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Pre-Administration of Medium Chain Triglycerides In Vivo Can Attenuate or Block the Effects of Recurrent Hypoglycemia

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Pre-Administration of Medium Chain Triglycerides *In Vivo* Can Attenuate or Block the Effects of Recurrent Hypoglycemia

An honors thesis presented to the
Department of Biological Sciences,
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and
graduation from The Honors College

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Abstract

Hypoglycemia is a state of abnormally low blood glucose. Many patients who use insulin, primarily for the treatment of diabetes, experience multiple bouts of hypoglycemia, termed recurrent hypoglycemia (RH). Because RH impairs cognitive function and ability to appropriately respond to a subsequent episode of hypoglycemia, it is critical to develop treatments. One approach, which we have taken here, is to attempt to preserve neuronal fuel supply during a hypoglycemic episode. Medium-chain triglycerides are medium-chain fatty acid (MCT) esters of glycerol that can provide an alternative fuel source to the brain via ketones; the hippocampus is known to express transporters for ketones and to be able to metabolize them. Hence, pre-administration of MCT prior to times of hypoglycemia could possibly prevent the deleterious effects of RH if they are due to loss of fuel generically rather than to specific loss of glucose. We have used a previously established three-day rat model of RH, in which rats are made hypoglycemic on each of three consecutive days and show both cognitive effects and impaired responses to hypoglycemia on the 4th day. Here, we gave Sprague-Dawley rats MCT (i.p.) prior to each episode of hypoglycemia, followed by cognitive testing, removal of the brain and analysis of brain proteins of interest: transporters for glucose and ketones as well as additional markers suggested by our prior studies. During cognitive testing, *in vivo* microdialysis was used to obtain real-time measures of hippocampal glucose and lactate; previous work showed that RH markedly affected local hippocampal metabolism during testing.

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List of Figures

Figure 1	14
Figure 2	15
Figure 3	16
Figure 4	17

List of Tables

Table 1	7
Table 2	11

Table of Contents

Abstract	ii
Acknowledgments	iii
List of Figures	iv
List of Tables	v
Introduction	1
Materials and Methods	6
A. Animals Used for Experiment	6
B. Surgery	6
C. Microdialysis.....	8
D. Spontaneous Alternation.....	8
E. Contextual Fear.....	9
F. Tissue Collection	9
G. Tissue Preparation.....	10
H. Western Blot	10
I. Statistical Analysis.....	12
Results	12
Discussion	18
References	21

Introduction

Hypoglycemia is the predominant side-effect of insulin therapy and is defined by abnormally low blood glucose, generally below 70 mg/dL. Cognitive impairment and neuronal death are results of severe hypoglycemia, when blood glucose levels are low and external assistance for recovery is required (Suh et al., 2003). A major drawback of insulin therapy, a common treatment for type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), is the increased risk of recurrent hypoglycemia (RH). RH refers to a patient experiencing multiple bouts of hypoglycemia over a short amount of time. Studies of RH in rat models, which focused on the impact of RH on hippocampal function, showed RH can affect subsequent cognitive performance as well as synaptic plasticity and brain metabolism (McNay & Sherwin, 2006).

A positive consequence of RH is it enhances euglycemic spatial memory performance, due to adequate glucose supply to the brain. However, RH impairs spatial memory performance during subsequent hypoglycemia (the clinically key state), when glucose supply to the brain depreciates. McNay et al. (2006), have shown cognitive performance is preserved if tested at euglycemia after prior RH. A previous study conducted by McNay and Sherwin (2004), used a model of RH, which consisted of three-hour injection periods daily for three consecutive days. The rats were either in a state of acute hypoglycemia or euglycemia during this period, and the effects of RH on subsequent hippocampally-dependent spatial memory were tested. The study showed improvements in subsequent cognitive performance at euglycemia due to RH. Similarly, changes in hippocampal glucose transport and metabolism, as well as in synaptic plasticity was observed (McNay & Sherwin, 2004). C-Fos expression, a marker of neuronal activation, increased by acute hypoglycemia but reduced after RH, indicating RH negatively influences subsequent neuronal function (Paranjape & Briski, 2005).

Additionally, it has been noted RH improves learning as well as increases the expression of glucocorticoid receptors, cyclic AMP response element binding (CREB) phosphorylation, serum/glucocorticoid-regulated kinase 1, *N*-Methyl-D-aspartic acid (NMDA) receptors, and plasma membrane levels of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in the hippocampus (Osborne et al., 2016). During periods of elevated glucocorticoid signaling during hypoglycemia there are changes in dorsal hippocampal physiology which enhance hippocampus dependent contextual learning (Osborne et al., 2016). For instance, Osborne et al. (2016) recognized the role of serum/glucocorticoid-inducible kinase 1 (SGK1) in mediating the cognitive enhancing effects of glucocorticoids. Changes to the dorsal hippocampus appear to be adaptive and may decrease any damage caused by exposure to severe hypoglycemia. Studies on the Diabetes Control and Complications Trial showed, specifically during testing at subsequent euglycemia, RH does not appear to produce any long-lasting cognitive deficits, indicating no association between hypoglycemia and baseline cognitive function and concluding repeated episodes of hypoglycemia are likely unrelated to cognitive decline (Austin & Deary, 1999). A study from The Diabetes Control Complications Trial/Epidemiology of Diabetes Interventions and Complications Study Research Group in 2007, further confirmed the conclusion of this study, stating moderate RH does not decline cognitive performance.

A major drawback of RH is that RH leads to *hypoglycemia unawareness*, a state in which cognitive impairments are less noticeable to the individual. In hypoglycemia unawareness, sympathoadrenal and neurogenic symptom responses which result from decreased sympathetic neural responses to diminishing levels of glucose, are lessened to a specified level of subsequent hypoglycemia (Cryer, 2005). RH also leads to *hypoglycemia-associated autonomic failure* (HAAF), a breakdown of autonomic counter-regulatory responses (Dagogo-Jack, Craft, & Cryer,

1993). HAAF mainly results in both defective glucose counterregulation and hypoglycemia unawareness. A key feature of the components of HAAF that is unknown is the mechanism by which the glycemic thresholds for sympathoadrenal activation is shifted to lower plasma glucose levels by hypoglycemia (Cryer, 2005). Cryer et al. (2005) proposes the mechanism is likely due to changes in brain metabolism, although this remains unclear as the specific changes are unidentified. At large, further research is necessary to eliminate hypoglycemia in those affected by diabetes.

RH is of clinical significance when a patient with a history of RH is about to engage in a task. For instance, if a patient with RH is about to drive, the patient is likely at risk because RH impairs the individual's cognition, decision-making, which is largely mediated by the prefrontal cortex as shown in Ragozzino (2007), and ability to detect hypoglycemia. Impaired judgment leads to impaired motor skills which could be unsafe to the patient. A study showed 43% of T1DM patients with hypoglycemia unawareness made the decision to drive despite having hypoglycemia (Stork, Haeften, & Veneman, 2007). Such a situation should be prevented, to preserve brain function and prevent the deleterious effects of RH, a focus of this present study.

Under hypoglycemic conditions there is neuronal metabolic challenge and a lack of glucose supplied to the brain. There continues to be metabolic deficiency unless an alternate fuel source to the brain is introduced. Although glucose has been viewed as the predominant energy source usable by neurons in the brain, Schurr et al. (1997), showed lactate could also play the role of a substrate for neuronal energy metabolism. Additionally, it is shown during episodes of hypoglycemia, lipids are used as an alternative to glucose, to supply fuel to the brain. Specifically, a study by Haywood et al. (2009) showed, increased lipids in the brain enhance sympathoadrenal response during hypoglycemia. These findings suggest the possibility of an

alternative fuel source to the brain using fatty acids, specifically medium-chain fatty acids (MCFA) to support neuronal metabolism during RH. Medium-chain triglycerides (MCT) are commonly found in mundane products like coconut and palm kernel oils and are a source of MCFA. There is interest in the potential to use MCT to offer a therapeutic advantage of preserving brain function under hypoglycemic conditions. We see the potential application of MCT in a situation where a patient is suffering from hypoglycemia and suffers a drop, in blood glucose causing neuronal metabolic deficiency. We believe MCT is a strong therapeutic approach because it provides both a direct and indirect fuel source to the brain through the production of ketones (Traul, Driedger, Ingle, & Nakhasi, 2000), which can be metabolized by the hippocampus. MCFA readily crosses the blood-brain barrier and is easily oxidized by the brain (Ebert, Haller, & Walton, 2003). Ketones are produced in the mitochondrial matrix of the liver during periods of low carbohydrates when energy must be obtained through the breakdown of fatty acids. The ketone bodies produced from acetyl-coA include: acetoacetate, β -hydroxybutyrate and acetone. During periods of insufficient glucose (2-3 days), energy is supplied to the brain via the ketone bodies. There is considerable evidence to suggest MCT has a strong potential to mitigate the effects of RH.

A study by Page et al. (2009), tested whether MCT could possibly provide an alternate fuel source to the brain, to prevent the deleterious effects of higher brain function caused by acute hypoglycemia in T1DM human subjects. The study showed without raising blood glucose levels, MCT can provide ketones to the brain and thus preserve neuronal function during hypoglycemic episodes in diabetic individuals. The study found through oral delivery of MCT in intensively treated T1DM patients, cognition improved without harmfully affecting counterregulatory hormones such as glucagon. Initiation of hormonal responses and hypothalamic glucose-sensing

was not affected by delivery of MCT. Likewise, MCT also completely prevented decline in short-term memory in hypoglycemic patients. A rat hippocampal slice model *in vitro* was also used in the same study. The hippocampal slice model prepared from nondiabetic rats was used to assess whether neuronal activity could be preserved in the presence of octanoate and β -hydroxybutyrate at times of glucose deprivation. The study found in hippocampal slices, under low exposure to glucose, β -hydroxybutyrate supported synaptic transmission. Octanoate did not support synaptic transmission under low glucose exposure, but rather, improved the rate of synaptic function when control glucose concentrations were restored. Importantly, from this study, it has been shown under hypoglycemic conditions, MCT offered the therapeutic advantage of preserving brain function.

A more thorough understanding of the effects of RH across different brain regions would be clinically valuable. It is essential to focus on the cognitive and neural effects of recurrent hypoglycemia. Literature suggests the brain responds to recurrent hypoglycemia by enhancing fuel supply to preserve cognitive functions. Further examining the brain's response to RH, may facilitate the development of preventative therapies such as the use of MCT as an additional fuel supply to the brain and as a therapy to reverse the effects of RH (McNay & Cotero, 2010).

In our study, we hope to show RH compensatory mechanisms do not set in when providing MCT. In the present study we have conducted behavior testing at euglycemia. We tested whether pre-administration of MCT in RH animals will impede neural compensatory mechanisms during hypoglycemia. To measure spatial memory processing and fear memory, spontaneous alternation and contextual fear testing were performed. We propose pre-administrating MCT will prevent changes to cognition and/or metabolic adaptation, hence RH/MCT animals will perform similarly to Euglycemic/Vehicle animals.

As an overview of the expectations for the animal groups, it is expected the Euglycemic/Vehicle animals will show no change in cognitive performance from baseline, as with the Euglycemic/MCT animals. Euglycemic/MCT animals, serve as a control to show MCT alone does not influence cognitive functioning without hippocampally mediated memory at euglycemia after recurrent hypoglycemia. Likewise, the RH/MCT animals would perform similarly to the Euglycemic/Vehicle animals since the pre-administration of MCT would prevent improved cognition. Artificial extracellular fluid (analogous to extracellular fluid produced in the rats) administration prior to insulin administration in the RH/Vehicle animals, should demonstrate improved performance in cognitive and behavioral tasks, due to hippocampally mediated memory after a period of recurrent hypoglycemia.

Materials and Methods

A. Animals Used for Experiment

The experiment was conducted with 34 male Sprague Dawley rats. The animals arrived at 11 weeks of age and were provided ample food and water. Animals were on a 12:12 hour light to dark schedule. The room temperature was constant at 25 °C. After 48 hours of the animal's arrival, the animals were handled for approximately 10 minutes each day. Post-surgery, and prior to behavioral testing the animals continued to be handled. On arrival the animals were housed in pairs. Post-surgery the animals were single-housed.

B. Surgery

A week post handling, the animals underwent a stereotaxic cannula implantation surgical procedure. In this procedure a microdialysis guide cannula was stereotaxically implanted into the left hippocampus of all animals at coordinates (in mm, relative to the bregma) -5.6 posterior,

+4.6 lateral, and -3.3 ventral. The nose-bar was adjusted to 5 mm above the interaural line. Prior to the start of surgery, oxygen was delivered, and the animals were anesthetized with isoflurane (5% by air) at the same time. Isoflurane to oxygen were maintained in a 3:2 ratio throughout the surgery. A 1 mL followed by 3 mL injection of sterile saline was administered before and after surgery respectively. To prevent bleeding, a 1:1 Epinephrine/Marcaine solution was administered as required during surgery. Following anesthetic removal, animals were kept in a warm incubator and monitored until recovery. The animals were handled for approximately five minutes each day post-surgery. Rimadyl tablets (5 mg/kg body weight) were provided to the animals for three consecutive days post-surgery.

Intraperitoneal Injections

Table1. The following groups of animals were handled and surgerized prior to receiving intraperitoneal injections (i.p. injection). Post i.p. injections the animals underwent behavioral testing and were sacrificed.

Group	Condition
MCT→Euglycemia	These animals received an i.p. injection of MCT oil 10 minutes prior to an i.p. injection of saline which was given during the RH induction period.
Vehicle→Euglycemia	These animals received an i.p. injection of artificial extracellular fluid 10 minutes prior to an i.p. injection of saline given during the RH induction period.
MCT→RH	These animals received an i.p. injection of MCT oil 10 minutes prior to an i.p. injection of insulin given during the RH induction period.
Vehicle→RH	These animals received an i.p. injection of artificial extracellular fluid 10 minutes prior to an i.p. injection of insulin given during the RH induction period.

Hypoglycemia was induced for a period of three hours daily for a total of three consecutive days. The animals received approximately 3 U/kg of insulin the first day, followed by 2 U/kg of insulin the second day, and 2 U/kg of insulin on the third day. The dosages were subject to

change based on the animal's glucose levels at the two-hour mark. A dosage of 2 U/kg, 1 U/kg, and 1 U/kg appeared to be more favorable. Animals that were not induced into hypoglycemia received a volume-matched intraperitoneal injection (i.p. injection) of saline. Blood was collected via a tail prick at every one-hour mark for the three-hour period after insulin or saline administration to test blood glucose levels. Blood glucose levels were tested using a glucometer. Animals, who did not return to euglycemia after the three-hour period, were given a volume-matched i.p. glucose injection. The animals were only provided with food after the three-hour period had been completed. To obtain an adequate dosage of MCT oil, a formula based on the weight of each animal was created in accordance with Page et al. (2009).

C. Microdialysis

Approximately 24 hours prior to microdialysis and on the day of the last i.p. injection, the animals were pre-probed for five minutes. A new probe was inserted on the day of the procedure, and the animals acclimated for an hour. The probes were perfused with artificial extracellular fluid composed of 153.5 mM Na, 4.3 mM K, 0.41 mM Mg, 0.71 mM Ca, 139.4 mM Cl, 1.25 mM glucose and a buffered pH for 7.4 at 1.5 uL/min (McNay & Sherwin, 2004). Acclimation, baseline (tubes 1-3), testing (tubes 1-2), and recovery (tubes 1-3) tubes of samples were collected in 20 min aliquots and later frozen at -20 °C.

D. Spontaneous Alternation

Animals were moved from the home cages into the center of a four-arm maze. Concomitant hippocampal microdialysis sampling was performed throughout spontaneous alternation (SA). All animals explored the maze for 20 minutes, before being placed back into their home cages. Each time the animal moved into an arm of the maze or placed a portion of their body in the arm,

it was noted. 70% ethanol was used to clean the maze prior to the testing of a new animal. In the process of exploring the maze, animals used spatial working memory to spontaneously alter between maze arms. An alternation was counted when the animal entered each of the four arm mazes within a span of five arm changes. Performance is expressed as a percentage, where the chance level is 44%. A measure of locomotor activity is the total number of arms entered, and this measurement can provide information on possibly confounding events, such as diet or treatment.

E. Contextual Fear

Animals underwent contextual fear (CF) training procedures three hours after the conclusion of microdialysis. The contextual fear procedure was adapted from Osborne et al. (2016). Animals were placed inside an open chamber that offered discrete cues to the animals about locations within the chamber. The animals were allotted 60s to explore the chamber, at which point an 80dB tone was sounded for 10s and followed by a 5s 0.8mA foot shock. The tone-shock pairing was repeated three times at one-minute intervals before the animals were returned to their home cage. Twenty-four hours after training, the animals were returned to the same chamber for testing. At this point the freezing behavior in response to the tone was noted for each one-minute interval for a total of three minutes. Evidence of a memory formed of the association between context and foot shock was assessed by time spent freezing without the tone, with freezing defined as a complete absence of movement in the chamber.

F. Tissue Collection

Twenty-four hours after contextual testing, animals were anesthetized with isoflurane decapitated once completely unconscious. Brains samples of the left and right hippocampus,

prefrontal cortex, amygdala, and hypothalamus were collected. Blood was centrifuged, and plasma samples were also collected. All samples were stored at -80°C in a freezer.

G. Tissue Preparation

The collected samples were transferred to $150\mu\text{L}$ of homogenization buffer and mixed with a tissue grinder. $30\mu\text{L}$ of the homogenized tissue was placed in $100\mu\text{L}$ of RIPA buffer to obtain the total sample. Plasma membrane samples were solely placed in homogenized buffer followed by membrane extraction.

H. Western Blot

Western blots were conducted to determine the expression of protein across the selected brain regions. BIO RAD Mini-PROTEAN TGX Gels were used and samples ran in 1x running buffer containing 0.25 M tris, 0.192 M glycine, and 0.1% SDS, pH 8.5. Lanes were loaded with biotinylated ladder from Cell Signaling and a sample from each different group along with 2X BIO RAD Laemmli Sample Buffer, 2.78% 2-beta mercaptoethanol, and RIPA buffer. Only the total samples were incubated at 95°C for five minutes prior to loading. Loading volumes were between $30\text{-}45\mu\text{L}$ depending on the sample amount. The samples ran on the gel for 60 minutes at 140V.

Through creating a “sandwich,” the gel was transferred to a PVDF membrane. The black side of the cassette consisted of sponge pre-soaked in the transfer buffer, a piece of filter paper also pre-soaked in the transfer buffer, the gel, methanol-activated PVDF membrane, and another piece filter paper pre-soaked in the transfer buffer. A pipette tip was rolled across the top of the filter paper to ensure there were no bubbles and the cassette was placed into the Bio-Rad buffer tank. The Bio-Rad buffer tank was kept in 4°C and the transfer ran for 60 minutes at 70V.

Proceeding the transfer to the membrane, the membrane was dipped in methanol, dried on a benchtop, and briefly reactivated with methanol. Ponceau S staining was done to ensure the transfer of proteins. The membrane was rinsed in 0.1% Triton X-100 containing tris-buffered saline (TBST), followed by blocking with a 1% TBST and BSA solution. All rinses were approximately 10 minutes. The membrane was rinsed with 0.1% TBST once more followed by overnight incubation in 4° C with primary antibody solution (varied based on antibodies). The antibodies of interest were GluT1, GluT3, GluT4, MCT1, MCT2, AMPK, and SGLT. All antibodies were from Abcam except MCT 1, MCT2 and AMPK which were from Biossusa and Cell Signaling respectively.

On the second day of the procedure, the membrane was rinsed with 0.1% TBST, followed by incubation in a secondary antibody solution (1: 20,000, Vector Labs). Another rinse with 0.1% TBST followed by incubation in the tertiary antibody, HRP Streptavidin (1: 5,000, Thermo Fisher Scientific) and 0.1% phosphate-buffered saline (PBS). After incubation, the membrane was rinsed with 0.1% TBST and 1X TBS and imaged with enhanced chemiluminescence (ECL) western blotting substrate detection reagent (1:1 light to non-light-sensitive) (Pierce). ImageLab software (BIO RAD) was used.

In summary, the purpose of the primary antibody is to bind to the protein of interest during overnight incubation. The secondary antibody will bind to the primary antibody if the secondary antibody is raised against the host animal of the primary antibody. The purpose of the tertiary antibody is to bind to the ladder as well as the antibodies which are bound to the protein of interest. After the addition of the chemiluminescent substrate, the substrate and HRP will react with each other and emit an ultraviolet light which is developed into an image upon being read by the BIO RAD imager.

I. Statistical Analysis

A Univariate Analysis of Variance (ANOVA) was used for both contextual fear and spontaneous alternation data. ANOVA was used for contextual fear behavioral data analysis to show differences between group means that were split on two independent variables (MCT and RH/Euglycemia or artificial extracellular fluid and RH/Euglycemia). Significance of data was taken <0.05 . The number (N) of each group for both contextual fear and spontaneous alternation data was between 5-8. ImageJ software was the software of interest to assess the results of western blots. ImageJ can make comparisons between different lanes and is a semiquantitative method to use to assess subtle differences between lanes.

Results

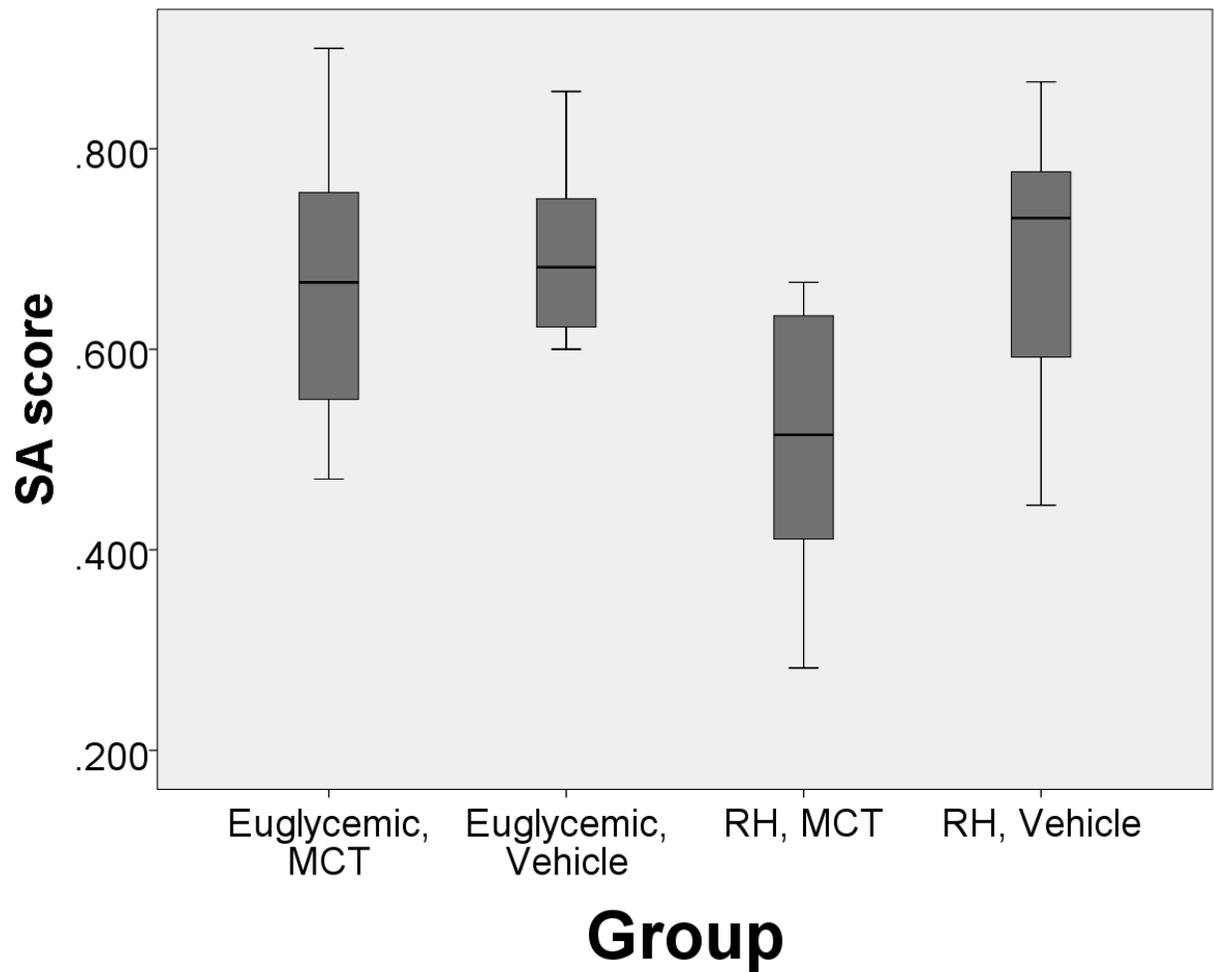
In this study we proposed pre-administrating MCT will prevent changes to cognition and/or metabolic adaptation, hence RH/MCT animals will perform similarly to Euglycemic/Vehicle animals. To measure spatial and fear memory processing, spontaneous alternation and contextual fear behavioral testing were performed. Figure1. and Figure2. presents behavioral data, with Figure1. representing data for SA and Figure2. representing data for CF. Neither SA scores or CF scores were significant ($p=0.064$ for SA; $p= 0.638$ for CF). We expected the Euglycemic/MCT group to perform similarly to the Euglycemic/Vehicle group which was the control group. We also expected to observe no changes to cognitive performance and metabolic rates. The data suggests that the detrimental effects of RH on SA or CF behavior were not rescued by injecting animals with alternate fuel namely, MCT. In the RH/Vehicle group we predicted this group would perform better on cognitive tasks due to metabolic adaptation which would allow for hippocampally-mediated memory after recurrent hypoglycemia. We observe the

percentage of non-random alternations is slightly higher in the RH/Vehicle group compared to the RH/MCT group, alike to the Euglycemic/MCT group but is lower than the Euglycemic/Vehicle group.

The RH/MCT group was expected to perform similarly to the control group, in that, as the RH/Vehicle group would show improved cognition, the pre-administration of MCT would prevent this from occurring. The behavioral data presented here suggests the RH/MCT group performed similarly to the RH/Vehicle group. However, it will be critical to evaluate by microdialysis analyses and western blot whether neural changes are happening although the behavior remains unaltered. A preliminary western blot (adopted from the first trial of this experiment) is shown in Figure3. and Figure4. An insignificant increase in GluT3 expression in the right hippocampus and prefrontal cortex in the Vehicle/RH and MCT/RH groups can be observed.

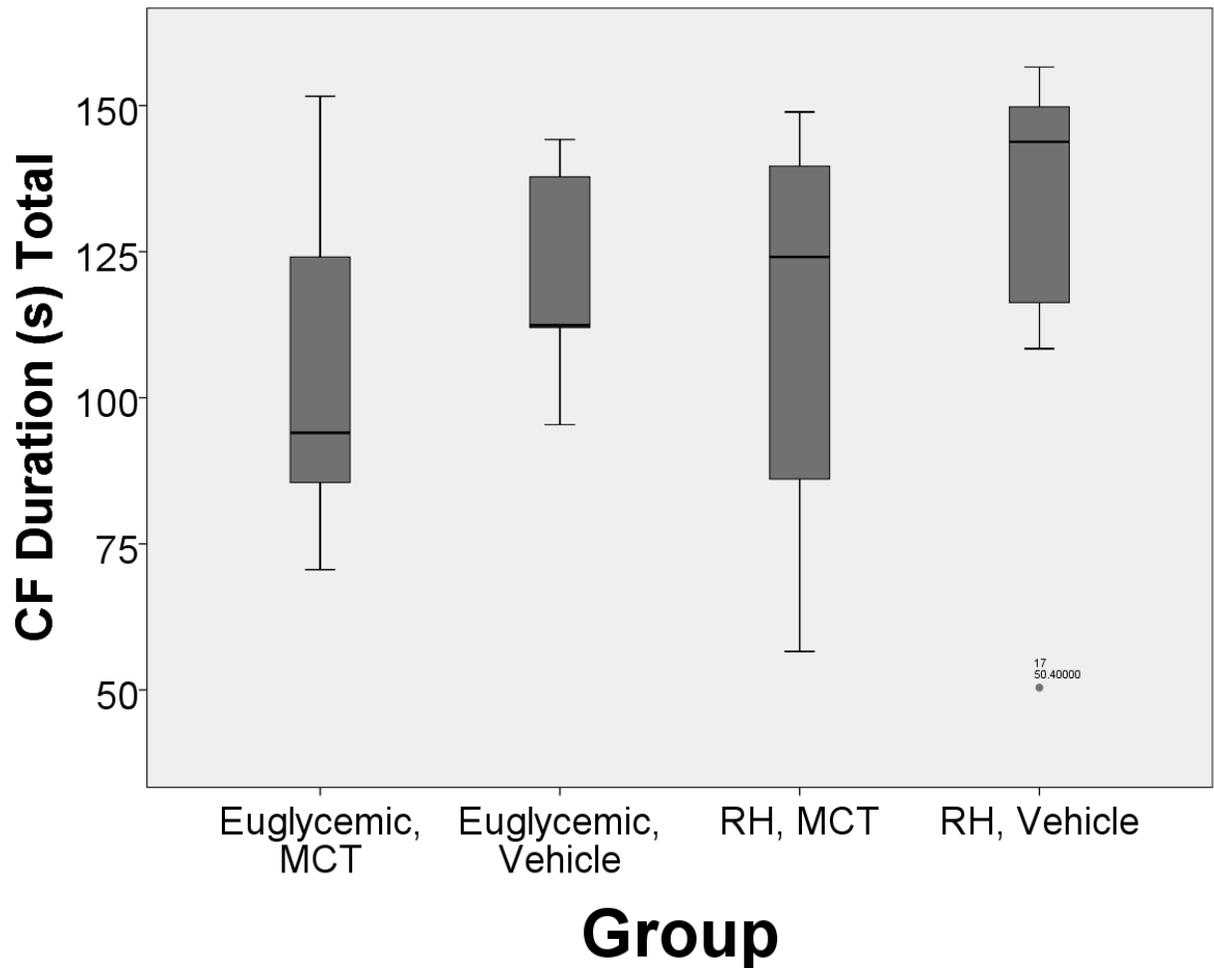
Generally, the RH appears to be beneficial in rescuing brain behavior even when MCT was administered leading us to speculate that regardless of MCT administration compensatory mechanisms may still occur due to RH in the brain.

Figure 1: Spontaneous Alternation Score



MCT treatment in RH rats does not alter SA behavior. Spatial memory was evaluated by alternations between maze arms when animals freely explored a four-arm maze. The box plot illustrates no significant difference between groups for SA scores. ANOVA showed, $F(3, 27) = 0.2775$ ($p=0.064$).

Figure 2: Contextual Fear Freezing Behavior



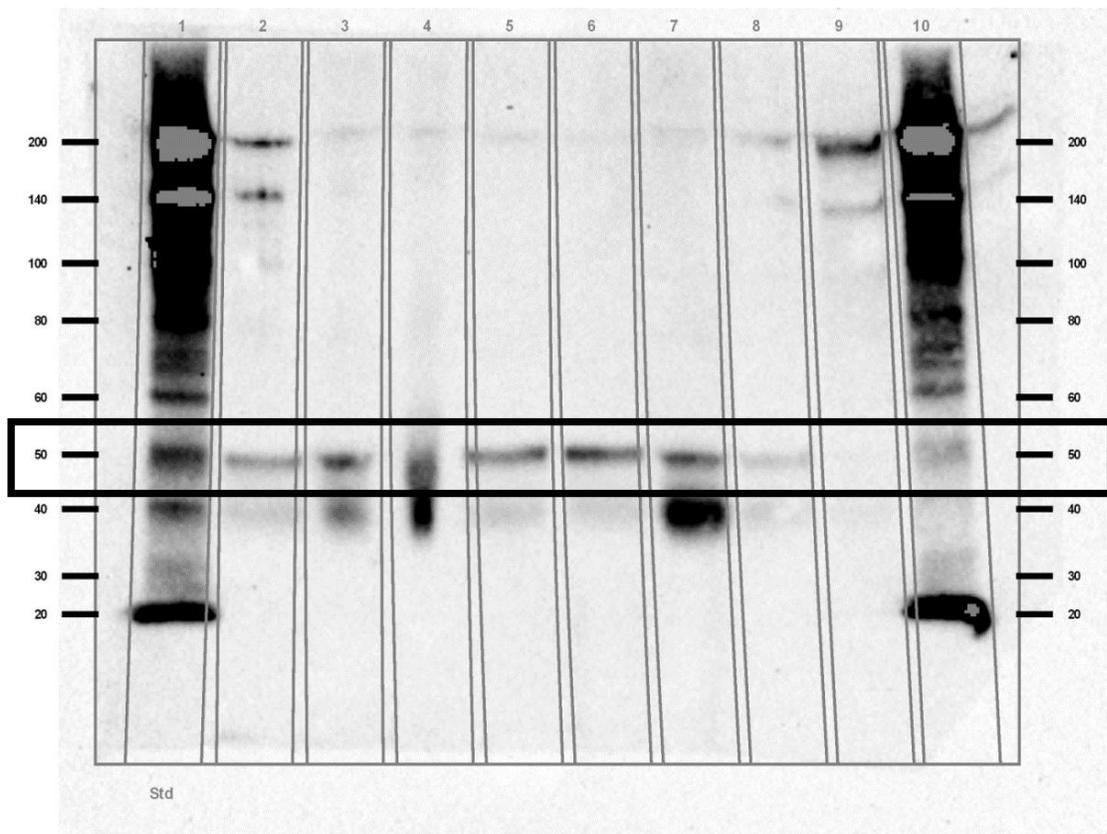
MCT treatment in RH rats does not alter CF freezing behavior. Evidence of a memory formed of the association between context and foot shock was evaluated by time spent freezing (complete absence of movement in the chamber) without the tone. The box plot illustrates no significant difference between groups for freezing behavior. ANOVA showed, $F(3, 27) = 0.574$ ($p = 0.638$).

Group Means

Table 2. The following represents the N-Value (number of animals) and mean scores for SA and freezing behavior.

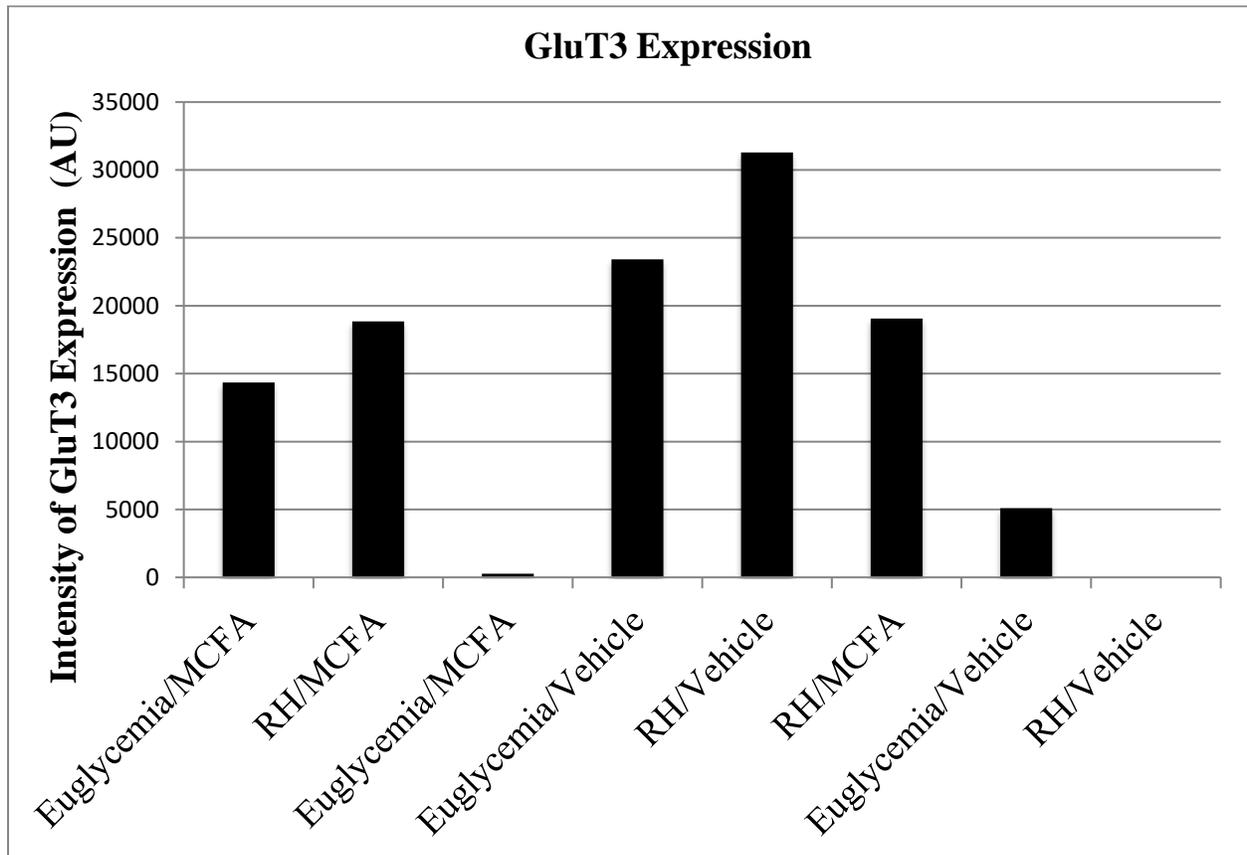
Group	N-Value	SA Score Means	Freezing Behavior Means
Euglycemic/ MCT	7	0.667569	105.057
Euglycemic/Vehicle	5	0.706046	120.36
RH/MCT	8	0.508278	113.15
RH/Vehicle	7	0.662682	126.143

Figure 3: GluT3 Blot Image



No changes in GluT3 (approximately 50 kD) expression in the right hippocampus and prefrontal cortex between groups as shown by western blot. There appears to be some background and cross reactivity in tissue samples. Western blotting was performed by gel electrophoresis, blotting, washing, blocking and antibody incubation.

Figure 4: GluT3 Expression Graph



No changes in GluT3 expression in the right hippocampus and prefrontal cortex between groups as shown in the bar graph (created based on the blot in Figure3.). ImageJ software was used for quantification.

Discussion

This study explored the effects of pre-administering MCT, on the neural mechanisms resulting from RH. Previous findings have shown when tested at euglycemia, RH enhances subsequent contextual fear memory (Osborne et al., 2017). Prior work also showed when tested at euglycemia, RH improves subsequent cognitive performance (McNay & Sherwin, 2004). Taken together, the present behavioral data show a lack of significance between groups in both behavioral tests. This could be due to the time of MCT administration and behavioral testing performed at euglycemia as opposed to hypoglycemia. It is unknown how long it may take for the liver to produce ketones and the brain to use ketones as an energy source after the administration of MCT. Behavioral testing was performed on the fourth day (after the last hypoglycemia bout) which may be insufficient time for the ketones to be adequately used as an energy source by the brain. Further experimentation will involve behavioral testing at hypoglycemia, which might demonstrate pre-administration of MCT will impede neural compensatory mechanisms during hypoglycemia in RH animals.

Another factor contributing to the present data is the age of the animals in the use of this study. It is possible the 12-15-week-old RH/MCT animals had an impairment, which could have contributed to the lack of attenuation of the effects of RH. Aged animals and/or lack of consistency in age throughout the experiment may have had an impact on the findings. There is evidence for a positive correlation between aging and both hippocampal and prefrontal system dysfunction. Numerous brain systems tend to deteriorate together with age (Zyzak, Otto, Eichenbaum, & Gallagher, 1995), possibly resulting in aged rats to lose the ability to respond to novelty in behavioral tests. In comparison, younger rats can react to novelty and spatial

displacement as shown in a repeated-trials test (Luparini, Del Vecchio, Barillari, Magnani, & Prosdocimi, 2000).

To investigate the expression of metabolism-related proteins such as GluT1, GluT3, GluT4, MCT1, MCT2, AMPK, and SGLT, across different brain regions such as, the prefrontal cortex, hypothalamus, and amygdala, but, particularly the left hippocampus (responds differentially in comparison to other brain areas), western blotting for protein expression is currently being conducted. We are particularly paying attention to monocarboxylate transporters MCT1 and MCT2 which serve to mediate the transport of ketones from the breakdown of MCT to the brain for use as a fuel source. Total cellular proteins and protein present on the plasma membrane will be measured.

The western blot and bar graph of GluT3 expression shown in Figures 3. and 4., was adopted from the first experimental trial (this present study was modified and repeated thereafter). The behavioral data was void in the first trial due to incorrect glucose concentrations of the artificial extracellular fluid, which provided surplus glucose that nullified any expected behavioral and microdialysis differences. However, the molecular data from this first trial may provide some insight to this present study. We observe from this preliminary blot, that there is an insignificant increase in GluT3 expression in the right hippocampus and prefrontal cortex in the Vehicle/RH and MCT/RH groups. Bands of around 50kD were visible and no changes in GluT3 intensity between groups was observed. We expected to observe an increased translocation of GluT3 to the plasma membrane in the RH animals. However, we did not observe any changes in expression. The western blots moving forward require extensive troubleshooting as they were hindered by cross reactivity with tissue samples, high background, and insufficient protein quantities loaded for detection.

Apart from analyzing protein expression, microdialysis and plasma membrane sample analysis will also be performed. The analytes of the microdialysis and plasma samples will include glucose, and lactate.

Furthermore, this was a rather small study. A larger sample size may be required to show increased significance in the performed tests. Significance at the metabolic and molecular level cannot be ruled out at this point; this data will only be available after the completion of biological sample analysis.

Lastly, the adequate dosage of MCT has presently not been determined. It is important to identify an effective dose of MCT in a pilot study or else the use of MCT as a therapeutic approach for the treatment of RH may not be as promising. Once an effective dosage has been determined, it would be insightful to explore the role of MCT in the context of other behavioral testing such as set-shifting, elevated-plus maze, and novel object recognition, which may provide novel perspectives to the effects of MCT treatment on the brain during RH.

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