, we expect that the lactate transport proteins will increase in content to facilitate increased demand for lactate shuttling, and will be associated with HAAF.

Specific Aim 2b) Confirm a reduced counter-regulatory response to antecedent recurrent insulin induced hypoglycemia among the treated rats compared to control rats.

Hypothesis 2b; It was hypothesized that the recurrently hypoglycemic rats will have an impaired counter-regulatory response compared to control rats.

Rationale; It was expected that recurrently hypoglycemic rats will have depressed epinephrine, and possibly glucagon response to insulin induced hypoglycemia compared with the control rats which has already been confirmed by several studies [23,29,30].

Significance

While rates of diabetes are increasing in the United States and globally, our current methods of treatment are not sufficient to reverse or even prevent the progression of the disease and its associated morbidities [11]. This is in part due to the inability to consistently reduce blood glucose concentrations to healthy levels without fear of inducing hypoglycemia. While T1D and T2D develop along fundamentally different pathophysiological pathways, they share several key issues, including the inability to aggressively manage blood glucose concentrations for fear of inducing potentially fatal hypoglycemic events. In both diabetic conditions, exercise and anti-hyperglycemic medications are used to manage blood glucose; however both treatments, under certain

circumstances, lead to hypoglycemia. In addition, they both may lead to subsequent impaired counter-regulatory response to hypoglycemia. This can become a vicious cycle, avoidable only by applying a less aggressive treatment paradigm. This study is significant because it ties together multiple independent areas of research pertaining to hypoglycemia, hypoglycemia associated autonomic failure (HAAF), and brain metabolism in an attempt to disentangle the underlying mechanisms relating to these two common diabetes therapies.

This research fills a gap in the current literature because it examines MCT protein content changes specifically within the VMH to both antecedent exercise and hypoglycemia. Although antecedent exercise and antecedent hypoglycemia may share characteristic depression of the counter-regulatory response to subsequent hypoglycemia, previous research has failed to recognize the potential common mechanisms and the research areas have remained independent. We will compare changes in MCT protein content in the VMH, which may partially moderate dysfunction in the counter-regulatory response. This has not been compared between antecedent exercise and hypoglycemia, and to our knowledge has not been examined at all in the recurrent hypoglycemia literature. While both recurrent hypoglycemia and antecedent exercise have been shown to lead to impairment of the counter-regulatory response, it is unknown whether the changes seen in the MCTs in the VMH are the same. Therefore as a starting point, we may be able to reveal a convergence point through which two different diabetes treatments affect subsequent autonomic failure/dysfunction. This may lead to future studies to develop strategies to prevent this dysfunction. If the risk of autonomic

failure/dysfunction were minimized or removed, individuals with diabetes could be treated more aggressively, with the ultimate goal to slow the progression of their disease and reduce the risk of co-morbidities by more tightly controlling blood glucose concentrations without fear of hypoglycemia and HAAF.

CHAPTER II

REVIEW OF LITERATURE

Overview

This review of the relevant literature will focus on several research areas related to the maintenance of blood glucose in healthy individuals compared to those with diabetes, including the normal mechanisms of blood glucose management, the counter-regulatory mechanisms used to respond to hypoglycemia, mechanisms within the central nervous system which help monitor glucose levels, response to changes in glucose and consideration of ways in which these mechanisms can be disrupted, including exercise and hypoglycemia. A detailed discussion of the astrocyte to neuron lactate shuttle (ANLS) hypothesis, and the lactate shuttling proteins (MCTs) will be included. Finally, the effects of exercise or hypoglycemia on MCTs will be discussed in both skeletal muscles as well as in brain. This review attempts to join together research from two separate areas; while both antecedent exercise and antecedent hypoglycemia have been examined, the former area focuses on the hormonal response systemically and the latter focuses on the changes in the CNS. In this review both will be discussed in an attempt to show that these different perturbations may be leading to similar changes both hormonally and within the brain.

Regulation of Blood Glucose Concentration

In healthy individuals blood glucose concentration is maintained within healthy parameters by several systems. Two very important organs involved in the regulation of blood glucose are the pancreas and the liver. Pancreatic islets of Langerhans are clusters of endocrine cells that secrete different hormones depending upon the metabolic condition of the body, as well as in response to neural stimulation. These clusters of cells include beta-cells, alpha-cells, delta-cells, pp cells, and epsilon-cells. Of these, alpha and beta cells account for the majority of cells within the pancreatic islets. The liver has the ability to not only store glucose as glycogen, but to release this glucose into the systemic circulation if blood glucose concentrations dip. These two organs work in concert, along with the CNS, to maintain circulating blood glucose concentrations.

Post-prandial Regulation of Glucose Concentration; After a meal is consumed, blood glucose concentrations are high, and need to be brought back to the normal physiologic range. The pancreatic beta-cells are the cells responsible for regulating blood glucose post-prandially, when blood glucose concentrations are high. Pancreatic beta-cells express both GLUT 1 and GLUT 3 glucose transport proteins [31]. These proteins allow blood glucose to enter the beta-cells where it can be used to produce ATP via aerobic glycolysis. Glucose that enters the cell must be phosphorylated before it can enter glycolysis. Pancreatic beta-cells express glucokinase (hexokinase IV), rather than hexokinase I and hexokinase II, which have lower kms for glucose by comparison [32,33]. This allows glucose to move into the cell easily, but limits movement into glycolysis at a high rate unless glucose concentration increases [32,33]. As more ATP is produced, the

ATP:AMP ratio is altered and ATP sensitive potassium channels (K_{ATP}) are stimulated to close, preventing potassium export from the cell. This results in a rise in the local membrane potential, which stimulates voltage gated calcium channels to open and allows calcium into the cell. The influx of calcium stimulates insulin-containing vesicles to translocate to the cell surface and release their contents into circulation. This chain of events leads to increases in blood insulin concentration after a meal is consumed.

Insulin has many actions throughout the body, although the most well-known is its role in regulating blood glucose (Figure 1). Insulin exerts its glucose regulatory effects in skeletal muscle, liver, pancreas, and adipose. In the post-prandial state, insulin binds to its receptor located on the cell membrane of a tissue (ie. skeletal muscle), to enable the tissue to take glucose from the circulation. When bound to the receptor in skeletal muscle, insulin stimulates the insulin receptor substrate (IRS) to phosphorylate phosphatidylinositol 3 kinase (PI3K), which converts phosphatidylinositol 2 phosphate (PIP2) to phosphatidylinositol 3 phosphate (PIP3) so that phosphoinositide-dependent kinase 1 (PDK) can dock on the intracellular portion of the membrane. PDK then interacts with protein kinase B (Akt), leading to inhibition of the Akt substrate of 160kDa (AS160, also known as TBC1D4). This pathway results in the translocation of intracellular GLUT 4 vesicles to the cell surface, increasing GLUT 4 protein at the cell membrane, and ultimately, increasing the movement of glucose down its concentration gradient in to the cell. While liver and adipose may also take up glucose, skeletal muscle is the primary "sink" for glucose in the post-prandial state.

The other major tissue where insulin exerts its effects is the liver. When insulin binds to its receptor in the liver, it stimulates the synthesis of glycogen. The insulin signaling pathway in hepatocytes is similar to that in skeletal muscle. However, the primary action from Akt is not on TBC1D4, but on glycogen synthase kinase (GSK). Once GSK is phosphorylated, it is in the inhibited form and decreases the rate of phosphorylating glycogen, thus allowing glycogen synthase to become active. Glycogen synthase promotes the formation of glycogen, the primary storage form of carbohydrate. Glycogen will remain stored in the hepatocytes until there is a need to increase blood glucose concentrations in the body, such as during a fast and/or with exercise, to maintain blood glucose concentrations.

Adipose tissue also responds to insulin stimulation, leading to glucose uptake and inhibition of lipolysis [34-36]. However, adipose tissue signals also plays a role in the regulation of insulin sensitivity, and the control of chronic blood glucose concentrations [34]. Adipose tissue, as well as the associated M1 macrophages, can secrete many different adipokines which influence metabolism and insulin signaling [34]. Certain adipokines may lead to a pro-inflammatory state and interfere directly or indirectly with insulin signaling in other tissues [34,35]. The chronic increase in TNF-α, IL-6, resistin, and leptin seen with obesity (often preceding T2D) can lead to serine phosphorylation of IRS1/2, decreased mRNA for adiponectin, upregulation of SOCS3, and activation of JNK and IKKβ signaling pathways [34,35]. All of these changes can lead to a reduced sensitivity to insulin signaling[34,37]. Regardless of adipokines or body mass, increased lipolysis can lead to reduced ability of insulin to properly reduce blood glucose concentrations [36]. This may

be related to the proportion of glucose to fat being used for oxidation, or potentially the buildup of intramyocellular or intrahepatic lipids [34,36,38,39]. However, because adipose tissue and adiposity affect the regulation of blood glucose concentrations over long periods of time, rather than in response to feeding or fasting, it will not be discussed further. Although it should be noted that body mass and adiposity do affect the ability of other systems to regulate blood glucose concentrations when interpreting data.

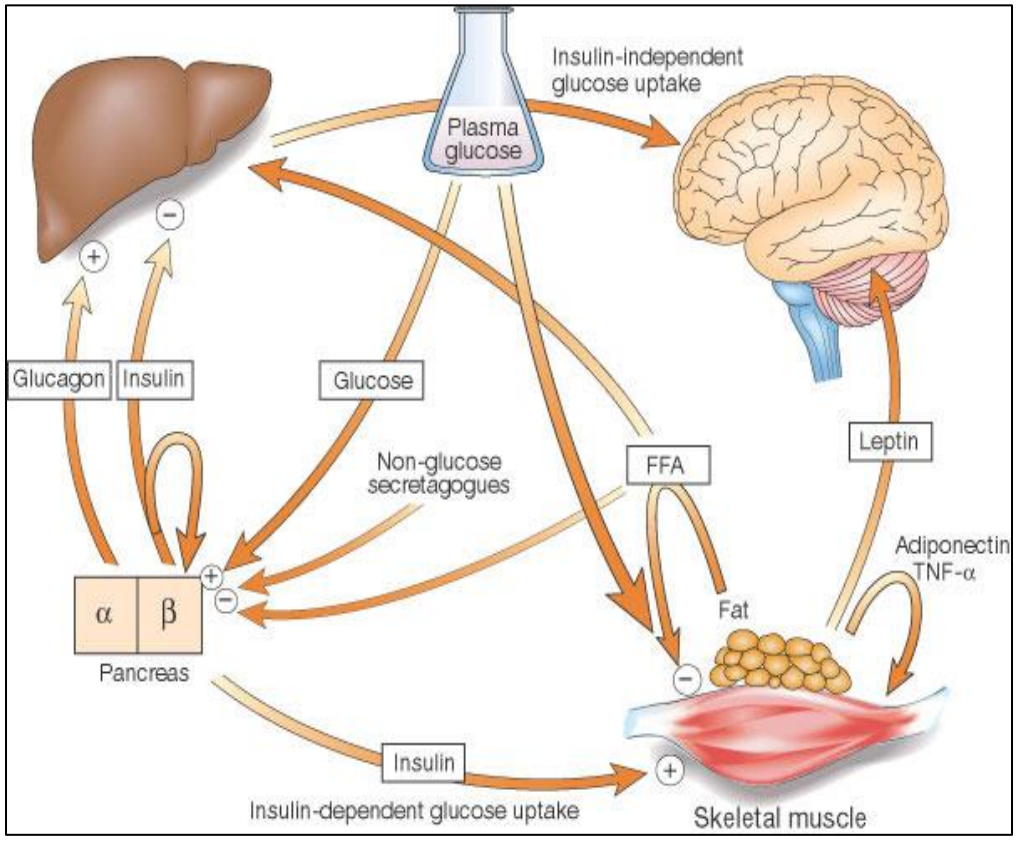

Figure 2.1. The Overall Regulation and Maintenance of Systemic Blood Glucose Concentrations in Healthy Individuals[40].

Maintenance of Glucose Concentration During a Fast; During fasting conditions, blood glucose must be maintained within the normal physiologic range from stored substrates within the body because no new glucose is entering circulation from the gut. The pancreatic alpha-cells are the cells primarily responsible for regulating blood glucose concentrations during fasting conditions. When blood glucose begins to decline during a fast, insulin levels also begin to decline while beta-endorphin levels rise [41,42]. The reduced insulin relieves the inhibition of glucagon release from alpha-cells, while increased beta-endorphins stimulate glucagon release [13,43,44]. The primary location where glucagon exerts its effects is the liver. When glucagon binds to its receptor in hepatocytes, it activates adenylate cyclase to produce cyclic AMP (cAMP), which activates protein kinase A (PKA). The activation of PKA converts glycogen phosphorylase B to phosphorylase A, as well as activating phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pc) transcription. The conversion of glycogen phosphorylase B to A increases its activity. Glycogen phosphorylase cleaves glycosidic 1,4 bonds removing glucose 1 phosphate from glycogen molecules. The glucose 1 phosphate can then be converted to glucose-6-phosphate by phosphoglucomutase. In the liver, glucose-6-phosphate can then be dephosphorylated by glucose-6-phosphatase, and the free glucose can leave the hepatocyte down its concentration gradient. This is the primary means of maintaining blood glucose during relatively short fasts.

In persistent fasting states, liver glycogen is depleted, so blood glucose concentrations are maintained by the production of glucose from non-carbohydrate

sources via gluconeogenesis. This requires the up-regulation of the gluconeogenic enzymes within the liver. PKA, besides its effect on glycogen phosphorylase, activates CREB and inhibits salt-induced kinases (SIKs). The SIKs then release their inhibition of CRTC2 (CREB-regulated transcription coactivator 2). CREB and CRTC2 form a dimer and translocate to the nucleus where they act as a transcription factor and bind to hormone response elements for gluconeogenic genes. In addition, the reduced insulin concentration (and therefore reduced Akt activation), leads to a reduced inhibition of forkhead box protein O1 (FOXO1), which also stimulates gluconeogenic gene transcription. The increase in gluconeogenic enzymes allows the liver to produce glucose from sources other than glycogen, primarily pyruvate produced from oxaloacetate or alanine.

Maintenance of Glucose Concentration During Exercise; Another common disruption in blood glucose homeostasis occurs when individuals exercise or perform physical activity [45,46]. Due to increased energy needs within active tissues, blood glucose needs to be maintained to prevent hypoglycemia. As an individual begins to exercise, there is a withdrawal of parasympathetic signaling which can lead to reduced insulin secretion, while increases in sympathetic signaling begin to increase circulating glucagon, epinephrine, and norepinephrine [45,47-49]. These hormones promote the same signaling cascades discussed in the section on fasting glucose control as well as inhibiting insulin release. This promotes the release of glucose from the liver as well as mobilization of non-esterified fatty acids from adipose to fuel working muscles [45,47-49].

The magnitude of the effect of physical activity is proportional to the intensity and duration of the activity. The signals can adjust as exercise intensity and duration change, but throughout, blood glucose concentrations are being maintained. Although it is not common for exercise to produce hypoglycemia in healthy individuals, mobilization of glucose within the body must be adequate to match the rate of glucose uptake and use by skeletal muscles. The systems compensating for perturbations in glucose homeostasis during exercise are similar to those seen during fasting or hypoglycemia, and are therefore potentially therapeutic or problematic for individuals with DM depending on how exercise is approached.

Because the maintenance of blood glucose concentration is so important to health, these processes are tightly regulated. Each signaling pathway is either activated or inhibited by many signals, including signals from the CNS. While insulin alone accounts for most regulation during the fed state, there are a multitude of factors such as glucagon, norepinephrine, beta-endorphins, and epinephrine, which activate the counter-regulatory response to low blood glucose concentrations.

Maintaining Blood Glucose in Diabetes; Compared to healthy individuals, diabetic individuals have difficulty maintaining their blood glucose concentration. Diabetes mellitus (DM) is the most notable disease in which the primary symptomology is a lack of control of blood glucose concentration. There are generally two different forms of DM, which have different root causes, but eventually converge upon a common condition. T2D is caused by the progressive loss of responsiveness of body tissues to the effects of insulin. This loss of tissue sensitivity results in progressive hyperinsulinemia

and the eventual deterioration of insulin production by the pancreatic β cells, a process termed 'beta-cell exhaustion'. T1D is caused by an autoimmune response which leads to the loss of pancreatic β cells. Because both forms of DM lead to the loss of pancreatic β cells, individuals with either condition may eventually use exogenous insulin to help maintain blood glucose concentration. At that point, these can both be considered IDDM. Regardless of the underlying cause of DM, both types have problems with the management of blood glucose concentration, which can lead to further health complications.

In both types of diabetes, the effectiveness of insulin is eventually decreased and the ability to produce adequate endogenous insulin is compromised. This leads to an inability to lower blood glucose concentration in the post-prandial state. This is usually referred to as impaired glucose tolerance, and leads to post-prandial glucose elevations that are greater and longer in duration compared to healthy individuals. In addition, the liver can become dysfunctional and increase hepatic glucose output when it is unnecessary[50]. This can lead to elevations in fasting glucose concentration, and is referred to as impaired fasting glucose. Because of insulin's effect on both liver and skeletal muscle, exogenous insulin can transiently help to correct both of these dysfunctions in individuals with diabetes. However, skeletal muscle can be stimulated to take glucose from circulation independent of insulin signaling under certain conditions, such as with exercise.

Exercise and Glucose Control; Exercise and physical activity have both been shown to help reduce fasting and post-prandial blood glucose concentration in T1D, T2D, and non-diabetic individuals [12,15,51-53]. Skeletal muscle contraction enhances glucose uptake through an insulin-independent mechanism, thus, the aforementioned beneficial effects of exercise and/or physical activity are reliant on this fundamental property related to skeletal muscle [54]. Interestingly, the effect of insulin and exercise on glucose uptake in skeletal muscle is synergistic rather than just additive [55,56]. In addition, exercise sensitizes the body to insulin, leading to increased potency of insulin post-exercise [54-56]. It has been shown that as little as 7 days of exercise training is sufficient to improve the post-prandial glucose regulation in individuals with T2D [52]. Indeed, even in individuals with T2D, a single bout of exercise can improve the regulation of post-prandial glucose concentration for that day [51]. However, these improvements are lost within a short time, usually 24-48 hrs.

The ability of muscle contractions to increase glucose uptake and enhance insulin sensitivity is a double-edged sword for individuals who rely on exogenous insulin to control blood glucose concentration. If carbohydrates are not properly balanced with insulin dose and physical activity, these individuals can become hypoglycemic post-exercise [12,15,57,58], and without intervention, it can lead to death in extreme cases. Therefore, individuals with diabetes are cautioned to error on the side of maintaining higher rather than lower glucose concentrations when treating with insulin, but this limits their ability to tightly regulate their blood glucose concentration.

Recurrent Hypoglycemia and HAAF

Counter-Regulatory Response to Hypoglycemia and Autonomic Failure;

Hypoglycemia is not normally a problem for healthy individuals. This is because initially, as blood glucose concentrations fall, insulin secretion decreases. This relieves the inhibition of insulin on α cells allowing glucagon to rise. The decrease in insulin reduces the uptake of glucose by skeletal muscle and adipose tissue. The withdrawal of insulin also disinhibits the release of glucose from the liver, while glucagon enhances both glycolysis and gluconeogenesis in the liver leading to enhanced hepatic glucose output. If these actions do not produce sufficient increases in blood glucose concentration, catecholamines (stimulated by the CNS), will further inhibit insulin's effect on the liver, enhance glucagon secretion, and reduce glucose utilization by skeletal muscle as seen in Figure 2. This is the normal counter-regulatory response to hypoglycemia and exercise. However, individuals with DM who routinely become hypoglycemic show a reduced responsiveness of this counter-regulatory response to subsequent hypoglycemia or exercise [59,60]. Unlike the underlying dysfunction within peripheral tissue that results in exacerbated post-prandial or fasting hyperglycemia, the dysfunction in the response to hypoglycemia seems to originate from the CNS. This common occurrence in individuals with IDDM has been termed hypoglycemia associated autonomic failure (HAAF) [12,13,61]. This central dysfunction results in a highly problematic situation for these patients, because they have a blunted response to low glucose and reduced awareness of hypoglycemia, but have to use some treatment to avoid hyperglycemia.

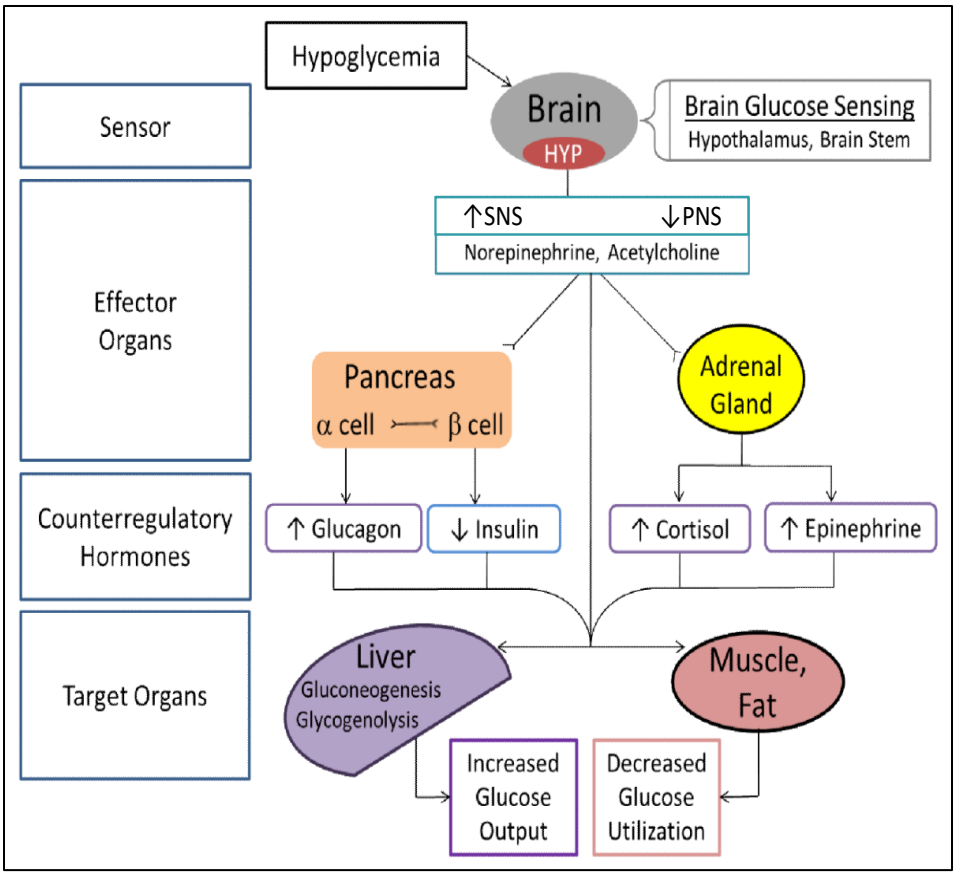

Figure 2.2. Interaction of the CNS with Peripheral Organs During a Counter-Regulatory Response to Hypoglycemia [44].

Antecedent Hypoglycemia and the Counter-Regulatory Response; One of the most common ways individuals with DM can control high blood glucose concentrations, either post-prandially, or fasting, is through the use of exogenous insulin injections [16,50,62,63]. Unfortunately, attempting to tightly control blood glucose concentrations though aggressive pharmacological treatment can result in an increase in the incidence of hypoglycemia [64,65]. After an individual has had an episode of hypoglycemia, they are more susceptible to subsequent hypoglycemia, because there is a loss of the counter-

regulatory response to hypoglycemia [59]. Interestingly, this effect is seen in individuals with DM as well as healthy individuals [15,59,61,66]. While it is unknown what the exact mechanisms are, they seem to be related to changes that occur within the hypothalamus [13,44,59,67,68]. This effect has been extensively studied in both diabetic and healthy populations.

A study by Boyle et al. (1994) used a progressive stepped hypoglycemic clamp in healthy humans for 4 consecutive days and measured the counter-regulatory response on days 1 and 4 [59]. This study showed that by day 4, there were reduced levels of norepinephrine, epinephrine, glucagon, as well as autonomic symptoms of hypoglycemia [59]. Another study by Davis et al (1997) used only one day of antecedent hypoglycemia, but divided their healthy participants into groups receiving different depths (3.9, 3.3, or 2.9mmol/l) of hypoglycemia on day 1 [66]. The second day, all participants received the same degree of hypoglycemia, and their counter-regulatory responses were measured [66]. Although all of the participants had the same degree of hypoglycemia and insulin concentrations on day 2, the lower the previous day's hypoglycemia, the lower the concentrations of epinephrine, norepinephrine, and glucagon [66]. Davis et al (2000) followed this study with another examining how the duration of antecedent hypoglycemia affects the counter-regulatory response [69]. Subjects were randomly selected to receive antecedent euglycemia or hypoglycemic clamp, allowing blood glucose to decline to 2.9mmol/l over 30 minutes, and then have euglycemia restored after 5 minutes, 30 minutes, or 90 minutes [69]. All participants remained on the clamp for a total duration of 2 hours [69]. The next day, all participants were given a hypoglycemic clamp, bringing blood

glucose concentrations to 2.9mmol/l over 30 minutes, and remaining at 2.9mmol/l for 90 more minutes [69]. Startlingly, unlike the dose response seen in their previous study, Davis et al. (2000) showed significantly decreased plasma glucagon and plasma epinephrine concentrations in all of the antecedent hypoglycemia groups, with no difference between these groups [69]. Although all hypoglycemia participants had a 30 minute duration of declining blood glucose concentration, only 5 minutes of sustained hypoglycemia was enough to cause a blunting of the counter-regulatory response to hypoglycemia on the subsequent day [69]. This gives a frightening impression that as incidences of hypoglycemia become more common and more severe, the chance of further autonomic failure increases.

While the reduced counter-regulatory response to hypoglycemia is an interesting phenomenon in the healthy population, it becomes a true impediment to tightly controlling blood glucose concentration within individuals with DM [16,64,70]. A study by Dagogo-Jack et al. (1993) used a stepped hypoglycemic clamp with healthy individuals or individuals with IDDM, and observed a significantly reduced epinephrine and glucagon response from the individuals with IDDM [71]. However, it is known that individuals with IDDM lose responsiveness of pancreatic α cells, and rely more heavily on epinephrine during counter-regulatory responses to hypoglycemia. Therefore, the researchers studied how individuals with IDDM responded to a stepped hypoglycemic clamp after either antecedent hypoglycemia or antecedent hyperglycemia [71]. Compared to individuals with IDDM who received antecedent hyperglycemia, the antecedent hypoglycemia group had a significantly blunted increase in plasma epinephrine

concentration [71]. This effect may even be related to long-term pharmacological suppression of blood glucose concentrations. A study by Davis et al. (2009) studied how 6 months of intensive therapy affected responses to 2 episodes of hypoglycemia [65]. In this study individuals with T2D were randomly grouped to either continue their normal diabetes treatment or undergo intensive treatment (combination therapy of metformin, glipizide XL, and acarbose) for 6 months [65]. The subjects were given 2 days of hypoglycemic clamps before, and 2 days of hypoglycemic clamps after the 6 month treatment, and compared to healthy individuals [65]. Before the intervention, the T2D group had no significant difference in epinephrine or glucagon compared to healthy controls, although all groups showed a blunted counter-regulatory response on day 2 [65]. However, after the 6 months of intensive therapy, the individuals with T2D had a significantly reduced epinephrine response on day 1, which was further suppressed on day 2 [65]. Thus an impairment of counter-regulatory responses to pharmacologically produced hypoglycemia is problematic for individuals with T1D and T2D, however, another avenue for management of blood glucose concentration which is commonly recommended is lifestyle management [72-74].

Antecedent Exercise and the Counter-Regulatory Response; Exercise is commonly recommended for health benefits as well as maintaining blood glucose concentrations for individuals with DM, but it has been shown that antecedent exercise can lead to inhibition of the counter-regulatory response to a subsequent hypoglycemic event [17,50,58,62,74,75]. This is similar to the suppression seen after antecedent hypoglycemia, which can also lead to the inhibition of the counter-regulatory response to a subsequent

bout of exercise [76-78]. These effects are not restricted to individuals with diabetes, but can also be seen in healthy individuals [61,78]. Although healthy individuals do not normally experience hypoglycemic excursions, individuals with diabetes are prone to them depending on their use of medication. For this reason, aggressive treatment of hyperglycemia, through both medication and lifestyle management, must be limited to prevent a vicious cycle of hypoglycemia leading to worsening hypoglycemia with insufficient counter-regulatory responses [13,15,64].

The mechanisms involved in antecedent exercise and the impact on subsequent hypoglycemia are not entirely understood. Galassetti et al. (2001) showed that in humans, 2 bouts of 90 minutes of exercise at 50% $VO_{2\ max}$ within the same day was able to decrease both glucagon and epinephrine response to a hyperinsulinemic hypoglycemic clamp 24 hours later [17]. In addition, the effects of the antecedent exercise were similar to antecedent hypoglycemia in both glucagon and epinephrine concentrations at the last 30 minutes of the hypoglycemic clamp [17]. These effects have been attributed to changes in many different substances, including: lactate, beta-endorphin, and cortisol, however the exact mechanisms are not understood [44,79].

The intensity and duration of antecedent exercise required to elicit the subsequent suppression of the counter-regulatory response has also been investigated, and seems to be more related to duration than intensity. A study by McGregor et al. (2002) used 2 bouts of 60 minutes of cycling at 70% VO_{2peak} or rest in humans the day before a hypoglycemic clamp. While epinephrine and glucose infusion rates were significantly different in the antecedent exercise group compared to antecedent rest group during the

hypoglycemic clamp, there was no difference in glucagon [80]. It was hypothesized that the higher intensity of this study, compared with previous work by Galassetti et al. (2001), would lead to greater cortisol release and exacerbate the suppression of the counter-regulatory responses [17,80]. The exercise sessions were sufficient to increase cortisol concentrations in the blood during the exercise, however, during the hypoglycemic clamp there were not any further reduced counter-regulatory responses compared to Galassetti et al. (2001) [17,80]. The authors suggested that the subjects who performed the 60 minutes at 70% were at a higher fitness level than most other studies, most of which have used lower intensities of exercise. This may indicate that some effects seen acutely with exercise are eventually compensated for though training adaptations. Another study in humans, by Sandoval et al. (2004), sought to test how two 90 minute bouts of exercise would affect the counter regulatory response at a lower intensity [18]. Sandoval et al. (2004) used the exercise protocol from Galassetti et al. (2001) at either 50% $VO_{2\ Max}$ or 30% $VO_{2\ Max}$. Regardless of the training intensity, there was a significantly depressed counter-regulatory response to a hypoglycemic clamp on the following day. Epinephrine, but not norepinephrine, was significantly lower during the hypoglycemic clamp. In addition, the glucose infusion rate and rate of disappearance were higher in the previously exercised humans while endogenous glucose production was lower [18]. Additionally, there was no difference in insulin concentrations between groups, suggesting that the difference was not accounted for by increased insulin stimulated glucose uptake [18]. Although, assuming exercise sensitizes tissues to insulin, the similar insulin concentration in the 2 groups may still have resulted in an increased responsiveness in tissues of the

previously trained group. Taken together, it seems as though antecedent exercise can lead to a diminished counter-regulatory response on a subsequent day. In addition, this effects seems less related to exercise intensity than to exercise durations, and potentially, the training state of the individuals engaged in exercise [17,18,80].

Hypoglycemia, Exercise, and HAAF; In addition to antecedent exercise affecting subsequent hypoglycemia, antecedent hypoglycemia affects subsequent exercise, suggesting that these systems are related. Davis et al. (2000) showed that healthy individuals, given antecedent hypoglycemia, had a reduced counter regulatory response to subsequent exercise compared to individuals given antecedent euglycemia [78]. During the subsequent exercise bout, glucagon, epinephrine, and norepinephrine were significantly depressed under the antecedent hypoglycemia condition compared to the antecedent euglycemia condition [78]. There was also a failure to reduce plasma insulin concentrations in the antecedent hypoglycemia group compared to antecedent euglycemia [78]. The suppression of the counter-regulatory response to exercise after antecedent hypoglycemia is also seen in individuals with T1D [76,77]. Galassetti et al (2003) showed that antecedent hypoglycemia blunted the increase in epinephrine, norepinephrine, and glucagon in response to 90 minutes of subsequent exercise (24 hours later) among individuals with T1D [77]. It was later shown by Galassetti et al. (2006) that the suppression of these counter-regulatory hormones respond in a stepwise fashion to deeper antecedent hypoglycemic excursions, at least within individuals with T1D, similar to what was shown by Davis et al. (1997) with different magnitudes of hypoglycemia [66,76].

This would lead to increased risk of exercise-induced hypoglycemia in individuals with IDDM who become hypoglycemic due to prior exogenous insulin administration [15,57,63].

The effects of antecedent exercise on subsequent hypoglycemia was also seen during the same day in individuals with T1D [19]. Humans with T1D who exercised for 90 minutes at 50% VO_{2Max} during euglycemia were given a hypoglycemic clamp 150 minutes after exercise. Similar to previous studies, the antecedent exercise group showed decreased epinephrine response to the hypoglycemic clamp [19]. In addition, the rate of endogenous glucose production was lower and glucose infusion rate was higher in the antecedent exercise group [19]. These results were compared to a morning euglycemia rest group, a morning hypoglycemia rest group, and a morning no treatment (control) group. The antecedent exercise group and antecedent hypoglycemia group showed similar depression of epinephrine in response to hypoglycemia compared to euglycemic and control groups [19]. This suggests that antecedent exercise and antecedent hypoglycemia may be affecting the counter-regulatory response through similar mechanisms.

It is interesting that there is a blunting of the counter-regulatory response to either exercise or hypoglycemia, if one condition precedes the other. It could be assumed that the counter-regulatory hormones (glucagon and epinephrine) are finite, and have become exhausted [81]. However, these hormones should be produced in adequate supply in alpha cells of the pancreas and the adrenal glands (respectively), and therefore it seems unlikely that they are the limiting factor. In addition, individuals who routinely exercise have increased catecholamines during each of their exercise sessions. Although there is a training response over time which requires less catecholamines to produce similar

responses. If peripheral glands, such as adrenal medulla and pancreatic islets, are functioning properly, it may be that the inhibition of the counter-regulatory response to hypoglycemia is a problem related to the CNS rather than acute changes to these peripheral organs. Therefore, it is important to understand the contribution of the CNS to the regulation of blood glucose concentration, and the counter-regulatory response to hypoglycemia.

In addition to the peripheral mechanisms, the CNS plays an important role in the regulation of blood glucose in healthy individuals. The pancreatic islets are innervated by both parasympathetic and sympathetic nerves (the vagus and splanchnic nerves, respectively) [82]. Stimulation of the vagus nerve leads to an increase in insulin secretion from pancreatic β cells, while stimulation of the splanchnic nerve leads to an increase in glucagon secretion from pancreatic α cells and an inhibition of insulin secretion [82]. While the pancreas has some autonomous ability to regulate its own function, due to fluctuations in blood glucose concentrations and glucokinase activity, if the pancreatic nervous innervation is obliterated, the responsiveness to changes in blood glucose concentrations is significantly diminished [29,82,83]. This suggests that there are areas within the CNS that respond to changes in glucose concentrations and stimulate peripheral responses to regulate these concentrations. Indeed, studies have shown that the counter-regulatory response can be blunted by increasing energy substrates, such as glucose or lactate, within the hypothalamus, despite systemic hypoglycemia [30,84]. This seems to suggest that glucose sensing portions of the hypothalamus may be able to regulate sympathetic efferents [24]. Therefore, the ability of neurons to alter their activity

in response to changes in blood glucose (or local glucose) concentration is important to the counter-regulatory response to hypoglycemia in both healthy individuals and individuals with DM.

Glucose Sensing in the Brain

The idea that the brain responds to the concentration of available glucose is not new and was established in the mid-1800s [85]. The glucose sensing activities in the brain encompass glucose-sensitive and glucose-responsive neurons, both of which alter their activity according to the concentration of glucose [86,87]. Glucose-responsive neurons [now referred to as glucose excitatory (GE) neurons], increase their rate of depolarization when glucose levels are high [86,87]. Glucose-sensitive neurons [now referred to as glucose inhibited (GI)], reduce their rate of depolarization while glucose is high, but increase activity when glucose becomes low [86,87]. These glucose sensing neurons are known to be present in several brain areas, specifically, the lateral hypothalamus, the arcuate nucleus, and the ventromedial hypothalamus (VMH) [82,83,86,88,89]. These areas of the brain are also thought to be associated with management of hunger and satiety. While either GE or GI neurons may dominate some brain areas, both types may exist in the same area. These neurons are known to produce changes in the systemic secretion of gluco-regulatory hormones by affecting both parasympathetic and sympathetic efferents.

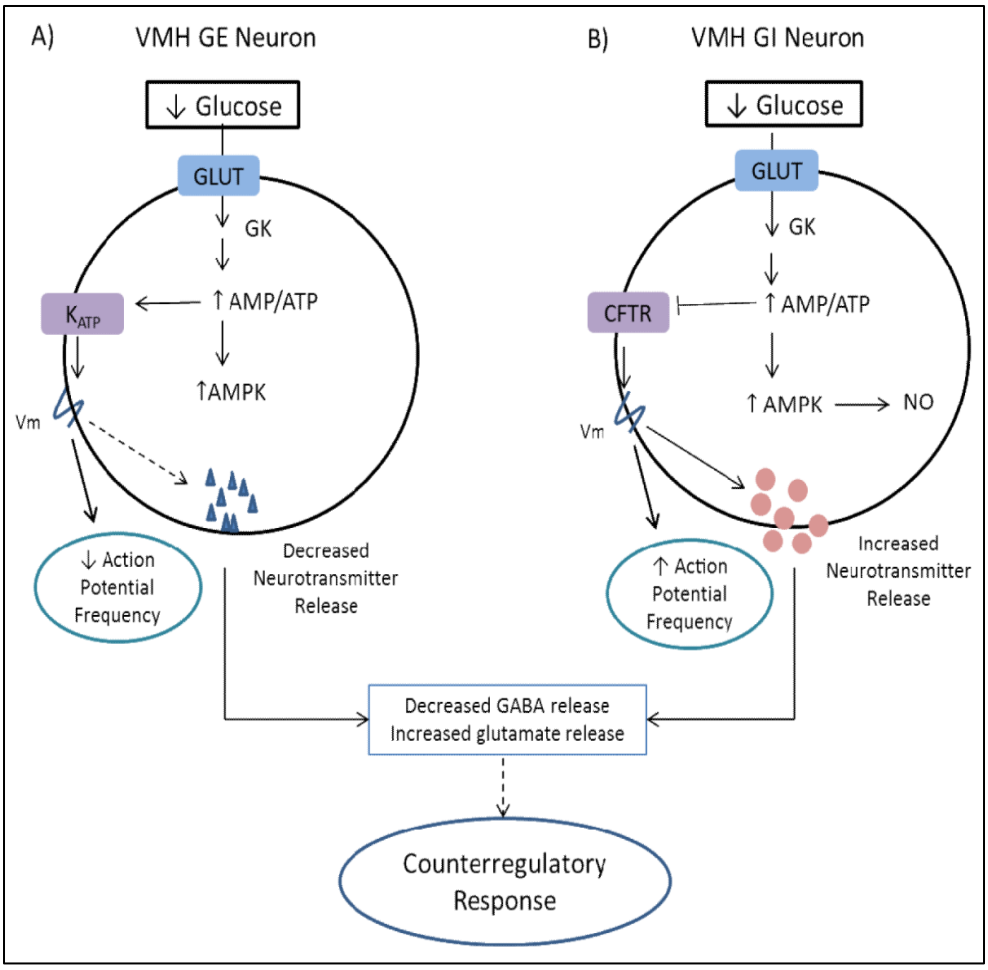

Figure 2.3. Proposed Mechanisms of Regulation of Both GE and GI Neurons [44].

Glucose sensing neurons have been studied within the VMH, which is widely recognized as one of the most important regions within the hypothalamus involved in glucose sensing and the counter-regulatory response to hypoglycemia. It is located near the third ventricle as well as the median eminence (see Figure 2.4), allowing it to sample both hormones which regulate glucose as well as glucose concentrations [30]. It is well established that both GE and GI neurons exist within this brain area [25,29,30,44,82-84,86,88-93]. Figure 2.3 illustrates several proposed mechanisms by which the glucose sensing neurons

are thought to alter their activity based on glucose concentrations. The current leading hypotheses related to the regulation and function of glucose sensing neurons focus on: 1) ATP sensitive potassium channels (K_{ATP}), 2) glucokinase and GLUT2, and 3) AMPK. The GE neurons contain GLUT3 and GLUT2 transporters, and rely on glucokinase to phosphorylate glucose entering the cell [30]. When local glucose concentrations increases, glucokinase activity increases which increases glycolysis and increases the ATP:ADP ratio causing the K_{ATP} channels to close which leads to extracellular Ca^{2+} entering the cell and stimulating the release of neurotransmitter containing vesicles [30]. AMPK activity is more commonly associated with GI neurons, and is thought to act on the cystic fibrosis transmembrane conductance regulator (CFTR) through NO and cGMP when ATP:AMP ratio is low [29,82,83,88,92]. It is important to note that the mechanistic pathway(s) for these glucose sensing neurons has not been fully elucidated, nor is it clear if associated glial cells are involved in the regulation [82]. It is also important to note that all of the proposed mechanisms rely on changes in energy availability, which suggests that the exact energy source driving the response may be substrates other than (or in addition to), glucose.

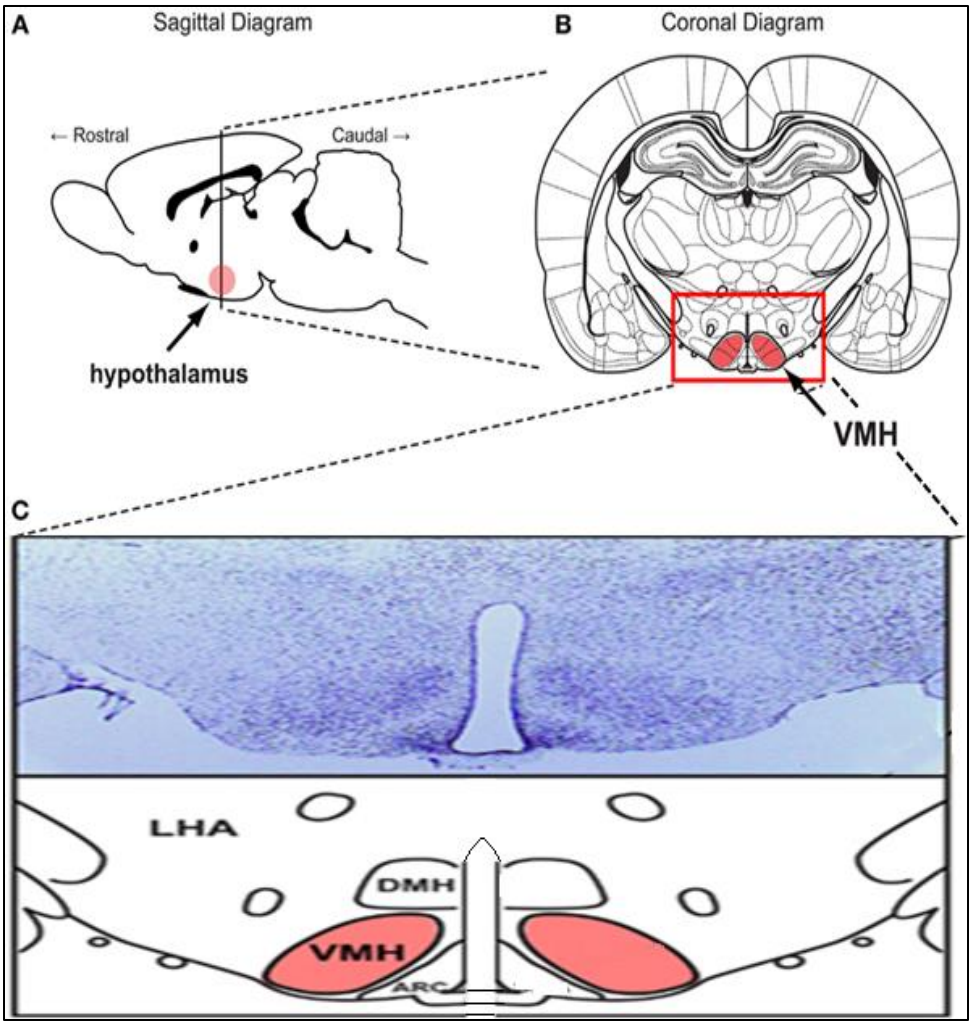

Figure 2.4. Location of the Ventromedial Hypothalamus [94].

Counter-Regulatory Response and the VMH

Human Studies; While glucose sensing neurons reside in many brain areas, an area that is frequently studied with respect to the counter-regulatory response to hypoglycemia is the hypothalamus. Human studies have used functional Magnetic resonance imaging (fMRI) and positron emission tomography (PET) scans to attempt to determine the areas in the brain that respond to changes in blood glucose concentrations.

Early studies using fMRI showed that ingestion of glucose results in a decrease in activity in the hypothalamus [95]. It was also shown that this decrease in activity was dose dependent, with less hypothalamic activity when greater amounts of glucose were ingested [96]. Interestingly, this effect is blunted in non-insulin dependent individuals with T2D compared to healthy controls [97]. The effect of hypoglycemia has also been tested using fMRI. These tests have shown that healthy individuals have an increase in activity in the hypothalamus in response to hypoglycemia [98]. Individuals with T1D also showed an increase in activity in the hypothalamus in response to hypoglycemia, but to an even greater extent than the healthy individuals [98]. However, the individuals with T1D still show reduced epinephrine and glucagon in response to hypoglycemia [98]. It is of note that, when fluorodeoxyglucose was used for PET scan of the brain, individuals with T1D and hypoglycemia unawareness showed reduced activation of the hypothalamus, and reduced blood epinephrine concentrations, compared to individuals with T1D with normal hypoglycemia awareness [99]. These studies cannot pinpoint the exact region of the hypothalamus that account for changes in brain activity in response to hypoglycemia, but the ventromedial hypothalamus is within the areas indicated. To test these mechanisms further, the brain must be manipulated and tested in ways that would not be permissible in humans; therefore, animal studies are required.

Animal Studies; Several studies have begun to unravel the role that glucose sensing neurons play in initiating the counter-regulatory response to hypoglycemia. It was initially shown that injecting glucose into the VMH of rats during systemic insulin-induced hypoglycemia would lead to a failure of the normal counter-regulatory

mechanisms [100]. This was attributed to the reduced concentration of norepinephrine in the VMH when glucose was administered [100]. However, norepinephrine is not the sole regulator of the counter-regulatory response in the VMH, as many other compounds have been implicated [30]. Under normal circumstances, it is thought that the neurotransmitter being released by GE neurons is GABA, inhibiting the sympathetic efferent innervation of pancreatic α cells and adrenal glands [30,101]. The changes in GABA concentrations in the VMH were linked to the state of the K_{ATP} channels [whether or not they were stimulated (closed)] [101]. When K_{ATP} were opened, there was low GABA release in the VMH and a systemic increase in epinephrine and glucagon in response to insulin-induced hypoglycemia [101]. However, during hypoglycemia performed in conjunction with GABA receptor agonists in the VMH, the counter-regulatory response was absent [101]. This effect was also observed when K_{ATP} channels were closed and then GABA receptor antagonists were applied [101]. These studies firmly establish the importance of glucose sensing neurons within the VMH in the counter-regulatory response, at least in rats. However, K_{ATP} channels respond to ATP:ADP ratio, not glucose directly. Therefore, it seems that other energy substrates could potentially stimulate similar responses.

Glucose or Lactate Sensing; The glucose sensing neurons in the VMH may be responsible for the diminished counter-regulatory response to hypoglycemia after either antecedent hypoglycemia or exercise, leading to autonomic failure. A current question of interest is whether these neurons are directly sensing *glucose*, or whether energy status is being signaled to the neurons in another way. Earlier research has shown that local L-lactate perfusion in the VMH reduced systemic glucagon, epinephrine, and

norepinephrine in response to a hypoglycemic clamp in rats [25]. To determine whether or not these changes were due to the metabolic role of L-lactate, D-lactate was also perfused in the VMH, showing no changes in counter-regulatory response, indicating that the level of metabolic fuels other than glucose, could potentially be indicative of energy status [25]. Since these findings were similar to effects seen in studies with GABA, it was further hypothesized that the effects of lactate were being mediated through the effects of GABA from GE neurons [24]. A recent study by Chan et al (2013), investigated the effects of L-lactate and GABA on the counter-regulatory response to hypoglycemia [24]. Previous studies have shown that increased GABA in the VMH leads to a reduced counter-regulatory response to hypoglycemia [101]. Utilizing microdialysis probes to inject certain compounds and assessing changes in GABA, hormones, and glucose via a carotid artery catheter, Chan et al. (2013) reported that when lactate was injected into the VMH prior to a hypoglycemic clamp, there was increased GABA present in the VMH [24]. Along with the increased GABA concentrations, blood concentrations of glucagon and epinephrine were lower compared to control animals [24]. When neuronal lactate transporters (MCTs) were inhibited by microinjection of 4CIN, GABA levels were reduced [24]. Finally, the researchers used a GABA receptor antagonist in addition to L-lactate infusion, and while GABA was still increased, the counter-regulatory response of glucagon and epinephrine was restored [24]. This suggests that, during hypoglycemia, glucose sensing neurons in the VMH can take up L-lactate from an extra-cellular pool to influence their activity. Additionally, the presence or absence of specific cellular membrane lactate transport proteins suggests L-lactate was not necessarily being produced within the neurons. For

this reason, the hypotheses for differential patterns of lactate flux have been suggested for the brain [102-107]. If lactate and/or local energy substrates are important to the HAAF response, and are potentially coming from outside the glucose sensing neurons, it is worth investigating mechanisms by which either hypoglycemia or exercise can influence energy availability and metabolism in the brain.

Brain Glycogen, and Impairment of the Counter-Regulatory Response to Hypoglycemia; The effect of antecedent exercise or hypoglycemia on subsequent hypoglycemic counter-regulatory responses may be driven by an increased concentration of glycogen within the brain. A recent study by Matsui et al. (2012) using rats, showed that there is an acute increase in brain glycogen after exercise in many different brain areas [108]. The animals ran on a treadmill at 20m/min until exhaustion (81.2 ± 2.7 min.). This was sufficient to decrease brain glycogen, and after 24 hours, many brain regions saw super-compensation of glycogen stores [108]. Significant changes were seen in whole brain as well as in the cortex, hippocampus, hypothalamus, cerebellum, and brainstem, however, the super-compensation in the hypothalamus was only seen at 6 hours post-exercise [108]. These researchers also tested whether 3 weeks of running at the same treadmill speed for 60 minutes 5 days each week would show even greater glycogen super-compensation in the brain. However, in this condition, only the hippocampus had increased glycogen. Unfortunately, the peak glycogen concentrations may have been missed, due to the timing of the measurement at 72 hours post-exercise [108]. Alternatively, super-compensation may be a transient adaptation that is lost once long-term adaptations occur [108].

In addition to super-compensation of brain glycogen in response to exercise, it has been shown that hypoglycemia can also lead to brain glycogen depletion, and subsequent super-compensation [109-112]. The effect of hypoglycemia on glycogen depletion and super-compensation has been measured in both human and rat models [109,112]. However, the super-compensation has not been shown to be equal within all regions of the brain, but seems to be more pronounced within the hypothalamus [111]. Of course, this is the region of the brain most associated with regulating the counter-regulatory response to hypoglycemia.

The changes seen in brain glycogen in both exercise and hypoglycemia suggest that this could be a common link by which both methods could provide an alternative substrate for neurons during a subsequent episode of hypoglycemia. However, a major problem is that brain glycogen exists within glial cells rather than neurons, and these glial cells, unlike hepatocytes, do not express glucose-6-phosphatase to allow glycogen derived glucose to exit the cell. However, as previously discussed, many researchers suggest that lactate could be the substrate providing the alternative fuel for neurons [104,105,113,114]. These lactate-related hypotheses may have significant implications for how glucose sensing neurons respond to hypoglycemia and ultimately, may provide insights into the coordinated effort, at the level of the brain, to maintain blood glucose concentrations within a physiologically acceptable range. This general hypothesis has become known as the astrocyte to neuron lactate shuttle (ANLS).

Astrocyte to Neuron Lactate Shuttle

The mechanism by which systemic glucose concentrations relate to brain lactate and glucose sensing is important for understanding the counter-regulatory response to hypoglycemia, as well as why there may be an inhibition of the counter-regulatory response after hypoglycemia or exercise. Glucose has long been known to be the preferred energy substrate of the brain; in fact, the brain uses about 25% of the total glucose consumed (at rest) during the day and around 20% of oxygen to meet its energy requirements [107]. However, the brain is composed of multiple cell types, mainly neurons and glial cells, the most abundant glial cells are astrocytes. Both neurons and astrocytes have been shown to increase their metabolic rates during brain activity [115,116]. During excitatory stimulation of brain activity, there is an increased need for ATP production; however, there is an observed mismatch in the location of increased cerebral blood flow and brain glucose uptake, and the location of oxygen utilization [107,117,118]. While the brain takes up large amounts of glucose, it is also capable of oxidizing many metabolic intermediates from glucose [102,107]. Due to the mismatch in the localization of glucose uptake and oxygen use, as well as the effects of lactate infusions in the brain, hypotheses have been developed to describe how energy substrates may be used within the brain.

The astrocyte to neuron lactate shuttle hypothesis (ANLS) suggests that when there is increased neuronal activity, astrocytes increase their glycolytic rate which produces lactate as shown in figure 2-5 [115,116,119]. The lactate is then shuttled out of the astrocyte and taken up by neurons, which can convert the lactate to pyruvate to enter the TCA cycle. Recently this model has been proposed to occur between oligodendrocytes

and neurons as well [120]. Several types of scientific evidence support this hypothesis; such as the relative expression of enzymes and their activity levels in astrocytes and neurons, the neuronal activity under different concentrations of metabolic intermediates, and the arrangement of cellular transport proteins. Each of these will be discussed in more detail in the following paragraphs. Based on current scientific evidence, the ANLS hypothesis seems likely to occur within at least some brain areas, however its relevance to the HAAF response is unknown.

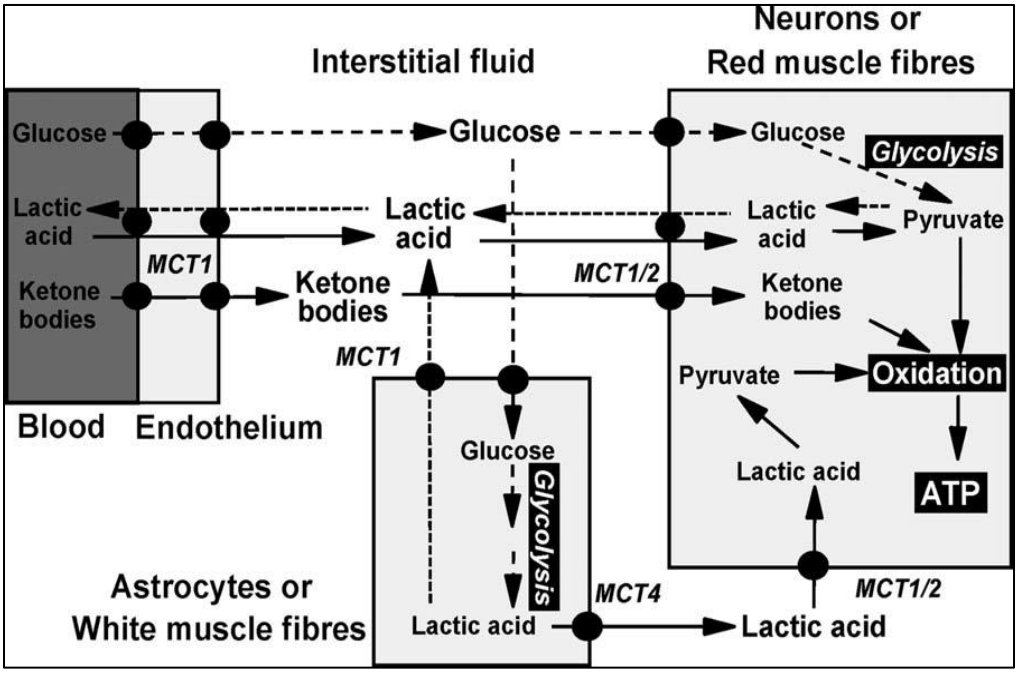

Figure 2.5. Model of the Lactate Shuttle Hypothesis[121].

Lactate production seems to be linked closely with brain activity, similar to observations for glucose and oxygen use. When neurons are stimulated to repeatedly depolarize, adjacent astrocytes produce lactate at a greater rate than at rest [116,122,123]. This appears to be mediated through the sodium dependent glutamate transport within

astrocytes, which have processes that enwrap most synapses [115,119,123]. Astrocytes take up glutamate via sodium dependent transporters, and the excess sodium is exported via Na/K ATPase pumps, leading to an increased rate of glucose uptake and glycolysis within the astrocyte [123]. There are also increases in blood flow to the active regions of the brain, possibly mediated via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on purkinje cells [122]. It has also been suggested that astrocytes independently regulate blood flow through the production of vaso-active molecules from aracadonic acid [116]. The increased blood flow and astrocytic glycolysis leads to the production of lactate which can be exported from the astrocyte and used as fuel by neurons [115,123]. Indeed, as AMPA receptors are stimulated on neurons there is an increase in lactate uptake due to increased lactate transporter translocation [124]. This seems to suggest a neural activity mediated mechanism for lactate shuttling from astrocytes, at least within some portions of the brain.

The ANLS hypothesis is also supported by the presence of certain enzymes within astrocytes and neurons. Recently, a study by Herrero-Mendez et al. (2009) showed that astrocytes express the enzyme 6-phosphofructo-2kinase/fructose-2,6-bisphosphatase-3 (Pfkfb3), which generates fructose-2,6-bisphosphate, an activator of phosphofructokinase 1 [125]. This enhances the rate-limiting step in glycolysis, allowing a higher glycolytic rate [107,125]. While neurons also express Pfkfb3, they express an ubiquitin ligase, anaphase-promoting complex/cyclosome-Cdh1 (APC/C), which marks Pfkfb3 for proteosomal degradation, thus reducing its activity [125]. Because astrocytes have low APC/C activity, they are not affected, and are capable of a higher rate of glycolysis [107,125]. Herrero-

Mendez et al. (2009) further tested the evidence for the ANLS by artificially increasing neuronal glycolytic rate by inhibiting APC/C in neurons, or up regulating Pfkfb3 in neurons [125]. In either case, neuronal glycolytic rates were enhanced, however this lead to increased oxidative stress and apoptosis [125]. The authors suggested that this increased oxidative stress is due to the redirection of glucose from the pentose phosphate shunt towards oxidative glycolysis [125]. This suggests that neurons require both high ATP and antioxidants for proper functioning. Additionally, it suggests that not all oxidative metabolism in neurons is from glucose, since glucose is required for the pentose phosphate shunt [107,125].

Additional evidence for the ANLS hypothesis comes from the documentation that the aspartate/glutamate carrier (part of the malate aspartate shuttle) is predominantly expressed in neurons but not in astrocytes, and that the pyruvate dehydrogenase complex is strongly inhibited in astrocytes, but active in neurons [126,127]. A study by Berkich et al (2007) used immunohistochemistry to examine the aspartate/glutamate carrier co-expression with glial fibrillary acidic protein (GFAP), and found no over lapping fluorescence [126]. They confirmed this finding showing that bulk preparation of synaptosomal (neuronal) mitochondria were capable of producing aspartate in the presence of glutamate at a higher rate than astrocytic mitochondria, which co-express branched chain aminotransferase and GFAP [126]. In addition, LDH isoforms have been compared between neurons and astrocytes, and while both contain the LDH1 isoform, the LDH-5 isoform was exclusive to astrocytes [119,128,129]. A study by Halim et al (2010) used immunohistochemistry in primary cell cultures and stained for LDH (both isoforms) and

observed a greater content of LDH within astrocytes than neurons [127]. They also measured greater pyruvate dehydrogenase complex activity in neurons [127]. Another study, by Bittar et al. (1996), used immunohistochemistry in human brain slices (collected 3-12 hour post-mortem) to stain for LDH1 and LDH5. While they observed that astrocytes, which also stain for GFAP, expressed LDH5, they noted that greater numbers stained for LDH1 [128]. While LDH1 favors conversion of lactate to pyruvate, LDH5 favors conversion of pyruvate to lactate [128-130]. This expression pattern supports the view that while neurons can oxidize fuel for energy, not all the fuel consumed is from glucose, and astrocytes may use glycolysis to provide substrates to neurons, as well as for oxidative phosphorylation.

Additionally, the presence of glycogen in the brain may also support the ANLS hypothesis. In adult animals, glycogen exists exclusively in astrocytes within the brain [28]. However, astrocytes do not express glucose-6-phosphatase to release glucose into the extracellular space [28]. Initially it would seem intuitive that astrocytes would use the glycogen to meet their own energy needs, a situation that parallels what is seen in skeletal muscle fibers. However, it was discovered that neurons, when cultured with astrocytes, could survive longer during glucose deprivation than when they were cultured alone [131]. In addition, this effect was only seen when the astrocytes were pretreated with insulin or methionine sulfoximine to induce enhanced glycogen content [28,131]. Neuronal overstimulation also showed that astrocytes helped to maintain the neuron's ability to produce compound action potentials [28,120]. When astrocytes with stored glycogen are present, the compound action potentials can be sustained for a longer duration before

failure [28,120]. Despite these studies, others have suggested that glycolysis within astrocytes is being sustained by uptake of glucose from circulation [122]. In either case this suggests that astrocytes are helping neurons to meet their metabolic needs, but evidence suggests that they must be transporting something other than just glucose. These findings appear to further support the ANLS hypothesis. But how is lactate movement coordinated within the brain?

Monocarboxylate Transport Proteins

Because of the potential importance of the movement of lactate in both brain metabolism and in the counter-regulatory response to hypoglycemia, monocarboxylate transport proteins (MCTs), has been heavily researched. One area of research has focused on the MCTs that facilitate the movement of lactate in and out of cells. More importantly, how expression of these transport proteins are affected under different conditions may improve knowledge of metabolic function in the brain and suggest novel targets for pharmacologic manipulations. Therefore, further investigation of these MCTs, and how they are regulated under different conditions, is necessary.

There are 14 identified MCTs, of which six have been functionally characterized and four have been shown to transport lactate coupled with a proton [121,132,133]. These are members of the Solute Carrier Family 16, but have different names because the MCTs were named based on their functional characterization, while SLC16 nomenclature was based on DNA sequencing [121]. The most important MCTs to the study of brain metabolism and lactate shuttling hypotheses are MCTs 1, 2, and 4. MCTs 1, 2, and 4 come from different genes [SLC16A1, SLC16A7, and SLC16A3 respectively] on

different chromosomes [1, 12 & 17, respectively (shown in figure 2-6) as red lines], and therefore have different properties.

The MCTs vary in their function according to the specific properties of each MCT, allowing differential monocarboxylate transport in different tissues and cell types. The MCTs have the capacity to transport many substrates, including lactate, pyruvate, and ketones, however they have different affinities for these substrates, shown in table 2-1 [134]. The MCTs were characterized in *Xenopus laevis* oocytes, which have no lactate transport activity and do not contain CD147 (Basigin, a chaperone protein used by some MCTs), allowing the functional characterization of the MCTs [134]. MCT's were characterized by microinjecting relevant cRNA for each MCT. The individual properties of MCT1, MCT2, and MCT4 are discussed below. The remaining characterized lactate transporting MCT, MCT3, is found only in the retinal epithelium and choroid plexus and does not seem to be associated with the central control of blood glucose concentrations or the ANLS, and therefore will not be discussed further [121,132].

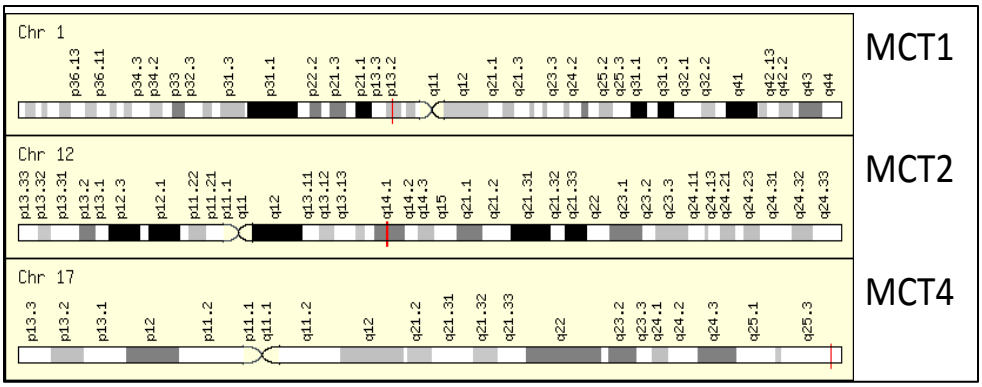

Figure 2.6. Location of the Genes for MCT1, MCT2, and MCT4.

Table 2.1 The Relative Km Values Each MCT has for Different Substrates, Adapted from Halestrap et al. 2013 [134].

Substrate	MCT1 K_m	MCT2 K_m	MCT4 K_m	Approximate Plasma conc.
L-lactate	3.5	0.74	28	1.5
D-Lactate	27.5	-	519	-
Pyruvate	1.0	0.08	153	0.06
D-β-Hydroxybutyrate	10.1	1.2	130	0.06
Acetoacetate	5.5	0.8	216	0.02

MCT1 is found in most tissues in all species in which it has been studied [132]. The K_m of MCT1 for lactate, 3.5mM, is between those of MCT2 and MCT4 [133,134]. MCT1 is not associated with CD147, and its activity is based solely on the substrate concentration and pH inside and outside the cell [134]. Therefore, the MCT is responsive to changes in substrate concentrations, but can also respond to changes in pH. The activity of MCT1 is very well characterized, and it is known that the co-transport of lactate and a proton must

begin with the MCT in an "open" conformation [134]. The proton must attach to its active site first, followed by the lactate. This induces a conformational change to the "closed" state, which allows the lactate and proton to be released on the opposite side of the membrane [134]. In the brain, MCT1 is the primary MCT on the endothelial cells which make up the capillaries of the blood brain barrier [133]. These transport proteins are present on both the lumen facing and basal lamina facing membranes of the endothelial cells [133]. This lends support to the idea that excess lactate and protons can be exported back and forth between the brain and circulation via the astrocyte end feet and endothelial cells. It has been suggested that this may be involved in activation dependent hyperemia in the brain, as well as maintaining pH by reducing excess protons from glycolysis [106,119,135]. Therefore, MCT1 can be thought of as a general, bi-directional, lactate transporter found in most tissues.

Not all MCTs seem to be bi-directional in nature like MCT1. Because of its ability to take up lactate at lower concentrations, MCT2 primarily imports lactate, and can be found in tissues which can use lactate for fuel, such as: testis, spleen, heart, kidney, liver, and brain [134,136]. In the brain, MCT2 is present exclusively in neurons [133]. MCT2 has the lowest K_m for lactate of the MCTs, about 0.7mM. Also, unlike MCT1, MCT2 is co-expressed with embigin, a chaperone protein that helps to integrate some MCTs into cell membranes [134]. It has even been suggested that MCT2 resides in the cell and is stimulated to translocate to the cell membrane during neuronal activation [124]. MCT2 translocation seems to be regulated by similar mechanisms as the glutamate receptors 2 and 3 (GluR2/3) such as glutamate, glycine, and tumor necrosis factor alpha

(TNFα). This allows neurons to increase MCT2 during times of increased stimulation and activity. This MCT appears to be used to import lactate into neurons for oxidation [134]. It has been suggested that MCT2 activity in the brain is controlled through translation rather than transcriptionally [137]. This may be accomplished through insulin and insulin like growth factor, which can access to the brain through the median eminence and third ventricle [137]. This lends support to the ANLS hypothesis due to MCT's presence in neurons, but not astrocytes, as well as its potential translocation to the cell surface during neuronal activation.

MCT4 is found predominantly in highly glycolytic tissues, and in the brain, MCT4 is almost exclusively present in astrocytes [133]. MCT4 has the highest K_m for lactate, about 35mM [133]. MCT4 is unique however, because its K_m for pyruvate is about 153mM, whereas the other MCTs have affinities for pyruvate that are more similar to their affinity for lactate [134]. This unique feature prevents the loss of pyruvate from the cells expressing MCT4, thus allowing glycolysis to continue by converting pyruvate to lactate and an NADH to NAD^+ [134]. It also functions to ensure that lactate transport only occurs when high concentrations of lactate are being produced in the cell via increased glycolysis. Additionally, the higher K_m for lactate can be overcome by the difference in pH that occurs as glycolysis continues to produce lactate and protons [129]. The proton gradient then pulls the transport of lactate, overcoming the higher K_m relative to the other MCTs [129]. This has been observed in skeletal muscle where glycolytic fibers export lactate via MCT4 and oxidative fibers take in lactate via MCT1 for oxidation [134]. This observed lactate shuttle in other tissues as well as the localization and functionality of the

MCTs in the brain have lent support to the ANLS hypothesis that: astrocytes export lactate via MCT4, while neurons import lactate via MCT2 to provide energy for neurons [133]

The arrangement of MCTs in the brain is intimately related to how energy substrates are being handled. Because MCT1 is present in the astrocytic end feet and on endothelial cells, it is plausible that lactate can be moved between the brain and circulation. The presence of MCT1 allows for lactate transport between the astrocytic end feet and circulation for either enhancement of hyperemia during increased brain activation, or for an additional energy substrate during physical activity or hypoglycemia, when systemic lactate is high [138]. However, there is also the presence of MCT2 on neurons, which makes it plausible that neurons are taking up lactate from the interstitial pool for oxidation. The presence of MCT4 on astrocytes makes it plausible that under certain conditions, such as intense exercise or hypoglycemia, astrocytes may be able to export lactate produced from increased anaerobic glycolysis, or being imported from circulation. It is unknown if the route of glycogen derived lactate efflux would lead to astrocytic end feet to promote hyperemia, interstitial lactate pools for neuronal uptake, or both, and is likely dependent on multiple factors. A deeper study of MCTs may help to elucidate these mechanisms. While different MCTs are differentially expressed in specific tissues and cell types, their total expression can be altered under different physiologic stimuli.

MCTs and Exercise

The MCTs exist in many tissues, and although it is already well established that they are altered by exercise in skeletal muscle, the effect of exercise on MCT content and function in the brain is also of interest. Due to the effects of lactate in the VMH on the counter-regulatory response to hypoglycemia, as well as the common recommendation of increased physical activity for individuals with DM, the effects of exercise on MCT expression may be important to understanding the HAAF response. First, this review will discuss the known effects of acute exercise on MCT expression in skeletal muscles and the brain, followed by the effects of chronic exercise on MCT expression in skeletal muscles and the brain. Also, the effects of diabetes on MCT expression will be briefly explored. In addition, potential mechanisms of MCT regulation will be briefly discussed.

Effects of Acute Exercise; It is well established that exercise can affect the expression of MCT proteins in skeletal muscles [22,26,27,134,139]. This is true for both acute exercise bouts as well as chronic exercise training [22,139]. Coles et al (2004), have shown that 2 hours of running on a treadmill at 21m/min and 15% grade increased MCT1 and MCT4 protein content in rat skeletal muscles [22]. Immediately after the single exercise session, MCT1 mRNA and protein increased in red and white gastrocnemius as well as the soleus [22]. However, MCT4 protein was only increased in red gastrocnemius and soleus muscles, this may have been due to the abundance of MCT4 already present in white gastrocnemius [22,136]. These increased protein contents were detected for 12 to 24 hours post exercise [22]. This shows that acute exercise is able to rapidly stimulate changes in MCT expression in certain skeletal muscles.

The effect of acute exercise on MCTs has not been thoroughly examined in the brain. To our knowledge, only one paper has studied this topic to date. A recent study by Takimoto et al. (2014), showed that acute exercise can have effects on MCT expression in various brain regions [21]. This study used Sprague-Dawley rats that had run on a treadmill for 2 hours at 20m/min on an 8% grade, which represents an intensity of about 50-70% of their VO_{2max}. The rats were sacrificed using focused microwave irradiation, which stops all enzymatic activity to prevent changes in lactate concentrations post-mortem. Different brain areas were removed and western blotted for MCT1, MCT2, and MCT4 and then compared to non-exercised rats [21]. Although MCT1 expression was increased in the cortex and hippocampus, there was no change in MCT1 in the hypothalamus [21]. MCT2 was increased in the hypothalamus at 5, 10, 18, and 24 hours post exercise as well as the hippocampus at 0, 5, and 10 hours post exercise and cortex at 5, 10, 18, and 24 hours post exercise [21]. MCT4 was increased only within the hypothalamus at 5 and 10 hours post exercise [21]. MCT2 and 4 are exclusively expressed in neurons and astrocytes respectively, and indicate a potential for increased lactate flux through the ANLS. This could provide a mechanism for the counter-regulatory response to hypoglycemia following exercise similar to that seen by infusions of lactate [24,25,90]. Lactate was also measured in the different brain regions (using a colorimetric assay) after varying exercise durations, and it was shown that lactate was increased in the hypothalamus after 90 minutes of exercise and after 120 minutes of exercise [21]. This paper does not, however, address whether this change in lactate was related to changes in catecholamines or glucagon peripherally (HAAF response). In addition, they did not test

the counter-regulatory response to a subsequent bout of hypoglycemia, which has been seen in other studies. Takimoto et al. (2014), also only measured MCTs in large brain areas, therefore, the increase may or may not have been localized to the VMH within the hypothalamus.

Effects of Chronic Exercise; The chronic effects of exercise training on MCT content in muscle has also been studied to determine if the changes seen acutely were transient or cumulative over time. A study by Dubouchaud et al (2000), used an exercise training model in humans [139]. These subjects exercised on cycle ergometers for 6 days a week at a heart rate estimated to be about 75% of VO_{2peak} [139]. The subjects were trained for nine weeks, and had pre and post muscle biopsies to ascertain the amount of MCTs in their skeletal muscle [139]. There was a significant increase in MCT1 and a non-significant increase in MCT4 in the vastus lateralis homogenates after training [139]. However, when sarcolemma-enriched samples were used in place of homogenates, there was a significant increase in both MCT1 and MCT4 [139]. In addition, a recent review by Thomas et al. (2011) noted that in many training studies that have examined MCT content in skeletal muscles, the more trained the individual, the greater the MCT content within their muscles [27]. However the review by Thomas et al. (2011) also suggested that MCT changes may be more related to higher intensity exercise, which may also be related to the level of training in individuals [27]. This suggests that chronic training adaptations may increase MCT protein content in skeletal muscle at least within humans.

Fewer studies have examined the effects of chronic exercise on skeletal muscle MCTs using rats (a model that could be expanded to examine brain MCTs). One

prominent study was performed by Baker et al. (1998) using 3 weeks of treadmill running at either high or moderate intensity [26]. Rats ran 5 days each week for 3 weeks at either 21m/min on an 8% grade (moderate intensity) or 31m/min on a 15% grade (high intensity) [26]. After 3 weeks the animals were sacrificed and western blotting analysis was used to compare MCT1 within the skeletal and cardiac muscles between both groups, and control animals. There was a 94% increase in MCT1 within the red gastrocnemius of the high intensity training group, but no change in the moderate intensity group [26]. However, there was no increase in MCT1 in the white gastrocnemius of either group [26]. Alternatively, MCT1 content within the cardiac muscle increased by 36% in the moderate exercise group and 44% within the high intensity group when compared to control animals [26]. A more recent study by Nikooie et al. (2013) used a 7 week training protocol to examine skeletal and cardiac MCT1 and MCT4 in rats [140]. Their training protocol consisted of speeds starting between15-20m/min for 20 minutes each day and increasing to 30m/min for 35 minutes each day by the last 2 weeks [140]. Unfortunately, the grade of the treadmill was not included in the methodology. The researchers found that exercise training had significantly increased MCT1 in sacrolemmal fractions of the extensor digitorumlongus (EDL) and soleus of the trained versus control animals, but saw no difference in cardiac muscle [140]. There were significant increases in MCT1 within the trained group for all three muscles when measuring mitochondrial fractions [140]. There was only a significant increase in MCT4 for the trained animals within the soleus [140]. Therefore, it seems that there are chronic training adaptations that increase MCT protein content within certain skeletal muscles as well as cardiac muscle.

If these acute changes accumulate over time in skeletal muscle there may also be a chronic training effect on MCT expression in the brain. A recent study by Aveseh et al. (2014), investigated the effects of 8 weeks of exercise training on MCT content in certain brain areas [20]. Using male Wistar rats, after running on treadmills 5 days/week for up to 60 minutes each day, MCT1 and MCT2 were measured via western blotting in the cortex, hippocampus, and cerebellum [20]. These were compared with sedentary rats, as well as diabetic rats that were either exercise trained or sedentary. This study showed no effect of training on MCT1 for the healthy rats, but there was an increase in MCT1 in diabetic exercise trained rats. Additionally, both diabetic groups showed higher MCT1 content in cortex and hippocampus compared to both exercise trained and sedentary control rats [20]. This same pattern was seen for MCT2. The researchers followed this by measuring the counter-regulatory response to insulin-induced hypoglycemia. The diabetic animals showed a HAAF response to the insulin-induced hypoglycemia compared to the non-diabetic animals [20]. The authors hypothesized that this blunted response was due to the increased MCT content in the diabetic animals leading to greater lactate uptake. They measured this by inducing hypoglycemia in the animals with insulin, and then giving an intraperitoneal injection of lactate. The lactate was measured in the blood and in the brain at 10, 20, and 30 minutes post-injection. At 30 minutes post-injection, there was significantly higher brain lactate in the diabetic animals compared to non-diabetic controls, and the trained diabetic animals had greater brain lactate than the diabetic controls [20]. This is congruent with studies that have shown lactate to blunt the counter-regulatory response to hypoglycemia and possibly with studies showing increased lactate

transport after antecedent recurrent hypoglycemia [23]. However, the brain lactate measures were done for the whole brain, while brain MCT measures were done in specific brain regions. Also, this may suggest that lactate comes from circulation rather than astrocytic glycogen. It is unfortunate that particular brain regions that are more integral to glucose sensing, such as the VMH, were not measured. While the HAAF response to antecedent hypoglycemia or antecedent exercise occur in both healthy and T1D individuals, there seems to be greater capacity for monocarboxylate transport among individuals with T1D compared to healthy controls [110,112]. If this is correct, then the ANLS in individuals with DM may have a higher capacity to shuttle lactate to glucose sensing neurons to increase GABA and prevent the counter-regulatory response to either hypoglycemia or exercise.

MCTs and Diabetes; There is also some evidence that MCT expression is altered in individuals with diabetes compared to healthy individuals regardless of exercise. Aveseh et al. (2014) showed that the STZ induced diabetic animals had elevated MCT content in the sampled brain regions compared to the control animals before any exercise intervention was given. Exercise training actually seemed to increase the MCT content slightly in both groups. The previous study showed an increase in brain MCT1 and MCT2 in diabetic animals following exercise training [20]. However, the change in MCT content may be due to chronic hyperglycemia or ketosis seen in STZ induced rats [141]. Another study by Canis et al. (2008) also used STZ induced diabetes to study the effect of hyperglycemia on MCT1 in the brain [141]. This study showed increases in MCT1 protein content in many brain areas of the hyperglycemic rats compared to

normoglycemic rats, including a 12.3% increase in the hypothalamus [141]. In addition, the researchers ran correlations between local cerebral glucose utilization and MCT1 content and showed an r=0.70 for hyperglycemic rats and r=0.66 for normoglycemic rats, suggesting that local glucose concentrations can influence changes in MCTs in the brain [141]. This suggests that glycemia may be more important for regulation of MCTs than diabetes per se, as other studies have induced changes in brain MCTs with high fat diets, but not diabetes [142]. A study by Pierre et al. (2007) fed mice either standard chow or high fat diet for 8 weeks and measured MCT1 and MCT2 in several brain areas [142]. The researchers measured increased MCT1 and MCT2 protein in the brain area containing the hypothalamus within the high fat diet animals compared to controls [142]. The weight, glucose concentrations, and insulin concentrations of the animals were tracked over the 8 week period, and, while the high fat diet rats were heavier and had greater glucose concentrations, they were still producing insulin, unlike STZ induced diabetic animals [142]. The researchers also examined control mice compared to db/db mice and ob/ob mice [142]. They showed, within the same brain region, that both db/db and ob/ob mice had significant increases in MCT1and MCT2 compared to controls [142]. It is also of note that the high fat diet mice, db/db mice, and ob/ob mice all had significantly increased body weight compared to controls, which may also play a role in MCT expression. Researchers also suggested that high fat diet induced some MCT4 expression in neurons, however, changes in MCT4 were much smaller than those of MCT1 and MCT2 [142]. These data seem to suggest that changes in the MCT content in brains of animals with

diabetes may be more related to chronic increased glucose concentrations in the blood and brain, or possibly increased body weight.

Other studies have shown reduced skeletal muscle MCT1 and MCT4 in STZ induced diabetic animals compared to control animals [140]. It is unknown why MCTs increase in the brains of diabetics, but decrease in muscle. It may be due to MCT's role in transporting other molecules such as β-hydroxybutyrate. In studies of exercise training and examining skeletal muscle, MCT1 content was increased in individuals and animals with diabetes after exercise training [140,143]. However, there is no consensus on whether MCT4 increases or decreases in skeletal muscle after exercise training in a diabetic model [140,143]. Again, these changes may be related to body weight or blood glucose concentrations. In the study by Nikooie et al. (2013), there was reduced MCT1 in EDL, soleus, and heart of diabetic animals, but trained diabetic animals' levels of MCT1 were preserved, and not significantly different from control animals [140]. However, the trained diabetic animals also had significantly lower blood glucose concentrations compared to the diabetic animals, and significantly lower body mass, however body mass was not different from control animals [140]. Because of the differential effects on MCTs in muscle or brain, and under different metabolic conditions, it is important to continue to investigate the effects of exercise training in different tissues.

Mechanisms of MCT Expression; The mechanisms behind the increase in MCTs in skeletal muscle are not entirely known. It has been proposed that AMPK signaling is one of the mechanisms leading to this up-regulation [144]. A study by Kitaoka et al. (2014) used rats with denervated gastrocnemius muscles, and then injected the muscle with

either saline or AICAR [AMPK activator] [144]. After seven days, the innervated muscles that had received AICAR had significantly higher MCT1 and MCT4 protein content compared to the innervated, saline treated, muscles [144]. Compared to control, there was no effect on MCT1 protein content when muscles were denervated regardless of whether AICAR treatment was applied [144]. However, the denervated muscle that received saline had significantly less MCT4 than the control, while the denervated muscle that received AICAR was not significantly different than control [144]. This indicates that AMPK may be one of the mediators for up-regulation of some MCTs, at least in skeletal muscle. It is unknown whether this would be true in brain. A study by Chenal et al. (2008) showed that IGF and insulin could increase MCT2 activity (measured by green fluorescent protein) in neuronal cell cultures [137]. This seemed to be mediated through the Akt, mTOR, p70S6K pathway [137]. However, this pathway would be inhibited by AMPK signaling via tuberous sclerosis complex 2, therefore, MCT2 (unexpressed in skeletal muscle) may be regulated differently than other MCTs or perhaps, brain MCTs may be regulated differently than muscle MCTs [145,146].

Lactate and hypoxia have also been suggested as mediators of changes in MCT protein content within skeletal muscles [147-149]. A study by Hoshino et al. (2014) examined 3 weeks of treadmill running at 25m/min for 40 minutes each day, 6 days a week in mice [149]. The mice were fed either saline or sodium lactate after each exercise session, and increased blood lactate levels were confirmed with a lactate meter [149]. The mice who received lactate showed greater MCT1 in white gastrocnemius as measured by western blotting [149]. Unfortunately, there was no sedentary group to compare, but it

seems that lactate concentrations were somewhat related to MCT1 expression in white muscle [149]. Another recent study by De Araujo et al (2015) used swim exercise in mice at the highest relative intensity to maintain blood lactate levels at a steady state [147]. After 25 minutes of swimming a group of mice were sacrificed either immediately, after 5 hours, or after 10 hours [147]. MCT1 in the heart was increased relative to control mice after 10 hours, while MCT1 in the soleus was increased at all three time points compared to control mice [147]. MCT1 in the red gastrocnemius did not change, and was only increased immediately after exercise in the white gastrocnemius [147]. MCT4 did not change in red or white gastrocnemius, but was increased in soleus immediately after exercise and 10 hours after exercise (but not 5 hours after exercise) in exercised compared to control mice [147]. However, again, the lack of a group that exercised at lower blood lactate levels makes it difficult to interpret the results of this study.

Another potential mediator of MCT expression in skeletal muscle during or post-exercise may be hypoxia. A recent study by Millet et al. (2014) compared human cyclists who trained under normoxic or hypoxic conditions (similar to altitude of ~3000 m) [150]. However, after 3 weeks of training, neither the normoxic nor hypoxic group showed significant differences in MCT1 or MCT4 within the vastus lateralis [150]. The authors suggested that there may not have been a strong enough stimulus for change in MCT content, or that the population studied (young, trained men with VO_{2Max}~58ml/kg/min) have limited room to further increase MCT content [150]. A more recent study by Saxena et al. (2016) used swim training in rats to examine the effects of exercise with or without pre-treatment with $CoCl_2$ (which stabilizes HIF-1α) [148]. The rats swam for 6 days each

week, starting at 30 minutes per day and increasing by 10 minutes each day until reaching 60 minutes [148]. The training continued for 15 days with one group receiving 10 mg/kg $CoCl_2$ 2 hours before their exercise session [148]. Only the red gastrocnemius was analyzed for mRNA as well as protein content. There was significantly more mRNA for MCT1 in the $CoCl_2$ control group compared to the saline control group, and significantly less mRNA for MCT2 and MCT4 [148]. The trained group had greater mRNA for MCT1 compared to controls, but the $CoCl_2$ trained group had significantly more than all other groups [148]. The trained group had more MCT2 mRNA than all other groups, while the $CoCl_2$ trained animals had greater than control, but less than the trained [148]. The trained animals had less MCT4 mRNA compared to controls, and the $CoCl_2$ trained animals had less MCT4 mRNA than all other groups [148]. When protein was assessed, similar patterns were seen, with the $CoCl_2$ controls having greater MCT1 than controls, and less MCT2 and MCT4 [148]. The trained animals showed greater MCT1 and MCT2 compared to controls, while $CoCl_2$ trained animals had greater MCT1 compared to all other groups, and MCT2 greater than control, but less than trained [148]. Finally, the trained animals had less MCT4 than controls, and the $CoCl_2$ trained animals had less MCT4 than all other groups [148]. The researchers also confirmed the increased amounts of HIF-1α and PGC-1α in the $CoCl_2$ treated animals [148]. Unfortunately, the researchers did not include other tissues, or even other muscles, as different adaptations may have been observed. The results are in partial agreement with other studies that examine MCTs and hypoxia. A recent study using human adipocytes showed increased mRNA for MCT1 and MCT4 but reductions in MCT2 when exposed to $CoCl_2$ [151]. This lends support to the idea that

exercise induced hypoxia may be partially responsible for increases in MCT proteins observed after exercise or exercise training.

Conclusions

There is little information about how exercise training or hypoglycemia affect the expression and overall protein content of MCTs in the VMH as well as how this may relate to the HAAF response. If there are increases in MCTs in the hypothalamus, as well as changes in glycogen content in astrocytes, there is reason to believe that the glucose sensing activities of the VMH may be affected via the ANLS. However, because lactate has been shown to blunt the counter-regulatory response to hypoglycemia, it is unknown whether these adaptations would positively or negatively affect individuals with T1D or T2D. While the adaptation seems negative, it is protective of the neurons when glucose becomes low.

While exercise is commonly prescribed to individuals with T2D to help regulate their blood glucose concentrations, individuals with DM often have problems with hypoglycemia after exercise [either immediately or delayed by hours]. In addition, tight pharmacological control of blood glucose concentrations is recommended to prevent diabetic complications [16]. Both of these treatment methods may potentially lead to increased incidence of hypoglycemia. This is due to increased glucose uptake in skeletal muscle via contraction stimulated or insulin stimulated GLUT4 translocation, as well as improved insulin sensitivity [54,152]. Whether or not some or all of the MCTs are up-regulated after either exercise or hypoglycemia may affect an individual's ability to respond to a subsequent episode of hypoglycemia. If this is the case it would give

another compelling piece of evidence for the recommendations for physical activity and exercise among individuals with T1D or T2D, as well as how glycemia should be pharmacologically managed. It may also offer a new target for pharmacological research for brain metabolism, as well as treatment of individuals with IDDM who are likely to experience HAAF in response to hypoglycemia or exercise.

CHAPTER III

CHRONIC AEROBIC TRAINING IN RATS DOES NOT ALTER MCT CONTENT WITHIN THE VENTROMEDIAL HYPOTHALAMUS

Abstract

While lactate was once considered to be a metabolic waste product, it is now well established that lactate can be used as a metabolic fuel by several tissues to produce pyruvate and enter the TCA cycle. Several tissues have been shown to "shuttle" lactate to other tissues for energy. This has been shown in skeletal and cardiac muscles as well as within the brain. Lactate is shuttled between cells via monocarboxylate transport proteins (MCTs). Acute aerobic exercise as well as short term training has been shown to increase MCTs within cardiac and skeletal muscles. The effects of exercise on MCTs within the brain, particularly the ventromedial hypothalamus (VMH) which helps regulate the energy status of the body, have been inconclusive. Therefore, the objective of the current study was to determine whether 6-7 weeks of aerobic exercise training would increase MCT protein content within the VMH as well as skeletal muscles. It was hypothesized that aerobic exercise training would increase MCTs in skeletal muscle, cardiac muscle, and within the VMH. Sprague-Dawley rats (males) were acclimated to treadmill running and trained for 6-7 weeks with a progressive protocol which plateaued at the end of week 4. After 6-7 weeks of training, animals were euthanized and had their VMH, red and white gastrocnemius, and cardiac muscle removed for western blotting of MCT1, MCT2, and MCT4. While training status was confirmed via increased

cytochrome C oxidase activity in the red and white gastrocnemius of the trained versus sedentary rats (Λ =0.521, p=0.01), there were no changes in MCT1, MCT2, or MCT4 within red or white gastrocnemius, cardiac muscle, or the VMH. These findings were somewhat surprising for muscle, which have previously been shown to increase MCT1 and MCT4 in response to exercise training. However, this was somewhat congruent with another study which showed no increase in MCTs within other brain areas after 8 weeks of training. Although it is uncertain whether MCTs change acutely after exercise, there seems to be no sustained chronic increase in MCTs within the VMH after 6-7 weeks of aerobic training.

Introduction

Aerobic exercise increases the metabolic demands on the body, requiring increases in ATP production to maintain muscle movements and cardiac output. As exercise intensity increases, increased glycolysis can lead to the production of lactate and protons, as well as local hypoxic conditions. Monocarboxylate transport proteins (MCTs) move a lactate molecule and a proton across cell membranes. Different MCTs are expressed in different cell types to enhance either uptake or efflux of lactate (and potentially other molecules such as ketones) [121,134,153-156]. Highly glycolytic tissues, such as white skeletal muscle, express more MCT4 to export lactate during high glycolytic activity while more oxidative muscle fibers express higher concentrations of MCT1 to allow import of lactate to be used for oxidative phosphorylation [121,134]. It has been shown that acute and short term exercise training leads to changes in MCT content in several tissues [22,26,27,139,157,158]. In addition, hypoxia or exercise with pretreatment with $CoCl_2$

increase the expression and protein content of MCTs 1 and 4 [148,151]. However, it remains controversial as to whether adaptations in MCTs occur with exclusively high intensity exercise or whether the exercise needs to be exhaustive, regardless of intensity [27].

MCTs are expressed in many tissues throughout the body in addition to skeletal muscles [134,136,159]. MCTs are even found within the central nervous system, where different cell types in the brain differentially express various MCTs [160,161]. Neurons primarily express MCT2 while astrocytes, and possibly oligodendrocytes, express MCT1 and MCT4 [104,114,160]. It has been suggested, based on the distribution of MCTs as well as other metabolic enzymes expressed in astrocytes or neurons, that lactate is produced in astrocytes via glycolysis and/or glycogenolysis and then shuttled via MCTs to neurons for oxidative phosphorylation [104,114,162]. This hypothesis has become known as the **a**strocyte to **n**euron **l**actate **s**huttle (ANLS).

Exercise has been shown to increase MCT mRNA and protein in skeletal and cardiac muscles [22,158]. Baker et al. (1998) showed that MCTs increase in several skeletal muscles, as well as cardiac muscle over a 3 week treadmill training protocol in rats [26]. Another study showed increased MCTs in skeletal muscle after only a single exercise session on a treadmill in rats [22]. However, few studies have investigated whether exercise has a similar effect on MCTs in the brain [20,21]. A study by Takimoto et al. (2014) showed rapid (within 5 hours) increases in MCT protein content after a single exhaustive exercise bout in several brain areas, including the hypothalamus [21]. However, a study by Aveseh et al. (2014) showed that exercise training did not cause increases in MCT1 or MCT2 in any areas of the brain measured in the study[20]. In consideration of these dichotomous

results, it must be noted that Aveseh et al. (2014) took measurements 48 hours after the last exercise session, which means they may have missed the more acute changes seen by Takimoto et al (2014) [20,21]. Unfortunately, while Aveseh et al. (2014) did not measure MCT changes in the hypothalamus as Takimoto et al. (2014) did, they did measured the counter-regulatory response to hypoglycemia, which Takimoto et al. (2014) did not. However, an important brain region involved in central regulation of blood glucose concentrations is located in the ventromedial hypothalamus (VMH), and this area was not assessed by Aveseh et al. (2014) [30]. Thus, a gap in the literature remains related to chronic aerobic training and the potential influence on MCT protein content within the VMH.

To fill this gap in the literature, the purpose of the current study was to determine whether 6 to 7 weeks of regular aerobic exercise leads to changes in MCT1, MCT2, or MCT4 within the VMH. In addition, this study will add to the general body of knowledge of how chronic aerobic exercise affects MCT content within the brain and skeletal muscles. It was hypothesized that aerobic exercise training would lead to increases in MCTs 1, 2, and 4, similar to observations by Takimoto et al. (2014) [21]. This was expected because the current investigation minimized the time between exercise and removal of the brain compared to the study by Aveseh et al. (2014) (5 hours post exercise versus 48 hours), and because Baker et al. (1998) showed that 3 weeks of training affected MCT1 and MCT4 protein content in cardiac and skeletal muscles [20,26].

Methods

Animal Acclimatization and Exercise Training; All male Sprague-Dawley rats were received and allowed to acclimate to the animal facility for a week before acclimation to the treadmill began. Rats were housed alone on a 12:12 hour light dark cycle. Throughout the study, the animals had ad libitum access to food from 4pm until 9pm (mid dark cycle until 1 hour before light cycle). The animals were also fed 90 minutes after their exercise (or sedentary) bout (1 hour after the beginning of the dark cycle). The acclimation to the treadmill lasted for 5 consecutive days (from Monday to Friday) and increased the duration and intensity as follows: Day1, 5min. 10m/min, Day2, 5min. 15m/min, Day3, 5min. 15m/min and 5min 20m/min, Day4, 10min. 20m/min, Day5, 15min 20m/min. After the week of acclimation to the treadmill, rats were separated into groups based on how well they acclimated to treadmill running. Therefore, rats were not truly randomized, rather, rats that would not run on the treadmill during acclimation became the sedentary group. The rats were then run for 6-7 weeks with increasing intensity until plateauing during week 4 (Table 3.1). The treadmill remained at an incline of 10.5% throughout the entire study. The sedentary rats were placed on a stationary treadmill, in the same room and at the same time of day, during the exercise period each day. The exercise sessions were scheduled to end at the same time each day (approximately 10 AM) as the duration increased. Therefore, the exercise occurred in the last hour of the rat's light cycle, while they were over-night fasted.

Table 3.1. The Progressive Endurance Exercise Training Protocol.

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Week 1	15min. 20m/min	25min. 20m/min	25min. 20m/min 5min. 25m/min	20min. 20m/min 10min. 25m/min	25min. 15m/min 15min. 25m/min
Week 2	25min. 15m/min 15min. 25m/min	30min. 25m/min	35min. 25m/min	40min. 25m/min	45min. 25m/min
Week 3	50min. 25m/min	55min. 25m/min	60min. 25m/min	60min. 26m/min	60min. 27m/min
Week 4	60min. 28m/min	60min. 29m/min	60min. 30m/min	60min. 30m/min	60min. 30m/min
Week 5	60min. 30m/min	60min. 30m/min	60min. 30m/min	60min. 30m/min	60min. 30m/min

Tissue Removal and Processing; The rats were sacrificed approximately 5 hours after their last exercise session by injection of pentobarbital and having their heart removed (part of another study). The choice of the 5 hours post-exercise timeframe for sacrifice was based on Takimoto et al. (2014) which reported increases of MCT2 and MCT4 by 5 hours after acute exercise. By sacrificing 5 hours after the last exercise

session, any acute increases in MCTs should be observed. The head was immediately removed, and the brain was extracted. The VMH (including the ventromedial nucleus, the arcuate nucleus, and the median eminence as shown in figure 3-1) was removed and placed in a -80°C freezer until tissue preparation. In addition gastrocnemius, was harvested. Following ischemia reperfusion testing of the hearts for another study, the cardiac tissue was also frozen and stored for analysis.

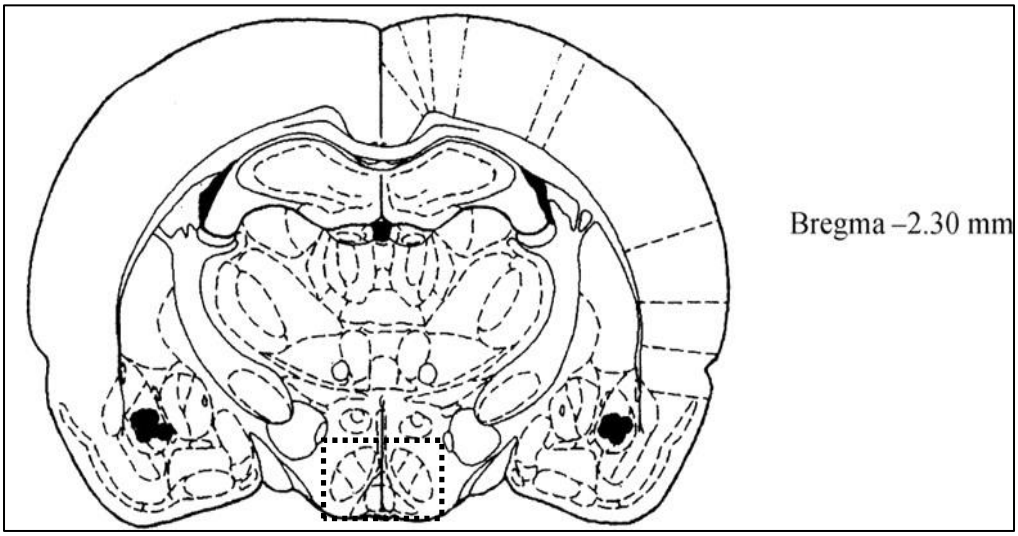

Figure 3.1 Area of Brain Taken for Analysis of MCTs within the VMH.

A sub-set from each group of rats had their gastrocnemius removed for the cytochrome c oxidase assay, as a verification that aerobic training adaptations occurred. The cytochrome c oxidase assay was performed with both red and white gastrocnemius. The muscles were homogenized in a buffer of 50mM KH_2PO_4, 0.1mM EDTA, and 0.1% Triton X-100 (pH7.4). The samples were kept constantly on ice until they were centrifuged at 4044 RPM (1,500 RCF) for 10 minutes. The supernatants were collected

and analyzed for cytochrome c oxidase activity using a Clark-type oxygen electrode and determined via polarography as performed by previous studies [163,164].

MCTs were assessed in brain tissues as well as skeletal and cardiac muscles of the trained versus sedentary rats. The VMH was homogenized with a glass mortar and pestle on ice using a RIPA buffer (appendix A). Protease inhibitors as well as phosphorylase inhibitors were also added to prevent any degradations or alterations of proteins in the lysate. After homogenization, the samples were sonicated 3 times each to further disrupt any cell membranes. The samples were centrifuged for 15 minutes at 14,000 RPM (18,407 RCF) afterward, supernatants were collected and stored at -80°C until used for western blotting. Gastrocnemius, soleus, and cardiac muscles were homogenized in RIPA buffer, including phosphatase and protease inhibitors, using a Pro Scientific homogenizer (Pro200, Pro Scientific Inc. Monroe, CT) and then sonicated 3 times each. The lysates were centrifuged for 15 minutes at 14,000 RPM (18,407 RCF). The supernatants were collected and stored at -80°C until used for western blotting analysis. Recovery rate for MCTs was estimated at 50% when comparing supernatant to re-suspended pellets.

Total Protein Assessment; A BCA assay (Pierce BCA Protein Assay Kit) was used to assess the total protein content within each sample. When running the western blotting assays, 50µg of total protein from each sample was added to each lane. Electrophoresis was run overnight at 60 volts. Proteins were then transferred to a PVDF membrane over 6 hours at 200 milliamps.

Western Blot Procedures; Samples were run for MCT1, MCT2, and MCT4 using primary antibodies purchased from Santa Cruz Biotechnology (sc-50325, sc-50323, and sc-50329 respectively). Antibodies were applied to VMH samples in 5% milk at 1:200 for MCT1, 1:500 for MCT2, and 1:75 for MCT4 on a rocker overnight in a 4°C cooler. Antibodies were applied to skeletal muscle samples in 5% milk at 1:200 for MCT1 and 1:250 for MCT4 on a rocker overnight in a 4°C cooler. Antibodies were applied to cardiac muscle samples in 5% milk at 1:500 for MCT1 on a rocker overnight in a 4°C cooler. A secondary antibody for all samples used anti-rabbit (Pierce 31460) and was applied in 5% milk at 1:5000 on a rocker for 2 hours at room temperature. Blots for VMH were exposed in a BioRad Chemidoc station for 140 seconds for MCT1, 200 seconds for MCT2, and 200 seconds for MCT4. Blots for red and white gastrocnemius were exposed for 160 seconds for MCT1 and 140 seconds for MCT4. Blots for cardiac muscle were exposed for 200 seconds for MCT1. After initial exposure, PVDF membranes were stripped and probed with primary antibody for GAPDH (sc-166545). Antibody was applied in 5% milk at 1:4000 on a rocker overnight in 4°C cooler. Secondary antibody for anti-mouse (Pierce 31450) was applied in 5% milk at1:2000 on a rocker for 2 hours at room temperature. Blots for GAPDH were exposed in a BioRad Chemidoc station for 40 seconds. In addition, there were a subset of rats whose white and red gastrocnemius and cardiac muscles were blotted with the same concentration of antibody and exposure time to examine MCT1 distribution across tissues with different oxidative capacity.

Bands from western blots were quantified using densitometry tools on Quality One software. The same researcher performed all densitometry without knowing which bands represented which animals. A ratio of MCT to GAPDH was determined, and a natural log transformation was used to normalize all data, to account for non-normal distribution of ratio data, and shifted up by 10 arbitrary units (ie, ln[(MCT:GAPDH)]+10). Further description of the western blotting procedure can be found in Appendix B.

Statistical Analyses; Statistics were run using SPSS version 23 (IBM, Armonk, New York). MANOVAs were used as an overall test of significance for the multiple dependent variables. If MANOVAs were significant, follow up ANOVAs were run for each dependent variable for interpretation. A MANOVA was run for cytochrome c oxidase activity in red and white gastrocnemius of exercise trained and sedentary rats. A MANOVA was run for MCT1 and MCT4 in red and white gastrocnemius in exercise trained and sedentary animals using body mass as a covariate. A MANOVA was run for MCT1, 2, and 4 in the VMHs of exercise trained or sedentary rats, using body mass as a covariate. Follow up ANOVAs were run for any significant MANOVAs. One-way ANOVAs were run for cardiac MCT1 and body mass between exercise trained and sedentary rats. An additional one-way ANOVA was run for a sub-sample of tissues of varying oxidative capacity (white and red gastrocnemius and cardiac muscle) for MCT1 with a Tukey post-hoc test. ANOVA, F and p values are reported for all tests, but only ANOVAs within significant MANOVAs should be interpreted. Significance was set at $p=0.05$ *a priori*.

Results

A total of 24 rats started the training paradigm. All but one of the rats completed the training protocol. A trained rat was injured and could not continue to run, this rat was excluded from the final analyses. There was not a statistically significant difference in body mass between the trained and sedentary group, although this comparison was close to significance (374.2 ± 7.4g vs.399.6 ± 10.3g respectively) ($F=3.95$, $p=0.053$). A sub-set of the trained group of rats (n=9) were shown to have increased cytochrome c oxidase activity within both the red and white gastrocnemius compared to a sub-set (n=9) of the sedentary group ($\Lambda=0.338$, $p<0.01$). There was a significant improvement in cytochrome c oxidase activity in the trained group in red gastrocnemius ($F=27.967$, $p<0.01$) as well as within the white gastrocnemius ($F=11.579$, $p<0.01$) as shown in Figure 3.2. When controlling for body mass, there was a significant overall effect on cytochrome c oxidase activity ($\Lambda =0.521$, $p=0.01$). However, when comparing this effect in individual tissues, the effect of body mass on cytochrome c oxidase activity was only significant within the white gastrocnemius ($F=12.537$, $p<0.01$). Finally, there was no difference in gastrocnemius mass : body mass ratio among this subset of runners (5.42) and sedentary rats (5.10) ($t=-1.13$, $p>0.05$).

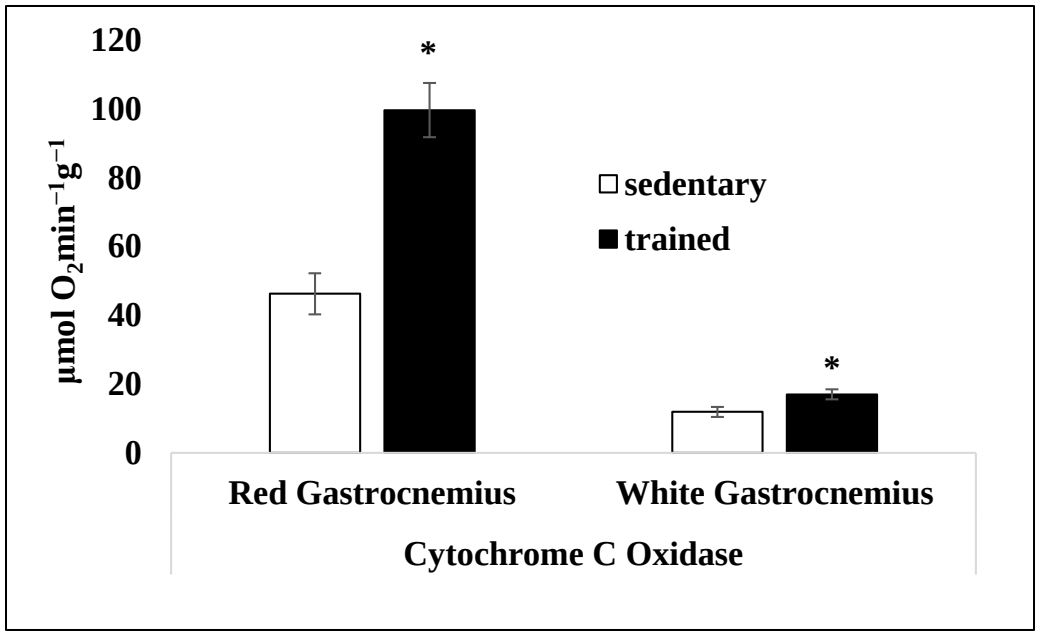

Figure 3.2. Cytochrome C Oxidase Activity in the Gastrocnemius.

(indicates difference from sedentary group, p<0.05)*

There was no overall (MCT1 red, MCT4 red, MCT1 white, MCT4 white = group | body mass) statistical difference between the trained and sedentary group for MCT1 and MCT4 protein concentration within the red and white gastrocnemius (Λ =0.924, p=0.508). Body mass was an overall significant covariate for both MCT1 and MCT4 within red and white gastrocnemius (Λ =0.611, p<0.01). The significant effect of body mass was seen in the follow up ANCOVA for MCT4 in both red and white gastrocnemius (F=14.217, p<0.01, F=7.242, p=0.01 respectively). Body mass was also significantly associated with MCT1 in red, but not white, gastrocnemius (F=18.266, p<0.01). While there was a statistically significant difference between red and white gastrocnemius for MCT1 protein content across both groups (p<0.01), there was no significant effect of training between the groups (F=0.182, p=0.672) as shown in Figure

3.3. There was no statistically significant difference in MCT1 in cardiac muscle between a sub-set of trained and a sub-set of sedentary rats (F=0.139, p=0.712) as seen in figure 3-3. However, there was a statistically significant difference between red gastrocnemius and white gastrocnemius for MCT1, and further significant difference between cardiac muscle and both red and white gastrocnemius (as shown in figure 3.4).

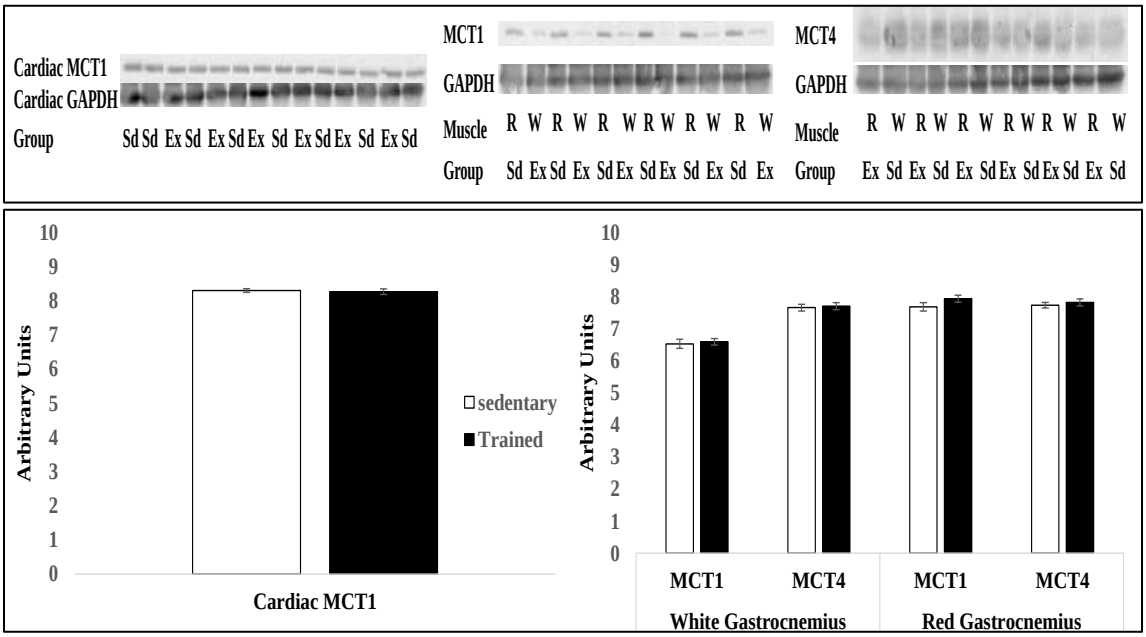

Figure 3.3. MCT Protein Content in Red and White Gastrocnemius and Cardiac Muscle between Trained and Sedentary Rats.

There were no overall significant group differences (MCT1, MCT2, MCT4 = group | body mass) in MCTs within the VMH between the trained and sedentary group (Λ=0.955, p=0.579), and body mass was not a significant covariate (Λ=0.984, p=0.878) as shown in Figure 3.5. There was no significant difference in follow up ANCOVAs in

MCT1, MCT2, or MCT4 in the VMH between exercise trained and sedentary animals (F=0.105, p=0.747, F=0.261, p= 0.612, and F=1.426, p=0.239 respectively).

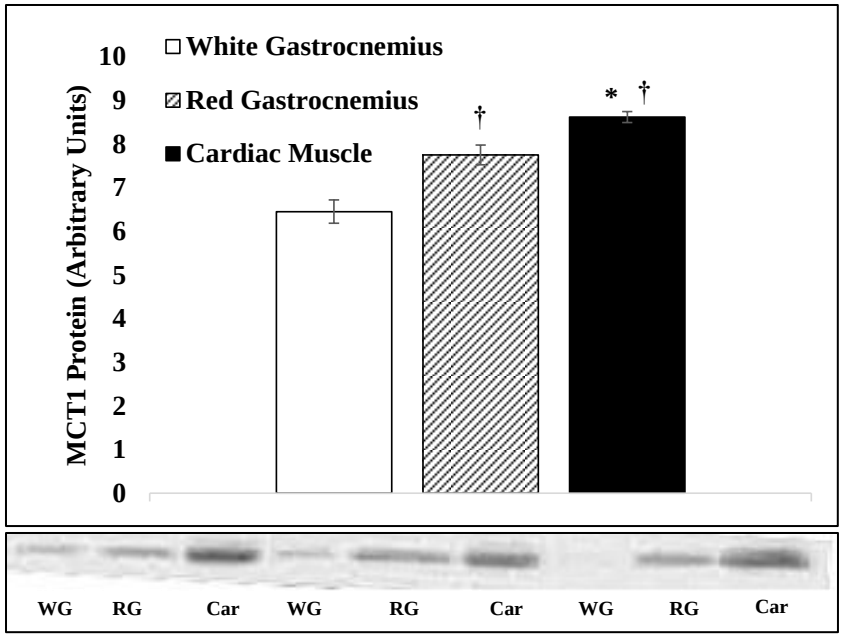

Figure 3.4. MCT1 Protein content in Red and White Gastrocnemius and Cardiac Muscle.

(indicates p<0.05 difference from RG, † indicates p<0.01 difference from WG)*

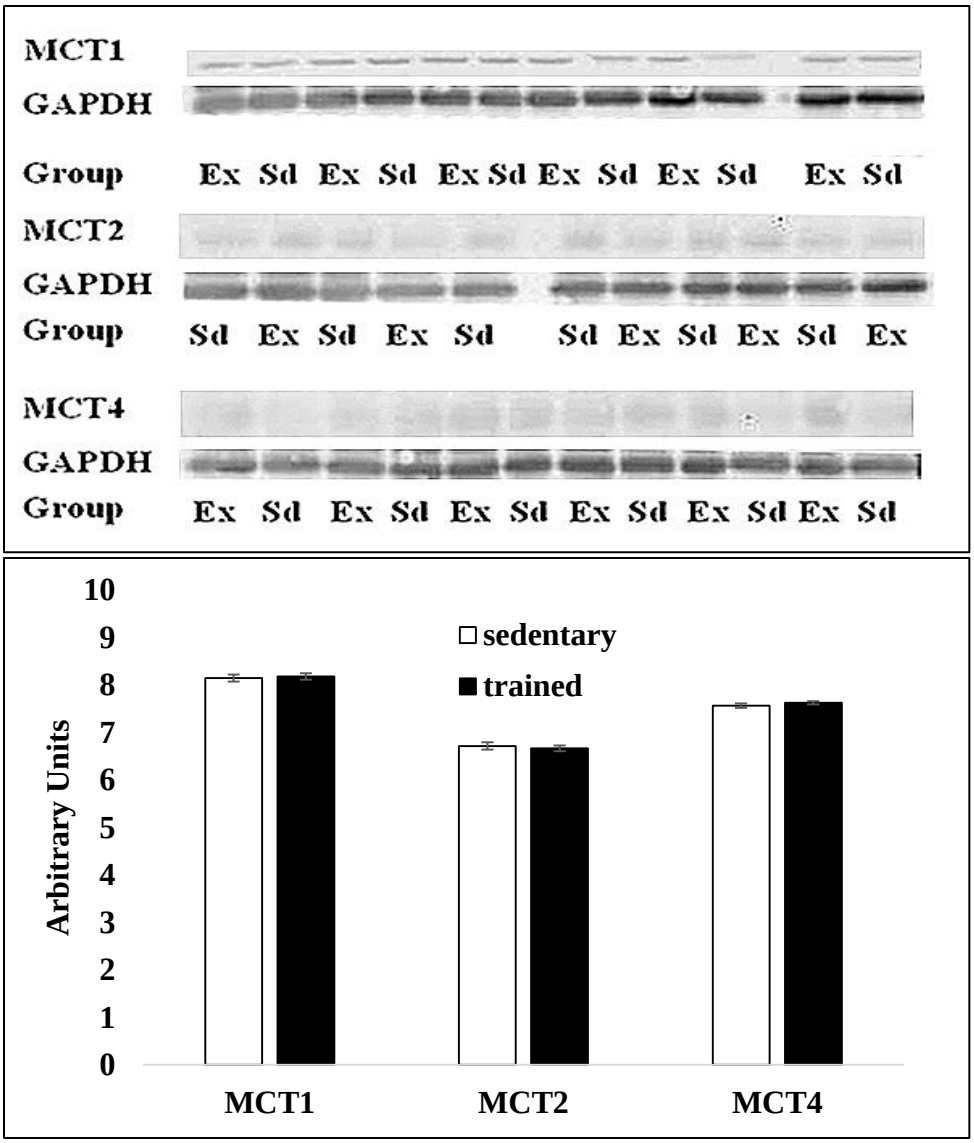

Figure 3.5. MCT Protein Content within the VMH between Trained and Sedentary Rats.

Discussion

In the current study, 6-7 weeks of progressive exercise training in rats did not result in significant differences in MCTs within the VMH of exercise trained animals compared to sedentary animals. These findings are congruent with those of Aveseh et al.

(2014) who showed no changes in MCT1 or MCT2 in cerebral cortex, cerebellum, and hippocampus after 8 weeks of aerobic training [20]. The current study's findings were in spite of strong evidence that aerobic adaptations had occurred, as determined by the increased cytochrome c oxidase activity in the trained group (figure 3.2). This made the findings that skeletal muscle MCTs were not affected by training even more surprising, as Baker et al. (1998) had previously reported that skeletal muscle MCTs were increased after 1, 2, and 3 weeks of aerobic training [26]. Because of these discrepancies, the current study workloads were compared to previous work in an attempt to understand the increased training, but lack of increased MCT content.

Exercise training has been shown in several studies to increase MCT content in skeletal and cardiac muscle, however no increases in MCTs were seen in either of these tissues in the current study [22,27,158]. The lack of increases in MCT protein content in brain, skeletal muscle, or cardiac muscle may be explained by the exercise intensity utilized in the current study. Treadmill speed and grade were used to estimate a workload in METS (using 3.5ml/kg/min as the equivalent of 1 MET) to compare between previous studies using treadmill training in rats, as seen in figure 3.6. The current study's intensity was approximately 3.49 METs at the end of the 3-week ramping protocol and this intensity was maintained over the next 3-4 weeks. A study by Baker et al. (1998) found that 2.46 METs was inadequate to increase MCT1 in red or white gastrocnemius over 3 weeks of training [26], but that approximately 4.29 METs would increase MCT1 in red, but not white gastrocnemius [26]. The lack of change in cardiac MCT1 was also unexpected due to the same study detecting increased MCT1 in cardiac muscle using only 2.46 METs

over 3 weeks [26]. However, the current study did find the stepwise increase in MCT1 in increasingly oxidative muscle tissue (Figure 3.5), which has been shown by other studies [114,136,157].

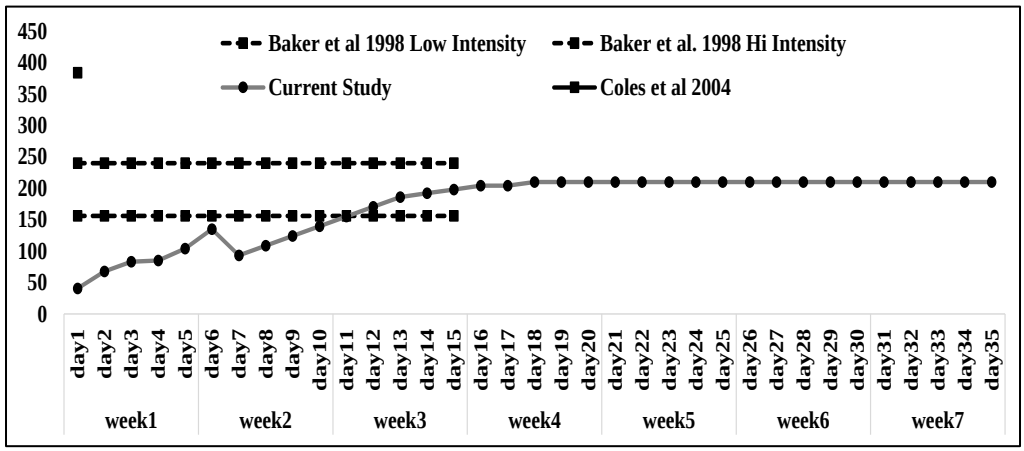

Figure 3.6. Comparison of Rat Treadmill Training Studies and MCTs in Muscles by Daily Workload Expressed in MET Minutes/Day.

Both Takimoto et al. (2014) and a more recent study have shown increased MCT expression in skeletal muscles after a single exercise bout [22]. A study by Coles et al (2004) showed an acute increase in MCT1 mRNA and protein in red and white gastrocnemius and MCT4 mRNA and protein only in red gastrocnemius using approximately 3.23 METs [22]. However, this acute study used an exhaustive exercise bout, and thus, a less intense effort may have been required to cause MCTs to be up-regulated. Although the METs in the study by Coles et al. (2004) were lower than in the current study, their longer duration translated to more MET minutes/day. These results suggest that when designing exercise training protocols to investigate MCT changes, the important factor may be the overall workload of the exercise.

While intensity and duration seem to be important for MCT adaptations in skeletal and cardiac muscle, METs may not be the best metric for comparing protocols. METs are an absolute measure of intensity, and 2 individuals, of differing fitness levels, performing exercise at the same MET are working at different relative workloads. Considering the proposed and documented role(s) of MCTs in various tissues, it is reasonable to assume that exercise workloads that do not induce lactate production or that do not require the tissue to rely on an alternate fuel source, are not likely to stimulate changes in MCT protein content. The lack of changes in MCTs within muscles between exercise trained and sedentary rats within the current study may have been due to early training adaptations that were lost after more robust training adaptations had occurred. Due to increases in cytochrome c oxidase activity, the muscles likely do not need to increase lactate shuttling, because it can more effectively perform oxidative metabolism and less lactate is produced. Therefore, ever increasing workload (either by increasing intensity or duration or both), may be necessary to see sustained alterations in MCTs.

While short-term training protocols in rats use a fixed workload, the rats' relative workload over the course of the study changes as they adapt (ie, high percentage of capacity at the beginning and a lower percentage at the end). It may be that the degree of physiologic overload is important to the expression of MCTs within skeletal and cardiac muscle [27]. For example, a study by Hoshino et al. (2014) showed that aerobic training supplemented with exogenous lactate produced greater increases in MCT1 than aerobic training alone [149]. Another study by Saxena et al. (2016) used rats to show that hypoxia preconditioning with cobalt chloride ($CoCl_2$) supplementation increased MCT1 in red

gastrocnemius significantly more than a group given distilled water [148]. In addition, a hypoxia preconditioned swim trained group increased MCT1 in red gastrocnemius to a greater extent than swim training alone [148]. This is in agreement with studies that have shown that HIF1α can stimulate MCT mRNA and protein, and suggests that higher intensity exercise, which increases lactate and causes hypoxia, may enhance MCT expression [151]. The current study may have elicited this response early in training (1-4 weeks), but without continuing to increase the intensity of exercise, the adaptations may have been lost. This "de-training" effect has been shown when hypoxia is removed from adipocyte cultures expressing MCTs [151].

There are far fewer studies that have investigated MCT protein content in the brain in response to exercise, which makes the current results difficult to interpret. The current study conflicts with one acute study by Takimoto et al. (2014), but is in agreement with a chronic training study by Aveseh et al. (2014). There are several reasons that these discrepancies may have occurred. First, it is possible that the increases in MCTs were present in the entire hypothalamus, as reported by Takimoto et al. (2014), but not to an appreciable degree within the specific region of the VMH. Another possibility is that the increase in hypothalamic MCTs occurs on an acute basis only, but then returns to baseline as animals adapt to training. In contrast, it is possible that the training protocol used in the current study was not of sufficient intensity to stimulate a response in the MCTs. The study by Takimoto et al (2014) used only a single bout of exercise at 20m/min and 8% grade, an intensity that is lower than the current study [21]. However, the single bout lasted for 120 minutes, twice as long as any single bout within

the current study's training schedule, among naive rats [21]. When considering the overall workload, the Takimoto et al (2014) study provided a significantly greater acute stimulus than any single exercise session in our study. Lastly, it is possible that the antibodies used by Takimoto et al. were more sensitive to their respective MCT, and were therefore able to measure a change in MCT content that the current study could not.

In addition to MCT protein content, a study by Aveseh et al. (2014) also examined the counter-regulatory response to hypoglycemia. While exercise training has not been shown to cause a blunting of a subsequent counter-regulatory response to hypoglycemia, acute exercise has been shown to produce this response. It is unknown whether acutely increased MCTs from exercise could contribute to this blunted response by allowing increased lactate shuttling to neurons, and preserving relative energy levels therein. It is known that lactate administration within the VMH can cause a blunted response to hypoglycemia [24,25,90]. The study by Aveseh et al. (2014) showed a blunted counter-regulatory response to hypoglycemia only among the diabetic animals, regardless of training. Both the trained and untrained (uncontrolled) diabetic animals had elevated MCTs within the brain, although their study did not measure MCTs within the hypothalamus [20]. It was also shown that the exercise trained animals and un-trained controls had a normal counter-regulatory response, and lower MCT content within the brain [20]. Therefore, MCTs and lactate may still be related to the antecedent exercise induced diminished response to subsequent hypoglycemia, but aerobic training does not seem to produce this effect.

It is of note that the total content of MCT protein does not indicate the activity of MCT proteins. MCT protein content within the VMH may already be adequate to shuttle lactate or other monocarboxylates without having to increase transcription and translation to produce more transporters. This is possible due to the fact that lactate shuttling should not be occurring under all conditions, but stimulated to occur by increased activity. Increased glutamatergic signaling leads to the stimulation of GluR on neurons which cause MCT2 to translocate to the cell surface. At the same time, Na coupled glutamate uptake by synaptisomal projections on astrocytes leads to increased glycolytic rate to increase NaK_{ATPase} pumps. Only under these high glycolytic conditions would the lower pH and increased lactate concentration overcome the km of MCT4 to export lactate. Because the lactate flux was not measured, it is unknown whether increased shuttling occurred during the current study.

Besides the potential changes in flux versus content, MCT proteins may be affected by other monocarboxylates which they can transport. The feeding protocol during the current study may have led to increased ketone body production in the animals during fasting conditions or after exercise. It has been shown that Sprague Dawley rats become ketotic after a 24 hour fast, just 10 hours more than was used in the current study [165]. This may have masked exercise induced changes in MCTs, due to the MCTs' role in transporting ketone bodies into the brain. However, overnight fasting combined with exercise leads to an exaggerated increase in ketone bodies post-exercise compared to overnight fasting and rest [166]. Therefore, the current study's groups should still have been

affected differently if the MCTs were being influenced by ketone body production in addition to exercise induced lactate and hypoxia.

A potential source of error when comparing the findings of the current study to other studies are the antibodies and procedures used for western blotting and quantifying data. The current study used commercially available antibodies for MCT1, MCT2, and MCT4; while most other studies use antibodies generated in their own lab. This makes data replication difficult, if not impossible, because other labs do not have access to the same materials. While all antibodies should specifically bind their target (MCT1, 2, or 4) there is marked differences in different antibodies [136]. This is particularly true for band intensity, which is used to quantify protein data via densitometry. In addition, different densitometry methods can be used which would affect the quantities obtained. However, the current procedures were able to measure differences in different muscle and cardiac tissue types (more or less oxidative) and changes up to 10-20% in controlled tests. Therefore, if the current study samples were analyzed with different methodology, slight differences may be expected, however, no differences between groups would likely be observed.

In summary, the data from the current study partially supports the findings of Aveseh et al. (2014) [20]. The findings by Takimoto et al. (2014), while not a training study, indicated increased MCTs in the hypothalamus 5 hours after an exercise bout [21]. Although the current study took brain samples approximately 5 hours post-exercise, no increase was apparent. This may indicate that MCT changes may occur acutely and eventually return to baseline. Future studies should attempt to determine whether acute

exercise increases MCT protein content within the VMH. In addition, rats that have acutely exercised (within 24 hours) should be given a hypoglycemic challenge to confirm that the antecedent exercise induced HAAF response is consistent in rats as it is in humans. Independent MCT inhibitors (such as oligodeoxynucleotides, or shRNAs) could be used to block specific MCTs to determine whether their removal corrects the HAAF response rather than using 4CIN, which is a less specific inhibitor. The VMH may also be tested after an animal is exposed to hypoxia or $CoCl_2$ to determine if VMH MCTs are responsive to HIF1α as in other tissues, and whether this produces a HAAF response.

CHAPTER IV

RECURRENT HYPOGLYCEMIA, LEADING TO HYPOGLYCEMIA ASSOCIATED AUTONOMIC FAILURE, DOES NOT ALTER MCT CONTENT WITHIN THE VENTROMEDIAL HYPOTHALAMUS OF RATS

Abstract

An increasing number of Americans suffer from diabetes mellitus, which leads to several diabetic health complications if blood glucose is not properly managed. The Diabetes Complications and Control Trial showed that tightly controlling blood glucose concentrations through aggressive medication can prevent several diabetic complications, however it also leads to increased incidences of hypoglycemia. If two hypoglycemic incidents occur in relatively short succession (24 hours) the second instance is likely to have a diminished hormonal response to correct the hypoglycemia. In addition, the deeper the antecedent hypoglycemia, the more severe the suppression of the subsequent counter-regulatory response. This can lead to a downward spiral of ever worsening hypoglycemia among individuals with insulin dependent diabetes. The loss of responsiveness to repeated episodes of hypoglycemia has been hypothesized to be related to changes in energy substrate utilization by neurons within the ventromedial hypothalamus. In particular, glial cells may use monocarboxylate transport proteins to shuttle lactate to neurons to supply them with energy when deprived of glucose. Therefore, the objective of the current study was to determine whether recurrent antecedent hypoglycemia leads to changes in monocarboxylate transport proteins within

the ventromedial hypothalamus. It was hypothesized that the monocarboxylate transport proteins would be increased by recurrent antecedent hypoglycemia. To determine whether monocarboxylate transport proteins change, male Sprague-Dawley rats were given subcutaneous injections of either insulin (RH) or saline (C) on 3 consecutive days before having their ventromedial hypothalamus removed. A subset of these animals from both groups were given a hypoglycemia challenge on day 4 and had blood samples taken at 0, 15, 30, 60, 90, and 120 minutes post insulin injection. These blood samples were analyzed for counter-regulatory hormones (glucagon, epinephrine, and norepinephrine), to assess whether the RH was sufficient to suppress the counter-regulatory response. It was shown that despite 3 days of recurrent hypoglycemia, there were no changes in the monocarboxylate transport proteins within the ventromedial hypothalamus. There was no significant difference in glucagon response among the subsets of rats on day 4, although there was a significantly lower glucose AUC for the RH group. Because there were no changes in monocarboxylate transport proteins after recurrent hypoglycemia, it seems unlikely that these transport proteins are required to change in order to suppress the counter-regulatory response to hypoglycemia.

Introduction

While many organs in the body contribute to glucose homeostasis, the central nervous system is integral to proper responses to hyperglycemia and hypoglycemia [30,68,84,88]. However, hypoglycemia poses an acute health risk, particularly for individuals with insulin dependent diabetes mellitus (IDDM) [12,15,70]. Severe hypoglycemia can lead to increased macro and micro vascular events as well as cardiovascular dysrhythmias and

death [70,167]. In addition to the acute risks of hypoglycemia, each hypoglycemic episode seems to reduce the responsiveness to a subsequent hypoglycemic episode [12,13,70,78]. The risk of subsequent reduced responsiveness seems to be related to the depth of the subsequent hypoglycemia rather than the duration [66,69]. This phenomenon has been referred to as hypoglycemia associated autonomic failure, or dysfunction, (HAAF) which can develop into a dangerous cycle of recurrent hypoglycemia [15].

While several brain areas have been identified as important to this process, there has been particular interest in studying the ventromedial hypothalamus (VMH) [25,30,67]. The VMH coordinates the response to hypoglycemia, however the exact mechanisms are currently unknown [30]. When the VMH is treated with 2deoxyglucose (2DG) to induce glucoprivic conditions locally, there is a systemic counter-regulatory response to increase blood glucose concentrations [82,168]. In contrast, a counter-regulatory response to systemic insulin induced hypoglycemia can be attenuated with local administration of glucose into the VMH [169]. Therefore, the VMH seems to respond to local glucose concentrations to regulate systemic glucose concentrations via sympathetic efferents [30,67,87,100]

Although the VMH responds to glucose concentrations, the response may not be specific to glucose. It has been shown that administration of lactate, rather than glucose, can lead to similar attenuation of the counter-regulatory response to systemic insulin induced hypoglycemia [24,25,90]. This is most likely due to the energy state of the neurons within the VMH, which can take up lactate via monocarboxylate transport proteins (MCTs) to be used for oxidative phosphorylation [30,87,88,90,93,170,171]. The sensing of the energy state could be mediated through K_{ATP} channels similar to pancreatic β–cells

[87,88,171]. Indeed, both neurons and glial cells express various MCTs which have different affinities for lactate and other monocarboxylates, which may favor lactate uptake by neurons.

The expression pattern of MCTs, as well as other metabolic enzymes, within the brain has led to the hypothesis that glycolysis or glycogenolysis derived lactate can be shuttled from astrocytes to neurons to support oxidative phosphorylation [113,133,162]. This model has become known as the astrocyte to neuron lactate shuttle hypothesis (ANLS). The ANLS model has been used to explain how neurons co-cultured with glycogen rich astrocytes can be stimulated longer than those co-cultured with glycogen depleted astrocytes, or how neuronal activity during hypoglycemia can be maintained in the presence of lactate [23,28,120,123,133,162]. This may mimic the glycogen depletion during hypoglycemia and the super-compensation that follows [109,111,112]. In addition others have recently reported that recurrent antecedent hypoglycemia can lead to enhanced lactate transport in the brain compared to control animals [23]. However, no measurement of lactate transporters or lactate-related metabolic enzymes were measured. It is, therefore, of interest to know whether lactate is shuttled to neurons from blood glucose or from astrocytic glycogen, leading to an attenuated counter-regulatory response to hypoglycemia after antecedent recurrent hypoglycemia.

To approach this question, the current study measured changes in the different MCTs (1, 2, and 4) within the VMH in response to 3 consecutive days of systemic insulin induced hypoglycemia. While this study was not able to quantify the actual lactate flux between astrocytes and neurons, total changes in MCT proteins were assessed.

Additionally, HAAF response was confirmed through hormonal changes throughout a hypoglycemic challenge. It was hypothesized that recurrent hypoglycemia would lead to increases in MCT2 and MCT4 to facilitate increased lactate shuttling from astrocytes to neurons.

Methods

All rats were received from Charles River and allowed to acclimate to the animal facility for one week before any hypoglycemia or surgeries began. Male Sprague Dawley rats were individually housed in shoebox cages on a 12 hour light / dark cycle and given ad libitum access to food and water 24 hours a day, except during hypoglycemia exposures and blood sampling. All rats received 3 days of either hypoglycemia or vehicle (saline), while a sub-group of rats were given a 4th day of hypoglycemia to measure blood glucose and hormone response.

Surgeries; A sub-group of rats had a jugular vein catheter surgically installed before beginning their 3 days of hypoglycemia (Appendix C). The procedure, briefly, entailed making a small incision in the chest to insert a catheter into the right jugular vein. Then a port was fixed to the top of the skull to allow access for blood sampling, and to avoid the rat damaging the port. After surgeries, the rats were moved to tall cages and allowed to recover for 3 days before beginning the hypoglycemia treatments.

Hypoglycemia; After the rats had acclimated to the animal facility, they were subjected to either hypoglycemia or sham treatment for 3 consecutive days. Midway through the light cycle the food was removed from the rats' cages. They were weighed and then given a subcutaneous injection of either insulin or saline at a dose of 4.5U/kg or

0.45ml/kg, respectively. The rats were then replaced in their cages and observed to ensure that no severe reactions occurred. After 30 minutes, the rats were removed from their cages and a small amount of blood was acquired through a pedal vein stick. In the sub-group of rats with jugular vein catheters, the catheter was used to collect blood samples 30 minutes after the injection. The blood was used to measure blood glucose concentration at 30 minute post-injection using an AccuChek Glucometer (ACCU-CHEK Aviva Plus, Roche Diabetes Care, Inc., Indianapolis, IN). After hypoglycemia was confirmed, food was replaced in the rats' cages. This procedure was repeated for 3 consecutive days. After 3 days of hypoglycemia, the rats were sacrificed on day 4, 24 hours after the last hypoglycemia.

Blood Sampling; After 3 days of hypoglycemia or sham, a sub-group of rats from each group were given an injection of insulin, regardless of their previous group, to produce hypoglycemia, and measure hormonal response (Appendix D). A length of tubing was attached to the catheter port fifteen minutes before blood sampling began to avoid measuring hormonal responses to handling. The hypoglycemia was achieved as previously described using insulin injections in both the previously hypoglycemia and previously sham animals. Blood samples were taken from the catheter without touching or handling the animal at time points: -10min, 0min, 15min, 30min, 60min, 90min, and 120 min relative to the injection at time zero. Animals were handled after time point 0min to administer insulin injections. Blood samples were heparinized and centrifuged immediately and plasma was stored with protease inhibitors and immediately frozen.

Once 2 hour blood sampling was completed, the rats were sacrificed via CO_2 chamber and decapitation.

Western Blot Procedures; After rats were sacrificed, their brains were removed, and the VMH was collected and immediately frozen. The VMH was homogenized with a glass mortar and pestle on ice using a RIPA buffer (Appendix A). Protease inhibitors as well as phosphorylase inhibitors were also added to prevent any degradations or alterations of proteins in the lysate. After homogenization, the samples were sonicated 3 times each to disrupt any cell membranes. The samples were centrifuged for 15 minutes at 14,000 RPM (18,407 RCF) afterward, supernatants were collected and stored at -80°C. A BCA assay (Pierce BCA Protein Assay Kit) was used to assess the total protein content within each sample. When running the western blotting assays, 20µg of total protein was added to each lane. Electrophoresis was run overnight at 60 volts. Proteins were transferred to PVDF membranes over 6 hours at 200 milliamps.

Samples were run for MCT1, MCT2, and MCT4 using primary antibodies purchased from Santa Cruz Biotechnology (sc-50325, sc-50323, and sc-50329 respectively). Antibodies were applied in 5% milk at 1:200 for MCT1, 1:250 for MCT2, and 1:100 for MCT4 on a rocker overnight in a 4°C cooler. Secondary antibody for anti-rabbit (Pierce 31460) was applied in 5% milk at 1:5000 on a rocker for 2 hours at room temperature. Blots for VMH were exposed in a BioRad Chemidoc station for 140 seconds for MCT1, 200 seconds for MCT2, and 300 seconds for MCT4. After initial exposure, PVDF membranes were stripped and probed with primary antibody for GAPDH (sc-166545). Antibody was applied in 5% milk at 1:4000 on a rocker overnight

in 4°C cooler. Secondary antibody for anti-mouse (Pierce 31450) was applied in 5% milk at1:2000 on a rocker for 2 hours at room temperature. Blots for GAPDH were exposed in a BioRad Chemidoc station for 40 seconds.

Band intensities from western blots were quantified using densitometry tools on Quality One software. All densitometry was performed by the same researcher in the same manner, and without knowledge of treatments per lane. A ratio of MCT to GAPDH was determined, and a natural log transformation was used to normalize all data, to account for non-normal distribution of ratio data. Further description of western blotting procedure can be found in appendix B.

Hormones; Glucagon was determined using Mercodia glucagon ELISA (10-1281-01, Mercodia Developing diagnostics, Uppsala, Sweden). Briefly, 10µl of calibrators and samples were added to a 96 well plate. Then, 50µl of enzyme conjugate was added and the plate was incubated on a plate shaker at 800rpm overnight in a 4°C cooler. The next day, the plate was removed and washed 6 times on an automatic plate washer. Then 200µl of TMB substrate was added to each well and the plate was incubated for 30 minutes before adding 50µl of stop solution. The plate was briefly shaken to ensure mixing, and read on a Gen5 plate reader at 450nm.

Statistics; All statistics were run using SPSS version 23 (IBM, Armonk, New York). MANOVAs were run for MCT1, 2 and 4 for recurrently hypoglycemic and control rats using body mass as a covariate. A MANOVA was also run for percent MCT1, 2, and 4 compared to control mean for recurrently hypoglycemic and control animals using body mass as a covariate. A repeated measures ANOVA was run for the 3

days of blood glucose monitoring using an independent T-test as a follow up to compare each day. A one way ANOVA was used to compare blood glucose concentration area under the curve for they hypoglycemic challenge and for body mass.

Results

Subcutaneous injections of insulin were sufficient to produce a significant reduction in blood glucose concentrations 30 minutes post injection compared to the group receiving saline injections ($F=385.11$, $p<0.01$) as shown in Figure 4-1. There was no significant within group variance for blood glucose concentrations 30 minutes post injection ($F=2.23$, $p=0.12$). Indeed, the subset of rats that were sampled for confirmation of HAAF response showed statistically significant reduction in blood glucose area under the curve (AUC) over the hypoglycemic challenge ($F=10.57$, $p=0.04$) as shown in Figure 4.2. However, there was no significant difference in blood glucose concentration between groups for the repeated measures ANOVA ($F=5.432$, $p=0.1$) as seen in figure 4.3A. There was no significant difference in glucagon between groups in the repeated measures ANOVA ($F=0.248$, $p=0.65$), however, both the control group and RH group seemed to have a blunted glucagon response seen in figure 4.3B compared with other studies measuring the HAAF response. There was also no significant difference in glucagon AUC between groups ($F=0.21$, $p=0.68$).

There was no statistical difference in body mass between the two groups (Control=351.1g ± 28.6, RH=333.8g ± 28.7; $F=0.18$, $p=0.67$), however, there was a large range of body mass within each group.

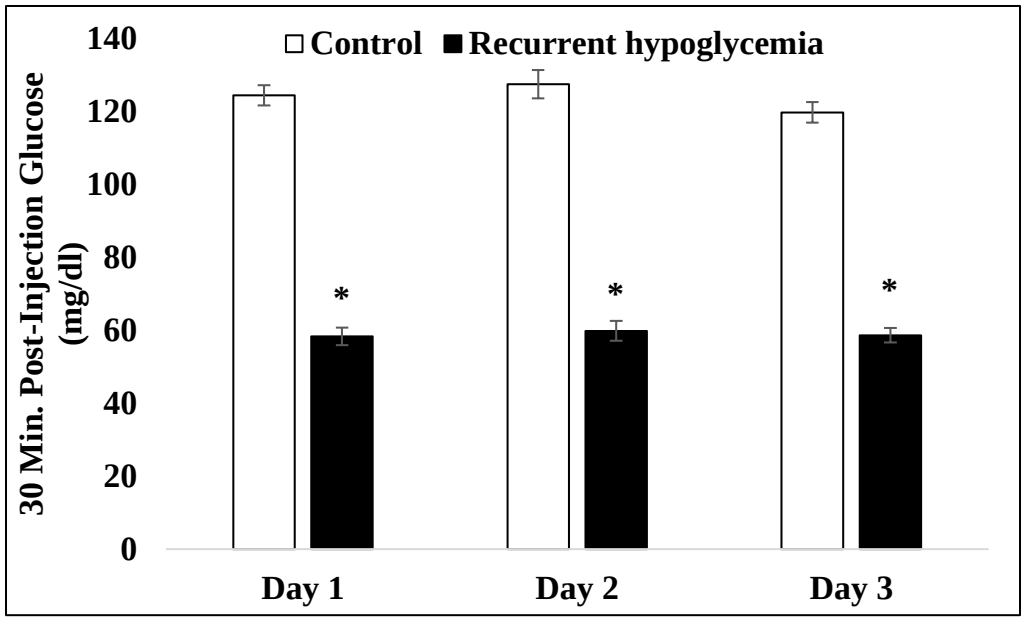

Figure 4.1. Thirty Minute Post-Injection between Control and RH Rats over 3 Days

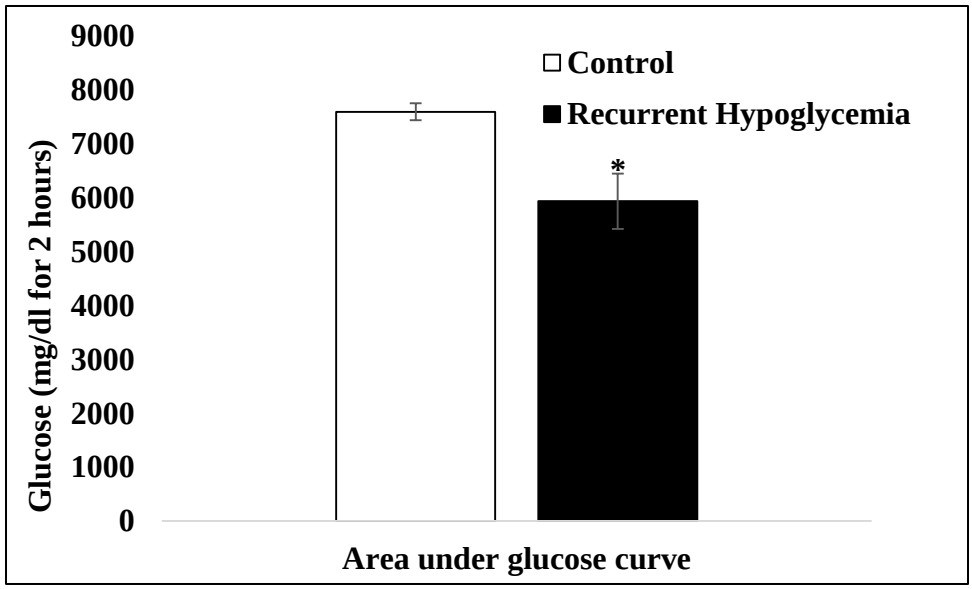

Figure 4.2. Glucose AUC for Subset of Blood Sampled Rats During Hypoglycemic Challenge. (indicates p<0.05)*

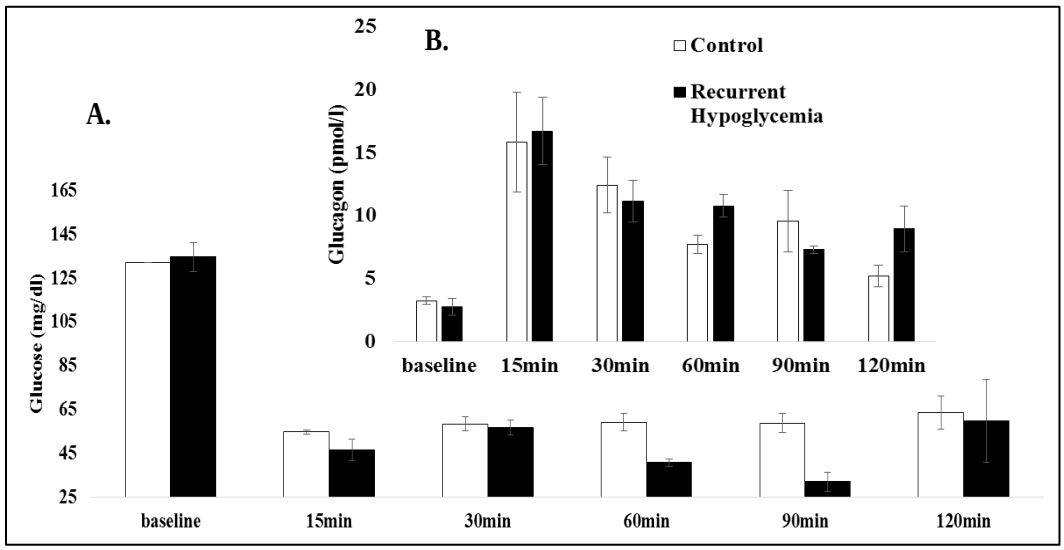

Figure 4.3. The Blood Glucose and Glucagon During the Hypoglycemic Challenge

There was no statistically significant difference in MCTs between recurrently hypoglycemic rats and control rats (Λ=0.993, p=0.95) as shown in Figure 4.4. However, there was a statistically significant difference in MCTs across different body mass (Λ=0.617, p<0.01). Follow up ANOVAs showed that there was a statistically significant effect of body mass for MCT1 (F=22.23, p<0.01), MCT2 (F=6.08, p=0.02), and MCT4 (F=15.00, p<0.01) as seen in Figure 4.5. As shown by the F statistic, the majority of variance accounted for by body mass is within MCT1 concentrations. This is also visible when the body mass and MCT concentrations are plotted out, as seen in Figure 4.5.

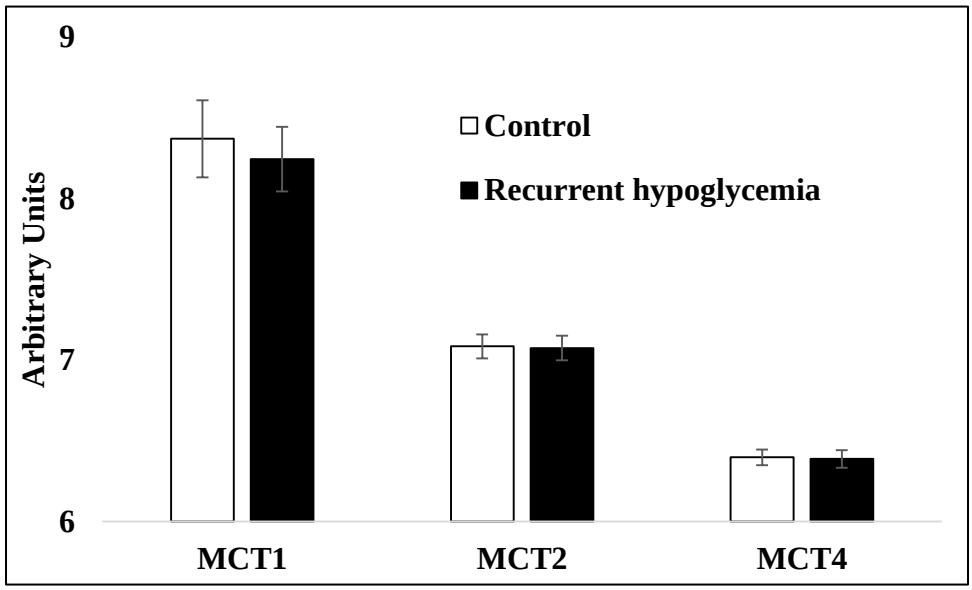

Figure 4.4. The MCT Protein Content within the VMH between RH and Control Rats.

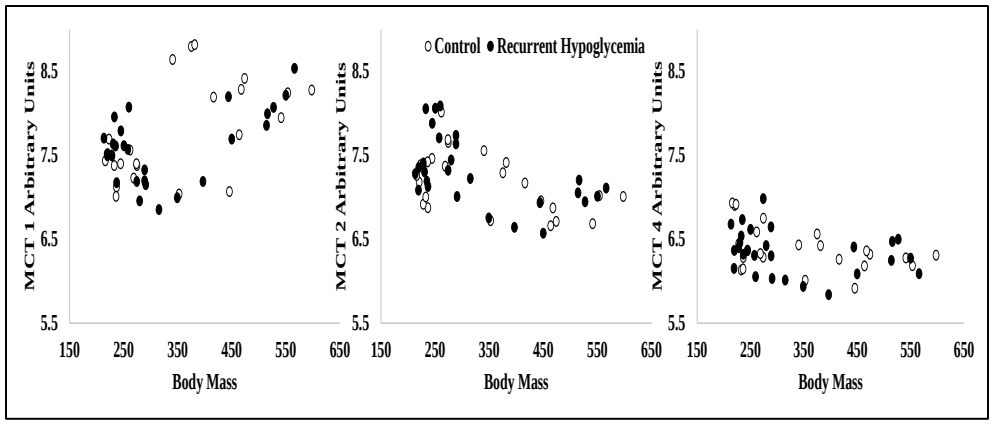

Figure 4.5. The Comparison of Body Mass and MCT Protein Content.

There was also no significant change in MCTs for recurrently hypoglycemic animals as a percent of the average control animal MCT content (Λ=0.98, p=0.86) as seen in Figure 4-6. However, body mass was still significant for percent of average control animal MCT content (Λ=0.68, p<0.01). Again, all follow up ANOVAs showed significant difference in percent of MCT1 (F=7.36, p=0.01), MCT2 (F=6.44, p=0.01), and MCT4 (F=16.44, p<0.01) across body masses.

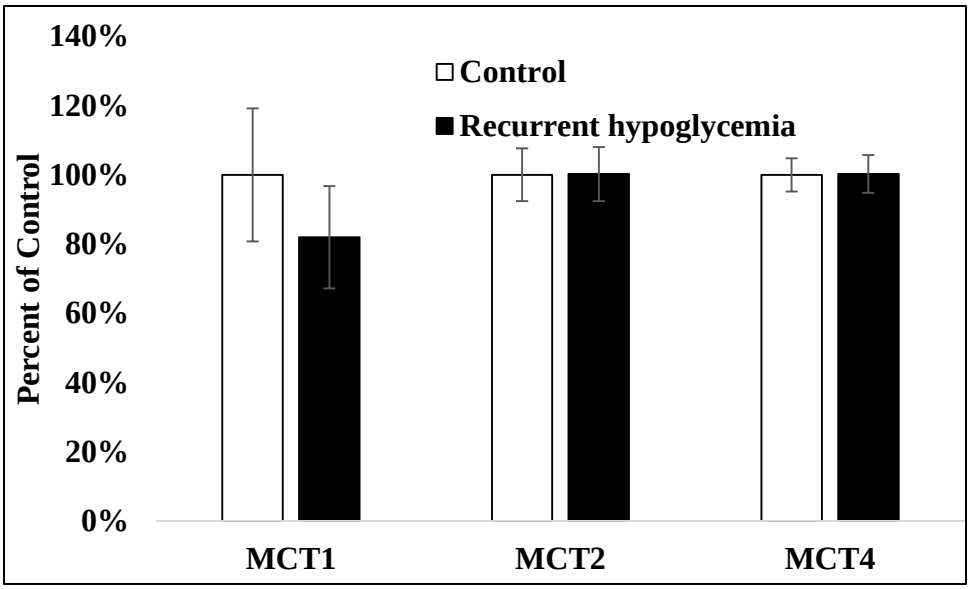

Figure 4.6. The MCT Percentage within the VMH between RH and Control Rats.

Discussion

Recurrent hypoglycemia leading to a HAAF response did not lead to changes in the overall protein content of any of the predominant MCTs within the VMH. Although it cannot be determined whether there was increased shuttling through the MCTs during the hypoglycemic episodes, there seemed to be no need for the cells to produce additional transport proteins. This finding does not confirm or deny the ANLS hypothesis. However, it seems that the lactate shuttling (if it occurs) does not require an increases in transport proteins in the VMH to cause a HAAF response to antecedent recurrent hypoglycemia. This may be because, under basal conditions, lactate concentrations do not overcome the high km of MCT4 in astrocytes. The lactate shuttling, if it occurs,

would only be under conditions of high glycolytic rates within astrocytes, potentially driven by increase glutamate and sodium uptake increasing Na/K_{ATPase} pumps.

It was reported by Herzog et al. (2013), that rats exposed to 3 days of antecedent recurrent hypoglycemia experienced a 2 fold increase in lactate transport in the brain during a fourth episode of hypoglycemia on the 4th day [23]. In addition, they showed that lactate infusion could rescue reduced brain activation during hypoglycemia in rats given antecedent recurrent hypoglycemia, but not control rats [23]. This seemed to suggest that the recurrently hypoglycemic rats had increased lactate uptake and/or metabolism compared to control animals as an adaptive response to recurrent episodes of hypoglycemia. However, there was no investigation of MCT mRNA or protein content. Despite these findings, the current study seems to suggest that, if these adaptations occur with antecedent recurrent hypoglycemia, this is more likely caused by changes in metabolic enzymes rather than MCT protein content.

It is possible that insulin induced hypoglycemia acutely affects MCT content, but then returns to basal levels [137]. It has been suggested that MCT2 expression was increased in neuronal cell culture when exposed to 1µg/ml insulin for 2 to 8 hours, but was not increased after 24 hours [137]. The authors measured mRNA and saw no changes, and concluded that MCT2 is regulated at the translational level [137]. However, the study involved insulin injections directly into the brain, rather than delivered systemically as with a hyperinulinemic hypoglycemic clamp, or normal insulin use by individuals with IDDM. Usually, insulin concentrations in plasma are about 10-20 fold higher than in cerebrospinal fluid (CSF) [172]. Insulin delivery to the brain seems to be saturable and,

regardless of insulin infusion rate, CSF insulin only reaches around 4μU/ml [173]. It is also possible that glucose sensing neurons within the VMH are responsive to insulin [170].

A recent study by Aveseh et al. (2014) did show increased MCT protein content within certain brain regions in diabetic animals compared to their healthy controls [20]. These animals showed a HAAF response to insulin induced hypoglycemia regardless of whether or not they were aerobically exercise trained. This suggested that the increased brain MCTs may play a role. However, MCTs within the hypothalamus were not measured, although the hypothalamus is known to be intimately related to the counter-regulatory response to hypoglycemia. In addition, the rats in their study were made diabetic through streptozotocin (STZ), which leads to chronic hyperglycemia [141]. It has been shown that STZ induced hyperglycemia can induce increases in MCT1 in many brain regions, including the hypothalamus [141,142,174]. Our current study showed no relationship between MCTs and a HAAF response induced with antecedent recurrent hypoglycemia. However, because our animals were not STZ treated, they never experienced the chronic hyperglycemia which may explain the differences in the MCTs in the Aveseh et al (2014) study.

The current study did show a strong relationship between body mass and the amount of MCT1, 2, and 4 proteins in the VMH. It is unknown what factors are affecting this relationship. MCT1 seemed to be positively associated with increased body mass, while MCT2 and MCT4 did not have as clear of an association. MCT1 has been previously shown to be associated with body mass as well as hyperglycemia [141,142,175]. However, it seems less likely that MCT1 causes these changes, and rather, that it is

responding to them. Other studies have shown that MCT1 content within the VMH and arcuate nucleus may play a role in feeding behaviors being regulated by multiple energy substrates including ketone bodies [142,153-156,175]. Therefore, the relationship between MCT1 and body mass may be regulated through many circulating substances that change as the body increases or decreases fat or lean mass. The use of rats of varying weights and ages may have led to increased variance in MCTs which was accounted for by the differences in body mass.

Due to the timing of sacrifices, some changes in protein content may have been missed. We would not have necessarily detected changes in protein levels if they occurred acutely after hypoglycemia. However, the HAAF response has been shown to be present 24 hours after an antecedent hypoglycemic event. Therefore, even if MCTs were only acutely changed, the fact that there were no significant differences in MCTs at 24 hours post-hypoglycemia suggests that they are not strongly related to the HAAF response. Another potential limitation was the depth of hypoglycemia which was achieved with the current study's protocol. While subcutaneous insulin injections are the most common route of administration in individuals with IDDM, the depth and duration of hypoglycemia achieved is not as precise as a hyperinsulinemic hypoglycemic clamp. Therefore, the exact glycemia of the rats throughout the study was not entirely uniform, and the same was true for the comparison between recurrently hypoglycemic and naive rats during hypoglycemia. While the current study achieved mild hypoglycemia, the HAAF response is associated with depth of hypoglycemia, and may not have been triggered.

Recurrent hypoglycemia and the associated hypoglycemia associated autonomic failure create a real problem for individuals with IDDM who must choose between long term benefits and short term risks. The changes which cause this dysfunction within the VMH are still unknown, however, it seems unlikely that they are mediated by changes in the MCT protein contents within this brain area. Lactate shuttling and ANLS may still play a role in HAAF, but the transport proteins are not required to change. Therefore, other components of the ANLS may warrant investigation.

CHAPTER V

OVERALL DISCUSSION

There are two common methods by which a hypoglycemia associated autonomic failure (HAAF) is likely to occur, antecedent exercise or antecedent hypoglycemia [12,13,15,17-19,66,78]. Insulin use by individuals with insulin dependent diabetes mellitus (IDDM) can cause antecedent hypoglycemia [16,64]. In addition, exercise in individuals with IDDM may itself cause hypoglycemia immediately, or delayed by several hours [57,63]. However, both of these treatments are widely recommended to control high blood glucose concentrations as well as to reduce the risk of diabetic complications [16,57,58,63]. Therefore, it is important to understand why these two common tools for diabetes treatment can become detrimental under certain circumstances, particularly when an individual with IDDM enters a cycle of hypoglycemia and HAAF [12,15,70]. Both of these treatment methods have been extensively studied in terms of their ability to cause a HAAF response, however, few attempts have been made to determine any common mechanisms. By examining both methods of inducing HAAF, new mechanisms of action may be understood. Once the underlying mechanisms are understood, steps can be taken to find a way to treat or prevent the HAAF response, allowing tighter regulation of blood glucose concentrations in individuals with diabetes mellitus.

The central purpose of these studies was to better understand the role of MCTs in the HAAF response from either antecedent exercise or antecedent hypoglycemia. It has been suggested that exercise can increase MCTs within the brain, and specifically, within the hypothalamus [21]. Additionally, there are suggestions that there are changes in lactate handling in the brain after antecedent recurrent hypoglycemia [23]. Furthermore, lactate injection or infusion into the brain has been used to induce a HAAF response, despite systemic hypoglycemia. However the concentrations used in most studies were greater than what would be present under normal physiologic conditions [24,25]. Studies using more physiologic concentrations of lactate show similar findings, although they were performed in brain slices rather than in a live animal [90]. The mechanisms of how lactate would increase in the brain during hypoglycemia following antecedent hypoglycemia or exercise is unknown. The most prevalent hypothesis is that there is a super-compensation of astrocytic glycogen that can be used for energy during the subsequent episode of glucoprivation [28,107-109,111,120].

It has been shown that lactate as well as ketone bodies (and possibly short and medium chain fatty acids) can elicit responses from either "glucose exited" or "glucose inhibited" neurons within the VMH [90,153,154]. In addition, these "glucose sensing" neurons have been shown to possess the enzymes necessary for an astrocyte to neuron lactate shuttle (ANLS) [170]. The MCTs are present within these neurons, although few studies have attempted to link them to a HAAF response [24,170]. The current 2 studies, taken together, suggest that, whether or not lactate or ANLS is involved in the HAAF

response, there are no changes in MCT content in the VMH in response to either antecedent recurrent hypoglycemia or chronic aerobic exercise training.

The current findings may be in line with other studies which have concluded that MCTs are not of particular importance for glucose sensing neurons in the hypothalamus [170]. A study by Kang et al. (2004) sought to characterize the glucose sensing neurons within the VMH by mRNA expression patterns [170]. While they measured MCT mRNA in most neurons, there was no difference between glucose inhibited neurons, glucose excited neurons, or non-glucose sensing neurons [170]. In addition, they found that most neurons, regardless of type, expressed both LDH1 and LDH5, indicating that most neurons can take up lactate and convert it to pyruvate for oxidative phosphorylation. This is in line with the ANLS, however, it appears that this would occur no differently between glucose sensing neurons and other neurons [170]. However, this study should be interpreted with caution because they used relatively young rats, and it is known that MCT expression changes with development [170,176]. It has also been suggested that MCTs are regulated translationally rather than transcriptionally [137]. Regardless, the current 2 studies have measured no changes in actual protein content of MCT's regardless of the level of transcription or translation. Therefore, it seems likely that MCTs or ANLS are not playing any larger role in the VMH and HAAF response to the metabolic processes of most other neurons in the brain.

Interestingly, the study by Kang et al. (2004) found that there was significantly more insulin receptor mRNA in glucose sensing neurons compared to non-glucose sensing neurons, suggesting that brain insulin may be partially regulating the activity of

these neurons [170]. Additionally, a study by Chenal et al (2008) which suggested MCT2 is regulated translationally, showed that insulin administration increased MCT2 protein expression [137]. The role of insulin signaling in the brain playing a role in circulating nutrient homeostasis has been suggested by experimental studies which have shown intranasal insulin to affect hepatic glucose output regardless of peripheral insulin concentrations [177]. Indeed, intranasal insulin has also been shown to have an effect on the regulation of triglyceride export from the liver [178]. In addition, the arcuate nucleus, which is in direct contact with the ventromedial nucleus (both within the VMH) has been shown to be affected by different hormones and nutrients to influence feeding behaviors [153,155,156,174,179]. Taken together this seems to suggest that the VMH is playing a larger role in metabolic sensing and regulation beyond simply glucose, and that MCTs may have an important role in the arcuate nucleus as well as ependymal cells of the median eminence.

There are three major points to be taken from the current studies: 1) the current findings neither confirm nor contradict an ANLS occurring within the VMH, 2) the current study does not confirm nor contradict a model that suggests increased lactate use by neurons in the VMH during hypoglycemia, but 3) the current study does show that whatever changes occur to produce a HAAF response, they are independent of changes in the MCT protein concentration which shuttle lactate.

Because all of the MCT isoforms were detected in varying amounts within the VMH, it is still physiologically possible for an ANLS to occur in this region. In addition the findings of previous researchers that indicate either changes in lactate flux in the

brain, or the ability of lactate to induce a HAAF response, may still be valid [23,24]. It is possible that MCT content is already sufficient to shuttle lactate at rates higher than observed under basal conditions. This is due to the high km of MCT4 which should not export lactate unless glycolytic rate increases. It is also possible that there are changes that occur in astrocytic glycogen, lactate metabolism, or oxidative enzymes rather than changes in MCT protein concentration [107,111,128]. Both exercise and hypoglycemia have been shown to deplete brain glycogen and induce subsequent super-compensation in different brain regions [108,111]. Although these points have not been confirmed or refuted by the current studies, the absence of any differences in MCTs 24 hours after antecedent recurrent hypoglycemia confirms that the HAAF response is not contingent on increases in MCT protein content. The HAAF response was measured, after antecedent recurrent hypoglycemia in the current study, and has been well documented after antecedent exercise in the literature [12,17-19,80]. If no change in MCT content is present within the VMH 24 hours post exercise or antecedent recurrent hypoglycemia, it can be concluded that their content does not have to change to induce a HAAF response which is present within this 24 hour window.

Further studies will be needed to determine whether lactate flux or lactate metabolizing enzymes are changed by either antecedent exercise or antecedent recurrent hypoglycemia. This could be accomplished by a specific knock down of astrocytic or neuronal MCT expression. Then interstitial lactate concentrations could be measured by microdialysis. If astrocytic MCT-KD or neuronal MCT-KD does not lead to decreased or increased (respectively) interstitial lactate concentrations, then it is unlikely that the

ANLS is occurring within the VMH. Furthermore, super compensation of astrocytic glycogen leading to ATP maintenance in neurons would not be related to the HAAF response observed after either antecedent exercise or antecedent recurrent hypoglycemia. This is because astrocytes lack glucose 6 phosphatase to allow glycogen derived glucose to leave the cell. Astrocytic glycogen can only provide fuel to neurons via other metabolic intermediates, such as lactate. If this were the case, then increased activity of GABAergic neurons due to increased lactate flux leading to increased ATP:ADP ratio and stimulation of K_{ATP} channels would seem less likely in mediating the HAAF response [24,30].

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APPENDIX A

CHEMICALS

Tris (pH 8.8)-
90.85g Tris Base
17ml HCl
In 500ml deionized water pH 8.8

Tris (pH 6.8)-
30.3g Tris Base
20ml HCl
In 250ml deionized water pH 6.8

APS (10%)-
4g Ammonium Persulfate
In 40 ml of deionized water

2x Loading Buffer-
25ml Tris 6.8
40ml SDS (4%)
10ml glycerol (10%)
8mg bromophenol blue (0.008%)
Makes 100ml

Electrophoresis buffer (10x)-
90.8g Tris Base
432.4g glycine
30g SDS
In 3 liters deionized water

Transfer buffer (10x)-
90.8g Tris Base
432.4g Glycine
20% methanol
Makes 3 liters

DTT-
154mg Dithiothreitol
In 10ml of deionized water

10x TBST –
24g Tris Base
88g NaCl
10ml Tween 20x
In 1000ml deionized water pH 7.6

Stripping buffer –

15g Glycine
1g SDS
10ml Tween 20x
In 1000ml deionized water pH 2.2

RIPA buffer –

50mM Tris Base
1mM EDTA
150mM NaCl
1% NP-40
1% Na Deoxycholate
1% SDS

Phosphatase inhibitor –

4.3mg NaF
43.2mg β-Glycerophosphate
In 1ml RIPA buffer

Catecholamine Extraction (CE) Tris Buffer-

45g Tris Base
5g Na_2EDTA
In 250ml deionized water pH to 8.6 with phosphoric acid.

HPLC Mobile Phase-

10.35g NaH2PO4•H2O
0.389g 1-Octanesulfonic Acid
0.009g Na EDTA
100ul Diethylamine (DEA)
70mL Acetonitrile (ACN)
In 1 liter of deionized water pH to 3.0 with phosphoric acid.

APPENDIX B

WESTERN BLOTTING

Sample preparation and Gel Electrophoresis

For determination of protein content of samples, a western blotting analysis was used. First a 12% acrylamide resolving gel was made for the gel electrophoresis. The 12% acrylamide gel consists of: 9.9ml of DI water, 12ml Acrylamide, 7.5ml Tris (pH 8.8), 300µl of SDS (10%), 300µl of APS, and 25µl of TEMED. This solution was poured into a cartridge to polymerize and capped with deionized water. Electrophoresis buffer was made from 100ml of 10x electrophoresis buffer and 900ml of deionized water. Electrophoresis buffer was placed in the bottom tank of the Hoeffer SE400 vertical electrophoresis unit. When resolving gel had polymerized, water cap was poured off, and the cartridge was gently dried with filter paper. The cartridge was placed the tank containing electrophoresis buffer.

A stacking gel was made and poured into the cartridge above the 12% resolving gel for the loading of the samples. The stacking gel consisted of: 6.8ml of DI water, 1.7ml of acrylamide, 1.25ml of Tris (pH 6.8), 100µl of SDS, 100µl of APS, and 15µl of TEMED. The stacking gel was poured into the cartridge above the resolving gel once the deionized water is removed. A comb is cleaned with 70% ethanol and dried before being inserted in the top of the cartridge to form the loading lanes. The stacking gel was then allowed to polymerize.

All samples were measured out by total protein and combined with an equal amount of 2x loading buffer and DTT. After loading, samples were gently vortexed to ensure mixing. All samples were kept on ice until they were loaded into the gel for electrophoresis. Before gel loading, all samples were briefly centrifuged to ensure all sample was at the bottom of the conical tube. Once the stacking buffer had polymerized, the comb was gently removed, and lanes were straitened (if necessary). Electrophoresis buffer was used to fill the lanes as well as to remove any bubbles. The samples were then pipetted into appropriate lanes. The Hoeffer SE400 electrophoresis top tank was then secured to the top of the cartridge. The electrophoresis buffer was added to a top and bottom tank for the vertical electrophoresis chamber (SE400 Air-Cooled Vertical Electrophoresis Unit, Hoefer, Inc. Holliston, MA) and the gel electrophoresis was run over night (approximately 15-16 hours) at 60 volts.

Transfer Electrophoresis

Once the electrophoresis has run, the gels were carefully removed from its cartridge. The stacking buffer was cut away, and any resolving buffer below the migration of the dye front was also removed. The gel was then placed in transfer buffer, sandwiched between 2 sponges, 2 pieces of filter paper, and flush against a piece of polyvinylidene fluoride (PVDF) membrane as shown in figure B.1. Together this was secured into a cartridge for transfer electrophoresis. The cartridge was placed into a transfer electrophoresis box and filled with transfer buffer. The transfer electrophoresis was then run, on ice, for 6 hours at 200 milliamps. Ice was added throughout the 6 hour transfer as necessary to keep the transfer buffer from being heated.

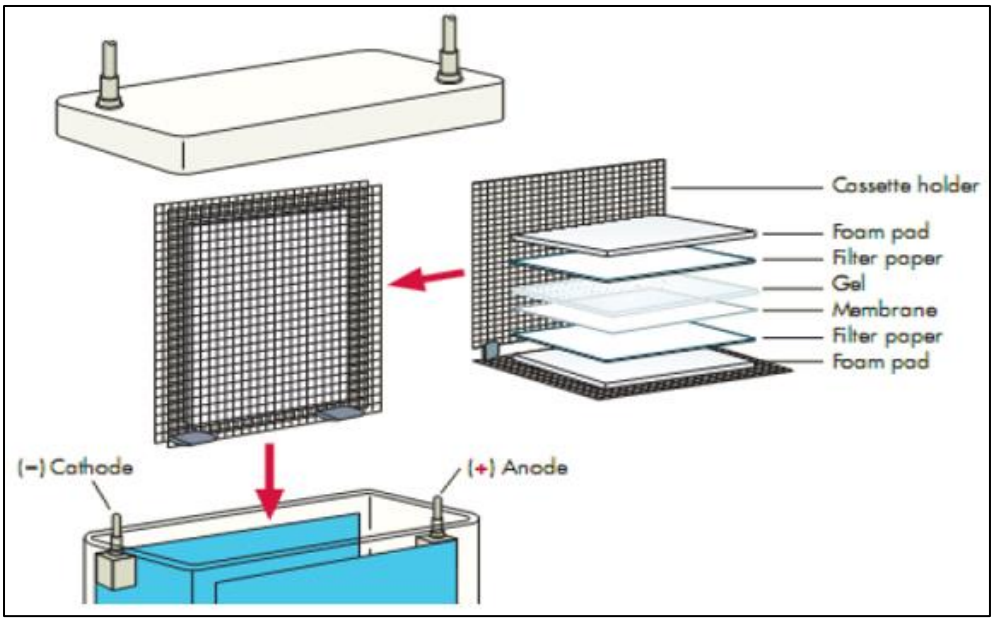

Figure B.1. Arragnement of Cartridge for Transfer Electrophoresis.

Blotting

After the transfer had completed, the gels were discarded and the PVDF membrane was washed in TBST for 5 minutes on an orbital shaker set at 100rpm. The TBST was discarded, and the PVDF membranes were blocked in a 5% milk solution (made with powdered milk and TBST) for 1 hour on an orbital shaker set at 100rpm. After the blocking step, the 5% milk was discarded. The membranes were washed again, 3 times for 5 minutes each, in TBST on an orbital shaker. The TBST was discarded after each wash and new TBST was added. Meanwhile, boxes for administration of primary antibodies were blocked for 15 minutes in 5% milk to reduce non-specific binding of the antibody to the plastic surface of the box. The 5% milk was then discarded. After washing, the membranes were placed in boxes on a rocker and 15ml of 5% milk with antibody was added. The membranes were then probed overnight with primary antibody

on the rocker at 4°C. Primary antibodies for MCT1, 2, and 4 were purchased from Santa Cruz Biotechnologies (sc-50325, sc-50323, and sc-50329 for MCTs 1, 2, and 4 respectively).

When probing with the primary antibody was done, the 5% milk and antibody was discarded. The membranes were washed again for 10 minutes in TBST. The TBST was then discarded. Then the membranes were washed 3 more times for 10 minutes each in 5% milk, discarding the used milk after each wash and adding fresh milk. Meanwhile, the boxes for the administration of secondary antibodies were blocked for 30 minutes in 5% milk. The 5% milk was then discarded. The membranes were then be put back into the boxes on the rocker. Then 15 ml of 5% milk and antibody was added to each membrane. The secondary antibody in 5% milk was put on the rocker at room temperature for 2 hours. Once the secondary antibody was done incubating, the secondary antibody was discarded. The membranes were then washed 4 times for 10 minutes each in TBST, discarding used TBST with each wash. The membranes were then soaked in ECL plus (PerkinElmer Inc. Waltham, Massachusetts) for 2 minutes and imaged on BioRad Chemidoc Station. An example of these images for the MCTs can be seen in Figure B.2. The band density was assessed using QuantityOne software on the same computer connected to the Chemidoc.

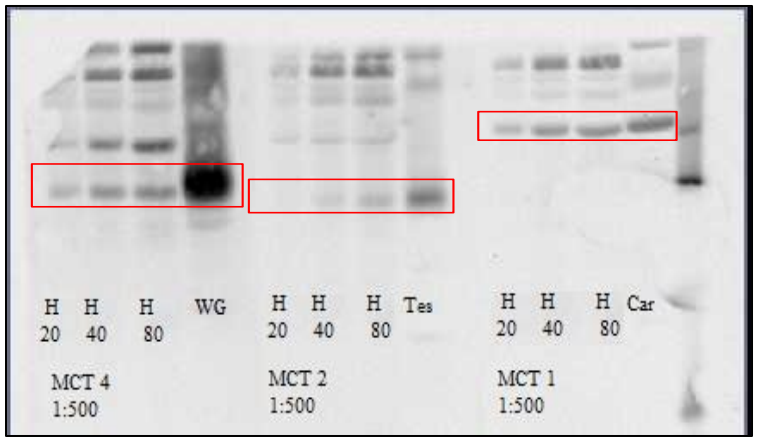

Figure B.2. Example Blot of MCTs 4, 2, and 1 within the Hypothalamus.

After the first exposure, the membranes were washed 2 times for 10 minutes each in stripping buffer to remove primary and secondary antibodies. Used stripping buffer was discarded after each wash. Once the stripping was completed, the membranes were washed 4 times for 10 minutes each in TBST, discarding used TBST each time. The PVDF membranes were then blocked again in a 5% milk solution for 1 hour on an orbital shaker set at 100rpm. The milk was then discarded. After the blocking step, the membranes were washed again 3 times for 5 minutes each in TBST on an orbital shaker. Meanwhile, boxes for administration of primary antibodies were blocked for 15 minutes in 5% milk. Used TBST and milk were then discarded. After washing, the membranes were each placed in a box on a rocker and had primary antibodies delivered in 15ml of 5% milk solution. They were then probed overnight with the primary antibody at 4°C. The primary antibody for GAPDH (the loading control protein) was also purchased from Santa Cruz Biotechnologies (sc-166545).

When probing with the primary antibody was completed the antibody and milk solution was discarded, and the membranes were washed again for 10 minutes in TBST. The TBST was then discarded. Then the membranes were washed 3 more times for 10 minutes each in 5% milk. Meanwhile, the boxes for the administration of secondary antibodies were blocked for 30 minutes in 5% milk. The milk used for blocking or washing was then discarded. The membranes were then put back on the rocker with 15ml of 5% milk with secondary antibody. They were then probed with secondary antibody at room temperature for 2 hours. Once the secondary antibody was done incubating, the secondary antibody and milk solution was discarded. The membranes were then washed 4 times for 10 minutes each in TBST. The TBST was discarded after each wash. The membranes were then soaked in ECL plus (PerkinElmer Inc. Waltham, Massachusetts) for 2 minutes and imaged on BioRad Chemidoc Station. The band density were assessed using QuantityOne software on the same computer connected to the Chemidoc. Finally, after this exposure, the membranes were discarded.

APPENDIX C

JUGULAR VEIN CATHETERIZATION PROTOCOL

Preparation

A jugular vein catheter was surgically implanted into some of the animals for multiple blood draws to assess changes in counter-regulatory hormones. Before the surgery began, necessary tools, instruments, and the surgical area will be sterilized. Tools were sterilized using the autoclave before the surgery, or using a glass bead sterilizer between animals. Immediately prior to the surgery, animals were injected with xylazine at a concentration of 1mg/ml and a dose of 1ml/kg body mass. This relaxes the animal and makes it easier to further anesthetize. After approximately 10 to 15 minutes, depending on the animal's behavior, the animal was placed in an anesthetization chamber to deliver approximately 4% isoflurane (depending on rat size and behavior) at 400 ml of oxygen per minute. Once the animal lost sternal recumbancy, it was moved from the chamber and fitted with a nose cone to continue to deliver isoflurane for the duration of the procedure; however the concentration was reduced to 2-3% depending on the size and needs of the animal. The animal's eyes were coated with ophthalmic lubricant to prevent them drying out during the procedure.

The animal's head and chest were shaved using clippers, and a damp towel was used to remove any excess hair. Once both sites were cleanly shaven, both sites were disinfected with 70% ethanol and povidone-iodine solutions, 3 times with each. Lidocaine was injected beneath the skin on top of the head, and allowed to absorb before the first incision was made. Then the animal was completely wrapped within a sterile wrapping to protect the surgical site from any external contamination. A hole was cut in the sterile wrappings above the head, and the chest to allow access to these areas during the surgery.

Surgery

First, a small incision along the mid-sagittal line was made on the top of the head to allow later access to the skull. A piece of sterile gauze was applied to the top of the head to contain any bleeding as well as to prevent contamination of the incision. The animal was then turned over to the supine position to expose the chest. The skin was gently pinched and pulled away from the body where a small incision was made using surgical scissors. The incision was then slowly spread by blunt dissection (repeatedly inserting closed scissors, and then gently spreading them). This was done as opposed to cutting the flesh with the scissors (sharp dissection) to avoid damaging any organs or major blood vessels. Once the jugular vein was revealed, it was carefully isolated using tweezers to slowly clean away fat and connective tissues. When the jugular vein was cleanly isolated, a 20 gage needle was inserted into the vein as far anterior (cranial) to the animal as possible. The needle was then be removed and a pair of sharp tweezers were inserted (closed) into the puncture hole. These tweezers were gently spread to allow

space for the catheter to be inserted using a second set of blunt tweezers. The catheter was then fed into the jugular vein until it was just above the right atrium. If the catheter was unable to be moved forward, it was backed out slightly to avoid placing the tip in the right atrium. Blood flow was checked using a 5ml syringe filled with saline to ensure that the tip of the catheter was not against a valve or vessel wall. Two sutures were used to secure the catheter in place as well as prevent bleeding from the jugular vein. Blood flow was checked using a 5ml syringe filled with saline to ensure that the sutures had not obstructed the flow.

The animal was then be turned onto its side and its eyes were re-lubricated. Using a cannula tunneling tool, the free end of the catheter was pushed through from the chest to the top of the skull. The catheter was passed between the ear and eye of the animal, to exit through the incision initially made on the head. Blood flow was then tested once more to ensure that the catheter is patent. The incision on the chest was then closed using a surgical staple. The animal was then be moved to the prone position with sterile gauze over the chest. The connective tissue was scraped away from the skull using a scalpel blade, while being careful not to cut the catheter tubing. Swabs were used to dry the area before drilling. Then, three to four holes were drilled into the skull of the animal, taking care not to drill into the brain. After drilling a hole, screws were inserted into the holes and screwed down about half way. A space was left between the skull and the head of the screws to allow anchoring points for the dental epoxy. Small pieces of gel foam clotting agent were placed under the skin along the sides of the work area to prevent any further bleeding or moisture entering the surgical area. A dental epoxy was then be used

to secure the port end of the catheter between the heads of the screws, being sure to coat the entire area with epoxy, including beneath the heads of the screws. The catheter was held in place until the epoxy dried to ensure that the port is accessible, and at an angle that the rats cannot remove the stylet. The catheter was then flushed and plugged with heparinized glycerol, and a stylet was used to cap the port. Once this was done, the animal was removed from the isoflurane and unwrapped from the sterile wrappings. The animal was then placed in a recovery cage which was placed on a heating pad. The animal was monitored until it has recovered sternal recumbency and was observed grooming and eating (approximately 60 minutes). The rat was then moved to a new tall cage, and is housed alone. The tall cage ensured that the catheter port would not be accidently caught on the ceiling. The animal would remain single housed in a tall cage for the remainder of the study.

APPENDIX D

BLOOD SAMPLE EXTRACTION PROCEDURE

Equipment

Per Animal;

16” silicone tubing (marked with ruler)

1 plugged needle

Hemostat

Rinse/waste cup

Syringes

5 mL waste

5 mL saline

1 mL hep. saline with 25G needle

1 mL waste

0.25 mL donor blood “x” number of samples

1 mL for blood sample “x” number of samples

Vials “x” number of samples

1.5 mL vial prepared with 25ul hep. saline

0.6 mL vial labeled

General;

Mini-centrifuge

Pipet at 100ul and pipet tips

Ice box for samples

Prior to Sampling

15 minutes prior to sampling, prepare rats with 16" tube on catheters and rest over edge of the cage with hemostat. Check plug for leaks before leaving unattended.

Blood samples taken at -10min, 0min, 15min, 30min, 60min, 90min, and 120min (relative to injection time) to obtain a total of 7 samples. First sample is taken ~30 minutes after food was removed from the cage. Total time for blood sample extraction is ~ 2.5 hours (150 minutes).

Sampling for each time point, for each rat

1. Clamp tubing with hemostat and remove plug
2. Use 5 mL syringe (waste) to draw blood to the end of tubing
3. Clamp with hemostat and remove waste syringe
4. Connect sterilized collection syringe
5. Draw 0.25 mL of blood with syringe held vertical
6. Clamp hemostat and remove collection syringe
7. Connect donor blood syringe
8. Inject 0.25 mL of donor blood or saline if first run
9. Clamp hemostat and remove donor syringe

10. Connect hep saline syringe and add small amount with fine needle at tube opening
11. Flick the end of tubing to create a small bubble in the tube
12. Clamp hemostat and remove hep saline syringe
13. Connect saline syringe and push bubble 3” down tube
14. Clamp hemostat and remove saline syringe
15. Flick the end of tubing to create a small bubble
16. Connect saline syringe again and push through the tube until 2nd bubble is at the top of rats metal catheter piece
17. Clamp and cap until next sample run time in ~30 minutes

Processing Each Sample

1. Gently introduce 0.25 mL sample into large vial prepped with 25ul of hep saline (hep saline should be 1/10th the amount of sample added)

   Centrifuge until plasma is separated (~1-2 minutes)
2. Pipet plasma and add to properly labeled vial
3. Store plasma vial in ice box
4. Add ~100ul of sterile saline to remaining sample and draw into a 1.0 mL syringe

   a. This will become the donor blood for the next sampling procedure

   Total sample time: Approximately 6 minutes

APPENDIX E

PLASMA CATECHOLAMINE EXTRACTION

Supplies

a) Costar Spin-X micro centrifuge filter tubes with 0.2 μm nylon filter. (Corning Inc., catalog nr. 8169), (Fischer # 07-200-389)

b) 2 mL flat cap microcentrifuge tubes (USA Scientific Inc, # 1620-2799)

c) Acid washed aluminum oxide (BAS # CF-8010)

d) 0.2 N $HClO_4$

e) CE Tris Base (Fischer # BP152-500)

f) DHBA solution for internal standard

g) Brass Poederschepje® scoop (patent pending) for dosing alumina

h) Multivortex

i) Centrifuge

Procedure

1. Label 24 (max) centrifuge filter tubes, the corresponding microcentrifuge tubes, and the corresponding autosampler vials. It is important to keep a record (on paper) where your samples go!
2. Weigh out 10 mg aluminum oxide into the filter tubes using the brass scoop.

3. Add 500 μL CE Tris buffer to each filter tube, make sure that the CE Tris buffer is at room temperature.
4. Pipette 80 μL DHBA standard (20 pg/20 μL) into each filter tube.
5. Pipette 100 μL plasma into each filter tube. Write down the exact sample volume when there is less than 100 μL available. Use a new yellow tip for each sample. Cap the tubes and vortex immediately (catecholamines are unstable at pH 8.6!) on the multivortex for 10 min. Make sure you observe the alumina dispersed within the tubes.
6. Centrifuge the tubes for 3 min at 12000 rpm and 4° C.
7. With each tube: Drain the filtrate, reassemble the tube, and add ~500 μL deionized water from the dispenser.
8. Centrifuge again for 1 min At 12000 rpm and 4° C. But extend the time as necessary for all of the water to clear the filter.
9. Repeat the wash procedure (steps 7 & 8) three more times.
10. Drain filtrate and centrifuge for 2 min at 12000 rpm to dry out the alumina.
11. With each tube: Transfer the filter assembly (top part) onto the new microcentrifuge tube with its corresponding label.
12. Pipette 80 μL $HClO_4$ 0.2 N into the filter tubes, and vortex briefly on the multivortex. Turn off the vortex and let the tubes incubate for 5 min.
13. Centrifuge the tubes for 2 min at 12000 rpm and 4° C.

14. Transfer the filtrate to the labeled autosampler vials and cap. Should be 80 µl. Refrigerate when not used immediately!

Remember to Include

1. vial of the DHBA internal standard used.
2. vial of the acid used.
3. vial of Aluminum + DHBA
4. vial of Aluminum.