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Homocysteine: A Potential Molecular Link Between Alzheimer's Disease and Type 2 Diabetes Mellitus

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"Homocysteine: A potential molecular link between Alzheimer's Disease and Type 2 Diabetes Mellitus"

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This Honors Thesis has been read and approved by the Undersigned and is hereby recommended for acceptance.

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Homocysteine: A potential molecular link between Alzheimer's Disease and Type 2 Diabetes Mellitus

Cyndel Carreau

In order to elucidate some of the mechanisms through which Alzheimer's disease (AD) and Type II Diabetes Mellitus (T2DM) are linked, this study investigated the effects of elevated plasma homocysteine levels – a risk factor for AD – in a rat model of T2DM. Both elevated plasma homocysteine levels and T2DM are associated with cognitive deficits and are recognized as strong risk factors for the development of AD. The present experiment examined the effects of diet-induced hyperhomocysteinemia on the development of cognitive impairments and insulin-resistance, as well as on the insulin signaling cascade, in a diet-induced obese rat model of T2DM. Hyperhomocyteinemia was induced in both control-fed and high-fat diet plus fructose-fed (T2DM) animals using dietary supplementation of 2% methionine. Homocysteine levels were dramatically elevated in animals receiving methionine supplementation and a high-fat diet plus fructose and were significantly increased in animals receiving either high methionine or high fat plus fructose. Behavioral impairments were observed in all groups receiving treatment. Impaired glucose tolerance was observed in animals receiving either high methionine or high fat plus fructose diets; glucose intolerance was observed in animals receiving both treatments. Akt phosphorylation was reduced by 50% in groups receiving high methionine, regardless of high fat plus fructose. These findings strongly suggest that there are mechanisms at work by which homocysteine modulates the insulin signaling pathway and/or vice versa.

Acknowledgements

The author would first and foremost like to thank Dr. Ewan McNay for his mentorship and, more importantly, for his patience, his insight, and his continuous willingness to help. The author also wishes to acknowledge Dr. Katy Gonder, who readily accepted to be a member of the author's thesis committee. The author would like to thank the McNay lab, particularly Andrew Byrne, Jiah Pearson-Leary, Christine Jorge, and Gregory Moy, for their great assistance and support. Additionally, the author wishes to thank Mrs. Lita Greenwald and her late husband, Mr. Stephen Greenwald, for their generous support through the University at Albany's Undergraduate Research Endowed Fellowship fund; the Albany Medical Center Department of Pathology and Laboratory Medicine and its client support manager, Ms. Diane Sisto; the director of the Molecular Core of the Life Science Research Building, Dr. Kim DeWeerd; and the Larsen lab. The author would also like to acknowledge Dr. Livingston Van De Water and the Van De Water lab at Albany Medical College for their moral support. Finally, the author would like to acknowledge her parents, her husband, and her family for being there when it was most needed.

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1. Introduction

Elevated plasma homocysteine levels are associated with several cognitive impairments, including age-related cognitive decline, cerebrovascular disease and stroke, and vascular dementia (Troen, 2005). More specifically, increased plasma homocysteine levels have been described as a strong, independent risk factor for Alzheimer's disease (Seshadri et al., 2002). Zhuo et al. (2010) demonstrated that a diet high in methionine (the metabolic precursor of homocysteine) used to induce hyperhomocysteinemia, a condition in which blood homocysteine levels are abnormally elevated, accelerated the development of Alzheimer's pathologies and behavioral impairments in APP transgenic mice, a mouse model of Alzheimer's disease.

Like elevated plasma homocysteine levels, type 2 diabetes mellitus is associated with cognitive deficits (Richardson, 1990) and is recognized as a strong risk factor for the development of Alzheimer's disease (Ott et al., 1999). There is currently a lack of consensus about the relationship between plasma homocysteine levels and type 2 diabetes in the literature. Several studies have reported a positive correlation between insulin levels or insulin resistance and plasma homocysteine levels (Gallistl et al., 2000; Sainani et al., 2009). Furthermore, insulin has been shown to inhibit the irreversible metabolism of homocysteine into cysteine through the trans-sulfuration pathway when methionine is in demand (Chieng et al., 2009).

To elucidate some of the relationships between Alzheimer's disease and type 2 diabetes, this study introduced the risk factor for Alzheimer's disease, hyperhomocysteinemia, into a diet-induced obese rat model of type 2 diabetes. Hyperhomocysteinemia was induced using 2% supplementation of methionine in the diet. Fructose was added to the high fat diet with the intention of breaking the body's correlation between food intake and caloric intake. Weight gain and food and water intake were monitored; body fat composition, serum homocysteine, and serum free fatty acids were quantitated; cognitive ability, glucose tolerance, and hippocampal protein activation were all measured.

2. Materials and Methods

2.1. *Animals*

All animal procedures were approved by the University at Albany IACUC and followed current protocol. Thirty-two male Sprague-Dawley rats (Charles River), 75 to 100 g (4 to 5 weeks old), were housed in pairs and kept on a 12h dark/light cycle with food and water available *ad libitum*. Following arrival in the Life Sciences Research Building Animal Care Facility, animals were given 2 to 6 days to acclimatize, after which diet administration began and continued for 10 weeks. Animal weights were obtained 1 to 2 times per week. Animals were handled regularly prior to testing.

2.2 *Diets*

Animals were randomly assigned to one of four diets: normal chow (Purina, Prolab RMH 3000) and normal water; normal chow plus 2% additional methionine (Open Source Diets, C15122) and normal water; high fat chow (31.8% kcal from fat; Open Source Diets, D12266B) and high fructose water (20% fructose by weight); and high fat chow plus 2% additional methionine (Open Source Diets, D10122701) and high fructose water. High fructose water was made by dissolving crystalline D-(–)-fructose (Sigma-Aldrich, F0127; MySpiceSage.com, #559) into tap water. Food and water intake were measured over a one-week period during the diet administration.

2.3 *Surgery*

After 8 weeks of diet administration, all animals underwent stereotactic surgery for placement of a microdialysis cannula into the left dorsal hippocampus. Animals were anesthetized using isofluorane. Upon exposure of the skull, a hole was drilled at coordinates x = +5.0 mm and y = –5.6 mm from bregma; a guide cannula (BASi, MD-2250; or CMA 12) was lowered to coordinate z = –3.0 mm from the dura. The guide cannula was sealed in place using dental cement. Animals were given 8 to 11 days post-surgery recovery prior to behavioral testing.

2.4 *Behavioral testing and microdialysis*

Animals were subjected to a spatial cognitive task during week 9 of diet administration. The task consisted of running a four-arm elevated plus-maze from which a spontaneous alternation score was generated. Animals were placed in the center of the maze and allowed to freely explore its arms for 20 minutes. Arm entries were recorded for each animal. Animals spontaneously alternate between arms, normally entering those which they have entered the least recently. An alternation is counted when the rat visits all four arms within any span of five consecutive arm choices; the maximum number of alternations is N – 4, where N is the total number of arms entered. The actual number of alternations made is expressed as a percentage of this number, with chance level at 44%. Animals which performed less than 14 arm entries during the task were omitted from the results.

Preceding, during, and following the course of the above-described spatial cognitive task, microdialysis samples from the hippocampus were collected using a 4 mm probe (BASi, IBR-4; or CMA 12), inserted into the guide cannula, for analysis of the metabolic state of the hippocampus during testing. Extracellular cerebrospinal fluid containing a physiological concentration of glucose (1.25mM) was used as a perfusate. Samples were collected at a rate of 2.0 µL/minute over 20 minute intervals, with baseline lasting 1 hour (3 samples), the maze task lasting 20 minutes (1 sample), and post-maze period lasting 1 hour (3 samples); a one-hour equilibrium sample was also collected prior to baseline. Samples were frozen at -80 °C until analyzed for glucose using a CMA 600 Microdialysis Analyser.

Following the spatial cognitive task and completion of microdialysis sample collection, animals were subjected to a second task, which consisted of running an open-field maze (1 m by 1 m box marked into 16 [12 peripheral and 4 center] equal-sized squares). Animals were placed in the center of the maze and allowed to freely explore the box. Crossings from square to square were recorded for each animal. An open-field score was generated by placing the sum of crossings into or between center squares over the sum total of crossings. Lower open-field scores are correlated with increased anxiety behavior.

2.5 *Glucose tolerance test*

Blood glucose was initially measured using a blood glucose meter (OneTouch Ultra Mini Monitor) during the week preceding surgery; blood was obtained by tail-prick.

Either the day of or the day following behavioral testing, a glucose tolerance test was performed on each animal. Animals were given an intra-peritoneal (I.P.) injection of 1g/kg body weight D-(+)-glucose (Sigma-Aldrich, G8270), dissolved at concentration of 20% by weight in dH_2O. Blood glucose was measured using a blood glucose meter at 0 minutes (baseline, prior to injection), 30 minutes after injection, and 60 minutes after injection. Blood was obtained by cutting the tip of the tail and "milking" the tail until a drop appeared at the tip.

2.6 *Brain, trunk blood, and epididymal fat pad collections*

Either the day of or the day following behavioral testing, animals were killed by decapitation following anesthesia by isoflurane. Whole brain was collected and immediately frozen on dry ice. Trunk blood was collected on ice and shaken to prevent clotting; within 30 minutes of collection, plasma was spun down at 3000 rpm for 15 minutes and the serum fraction was collected for analysis. Epididymal fat pads were harvested from the carcass following trunk blood collection and weighed.

2.7 *Serum analyses*

Serum samples from each animal were sent to the Albany Medical Center Department of Pathology and Laboratory Medicine for analysis of blood homocysteine and free fatty acid levels. Homocysteine was detected using the ARCHITECT Homocysteine immunoassay. Free fatty acids were detected using spectrophotometry.

2.8 *Western blotting*

Hippocampal brain tissue was dissected from frozen whole brain then homogenized using a Polytron homogenizer into three volumes of homogenization buffer (BioVision, from kit K268-50) containing phosphotase and protease inhibitors. For total homogenate fraction, 30µL of homogenate was added to

200 µL of RIPA buffer containing phosphotase and protease inhibitors. The remaining non-total fraction homogenate was used to obtain the plasma membrane fraction. Plasma membrane protein extraction was carried out using the BioVision Plasma Membrane Protein Extraction kit (K268-50) according to the instructions provided with the kit. A BCA assay (Pierce, 23225) was carried out on both total and plasma membrane fractions.

Precast 10% polyacrylamide gels (Biorad, Mini-PROTEAN TGX) were loaded with 20 µg of protein from each sample – to which 10 µL sample buffer containing 5% mercaptoethanol was added – per well and run in tris-glycine running buffer at 120V for 80 minutes. Total fraction samples to be probed for pAkt, Akt, and GluT4 were microwaved in boiling water for 2 minutes prior to loading into gel; total fraction samples to be probed for GluT3 and plasma membrane fraction samples were not microwaved. Following electrophoresis, gels were transferred to PVDF membranes at 350 mA for 1 hour at 4 °C. Membranes were blocked in 5% milk for 1 hour then were probed for GluT3 (Abcam, ab41525-100), GluT4 (Millipore, 07-1404), pAkt (Cell Signaling, 4060L), Akt (Cell Signaling, 4691L), or β-actin (Sigma, A1978) using monoclonal primary antibodies. Following primary incubation overnight at 4 °C, membranes were probed with the appropriate biotinylated secondary antibodies (Pierce) for 1 hour at room temperature and then with HRP strepdavidin (Pierce, 21130) for 1 hour at room temperature. Membranes were then exposed to chemiluminescent substrate (Pierce, 34078) before being developed. Analysis of western blots was done using ImageQuant TL software.

2.9 *Statistical analyses*

Statistical significance was determined using a two-tailed, two-sample equal variance Student's t-Test. The test was performed using the TTEST function on Microsoft Excel. Data points which were more than two standard deviations away from the mean were deemed to be outliers and omitted from data presentation, with the exception of behavioral data points, which were omitted if more than one standard deviation away from the mean.

3. Results

3.1 *Animal weights*

Animal weights were monitored over the course of diet administration in order to observe the effects of the four diets on weight gain. Figure 1 shows weight gain from day 0 to day 59.5 of diet administration. Groups receiving high methionine (Hmet) and high methionine plus high fat/fructose diet (Hmet + HFD) were significantly ($p < 0.005$) lighter on day 59.5 than groups receiving either normal diet or HFD, but were not significantly different from each other. Similarly, groups receiving normal diet and HFD were not significantly different from each other on day 59.5.

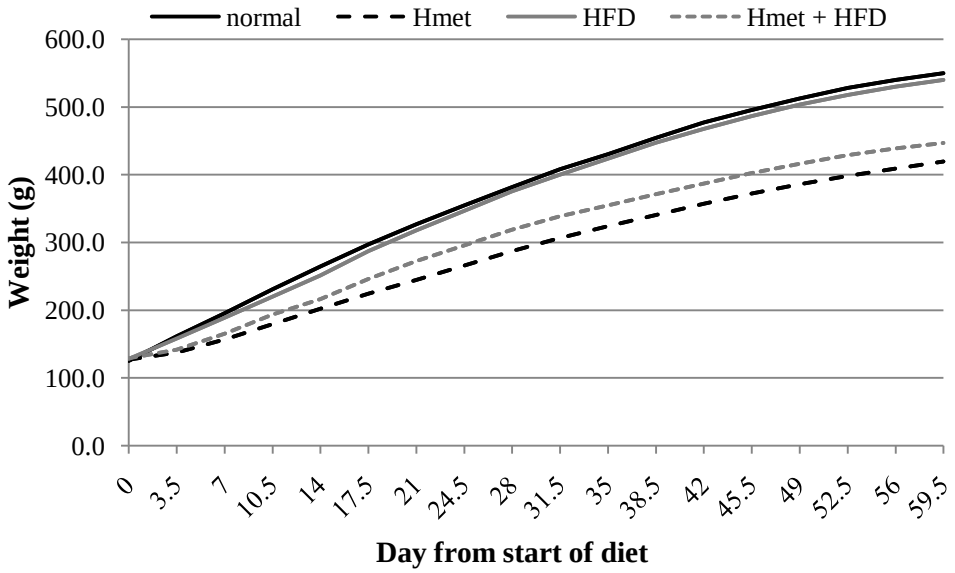

Figure 1. Animal weights from the first day of diet administration to day 59.5 of diet administration. High (2%) methionine is denoted "Hmet" (high methionine) and high fat diet plus high (20%) fructose water is denoted "HFD" (high fat/fructose diet). Animals on Hmet diet and Hmet + HFD were significantly lighter on day 59.5 of diet administration than animals on normal diet or HFD. N = 7-8 per group.

3.2 *Food and water intake*

Given early differences in weight between groups receiving high methionine and groups not receiving high methionine, and a lack of expected difference between the group receiving normal chow and the

group receiving HFD, food and water intake were monitored for a one week period during food administration. Figure 2a shows food intake in grams per animal per day. Animals receiving normal diet had the highest food intake whereas animals receiving Hmet + HFD had the lowest intake. All groups had significantly different intakes ($p < 0.05$). Figure 2b shows food intake in grams normalized to 100g body weight per day. Animals receiving normal diet and Hmet diet had a significantly – almost 2-fold – higher intake than animals receiving HFD and Hmet + HFD ($p < 0.005$); however, there was no significant difference in intake between either normal diet and Hmet diet or HFD and Hmet + HFD.

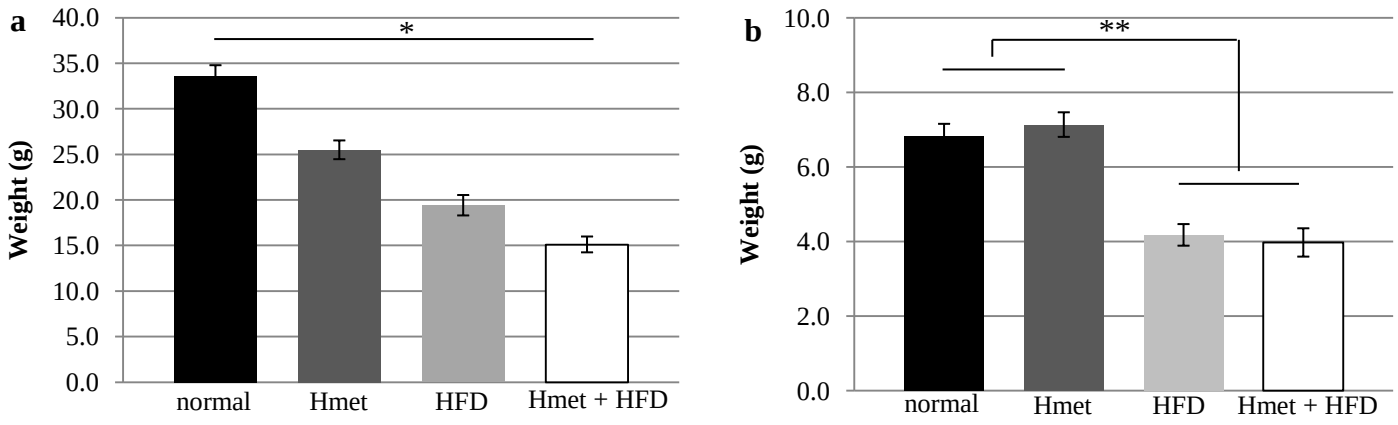

Figure 2. **a.** Food intake in grams per animal per day. All groups were significantly different from each other (* $p < 0.05$). N = 4 (animals housed in pairs) per group. **b.** Food intake in grams per 100g body weight per day. Normal diet and Hmet were significantly higher than HFD and Hmet + HFD (** $p < 0.005$). N = 4 per group.

In order to investigate the observed 2-fold higher food intake in animals not receiving high fat/fructose diet, water intake was measured to determine the number of calories obtained from fructose in the fructose drinking solution. Figure 3a shows water intake in grams normalized to 100g body weight per day. There were no significant differences in drinking water intake between groups. Figure 3b shows total caloric intake (food + drinking water, where applicable) normalized to 100g body weight per day.

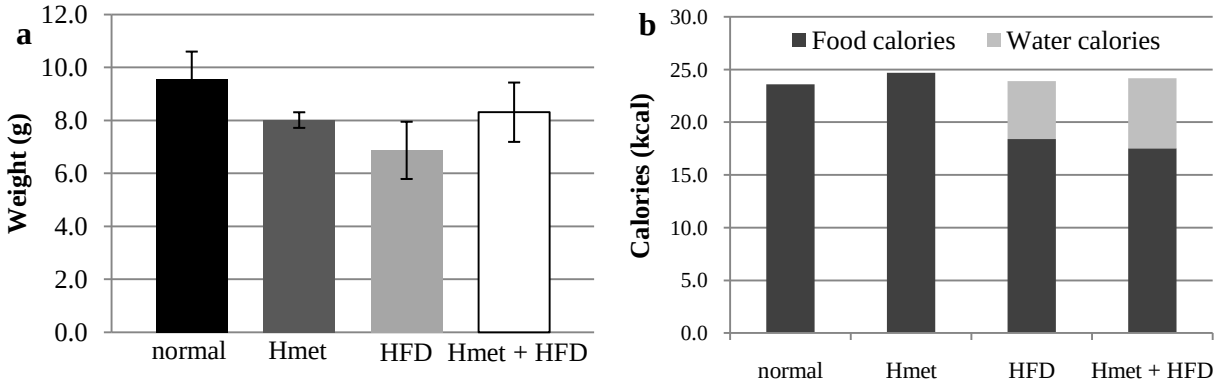

Figure 3. **a.** Water intake in grams per 100g body weight per day. N = 4 per group. **b.** Total caloric intake per 100g body weight per day. N = 4 per group.

Calories from fructose drinking water filled in the gap in food intake between animals not receiving fructose and animals receiving fructose in the drinking water. There were no significant differences in total caloric intake per day between groups.

3.3 *Body fat composition*

Epididymal fat pad was collected and weighed after decapitation to determine body fat composition. Body fat composition was calculated by creating a ratio of epididymal fat pad weight over total animal weight. Figure 4 shows the average ratio of epididymal fat pad weight over total animal weight 14 days prior to killing.

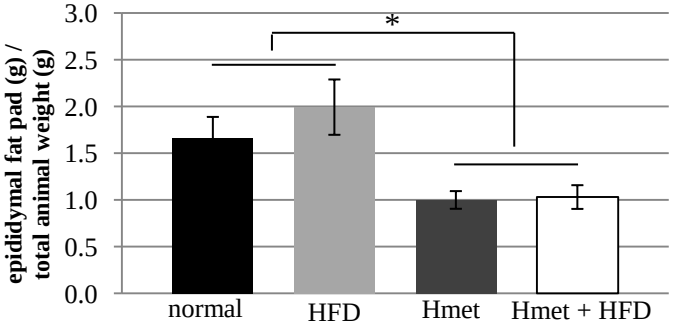

Figure 4. Epididymal fat pad weight over total animal weight 14 days prior to killing. Normal diet and HFD had significantly more fat than Hmet and Hmet + HFD (* $p < 0.05$). N = 7-8 per group.

Animals receiving either of the high methionine diets had significantly ($p < 0.05$) – up to 2-fold – less fat than animals not receiving high methionine. There were no significant differences in body fat composition between Hmet and Hmet + HFD groups or between normal and HFD groups, although animals receiving HFD tended to have a higher body fat composition than animals receiving normal diet.

3.4 *Serum analyses*

To verify that hyperhomocysteinemia was induced in animals receiving a high methionine diet and to examine any differences in homocysteine levels between groups, serum homocysteine levels were determined. Figure 5 shows the group average concentrations of homocysteine in serum.

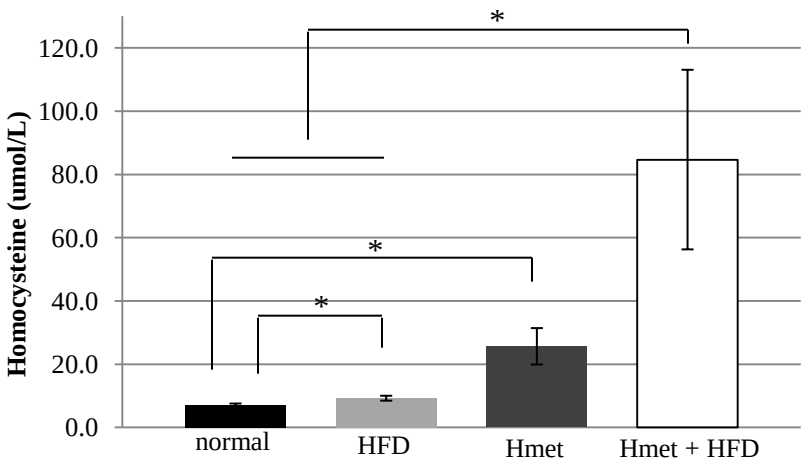

Figure 5. Serum homocysteine concentrations. Animals on Hmet diet had significantly higher serum homocysteine levels than animals on normal diet, as did animals on HFD (* $p < 0.05$). Animals on Hmet + HFD had significantly higher serum homocysteine levels than animals on either normal diet or HFD (* $p < 0.05$). N = 7-8 per group.

Animals on Hmet diet had significantly ($p < 0.05$) – over 3-fold – higher serum homocysteine levels than animals on normal diet. Animals on HFD also had significantly ($p < 0.05$) – about one-third – higher serum homocysteine levels than animals on normal diet. Animals on Hmet + HFD had significantly ($p < 0.05$) – about 10-fold, on average – higher homocysteine levels than animals on normal diet or HFD, but did not have homocysteine levels that were significantly different from animals on Hmet diet.

Serum free fatty acids were also determined. Figure 6 shows average concentrations of free fatty acids in serum. There were no significant differences across groups.

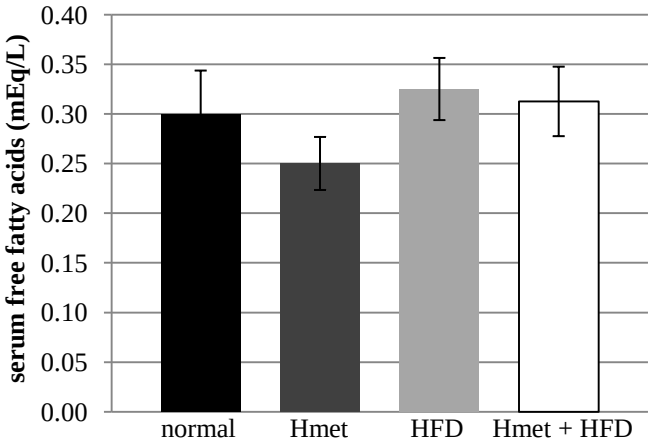

Figure 6. Serum free fatty acid concentrations. N = 7-8 per group.

3.5 *Glucose tolerance test*

During the week prior to surgery, all animals were tested for blood glucose levels to determine if the diets had any effect on blood glucose levels. Figure 7 shows average blood glucose levels, measured using blood obtained by tail-prick, for each group during week 7 of diet administration. Animals receiving Hmet diet had significantly higher blood glucose levels than animals receiving Hmet + HDF.

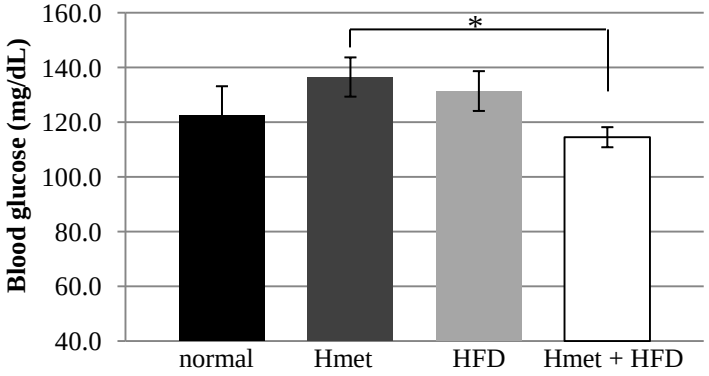

Figure 7. Average blood glucose levels of each group during week 7 of diet administration. Animals on Hmet had significantly higher blood glucose levels than animals on Hmet + HFD (* $p < 0.05$). N = 7-8 per group.

To further investigate the effect of each diet on insulin signaling, a glucose tolerance test was performed. Figure 8 shows the results of the glucose tolerance test for each group.

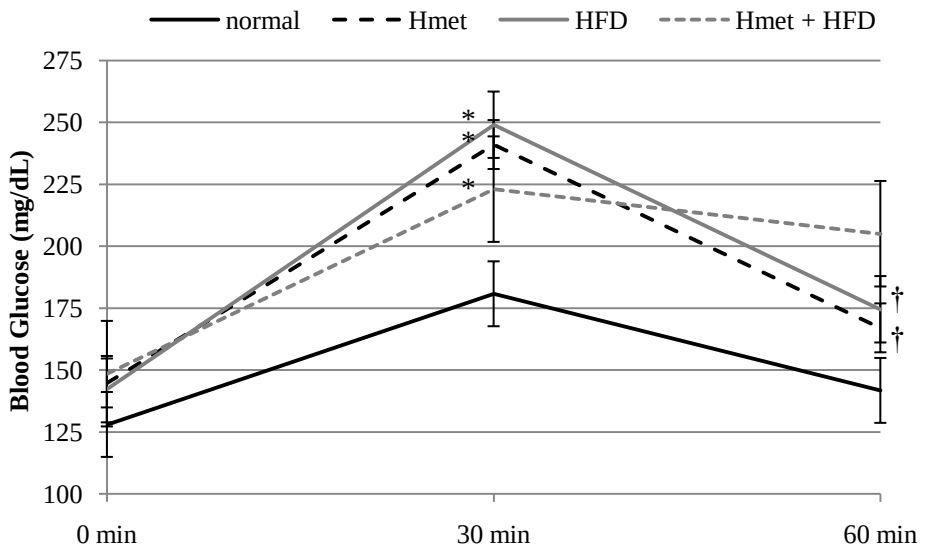

Figure 8. Glucose tolerance test results per group. Animals on Hmet diet, HFD, and Hmet + HFD had significantly elevated blood glucose levels at 30 minutes after injection (* $p < 0.05$); blood glucose levels at 60 minutes for animals on Hmet and HFD fell significantly from 30 minutes († $p < 0.05$). N = 4 per group.

Animals receiving Hmet diet, HFD, and Hmet + HFD all experienced significantly ($p < 0.05$) elevated blood glucose levels from baseline 30 minutes after injection of glucose. At 60 minutes after injection, blood glucose levels had fallen significantly ($p < 0.05$) from 30 minutes in animals receiving Hmet diet and HFD but not in animals receiving Hmet + HFD, in which blood glucose remained elevated.

3.6 *Western blots*

To determine if the diets had any effect on the activity of hippocampal proteins involved in the insulin signaling cascade, western blots were performed on hippocampal brain tissue homogenate from each animal. Blots were probed for GluT3, GluT4, and Akt. Figure 9 shows western blot results for GluT3 (a) and GluT4 (b) translocation and Akt (c) phosphorylation (in total fraction), with each group expressed as a percentage of control (normal diet) group.

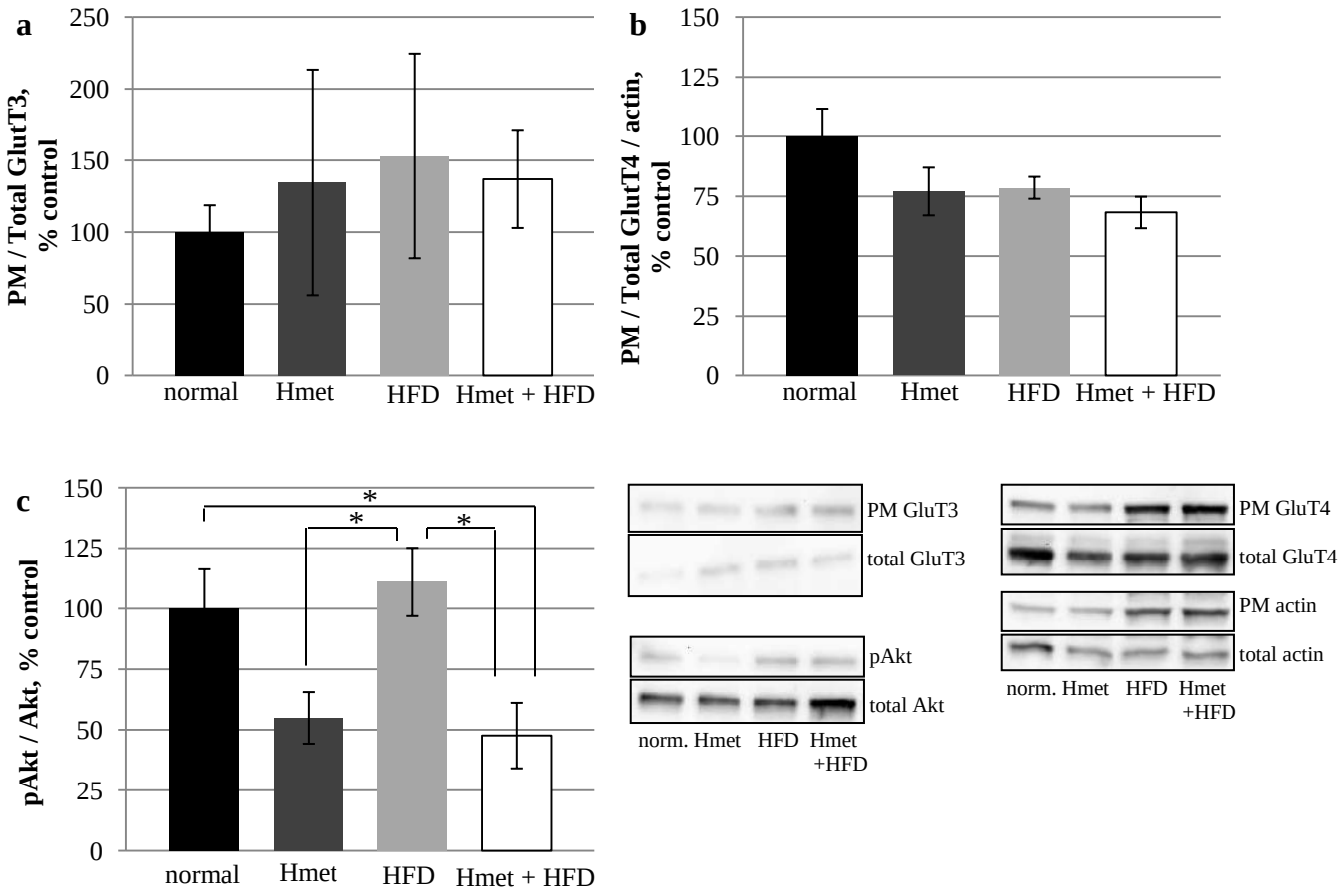

Figure 9. **a.** Ratio of plasma membrane (PM) fraction GluT3 over total fraction GluT3. N = 4 per group. **b.** Ratio of PM fraction GluT4 over total fraction GluT4, normalized over PM fraction over total fraction actin. N = 4 per group. **c.** Ratio of phosphorylated Akt (pAkt) over total Akt. Animals on Hmet + HFD had significantly lower levels of Akt phosphorylation than animals on either normal diet or HFD (* $p < 0.05$); animals on Hmet diet had significantly lower levels of Akt phosphorylation than animals on HFD (* $p < 0.05$). N = 4 per group.

There were no significant differences in GluT3 or GluT4 translocation between groups, although there was a trend toward higher levels of GluT3 translocation in groups receiving Hmet diet, HFD, and Hmet + HFD; correlated with this, there was also a trend toward lower levels of GluT4 translocation in groups receiving Hmet diet, HFD, and Hmet + HFD. Animals receiving Hmet + HFD had significantly ($p < 0.05$) – about 2-fold – lower levels of Akt phosphorylation than animals receiving normal diet or HFD.

Similarly, animals receiving Hmet diet had significantly ($p < 0.05$) lower levels of Akt phosphorylation than animals receiving HFD, although the levels were not significantly different from those of animals receiving normal diet.

3.7 *Behavioral testing*

All animals were subjected to a spatial cognitive task in the form of an elevated four-arm plus-maze during which arm entries were recorded for calculation of a spontaneous alternation score. The objective of performing this cognitive task was to determine how each diet affected spatial memory and cognitive ability. Figure 10 shows the average spontaneous alternation scores for each group.

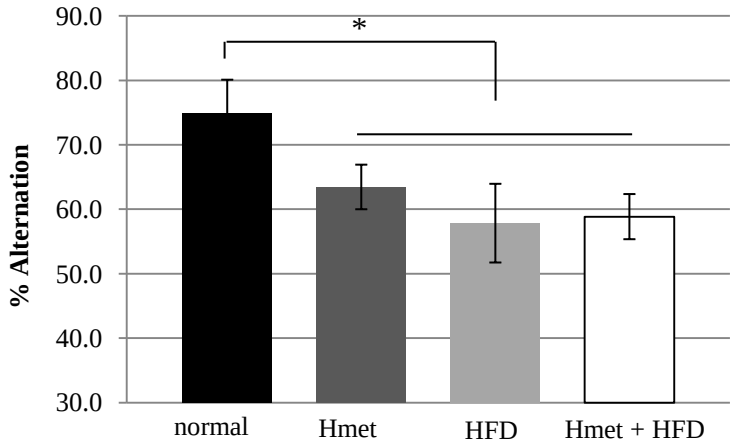

Figure 10. Spontaneous alternation scores for each group. Animals on Hmet diet, HFD, and Hmet + HFD had significantly lower scores than animals on normal diet (* $p < 0.05$). N = 5 for normal diet; N = 6 for Hmet diet; N = 7 for HFD; and N = 6 for Hmet + HFD.

Animals were subsequently subjected to the open-field maze task to observe how each diet affected anxiety behavior. Figure 11 shows the average open-field scores for each group. Animals receiving Hmet + HFD made significantly ($p < 0.05$) less center crossings than animals receiving either Hmet diet or HFD, but did not have significantly different numbers of center crossings from animals on normal diet. There was a non-significant trend toward more center crossings in animals receiving Hmet diet or HFD than in animals receiving normal diet.

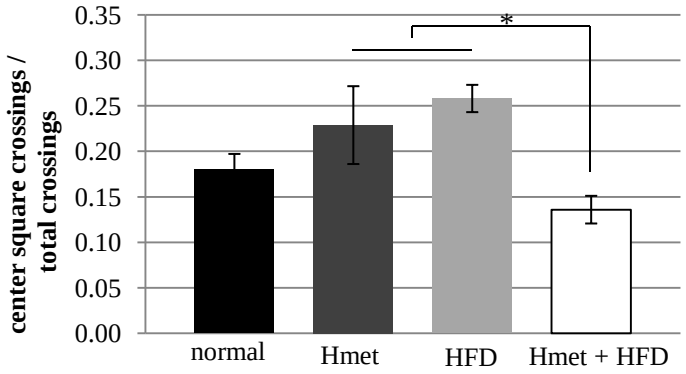

Figure 11. Open-field score for each group. Animals on Hmet + HFD made significantly less center crossings than animals on either Hmet diet or HFD (* $p < 0.05$). N = 6 for normal diet, HFD, and Hmet + HFD; N = 4 for Hmet diet.

3.8 *Microdialysates*

Microdialysis was performed during the elevated four-arm plus-maze task to examine the metabolic state of the hippocampus prior to, during, and following the task. Figure 12 shows average glucose levels prior to (baselines, B1 – B3), during (maze, M), and following (post-maze, P1 – P3) the maze task for each group. There were no significant differences between groups. A significant ($p < 0.05$) "dip" in glucose levels during the maze task was observed only in animals receiving Hmet + HFD, although a small, non-significant "dip" in glucose during the task did occur in animals receiving normal diet and Hmet diet.

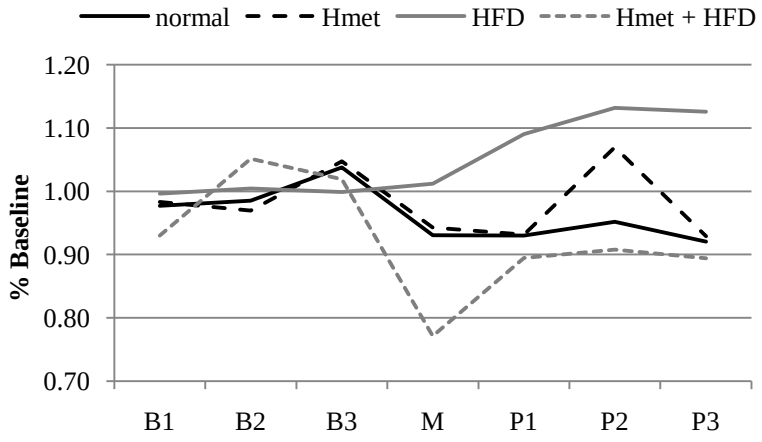

Figure 12. Hippocampal microdialysate glucose levels, expressed as a percent of baseline. N = 4 for normal diet; N = 3 for Hmet diet; N = 4 for HFD; and N = 7 for Hmet + HFD.

4. Conclusions

In a rat model, a high methionine diet – with or without a high fat diet – is correlated with lower body weight and decreased food intake per animal. A high methionine diet coupled with a high fat diet results in a dramatically decreased food intake, with animals receiving Hmet + HDF eating almost 2-fold less than animals receiving a normal diet. Reduced food intake in animals receiving either of the high methionine diets was foreseen before the experiment (Fau et al., 1988) was started, but lower food intake in animals receiving Hmet + HFD had not been predicted. Results additionally showed that animals receiving HFD ate significantly less than animals receiving normal diet, which had also not been predicted nor observed by our lab before. When food intake was normalized to 100g of body weight, food intake was only significantly reduced in animals receiving a high fat diet, which prompted us to calculate the number of calories being obtained from high fructose drinking solution coupled to the high fat diet. Interestingly, total caloric intake was the same across all groups, indicating that reduced food intake in animals receiving a high fat diet was due to caloric intake from the drinking water. Fructose was initially added to the diet with the intention of de-regulating the body's correlation between food intake and measured caloric intake, as the hypothalamus can neither uptake nor sense calories obtained from fructose, in order to maximize food intake and prevent the separation of diet-resistant animals from diet-induced obese animals (Levin et al. 1997). Although separation within the group of animals receiving HFD did not occur, the results from this experiment suggest that fructose did not cause the desired de-regulation, as caloric intake from food was adjusted by groups receiving a high fat diet to compensate for calories obtained from the drinking solution. These findings suggest a review of the known mechanisms by which the body senses caloric intake.

Hyperhomocysteinemia (> 20 μmol/L serum homocysteine levels) was successfully induced in animals receiving either of the high methionine diets. Although hyperhomocysteinemia was not induced in animals receiving HFD, the serum homocysteine levels of these animals were significantly higher than those of animals receiving normal diet, suggesting that the high fat plus high fructose diet played a role in the accumulation of free homocysteine in the blood stream. Indeed, animals receiving Hmet + HFD had

dramatically elevated serum homocysteine levels – more than 10-fold higher than those of animals receiving normal diet. However, the serum homocysteine levels of animals receiving Hmet + HFD were also highly variable and thus not significantly different from those of animals receiving Hmet diet. These results suggest that a high fat plus high fructose diet, or some consequence of this diet, can modulate the levels of serum homocysteine and, in some cases, yield dramatically elevated levels of homocysteine.

Hyperhomocysteinemia was correlated with a decreased body fat composition, which was similar in both groups receiving high methionine, regardless of a high fat plus high fructose diet. Interestingly, the high fat plus high fructose diet did not induce body fat compositions that were significantly different from those of animals receiving a normal diet, although the animals receiving HFD tended to have a higher body fat composition. The high fat plus high fructose diet was expected to produce significant obesity in animals receiving this diet. It is possible that the type of fat used in the high fat diet does not produce obesity readily. Crude calculations of the fat composition of the high fat diet revealed that about 25% of the fat, obtained from butter and corn oil, is saturated fat, comprising about 4% of the weight of the diet. In contrast, about 30% of the fat in the normal diet is saturated fat, comprising about 1.75% of the weight of the diet. When food intake per 100g body weight of animal is taken into consideration, animals receiving HFD took in a significant 1.5 times more saturated fat, per weight, than animals on normal diet (data not shown). However, animals receiving HFD were not 1.5 times more obese than animals receiving normal diet, which indicates that other factors, such as genetics, were at play in their lack of obesity.

Similarly, no significant trends in serum free fatty acid levels were observed, although these levels tend to be elevated in type 2 diabetes (Reitsma, 1967). This result may be related to the lack of obesity in animals receiving HFD.

Blood glucose levels, like serum free fatty acid levels, also tend to be elevated in type 2 diabetes and this measure is commonly used to diagnose and monitor diabetes. However, after 7 weeks of diet administration, blood glucose levels were not significantly elevated in any of the groups. To determine insulin sensitivity, a glucose tolerance test was performed during week 9 of diet administration. Animals receiving Hmet diet, HFD, and Hmet + HFD were shown to have significantly elevated blood glucose

levels 30 minutes after I.P. injection of 1g/kg body weight glucose, whereas animals receiving normal diet did not show significantly elevated blood glucose levels 30 minutes after injection, suggesting impaired glucose tolerance and some level of deregulation in the insulin signaling cascade given the delayed response. Sixty minutes after injection, animals receiving Hmet diet and HFD had blood glucose levels that were significantly lower than those at 30 minutes after injection. This pattern of blood glucose levels following an injection of glucose is representative of a pre-diabetic phenotype. Finding an impaired glucose tolerance similar to animals receiving HFD in animals receiving Hmet diet suggests that hyperhomocysteinemia may play a role in modulating insulin sensitivity. Indeed, in animals receiving Hmet + HFD, blood glucose levels at 60 minutes were not significantly lower than those at 30 minutes after injection. This result suggests that animals on Hmet + HFD developed insulin resistance and were therefore glucose intolerant. More interestingly, taken as a whole, these results suggest that, by themselves, a high methionine diet and a high fat plus high fructose diet can lead to an impaired glucose tolerance condition after 9 weeks of diet administration but, when administered together, these diets accelerate the rate of development insulin resistance.

Molecular data from western blots suggests that GluT4 translocation may be decreased in the hippocampus of animals receiving Hmet diet, HFD, and Hmet + HFD, although differences were not significant. This finding is correlated with findings from the glucose tolerance test and previous findings that GluT4 translocation is decreased in a state of impaired glucose tolerance and insulin resistance (James & Piper, 1994). Molecular data also showed that hippocampal Akt phosphorylation was 50% reduced in animals receiving either of the high methionine diets but was not impaired in animals receiving HFD. This result contradicts several others which have previously found that homocysteine activates the PI3K/Akt pathway (Chiang et al., 2011). However, these studies used acute exposure of non-neuron cell cultures to homocysteine. The present study used chronic exposure to homocysteine and suggests that chronic hyperhomocysteinemia may promote decreased Akt activation. Further investigation of this finding is necessary to reach a more conclusive understanding of the relationship between hyperhomocysteinemia and Akt phosphorylation.

Results from the elevated four-arm plus-maze task showed that cognitive spatial ability was impaired in animals receiving Hmet diet, HFD, and Hmet + HFD. No significant differences between these groups were observed, however, suggesting that the combination of high methionine and high fat plus high fructose does not cause a further cognitive impairment in animals receiving this treatment than that observed in animals receiving just one of these treatments. This result was surprising; it would be worthwhile to investigate how the combination of high methionine and high fat plus high fructose affects the physiology of the hippocampus. Results from the open-field task suggest that a high methionine diet and a high fat plus high fructose diet may lead to decreased anxiety, although the combination of the two seems to lead to increased anxiety; none of these results were significantly different from those of animals receiving normal diet. Decreased anxiety in animals receiving Hmet diet and HFD may be the result of decreased cognitive ability; increased anxiety in animals receiving Hmet + HFD may have been a shift to the right side of an inverted U-curve for cognitive ability and anxiety levels.

Overall, the results from this study suggest there is a molecular relationship between homocysteine levels and insulin signaling. Serum homocysteine levels were significantly elevated in both animals receiving high methionine and in animals receiving high fat plus high fructose, although they were not elevated enough to diagnose hyperhomocysteinemia in the latter. In animals receiving Hmet + HFD, homocysteine levels were dramatically elevated, although highly variable; these findings, along with results from animals on HFD, suggest that the high fat plus high fructose diet, or a consequence of it (impaired glucose tolerance?) may modulate homocysteine levels. The finding of impaired glucose tolerance in animals receiving a high methionine diet seems to suggest that serum homocysteine levels can also modulate insulin sensitivity, yielding insulin resistance when combined with a high fat plus high fructose diet. Furthermore, hyperhomocysteinemia was correlated with a significant and dramatic decrease in Akt phosphorylation, which plays a major role in the insulin signaling pathway. Future studies should focus on determining the particular mechanisms by which homocysteine modulates the insulin signaling pathway and/or vice versa.

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