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The human body seeks to maintain a stable physiological blood glucose concentration, despite a constantly changing availability of energy from eating. During food consumption, blood sugar would typically increase with time and during fasting, the opposite would be true. The body is able to release specific hormones to bring about either the storage or release of glucose from specific sites in the body. While this is all well and good, the body needs a sensor of sorts to determine when these actions need to take place. The brain, being the complex control center of the body, has such a site. The ventromedial hypothalamus is responsible for determining systemic glycemic levels. The exact mechanism that exists for the sensing isn't fully understood, but it involves the VMH neurons. The loss of glucose, possibly due to fasting and a depleted amount of stored glucose, would lead to a response in the neurons that now lack fuel. While in a state of hypoglycemia, the neurons still need to function, and that is where the proposed astrocyte to neuron lactate shuttle hypothesis comes into play.

This hypothesis suggests that glycogen stores in the astrocytes would be mobilized and converted to lactate, where it would be exported into the interstitial space and taken up via MCT2 proteins into the neurons to provide energy. In my study, we hypothesized that the knockdown of MCT2 proteins in the VMH will lead to an increased epinephrine response with recurrent bouts of exogenous-insulin induced hypoglycemia. Our results

showed that MCT2 protein is very important to the function of energy sensing in the VMH and the loss of available lactate might simulate a bout of hypoglycemia

MCT2 EXPRESSION IN THE HYPOTHALAMUS AND THE COUNTERREGULATORY RESPONSE TO HYPOGLYCEMIA

by

Vincent Porcelli

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Approved by

Dr. Joseph Lee Beverly
Committee Chair

APPROVAL PAGE

This thesis written by Vincent Porcelli has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair Dr. Joseph Lee Beverly

Committee Members Dr. Steve Fordhal

Dr. Joseph Starnes

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CHAPTER I

REVIEW OF LITERATURE

Hypoglycemia

The brain is a vital organ that regulates physiological change in the body. Changes in temperature, pH and blood sugar are just a few variables that are monitored and adjusted by the central nervous system. The ventromedial hypothalamus (VMH) is a primary brain area for controlling energy balance as well as the levels of circulating glucose through its influence on plasma catecholamines, insulin, and glucagon. These hormones are the major players in the normal control of blood glycemic levels. During hypoglycemia, the VMH senses a drop-in blood glucose below the normal functioning range. A variety of systemic changes return the body to a euglycemic state by activating redundant physiological systems. Two that I will highlight include the adrenomedullary response and the glucocorticoid responses. During a bout of hypoglycemia, the sympathoadreanal system releases norepinephrine(NE) and epinephrine(Epi) into the blood from the adrenal medulla, located near the kidneys. The VMH contains a variety of cell types. Two of interest are the glucose excitatory neurons (GE) and the glucose inhibitory neurons (GI). GE neurons increase activity during hyperglycemia. It has been suggested that GE neurons sense glucose in a manner similar to pancreatic beta cells: K_{ATP} channel that plays an important role in glucose sensing and blood sugar maintenance (28). Rats with these K_{ATP} channels (Kir6.2) were blocked in the VMN lost

some of their glucose sensitivity (43). Conversely, GI neurons increase their rate of activity with a drop in ambient glucose levels. Murphy et al. 2009 collected brain slices to better understand the pathway by which the VMH GI neurons recognize hypoglycemia. They hypothesized that AMP activated protein kinase(AMPK) phosphorylates eNOS to increase nitric oxide production in VMH GI neurons due to a drop-in blood glucose (39). Maintaining sensitivity of GI neurons is important to preventing large downward swings in blood glucose. The loss of functionality by these cells in the VMH could lead to loss of hypoglycemic awareness.

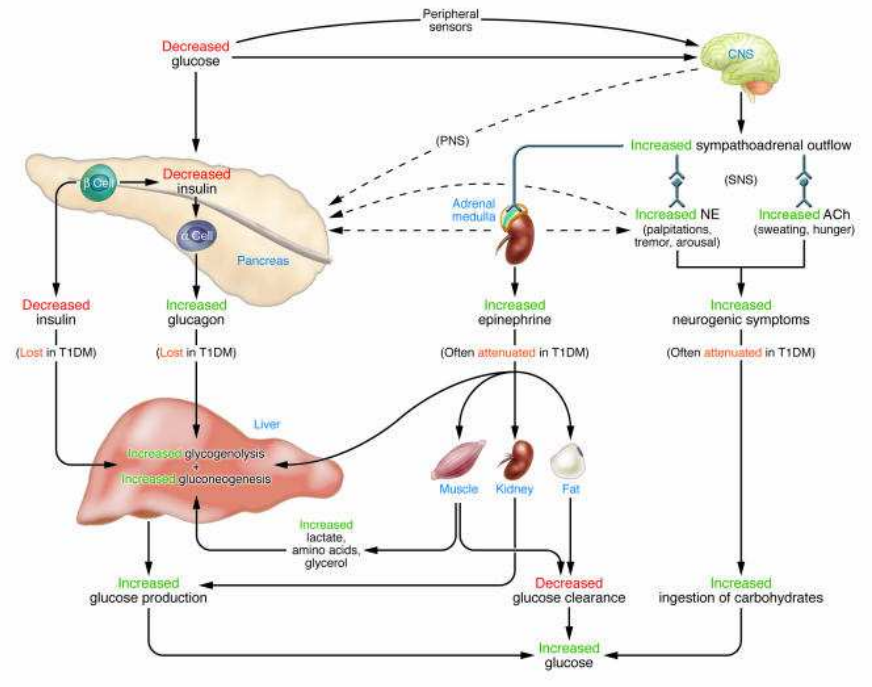


Figure 1. Cognitive and Metabolic Brain. The complex system of checks and balances to maintain euglycemia is accomplished through multiple endocrine signals. (14)

Initial glucose sensing studies revealed a role of brain sensing areas for blood sugar control. Damage to the fourth of a dog brain resulted in the onset of diabetes, leading scientists to believe that the brain played an important role in the body's metabolic systems (3). The hypothalamus came to light as a brain area where major metabolic shifts occur when this brain area is manipulated. For example, destruction of the VMH brings about hyperphagia and obesity (6). The VMH is one area in the brain that contains both glucose excitatory cells and glucose inhibitory cells (62). The brain consumes far more glucose then any organ in the body. Thus, a proper glucose level is required to maintain brain function.

The hypothalamus contains glucose inhibitory neurons, which could help to sense and ultimately maintain proper glucose levels for the central nervous system (31). They also have been suggested to participate in the portioning of glucose throughout the endocrine system (27). During bouts of hyperglycemia, brain glucose levels were monitored and found to remain at a lower level despite 20mM systemic glycemic levels (52). Higher levels of glucose have been shown to hinder glucose sensing (9). Further work has been done to show that glucose in the VMH is similar to that of the arcuate nucleus, another portion of the brain which in encompassed in the hypothalamus. The arcuate nucleus, a portion of the VMH, does not contain GI or GE neurons. The VMH has been historically considered the satiety center of the brain, with the lateral hypothalamus being the feeding center. The administration of glucose *in vivo* into these two hypothalamic areas had a notably different effect, with glucose given to the VMH increased neuronal activity, and decreased when injected into the lateral hypothalamus

(42) Currently there are two types of established glucose sensing neurons: glucose-excited (GE) and glucose-inhibited (GI) (55). The VMH contains GE neurons that are similar in function to the insulin secreting beta cells of the pancreas (2). A variety of hormones also influence the activity of glucose sensing neurons, with both insulin and ghrelin inhibiting GE neurons (58). Hypoglycemia did not have the repressing effect on GE neurons when insulin was administered (12). Hypoglycemia is readily detectable in the VMH, based upon the high levels of sensitivity by GE and GI neurons to a drop in physiological glucose (55).

The counter regulatory physiological response (CRR) to hypoglycemia has three distinct components. A decrease in circulating insulin, an increase in glucagon, and the adrenomedullary release of epinephrine; the latter promote hepatic glycogen breakdown needed to maintain euglycemic and maintain glucose availability to the brain (13). VMH GI neurons have been shown to play a role in the CRR (56). A slight drop in blood glucose levels in a typically healthy individual is not a major concern and will not elicited these responses, as the body can quickly replenish circulating blood glucose concentration; however, insulin treatments may cause blood glucose to rapidly drop to levels that could cause permeant irreversible damage to portions of the brain which won't properly function when deprived of glucose for even a short period of time. Chronic states of hypoglycemia can lead to coma and death. In advanced societies use of exogenous insulin is the leading cause of hypoglycemia.

Fuel Use in the Brain

The brain requires a consistent supply of glucose to maintain its function. Activation within a brain area causes increased blood flow, and thus increased available glucose for energy production to support increased activity (30). During activation, glycolytic and oxidative metabolism was demonstrated to increase in synaptosomes *in vitro* (29). The use of glucose transporter (GLUT) proteins allows the brain to uptake available glucose from the blood (53). During prolonged hypoglycemia, the body enters a ketogenic state driven by a breakdown of long-chain fatty acids for oxidation into ketone bodies (35). the increase in fatty acid oxidation is supported by an increased ratio of glucagon to insulin. This could be due to loss of insulin production, the main physiological issue with diabetes mellitus type 1 (35). Ketone body production is not strictly due to an increase in FA availability, but rather the need for energy (35). The production of ketone bodies as an outlet for fuel production and utilization is important as it allows the body to spare what glucose is left for euglycemic maintenance. Acetone, B-hydroxybutyrate and acetoacetate are produced in the liver. Ketone bodies are converted to actyl-CoA, where they can be utilized as substrate in the TCA cycle.

The consumption of fuels by the brain has been termed flow dependent. Imaging studies documented changes in cerebral oxygen consumption, blood flow and glucose utilization are altered with brain activation (25). Increased blood flow paralleled glucose availability and oxygenation, but oxygen consumption in not as greatly increased compared to the uptake of glucose and increased regional cerebral blood flow (30). A variety of studies have discussed possible sources of energy during this activation. Early

work with H_1 NMR *in vivo* showed that physiological lactate increases in the visual cortex with photic stimulation (48). It could be assumed that brain activation causes an increase in lactate to provide additional energy to neurons. Later, it was suggested that lactate was released from astrocytic cells via the signaling functions of glutamate (24). This sets the basis for the astrocyte to neuron lactate shuttle (ANLS) hypothesis (described in more detail below). Monocarboxylic transporters (MCTs) are proteins that transport lactate across membranes. Monocarboxylic transporter 2 (MCT2, primarily expressed in the neurons, is a proton driven membrane protein which is affected little by electrical membrane potential (8). MCT2 is considered to have a much higher affinity for lactate and pyruvate compared to MCTs 1 and 4, which are associated with glia (7). There may be a transient increase in lactate uptake by activated neurons, although astrocytes may also be involved in uptake and utilization of free lactate (54). The infusion of lactate into the VMH was shown to inhibit the CRR *in vivo*, (5) while the administration of lactate *in vivo* blunts VMH GI neurons during a drop-in glucose levels (56).

Recurrent Hypoglycemia

While the brain has multiple levels of protection to prevent large and prolonged changes from euglycemic, hypoglycemia can still become a problem. Incidents of hypoglycemia are noted in overall healthy individuals who exercise without proper glycogen stores, such as one who undergoes intensive training in the morning after an over-night fast (24). Individuals who suffer from diabetes mellitus type I are prescribed

exogenous insulin injections to lower blood glucose. This is a great treatment for correcting hyperglycemia, but improper dosages or lack of physiological correction for the excess insulin can lead to hypoglycemia. Diabetes mellitus type II patients are typically advised to make life style modifications to better their overall health, but drugs and insulin therapy are becoming a mainstream way of controlling blood sugar levels. In fact, twice as many diabetic individuals suffer from hypoglycemia each year when taking insulin compared to those who primarily practice good lifestyle choices (16).

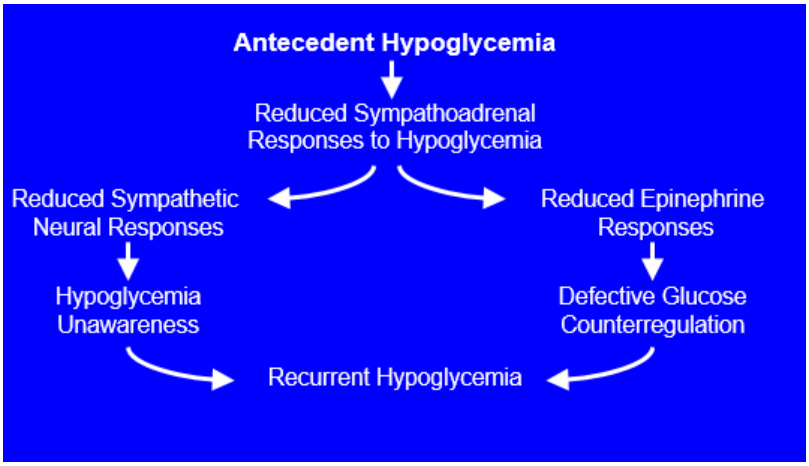


Figure 2. Integration of Brain Function. Antecedent hypoglycemia leads to recurrent hypoglycemia bouts via defective glucose counterregulation and philological hypoglycemia unawareness. (14)

In type I diabetic individuals, the loss of glucagon production with the progression of the disease make the release glucose via glycogenolysis and glycogenesis mediated by epinephrine even more important. These individuals may have a compromised response to hypoglycemia. Hypoglycemia could become a major issue

when combined with fasting and/or inaccurate insulin injection. Individuals who 'overdose' themselves with insulin, possibly due to either miscalculations on the administered amount, are at risk of prolonged severe hypoglycemia. Recurrent bouts of hypoglycemia have been shown to decrease the scope of the counterregulatory responses to restore normal glucose concentrations. Repeated hypoglycemia can numb the individual to being aware of their hypoglycemic status and lead to a diminished autonomic response, coined hypoglycemic associated autonomic failure (HAAF). HAAF decreases the typical physiological responses that are clinical markers of hypoglycemia. This includes fatigue, anxiety, heart palpitations and a 'cloudy head.' This hypoglycemic unawareness presents a positive feedback loop that develops with repeated hypoglycemic episodes.

Brain Glycogen

The utilization of brain bound glycogen by the neurons is receiving increased attention. As previously stated, during activation, the brain consumes glucose at a higher rate. This is possibly augmented by stores of glycogen in the astrocytes. The storage of glycogen in the brain was originally thought to be primary to provide glucose and energy allotment during situations where available glucose was limited. The glycogen could act as a short-term pool of glucose to allow continued function (51). Later literature showed that this glycogen was consumed at such a rate as to be inefficient for function over long periods of time (32). Later studies conducted by Swanson et al. determined that glycogen was consumed during whisker stimulation in the barrel cortex region of the brain (61).

Thus, glycogen was utilized in specific scenarios, but how it was being used was still unknown. A variety of pathways are applicable for glycogen. A proposed pathway by Dringen et al. points to the generate lactate which is then exported from the astrocytes into the blood or neuronal cells to provide a source for ATP generation (18).

The synthesis and breakdown of glycogen is heavily controlled. Glycogen in the brain is concentrated in the astrocytes, as is glycogen phosphorylase needed to utilized the stores (40). Although studies show insignificant glycogen stores for brain function during oxygen starvation, DiNuzzo et al. proposed that astrocytic glycogen could be useful for situations of brain activation (17). Knockout of glycogen synthase reduced learning abilities, and available glycogen plays a hardy role when brain areas are stimulated (20). Though this work was done in the hippocampus, it could be a similar response to glucose sensing activities in the hypothalamus.

A single bout of severe hypoglycemia leads to increased glycogen storage (19). This was evident regardless of blood glucose levels during recovery, and supercompensation was shown in the hippocampus, cortex and striatum, but was absent in the hypothalamus (19). Episodes of hypoglycemia have been touted as leading to an increased expression of glucose carriers (53). This would theoretically lead to increased glycogen stores with greater transport faculties. Increased glycogen concentration was studied as a possible link to hypoglycemia unawareness. Supercompensation of brain glycogen was studied by Canada et al. 2011 where brain glycogen amounts were determined in mice after bouts of acute or recurrent hypoglycemia (10). Their findings suggest that glycogen stores increase after hypoglycemia, and that the change in glycogen

degradation and reaccumulation occur to the same extent in instances of acute or recurrent hypoglycemia (9).

Astrocyte to Neuron Lactate Shuttle

Glutamate is a major signaling neurotransmitter in the brain involved with the activation of neurons. Pellerin and Magistretti 1994. surmised that glutamate ultimately leads to glycolysis in the astrocytes to provide energy, via glycogen stores, to the neurons when increased energy is needed. Neuron function in brain slices was restored with the administration of L-lactate (60). The ANLS hypothesis is supported by the idea that all neurons contain Lactate dehydrogenase 1 (LDH1) and Lactate dehydrogenase-5 (LDH5) proteins which allow the inter-conversion of lactate and pyruvate.

The hypothesized astrocyte to neuron lactate shuttle (ANLS) suggests that the neuron in the brain receives lactate via the astrocyte through monocarboxylic transport proteins (MCTs) (46). The movement of lactate out of the astrocyte via MCT1 and MCT4 proteins allows lactate to move into the intracellular space to be taken up by neurons through MCT2 membrane proteins. This has been the driving idea, with MCT4 being primarily expressed on the astrocyte, and MCT2 primarily found on the neuron (47). Lactate can then be converted to pyruvate through the Cori cycle for ATP generation. It has also been hypothesized that the generation of lactate from glycogen stores in the astrocytes works to spare remaining glucose for neurons (17). Suzuki et al reviewed past experiments which sought to determine the role of lactate and proposed the lactate to neuron shuttle facilitates long-term memory formation in rat hippocampus (59).

These authors hypothesized that increases in lactate during memory formation, specifically long term, provide extra fuel for neurons in the hippocampus. They used Oligodeoxynucleotides (ODN) to knockdown specific MCT protein in the hippocampus. During memory formation, rats given MCT4 and MCT2 ODNs exhibited notable lack of memory formation, possibly due to the lack of lactate shuttling. Memory formation was ultimately restored in groups given the MCT4 and MCT1 ODNs through the administration of exogenous lactate. This was not the case for MCT2 ODN groups, where the administration of lactate did not restore cognitive faculties.

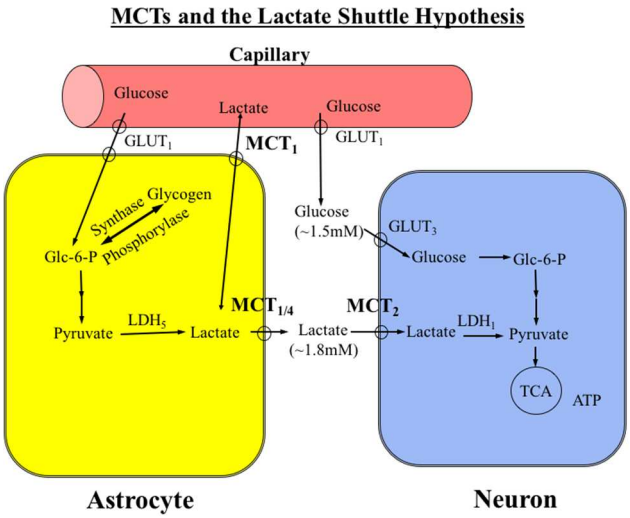


Figure 3. Astrocyte to Neuron Lactate Shuttle. MCT1 and MCT4 move lactate out of the astrocyte, while MCT2 moves interstitial lactate into the neurons.

Oberlin et al (unpublished studies) measured the effect of recurrent verses acute hypoglycemia on MCT protein expression in various tissues. The study found no increase in MCT in the hypothalamus. MCTs were also not shown to change with bouts of

exercise induced HAFF. MCTs may not be important to maintaining glucose through sensing neurons, but their role in lactate transport still carries itself as its primary role (40).

Availability of Lactate and Study Design

Our study seeks to investigate the mechanisms behind glucose sensing, counter regulation and brain function during hypoglycemia. The proposed increase in brain glycogen after a single bout of hypoglycemia could lead to increased levels of available lactate to the neurons. The increased levels of glycogen in the astrocytes would provide alternative available substrate to provide energy for the neurons, despite a decrease in glucose levels. The VMH was the brain area targeted, and the adrenal response analyzed and interpreted. This study focuses in on the effects of MCT2 knockdown, and the sub sequential theoretical decrease in available lactate, and the change in epinephrine released due to hypoglycemia.

CHAPTER II

METHODS

Male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing approximately 200g were single housed in a light (12 hour on off cycle) and temperature (25+/- 2 C) controlled vivarium and given unlimited access to food (18% protein global chow, Harlan Teklad Labs, Dublin VA) and water. All animal studies were approved by the University of North Carolina at Greensboro's Institutional Animal Care and Use Committee (IACUC).

Animal Surgery

Rats were administered Xylazine at 10mg/kg before being placed in anesthesia chamber and administered vaporized isoflourene at 3.2% until sedated. Rat were the removed and a nosecone placed to anesthesia via a Kent Scientific Sumnosuite low-flow anesthesia delivery system (2014, Kent Scientific Corporation, Torrington CT). Isoflurane flow rate was reduced to 1.6% and held there until the completion of the surgery. The rats were prepared by shaving the crown of the head and the area of the neck where the jugular vein was located, while keeping the eyes properly lubricated to prevent drying and damage. The areas were cleaned with a combination of ethanol and iodine to prevent infection. Carprofen was administered at 5mg/kg into the hindquarters, and a bolus of 0.25% bupivacaine was injected under the skin of the scalp to decrease post-surgical pain.

The surgical field was isolated using cling wrap and an incision was made on top of the skull with the scalpel. The rat was then flipped over and a similar incision was made in the neck. The jugular vein was identified and a silastic catheter was inserted into the vein. The other end of the catheter was then run along the neck under the skin and extracted from the incision site on the top of the head. The rat was then moved to the stereotaxic and held in place via two ear canal anchors to prevent misalignment. The skull was scraped with the scalpel to debride the surface and prevent bleeding. Gelfoam was used liberally to curb bleeding. Three holes were drilled in the skull of the subject with an electric surgical drill and fitted with 1.25mm screws to act as anchors. Two smaller holes were drilled 0.8mm to the right and left of centerline. Another 2.4mm to the posterior of bregma was then marked and cannula guides were inserted 6.0mm into the brain to position them 2mm above the VMN. Once placed, dental acrylic cement was applied to the skull and allowed to solidify. The cannulas and the port for the catheter were filled with a stylet to prevent coagulation and infection. The catheter was filled with heparin glycerol solution to prevent coagulation in the tubing. After the surgery, Carprofen was administered at 5mg/kg/d to reduce pain and anxiety.

	Day 1	Day 2	Day 3	Day 4
Scrambled Acute	Scrambled ODN	Saline	Saline	Insulin
Scrambled Recurrent	Scrambled ODN	Insulin	Insulin	Insulin
Anti-MCT2 Acute	MCT2 ODN	Saline	Saline	Insulin
Anti-MCT2 Recurrent	MCT2 ODN	Insulin	Insulin	Insulin

Figure 4. Experimental Design. This protocol schedules the ODN injections and recurrent hypoglycemic events in our rat models. Days 1 involved VMH injection while days 2-4 are either saline or insulin injections.

ODN Injections

All animals were given two days to recover after the surgery before the ODN injections. On the third day, rats were no longer dosed with carprofen. The Harvard PicoPlus perfusion pump was utilized, and injection cannula were fitted with a prefilled PE tubing containing either MCT2 antisense (5'-GACTCTGATGGCATTTCTGAG-3') or scrambled control (5'-GGTTTACGAGTCGTCCGTAAT-3') , and delivered at 0.1uL per min over 10mins. The rats were allowed to roam freely during administration of the ODNs, while taking care to prevent dislocation of injection needles. once complete,

injection needles were left in place for 5 minutes before being slowly removed and the stylets reinserted.

Blood Sampling and Tissue Extraction

Blood samples were collected over three consecutive days, with the first day starting the day after ODN injections (day 2). Food dishes for the rats were removed each morning before blood sampling. To minimize stress, blood samples were collected from an animal's home cage via tubing connected to the jugular vein access port. Two baseline samples were collected at -10 and 0 minutes before the administration of either insulin (3 units/kg) or saline. On days 2 and 3, additional blood sample was collected 30 and 60 minutes after insulin or saline. On day 4 all animals received insulin and blood was collected 15, 30, 60, 90, and 120 minutes after insulin. Approximately 250 microliters of whole blood were collected at each time point. Each sample was briefly centrifuged for 10-15 seconds at 6000 rpm and 100 microliters of plasma were harvested. Blood volume was maintained by resuspending harvested red blood cells in a volume of 0.9% sterile saline solution equal to the amount of plasma removed at each time point. The resuspended red blood cells were readministered to each animal through the jugular vein at the time of sampling. plasma was stored at -80^{o}C for later analysis.

Rats were euthanized in a CO_2 chamber; the brain was taken out and a 2mm slice containing the VMH isolated. Punches were taken from the ventromedial nucleus with 18gauge blunted needle. The remainder of the ventromedial hypothalamus was taken and stored in a separate tube. Both were stored at -80^{o}C until thawed for analysis.

HPLC for Catecholamine Analysis

High performance liquid chromatography was used to determine plasma concentrations of norepinephrine and epinephrine using a ESA Coulochem II detector and Thermo Scientific UltiMate 3000 system (Fisher Scientific). All samples were thawed on ice while initial preparation in Eppendorf filter tubes. 10µg of aluminum oxide were added to each tube. Tris buffer was added at 500µL per tube, followed by 80µL of DHBA standard at 1pg/µL concentration. Finally, 100µL of plasma was added to each tube, after being lightly vortexed. Tubes were then agitated for 10min at room temperature. All tubes were centrifuged at 12000rpms for 3mins at 4^{o}C, or until all of the tris buffer had cleared the filter. From there, each sample was washed with 500µL of water for 1min in the centrifuged at 12000rpm, or until the water completely cleared the filter. This was repeated a total of four times. The filter was then transferred to another 150µL sample tube, and 80µL of 0.2N perchloric acid was placed in each filter. Samples were vortexed briefly (1-2min) and allowed to incubate at room temperature for 5min. Tubes were centrifuged at 12000rpm for 2 minutes, or until the acid had completely cleared the filter. Samples were then placed in HPLC sampling tubes and analyzed. All samples were run at 0.275mL/min with column pressure between 2000psi and 2200psi.

Western Blotting

Brain punch samples were removed from -80^{o}C freezer and homogenized in 150µL of a 10:1 solution comprised of RIPA buffer and phosphatase inhibitor. Specific protease inhibitors were added at 100:1 ratio of total volume. Samples were sonicated on ice using a Fisher Scientific Sonic Dismembrator Model 50 at the lowest setting. Three

hits of the sonicator were used for each tube. Tubes were then centrifuged for 15min at 14000rpms at 4ºC. All sample were aliquoted out at 20µL per tube edit one frozen at -80ºC until ready to use. Total protein was assessed using a Pierce BCA kit and a BioTek Epoch plate reader.

On day one of western blotting, homogenized VMN brain samples were thawed on ice. Western blots were performed to determine the presence and amount of MCT2 protein. Plates and holders were cleaned with 70% ethanol and allowed to dry. Plates were inserted into holders, using spacers to allow for internal area. The plate was set on a Hoffman electrophoresis stand, and anchored in placed via attachment screws. Plates were marked as a reference to where the stand would be filled with resolving gel. The 12% resolving gel (see formula appendix) was made and placed in between the plates 3mL of DI water was pipetted on top to prevent drying. The gel was allowed to sit for 30min to completely set up, while being watched for leaks. After 30min, the water was poured off, and a stacking gel was made and pipetted on top of the resolving gel. The bottom of the stand was filled with electrophoresis buffer, prepared fresh for each run. A fifteen well fork was cleaned with 70% ethanol and placed in the stacking gel while it set up over the next 30min. Once the stacking gel was established, VMH homogenate was combined with an equal part of loading buffer (see formula appendix) and was lightly vortexed and pipetted into each lane at 50µg per lane with volume determined from the Pierce BCA. If the total volume to make 50µg was too large for the well, a lesser volume was pipetted and the volume was recorded. Samples were kept on ice throughout process. A lane marker (MagicMark) at 2µl was run alongside samples to act as a weight marker

for protein migration. The set up was filled with electrophoresis buffet and run over night (15-16hr) at 65mV.

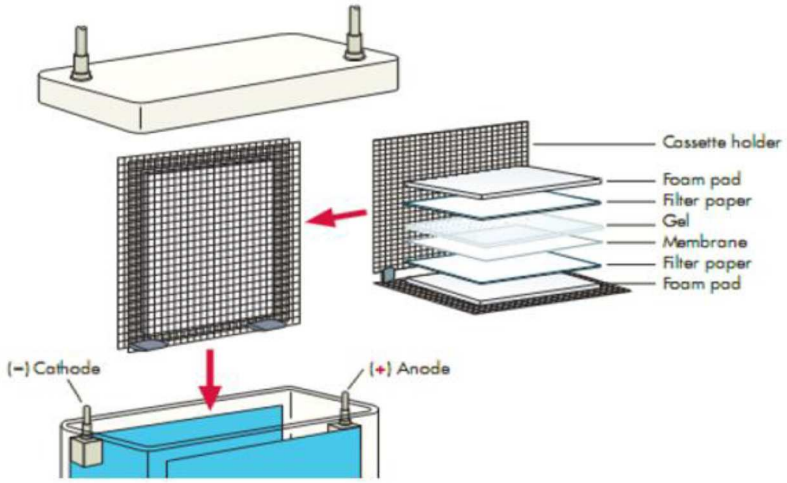


Figure 5. Western Blotting Setup. This shows the device breakdown for the running of gel transfer electrophoresis, giving appropriate positioning of the pads and filters needed to establish protein bands on PVDF membrane. (Image via Bolster Bio, Pleasanton CA)

On the second day, the protein bands were transferred to a PVDF membrane. The membrane was soaked in methanol and sandwiched between 2 sponges and 2 pieces of filter paper while being pressed against the gel. The plate was then placed in a transfer cassette for 6hr at 200mA. The cassette was buried in ice to prevent excess heat generation. After the transfer was completed, The PVDF membrane was washed in TBST for 5min on a Bigger Bill orbital shaker at 100rpm. The TBST was then discarded and a solution of 5% Saco mix and drink powdered milk and TBST was used to wash the membrane for 1hr at 100rpm. The membrane was then washed in TBST three times for 5min, while boxes for primary antibody were washed for 15min in 5% milk. The

membrane was then placed in the box with 15mL of 5% milk and primary antibody for MCT2 (Santa Cruz sc-50232) at a 1:250 ratio. The primary antibody was allowed to block overnight (15-16hr) on a Speci-Mix rocker at 4ºC.

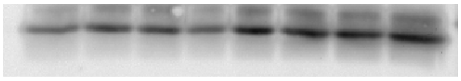


Figure 6. Western Blot for MCT1 Protein. Dark bands in the middle represent the protein, the right most well is the Magicmark ladder for determining weight of proteins.

The following day, the milk was discarded and the membrane was washed in TBST for 10min. The membrane was then washed again with TBST and 5% milk three times for 30min, discarding and replacing milk every 10min. During this phase, boxes for secondary antibody binding were blocked for 30min in 5% milk. The milk was discarded, and the secondary anti-rabbit (Pierce 31460) was applied in 15mL of milk at 1:5000 for 2 hours at room temperature. The milk was discarded, and the membrane was washed in TBST four times for 10min each, discarding after each wash. ECL plus was used to soak the membrane before exposure. The membrane was exposed on a BioRad Chemidoc Station eight times over 300s. The band densities were determined using the densitometry tool in the QuanitiyOne software. Once the exposure had finished, the membrane was washed two times for 10min per wash in stripping buffer. Once the stripping was complete, the membrane was washed for 10min four times in TBST. The membrane was then blocked in 5% milk for 1hr on the orbital shaker. The milk was discarded after an hour, and the membrane was washed three times for 5min per wash in TBST. Concurrently, the box for administration of secondary antibody was washed in 5% milk

for 15min. The membrane was placed in the box with 15mL of 5% milk and primary antibody for GAPDH (Santa Crux sc-166545) at a ratio of 1:4000, and left on a rocker overnight at 4ºC.

Figure 7. Western Blot for GAPDH Control. This gives the ratio of MCT proteins to unaffected control to determine if the amount of protein was affected during ODN treatment.

After the overnight probe with GAPDH, the milk was discarded and the membrane was again washed in TBST. The membrane was then washed three times in 5% milk for 10min per wash. While this was occurring, the box for secondary antibody was washed for 30min with 5% milk. The membrane was then place in this box with 15mL of 5% milk and secondary anti-mouse (Pierce 31450) at 1:2000 for 2hr at room temperature. Once the secondary anti-body had finished, the membrane was washed four times for 10min each in TBST. The membrane was again soaked in ECL solution and exposed eight times for 300s on the Chemidoc Station. Densities were determined using the densitometry tool on the QuantityOne software. Densities of MCT2 and GAPDH were compared to determine the ratio between two proteins, and relative MCT2 expression in the VMN.

Statistical Analysis

Data analysis and graphing was completed using Prism (GraphPad Software, Inc., La Jolla, CA). I performed a two-way repeated measures ANOVA to compare plasma epinephrine to blood glucose levels. The was also used to analyze the changes in plasma epinephrine and blood glucose over the experimental time periods for group statistical significance. A one-way ANOVA was used for the majority of data collected. I used this test for MCT2/GAPDH ratio and compare vs experimental groups. A post-hoc T-test of variance was used when significance was determined. A linear correlation was implemented to set MCT2/GAPDH ratio verses baseline and peak plasma epinephrine levels.

CHAPTER III

RESULTS

MCT2 protein expression in the VMH (Figure 8) of rats administered the MCT2 antisense ODN was reduced to ~57% of non-injected and scrambled ODN- administered animals ($F_{2, 35} = 33.93$, $P<0.001$). Values are presented as percent of non-injected. No statistical differences were seen between the control (scrambled ODN) and non-injected groups.

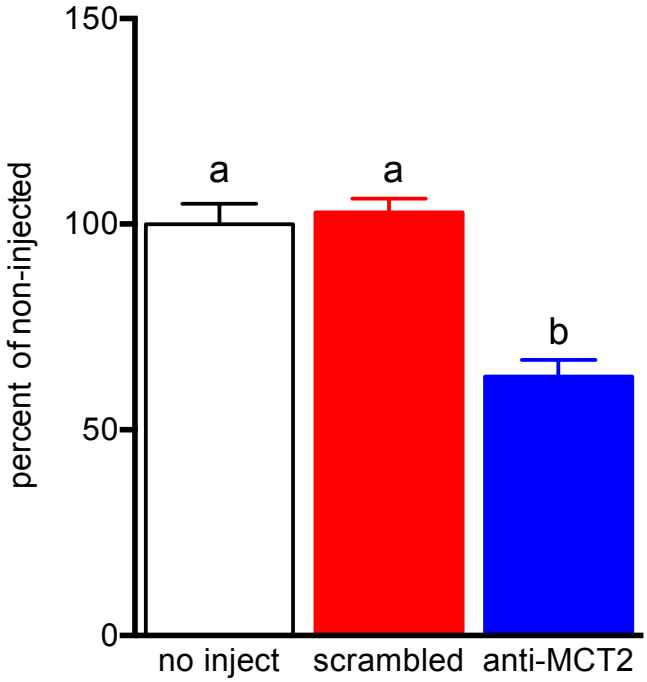


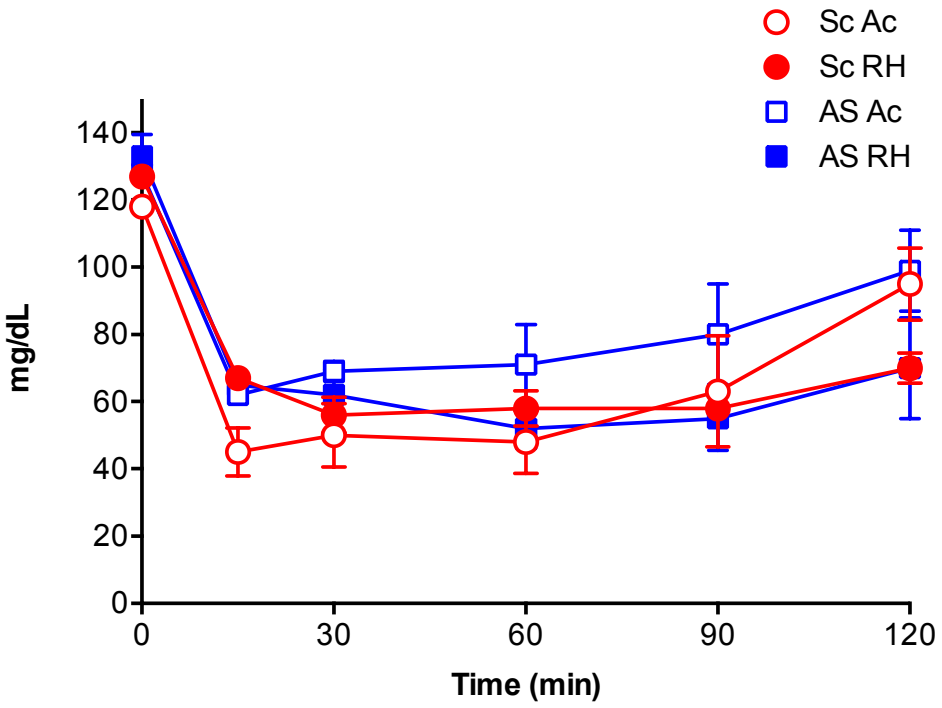
Figure 8. MCT2 Expression in the VMH. MCT2 protein in the ventromedial hypothalamus (VMH) 4 days following injection of scrambled (n=16) or anti-MCT2 (n=14) oligodeoxynucleotide into the VMH. Values are as a percent of non-injected (n=6) rats. Bars with same superscripts are not different.

Plasma glucose decreased ($F_{3, 30} = 44.12$, $P<0.001$) similarly across groups in response to the first 2 bouts of insulin-induced hypoglycemic episodes (days 2 and 3, Table 1). The increase in plasma epinephrine concentration following insulin was apparent only after the first episode of hypoglycemia in the scrambled ODN group ($F_{3, 30} = 5.08$, $P<0.001$).

Table 1. Days 2 and 3 Blood Glucose and Plasma Epinephrine Levels. Plasma glucose (mg/dL) and epinephrine (credit pg/mL) 60 minutes after administration of saline (acute) or insulin (recurrent).

		day 2	**day 3**
Glucose (mg/dL)			
Scrambled	acute	115 ± 8 [a]	118 ± 12 [a]
	recurrent	62 ± 7 [b]	50 ± 2 [b]
Anti-MCT2	acute	121 ± 4 [a]	128 ± 4 [a]
	recurrent	55 ± 10 [b]	59 ± 2 [b]
Epinephrine (pg/mL)			
Scrambled	acute	161 ± 29 [a]	114 ± 56 [a]
	recurrent	953 ± 54 [b]	475 ± 90 [a]
Anti-MCT2	acute	252 ± 6 [a]	278 ± 62 [a]
	recurrent	410 ± 76 [a]	366 ± 95 [a]

Rats were administered oligodeoxynucleotide targeting MCT2 or a scrambled sequence into the ventromedial hypothalamus on day 1. Values with similar superscripts are not different, as determined by two-way ANOVA and tukey's post hoc analysis. (Scrambled acute: n=6, Scrambled recurrent: n=6, anti-MCT2 acute: n=5, anti-MCT2 recurrent: n=5).



Blood glucose (Figure 9) was reduced from baseline ($F_{5, 90} = 44.46$, $P<0.001$) to a similar degree in all groups following insulin administration on day 4.

Figure 9. Change in Blood Glucose Over Time on Day 4. Blood glucose following insulin administration, at time= 0, on experimental day 4. means ± standard error. all points were different ($P< 0.05$) from baseline (time= 0) but there were no differences between treatment group as determined by repeated measure two-way ANOVA. (Sc Ac: Scrambled Acute: n=6, Scrambled recurrent: n=6, anti-MCT2 acute: n=5, anti-MCT2 recurrent: n=5)

The increase ($F_{5, 90} = 6.82$, $P<0.001$) in plasma epinephrine (Figure 10) following insulin administration on day 4 was limited to the rats receiving scrambled ODN.

As expected, the epinephrine response was attenuated in the recurrent group; total response, e.g. area under the curve, was 52% of acute group ($F_{1,18} = 4.58$, $P<0.05$). Significant changes were seen in the scrambled acute group at 15min and 60min compared to all other groups. The scrambled acute group was significant from both antisense groups and the scrambled recurrent group. at the 30min time point. The scrambled recurrent group was significantly different from the scrambled acute group and both of the antisense groups at the 30min time point. The scrambled acute and scrambled recurrent group showed statistical significance between each other and against both antisense groups. Significance was noted at the 90min time point between the scrambled and antisense groups, but not between scrambled and acute of the same group. No significant difference was noted between the antisense groups against each other at any time point. P=0.05

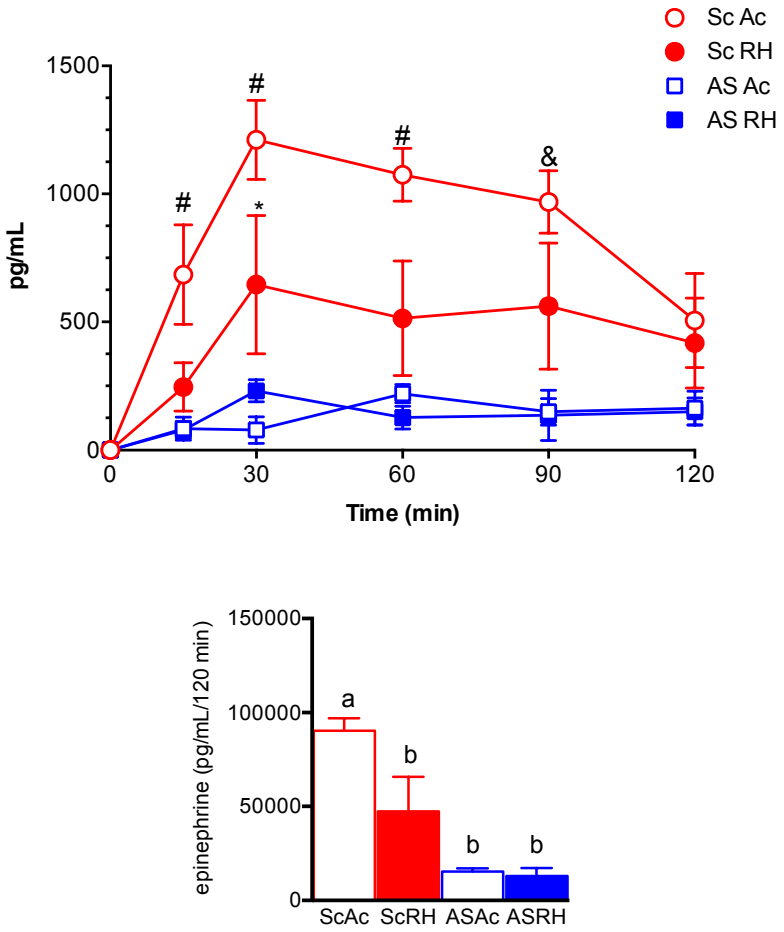


Figure 10. Change in Plasma Epinephrine Over Time on Day 4. Changes in plasma epinephrine following insulin administration, at time= 0, (top panel) and area under the curve (bottom panel) on experimental day 4. Values are means ± standard error. Top panel: # Sc Ac different ($P< 0.05$) from all other groups, *Sc RH different from antisense group, &scrambled groups different from antisense groups as determined by repeated measure two-way ANOVA and Tukey's post hoc analysis. Bottom panel: bars with similar superscripts are not different as determined by one-way ANOVA and Tukey's post hoc analysis. (Sc Ac: Scrambled Acute: n=6, Scrambled recurrent: n=6, anti-MCT2 acute: n=5, anti-MCT2 recurrent: n=5)

One cohort of rats receiving the antisense responded differently to insulin on day 4 (Figure 4). Blood glucose recovered quickly in this group despite receiving the same dose of insulin and the absence of an increase in plasma epinephrine. Baseline concentrations of epinephrine were almost threefold higher ($F_{3,20} = 12.74$, $P<0.001$) and MCT2 expression in the VMH (Figure 11) fourfold lower ($F_{3,36} = 70.42$, $P<0.001$) in these animals which we termed 'nonresponders'.

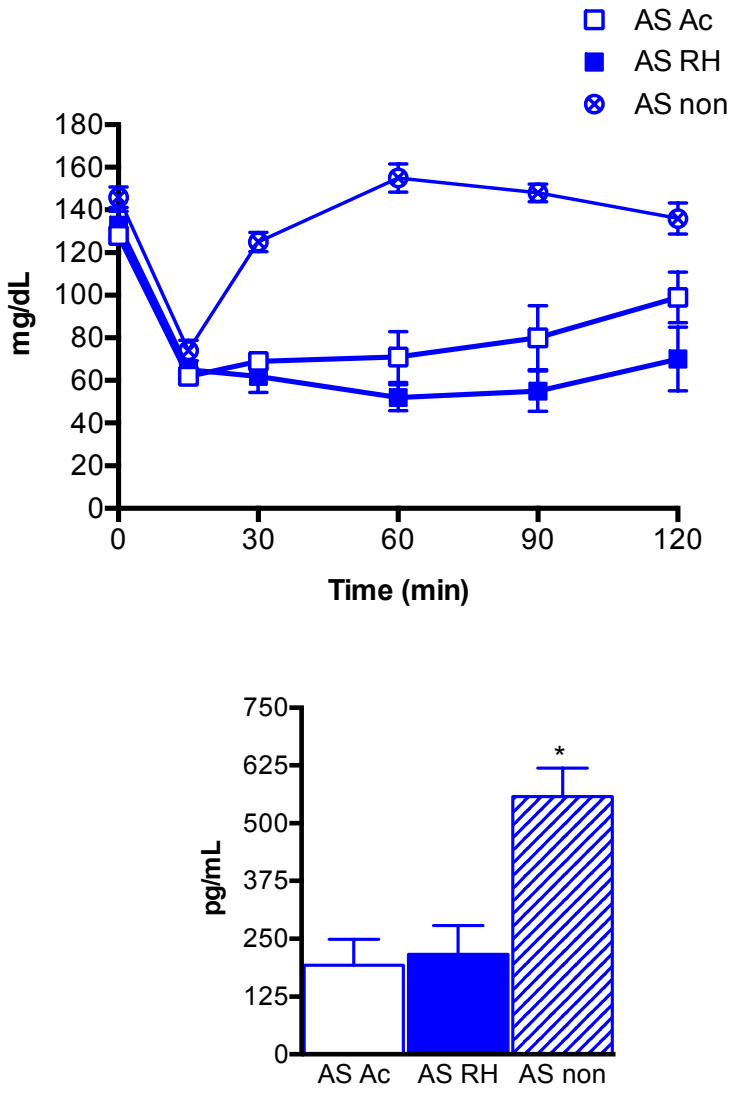


Figure 11. Anti-sense Day 4 Blood Glucose and Baseline Epinephrine. top panel: change in blood glucose levels in antisense groups on day 4. bottom panel: baseline epinephrine level on day 4 baseline, at time point zero. P =0.05 (Sc Ac: Scrambled Acute: n=6, Scrambled recurrent: n=6, anti-MCT2 acute: n=5, anti-MCT2 recurrent: n=5)

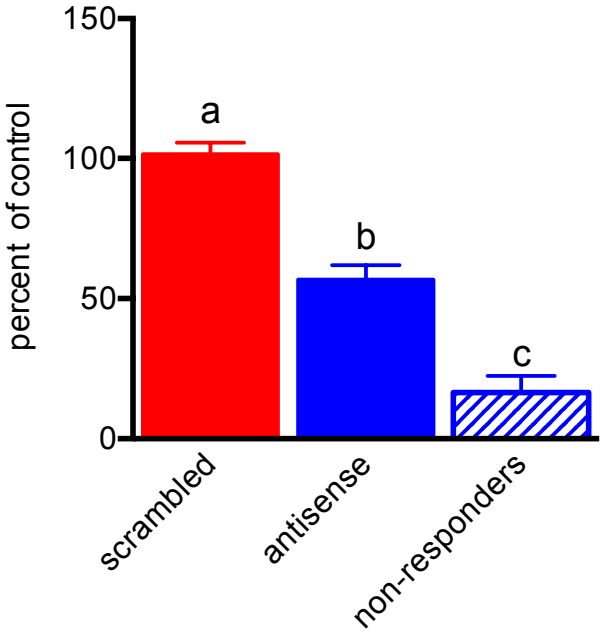


Figure 12. MCT2 Levels in Scrambled, Anti-sense and Non-respondents. MCT2 expression in the VMN following injection of scrambled or anti- MCT2 ODN into the VMH. The level of expression in antisense and non-responders is compared to the scrambled group. All groups were analyzed via Chemidoc densitometry, and all groups show statistically significance from all other groups. P=0.05 (scrambled: n = 12, antisense: n = 8, non-responders: n = 5)

There was a negative correlation ($r^2 = 0.68$; $F_{1,16} = 34.34$, $P<0.001$) between baseline epinephrine concentrations and MCT 2 expression in the VMH (Figure 13). There was also positive correlation ($r^2 = 0.20$; $F_{1,16} = 3.96$, $P = 0.06$) between peak epinephrine response (on day 4) and MCT 2 expression.

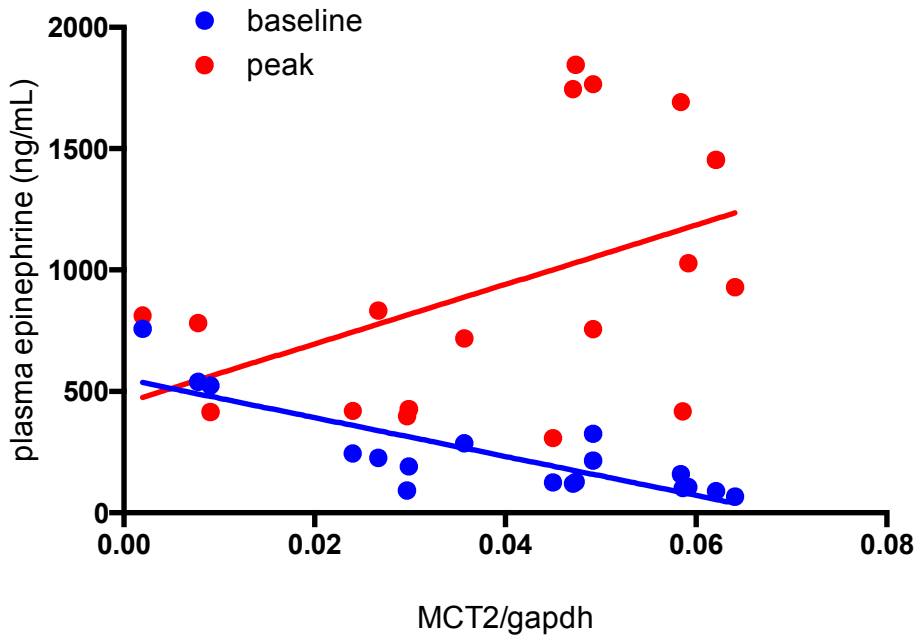


Figure 13. MCT2/GAPDH Expression Correlation to Epinephrine Levels. Scatter plot comparing ratio of MCT2/GAPDH to baseline and peak plasma epinephrine response to hypoglycemia on day 4. Blue dots represent correlation between baseline plasma epinephrine levels and MCT2 expression ratios. Red dots indicate correlation between peak levels of epinepherine on day 4 and MCT2 expression ratios. P=0.05 (peak: n=18, baseline: n=18)

There was a 54% decrease ($F_{2,20}$ = 18.37, P<0.001) in MCT1 protein expression levels in the VMH only in the "non-responders" group (Figure 14). This decrease was not apparent in all rats receiving the anti-MCT2 ODN, as MCT1 protein expression was similar to rats receiving the scrambled ODN and non-injected animals.

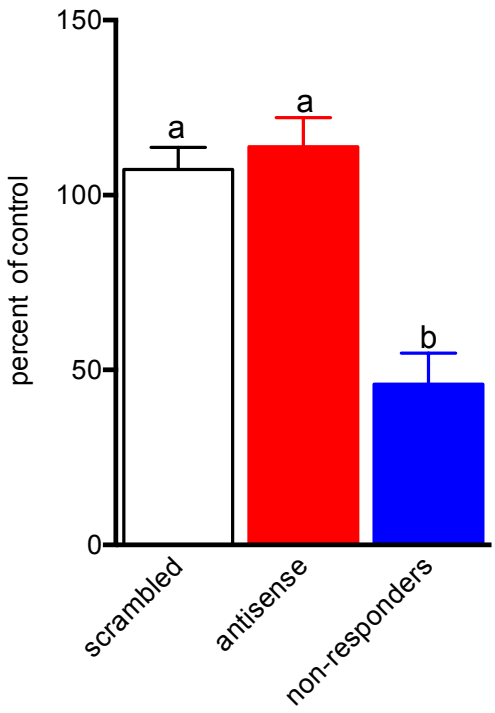


Figure 14. MCT1 Expression Levels in the VMH. MCT1 protein expression levels in the VMH. The white bar represents scrambled rats, the red bar represents the antisense group and the blue bar represents the antisense nonreponders. A one-way ANOVA was completed to determine significance. P=0.05 (scrambled: n = 12, antisense: n = 8, non-responders: n = 5)

CHAPTER IV

DISCUSSION

Normal lactate transport in neurons of the VMH is necessary for the sympathoadrenal response to hypoglycemia. The customary increase in plasma catecholamines during insulin-induced hypoglycemia was absent when MCT2 protein expression in the VMH was reduced by MCT2 antisense ODN. The influence of lactate availability to neurons in the VMH on CRR demonstrated that lactate is probably an important fuel source for the brain. This is not only during bouts of hypoglycemia, but for situations when energy is needed beyond the available glucose. In the VMH, neuronal lactate uptake influences GABAergic activity, which has been associated with the CRR. Chan and associates reported increase in GABA concentrations in the VMH to exogenous lactate was blocked by the MCT inhibitor α-cyano-4-hydroxy-cinnamate (4-CIN). Exogenous lactate in the VMH during hypoglycemia reduced the CRR (Borg and Chan papers) and inhibiting lactate transport with 4-CIN restored the blunted CRR during recurrent hypoglycemia (11). The authors suggested lactate serves as an alternative fuel, and perhaps a preferential fuel following antecedent hypoglycemia, to glucose sensing neurons. During recurrent hypoglycemia, the lack of available glucose could cause the increased utilization of lactate for neuronal fuel (21). Song and Routh (52) reported convincing evidence of an effect of lactate on glucose sensing neuron activity in the VMH. The same authors noted a similar decrease in glucose sensitivity of GI neurons

following recurrent hypoglycemia was induced by supplemental lactate (53). The complete absence of a plasma epinephrine response to hypoglycemia when MCT2 expression was reduced in the VMH was surprising. Given the K_M for lactate by MCT2 is ~ 0.75uM and baseline interstitial lactate concentrations 1.5-2.0 uM in the VMH (11) we expect the 50% decrease in MCT2 protein expression resulted in a decrease in lactate transport into neurons. 4-CIN administered into the VMH restored plasma epinephrine response following recurrent hypoglycemia (11); however, these authors did not measure the CRR when 4-CIN was administered prior to acute hypoglycemia. One response to 4-CIN, and disruption of lactate supply, may be an increase in glucose uptake by neurons (21) and it is possible the chronic decrease in lactate uptake induced by reduced MCT2 expression was compensated by an increase in the capacity for glucose uptake. Fan and colleagues (22) used in shRNA approach to reduce expression of the glucose transporter SGLT1 in the VMH and noted an improved the CRR following recurrent hypoglycemia. The ~53% decrease in SGLT1 was similar in magnitude to the decrease in MCT2 expression of the present study; however, unlike the present study the CRR to acute hypoglycemia was augmented instead of suppressed.

It is unlikely the expected reduction in MCT2 activity in the VMH itself prevented an increase in plasma epinephrine. A cohort of animals with a more severe reduction (~80% below controls) exhibited baseline plasma epinephrine concentrations that were fivefold higher. It is unclear if the higher baseline epinephrine levels were a direct effect of the reduced MCT2 on neurons regulating sympathoadrenal response. Although we did not observe any unusual behavior this subgroup of animals may have

been more sensitive to stress, for example associated with handling at the time of blood sampling. The elevated baseline level of epinephrine may account for the rapid rebound in plasma glucose in these rats. The high levels of epinephrine could have jump started glycogenolysis and gluconeogenesis. In addition, it is unlikely disruption in normal lactate transport resulted in general dysfunction in the VMH. Lesions to the VMH result in hyperphagia and obesity (4) and those rats receiving the antisense treatment maintained normal body weights and blood glucose levels. While the CRR to hypoglycemia was absent in rats with VMH lesions baseline epinephrine was not elevated in VMH-lesioned rats (4). In addition, western blotting of MCT1 revealed that the ODN for MCT2 did not have a deleterious effect on MCT1 expression.

A blunted plasma epinephrine response would not be expected with the first day of hypoglycemia; however, this appeared to be the case in the antisense group. The increase in plasma epinephrine observed in the scrambled ODN group 60 minutes after the initial hypoglycemic episode was absent in both groups of rats receiving the antisense ODN. An interesting possibility is whether inhibition of MCT2 protein expression up to 50% would imitate a state of hypoglycemia by reducing fuel availability to neurons, including glucose sensitive neurons, in the VMH. It may be the initial response, during the first 24 hours, after antisense administration was a disruption in fuel supply in the VMH analogous to what would occur during hypoglycemia. We did not evaluate for a counter regulatory response during this period. The decline in protein expression would be expected to limit to, a certain extent, the flow of lactate into the neurons and could initiate an adrenergic medullary response. If so, this would be expected to lead to a

blunted CRR to subsequent hypoglycemia. We are unaware of any reports for antecedent lactate deprivation and normal CRR to hypoglycemia.

MCT proteins are located on both the astrocytes and the neurons in the VMH. MCT2 has been experimentally determined to be expressed in the neurons of whole brain. MCT4 has been shown to export lactate from the cell it is located on, and is found primarily on the astrocytes in rodent brains. MCT1 is prolific throughout the cells of a postnatal developing rat. The levels of expression decrease over time but are never found in the neurons in large quantity. our methods did not differentiate where MCT2 proteins were located in the VMH, whether or not the reduction in MCT2 proteins were in the neuronal versus mitochondrial membrane. Although expression of MCT2 protein has been shown to be primarily expressed in the neurons within the VMH, homogenization makes it impossible to determine the exact location of expression and function. Throughout our work and the work of Suzuki et al. the potency of the MCT2 ODN was shown at varying levels and the effects that they had on the ability to bring lactate into the neuron. The lack of learning present in the rats given the ODN, followed by the infusion of lactate to restore it provides solid evidence that MCT2 is providing lactate to the neurons to aid in learning. Whereas MCT1 and MCT4 knockdown rats could be rescued via the infusion of lactate, MCT2 knockdown rats had no change verses the rats not given lactate (60.) The Western blotting of MCT2 shows that it was decreased in both our study and Suzuki et al. Our blotting of MCT1 shows the specificity of the ODN, as no change in expression was noted, albeit from the non-respondent group, whose members showed different outcomes all together.

In the present study, reducing MCT2 expression in the VMH, using specific antisense ODN, suppressed the plasma epinephrine response to both an acute episode and recurrent episodes of hypoglycemia. There were no obvious behavioral or physiological indicators of impairment to this brain area by the treatment. These results support the importance of these transporters and the potential significance of changes in lactate availability on the physiological response to changes in glycemic status.

General Discussion

Animals were familiarized with handling before the treatment began. Even so, future studies could give a long period of recovery after implantation of the catheter and brain cannulas. This could further reduce anxiety associated with handling and blood draws. Bloods draws were done in the least anxiety-inducing manner, to prevent confounding of the data. Changes in blood glucose levels were adequate in the animals included in the study. A portion of the rats could not be included due to their lack of hypoglycemia on the days leading up to the final day and tissue euthanasian. Rats included typically had experimental blood glucoses levels below 60mg/dl, and showed physical signs of hypoglycemia. Blood glucose testing was done either during the administration of insulin and saline, or after the experimental period using a glucose analyzer. This should not give skewed results. Another limitation includes the small sample size. A clear statistical trend was noted, despite the low number of rats. Future studies will include increased sample size to meet the initial desired numbers.

One additional change to be made would be to take a plasma sample on the day of the ODN injection. This would give an idea if elevated catecholamine levels were detected in the antisense rats verses the control rats. Time would need to be taken after injections, as the handling of the rats would cause a large spike in stress hormones. It would need to be prompt enough so that the level would not have decreased, if a change did occur due to the injection of the ODN and the limited available lactate into the neuronal cells. Insulin could possibly be administered to the rats on day 1, soon after the ODN injections, with the only limiting factor being the rate at which the protein is turned over and a notable decrease in expression is present.

Western blotting of the VMH tissues showed that the ODN for MCT2 knockdown was effective. For the majority of rats, the expression of MCT2 was reduced by about 50%, while the non-respondent group showed much higher levels of knockdown. The ODNs exceeded our presumed level of effectiveness. This could be due to the larger injected amount compared to other studies that have used the same ODN for knockdown. The western blotting of MCT1 showed that for the majority the ODN for MCT2 did not have a deleterious effect on its expression, as expect. The western blotting for rats who were non-respondents showed a statistical knockdown in MCT1 level. This could indicate that high concentrations of ODN have a disruptive effect on all proteins. Further research into this area should look at the effects of MCT1 and MCT4 knockdown via ODNs. They should be administered separately and together, as to determine the effect on the stress response and changes in the CRR. Measured changes in steady-state lactate could give a clue if the flow of lactate into the neuron is possibly coming into the

interstitial space from somewhere other than the astrocytes. Rats VMH could be taken and ultimately and glycogen asseys could give a better clue into the possibility of supercompensation. It would be impossible without tagging and imaging to determine where in the VMH the glycogen was forming. Tagging glucose and scanning for this would be the best option for following the path of the glucose after absorption. Preventing excess glycogen formation could prevent the development of hypoglycemic unawareness.

Further work in monocarboxylic rescue with lactate could provide some further depth into how MCT2 acts during knockdown. Neurons would theoretically not be able to take in much lactate after ODN knockdown, but this may help to overcome the effects of the blunted CRR in both the antisense and non-respondent groups. If rats were given a large dose of the different ODNs, it could be determined the threshold they knockdown their target protein, and at what point they being to effect other proteins. This would help to facilitate further studies in MCT knockdown. The use of ODNs to monitor change in the MCT levels could ultimately bring about a way of reducing or eliminating the blunting CRR to hypoglycemia. Providing a therapeutic treatment that could eliminate lactate availability, or glycogen utilization could prevent the lack of physiological awareness of the dip in glucose. This would allow the individual to consume glucose to bring the body back to euglycemia. This would also be useful for diabetic individuals who may not possess the insulin production needed to take up circulating glucose. The lack of initial unawareness would prevent further instances of this and allow better glycemic control overall

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APPENDIX A

FORMULAS AND RECIPIES

Mobile phase for catecholamine analysis

Components	Amount per 1L DI water
75mm NaH_2PO_4 H_2O (MW=137.99)	10.35g
1.8mM 1-Octanesulfonic Acid (MW=216.3)	0.389g
25uM Na EDTA (MW=372.24)	0.009g
Diethylamine (DEA)	100uL
7% Acetonitrile (ACN)	70mL

Tris Buffer: 45g Tris base, 5g Na EDTA in 200mL DI H_2O. Bring pH to 8.6 with phosphoric acid. Bring total volume to 250mL and refrigerate up to 3 weeks.

RIPA Buffer: 50mM Tris base, 1mM EDTA, 150mM NaCl, 1% NP-40, 1% deoxycholate, 1% SDS.

10x TBST: 24g Tris base, 88g NaCl, 10mL Tween 20x in 1L H_2O with pH adjusted to 7.6 with phosphoric acid.

12% Acrylamide Resolving gel: 9.9ml of DI water, 12ml Acrylamide, 7.5ml Tris (pH8.8), 300µl of SDS (10%), 300µl of APS, and 25µl of TEMED.

12% Acrylamide Stacking gel: 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.

Electrophoresis buffer 10x: 90.8g Tris Base, 432.4g glycine and 30g SDS in 3L DI H_2O.

Transfer buffer: 90.8g Tris Base, 432.4g glycine and 20% methanol in 3L DI H_2O.

Stripping buffer: 15g Glycine, 1g SDS, 10mL Tween 20x. All in 1L DI H_2O with pH adjusted to 2.2 with phosphoric acid.