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Thesis Acknowledgments

This thesis would not have been possible without the guidance of Dr. Vaishali Jahagirdar and Dr. Ewan McNay. One of Dr. Jahagirdar's passions is mentoring students. Not only has she been an immense help by providing invaluable guidance throughout the composition of this manuscript, she has also bestowed upon me countless life lessons. Before I started research in Dr. McNay's lab, I believed I would gain in depth knowledge, have the opportunity to interact with experts, and achieve a sense of pride and accomplishment. What I have gained through my experience has been more than I could have imagined. I would like to thank both Dr. Jahagirdar and Dr. McNay for all their time, help, and encouragement.

Abstract

A major side-effect of intensive insulin replacement therapy, is recurrent hypoglycemia (RH). Clinical and experimental evidence suggest that RH may be associated with deficits in higher cognitive processes; specifically, in judgment and mental flexibility, processes believed to be mediated in large part by the medial prefrontal cortex. The present study investigates the effects of short-term RH on mental flexibility in rats. Animals underwent food restriction and were extensively handled and habituated. Prior to testing animals were randomly assigned to one of four groups with varying levels of RH. Animals were then tested for two consecutive days on a set-shift maze task designed to be analogous to the Wisconsin-Card Sorting Task used in humans as a task of frontal cortex-based rule-learning and flexibility processes. The Set-Shift maze is a rotating plus maze with a food well at the end of each arm, where each arm varies with respect to two stimulus dimensions: brightness (smooth or dark) and texture (rough or smooth). Day 1 of testing required each animal to learn a contingency rule: food reward is associated with one value of a single dimension. On day two, the reward contingency switched dimensions such that for example on day 1, if brightness was rewarded, on day 2, an aspect of texture would be rewarded. Performance on day 2 requires the animal to both (i) inhibit the previously learnt behavior, and (ii) employ a novel mental strategy to acquire the new rule, testing mental flexibility. We investigated the effects of RH on brain metabolite and neurotransmitter levels during behavioral testing using *in vivo* microdialysis. Post-testing, brain tissue was harvested, sectioned, and stained for proper probe placement verification and future analysis. Results indicate that on day two of testing, animals tested while euglycemic but with prior RH required significantly more trials to reach criterion as compared to controls ($f=3.673$; $p=.029$). Acute hypoglycemic status had no effect on maze performance, possibly because of the increased

motivation for receiving a food reward while hypoglycemic. These findings suggest that a history of prior hypoglycemia may lead to impaired mental flexibility and decision-making, a result which is consistent with anecdotal clinical findings and which has important implications for diabetics undergoing insulin replacement therapy. Further research may be necessary to develop more effective treatment regimens that reduce the risk of RH.

The Effect of Recurrent Hypoglycemia on Mental Flexibility

Patients with Type 1 Diabetes Mellitus (T1DM), as well as some patients with Type 2 Diabetes Mellitus (T2DM) must undergo lifelong insulin replacement therapy in order to prevent chronic hyperglycemia, which if left untreated causes a range of pathologies (Stolar, 2010). Intensive insulin therapy, with the aim of aggressively preventing hyperglycemia, has been the gold standard treatment for T1DM over the past 20 years, based largely on the results of large scale clinical trials (DCCT, 1991; DCCT, 1993): Intensive insulin treatment minimizes long term complications as a result of vascular problems associated with hyperglycemia (DCCT, 1993; Morales, 2009). However, long-term intensive insulin replacement therapy increases the propensity for recurrent hypoglycemia (RH) (DCCT, 1991; Tamborlane & Ahern, 1997): A follow up analysis of the DCCT has shown that intensive insulin therapy significantly increases the risk of developing hypoglycemia by as much as 3 fold (DCCT, 1993). Both experimental and clinical evidence suggests that RH may alter subsequent cognitive performance, and may perhaps affects areas of the brain that mediate cognitive function (Cox, Gonder-Frederick, Kovatchev, Julian, & Clarke, 2000; Fruehwald-Schultes, Born, Kern, Peters, & Fehm, 2000; Langan, Deary, Hepburn, & Frier, 1991; McNay, 2005; McNay & Sherwin, 2004; McNay, Williamson, McCrimmon, & Sherwin, 2006).

One of the dangers of RH, and a barrier to effective glycemetic control, is hypoglycemic unawareness (Smith CB, 2009). In normal individuals, low blood glucose levels trigger a counter regulatory response in order to restore normal glucose levels. Epinephrine and glucagon, which function to raise blood glucose levels and heighten hypoglycemic awareness, are released. Individuals who experience RH require lower blood glucose levels to trigger this response, and have diminished epinephrine and glucagon release as a result of future instances of

hypoglycemia. This breakdown of the body's counter regulatory response is referred to as hypoglycemia-associated autonomic failure (HAAF) (Dagogo-Jack, Craft, & Cryer, 1993). This blunted physiological response leads to hypoglycemia unawareness, where the body is unable to recognize a hypoglycemic episode and has an impaired ability to restore normal blood glucose levels (Diedrich, Sandoval, & Davis, 2002). Effective treatment for both T1DM and T2DM must take these complications into consideration and focus on preventing RH. Moreover, epinephrine in particular is a known cognitive enhancer (Cahill & Alkire, 2003) and such reduced levels of epinephrine secretion might also contribute to impairments in cognitive function following RH.

The impact of RH on cognitive function has recently been the subject of initial animal-model studies, examining the impact of RH on spatial memory performance and hippocampal function (McNay et al., 2004; McNay et al., 2006). These initial studies have established the value of rodent models of RH in cognitive testing, in particular due to the ability to study RH without the associated complication of underlying diabetes. Further validating the use of animal models of RH, neuronal circuitry mediating executive functioning in rats is analogous to that of humans (Cohen, et al., 1997; Floresco & Magyar, 2006; Heidbreder CA, 2003; Seamans, Lapish, & Durstewitz, 2008). Clinical studies have suggested that following RH, executive processes such as judgment and decision-making abilities may be impaired (Clarke, Cox, Gonder-Frederick, & Kovatchev, 1999; Cox, et al., 2000). It has been established that hypoglycemia impairs driving ability, and hypoglycemic individuals are aware of their driving impairment (Cox, et al., 2000). In one study, participants with T1DM estimated their blood glucose level, and then were asked whether or not they would drive. 38% of the participants stated they would drive when their actual blood glucose levels were severely low, below 40mg/dL (Clarke, et al.,

1999). In a replication of these findings, 47% of participants stated that they would drive while their blood glucose levels were below 40mg/dL (Clarke, et al., 1999). Another study found patients with T1DM and impaired awareness of their hypoglycemic state would decide to drive, whereas only 4% of participants who were aware of their condition would decide to drive (Alexander D.M. Stork & Veneman, 2007). That same study also included participants with T2DM, and found that although they were aware of their hypoglycemia, 25% of them stated they would drive. This data suggests that not only does HAAF associated hypoglycemia unawareness pose a barrier to glycemic control, both HAAF and a history of RH even in the absence of HAAF may influence judgment and decision-making.

RH has also been linked to other deficits in executive functioning. Like judgment and decision-making, the prefrontal cortex (PFC) also mediates self-control. One study compared delinquent to non-delinquent juveniles. Delinquency is one indicator of poor self-control (Gans, et al., 1990). After sucrose administration, fasting blood glucose levels were measured at 6 time intervals over three hours. The participants in the delinquent group had slightly lower blood glucose levels at four of the six time points, and a higher insulin response at the 30 minute time point. Low blood glucose levels in delinquents indicate brain glucose supply may be important in regulating self-control pathways.

Another double blind clinical study looked at the effect of sucrose on delinquent behavior (Bachorowski, et al., 1990). The author found that when a high sucrose breakfast was administered as opposed to an aspartame sweetened no-sucrose breakfast, those children with the greatest behavior problems showed the most improvement as compared to less problematic delinquents. This finding suggests sucrose ingestion promoted self-control by preventing low blood glucose. The adolescent PFC is not fully developed, and low systemic glucose levels may

not provide enough energy within the immature PFC to mediate self-control (Steinberg, 2008). This indicates that glucose metabolism within the PFC may be necessary in regulating self-control.

Experimental studies using animal models have found that RH causes changes in cognition as a result of altered neuronal metabolism with concomitant changes in brain glucose uptake. A study done by McNay and Sherwin looked at the effect RH had on hippocampal-mediated spatial working memory (McNay & Sherwin, 2004). RH was found to improve spatial working memory at euglycemia, with respect to control animals, but impaired performance in animals that were tested while hypoglycemic. A task-associated dip in hippocampal extra cellular fluid (ECF) glucose concentrations was observed in control animals but the dip was absent in RH treated animals. RH animals were also observed to have significantly greater hippocampal ECF glucose levels in response to a peripherally administered glucose bolus as compared to controls. These data suggests RH may cause an increase in glucose transporters to facilitate the delivery of glucose to the hippocampus. The absence of a task-associated dip in hippocampal ECF glucose in animals with a prior history of hypoglycemia may indicate that RH causes facilitation of glucose transport to meet cognitive demand (McNay & Sherwin, 2004; Rao, Oz, & Seaquist, 2006). Another *in vivo* study in rats found that a prior history of hypoglycemia caused increased basal brain-glucose metabolism while tested when euglycemic, but decreased brain glucose metabolism when tested while hypoglycemic as compared to controls (Jiang, et al., 2009). This evidence further supports the idea that a history of RH alters brain glucose metabolism. The facilitation of glucose transport is mediated by a family of glucose transporter proteins (GLUTs), which are membrane bound proteins that allow for facilitated diffusion of glucose into cells.

Behavioral and metabolic effects of RH are further supported with evidence that RH causes biochemical changes within the brain. One study found following RH, there is a significant up-regulation in GLUT-3, the GLUT found in neurons (Uehara, Nipper, & McCall, 1997). Another study found an up-regulation in GLUT-1, the protein responsible for glucose transport across the blood brain barrier, after RH (Kumagai, Kang, Boado, & Pardridge, 1995). Other researchers looked at the genes associated with the production of glucose transporter proteins in the hypothalamus and cortex (Mastaitis, Wurmbach, Cheng, Sealfon, & Mobbs, 2005). It was found that hypoglycemia induced expression of a gene that codes for the production of GLUT-1. Fasting alone caused induced expression of the gene that codes for GLUT-4, found primarily in cells of muscle tissue. Transcriptional factors for the immediate early genes (IEG), which are markers of neuronal activity and cell proliferation, are also expressed following RH. This includes the protein FosB, a c-fos isoform (Al-Noori, et al., 2008; Paranjape & Briski, 2005). These data suggests RH may have lasting neural and cognitive consequences (McNay, et al., 2006).

Other cognitive impairments are seen in individuals prone to RH. One study looked at factors that influence academic performance in children with T1DM (McCarthy, Lindgren, Mengeling, Tsalikian, & Engvall, 2003). Participants with the lowest metabolic control who experienced the most frequent hypoglycemic episodes had the worst academic performance. Another study looked adults over 70 with T2DM (Munshi, 2006). Participants with lower cognitive ability were shown to have poor glycemic control, and were more likely to be on insulin therapy. A recent meta-analysis of T1DM patients and their cognitive performance revealed that while learning and memory are not affected, mental speed and flexibility are impaired (Brands, et al., 2005). Although clinical evidence as well as the little experimental data

available suggests that RH may impair executive functioning, little research has been devoted to elucidate the mechanisms underlying these cognitive deficits.

Dopamine has been shown to affect medial prefrontal cortex (mPFC) mediated executive functioning and working memory in rats (Floresco, Magyar, Ghods-Sharifi, Vexelman, & Tse, 2006; Seamans, Floresco, & Phillips, 1998). The mPFC is the brain region associated with mental flexibility (Birrell & Brown, 2000; de Bruin JP, 1994). Mental flexibility is the rat analog to judgment and decision-making in humans. Specific dopamine receptors subtypes (D) have been implicated in modulating mental flexibility. Blocking D2 receptors, cause decline in the ability to shift mental processes, a measure of mental flexibility (Floresco, et al., 2006). Interestingly, blocking D4 receptors caused dose dependent facilitation of set-shifting ability, while promoting D4 subtype expression caused impairment (Floresco, et al., 2006). These data suggests dopamine plays a significant role in executive functioning. Given the clinical significance that RH has on executive functioning, specifically with respect to mPFC mediated mental flexibility, and the role dopamine plays in the mPFC, we propose that there may be an interaction between RH, D, brain glucose metabolism, and executive functioning.

Methods

Subjects

Upon arrival, male Sprague-Dawley rats ($n=46$), 275-300gms, from Charles River Laboratories, Wilmington, MA, were housed two per cage. The animals were maintained on a 10:14 light dark cycle with lights on at 0600 hrs. Animals were allowed one week to acclimate to the facility and were given rat chow (Purina Prolab RMH 3000, 4%fat) and water ad libitum

until diet-restriction commenced. Diet restriction was initiated on the third day of handling. The goal of the food restriction was to maintain the weight of the animals to 85% of their free-feeding weight, calculated using a growth chart provided by Charles River Laboratories. This was usually achieved by feeding the animals 18gms of rat chow and some reward pellets (Dustless Precision Pellets, purified formula, 45mm; BioServ, Frenchtown, NJ) each day until testing commenced. Food quantity was adjusted as necessary to maintain the target weight. The restricted diet paradigm increases the animal's motivation for obtaining reward pellets during the maze testing over extended number of trails. The University at Albany's Institutional Care and Use Committee approved all methods and procedures.

Materials

Set-Shift Maze. This is a rotating plus maze with a distinct center square. Each arm of the maze can be blocked individually. During testing the maze is set up in a T-configuration. At the end of each arm is a food well. Each arm varies with respect to two dimensions, color and texture, such that the arms are: light smooth, light rough, dark smooth, and dark rough. A dark wooden holding chamber is used to place the rat in when the maze is being set up and rotated in between trials.

Procedures

Each animal underwent a 3-stage acclimation and habituation process, followed by treatment and testing.

Handling. Animals were extensively handled for at least 5 minutes per day for one week. Each day, animals were given 10-15 reward pellets in their home cages to familiarize them with the taste.

Open arm habituation. After one week of handling, animals were acclimated to all arms of the maze for a week. Briefly, during open-arm habituation, all four maze arms were open to allow the animals to explore freely. On the first day, the food well at each maze arm was baited with four reward pellets. Paired cage mates were placed in the center of the maze and allowed to explore the maze together until all the pellets were consumed. From the second day onwards, each arm was baited with one pellet, and rats were individually placed in the center of the maze and explored until all the pellets were eaten, or until they exceeded their time limit of five minutes. Immediately after the rats had finished in the maze, they were placed in the holding chamber for one minute to familiarize them with the environment, and then returned to their home cage.

Closed arm/trial reward habituation. Following the week of open arm habituation, animals went through a week long closed arm habituation stage. During this time the maze was configured in a T-shape. Animals were habituated individually for 8 trials. Trials were arranged pseudorandomly such that each start arm was used twice. For each trial, the arm directly opposite the start arm was blocked using a removable door. For the first trial, both arms were baited with one pellet. Depending on whether the animal turned left or right, the opposite arm was baited on subsequent trials. On each trial, the animal was placed into the start arm, the stem of the T, and allowed to enter a second arm and eat the reward pellet if the arm was baited. The animals were then taken out of the maze, placed in the holding chamber, and the maze was rotated and baited for the next trial. The inter trial interval was approximately 22 seconds.

RH treatment, experiment 1. After completion of handling and habituation, animals were treated based on our 3-day model of RH ($n=25$) (McNay & Sherwin, 2004). Briefly, RH was induced by insulin injections administered intraperitoneally (i.p.) targeted to lower plasma

glucose to ~50mg/dL, in line with previous data using this model. Animals were induced with RH on the three days prior to testing ($n=6$) (RH3: 3 days), or on the two days of testing ($n=6$) (RH2: 2 test days), or three days prior as well as on the two days of testing ($n=6$) (RH5: 3 days + 2 test days). Control animals received i.p. saline injections rather than insulin. Animals in the RH3 group received insulin doses of 10, 8, and 6 U/kg respectively for three days prior to testing: The reduction in dose is because of reduced counter-regulation following the prior hypoglycemic episodes. RH2 group animals received doses of 10, and 8 U/kg, and those in the RH5 group received doses of 10, 8, 6, 6, 6, and 6 U/kg respectively. The volume of saline administered for control animals was matched to the volume of insulin injections. Animals' food was removed for 3 hours post injection of insulin or saline to induce hypoglycemia, and then replaced. Animals were closely observed, and those with severe adverse reactions to insulin treatment such as seizures or coma were immediately euthanized ($n=1$). Those animals that were immobile and unresponsive to a mild tail pinch during hypoglycemia were quickly administered 1g of i.p. Dextrose. The efficacy of the RH paradigm was tested in a separate cohort of animals; blood glucose levels both pre and post-insulin treatment were measured via the tail vein using a One Touch Ultra Mini Blood Glucose Meter Kit.

Set-shift task. Testing took place in two separate sessions separated by a 24-hour period. Initial reward contingencies were determined in a random, counterbalanced fashion [either color (light or dark) or texture (smooth or rough)]. The chosen contingency was always baited during each testing session. During the first session, animals were tested until they met the pre-established criterion of choosing the correct arm on 8 consecutive trials, and then returned to their home cage. On the second testing session, each animal's reward contingencies switched dimensions. For example, if during session one, the reward contingency were color, session two

would use texture. As in session one, during the second session animals were tested to criterion then returned to their home cage. For each trial, the start and end times were recorded.

Immediately after session two, animals are euthanized, and tissue for histology was collected.

Behavioral analysis included the number of trials needed to reach criterion on day one (measure of ability to acquire a contingency rule), the number of trials needed to reach criterion on day two (measure of mental flexibility), and the average trial latency to enter a choice arm (a measure of motor activity). The set shift paradigm tests the animals' ability to associate a reward with specific environmental cues, shift strategies when the cues change, and inhibit previously learnt behavior.

Surgery. A separate cohort of animals ($n=16$) (Experiment 2) was used to determine changes in local neuronal metabolism and dopamine levels. Animals went through handling and habituation as described above, following which a microdialysis probe was implanted using stereotaxic surgery as described previously (Stefani, & Moghaddam, 2006). During recovery, animals received food and water ad libitum for four days post surgery and were handled at least five minutes per day. Briefly, rats were anesthetized using 2% isoflurane + O₂ (2 liter per minute flow rate). A heating pad was used to maintain body temperature at 37°C. An incision was made in the skin over the skull, the scalp retracted, and three holes were drilled; two for skull screws, and one for the guide cannula. A microdialysis guide cannulae (CMA12, CMA/Microdialysis) was inserted into the mPFC with stereotaxic coordinates as follows: +/- 3.7mm anterior to bregma, +/-0.6mm lateral, and -2.5mm ventral from dura. The microdialysis guide cannulae was secured to the skull using skull screws and dental acrylic, and stylet inserted to maintain patency. Post surgery, animals received a subcutaneous 1mL saline injection to prevent dehydration. They were then placed in a recovery room and allowed to recover on a

heating pad in a plexiglass cage. Animals received acetaminophen (50mg /L) in their drinking water for at least two days post surgery.

Microdialysis. 24 hours prior to testing, a microdialysis probe was inserted into the guide cannula for 5 min, then removed and the stylet replaced. This has been shown to optimize measurement on the day of testing (McNay, Fries, & Gold, 1999). On the morning of set shift day one, a fresh probe was inserted into the guide cannula of the animal and the probe was equilibrated for one hour. Animals moved freely during this period, and throughout testing. During equilibration and throughout testing, including post-test recovery, the probe was perfused with artificial extracellular fluid (aECF) at 1.5 μ L/min. The composition of aECF is based on previous studies from our lab (McNay & Sherwin, 2004).

Sample analysis. Microdialysis samples were assayed for glucose, lactate, pyruvate, and glutamate using a CMA600 analyzer. Metabolite concentrations were corrected for in vivo probe recovery using the slope of a mPFC zero net flux plot for glucose under the same experimental conditions. Analysis for catecholamines (norepinephrine, and dopamine) was performed using a High Performance Liquid Chromatography device. (HPLC; ESA, Acton, MA).

Histology. Upon completion of testing, animals were euthanized and their brain tissue was collected. Brains from the microdialysis cohort were visually inspected for proper placement of the cannula. Brains from the behaviorally tested cohort were immersion fixated in formalin for seven days at 4°C and then stored in 30% sucrose solution until processed for future protein analyses.

Statistical Analysis. Blood glucose, average latency and behavioral performance data are

expressed as means \pm standard error of mean (SEM). Microdialysis data are expressed as a percentage of baseline for each subject, where the pretest baseline sample was 100%. Statistical analysis was performed using one and two way ANOVA. Significance level was $\alpha = 0.05$.

Results

Effect of insulin treatment on blood glucose levels. Insulin treatment effectively lowered animal's blood glucose levels one-hour post injection across all groups to moderately hypoglycemic levels (Table 1). On an average, baseline glucose levels across all groups were 118.6mg/dL. One-hour post insulin treatments, glucose levels across all groups were 34.2mg/dl. No animals in the blood glucose testing cohort experienced coma or seizure. Because these values were obtained from tail-vein sampling, the core plasma glucose level is approximately 10mg/dL (Kelkar, & Kaklij, 1994) higher, or 44.2mg/dL, in line with our targeted hypoglycemia range.

Average latency. There was no effect of average latency, a measure of motor activity, as a function of treatment on either day one ($F=0.988$; $p>0.05$) or day two of testing ($F=1.045$; $p>0.05$) (Table 2).

Maze performance, experiment 1. Figure 1 shows the behavioral effect of RH on day one and two of testing. RH did not affect performance in any groups on Set-Shift task on day 1 of testing ($F=2.56$; $p>0.05$). However, on day two of testing, animals in the RH3 group took significantly more trials to reach criterion as compared to controls ($F=3.673$; $p<0.05$). No significance was observed with respect to the amount of trials taken to reach criterion by the animals in RH2 ($p>0.05$) and RH5 ($p>0.05$) groups as compared to controls. Because the

primary result was a deficit on day two in RH3 animals as compared to controls, the microdialysis studies focused on these two groups.

Microdialysis testing, and mPFC Metabolism of glucose, lactate, pyruvate, and dopamine during the Set-Shift task.

Maze performance, experiment 2. In contrast to the data obtained in our first cohort, RH3 animals tested during microdialysis performed significantly better than controls on day one (Figure 2) of testing ($p < 0.05$). This finding is in fact consistent with our previous work, which suggested that the RH3 protocol enhanced the ability to learn a spatial task (McNay & Sherwin, 2004). On day two of testing (Figure 2), RH3 animals performed significantly worse than controls, replicating and confirming the result observed in our behaviorally tested group ($p < 0.05$).

Glucose. ECF glucose concentrations did not differ significantly from controls with respect to ECF glucose levels (Figure 3a; Figure 3b).

Lactate. On day one (Figure 4a), ECF lactate levels in RH3 animals (but not controls) were significantly higher towards the end of testing with respect to their baseline ($p < 0.05$). On day 2 (Figure 4b), there was a significant interaction between treatment and stage of testing ($F = 4.028$; $p < 0.05$). There were also main effects on both treatment ($F = 10.40$; $p < 0.05$) and stage of testing ($F = 4.16$; $p < 0.05$) on ECF lactate levels on day two. As opposed to the trend in day one, day two ECF lactate levels were significantly higher toward the end of testing in control (but not RH3) animals as compared to baseline.

Pyruvate. Changes in pyruvate levels seem to reflect the changes in lactate levels on both days one (Figure 5a) and two (Figure 5b) of testing. On day one, there are main effects of

both treatment ($F=5.75$; $p<0.05$) and stage of testing ($F=5.00$; $p<0.05$) on ECF lactate levels.

Towards the end of testing, pyruvate levels are significantly higher in RH3 animals as compared to controls ($p<0.05$) and pyruvate concentration was higher than baseline. On day two there was a main effect of testing ($F=6.07$; $p<0.05$), and pyruvate ECF levels in controls were significantly more than RH3 treated animals ($p<0.05$).

Glutamate. On both day one (Figure 6a) and day two (figure 6b) of testing, RH3 animals did not differ significantly from controls.

Dopamine. Results from the microdialysis cohort show a significant spike ($p<0.05$) in Dopamine levels towards mid-testing in the RH3 group as compared to controls on day one (Figure 7a). Following the spike, the remaining test samples as well as post test baselines were not significantly different as compared to controls, and reduced to levels lower than baseline. In controls, the dopamine levels don't spike, rather a small increase is observed during mid testing followed by a return to baseline. Day two results (Figure 7b) from the revealed no spike in dopamine levels in either control or RH3 groups during mid-testing, as in day one. At the end of testing, there is a drop in dopamine levels in the control group, but not in the RH3 group. In the post-test baselines, there is a return to baseline dopamine levels in controls, but levels are elevated in the RH3 group.

Discussion

These data present an interesting picture. Results indicate that a prior history of hypoglycemia enhanced performance in animals when they learned a contingency rule, but impaired performance in mental flexibility when the rule changed. However, this was true only in animals tested while euglycemic; animals that experienced acute hypoglycemia were

surprisingly unaffected by either such hypoglycemia *or* RH, likely due to increased motivation to receive sugar reward pellets driving a level of performance akin to that of controls. As the animals learned a contingency rule (day one of testing), animals with a history of RH had higher lactate, pyruvate, and dopamine levels during testing as compared to controls. When the contingency rule changed (day two of testing), RH animals had lower lactate and pyruvate levels, with no significant difference in dopamine during testing. These data suggest that animals with prior RH may have increased activity, both in terms of neuronal transmission and metabolism, on day one. This is in line with results seen in prior work with the hippocampus (McNay et al., 2004; McNay et al., 2006). In contrast, on day two, the impaired performance seen in RH3 animals is accompanied by an apparent decrease in local mPFC glucose metabolism and dopaminergic activity. The elevated mPFC activity seen in day 1 suggest that the requirement for learning an association between a particular maze attribute and reward places a significant load on the mPFC, consistent with literature findings (McNay et al., 2004; McNay et al., 2006).

In contrast to the interaction seen between RH and day of testing (hence varied task demand and presumed involvement of mPFC) in animals tested when euglycemic, animals tested when acutely hypoglycemic showed neither an effect of prior RH nor any impairment associated with the acute hypoglycemia. This finding may appear surprising, but is likely due in large part to the nature of the reward motivation used: animals experiencing hypoglycemia may have been more motivated to find the sugar reward pellets than their euglycemic counterparts. It is also possible that the elevation in systemic epinephrine that accompanies acute hypoglycemia and is known to improve memory performance in a range of tasks, likely in part via stimulation of the vagus nerve (Roosevelt, Smith, Clough, Jensen, & Browning, 2006; Zuo & Jensen, 2007) may have contributed to the failure to observe an impairment with acute hypoglycemia, although in

most experiments any such elevation in epinephrine has been dominated by the drop in circulating glucose. One approach to extending the present findings might be to use a task with aversive motivation, such as foot shock, rather than sugar pellet reward, and determine whether an identical pattern of results is obtained.

mPFC mediated mental flexibility was impaired as a result of past history of hypoglycemia. These results are contestant with, but significantly extend, past findings from our lab. In a treatment model essentially identical to our RH3 treatment, McNay & Sherwin (McNay & Sherwin, 2004) showed that prior hypoglycemia enhanced both performance and local neuronal metabolism when tested on a hippocampally-mediated task while euglycemic, but *impaired* when measured during subsequent hypoglycemia. Hence, the present results from Day 1 are to some extent a replication of those findings; the key finding from the present experiment is that when the task demands are altered to require mental flexibility and inhibition of prior learning, a history of RH *impairs*, rather than enhances, performance even at euglycemia. The impact of RH on subsequent cognitive and neural performance hence varies by brain region and the details of cognitive demand. Dopamine levels were higher on day one of testing in RH3 animals than in controls. Given the role dopamine has in mediating working memory (Floresco, et al., 2006; Seamans et al., 1998), the increased dopamine release may account for the enhancement in maze performance in this group. Furthermore, the increased dopamine may function to enhance consolidation of rule learning on day one, making it difficult on day two to inhibit previously learnt associations and adopt a changed strategy.

No change in glucose levels in the mPFC on either day one or two of testing indicate prior history of hypoglycemia does not affect glucose supply and demand in this area. Significant differences in lactate and pyruvate across testing days signify that the brain may be

utilizing alternate energy sources. The higher lactate and pyruvate levels on day one towards the end of testing in RH treated animals indicate the brain was using these metabolites for energy, explaining the day one performance enhancement. Lactate and pyruvate were lower in RH treated animals than controls animals on day two of testing. This may indicate that RH may cause the brain to conserve energy when set shifting, a higher executive function, as to preserve lower order functions.

These data show that RH impairs mental flexibility and ability to accurately judge appropriate strategy. Diabetic patients undergoing intensive insulin replacement therapy are not only at increased risk for developing recurrent hypoglycemia, but as a consequence, may also be at risk for impaired judgment, consistent with clinical reports. More research in this area is needed both to further explore the impact of RH on cognitive function across brain regions and task demand modalities, but also to investigate potential alternate treatment regimens that may reduce the risk of RH while maintaining optimal avoidance of hyperglycemia.

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Table 1

Blood Glucose Levels One hour Post Treatment

Group	Average Baseline (mg/dl)	1-hr Post Insulin Treatment (mg/dl)
RH2	114.0	35.2
RH3	103.1	35.3
RH5	138.7	32.2

Note. RH2 animals have been made recurrently hypoglycemic for two days just prior to testing. RH3 animals have been made recurrently hypoglycemic on the three days prior to testing. RH5 animals were made recurrently hypoglycemic on the three days prior to testing, as well as two days just prior to testing. “Average Baseline (mg/dl)” was a blood sample taken via the tail vein collected just prior to insulin treatment and it reflects blood glucose levels in mg/dl. “1-hr Post Insulin Treatment” was a blood sample taken via the tail vein collected one hour post insulin treatment and it reflects blood glucose levels in mg/dl. “Hypoglycemic” denotes a condition the animals were in characterized by low blood glucose levels.

Table 2

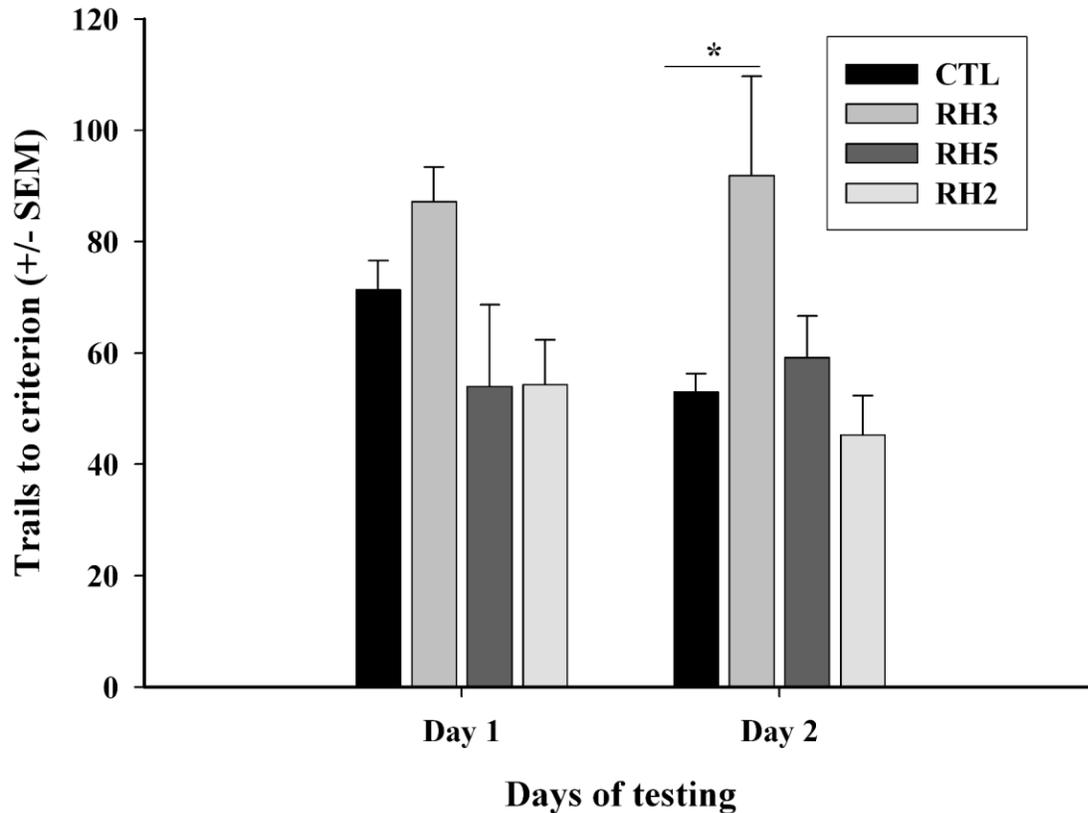
Average Latency on Day One and Two of Testing

Group	Mean Latency on Day 1 in sec (+/- SEM)	Mean Latency on Day 2 in sec (+SEM)
CTL	28.71 (+/- 12.31)	57.75 (+/- 24.67)
RH3	16.11 (+/- 7.37)	24.49 (+/- 6.10)
RH2	35.10 (+/- 4.86)	42.41 (+/- 29.88)
RH5	30.57 (+/- 6.12)	29.36 (+/- 7.48)

Note. CTL animals have not been made hypoglycemic. RH2 animals have been made recurrently hypoglycemic for two days just prior to testing. RH3 animals have been made recurrently hypoglycemic on the three days prior to testing. RH5 animals were made recurrently hypoglycemic on the three days prior to testing, as well as two days just prior to testing. “Mean Latency” is a measure of motor activity. Day 1 denotes the first day of testing. Day 2 denotes the second day of testing. “Hypoglycemic” refers to a condition the animals were in, characterized by low blood glucose levels.

Figure 1

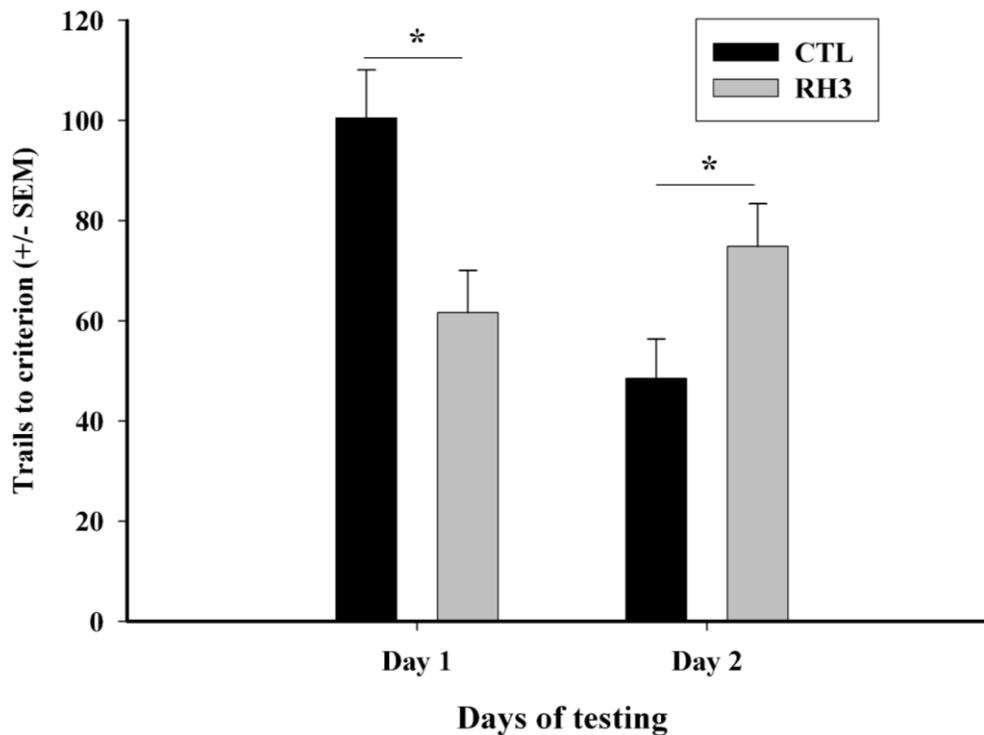
Behavioral Effect of RH on Day One and Two of Testing (behavioral cohort)



Note. CTL animals have not been made hypoglycemic. RH2 animals have been made recurrently hypoglycemic for two days just prior to testing. RH3 animals have been made recurrently hypoglycemic on the three days prior to testing. RH5 animals were made recurrently hypoglycemic on the three days prior to testing, as well as two days just prior to testing. Mean Latency is a measure of motor activity. Day 1 denotes the first day of testing. Day 2 denotes the second day of testing. Trials to criterion refer to the amount of trials necessary for the animal to acquire a contingency rule. “Hypoglycemic” refers to a condition the animals were in, characterized by low blood glucose levels.

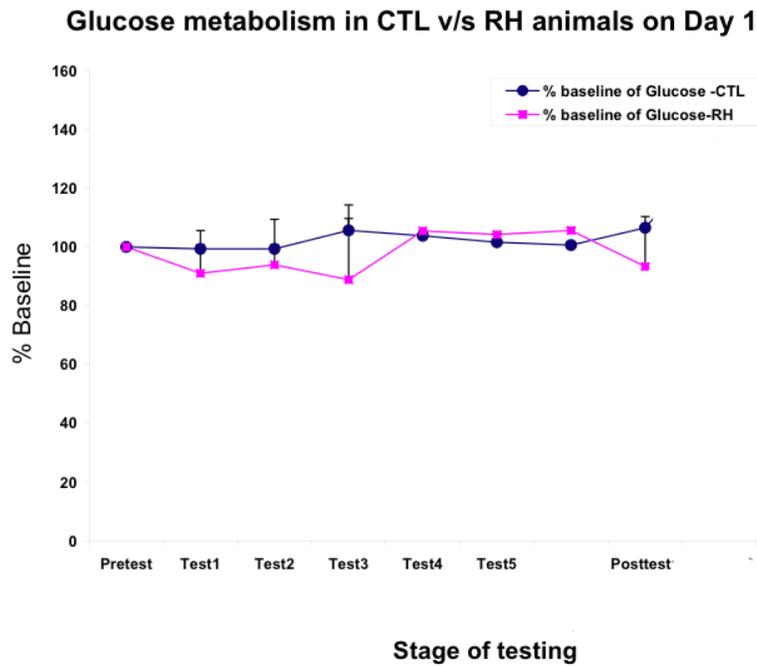
Figure 2

Behavioral Effect of RH on Day One and Two of Testing (microdialysis cohort)



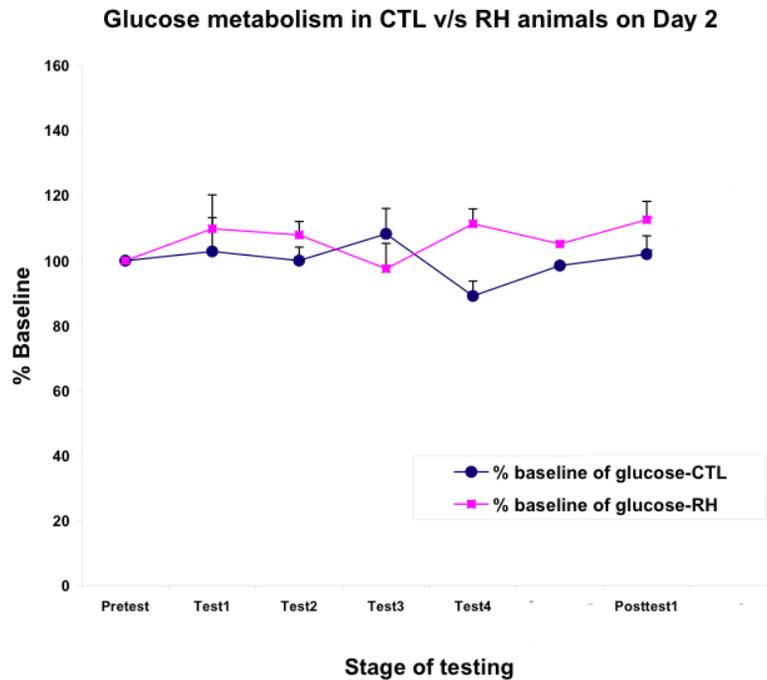
Note. CTL animals have not been made hypoglycemic. RH3 animals have been made recurrently hypoglycemic on the three days prior to testing. Mean Latency is a measure of motor activity. Day 1 denotes the first day of testing. Day 2 denotes the second day of testing. Trials to criterion refer to the amount of trials necessary for the animal to acquire a contingency rule. “Hypoglycemic” refers to a condition the animals were in, characterized by low blood glucose levels.

Figure 3a

mPFC ECF Glucose Levels on Day One of Testing

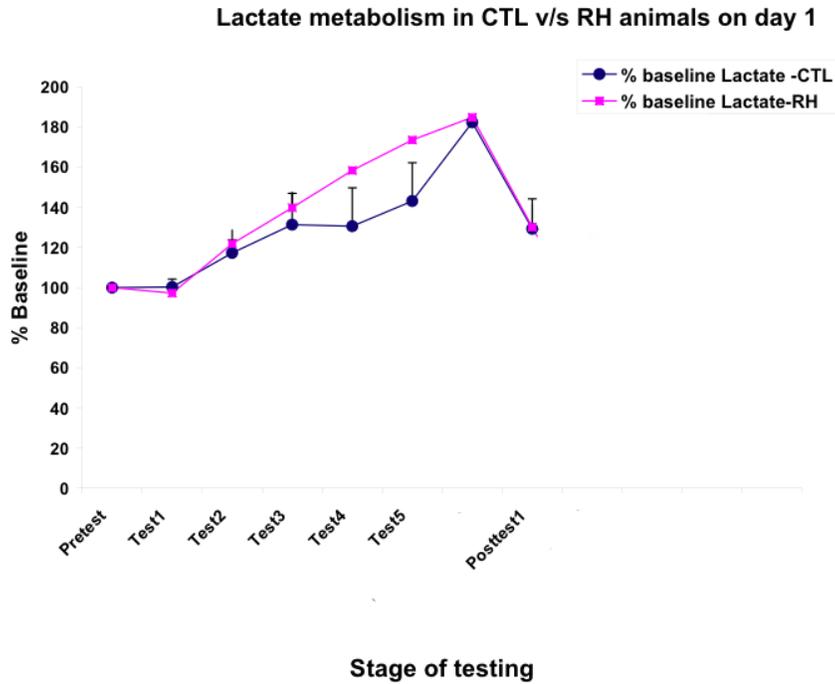
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 3b

mPFC ECF Glucose Levels on Day Two of Testing

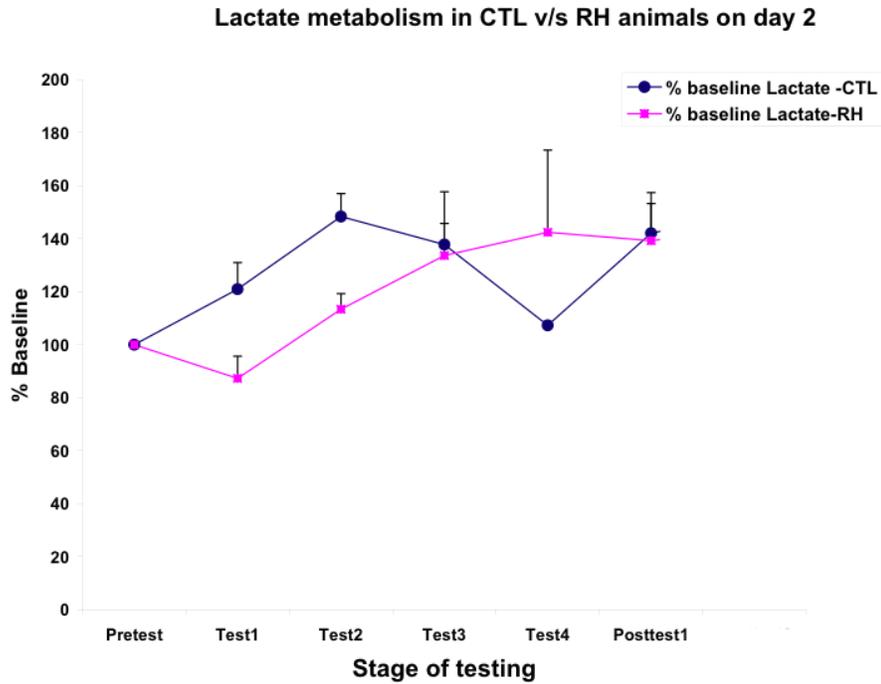
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 4a

mPFC ECF Lactate Levels on Day One of Testing

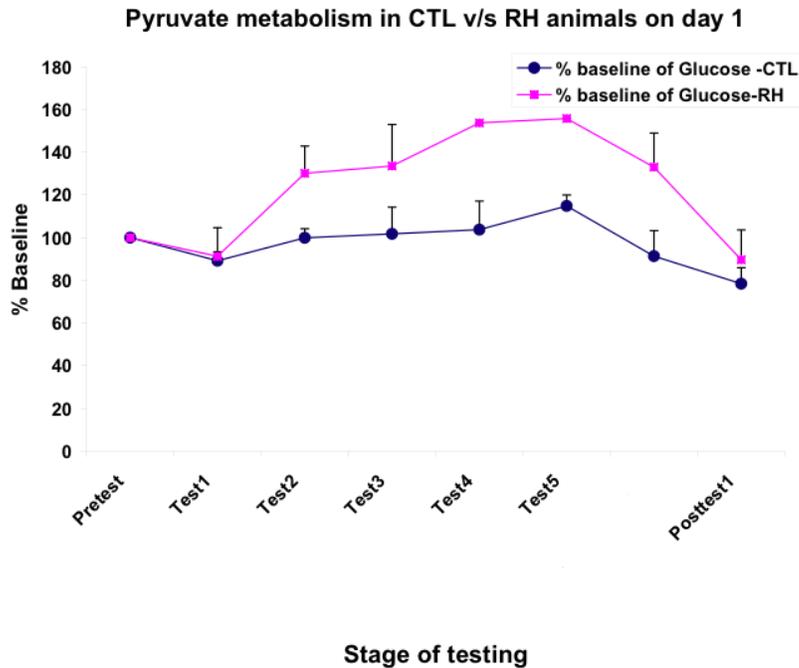
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 4b

mPFC ECF Lactate Levels on Day Two of Testing

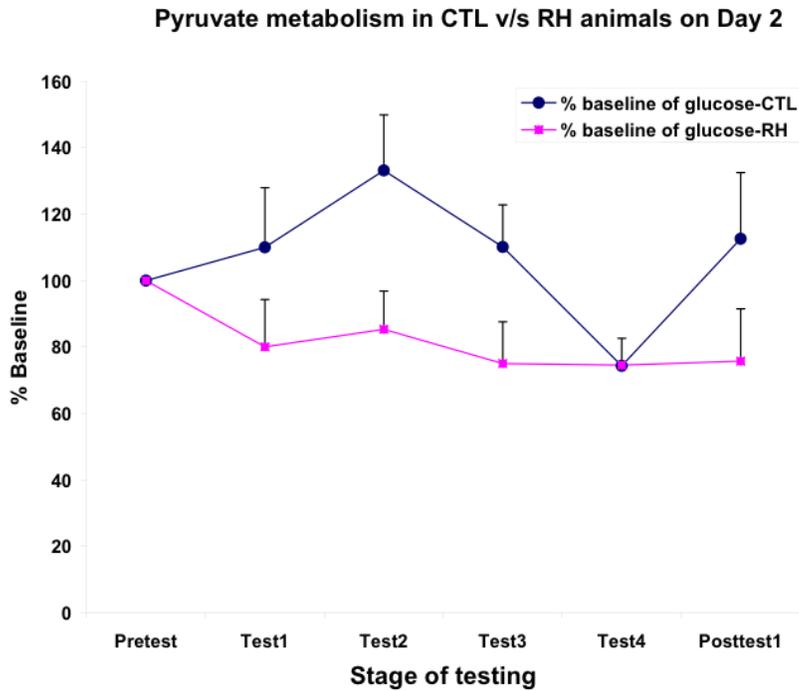
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 5a

mPFC ECF Pyruvate Levels on Day One of Testing

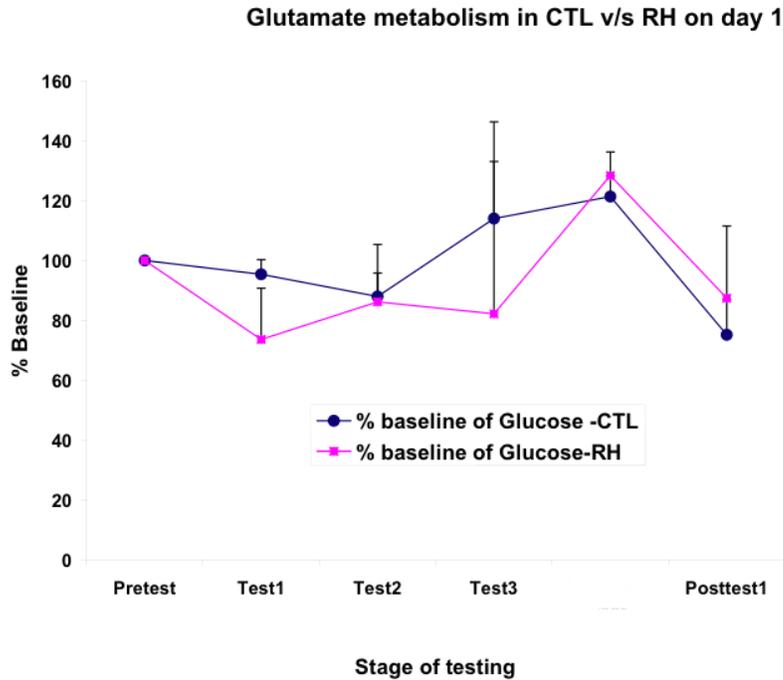
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 5b

mPFC ECF Pyruvate Levels on Day Two of Testing

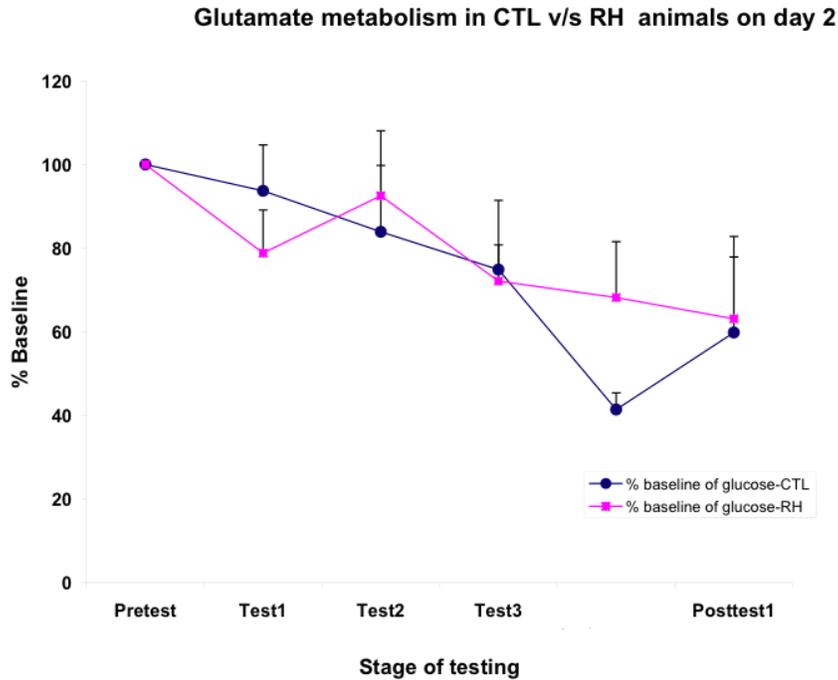
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 6a

mPFC ECF Glutamate Levels on Day One of Testing

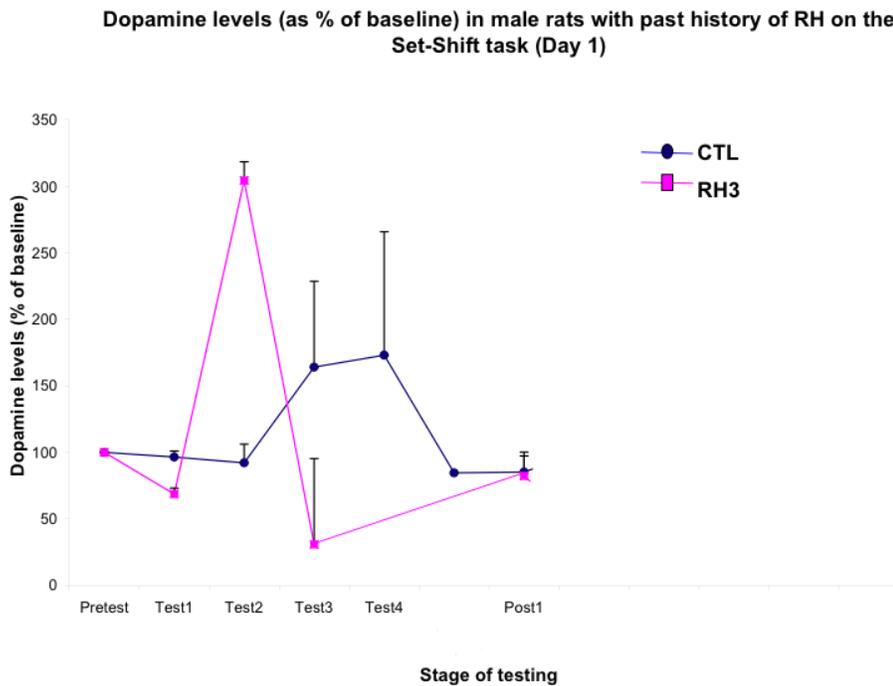
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 6b

mPFC ECF Glutamate Levels on Day Two of Testing

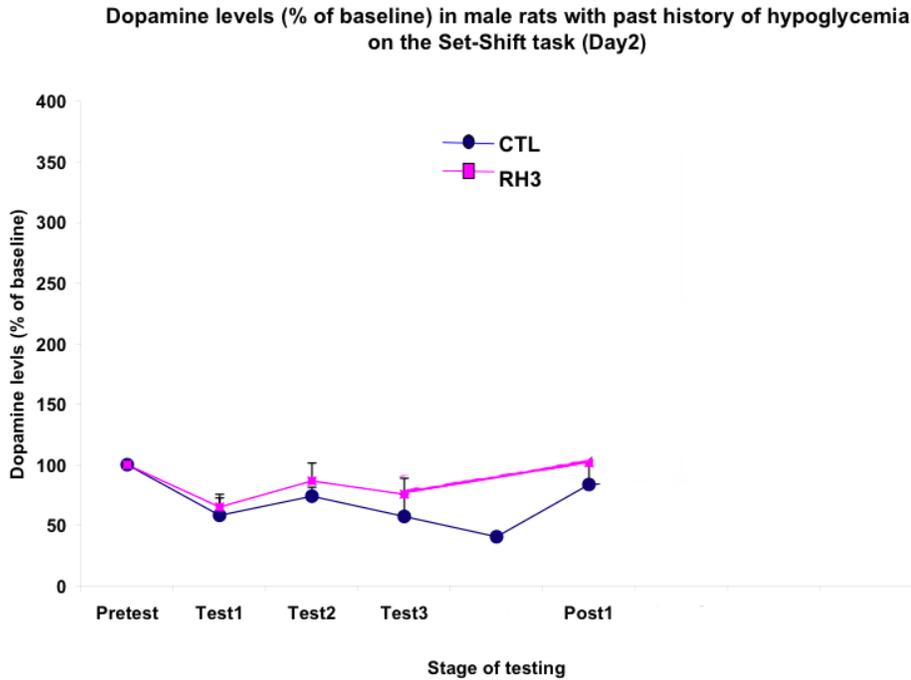
Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 7a

mPFC ECF Dopamine Levels on Day One of Testing

Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.

Figure 7b

mPFC ECF Dopamine Levels on Day Two of Testing

Note. CTL animals have not been made hypoglycemic. RH animals have been made recurrently hypoglycemic for three days prior to testing. All samples are reported as a percentage of baseline, where the pretest baseline sample represents 100%. “Test” samples were collected as the animals were undergoing testing. “Posttest” samples were collected after testing was complete.