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## Peripheral and central mechanisms through which high energy diets impair hippocampal-dependent memory in male rats

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PERIPHERAL AND CENTRAL MECHANISMS THROUGH WHICH  
HIGH ENERGY DIETS IMPAIR HIPPOCAMPAL-DEPENDENT MEMORY  
IN MALE RATS

by

AMY PATRICIA ROSS

Under the Direction of Dr. Marise Parent

ABSTRACT

Over the past five decades, *per capita* caloric intake has increased by approximately 28% in the United States. A hallmark of the current standard American diet is an excess of energy sources from saturated fat and refined carbohydrates. High energy diets such as the “Western” diet cause numerous pathologies, including non-alcoholic fatty liver disease (NAFLD), high blood pressure, dyslipidemia, and peripheral insulin resistance. High energy diets also negatively impact the hippocampus, a brain area important for learning and memory. It is not surprising, then, that high energy diets impair hippocampal-dependent memory. The experiments in this dissertation investigate possible diet-induced consequences that may contribute to the impairing effects of high en-

ergy diets on hippocampal-dependent memory. Our initial experiments found that diet-induced NAFLD impairs hippocampal-dependent memory, but these cognitive deficits were not due to decreases in insulin-like growth factor-1 (IGF-1) or hippocampal insulin signaling. Next, we found that a high energy diet increased the ability of epinephrine to increase blood glucose concentrations, indicating a novel way in which high energy diets impair liver function. The final set of experiments found that high energy diets do not necessarily impair memory but instead may prevent the memory-enhancing effects of acute stress. Taken together, these results indicate that high energy diets interact with acute stress to negatively impact hippocampal-dependent memory, and that hippocampal insulin resistance and IGF-1 are not likely involved.

**INDEX WORDS:** Memory, Hippocampus, Diet, Liver, Fructose, Rat, Stress, Epinephrine, Corticosterone, Glucose, Non-alcoholic fatty liver disease, Water maze, Object recognition, Western diet, Cafeteria-style diet, High energy diet

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Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2012

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**LIST OF ABBREVIATIONS**

aCSF, artificial cerebral spinal fluid

BBB, blood brain barrier

BDNF, brain-derived neurotrophic factor

ELISA, enzyme-linked immunosorbent assay

HED, high energy diet

HPLC, high performance liquid chromatography

IGF-1, insulin-like growth factor-1

IR, insulin resistance

IR- $\beta$ , insulin receptor beta-subunit

kcal, kilocalorie

LTD, long term depression

LTP, long term potentiation

NAFLD, non-alcoholic fatty liver disease

NMB, non-fat milk blocker

PKB/AKT, protein kinase B

RIA, radioimmunoassay

SC, standard chow

SOR, spatial object recognition

TBS-T, tris-buffered saline with Tween 20

TGs, triglycerides

## CHAPTER 1: GENERAL INTRODUCTION

Over the past five decades, *per capita* caloric intake has increased by approximately 28% in the United States (USDA 2011). A hallmark of the current standard American diet is an excess of energy sources from saturated fat and refined carbohydrates (Grotto and Zied 2010; Hu, Rimm et al. 1999; Iqbal, Anand et al. 2008). High energy diets such as the “Western” diet cause numerous pathologies, including non-alcoholic fatty liver disease (NAFLD; Ackerman, Oron-Herman et al. 2005; Fu, Sun et al. 2009), high blood pressure (Hwang, Ho et al. 1987; Elliott, Keim et al. 2002; Catena, Giacchetti et al. 2003; Delbosc, Paizanis et al. 2005; Panchal, Poudyal et al. 2010; Poudyal, Campbell et al. 2010), dyslipidemia (Sleder, Chen et al. 1980; Kelley, Allan et al. 2004; Couchepin, Le et al. 2008), and peripheral insulin resistance (Zavaroni, Sander et al. 1980; Tobey, Mondon et al. 1982; Bezerra, Ueno et al. 2000; Panchal, Poudyal et al. 2010). Many of these pathologies are components of the metabolic syndrome, which now affects both children and adults (Kohen-Avramoglu, Theriault et al. 2003). Although most research has focused on the peripheral consequences of high energy diets, there is a growing body of literature demonstrating high energy diets impact the brain (Greenwood and Winocur 1990; Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009). The goal of the following studies was to determine if certain peripheral and central effects of high energy diets are involved in the memory-impairing effects of these diets.

*High energy diets negatively impact the hippocampus.*

Previous research has identified a number of ways in which high energy diets affect the structure and function of the hippocampus, a brain region essential for learning

and memory (Ergorul and Eichenbaum 2004). High energy diets decrease brain derived neurotrophic factor (BDNF; Molteni, Barnard et al. 2002), synaptic communication (Stranahan, Norman et al. 2008), and neurogenesis (Lindqvist, Mohapel et al. 2006) in the hippocampus. In addition, high energy diets decrease long-term potentiation (LTP; Stranahan, Norman et al. 2008) and long-term depression (LTD; Mielke, Taghibiglou et al. 2005), cellular mechanisms of plasticity that are thought to underlie learning and memory processes (Malenka and Bear 2004; Massey and Bashir 2007).

It is not surprising, then, that high energy diets impair performance in memory tasks that depend on the hippocampus. For example, feeding rats a high fructose diet (Ross, Bartness et al. 2009) or a high fat, high sugar diet (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Darling et. al unpublished) causes memory impairments in the water maze task. In addition, fat-fed rats are impaired in the water maze task (Pathan, Gaikwad et al. 2008), radial arm maze, and a variable-interval delayed alternation task (Greenwood and Winocur 1990).

*High energy diets may impair memory via central insulin resistance.*

It has been well documented that high energy diets cause peripheral insulin resistance (Zavaroni, Sander et al. 1980; Tobey, Mondon et al. 1982; Bezerra, Ueno et al. 2000; Panchal, Poudyal et al. 2010). Of interest, impaired insulin signaling in the periphery is related to cognitive deficits in rats fed high energy diets (Chen, Xie et al. 2009). High energy diets also cause central insulin resistance (Mielke, Taghibiglou et al. 2005). Mielke and colleagues (2005) found that feeding hamsters a diet of 60% fructose for 6 weeks decreases phosphorylation of the insulin receptor- $\beta$  subunit and protein kinase B in the hippocampus. This is significant because hippocampal insulin signaling is

important for learning and memory (Moosavi, Naghdi et al. 2007; Moosavi, Naghdi et al. 2007; McNay, Ong et al. 2010). In addition, diet-induced insulin resistance reduces the ability of insulin to stimulate LTD (Mielke, Taghibiglou et al. 2005), and high energy diets prevent exogenous insulin from enhancing memory (McNay, Ong et al. 2010). These findings raise the possibility that high energy diets may impair memory by causing hippocampal insulin resistance.

*High energy diets may impair hippocampal-dependent memory via non-alcoholic fatty liver disease-induced decreases in insulin-like growth factor-1.*

Chronic consumption of high energy diets causes NAFLD (Ackerman, Oron-Herman et al. 2005; Ahmed, Redgrave et al. 2009; Panchal, Poudyal et al. 2010). The liver sends excess energy from high energy diets through the bloodstream to adipose tissue (Flint 1998) or stores them as lipids (Stein and Shapiro 1960). NAFLD is an accumulation of lipids in the liver, ranging from simple steatosis to fibrosis (McCullough 2004). Hepatic lipid accumulation causes insulin resistance (Samuel, Liu et al. 2004), oxidative stress (Raso, Esposito et al. 2009; Ruiz-Ramirez, Chavez-Salgado et al. 2011) and impairs liver functioning (Ai, Zhu et al. 2011; Ha, Kim et al. 2011; Pasarin, Abrales et al. 2011).

It is possible that the NAFLD-induced disturbances in liver functioning could result in impaired cognition. NAFLD could impair hippocampal-dependent memory by reducing hepatic insulin-like growth factor-1 (IGF-1) production and/or by decreasing the amount of IGF-1 transported across the blood brain barrier (BBB). The liver is the main source of IGF-1 (Yakar, Liu et al. 1999), a hormone capable of crossing the BBB (Reinhardt and Bondy 1994) and impacting cell growth and repair (Aberg, Aberg et al. 2000;

O'Kusky, Ye et al. 2000; Lichtenwalner, Forbes et al. 2001). In addition, IGF-1 is important for learning and memory. For example, spatial memory deficits observed in rodent models of type 2 diabetes are prevented by exogenous administration of IGF-1 (Lupien, Bluhm et al. 2003), and blocking IGF-1 receptors in the brain impairs performance of hippocampal-dependent memory tasks (Lupien, Bluhm et al. 2003). Increases in endogenous IGF-1 cause exercise-induced memory enhancement in rats (Ding, Vaynman et al. 2006; Cassilhas, Lee et al. 2012). In addition, decreases in IGF-1 concentrations are correlated with cognitive function in aging individuals (Tan and Baxter 1986; Aleman, de Vries et al. 2000; Bellar, Glickman et al. 2011). Of interest, patients with NAFLD exhibit decreases in circulating IGF-1 (Arturi, Succurro et al. 2011) and experience cognitive impairments (Newton 2010).

*High energy diets may impair memory through non-alcoholic fatty liver disease-induced decreases in the ability of epinephrine to release hepatic glucose.*

Diet-induced NAFLD may interfere with the capacity of the liver to mobilize glucose during the fight or flight response. During this response, stress stimulates the adrenal gland to release epinephrine. Epinephrine, in turn, stimulates the liver to release glucose, which then circulates throughout the body (Sacca, Vigorito et al. 1983). Given that hepatic lipid accumulation impedes the ability of insulin to decrease hepatic glucose secretion (Yadav, Jain et al. 2009), these excess lipids may interfere with the ability of epinephrine to increase hepatic glucose release.

Emotionally arousing events enhance memory, at least in part, via the release of endogenous epinephrine (Gold and van Buskirk 1978; King and Williams 2009). Stressful events, such as swimming in the water maze task (Mabry, Gold et al. 1995), receiv-

ing a footshock in an inhibitory avoidance task (McCarty and Gold 1981), and being placed in a novel environment (de Boer, Koopmans et al. 1990) cause the release of endogenous epinephrine. Given that epinephrine cannot cross the BBB (Weil-Malherbe, Axelrod et al. 1959), it is thought that epinephrine enhances memory, in part, through the hepatic release of glucose (Sacca, Vigorito et al. 1983; Hall and Gold 1986). This idea is supported by the fact that epinephrine and glucose both impact memory in a similar dose-dependent manner. To be specific, optimal memory performance occurs with mid-range doses of either hormone, but too little or too much glucose or epinephrine has no or negative effects on memory (Gold and Van Buskirk 1975; Gold, van Buskirk et al. 1977; Gold 1986; Gold, Vogt et al. 1986). In addition, eliminating endogenous epinephrine release through adrenalectomy reduces stress-induced increases in blood glucose concentrations and impairs performance in an inhibitory avoidance task (Hall and Gold 1990). Injections of exogenous glucose restore memory performance in adrenalectomized rats (Hall and Gold 1990). Emotionally arousing events also enhance memory through the release of endogenous corticosterone (Sandi, Loscertales et al. 1997). Stressful events stimulate the adrenal gland to secrete corticosterone, which, unlike epinephrine, crosses the BBB (McEwen, Weiss et al. 1968; McEwen, Weiss et al. 1969). Once corticosterone is in the brain, limbic structures, including the hippocampus, absorb and retain the hormone (McEwen, Weiss et al. 1968; McEwen, Weiss et al. 1969). Corticosterone injections enhance memory in object recognition (Okuda, Roozendaal et al. 2004) and the spatial water maze (Sandi, Loscertales et al. 1997). In addition, eliminating endogenous corticosterone through adrenalectomy impairs water maze memory performance (Oitzl and de Kloet 1992).

It is possible that high energy diets only impair memory in tasks that stimulate the release of stress hormones. High energy diets increase epinephrine (Kaufman, Li et al. 1993) and corticosterone (Tannenbaum, Brindley et al. 1997; Legendre and Harris 2006; Cano, Jimenez-Ortega et al. 2008) secretion and decrease adrenal gland weight (Boukouvalas, Antoniou et al. 2008). In addition, behavioral data from our lab and others suggest that rats fed a high fructose or high fat/high sugar diet have impaired memory during tasks that are stressful, such as the water maze task (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009), but not during less stressful tasks such as object recognition (Darling et al., unpublished) or spontaneous alternation (Ross 2008). The existing data do not directly address the possibility that high energy diets only impair memory in stressful tasks because there are many experimental differences in these studies in addition to stress, such as different handling and training protocols, testing apparatus, retention intervals, motivation, performance requirements, and light cycle.

*Are the high energy diet-induced memory impairments reversible?*

To our knowledge, there are no studies that have investigated whether removing the high energy dietary components reverses the diet-induced memory impairments. One study has found that giving rats an antidiabetic drug reverses the cognitive deficits induced by a high energy diet (Pathan, Gaikwad et al. 2008). Beyond this finding, most research in this area has been dedicated to determining whether the peripheral effects of high energy diets can be attenuated with interventions such as exercise (Brandt, De Bock et al. 2010; da Luz, Frederico et al. 2011) or dietary supplements (Shetty, Mengi et al. 2010; Marsman, Heger et al. 2011; Panchal, Poudyal et al. 2011; Tan, Kamal et

al. 2011). Such interventions reverse diet-induced non-alcoholic fatty liver disease (Shetty, Mengi et al. 2010; Marsman, Heger et al. 2011; Panchal, Poudyal et al. 2011) and peripheral insulin resistance (Brandt, De Bock et al. 2010; da Luz, Frederico et al. 2011; Tan, Kamal et al. 2011) in high energy-fed rats. Given that the high energy diet-induced metabolic and cognitive consequences are not permanent, it is possible that the diet-induced memory impairments could be overturned by simply removing the high energy dietary components.

### 1.1 Specific Aims

The evidence reviewed above suggests that the high energy diet-induced memory impairments are caused by metabolic disturbances in the periphery and in the brain. Therefore, the experiments in this dissertation investigate possible diet-induced consequences that may contribute to the impairing effects of the diets on hippocampal-dependent memory. These experiments are outlined in the following aims:

*Specific Aim 1: Are the memory deficits induced by high energy diets caused, at least in part, by hippocampal insulin resistance?*

High energy diets produce hippocampal insulin resistance in *hamsters* (Mielke, Taghibiglou et al. 2005); however, it is not known if these diets impair central insulin signaling in *rats*. Given that hippocampal insulin signaling is important for learning and memory (Moosavi, Naghdi et al. 2007; Moosavi, Naghdi et al. 2007; McNay, Ong et al. 2010), and high energy diets impair hippocampal-dependent memory (Greenwood and Winocur 1990; Molteni, Barnard et al. 2002; Pathan, Gaikwad et al. 2008; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009), this experiment tested whether the

high energy diet decreases phosphorylation of the insulin receptor- $\beta$  subunit and protein kinase B/AKT in rats, and if so, whether this insulin resistance is related to the diet-induced memory impairments.

*Specific Aim 2: Are the memory deficits produced by high energy diets caused, at least in part, by NAFLD-induced decreases in IGF-1?*

High energy diets are used to create models of non-alcoholic fatty liver disease (NAFLD; Ackerman, Oron-Herman et al. 2005; Fu, Sun et al. 2009), a metabolic disturbance that results in impaired liver function (Samuel, Liu et al. 2004; Ai, Zhu et al. 2011; Ha, Kim et al. 2011; Pasarin, Abalde et al. 2011). Of importance, the liver is the main source of insulin-like growth factor-1 (IGF-1; Yakar, Liu et al. 1999), a hormone important for learning and memory (Tan and Baxter 1986; Aleman, de Vries et al. 2000; Lupien, Bluhm et al. 2003). In addition, NAFLD increases circulating triglycerides (TGs; Ackerman, Oron-Herman et al. 2005), which prevent IGF-1 from crossing the BBB (Dietrich, Muller et al. 2007). It is possible that high energy diets impair memory through NAFLD-induced decreases in hepatic IGF-1 production and/or the amount of IGF-1 transported to the brain. Therefore, Experiment 1 tested whether high energy diet-induced NAFLD decreases hepatic IGF-1. A second experiment tested whether NAFLD decreases hippocampal IGF-1.

*Specific Aim 3: Do high energy diets impair epinephrine-stimulated glucose release?*

Sympathetic nervous system activation mobilizes resources for the fight or flight response, in part, through the release of epinephrine, which increases hepatic glucose release into the bloodstream (Sacca, Vigorito et al. 1983). In addition, the ability of emotion to modulate memory is thought to occur, in part, through this epinephrine-

stimulated glucose release (Hall and Gold 1986). High energy diets produce memory impairments (Greenwood and Winocur 1990; Molteni, Barnard et al. 2002; Pathan, Gaikwad et al. 2008; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009) and NAFLD (Ackerman, Oron-Herman et al. 2005; Svegliati-Baroni, Candelaresi et al. 2006; Ahmed, Redgrave et al. 2009), and NAFLD impairs liver functioning (Ai, Zhu et al. 2011; Ha, Kim et al. 2011; Pasarin, Abraldes et al. 2011). Therefore, this experiment tested whether a high energy diet impairs the ability of epinephrine to increase blood glucose concentrations. Given that previous studies demonstrate that the effects of high energy diets, such as NAFLD, are not permanent, (Shetty, Mengi et al. 2010; Marsman, Heger et al. 2011; Panchal, Poudyal et al. 2011), a second experiment tested whether removing excess fat and sugar from the diet reverses hepatic lipid accumulation and restores the ability of epinephrine to increase blood glucose concentrations.

*Specific Aim 4: Do high energy diets impair the stress hormone modulation of memory?*

High energy diets increase the secretion of epinephrine (Kaufman, Li et al. 1993) and corticosterone (Tannenbaum, Brindley et al. 1997; Legendre and Harris 2006; Cano, Jimenez-Ortega et al. 2008), which are two hormones important for the modulation of memory (Gold and Van Buskirk 1975; Gold and van Buskirk 1978; Sandi, Loscertales et al. 1997; King and Williams 2009). Given that rats fed high energy diets are impaired on stressful memory tasks (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009), but not on relatively unstressful memory tasks (Ross 2008; Darling et al., unpublished), it is possible that high energy diets impair the stress hormone modulation of memory. Experiment 1 tested whether a high energy diet impairs memory in a high stress version of a spatial object recognition (SOR) task, but not

in a low stress version of the same task. Experiment 2 tested whether the diet-induced memory impairments could be replicated by injecting the rats with epinephrine in a moderate stress version of the SOR task, and Experiments 3 and 4 tested whether the diet-induced memory impairments could be prevented by blocking the effects of epinephrine and corticosterone in the high stress version of the SOR task.

*Specific Aim 5: Are the memory-impairing effects of high energy diets reversible?*

Thus far, to our knowledge, no one has tested whether eliminating access to fat and sugar reverses the cognitive effects of high energy diets. Therefore, this experiment tested whether removing excess fat and sugar from the diet restores the ability of stress to enhance hippocampal-dependent memory.

In summary, the following experiments use behavioral, biochemical, and histological techniques to test whether the memory-impairing effects of high energy diets are caused by (1) hippocampal insulin resistance and (2) NAFLD-induced decreases in hippocampal and/or hepatic IGF-1, (3) NAFLD-induced decreases in epinephrine-stimulated glucose release, (4) the result of altered adrenal hormone modulation of memory and (5) whether the diet-induced memory impairments are reversible. These findings add to the growing body of literature demonstrating that high energy diets are harmful to the brain as well as the body.

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## CHAPTER 2: NON-ALCOHOLIC FATTY LIVER DISEASE IMPAIRS HIPPOCAMPAL-DEPENDENT MEMORY IN MALE RATS

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### 2.1 Abstract

Non-alcoholic fatty liver disease (NAFLD) is a disorder observed in children and adults characterized by an accumulation of liver fat (> 5% wet weight) in the absence of excessive alcohol intake. NAFLD affects 10 to 30% of the American population and is the most common cause of liver disease in the United States. NAFLD leads to serious disturbances in cardiovascular and hormonal function; however, possible effects on brain function have been overlooked. The aims of the present study were to test whether diet-induced NAFLD impairs hippocampal-dependent memory and to determine whether any observed deficits are associated with changes in hippocampal insulin signaling or concentrations of brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1). Post-weanling male Sprague-Dawley rats were fed a high fructose (60% of calories) or control diet for 12 weeks and then trained and tested in a spa-

tial water maze. NAFLD was confirmed with postmortem measures of liver mass and liver lipid concentrations. NAFLD did not affect acquisition of the spatial water maze, but did impair retention tested 48 h later. Specifically, both groups demonstrated similar decreases in latency to swim to the escape platform over training trials, but on the memory test NAFLD rats took longer to reach the platform and made fewer visits to the platform location than control diet rats. There were no differences between the groups in terms of insulin-stimulated phosphorylation of insulin receptor  $\beta$  subunit (IR- $\beta$ ) and protein kinase B (PKB/AKT) in hippocampal slices or hippocampal BDNF or IGF-1 concentrations. Thus, these data indicate that NAFLD impairs hippocampal-dependent memory function and that the deficit does not appear attributable to alterations in hippocampal insulin signaling or hippocampal BDNF or IGF-1 concentrations.

## **2.2 Introduction**

Non-alcoholic fatty liver disease (NAFLD) is observed in children and adults and is characterized by an accumulation of liver fat (> 5% wet weight) in the absence of excessive alcohol intake (Neuschwander-Tetri and Caldwell 2003; Patton, Sirlin et al. 2006). The prevalence of pediatric and adult NAFLD is alarmingly high, and its incidence is increasing worldwide. For example, in the United States, over 30% of adults and 10% of children suffer from NAFLD (Bloomgarden 2005; Schwimmer, Deutsch et al. 2006). In fact, NAFLD is the most common cause of liver pathology in adults and children and causes serious disturbances in cardiovascular and hormonal function (Pacifi-co, Cantisani et al. 2008; Mager, Patterson et al. 2010; Lee, Shim et al. 2011; Lucero, Zago et al. 2011; Nseir, Shalata et al. 2011).

Over-nutrition is considered the main cause of NAFLD (Ackerman, Oron-Herman et al. 2005; Ahmed, Redgrave et al. 2009; Safwat, Pisano et al. 2009; Larter, Chitturi et al. 2010). In the US, children and adults over consume high energy foods, particularly those that are high in fat and sugar (Hu, Rimm et al. 1999; Iqbal, Anand et al. 2008; Mager, Patterson et al. 2010). These macronutrients are metabolized mainly by the liver and converted to energy, and the surplus is stored by the liver as lipids. Given that NAFLD is primarily a disorder of over-nutrition, feeding rats high-energy diets is a commonly accepted animal model of NAFLD (Ackerman, Oron-Herman et al. 2005; Svegliati-Baroni, Candelaresi et al. 2006; Ahmed, Redgrave et al. 2009). For example, Sprague-Dawley rats fed a 60% fructose diet or a diet of at least 57% fat develop symptoms of NAFLD, including hepatomegaly (Ross, Bartness et al. 2009), hypertriglyceridemia (Ackerman, Oron-Herman et al. 2005; Ross, Bartness et al. 2009), and increased hepatic lipids (Ackerman, Oron-Herman et al. 2005; Svegliati-Baroni, Candelaresi et al. 2006; Ahmed, Redgrave et al. 2009).

Virtually all research on NAFLD thus far has focused on the peripheral effects of the disease, whereas the effects of NAFLD on central function have been largely overlooked. For a number of reasons, we hypothesized that NAFLD impairs hippocampal-dependent memory. For instance, NAFLD is characterized by disruptions in lipid metabolism, which result in increased circulating triglycerides (TGs; Kelley, Allan et al. 2004). Elevated TGs, in turn, prevent substances important for learning and memory, such as leptin (Farr, Banks et al. 2006; Oomura, Hori et al. 2006) and insulin-like growth factor-1 (IGF-1; Lupien, Bluhm et al. 2003; Ding, Vaynman et al. 2006), from crossing the blood brain barrier (BBB; Banks, Coon et al. 2004; Dietrich, Muller et al. 2007). In

addition, direct administration of TGs into the brain impairs hippocampal-dependent memory (Farr, Yamada et al. 2008). Diet-induced NAFLD could also impact cognition by impairing hepatic glucose control, or by producing central insulin resistance. Our data indicate that NAFLD potentiates epinephrine-induced hepatic glucose release (Ross, Darling et al. 2010), which is significant because acute and chronic hyperglycemia are associated with memory deficits (Gold, Vogt et al. 1986; Meneilly, Cheung et al. 1993; Rodriguez, Horne et al. 1994; Vanhanen, Koivisto et al. 1997; Awad, Gagnon et al. 2002). In addition, elevations in glucose result in simultaneous increases in circulating insulin, and hippocampal infusions of insulin enhance memory (Moosavi, Naghdi et al. 2007; Moosavi, Naghdi et al. 2007; McNay, Ong et al. 2010). Given that insulin signaling in the hippocampus is important for learning and memory (Moosavi, Naghdi et al. 2007; Moosavi, Naghdi et al. 2007; McNay, Ong et al. 2010), NAFLD-induced interruptions in hippocampal insulin signaling could result in learning and memory deficits. Notably, rats fed a high fructose diet and patients with NAFLD often experience peripheral insulin resistance (Zavaroni, Sander et al. 1980; Tobey, Mondon et al. 1982; Bezerra, Ueno et al. 2000; Manco, Marcellini et al. 2008; Manco, Giordano et al. 2009), but it is not known if they also suffer from central insulin resistance.

Diet-induced NAFLD could also impair cognition through changes in the levels of growth hormones found in the hippocampus. Over-nutrition is often associated with decreases in hippocampal brain-derived neurotrophic factor (BDNF; Molteni, Barnard et al. 2002; Kanoski, Meisel et al. 2007; Stranahan, Norman et al. 2008), which is significant because BDNF promotes hippocampal cell growth and is involved in learning and memory (Barde 1994; Linnarsson, Bjorklund et al. 1997; Ma, Wang et al. 1998). In addi-

tion, the liver is responsible for releasing IGF-1 (Yakar, Liu et al. 1999), a hormone also important for the growth and repair of hippocampal neurons and learning and memory (Aleman, de Vries et al. 2000; Lupien, Bluhm et al. 2003). As a result, NAFLD-induced decreases in the availability of IGF-1 and/or BDNF could negatively impact hippocampal-dependent learning and memory.

Given the extensive plasticity that takes place in the brains of developing animals (Eayrs and Goodhead 1959; Aghajanian and Bloom 1967; McIlwain and Bachelard 1971), and the high prevalence of NAFLD in childhood and adolescence (Schwimmer, Deutsch et al. 2006), we hypothesized that over-nutrition-induced NAFLD during development has long term consequences on brain function. As a result, we induced NAFLD in post-weanling rats using a diet high in fructose and then tested hippocampal-dependent learning and memory once they reached adulthood. NAFLD was confirmed with postmortem measures of liver mass and liver lipid concentrations. We also determined whether NAFLD impaired the ability of insulin to stimulate phosphorylation of the insulin receptor  $\beta$ -subunit (IR- $\beta$ ; the first protein in the signaling cascade; Kahn, White et al. 1993) and protein kinase B (PKB/AKT; a downstream molecule involved in learning and memory; Dou, Chen et al. 2005). Finally, we determined whether NAFLD decreased hippocampal BDNF or IGF-1 concentrations.

## **2.3 Materials and Methods**

### *Animals and housing*

Twelve timed-pregnant dams (Sprague-Dawley rats, Charles River, Wilmington, MA) arrived 7-10 days before delivering litters. The animal care facility was controlled for temperature and humidity, and rats were maintained on a 12 h light/dark schedule.

All procedures were approved by Georgia State University's Institutional Animal Care and Use Committee and were in accordance with PHS guidelines.

### *Diets*

On postnatal day 21, male pups were weaned and placed singly in suspended, wire-bottom cages (Allentown, Allentown, NJ) with plastic inserts that covered half the cage floor. The rats were weighed, matched on absolute body mass, and then assigned to the control or diet-induced NAFLD (NAFLD) groups. To control for possible litter effects, both the control and NAFLD groups were comprised of rats from each litter. The NAFLD group was provided *ad libitum* with pellets composed of 60% fructose (Research Diets, New Brunswick, NJ). The 60% fructose concentration was chosen because this amount is an established model of NAFLD (Ackerman, Oron-Herman et al. 2005; Mohamed, Nallasamy et al. 2009; Chen, Fang et al. 2010), produces hippocampal insulin resistance in hamsters (Mielke, Taghibiglou et al. 2005), and causes memory deficits in rats fed fructose starting in adulthood (Ross, Bartness et al. 2009). The control group was fed *ad libitum* with isocaloric pellets that contained no fructose (60% vegetable starch; Research Diets, New Brunswick, NJ), but an equal percentage of carbohydrates (70%), proteins (20%), and lipids (10%; see Table 1). Rat body mass and food intake were recorded daily for 1 week out of every 3 weeks until behavioral tests were performed. To measure food intake, pellets in each hopper and dried spillage from under each cage were weighed and then subtracted from the amount placed in the hopper the previous day. Average daily caloric consumption was calculated by multiplying the average grams of food consumed daily by kilocalorie (kcal) per gram of food.

### *Spatial water maze*

After 12 weeks on the diet, the spatial water maze task was used to assess learning and memory. The task depends on the integrity of the hippocampus for successful performance (Morris, Garrud et al. 1982; Clark, Broadbent et al. 2005), and rats fed fructose during adulthood have impaired retention performance in the spatial water maze (Ross, Bartness et al. 2009). For water maze acquisition, the rats were trained to locate a clear, Plexiglas platform (26 cm in height and 11.5 cm in diameter) that was submerged 1 cm below the surface of the water ( $20 \pm 3^\circ\text{C}$ ) in a circular pool (0.46 m in depth and 1.35 m in diameter). Curtains were hung on two sides of the pool, and one visual pattern was pasted on each curtain. For purposes of analysis, the water maze was divided into four virtual quadrants, with one quadrant containing the platform. Acquisition training began by placing the rat on the platform for 30 s and then placing it in the water in each non-platform containing quadrant in a random order and allowing it a maximum of 60 s to reach the platform. Latency to reach the platform on each training trial was used as the measure of acquisition. If the rat did not reach the platform within 60 s, it was guided gently by hand to the platform. The rat was allowed to remain on the platform for 15 s at the end of each trial and was then placed in an empty cage with a heat lamp for a 30 s inter-trial interval. All rats were given 8 training trials on the first day. On the second day, they were trained until they swam to the platform in less than 8 s for 3 consecutive trials for a maximum of 8 trials. Rats that did not reach the criterion on the second day were given a third day of training with the same criterion for a maximum of 8 trials. This criterion was determined on the basis of pilot studies in which control rats were given 8 training trials per day for 3 days, and the results indicated that

group performance plateaued at 8 seconds. This training protocol was adopted in order to ensure that learning occurred and to avoid overtraining, which could mask any memory impairments. Eight NAFLD and 9 control rats did not meet the criterion by the end of the third day and thus were excluded from all of the memory and biochemical measures, resulting in 19 NAFLD and 21 control rats.

Retention of the training was tested 48 h after the last training day. Each rat was placed in the pool facing the wall in a randomly determined non-platform quadrant and allowed to swim for 20 s. The platform was not present, and memory measures during the probe test included: 1) latency to cross the target (previous platform location), 2) time spent in the target quadrant, 3) number of target approaches and 4) average proximity to the target. Swim speed was also measured. All trials were recorded and analyzed using TopScan software (CleverSys, Reston, VA).

#### *Tissue collection and assays*

Forty-eight hours after water maze testing, rats were fasted for 4 h, anesthetized with isoflurane (5% in 95% O<sub>2</sub>) and then euthanized by decapitation. Brains were removed, and in one subset of rats (NAFLD: n = 6; control: n = 6), the hippocampus was extracted and frozen on dry ice for measures of IGF-1 and BDNF. In the other group (NAFLD: n = 7; control: n = 7), hippocampal tissue was collected and sliced for measures of hippocampal insulin signaling (see below). After decapitation, trunk blood from all rats was collected in heparinized tubes and centrifuged to obtain plasma for measures of IGF-1, TGs, and glucose. Livers from all rats were removed, weighed, and flash frozen for measures of lipids and IGF-1. All tissue was stored at -80°C until assays were performed. To verify the presence of NAFLD, hepatic lipids were extracted using the

Folch method (Folch, Lees et al. 1957), and a subset of liver samples were sectioned and stained with oil red-O and hematoxylin for assessment of lipid droplets (Ackerman, Oron-Herman et al. 2005).

Following the manufacturer's recommended protocols, enzyme-linked immunosorbent assays (ELISAs) were used to measure IGF-1 (R&D Systems, Minneapolis, MN) and BDNF (Promega, Madison, WI). In addition, a protein assay (BioRad, Hercules, CA) was used to measure the total protein concentration present in the samples. Plasma samples were assayed for TGs (Sigma, St. Louis, MO) using a colorimetric assay and spectrophotometry. Glucose was measured using an Accu-Chek glucose meter (Roche, Indianapolis, IN).

#### *Hippocampal insulin signaling*

##### *Preparation of homogenates*

The solutions used for the preparation of hippocampal slices and homogenates were as described by Mielke and colleagues (Mielke, Taghibiglou et al. 2005). Each brain was dissected in chilled artificial cerebrospinal fluid (aCSF). The hippocampus was then sectioned into 12 to 16 400  $\mu\text{m}$  transverse slices, which were suspended on mesh in oxygenated aCSF (5%  $\text{CO}_2/95\%\text{O}_2$ ; 33 – 34°C). After a 60 min post-slicing recovery period, half the slices were placed in aCSF plus insulin (100 nM) for 10 min, and the other half remained in aCSF. Slices from both conditions were then manually homogenized over ice in separate tubes containing 5 mL of lysis buffer. The homogenates were centrifuged at 1000 x  $g$  for 10 min at 4°C, and a detergent-compatible protein assay (BioRad, Hercules, CA) was used to measure the protein concentration present in the supernatants.

### Electrophoresis and immunoblotting

To measure the effect of NAFLD on insulin-stimulated phosphorylation of IR- $\beta$  and PKB/AKT, the protein samples were separated electrophoretically on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. All membranes were blocked in 5% non-fat milk blocker (NMB)/tris-buffered saline with Tween-20 (TBS-T) and probed with primary antibodies against phospho-IR- $\beta$  (mouse monoclonal directed against Tyr1150 and Tyr1151; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-PKB/AKT (rabbit monoclonal directed against Ser473; 1:6,000; Cell Signaling Technology, Danvers, MA) followed by the appropriate horseradish peroxidase (HRP) linked secondary antibody (goat anti-mouse or goat anti-rabbit; 1:20,000). All antibody solutions were prepared in 5% NMB/TBS-T. The membranes were stripped and re-probed for total IR- $\beta$  (rabbit polyclonal; 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA), PKB/AKT (rabbit monoclonal; 1:6,000; Cell Signaling Technology, Danvers, MA), and  $\beta$ -actin (rabbit polyclonal; 1:50,000; Novus Biologicals, Littleton, CO). Membranes were then incubated in chemiluminescent HRP substrate (Millipore, Billerica, MA) to activate the secondary antibody. Densitometry was performed using the FluorChem 8800 Imaging System (Alpha Innotech, Santa Clara, CA).

### *Data analysis*

The data were stored and analyzed using Microsoft Excel, Version 5.0 and Statistical Package for the Social Sciences (SPSS), Version 18.0. A Student's t-test was performed to determine whether there were differences between the means of the control and NAFLD rats for acquisition, time spent in the target quadrant, average proximity to the target, average kcal consumption, liver mass, hepatic lipid, TG, glucose, IGF-1,

and BDNF concentrations, and IR- $\beta$  and PKB/AKT levels. Trials to criterion, latency to cross the target, the number of target approaches, and swim speed were not normally distributed (*Kolmogorov-Smirnov test*). As a result, a Mann-Whitney U-test was used to analyze these measures. A repeated measures analysis of variance was performed to determine whether there were differences in body mass across time between the control and NAFLD rats. Differences among groups were considered statistically significant if  $p < 0.05$ .

## 2.4 Results

### *Chronic high fructose consumption increased liver mass and lipid content*

Body mass across the experiment [ $F(1, 38) = 0.05, p = 0.83$ ; Figure 2.1A] and average kcal consumption [ $t(38) = -1.63, p = 0.11$ ; Figure 2.1B] did not differ between groups. Liver mass [ $t(38) = -4.65, p = 0.00$ ; Figure 2.2A] and hepatic lipid concentrations [ $t(37) = -3.49, p = 0.00$ ; Figure 2.2B] were significantly higher in the NAFLD rats than in control rats. In addition, visual inspection of oil red-O staining suggests that lipid droplets were larger and more numerous in the NAFLD rats [Figures 2.2C and 2.2D].

NAFLD significantly increased circulating TGs [ $t(38) = -2.07, p = 0.05$ ; Figure 2.3A], but did not significantly affect plasma glucose concentrations [ $t(32) = -0.45, p = 0.66$ ; Figure 2.3B] or hepatic IGF-1 concentrations [ $t(10) = 0.20, p = 0.84$ ; Figure 2.3C].

### *NAFLD impaired retention performance in a spatial water maze*

NAFLD did not impair acquisition of the spatial water maze task. Decreases in the latency to reach the platform across trials were comparable across groups [all  $p > 0.05$ ; Figure 2.4]; moreover, the number of trials to criterion did not differ between groups [ $U(38) = 170.00, p = 0.42$ ; Figure 2.4 inset].

NAFLD impaired performance on the memory test. NAFLD rats displayed significantly longer latencies to reach the target on the retention test [ $U(38) = 279.00$ ,  $p = 0.03$ ; Figure 2.5A] and made significantly fewer target approaches [ $U(38) = 121.00$ ,  $p = 0.03$ ; Figure 2.5B] than did control rats. In addition, NAFLD rats tended to spend less time in the target quadrant [ $t(38) = 1.80$ ,  $p = 0.07$ ; Figure 2.5C] and tended to swim farther away from the target [ $t(38) = -1.78$ ,  $p = 0.07$ ; Figure 2.5D] than did control rats. Swim speed did not differ significantly between the two groups on the retention test [ $U(38) = 202.00$ ,  $p = 0.95$ ; Figure 2.5E].

#### *NAFLD altered neither hippocampal insulin signaling nor growth hormone levels*

NAFLD did not significantly affect IR- $\beta$  phosphorylation [ $t(9) = 1.09$ ,  $p = 0.30$ ; Figure 2.6A] or PKB/AKT phosphorylation [ $t(9) = 0.41$ ,  $p = 0.69$ ; Figure 2.6B]. In addition, the total amount of IR- $\beta$  [ $t(9) = 0.59$ ,  $p = 0.57$ ; Figure 2.6C] and PKB/AKT protein [ $t(9) = 1.55$ ,  $p = 0.69$ ; Figure 2.6D] was not significantly different between control and NAFLD rats. NAFLD also did not significantly affect hippocampal BDNF [ $t(10) = 1.16$ ,  $p = 0.27$ ; Figure 2.7A] or IGF-1 concentrations [ $t(10) = -1.19$ ,  $p = 0.26$ ; Figure 2.7B].

## **2.5 Discussion**

In this study, we replicated the finding that rats fed a high fructose diet displayed higher plasma TG concentrations, larger livers, and more hepatic lipids than animals fed a control diet. Of importance, the present results indicate that NAFLD impairs hippocampal-dependent memory. Although rats fed the high-fructose diet required the same number of trials to learn the location of the submerged platform in the spatial water maze task, they did not remember this location as well as control rats 48 h later. To be specific, on the memory test it took them longer to swim to the previous platform loca-

tion and they made fewer visits to this area. In addition, the impaired rats had a tendency to spend less time in the quadrant of the pool that previously contained the platform and to swim farther away from this area. None of these differences in retention performance could be attributed to differences in swim speed, body weight, or caloric intake. Also, the finding that the NAFLD rats were not impaired during training indicates that impairments observed on the memory test are not likely due to sensory, motor, or motivational deficits. The present results also show that the diet-induced hippocampal-dependent memory impairments do not appear to be a result of changes in plasma glucose or liver IGF-1 concentrations, decreased IR- $\beta$  or PKB/AKT phosphorylation, or a change in liver or hippocampal IGF-1 or hippocampal BDNF concentrations.

Both the control and NAFLD rats had concentrations of hepatic lipids that met the diagnostic criterion for NAFLD (i.e., > 5% wet weight). In addition, some lipid droplets were observed in the liver sections obtained from the control rats, which is not typical in healthy control rats (Ackerman, Oron-Herman et al. 2005; Wang, Wang et al. 2008). The pattern of findings suggests that the control diet produced NAFLD, although the severity was significantly less than that produced by the fructose-containing diet. Quite likely, the high percentage of cornstarch, which is the typical control used to match the carbohydrate concentration produced by the fructose (Molteni, Barnard et al. 2002; Mohamed, Nallasamy et al. 2009; Kannappan, Palanisamy et al. 2011; Palanisamy and Venkataraman Anuradha 2011; Rickman, Iyer et al. 2011; Taleb-Dida, Krouf et al. 2011), increased liver lipids. The presence of NAFLD in rats fed the control diet has some important implications. For instance, the memory-impairing effects of NAFLD may be more pronounced when standard rat chow is used as a control, and the degree of

memory impairment may depend on the stage of NAFLD. Given that NAFLD can range from simple steatosis to steatohepatitis (fatty liver with cell injury and inflammation; NASH) to hepatic fibrosis and cirrhosis (McCullough 2004; Schwimmer, Behling et al. 2005; Mager and Roberts 2006), future studies should use standardized criteria for histological analysis of liver sections for presence of fatty liver and stage of disease (Brunt, Janney et al. 1999) to determine whether NAFLD-induced memory deficits depend on or vary as a function of the particular stage of the disease.

Our results are inconsistent with previous experiments demonstrating that high-energy diets negatively impact hippocampal insulin signaling (Mielke, Taghibiglou et al. 2005) or significantly decrease BDNF concentrations (Molteni, Barnard et al. 2002; Kanoski, Meisel et al. 2007; Stranahan, Norman et al. 2008). There are, however, several important differences that could account for the variation in the results. For instance, in the present study, the diets were given to the rats early in development; whereas, in the previous studies the diets were given to animals that had already reached adulthood. In addition, the duration of feeding differed between the studies. Moreover, the study that found fructose feeding (60%) impaired insulin signaling was conducted in hamsters (Mielke, Taghibiglou et al. 2005), which metabolize fructose slightly differently from rats (Thomson, Hotke et al. 1982). Although the BDNF studies were conducted in rats, the experimental diet in that study contained fat in addition to sugar (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008), raising the possibility that the fat in the diet could be responsible for the effects on hippocampal BDNF. In addition, it is possible that the 4-hr fast may have impacted hippocampal BDNF concentrations as there are some reports indicating that fasting can affect hippocampal BDNF levels; however, these stud-

ies employed chronic or intermittent calorie restriction and found that BDNF levels were increased (Duan, Lee et al. 2001; Lee, Seroogy et al. 2002; Del Arco, Segovia et al. 2011) or unchanged (Khabour, Alzoubi et al. 2010). It also is important to mention that although it appeared as though there was a trend for NAFLD to decrease BDNF concentrations and insulin-stimulated phosphorylation of IR- $\beta$ , the differences did not approach significance ( $p = 0.272$  for BDNF;  $p = 0.304$  for phosphorylation of IR- $\beta$ ). The effect would not have likely reached statistical significance if the sample sizes has been greater, because a power analysis (Faul, Erdfelder et al. 2007) indicates sufficient power with the current sample and effect sizes ( $1-\beta = 0.84$  for BDNF;  $1-\beta = 1.0$  for phosphorylation of IR- $\beta$ ).

How NAFLD impacts hippocampal-dependent memory remains to be determined. One possibility is that NAFLD impairs memory by interfering with hippocampal neurogenesis. The dentate gyrus region of the hippocampus is one of only two brain areas in which new neurons are created throughout the lifespan (Eriksson, Perfilieva et al. 1998; Gould, Beylin et al. 1999; Lledo, Alonso et al. 2006). Both high fat (Lindqvist, Mohapel et al. 2006) and 23% fructose diets (van der Borght, Kohnke et al. 2011) decrease cell proliferation in the hippocampus. In addition, when hippocampal neurogenesis is pharmacologically prevented (Shors, Townsend et al. 2002), spatial *learning* is not affected, but *memory* is impaired in the water maze task, which suggests that the new neurons are essential for the storage and/or retrieval of new spatial memories. This pattern of effects (i.e., spared acquisition; impaired retention) parallels those seen in the current study. The finding that decreases in BDNF are often concurrent with impaired neurogenesis (Xu, Chen et al. 2006; Umka, Mustafa et al. 2010) and the fact that we did

not observe a decrease in BDNF raises the possibility that the NAFLD did not impair memory by decreasing neurogenesis in the present experiment. On the other hand, there are instances in which the effects of a manipulation on BDNF and neurogenesis are dissociated (Greisen, Altar et al. 2005; Nygren, Kokaia et al. 2006; Wolf, Kronenberg et al. 2006; Xu, Li et al. 2011). Future studies should test directly whether NAFLD impairs hippocampal neurogenesis.

In summary, the present findings indicate that fructose-induced NAFLD impairs hippocampal-dependent memory, but not learning, in young male rats. These results demonstrate that over-nutrition starting in childhood and adolescence impacts adult cognitive functioning. The findings also suggest that the memory impairment does not result from increases in plasma glucose concentrations, decreases in hepatic IGF-1 concentrations, hippocampal insulin resistance, or reduced hippocampal IGF-1 or BDNF levels. As a result, additional studies are required to identify the underlying mechanisms. Finally, it is important to note that hippocampal lesions are associated with alterations in meal patterns and food intake (Clifton, Vickers et al. 1998; Davidson, Chan et al. 2009), which raises the possibility that NAFLD-induced hippocampal dysfunction could lead to over-nutrition, thereby exacerbating NAFLD. Therefore, our findings that NAFLD impairs hippocampal-dependent memory have important implications for both cognition and for the development and maintenance of NAFLD. Interventions are needed to end this potentially vicious circle in order to maintain healthy liver and brain function.

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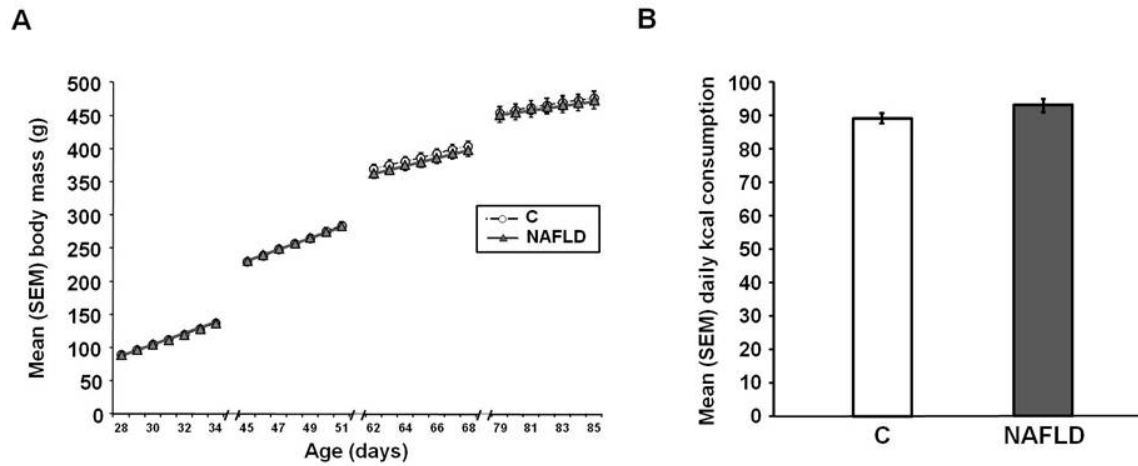
**2.8 Chapter 2 Table****Table 2.1:** Composition (kcal) of the control and experimental diets.

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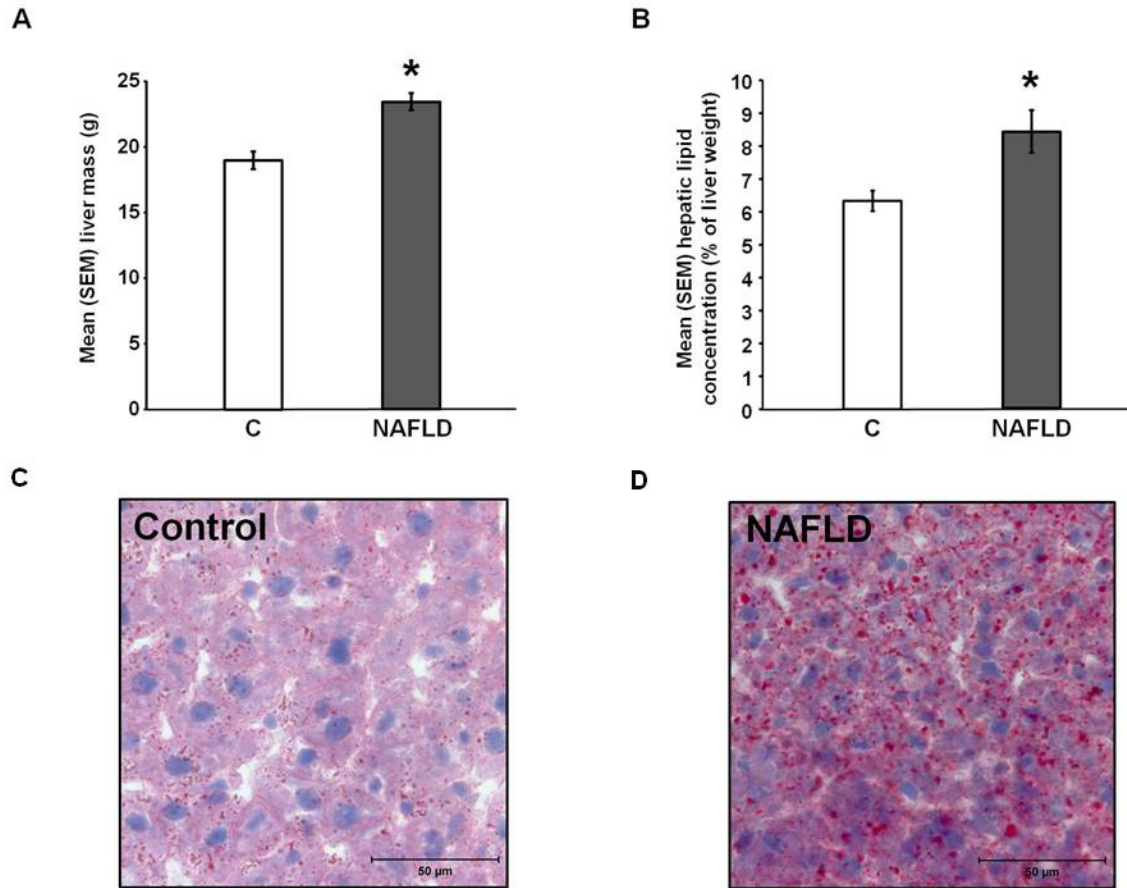
Diet component	Control diet	60% Fructose diet
Fructose	0	2220
Corn starch	2300	80
Maltodextrin 10	500	500
Soybean oil	225	225
Lard	180	180
Casein	800	800
L-Cystine	12	12
Vitamin mix	40	40
<b>Total kcal</b>	<b>4057</b>	<b>4057</b>

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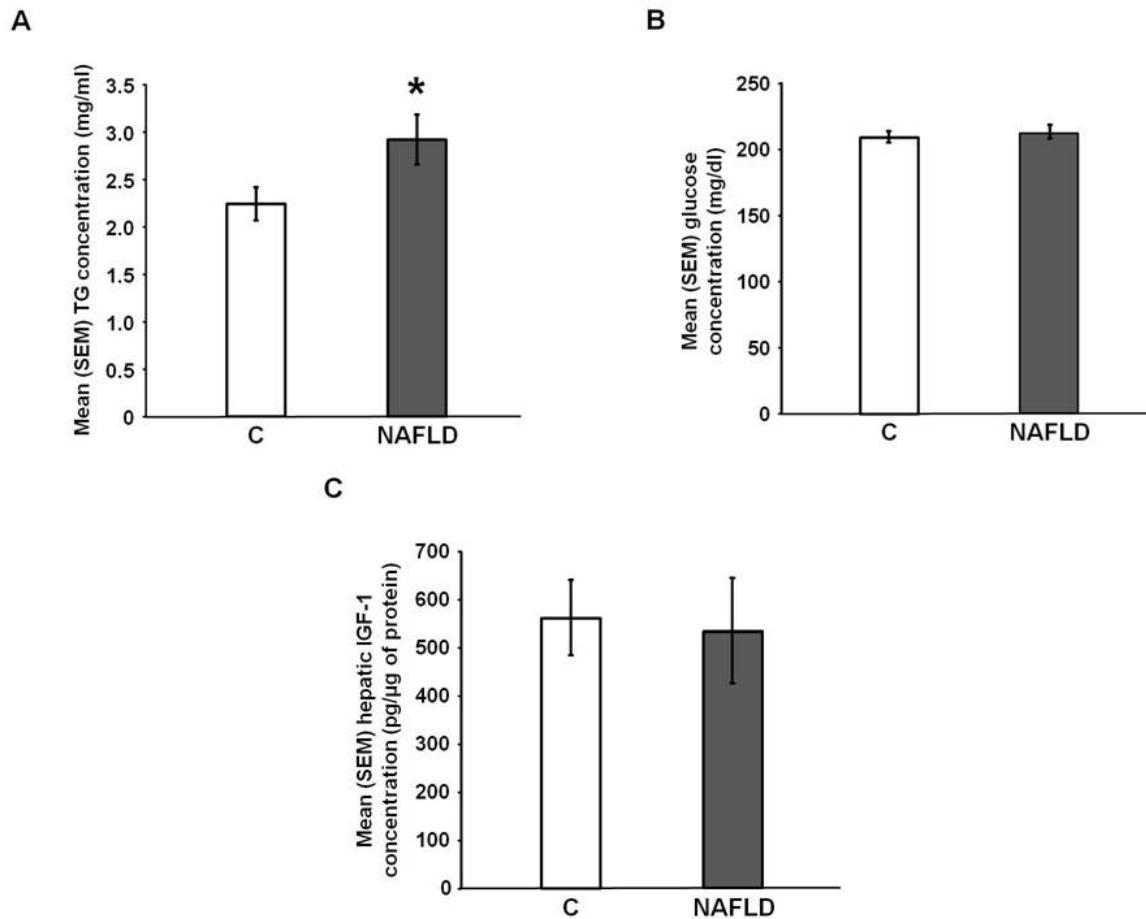
## 2.9 Chapter 2 Figures



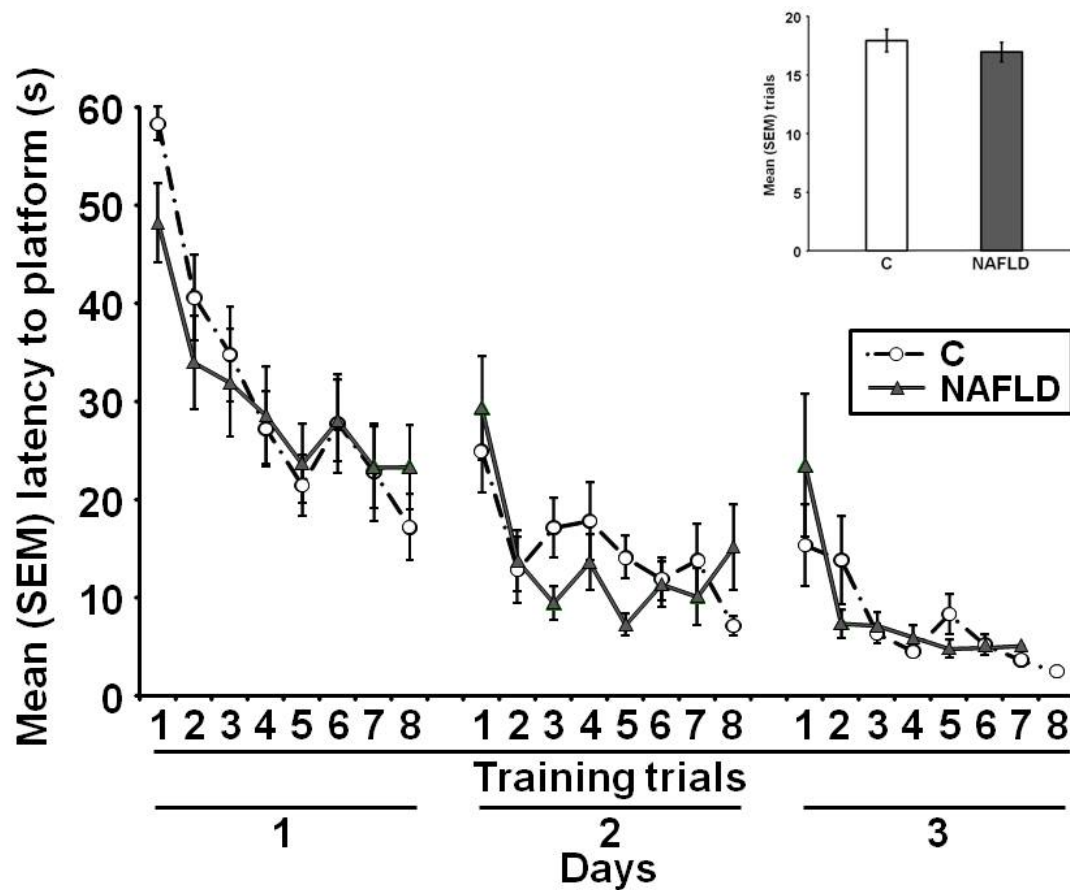
**Figure 2.1: Body mass and caloric consumption.** Mean (+/- SEM) **(A)** body mass across the experiment and **(B)** average calorie consumption for control rats (n = 21) and rats with fructose-induced non-alcoholic fatty liver disease (NAFLD; n = 19).



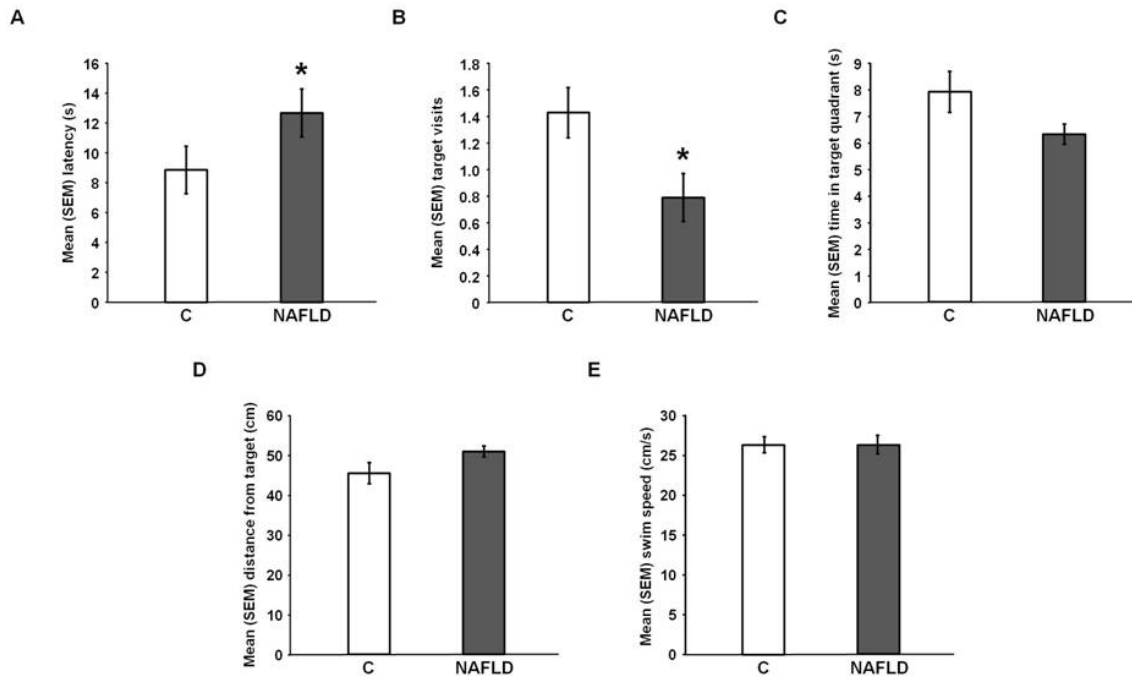
**Figure 2.2: Confirmation of non-alcoholic fatty liver disease.** Mean ( $\pm$  SEM) **(A)** liver mass (C:  $n = 21$ , NAFLD:  $n = 19$ ) and **(B)** hepatic lipid concentration (C:  $n = 21$ , NAFLD:  $n = 18$ ; \* $p < 0.05$  vs. control rats). Representative liver sections stained with hematoxylin and oil red-O from **(C)** a control rat and **(D)** a rat with fructose-induced NAFLD.



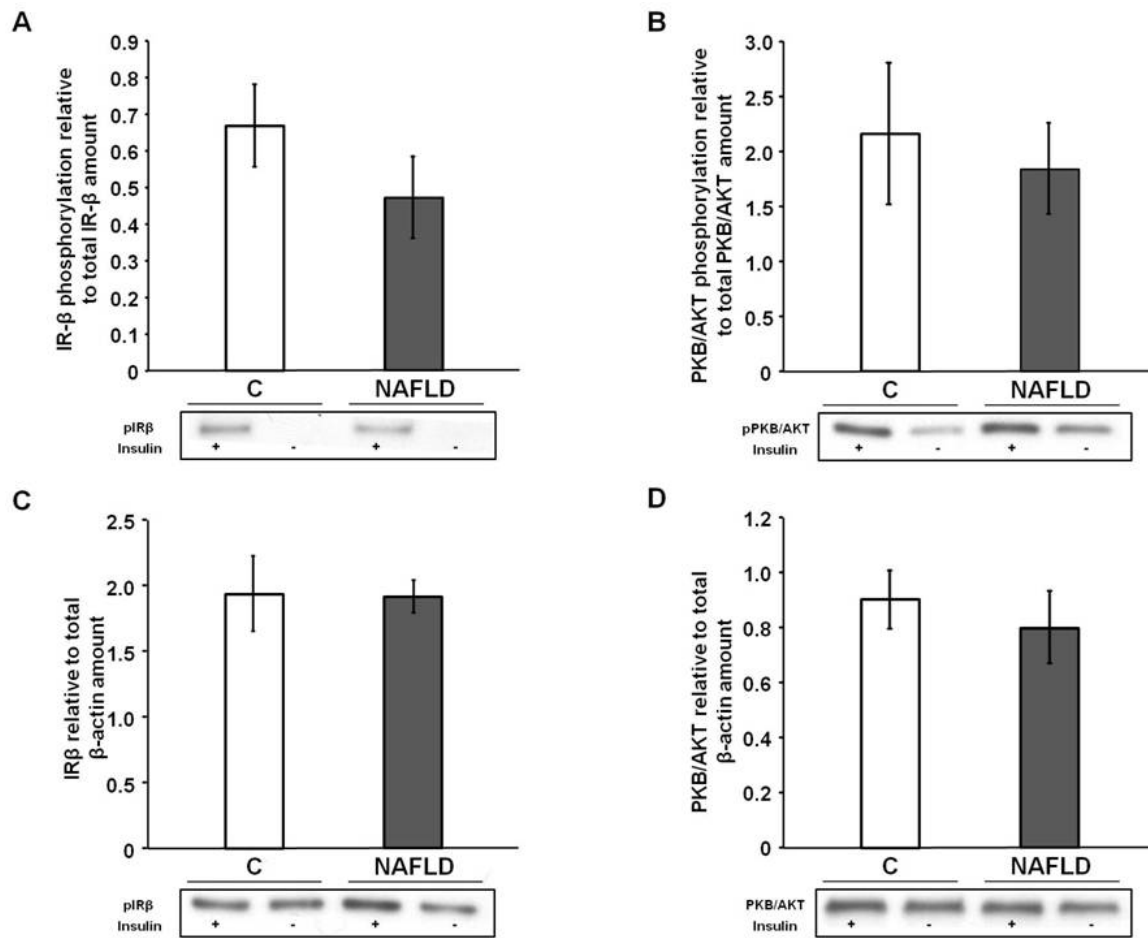
**Figure 2.3: Plasma triglyceride and hormone concentrations.** Mean ( $\pm$  SEM) (A) plasma TG concentrations (C:  $n = 21$ , NAFLD:  $n = 19$ ), (B) plasma glucose concentrations (C:  $n = 18$ , NAFLD:  $n = 16$ ), and (C) hepatic insulin like growth factor-1 (IGF-1) concentrations (C:  $n = 6$ , NAFLD:  $n = 6$ ; \* $p < 0.05$  vs. control rats).



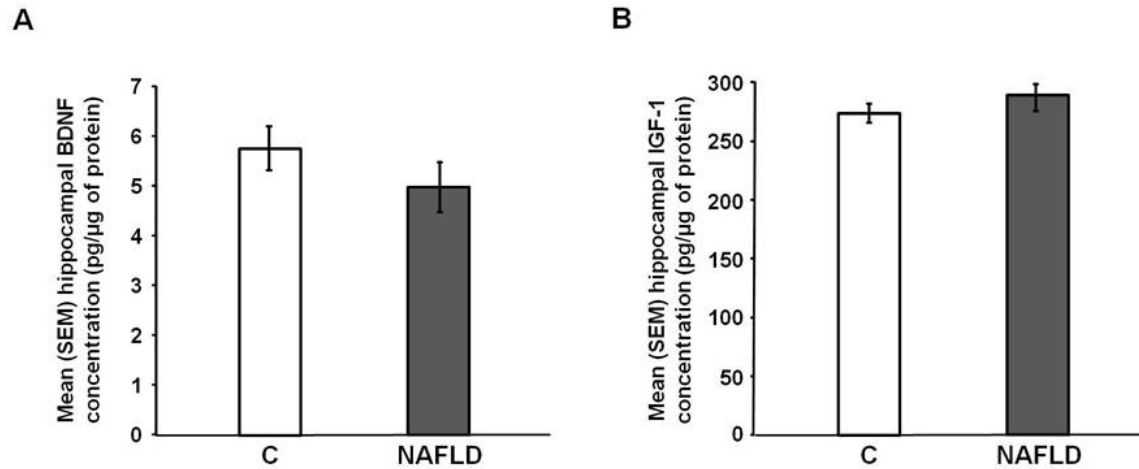
**Figure 2.4: Spatial water maze training.** The effects of NAFLD on the mean ( $\pm$  SEM) latency to reach the platform during spatial water maze training and (**inset**) the number of trials to reach the learning criterion (Day 1 and Day 2; C:  $n = 21$ , NAFLD:  $n = 19$ . Day 3; C:  $n = 13$ , NAFLD:  $n = 9$ ).



**Figure 2.5: Spatial water maze testing.** The effects of NAFLD on the mean (+/- SEM) (A) latency to reach the target, (B) number of target approaches, (C) amount of time spent in the target quadrant, (D) proximity to the target during the water maze retention test and (E) swim speed (C: n = 21, NAFLD: n = 19; \*p < 0.05 vs. control rats).



**Figure 2.6: Insulin-stimulated phosphorylation of IR- $\beta$  and PKB/AKT.** The effects of NAFLD on **(A)** phosphorylation of hippocampal insulin receptor  $\beta$  subunit (IR- $\beta$ ; C: n = 7, NAFLD: n = 7), **(B)** phosphorylation of hippocampal protein kinase B (PKB/AKT; C: n = 5, NAFLD: n = 6), **(C)** total IR- $\beta$  (C: n = 7, NAFLD: n = 7), and **(D)** total PKB/AKT (C: n = 5, NAFLD: n = 6). Plus signs located below representative blots indicate insulin-stimulated versus basal (minus signs).



**Figure 2.7: Hippocampal growth factor measurements.** The effects of NAFLD on **(A)** hippocampal brain derived neurotrophic factor (BDNF) and **(B)** hippocampal IGF-1 (C: n = 6, NAFLD: n = 6).

## CHAPTER 3: A HIGH ENERGY DIET, BUT NOT STEATOSIS, POTENTIATES THE ABILITY OF EPINEPHRINE TO INCREASE BLOOD GLUCOSE CONCENTRATIONS

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### 3.1 Abstract

In humans, non-alcoholic fatty liver disease (NAFLD) is characterized by an accumulation of lipids in the liver (> 5% wet weight; a.k.a. steatosis) in the absence of excessive alcohol intake. In rodents, hepatic steatosis has severe consequences, including oxidative stress and hepatic insulin resistance. The liver plays an important role in emotional arousal; epinephrine from the adrenal medulla stimulates the liver to release glucose, which is then used as energy in conditions of immediate need. The purpose of this experiment was to test whether hepatic steatosis compromises epinephrine-induced hepatic glucose release. Adult male Sprague-Dawley rats were fed a high energy cafeteria-style diet (HED) to induce hepatic steatosis. Weight gain during 5 days on the diet was used to divide the rats into a HED-Lean group (bottom 33%) and HED-Obese group (top 33%). After 9 weeks, the rats were injected with epinephrine, and blood glucose concentrations were measured. To assess if the effects of the HED could be reversed, the high energy components of the diet were removed for 4 weeks, and then the injections and blood glucose measurements were repeated. The results indicate that the HED induced hepatic steatosis and, surprisingly, *potentiated* epinephrine-induced increase in blood glucose. In addition, removing the high energy dietary components re-

versed the effects of the HED on epinephrine-induced glucose release, but did not reverse the hepatic steatosis. These results suggest that steatosis does not contribute to the potentiated ability of epinephrine to increase blood glucose levels. Future experiments are needed to determine whether these diet-induced increases in blood glucose contribute to diet-induced memory impairments.

### **3.2 Introduction**

In humans, non-alcoholic fatty liver disease (NAFLD) is characterized by an accumulation of lipids in the liver (> 5% wet weight; a.k.a. steatosis) in the absence of excessive alcohol intake (Neuschwander-Tetri and Caldwell 2003). NAFLD is the most common form of liver disease in the United States, and it is estimated that it affects at least 30% of the American population (Lazo and Clark 2008). Non-alcoholic fatty liver disease encompasses a wide range of disease states, including simple steatosis, steatohepatitis and fibrosis (McCullough 2004). Hepatic lipid accumulation has severe consequences. These include oxidative stress (Raso, Esposito et al. 2009; Ruiz-Ramirez, Chavez-Salgado et al. 2011), which can cause cellular inflammation and damage to DNA (Milne 2008), and hepatic insulin resistance (IR; Samuel, Liu et al. 2004), which leads to vascular dysfunction (Pasarín, Abalde et al. 2011) and impaired lipid metabolism (Ai, Zhu et al. 2011; Ha, Kim et al. 2011).

It is possible that hepatic steatosis could have other negative effects on liver function. For example, it is well established that sympathetic nervous system activation mobilizes energy reserves during conditions of immediate need such as 'flight or fight' and stress responses, in part, through the hepatic release of glucose. Epinephrine release, from the adrenal medulla or exogenous administration of epinephrine, increases

hepatic glucose release into the blood (Sacca, Vigorito et al. 1983). Specifically, epinephrine activates glycogenolysis (Berg, Tymoczko et al. 2002) and gluconeogenesis (Sacca, Vigorito et al. 1983), which increase circulating glucose concentrations. To the best of our knowledge, whether hepatic lipid accumulation disrupts the ability of epinephrine to increase blood glucose concentrations has not been determined. Given that hepatic steatosis disrupts liver function (Samuel, Liu et al. 2004; Ai, Zhu et al. 2011; Ha, Kim et al. 2011; Pasarin, Abalde et al. 2011), and an important function of the liver is to release glucose in response to stress (Sacca, Vigorito et al. 1983; Berg, Tymoczko et al. 2002), we hypothesized that diet-induced hepatic lipid accumulation decreases the ability of epinephrine to release hepatic glucose.

Over-consumption of high energy foods, particularly fats and sugars, is considered the major cause of hepatic lipid accumulation (Ackerman, Oron-Herman et al. 2005; Ahmed, Redgrave et al. 2009; Larter, Chitturi et al. 2010); thus, high energy diets (HEDs) are used commonly to create animal models of steatosis (Ackerman, Oron-Herman et al. 2005; Svegliati-Baroni, Candelaresi et al. 2006; Ahmed, Redgrave et al. 2009). Typically, the diets employed to induce steatosis contain fat and/or sugars in a single pellet and nutritional alternatives are not provided (Ackerman, Oron-Herman et al. 2005; Ahmed, Redgrave et al. 2009; Ross, Bruggeman et al. 2012). To test the effects of steatosis on epinephrine-induced hepatic glucose release, we elected to induce steatosis using a cafeteria-style diet in which the rats were offered standard chow and tap water in addition to sources of fat and sugar. This diet is relevant to the “Western” diet consumed by most Americans (Hu, Rimm et al. 1999; Iqbal, Anand et al. 2008). The animal determines how much of the high energy foods to consume, and healthy alterna-

tives (standard chow and water) are available. Initial weight gain on a cafeteria-style diet *predicts* the later development of lean and obese phenotypes (Dourmashkin, Chang et al. 2006) and diet-induced hepatic steatosis (Darling et al, unpublished). Thus, in the present experiment, rats were fed a high energy cafeteria-style diet for 9 weeks, injected with epinephrine, and blood glucose concentrations were measured.

To our knowledge, no studies have examined whether the effects of hepatic lipid accumulation can be reversed by removing the high energy dietary components. Rather, research has been dedicated to studying how diet-induced steatosis and its consequences can be overturned using interventions such as growth hormones (Qin and Tian 2010) or plant extracts (Shetty, Mengi et al. 2010; Panchal, Poudyal et al. 2011). Therefore, we also tested whether the effects of steatosis on epinephrine-stimulated glucose release are reversible by removing the experimental fat and sugar sources. After 4 weeks on this standard chow diet, the epinephrine injections and blood glucose measurements were repeated.

### **3.3 Methods**

#### *Animals and housing*

Male Sprague-Dawley rats (Charles River, Wilmington, MA), aged 53 days, were housed individually in an OptiRat® cage system (Animal Care Systems, Centennial, CO) and maintained on a 12 h light cycle (lights on at 7:00 am). They were weighed upon arrival to the lab and allowed to acclimate for 1 week. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and are in accordance with PHS guidelines.

### *Diets*

All rats were fed standard chow (3.01 kcal/g; LabDiet 5001, Purina Mills, Gray Summit, MO) during the acclimation week. On the seventh day, they were weighed again, matched on percent change in body mass, and assigned to either the control diet (C; n = 28) or the high energy diet (HED; n = 80) group. The HED consisted of a bottle of tap water, a bottle of 32% sucrose solution (3.75 kcal/g), standard rat chow, and a glass petri dish containing animal lard (9.0 kcal/g; Armour, Omaha, NE; Pecoraro, Reyes et al. 2004). The control diet consisted of two bottles of tap water and standard rat chow, and an empty glass petri dish was placed in the cage. Both the control and HEDs were fed ad libitum.

To examine obese and lean phenotypes in response to the HED, a tertile split was used to divide HED rats based on the percent change in body mass during the first 5 days on the diet (Dourmashkin, Chang et al. 2006). Rats in the bottom 33% (i.e., those with the smallest percent change in body mass) were defined as HED-Lean (n = 15). The rats in the top 33% (i.e., those with the largest percent change in body mass) were labeled HED-Obese (n = 25). Forty rats from the middle 33% were removed from the study. The remaining rats were maintained on their respective diets for 9 weeks before the epinephrine injections. Food intake measurements were not collected because there is a possibility that this process can be stressful, and the rats in this experiment were used in a second experiment involving stress manipulations.

### *Epinephrine injections/Blood glucose sampling*

For 2 days before the injection, the rats were handled by placing them into a folded towel leaving only the tail exposed for 2 min each day. On the injection day, each

rat was given a subcutaneous injection of epinephrine (0.05 or 0.1 mg/kg) or saline (0.9% w/v NaCl). Blood glucose was measured from a nick in the tail vein using an Accu-Chek glucose meter (Roche, Indianapolis, IN) immediately before the injection and 5, 10, 30, 60, 120 and 180 min after the injection. The experimenter giving the injections and measuring blood glucose concentrations was blind to the diet condition of each rat.

#### *Diet reversal*

Three days after the injection and blood glucose sampling, the 32% sucrose solution and lard were removed from the HED rat cages. A subset of rats was euthanized at this time (see below) to verify the presence of steatosis. The remaining rats were maintained on standard chow and tap water for an additional 4 weeks, and then the epinephrine/saline injections and blood glucose sampling were repeated.

#### *Tissue collection and lipid assays*

For tissue collection, all rats were fasted for 4 h, anesthetized with isoflurane (5% in 95%O<sub>2</sub>), and then killed by decapitation. The liver was extracted, weighed, flash frozen, and placed at -80°C until the lipid assay was performed. To verify the presence of steatosis, hepatic lipids were extracted using the Folch method (Folch, Lees et al. 1957). In addition, liver samples were sectioned and stained with oil red-O for visual assessment of lipid droplets.

#### *Data Analysis*

The data were stored and analyzed using Microsoft Excel, Version 5.0 and Statistical Package for the Social Sciences (SPSS), Version 18.0. An analysis of variance (ANOVA) and Tukey post hoc tests were performed to determine if there were differences in percent change in body mass, baseline blood glucose concentrations, and he-

patic lipids between the control, HED-Lean and HED-Obese rats. A mixed ANOVA and Tukey post hoc tests were performed to determine if there were differences in blood glucose concentrations across time following the injections in the control, HED-Lean and HED-Obese rats. To increase statistical power, changes in blood glucose concentrations were analyzed separately for saline, 0.05 mg/kg epinephrine, and 0.1 mg/kg epinephrine. Differences were considered statistically significant if  $p \leq 0.05$ .

### 3.4 Results

#### *Voluntary consumption of a high energy diet increased body mass.*

The tertile division revealed a significant effect of diet on percent change in body mass during the first 5 days on the diet [ $F(2, 61) = 60.59, p = 0.00$ ; see Figure 3.1A]. Post hoc tests indicate that HED-Obese rats gained more weight and HED-Lean rats gained less weight than control rats (both  $p = 0.00$ ). At the time of the first series of epinephrine injections 9 weeks after the start of the diet, there was still a significant effect of diet on percent change in body mass [ $F(2, 65) = 32.50, p = 0.00$ ; see Figure 3.1B]. Post hoc tests show that HED-Obese rats gained more weight than control rats ( $p = 0.00$ ), but percent change in body mass for HED-Lean rats did not significantly differ from that of control rats at this time ( $p = 0.65$ ).

#### *Voluntary consumption of a high energy diet caused hepatic steatosis.*

There was a significant main effect of diet on hepatic lipid concentrations [ $F(2, 19) = 4.44, p = 0.03$ ; see Figure 3.2A] in rats euthanized 9 weeks after the start of the diet. To be specific, hepatic lipids were significantly increased in the HED-Obese rats compared with control rats ( $p = 0.03$ ). Hepatic lipids did not differ between the HED-Lean and control ( $p = 0.81$ ) or HED-Lean and HED-Obese rats ( $p = 0.13$ ). In addition,

visual inspection of the liver tissue stained with oil red-o suggests that the lipid droplets were larger and more numerous in HED-Obese rats than in HED-Lean and control rats (see Figures 3.2B-D).

*A high energy diet potentiated the ability of epinephrine to release hepatic glucose.*

Diet increased baseline blood glucose concentrations [ $F(2, 65) = 5.44, p = 0.01$ ; see Figure 3.3]. Post hoc tests indicated that the HED-Obese rats had higher baseline blood glucose concentrations than HED-Lean and control rats (both  $p = 0.02$ ). For the saline injections, there was a significant main effect of time [ $F(6, 114) = 7.67, p = 0.00$ ] and a main effect of diet [ $F(2, 114) = 19.58, p = 0.00$ ; see Figure 3.4A], indicating that the injection and/or tail blood collection procedures increased blood glucose concentrations in HED-Obese rats, but not in HED-Lean or control rats. For the 0.05 mg/kg epinephrine injections, there was a significant interaction such that the 0.05 mg/kg epinephrine dose increased blood glucose concentrations, but the effect depended on diet group and time after injection [ $F(2, 19) = 6.39, p = 0.01$ ; see Figure 3.4B]. Specifically, compared with control rats, the 0.05 mg/kg epinephrine dose increased glucose concentrations in the HED-Obese rats at 120 min post-injection ( $p = 0.01$ ) and at 180 min post injection ( $p = 0.01$ ), but did not affect glucose concentrations in HED-Lean rats at any time (all  $p > 0.05$ ). There also was a significant interaction for the 0.1 mg/kg epinephrine injections such that the 0.1 mg/kg epinephrine dose increased blood glucose concentrations, but the effect depended on diet group and time after injection [ $F(2, 21) = 11.96, p = 0.00$ ; see Figure 3.4C]. Post hoc tests indicated that compared with control rats, the 0.1 mg/kg epinephrine dose increased glucose concentrations in HED-Obese rats at 30 min, 60 min, 120 min, and 180 min post-injection ( $p < 0.01$  for all). The 0.01

mg/kg epinephrine dose increased glucose concentrations in HED-Lean rats only at 60 min ( $p = 0.05$ ) and 120 min post-injection ( $p = 0.02$ ).

*Four weeks of standard chow feeding did not reverse HED-induced hepatic steatosis, but did reverse HED-induced potentiation of epinephrine-induced increases in blood glucose concentrations.*

There was still a significant effect of diet on percent change in body mass after the high energy components of the diet were removed [ $F(2, 65) = 34.03, p = 0.00$ ; see Figure 3.5A]. Post hoc tests show that HED-Obese rats gained *less* weight than control rats ( $p = 0.00$ ) during the diet intervention, but percent change in body mass for HED-Lean rats did not significantly differ from that of control rats during this time ( $p = 1.00$ ).

In addition, the diet intervention did not affect steatosis; there was no significant difference between the percent of hepatic lipids within each diet group before and after 4 weeks of standard chow feeding [control:  $t(33) = 1.34, p = 0.07$ ; HED-Lean:  $t(19) = -0.56, p = 0.58$ ; HED-Obese:  $t(31) = 0.37, p = 0.71$ ]. Therefore, it is not surprising that there was still a significant effect of diet on hepatic lipid accumulation [ $F(2, 64) = 9.71, p = 0.00$ ; see Figure 3.5B]. Post hoc tests show that the hepatic lipids were still higher in HED-Obese rats than in control rats ( $p = 0.00$ ), and there was still no difference between HED-Lean and control rats ( $p = 0.45$ ). Visual inspection of the tissue stained with oil red-o suggests that the lipid droplets were still larger and more numerous in HED-Obese rats than in HED-Lean and control rats (see Figures 3.5C-E).

After the diet intervention, blood glucose measures were comparable across all diet groups. Of interest, 4 weeks of standard chow feeding did not affect baseline blood glucose concentrations in the control rats ( $t(54) = 1.45, p = 0.15$ ) or HED-Lean rats

[ $t(28) = -0.31, p = 0.76$ ]; however, baseline blood glucose concentrations decreased in the HED-Obese rats [ $t(48) = 2.84, p = 0.01$ ]. There was no longer a significant effect of diet on baseline blood glucose concentrations [ $F(2, 65) = 0.31, p = 0.74$ ; see Figure 3.6], nor on the saline- and epinephrine-induced changes in blood glucose concentrations [saline:  $F(2, 17) = 0.05, p = 0.95$ : see Figure 3.7A; 0.05 mg/kg epinephrine dose:  $F(2, 18) = 0.05, p = 0.96$ : see Figure 3.7B; 0.1 mg/kg epinephrine dose:  $F(2, 20) = 1.67, p = 0.21$ : see Figure 3.7C].

### 3.5 Discussion

To the best of our knowledge, the current study is the first to test the effects of diet-induced hepatic steatosis on the ability of epinephrine to increase blood glucose concentrations. The surprising results indicate that the HED *potentiated* the ability of epinephrine to increase circulating glucose. To be specific, the rats that gained the most weight during the first 5 days of consuming the HED (HED-Obese) displayed the greatest increase in epinephrine-induced glucose release 9 weeks later. Only the larger dose of epinephrine potentiated glucose release in HED-Lean rats, suggesting that the HED shifted the dose response properties of epinephrine. In addition, the HED increased baseline blood glucose concentrations and hepatic lipid content in HED-Obese rats but not in HED-Lean rats. Removing the lard and sugar from the HED for 4 weeks restored the ability of epinephrine to increase circulating glucose in both the HED-Lean and Obese rats to control levels. Baseline blood glucose concentrations in the HED-Obese rats also returned to control levels. Of interest, the diet intervention did not reverse the steatosis in these rats.

The current results suggest that hepatic steatosis is not the mechanism underlying the potentiated ability of epinephrine to increase blood glucose concentrations. First, the larger epinephrine dose produced a significant increase in blood glucose concentrations in the HED-Lean rats even though the HED did not increase hepatic lipids. Second, after removing the high energy components of the diet, the ability of epinephrine to increase circulating glucose was restored in the HED-Obese rats, but the amount of hepatic lipids remained elevated at the same magnitude as before the diet intervention.

Insulin resistance (IR) may have caused the potentiated ability of epinephrine to increase blood glucose. High energy diets cause IR (Sampey, Vanhooose et al. 2011). Given that baseline blood glucose concentrations were increased in the HED-Obese rats after 9 weeks on the diet, and that elevated glucose concentrations are a hallmark of IR, it is possible that IR may have been present in these rats. In addition, after removing the lard and sugar from the diet for 4 weeks, the blood glucose concentrations in HED-Obese rats were no longer increased, suggesting that the IR may have been corrected. Additional experiments are required to confirm the presence of IR and to investigate the relationship between IR and the ability of epinephrine to release hepatic glucose.

The current study does not identify the HED-induced changes within the liver that may be responsible for the potentiated ability of epinephrine to increase circulating blood glucose. One possibility is that the HED increased the number of hepatic adrenergic receptors. Of interest, these receptors are increased threefold in the livers of *ob/ob* mice, a genetic model of obesity and Type 2 diabetes (Begin-Heick 1994). In addition, there is a glucocorticoid-induced increase in activity of adenylyl cyclase (Begin-Heick

1994), which could translate into an increase in hepatic glucose release (Berg, Tymoczko et al. 2002). The HED also may have increased hepatic glucose stores. In the liver, glucose is stored as glycogen, and HEDs augment hepatic glycogen concentrations (Hoefel, Hansen et al. 2011), indicating that there would be more stored glucose available for epinephrine to release into the bloodstream in the HED-Obese rats.

The potentiated ability of epinephrine to increase blood glucose concentrations may be related to HED-induced memory impairments. It is well established that HEDs impair hippocampal-dependent memory (Greenwood and Winocur 1990; Molteni, Barnard et al. 2002; Pathan, Gaikwad et al. 2008; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009). In addition, glucose and epinephrine influence memory (Gold and Van Buskirk 1975; McCarty and Gold 1981; Gold 1986) in a dose-dependent manner such that excessive amounts can impair memory (Gold, van Buskirk et al. 1977; Gold 1986). Given that epinephrine cannot cross the blood brain barrier in any appreciable amount (Weil-Malherbe, Axelrod et al. 1959), it is thought that epinephrine affects memory, in part, via the hepatic release of glucose (Sacca, Vigorito et al. 1983; Hall and Gold 1986; Talley, Kahn et al. 2000). Additional studies are required to determine whether the potentiated ability of epinephrine to increase blood glucose increases circulating glucose concentrations into the range that impairs memory.

In summary, a HED potentiates the ability of epinephrine to increase blood glucose concentrations, and this effect can be reversed by removing the high energy components of the diet. In addition, the current results suggest that hepatic steatosis is not the mechanism underlying the ability of HEDs to potentiate epinephrine-induced glucose release.

### 3.6 Acknowledgements

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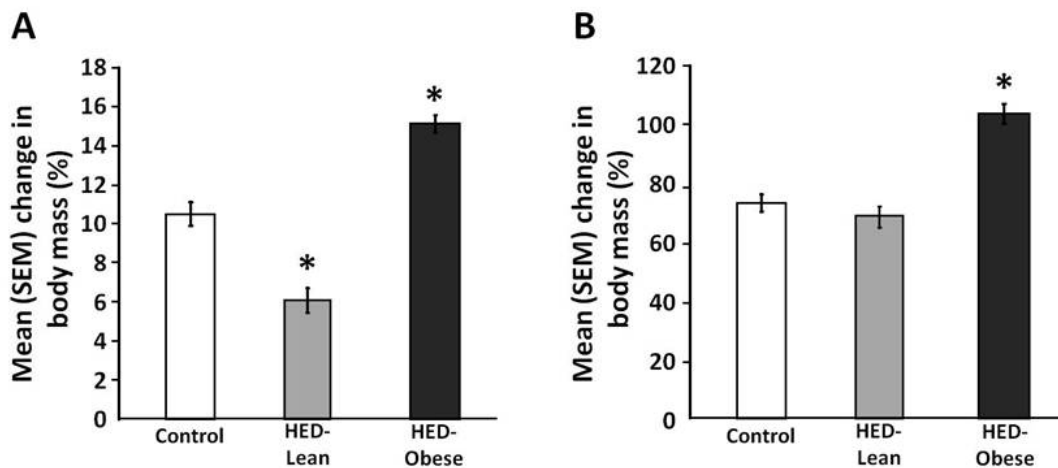
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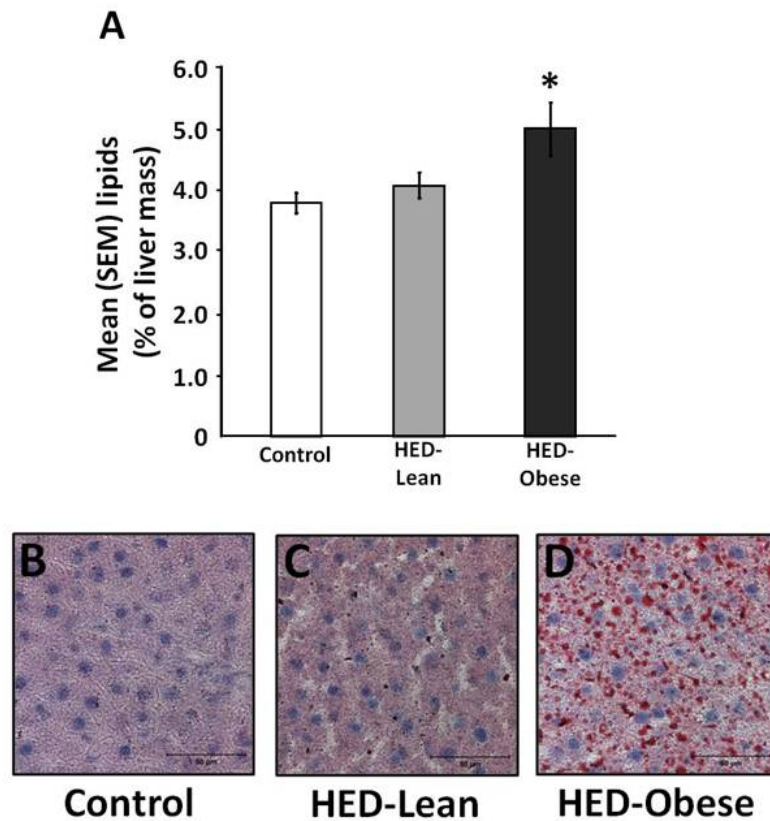
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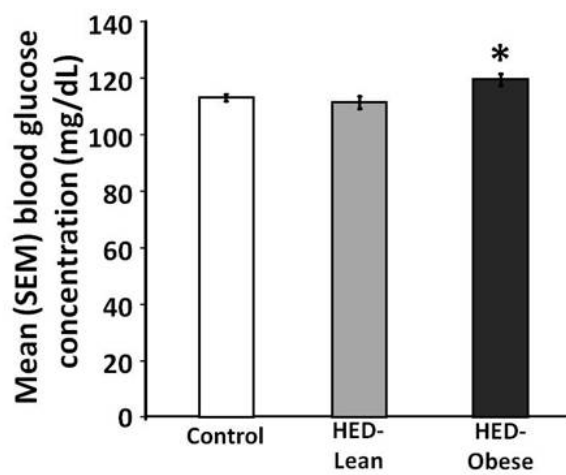
## 3.8 Chapter 3 Figures



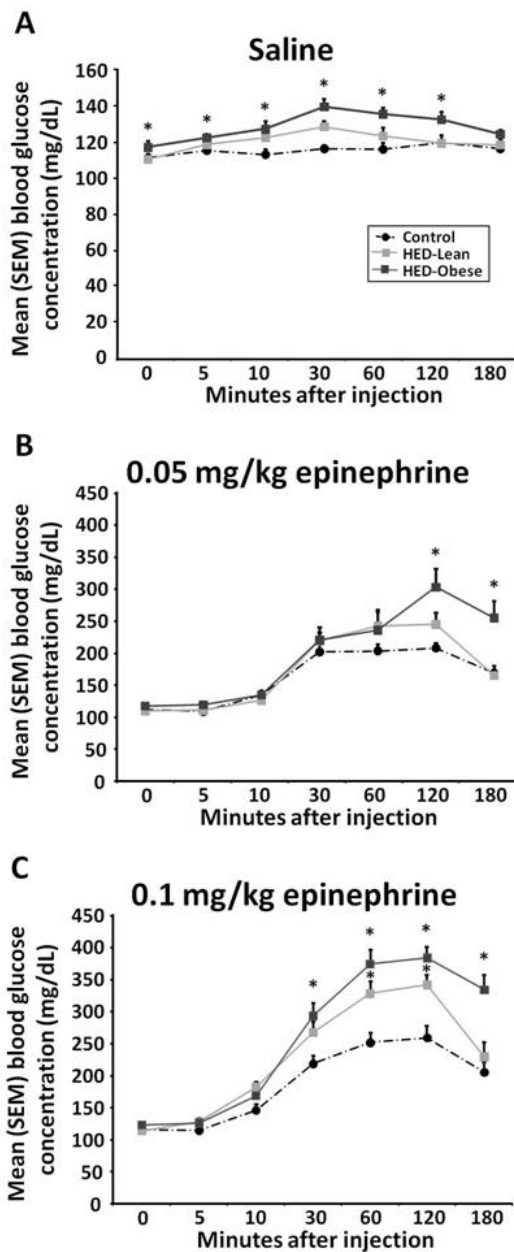
**Figure 3.1: Percent change in body mass.** Mean ( $\pm$  SEM) percent change in body mass after (A) 5 days and (B) 9 weeks for control rats, HED-Lean (rats that gained the least amount of weight during the first 5 days on the HED) and HED-Obese (rats that gained the most amount of weight during the first 5 days on the HED;  $*p < 0.05$  vs. control).



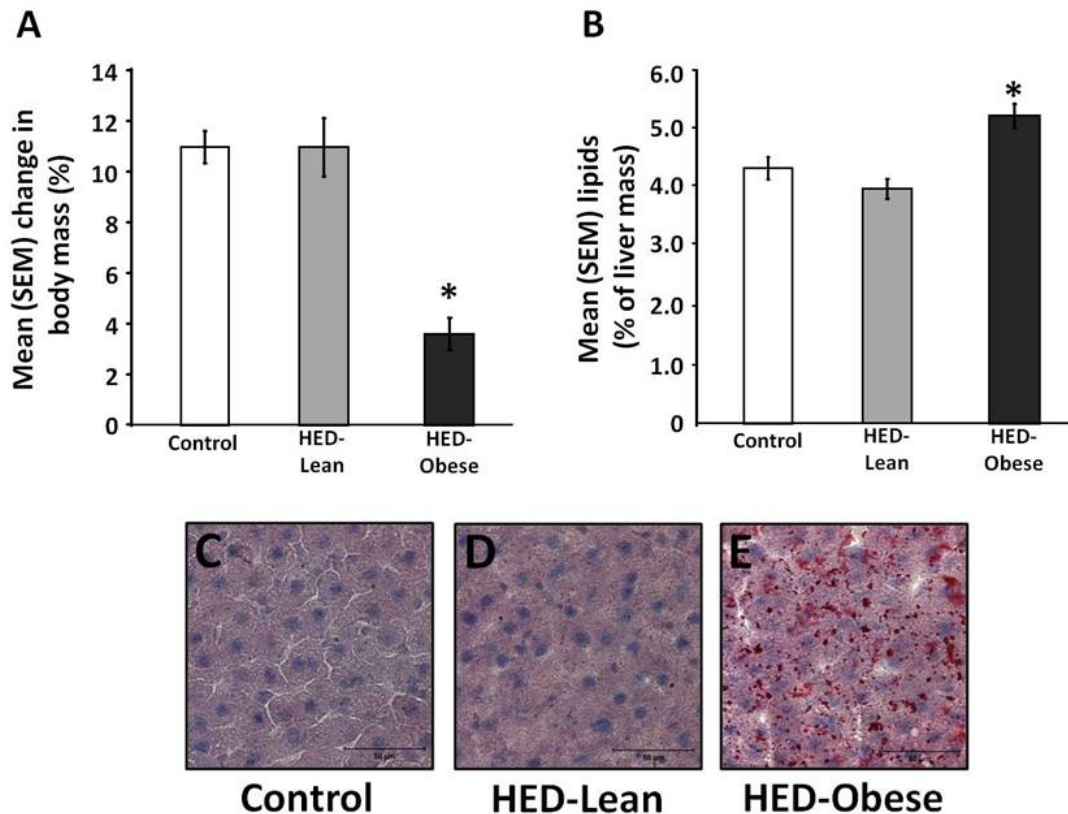
**Figure 3.2: Confirmation of non-alcoholic fatty liver disease.** Mean ( $\pm$  SEM) **(A)** hepatic lipid concentrations and representative liver sections stained with hematoxylin and oil red-O from **(B)** a control rat, **(C)** an HED-Lean rat, and **(D)** an HED-Obese rat after 9 weeks on the control diet or HED ( $*p < 0.05$  vs. control).



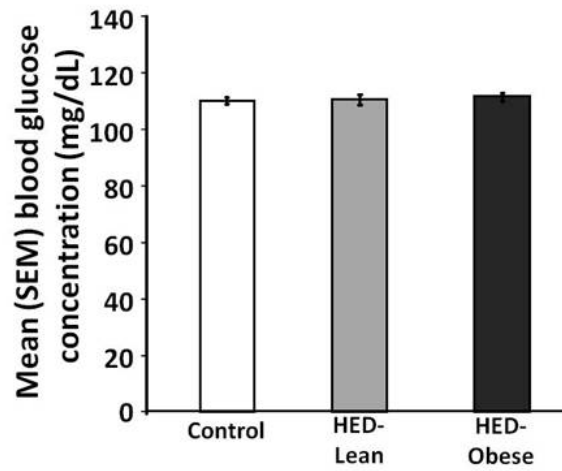
**Figure 3.3: Baseline blood glucose concentrations.** The effects of 9 weeks of HED feeding on mean ( $\pm$  SEM) baseline blood glucose concentrations ( $*p < 0.05$  vs. control).



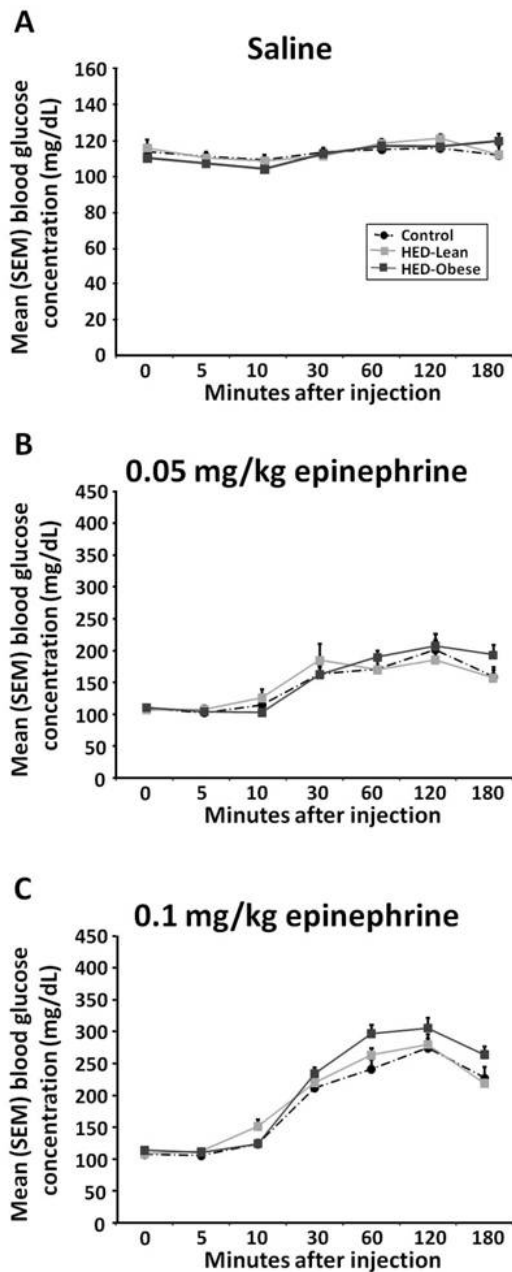
**Figure 3.4: Epinephrine-stimulated blood glucose concentrations.** The effects of (A) saline injection, (B) 0.05 mg/kg epinephrine injection, and (C) 0.1 mg/kg epinephrine injection on mean ( $\pm$  SEM) blood glucose concentrations after 9 weeks on the control diet or HED ( $*p < 0.05$  vs. control).



**Figure 3.5: Percent change in body mass and lipid accumulation reversal.** Mean (+/- SEM) **(A)** percent change in body mass during the diet intervention **(B)** hepatic lipid concentrations and representative liver sections stained with hematoxylin and oil red-O from **(C)** a control rat, **(D)** an HED-Lean rat, and **(E)** an HED-Obese rat after 9 weeks on the control diet or HED followed by 4 weeks of standard chow feeding (\* $p < 0.05$  vs. control).



**Figure 3.6: Baseline blood glucose concentrations.** The effects of 9 weeks of HED feeding followed by 4 weeks of standard chow feeding on mean ( $\pm$  SEM) baseline blood glucose concentrations ( $*p < 0.05$  vs. control).



**Figure 3.7: Epinephrine-stimulated blood glucose concentrations.** The effects of (A) saline injection, (B) 0.05 mg/kg epinephrine injection, and (C) 0.1 mg/kg epinephrine injection on mean ( $\pm$  SEM) blood glucose concentrations after 9 weeks on the control diet or HED followed by 4 weeks of standard chow feeding ( $*p < 0.05$  vs. control).

**CHAPTER 4: THE INTERACTION BETWEEN THE EFFECTS OF A HIGH ENERGY  
DIET AND ACUTE STRESS ON HIPPOCAMPAL-DEPENDENT MEMORY  
IN MALE RATS**

A.P. Ross, J.N. Darling, and M.B. Parent

**4.1 Abstract**

Over the past five decades, *per capita* caloric intake has increased by approximately 28% in the United States. High energy diets (HEDs), or those diets that contain excessive calories, negatively impact the hippocampus and hippocampal-dependent memory by decreasing neuronal growth factors, neurogenesis, and synaptic communication. In addition, HEDs increase secretion of both epinephrine and corticosterone. Given that previous studies from our lab indicate rats fed HEDs display impaired performance during stressful tasks, but not during less stressful tasks, high energy diets may also impair hippocampal-dependent memory through excessive increases in epinephrine and corticosterone. The following experiments tested the hypothesis that HEDs impair hippocampal-dependent memory, in part, through diet-induced increases in the acute stress response. Rats were fed a high energy cafeteria-style diet for 4 weeks and then trained and tested in a low stress or high stress version of a spatial object recognition (SOR) task. Experiment 2 tested whether injecting rats with epinephrine (0.1 mg/kg) in a moderate stress version of the SOR task would impair memory in high energy-fed rats. Experiments 3 and 4 tested whether blocking the effects of epinephrine with the adrenergic antagonist sotalol (4 mg/kg) or preventing corticosterone synthesis

with metyrapone (50 mg/kg) would enhance memory in high energy-fed rats in a high stress version of the same task. Minimal research has focused on reversing the cognitive effects of HEDs; therefore, an additional experiment tested the hypothesis that the effects of HEDs on memory are reversible through diet intervention. After 4 weeks on the high energy cafeteria-style diet, the high energy components of the diet were removed, and the rats were fed standard chow and tap water for an additional 4 weeks. They were then trained and tested in the SOR task. Results from the first experiment were surprising because the HED did not impair memory. Instead, it prevented the memory-enhancing effects of stress. In the follow-up experiments, epinephrine did not enhance memory, and blocking the effects of epinephrine or corticosterone did not impair memory in the control rats. Therefore, the results of these studies were difficult to interpret. In addition, results of the reversal experiment were inconclusive, because the memory-enhancing effect of stress was not replicated in control rats and stress *impaired* memory in both diet groups. In summary, it appears that HEDs do not necessarily impair memory, but instead prevent the memory-enhancing effects of acute stress. Future experiments should further investigate the role of epinephrine and corticosterone in the interaction between HEDs and stress on hippocampal-dependent memory.

## **4.2 Introduction**

Over the past five decades, *per capita* caloric intake has increased by approximately 28% in the United States (USDA 2011). A hallmark of the current standard American diet is an excess of energy sources from saturated fat and refined carbohydrates (Hu, Rimm et al. 1999; Iqbal, Anand et al. 2008; Grotto and Zied 2010). High energy diets (HEDs) such as the “Western” diet cause numerous pathologies, including

non-alcoholic fatty liver disease (NAFLD; Ackerman, Oron-Herman et al. 2005; Fu, Sun et al. 2009), high blood pressure (Hwang, Ho et al. 1987; Elliott, Keim et al. 2002; Catena, Giacchetti et al. 2003; Delbosc, Paizanis et al. 2005; Panchal, Poudyal et al. 2010; Poudyal, Campbell et al. 2010), dyslipidemia (Sleder, Chen et al. 1980; Kelley, Allan et al. 2004; Couchepin, Le et al. 2008), and peripheral insulin resistance (Tobey, Mondon et al. 1982; Zavaroni, Chen et al. 1982; Bezerra, Ueno et al. 2000; Panchal, Poudyal et al. 2010). Although most of the research has focused on the peripheral consequences of HEDs, there is growing concern regarding the effects of HEDs on the brain and behavior.

High energy diets negatively impact the hippocampus and hippocampal-dependent learning and memory. These diets decrease brain derived neurotrophic factor (Molteni, Barnard et al. 2002), synaptic communication (Stranahan, Norman et al. 2008), and neurogenesis (Lindqvist, Mohapel et al. 2006) in the hippocampus. High energy diets also decrease long-term potentiation (LTP; Stranahan, Norman et al. 2008) and long-term depression (LTD; Mielke, Taghibiglou et al. 2005), cellular mechanisms thought to underlie learning and memory processes (Malenka and Bear 2004; Massey and Bashir 2007). Therefore, it is not surprising that fructose-fed rats (Ross, Bartness et al. 2009), fat-fed rats (Pathan, Gaikwad et al. 2008), and rats fed a high fat, high sugar diet (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Darling, et al., unpublished) are impaired in the spatial version of the water maze memory task, which depends on an intact hippocampus for successful completion (Morris, Garrud et al. 1982; Clark, Broadbent et al. 2005). Fat-fed rats are also impaired in the radial arm maze and

a variable-interval delayed alternation task, both of which are also hippocampal-dependent (Greenwood and Winocur 1990).

High energy diets may also impair hippocampal-dependent memory by excessively increasing stress hormone levels. These diets decrease adrenal gland weight in male rats (Boukouvalas, Antoniou et al. 2008), increase epinephrine secretion (Kaufman, Li et al. 1993), and potentiate the ability of epinephrine to release hepatic glucose (see Chapter 3). Rats fed high fat diets have increased basal (Tannenbaum, Brindley et al. 1997; Cano, Jimenez-Ortega et al. 2008) and stress-induced (Tannenbaum, Brindley et al. 1997; Legendre and Harris 2006) levels of corticosterone. Epinephrine and corticosterone, in turn, influence hippocampal-dependent learning and memory (Gold and Van Buskirk 1975; Gold, van Buskirk et al. 1977; Cahill, Prins et al. 1994; Sandi, Loscertales et al. 1997; Jurado-Berbel, Costa-Miserachs et al. 2010). The effects of epinephrine on memory are dose-dependent such that optimal memory performance occurs with mid-range doses of either hormone; too little or too much of the hormone can have no effect or impair memory (Gold and Van Buskirk 1975; Gold, van Buskirk et al. 1977). Like epinephrine, the dose-response properties of corticosterone on memory follow an inverted U shape (Joels 2006).

Rats fed HEDs display impaired performance during stressful tasks such as the water maze (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009), but not during less stressful tasks such as spontaneous alternation (Ross 2008) or spatial object recognition (Darling, unpublished). Given that acute stress enhances hippocampal-dependent memory in healthy individuals (Cahill, Prins et al. 1994; Sandi, Loscertales et al. 1997), Experiment 1 tested the hypothesis that HEDs

impair hippocampal-dependent memory, at least in part, through diet-induced excessive increases in the acute stress response. If a HED impairs memory by augmenting the acute stress response, then stress will enhance memory in control diet rats, but will impair memory in HED rats. Rats were fed a high energy cafeteria-style diet for 4 weeks and then trained and tested in the spatial object recognition task (SOR). This task was chosen because it is hippocampal-dependent (Mumby, Gaskin et al. 2002), and the stressfulness of the task can be manipulated by familiarizing the rats with the testing apparatus prior to training (Okuda, Roozendaal et al. 2004). Being placed in a novel environment, such as the SOR box, stimulates the release of epinephrine and corticosterone in rats, and repeated exposure to an environment decreases this novelty-induced stress (de Boer, Koopmans et al. 1990). Thus, placing the rats into the testing environment repeatedly before training creates a low stress version of the SOR task, whereas withholding this familiarization to the testing environment creates a high stress version of the same task (Okuda, Roozendaal et al. 2004). Using different versions of the same task allows us evaluate the effects of diet and stress on memory while keeping all other experimental parameters constant, such as performance requirements, motivation, handling, testing apparatus, and lighting.

Experiment 2 tested the prediction that if the memory-impairing effects of HEDs are caused by too much circulating epinephrine, then injecting high energy-fed rats with a dose of epinephrine known to enhance memory in control rats will impair memory in a moderately stressful version of the SOR task. Experiments 3 and 4 tested the predictions that if the memory-impairing effects of HEDs are caused by too much circulating epinephrine or corticosterone, then blocking epinephrine receptors with sotalol or pre-

venting corticosterone synthesis with metyrapone will impair memory in control diet rats but enhance memory in rats fed a HED in the high stress version of the SOR task.

To our knowledge, there are no studies that have focused on reversing the diet-induced memory impairments by simply removing the high energy dietary components. Previous results from our lab, however, indicate that 9 weeks of HED followed by 4 weeks of standard chow feeding restored the ability of epinephrine to increase blood glucose concentrations (see Chapter 3). These results suggest that if the memory-impairing effects of a HED are caused by an increased acute stress response, then it is possible the diet-induced effects on cognition may be reversible through diet intervention. Therefore, Experiment 5 tested the hypothesis that the effects of HEDs on cognition are reversible through diet intervention. After 4 weeks on the high energy cafeteria-style diet, the high energy sources were removed, and the rats were fed standard chow and tap water for 4 weeks. They were then trained and tested in either the high stress or low stress version the SOR task.

To validate the stressfulness of the different versions of the SOR task, Experiment 6 measured plasma concentrations of epinephrine and corticosterone. Glucose was also measured because epinephrine increases hepatic glucose release (Sacca, Vigorito et al. 1983), and HEDs potentiate this effect (see Chapter 3). Rats were fed the high energy cafeteria-style diet and then trained in either the high stress or low stress version of the SOR task. After training, the rats were immediately euthanized, and plasma was collected to quantify hormone concentrations. Collecting post mortem samples ensured that there was enough plasma to measure epinephrine, corticosterone and glucose; however, this limited us to collection at only one time point. The possibility that

this time point would be optimal for measuring all three hormones is unlikely. Novelty-induced stress increases circulating epinephrine immediately (de Boer, Koopmans et al. 1990), but the effects of stress on glucose can be observed after approximately 10-15 min (de Boer, Koopmans et al. 1990; see Chapter 3). Corticosterone concentrations reach a peak around 45 min after the onset of the stressor (de Boer, Koopmans et al. 1990). Therefore, Experiment 7 measured the changes in the hormones over time. Rats were trained in either the high stress or low stress version of the SOR task, and hormone measurements were collected at regular intervals for 2 h after removal from the testing apparatus. Epinephrine was not measured in this experiment because the amount of plasma collected at each time point was not sufficient.

### **4.3 Experiment 1 Methods**

#### *Animals and housing*

Male Sprague-Dawley rats (Charles River, Wilmington, MA), aged 53 days, were housed individually in an OptiRat® cage system (Animal Care Systems, Centennial, CO) on a 12 h light cycle (lights on at 7:00 am). They were weighed upon arrival to the lab and allowed to acclimate for 1 week. After the acclimation week, all rats were handled for 1 min twice a week until the start of the behavioral experiments. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and are in accordance with PHS guidelines.

#### *Diets*

All rats were fed standard chow (3.01 kcal/g; LabDiet 5001, Purina Mills, Gray Summit, MO) during the acclimation week. On the seventh day, they were weighed again, matched on percent change in body mass, and assigned to either the control diet

or the high energy diet (HED) group. The HED consisted of a bottle of tap water, a bottle of 32% sucrose solution (3.75 kcal/g), standard rat chow, and a glass petri dish containing animal lard (9.0 kcal/g; Armour, Omaha, NE; Pecoraro, Reyes et al. 2004). The control diet consisted of two bottles of tap water and standard rat chow, and an empty glass petri dish was placed in the cage. Both the control and HED were fed *ad libitum*. All rats were fed their respective diets for 4 weeks before training and testing began. This diet duration was chosen because the high energy cafeteria-style diet impairs hippocampal-dependent memory in the water maze task in rats fed for 4 weeks (Darling, et al., unpublished). Body mass and food intake measurements were collected every other week from a group of rats that were not included in the behavioral experiments. Given that measuring body mass and food intake requires the use of a raised metal floor in the bottom of the cage and handling the rats daily, there is a possibility this process can be stressful. Therefore, to minimize the stress confound created by collecting these measurements, these rats were not tested in the SOR task. To measure food intake, pellets in each hopper and dried spillage from the bottom of each cage were weighed and then subtracted from the amount placed in the hopper the previous day. In a similar manner, lard dishes and water bottles were weighed individually, and then subtracted from the weight of the dish or bottle from the previous day.

### *SOR apparatus*

The spatial object recognition (SOR) apparatus was a white plexiglass box (60 cm x 70 cm x 70 cm) containing a layer of corncob bedding. After each trial, the sides of the SOR box were cleaned with 70% reagent alcohol, and the bedding was stirred. The bedding was replaced after every 5 trials.

### *SOR handling and habituation*

All rats were brought to the testing suite and handled for 1 min twice a day for the 7 days prior to SOR training. For the low stress version of the SOR task, half of the rats from each diet group were familiarized to the testing apparatus by placing them into the SOR box for 3 min immediately after each of the 14 handling sessions (Okuda, Roozendaal et al. 2004). The remaining rats were not given this familiarization period. Rather, they were returned to their home cages immediately after each handling session, creating a high stress version of the SOR task (Okuda, Roozendaal et al. 2004).

### *SOR training*

Two identical soda cans were placed towards the center of the SOR box. The cans were located 16 cm from the sides of the SOR box and 22 cm from each other. Each rat was placed into the SOR box facing the wall, equidistant from each soda can. The rats in both stress conditions were given 5 min to explore the SOR box and the cans.

### *SOR testing*

Twenty-four h after the training session, one of the soda cans was moved to the corner of the SOR box, 10 cm from the side of the SOR box and 34 cm from the other can, which remained in the same position as the day before. Each rat was placed in the SOR box in the same location as the previous day and allowed to explore for 1 min. This task exploits the rats' inherent interest in novelty. Control rats spend more time exploring the object in the novel location, indicating that they remember the placement of the objects from the previous day (Mumby, Gaskin et al. 2002). This spatial version of

the object recognition task is considered hippocampal-dependent because lesions of the hippocampus impair retention performance in this task (Mumby, Gaskin et al. 2002).

### *SOR scoring*

A camera mounted to the ceiling above the SOR box recorded the training and testing sessions. Time spent investigating the objects during training and testing was measured using real-time ethological recording and analysis software (Hindsight for MS-DOS, version 1.5, programmed by Scott Weiss, UK). Training data were analyzed to verify that the rats did not display an object bias by exploring one object three times more than the other object. For all experiments, rats that displayed an object bias were eliminated from the analyses. Therefore, a total of 22 control rats and 24 HED rats were excluded. In addition, rats that explored the objects less than 10 sec during training also were eliminated from the analyses. Only one control rat and no HED rats were eliminated based on this criterion. A discrimination index was calculated for the testing data by subtracting the amount of time spent investigating the non-moved object from the time spent investigating the moved object and then dividing this value by the total investigation time. To measure locomotor activity, the SOR box was divided into four virtual quadrants, and the number of times each rat crossed into a different quadrant was scored using TopScan (CleverSys, Reston, VA).

As an additional verification of the stress manipulation, the amount of time the rats spent in the outer 50% of the SOR box versus the inner 50% of the SOR box was recorded. Previous research suggests that rats experiencing high levels of stress spend most of their time in the outer 50% of the testing area, close to a wall. In contrast, rats that are experiencing a low amount of stress will venture into the inner 50% of the box,

away from the walls and into the open area (Prut and Belzung 2003). As a result, a difference score was calculated by subtracting the amount of time spent in the inner 50% of the box from the outer 50% of the box. A high difference score indicates that the rat spent more time in the outer 50% of the box than the inner 50%, and suggests that the rat experienced high levels of stress.

### *Statistical analysis*

The SOR training and testing procedure does not provide a measure to confirm whether or not each rat has learned the placement of the objects during SOR training. Thus, the low discrimination index scores of rats that did not learn the task may not reflect the effects of the diet or stress manipulations but instead a lack of learning. Including the memory scores of these rats in the analyses could mask the impairing effects of diet and stress on memory. In order to decrease the risk of a Type II error, a median split was performed on the discrimination index scores for each group. The top 50% of the scores from each group (i.e. those scores from rats who most likely learned the task) were analyzed separately from the bottom 50% of the scores.

Data were stored and analyzed using Microsoft Excel, Version 5.0 and Statistical Package for the Social Sciences (SPSS), Version 18.0. A Student's t-test was used to determine whether there were differences between HED rats and control diet rats in percent change in body mass and caloric consumption. A Student's t-test also was used to determine whether there were differences within each group in the amount of time spent exploring the two objects during training. In addition, a univariate analysis of variance (ANOVA), Tukey post hoc tests and Student's t-tests were used to determine if there were differences between the groups in the amount of time spent exploring the

objects, discrimination index scores, number of quadrant crossings, and open field difference scores. Differences between groups were considered statistically significant if  $p \leq 0.05$ .

#### 4.4 Experiment 1 Results

##### *The effects of a HED on body mass and food intake*

The HED significantly increased percent change in body mass [ $t(106) = -2.35, p = 0.02$ ; see Figure 4.1A] and caloric consumption [ $t(106) = -24.83, p = 0.00$ ; see Figure 4.1B]. In addition, the HED rats consumed fewer calories from chow than did control diet rats [ $t(106) = 40.40, p = 0.00$ ; see Figure 4.1B].

##### *The effects of a HED and stress on SOR training in rats with SOR scores above the median*

To simplify the presentation of the results, only interactions between variables that were significant are presented. In addition, there was no indication of an object bias in any of the experiments; therefore, these results are not presented. When the 5 min training session was analyzed for rats that were considered to have learned the task (i.e. the top 50% of the discrimination index score), stress decreased the amount of time spent investigating objects [ $F(1, 24) = 35.40, p = 0.00$ ] and open field activity [ $F(1, 24) = 7.02, p = 0.01$ ]. Rats in the high stress condition spent less time exploring the objects [ $t(26) = 6.15, p = 0.00$ ; see Figure 4.2A] and more time in the outer 50% of the SOR box [ $t(26) = -2.71, p = 0.01$ ; see Figure 4.2B] than did rats in the low stress condition. There was no significant effect of stress on locomotor behavior [ $F(1, 22) = 0.54, p = 0.47$ ].

Diet did not affect object investigation [ $F(1, 24) = 0.11, p = 0.74$ ] nor the amount of time spent in the outer 50% of the SOR box [ $F(1, 24) = 0.79, p = 0.38$ ]. The HED did

increase locomotor activity [ $F(1, 22) = 4.46, p = 0.05$ ]. The HED rats made more quadrant crossings than did the control rats [ $t(24) = -2.12, p = 0.05$ ; see Figure 4.2C].

*The effects of a HED and stress on SOR memory in the rats with scores above the median*

As predicted, the effects of stress on memory during the 1 min retention test 24 h later were dependent on diet group [ $F(1, 24) = 7.85, p = 0.01$ ; see Figure 4.3]. To be specific, stress increased memory performance only in the control rats. Control diet high stress rats had higher discrimination index scores than did control diet low stress rats, HED low stress rats and HED high stress rats (all  $p = 0.00$ ). The discrimination index scores of HED high stress rats did not differ from those of HED low stress rats ( $p = 0.99$ ). Although the HED prevented stress from enhancing memory, it did not impair memory. The discrimination index scores of HED low stress rats ( $p = 0.48$ ) and HED high stress rats ( $p = 0.65$ ) did not differ from those of control diet low stress rats. There were no significant effects of diet [ $F(1, 23) = 1.18, p = 0.29$ ] or stress [ $F(1, 23) = 2.60, p = 0.12$ ] on the number of quadrant crossings made during the retention test.

*The effects of a HED and stress on SOR memory in the rats with scores below the median*

The pattern of results during *SOR training* was similar for the rats with discrimination index scores above the median compared to rats with scores below the median (results not presented). A different pattern of effects was observed, however, during the memory test. To be specific, the HED [ $F(1, 21) = 7.46, p = 0.01$ ], but not stress [ $F(1, 21) = 0.09, p = 0.76$ ], affected discrimination index scores in this group. HED rats had lower discrimination index scores than control diet rats [ $t(23) = 2.69, p = 0.01$ ; see Fig-

ure 4.4A]. In contrast, stress [ $F(1, 21) = 5.55, p = 0.03$ ] but not diet [ $F(1, 21) = 1.92, p = 0.18$ ] decreased locomotor activity. Rats in the high stress condition made fewer quadrant crossings than did the rats in the low stress condition [ $t(23) = 2.30, p = 0.03$ ; see Figure 4.4B].

#### *The effects of a HED and stress on SOR memory in all rats*

This analysis revealed only a significant effect of diet on memory [ $F(1, 49) = 7.15, p = 0.01$ ], wherein rats fed the HED had lower discrimination index scores than did rats fed the control diet [ $t(51) = 2.68, p = 0.01$ ; see Figure 4.5]. This indicates that performing the median split on the discrimination index scores revealed an interaction between the HED and stress that otherwise would have been overlooked, constituting a Type II error. Therefore, for the remaining experiments, only the data from rats with discrimination index scores above the median will be presented.

### **4.5 Experiment 2 Methods**

This experiment tested the prediction that epinephrine would enhance memory in control diet rats but impair memory in HED rats. To increase the likelihood of observing both a memory enhancement and impairment in the same experiment, we modified the familiarization protocol to create a moderate stress version of the SOR task. The rats in this experiment were given 7 familiarization sessions, which is half the amount given to rats in the low stress version of the SOR task. Therefore, during the 4<sup>th</sup> week on the diet, all rats were handled for 1 min twice a day for 3 days and once on the fourth day, and *all* rats were placed in the SOR box for 3 min after each of the 7 handling sessions. On the training day, each rat was given a subcutaneous injection of epinephrine (0.1 mg/kg; IMS Limited, El Monte, CA) or saline (0.9% w/v NaCl) immediately after training.

This dose of epinephrine was chosen because it enhances memory in other hippocampal-dependent tasks (Gold and Van Buskirk 1975; Gold and van Buskirk 1978; Talley, Kahn et al. 2000) and potentiates the ability of epinephrine to release hepatic glucose in HED rats (see Chapter 3). A posttraining injection was given because this protocol enhances memory in the SOR task (Jurado-Berbel, Costa-Miserachs et al. 2010).

#### **4.6 Experiment 2 Results**

##### *The effects of the HED and epinephrine on SOR training*

Given that the epinephrine injection was given after the 5 min training session, it was not surprising that there were no group differences observed during training (data not presented).

##### *The effects of the HED and epinephrine on SOR memory*

During the 1 min retention test 24 h later, neither epinephrine [ $F(1, 16) = 0.03, p = 0.88$ ; see Figure 4.6] nor diet [ $F(1, 16) = 0.06, p = 0.82$ ] affected memory.

#### **4.7 Experiment 3 Methods**

To investigate the impact of blocking epinephrine receptors on the effects of a HED and stress on hippocampal-dependent memory, only the high stress version of the SOR task was used. Therefore, *the rats were not exposed to the apparatus prior to training*. Five min before training, each rat was given an intraperitoneal injection of the peripheral  $\beta$ -adrenergic receptor antagonist sotalol (4 mg/kg; Sigma-Aldrich, St. Louis, MO) or saline (0.9% w/v NaCl). This dose of sotalol and the timing of the injection were chosen because this protocol prevents the memory-enhancing effects of novelty-induced stress (King and Williams 2009).

## 4.8 Experiment 3 Results

### *The effects of a HED and sotalol on SOR training*

During the training session, sotalol did not affect object investigation [ $F(1, 16) = 1.40, p = 0.25$ ; see Figure 4.7] or locomotor activity [ $F(1, 26) = 1.10, p = 0.30$ ]. There was no significant effect of diet on total object investigation time [ $F(1, 16) = 0.01, p = 0.94$ ] or on the number of quadrant crossings [ $F(1, 26) = 0.35, p = 0.56$ ].

### *The effects of a HED and sotalol on SOR memory*

Contrary to the predicted results, neither the HED [ $F(1, 26) = 2.88, p = 0.10$ ] nor sotalol [ $F(1, 26) = 0.11, p = 0.74$ ] affected memory performance during the 1 min retention test (see Figure 4.8).

## 4.9 Experiment 4 Methods

Ninety min before training in the high arousal version of the SOR task, each rat was given a subcutaneous injection of the corticosterone synthesis inhibitor metyrapone (50 mg/kg; Enzo, Farmingdale, NY) or vehicle (polyethylene glycol, Sigma, St. Louis, MO in saline). This dose of metyrapone and the timing of the injection was chosen because this is when it impairs memory in stressful tasks such as the water maze (Roozendaal, Bohus et al. 1996; Akirav, Kozenicky et al. 2004) and inhibitory avoidance (Roozendaal, Bohus et al. 1996).

## 4.10 Experiment 4 Results

### *The effects of a HED and metyrapone on SOR training*

Metyrapone decreased object investigation [ $F(1, 45) = 6.18, p = 0.02$ ] and locomotor activity [ $F(1, 44) = 5.97, p = 0.02$ ]. Rats injected with metyrapone explored the objects less [ $t(46) = 2.47, p = 0.02$ ; see Figure 4.9A] and made fewer quadrant cross-

ings [ $t(46) = 2.52, p = 0.01$ ; see Figure 4.9B] than did rats injected with vehicle. The HED did not affect object investigation [ $F(1, 45) = 0.08, p = 0.78$ ] or locomotor activity [ $F(1, 44) = 0.31, p = 0.58$ ].

#### *The effects of a HED and metyrapone on SOR memory*

Metyrapone did not affect memory performance [ $F(1, 45) = 0.43, p = 0.52$ ]. To our surprise, in this experiment, the HED enhanced memory [ $F(1, 45) = 9.50, p = 0.00$ ]. HED rats had higher discrimination index scores than control diet rats [ $t(46) = -2.71, p = 0.01$ ; see Figure 4.10]. This memory enhancement cannot be attributed to differences in locomotor activity, because there were no significant effect of diet [ $F(1, 44) = 0.38, p = 0.54$ ] on the number quadrant crossings made during the retention test.

### **4.11 Experiment 5 Methods**

To test whether the effects of a HED and stress can be reversed by simply removing the high energy dietary components, the rats were fed the HED or control diet for 4 weeks, then the lard and sucrose solution were taken from the cages. After 4 weeks of standard chow (SC) feeding, hippocampal-dependent memory was tested using the high stress and low stress versions of the SOR task. This timing for the diet intervention was chosen because 4 weeks of standard chow feeding reverses the diet-induced increases in baseline glucose concentrations and the potentiated ability of epinephrine to increase circulating blood glucose (see Chapter 3).

### **4.12 Experiment 5 Results**

#### *The effects of diet intervention on SOR training*

As in Experiment 1, stress decreased object investigation [ $F(1, 22) = 12.18, p = 0.00$ ] but did not affect locomotor activity [ $F(1, 21) = 2.57, p = 0.12$ ]. Rats in the high

stress conditions explored the objects less than rats in the low stress condition [ $t(24) = 3.19, p = 0.00$ ; see Figure 4.11]. In addition, the HED/standard chow (HED/SC) feeding did not affect object investigation [ $F(1, 22) = 1.87, p = 0.19$ ] or locomotor activity [ $F(1, 21) = 0.99, p = 0.33$ ].

#### *The effects of diet intervention on SOR memory*

There was no significant effect of diet [ $F(1, 22) = 0.03, p = 0.86$ ] on discrimination index scores. Contrary to the predicted results, stress *impaired* memory in both diet groups [ $F(1, 22) = 22.27, p = 0.00$ ]. Rats in the high stress condition had lower discrimination index scores than did rats in the low stress condition [ $t(24) = 4.73, p = 0.00$ ; see Figure 4.12]. This memory impairment cannot be attributed to differences in locomotor activity, as there was no significant effect of stress [ $F(1, 22) = 0.15, p = 0.70$ ] on the number of quadrant crossings made during the test.

### **4.13 Experiment 6 Methods**

The goal of the following experiment was to validate the stressfulness of the high and low stress versions of the SOR task by measuring stress hormone concentrations. Ten min after the SOR training session, low and high stress HED and control rats were killed by decapitation and trunk blood was collected. Blood was centrifuged to collect plasma, which was stored at  $-80^{\circ}\text{C}$  until assays were performed. Epinephrine was measured using high performance liquid chromatography (HPLC; Thermo Scientific, Sunnyvale, CA). Corticosterone was measured using a radioimmunoassay (MP Bio-medicals, Solon, OH), and glucose was measured using an AccuChek glucose meter (Roche, Indianapolis, IN). An ANOVA and Student's t-tests were used to determine if

there were differences between the groups in plasma concentrations of epinephrine, corticosterone and glucose.

#### 4.14 Experiment 6 Results

Contrary to the predicted results, there were no significant effects of stress [ $F(1, 19) = 0.12, p = 0.73$ ] or diet [ $F(1, 19) = 0.37, p = 0.55$ ] on epinephrine concentrations (see Figure 4.15A).

To our surprise, the HED decreased corticosterone [ $F(1, 24) = 5.44, p = 0.03$ ]. Rats fed the HED had lower plasma corticosterone concentrations than did control diet rats [ $t(26) = 2.41, p = 0.02$ ; see Figure 4.15B]. The stress manipulation, however, did not increase corticosterone [ $F(1, 24) = 0.14, p = 0.71$ ].

Of interest, both the stress manipulation and the HED affected plasma glucose. Stress increased glucose [ $F(1, 24) = 5.23, p = 0.03$ ]; rats in the high stress condition had higher plasma glucose concentrations than did rats in the low stress condition [ $t(26) = -2.57, p = 0.02$ ; see Figure 4.15C]. In addition, the HED increased glucose levels [ $F(1, 24) = 7.42, p = 0.01$ ]; HED rats had higher plasma glucose concentrations than did control diet rats [ $t(26) = -2.08, p = 0.05$ ; see Figure 4.15D].

#### 4.15 Experiment 7 Methods

To measure the effects of the high and low stress versions of the SOR task on stress hormone concentrations across time, immediately after the training trial, blood glucose was measured from a nick in the tail vein using an Accu-Chek glucose meter. Blood was then collected from the tail vein and centrifuged to obtain plasma, which was stored at  $-80^{\circ}\text{C}$  until the corticosterone assay was performed. This process was repeated at 10 min, 20 min, 40 min, 70 min, 100 min, and 130 min after the end of the

training trial. Epinephrine was not measured in this experiment because the amount of plasma collected at each time point was not sufficient for the HPLC. In addition, HED rats were not included in this experiment because the timing of the plasma collections limited the amount of rats that could be tested at one time. Finally, open field behavior was analyzed because it is an additional indicator of stress (Prut and Belzung 2003), and there was a significant effect of the stress manipulation on this measure in Experiment 1. A mixed ANOVA was used to determine whether there were differences in corticosterone and glucose across time between the rats in the low stress and high stress conditions. A Student's t-test was used to determine whether there were differences between the rats in the low stress and high stress conditions in the open field.

#### **4.16 Experiment 7 Results**

##### *The effects of stress on plasma corticosterone concentrations across time*

The stress manipulation did not affect corticosterone concentrations [ $F(1, 12) = 0.34, p = 0.57$ ], but corticosterone concentrations did increase across time for both groups [ $F(6, 72) = 65.54, p = 0.00$ ; see Figure 4.16A].

##### *The effects of stress on blood glucose concentrations across time*

Unlike the previous experiment, the stress manipulation did not affect glucose concentrations [ $F(1, 12) = 2.01, p = 0.18$ ]. Glucose levels did change over time for both groups [ $F(6, 72) = 16.15, p = 0.00$ ; see Figure 4.16B].

##### *The effects of stress on open field activity*

Despite the findings that corticosterone and glucose did not differ between the groups, there was a trend for stress to decrease open field activity. Rats in the high stress condition had a tendency to spend more time in the outer 50% of the SOR box

than did the rats in the low stress condition, but the effect did not reach statistical significance [ $t(1) = -1.99, p = 0.07$ ; see Figure 4.16C].

#### **4.17 Discussion**

To our knowledge, this is the first study to examine the interaction between the effects of a HED and stress on hippocampal-dependent memory. Results from Experiment 1 suggest that the HED prevented the memory-enhancing effects of acute stress. Stress improved memory scores of control rats, but did not affect the memory scores of the rats fed the HED. These results cannot be explained by differences in exposure to the objects during training because the HED did not affect object investigation. These results are particularly interesting because it was predicted that the HED would impair memory in the stressful version of the SOR task. Instead, the HED prevented the emotional modulation of memory. This finding is important because the results of many previous experiments suggest HEDs impair memory; however, these studies include only a stressful version of a memory task (Pathan, Gaikwad et al. 2008; Ross 2008; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009; see Chapter 2). The results from the current experiment suggest that these interpretations may be incorrect, because the HEDs in these experiments may actually be preventing the memory-enhancing effects of stress.

The current experiments are unique in that the effects of HEDs on hippocampal-dependent memory were examined while manipulating the stress level in a single task. Previous experiments tested the effects of HEDs in tasks that could be considered either high or low stress (Molteni, Barnard et al. 2002; Ross 2008; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009; Darling, unpublished). Using the SOR task allowed

us to keep all experimental parameters constant. The only difference between the two versions of the task was the familiarization to the testing apparatus. This also allowed us to observe that stress only enhanced memory in the control rats. The memory performance of the HED rats in the high stress condition was comparable to the performance of the control diet rats and the HED rats in the low stress condition, indicating that the HED did not impair memory.

Experiments 2-4 manipulated epinephrine and corticosterone concentrations to further test the hypothesis that HEDs impair memory by excessively increasing the acute hormonal response to stress. For a few reasons, the roles that these stress hormones may play in the diet-induced memory impairments were not determined. First, the HED did not impair memory. Therefore, there was no deficit to block with the adrenergic antagonist sotalol and the corticosterone synthesis inhibitor metyrapone. Second, the 0.1 mg/kg epinephrine dose did not enhance memory in control rats. One possible explanation for these results is that we did not use the correct dose of epinephrine. It is difficult to determine whether this dose was too high or too low to enhance memory, given that the effects of epinephrine on memory follow an inverted U curve (Gold and Van Buskirk 1975; Gold, van Buskirk et al. 1977). The combination of endogenous and exogenous epinephrine in this task may have been too much or too little to influence memory. Additional experiments are required to determine the amount of endogenous epinephrine released in the moderate stress version of the SOR task and the appropriate dose of exogenous epinephrine needed to enhance memory. In addition, sotalol did not impair memory in control rats as predicted. It is possible that the 4 mg/kg dose was not high enough to completely block the memory-enhancing effects of stress, and a lar-

ger dose of sotalol would have been effective. The only published experiment demonstrating that sotalol impairs memory used the 4 mg/kg dose but involved a memory task that was not hippocampal-dependent (King and Williams 2009). Therefore, a direct comparison between the findings of this previous study and the current results cannot be made. Finally, we were unable to replicate the finding that metyrapone impairs memory in control rats. It appears that the vehicle injections of polyethylene glycol impaired memory, causing a floor effect and preventing us from observing a memory impairment in rats given metyrapone. In the current studies, *rats injected with saline tested at the same time* (as part of the sotalol experiment) had a tendency to have better memory scores (mean discrimination index = 0.88) than rats injected with vehicle (mean discrimination index = 0.38;  $t(5) = -2.44$ ,  $p = 0.06$ ). To our knowledge, there are no reports of polyethylene glycol producing memory deficits, but most experiments do not include a saline control.

Experiment 5 tested whether the effects of the HED on cognition were reversible. The results of this experiment are inconclusive because stress *impaired* memory in both diet groups. This effect was the opposite of both what was predicted and also what was observed in Experiment 1 and previously published studies (Cahill, Prins et al. 1994; Sandi, Loscertales et al. 1997; Okuda, Roozendaal et al. 2004). One possible explanation is that before the behavioral experiments began, these rats were handled more than the rats in the previous experiments. The rats in Experiment 5 lived in the vivarium for an additional 4 weeks (for the diet intervention) and were therefore handled twice as much as the rats in Experiments 1-4. Although frequent handling is meant to reduce stress during behavioral manipulations, the handling procedure involves removing the

rat from its home cage, which is stressful (Balcombe, Barnard et al. 2004; Reinhardt 2004). The handling may then have become a chronic stressor, and chronic stress impairs hippocampal-dependent memory (Conrad, Galea et al. 1996; Kleen, Sitomer et al. 2006).

Experiments 6 and 7 attempted to validate the stress level in the two versions of the SOR task by measuring stress hormone concentrations. Previous research has established that placing rats in a novel environment increases circulating epinephrine, corticosterone and glucose (de Boer, Koopmans et al. 1990). In addition, repeated exposure to a particular environment decreases the hormonal response to this novelty-induced stress (de Boer, Koopmans et al. 1990). Previous research also has demonstrated that using the same high stress SOR protocol used in the current experiments increases circulating corticosterone (Okuda, Roozendaal et al. 2004). We were unable to replicate the corticosterone response in the low stress version of the SOR task. One likely explanation for this inability to replicate may be that in Experiment 6, plasma samples were collected at a single time point (based on (de Boer, Koopmans et al. 1990)). It is likely that this sampling time occurred too late to observe the effects of stress on epinephrine, which increases immediately following the onset of a stressor (de Boer, Koopmans et al. 1990). This interpretation is supported by the observed increase in glucose. Given that epinephrine induces hepatic glucose release (Sacca, Vigorito et al. 1983), these results indicate that stress from the SOR task may have increased epinephrine, which in turn increased blood glucose levels. In addition, the observed increase in corticosterone concentrations in both stress groups suggest that all rats experienced stress. In fact, the corticosterone concentrations for all groups were at least

three times greater than average baseline levels (de Boer, Koopmans et al. 1990; Cordero, Kruyt et al. 2002; Legendre and Harris 2006; Cano, Cardinali et al. 2008). Experiment 7 attempted to resolve the timing issue by measuring glucose and corticosterone across time following the stressor. Again, corticosterone concentrations increased equally in both groups, indicating that all rats experienced stress. In addition, stress increased glucose concentrations equally in rats in both the low and high stress conditions. One possible reason that both of these groups appear stressed is that the blood sampling procedure may have been stressful. The tail nick involves a short period of restraint and a small incision near the tip of the tail, both of which are stressful (Balcombe, Barnard et al. 2004). It is likely that the stress-induced increases in corticosterone and glucose were caused by this procedure, and masked the effects of the familiarization to the SOR box. The results of the open field test support this interpretation because they suggest that the high stress version was indeed more stressful than the low stress version of the SOR task. To be specific, there was a tendency for the rats in the high stress condition to spend more time in the outer 50% of the SOR box than did rats in the low stress condition, which is an indication of stress (Prut and Belzung 2003). In addition, contrary to our prediction, the HED decreased corticosterone concentrations in Experiment 6. These results are consistent, though, with those of previous studies that have demonstrated the cafeteria-style HED, as opposed to involuntary consumption of a HED, decreases corticosterone response to an acute stressor (la Fleur, Houshyar et al. 2005).

In summary, a HED impaired the emotional modulation of memory by preventing the memory-enhancing effects of acute stress. Future experiments should focus on

identifying how HEDs impact the acute stress response by further investigating the relationship between hippocampal-dependent memory and stress hormones. The results of the current experiments add to the growing body of literature demonstrating that HEDs are harmful to cognition.

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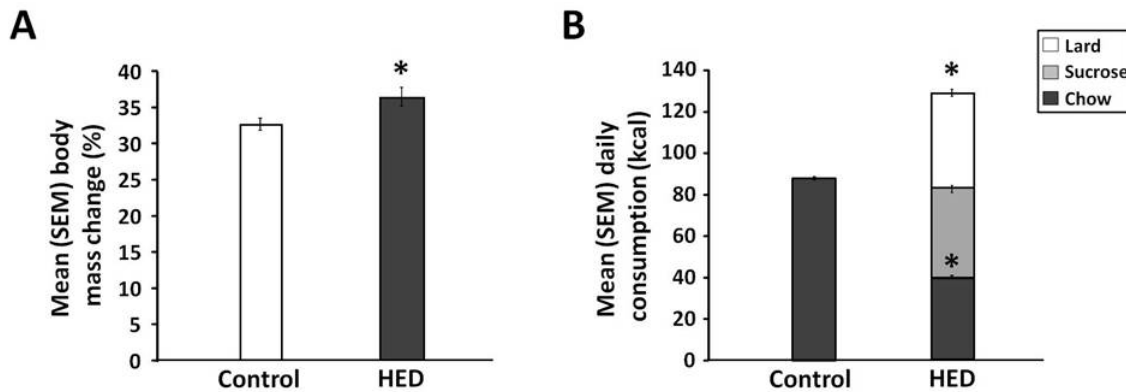
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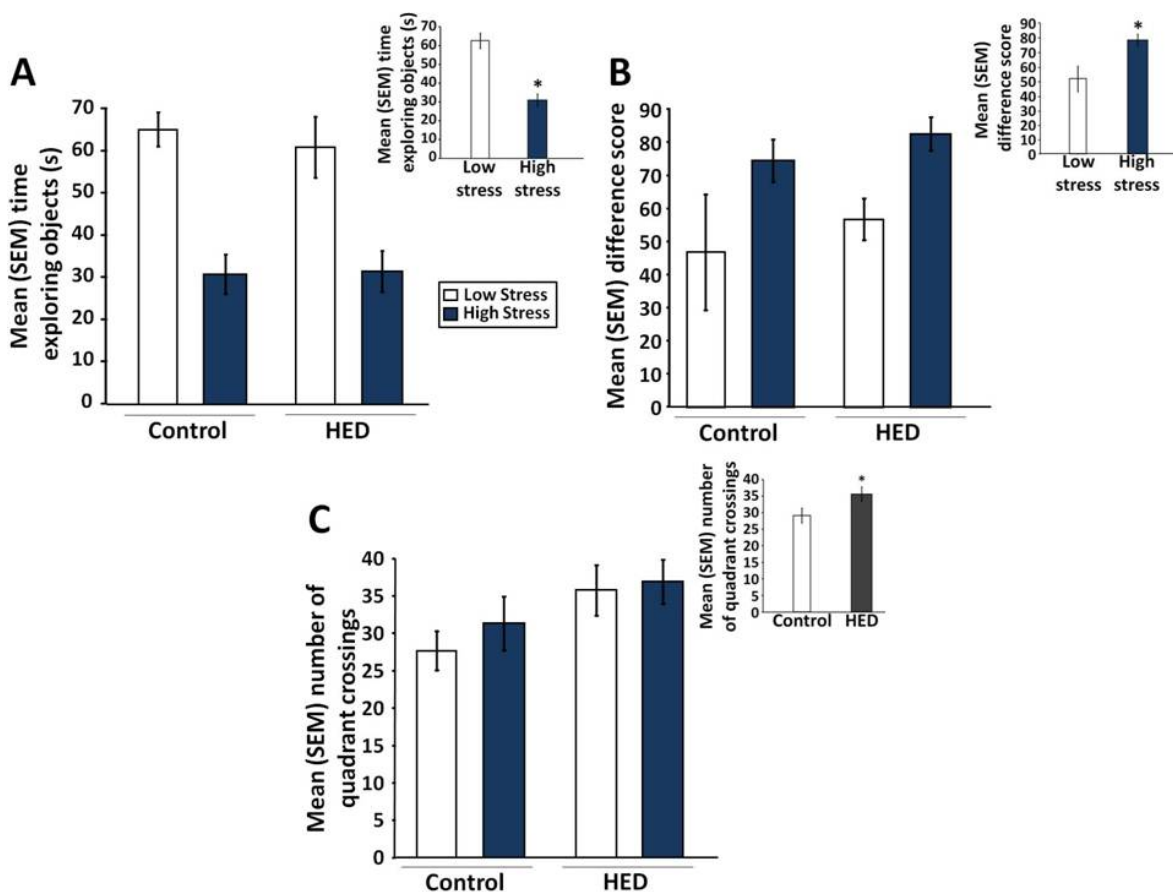
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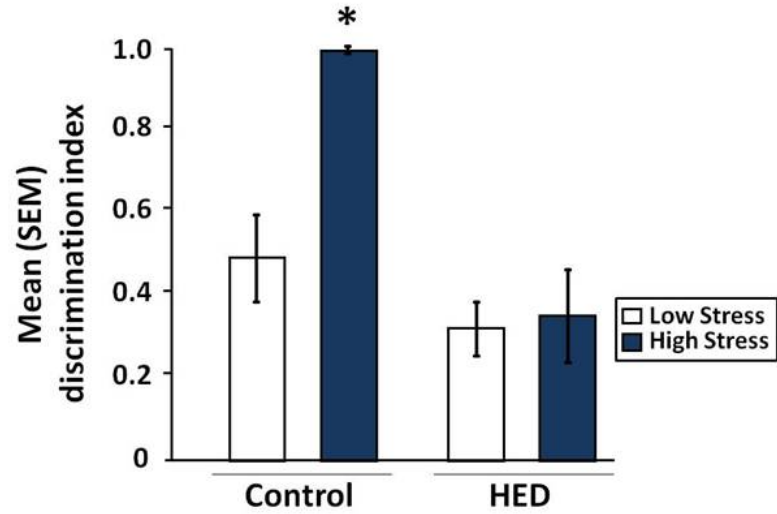
## 4.20 Chapter 4 Figures



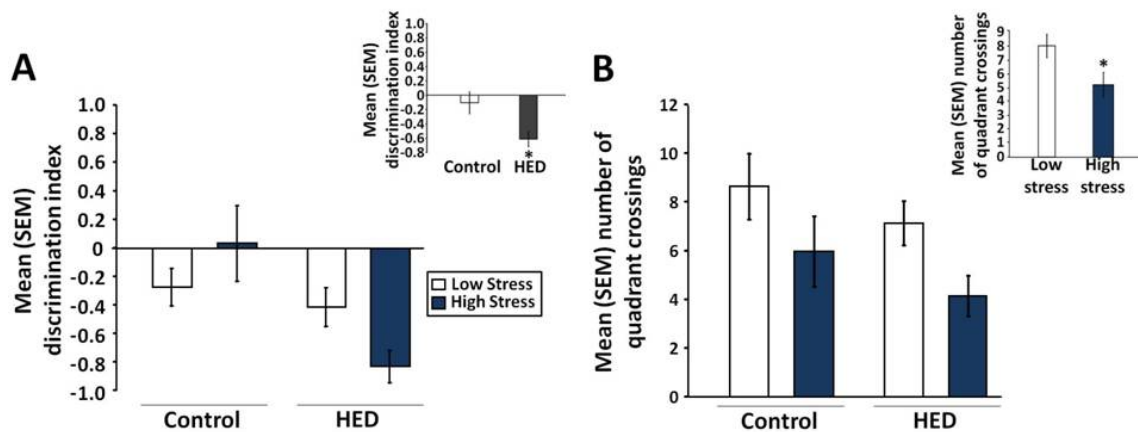
**Figure 4.1: Percent change in body mass and caloric consumption.** Mean ( $\pm$  SEM) (A) percent change in body mass and (B) average caloric consumption for control rats ( $n = 54$ ) and rats fed a high energy diet (HED;  $n = 54$ ;  $*p < 0.05$  vs. control).



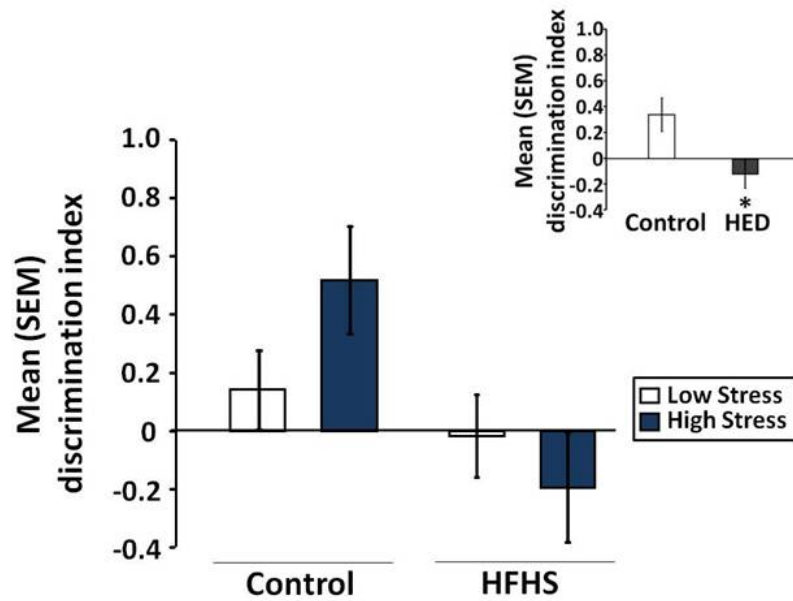
**Figure 4.2: Spatial object recognition training in rats with scores above the median.** The effects of stress and/or consumption of a HED on mean ( $\pm$  SEM) **(A)** time spent exploring objects (control diet low stress:  $n = 7$ ; control diet high stress:  $n = 7$ ; HED low stress:  $n = 7$ ; HED high stress:  $n = 7$ ), **(inset)** time spent exploring objects (low stress:  $n = 14$ ; high stress:  $n = 14$ ;  $*p < 0.05$  vs. low stress), **(B)** open field difference scores;  $*p < 0.05$  vs. low stress **(inset)** open field difference scores, **(C)** number of quadrant crossings, and **(inset)** number of quadrant crossings (control:  $n = 14$ ; HED:  $n = 14$ ;  $*p < 0.05$  vs. control) during spatial object recognition training.



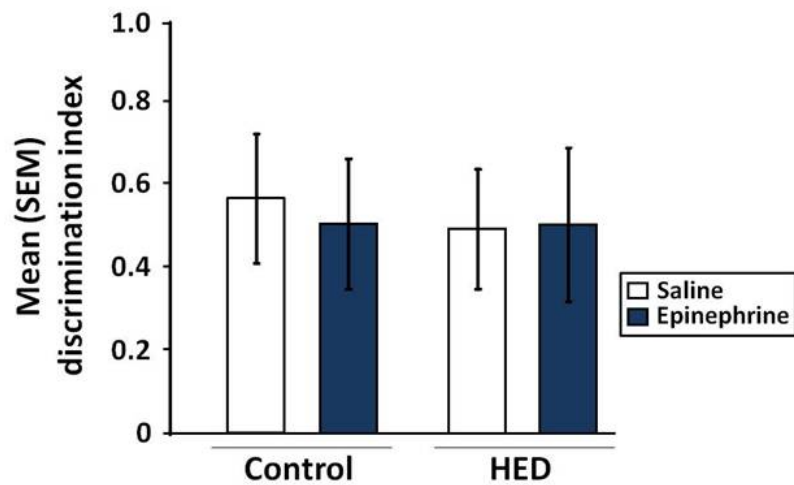
**Figure 4.3: Spatial object recognition testing in rats with scores above the median.** The effects of stress and consumption of a HED on mean ( $\pm$  SEM) discrimination index scores during the spatial object recognition memory test;  $*p < 0.05$  vs. all groups.



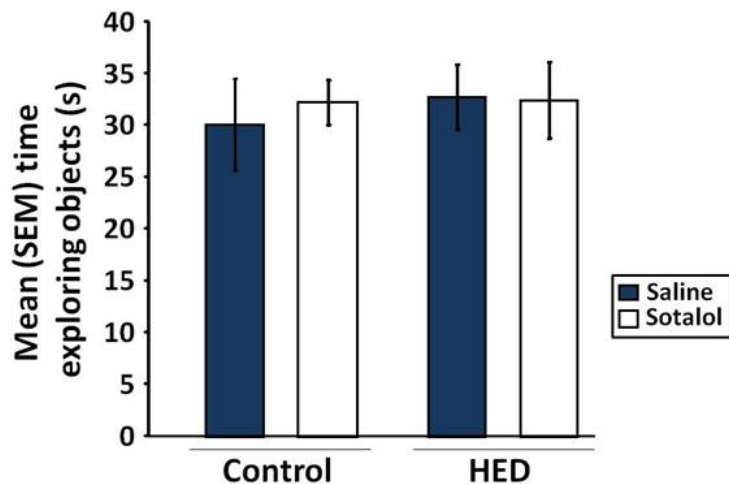
**Figure 4.4: Spatial object recognition testing in rats with scores below the median.** The effects of stress and/ or consumption of a HED on mean ( $\pm$  SEM) **(A)** discrimination index scores (control diet low stress:  $n = 6$ ; control diet high stress:  $n = 7$ ; HED low stress:  $n = 6$ ; HED high stress:  $n = 6$ ), **(inset)** discrimination index scores (control:  $n = 13$ ; HED:  $n = 12$ ;  $*p < 0.05$  vs. control), **(B)** number of quadrant crossings, and **(inset)** number of quadrant crossings (low stress:  $n = 12$ ; high stress:  $n = 13$ ;  $*p < 0.05$  vs. low stress) during the spatial object recognition memory test.



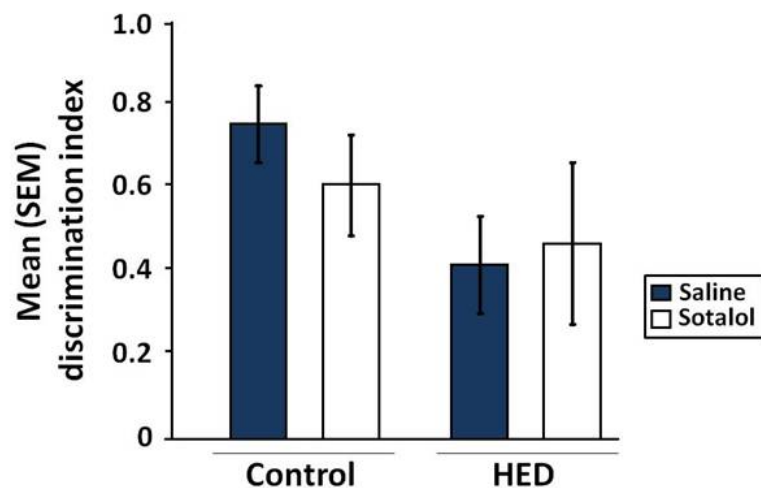
**Figure 4.5: Spatial object recognition testing in all rats.** The effects of stress and/ or consumption of a HED on mean (+/- SEM) discrimination index scores (control diet low stress:  $n = 15$ ; control diet high stress:  $n = 14$ ; HED low stress:  $n = 15$ ; HED high stress:  $n = 15$ ) and (**inset**) discrimination index scores (control:  $n = 29$ ; HED:  $n = 30$ ;  $*p < 0.05$  vs. control) during the spatial object recognition memory test.



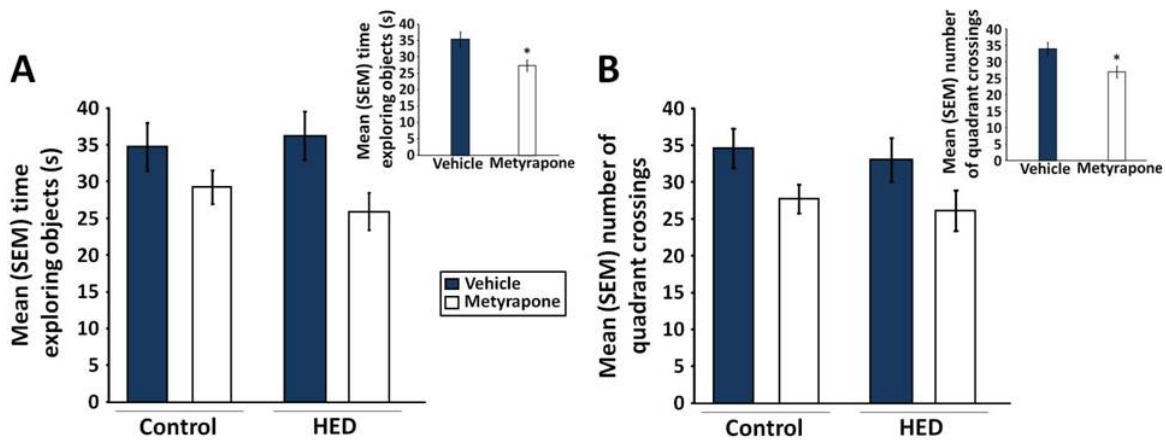
**Figure 4.6: Spatial object recognition testing in rats injected with epinephrine.** The effects of epinephrine (0.1 mg/kg) and consumption of a HED on mean ( $\pm$  SEM) discrimination index scores during the spatial object recognition memory test (control diet saline:  $n = 5$ ; control diet epinephrine:  $n = 5$ ; HED saline:  $n = 5$ ; HED epinephrine:  $n = 5$ ).



**Figure 4.7: Spatial object recognition training in rats injected with sotalol.** The effects of sotalol (4 mg/kg) and consumption of a HED on mean ( $\pm$  SEM) time spent exploring objects during spatial object recognition training (control diet saline:  $n = 7$ ; control diet sotalol:  $n = 8$ ; HED saline:  $n = 7$ ; HED sotalol:  $n = 8$ ).

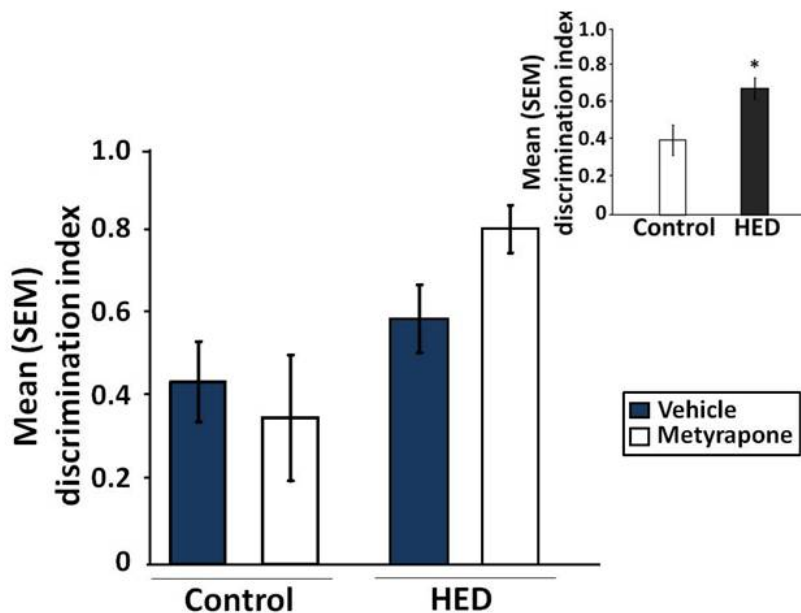


**Figure 4.8: Spatial object recognition testing in rats injected with sotalol.** The effects of sotalol (4 mg/kg) and consumption of a HED on mean ( $\pm$  SEM) discrimination index scores during the spatial object recognition memory test.



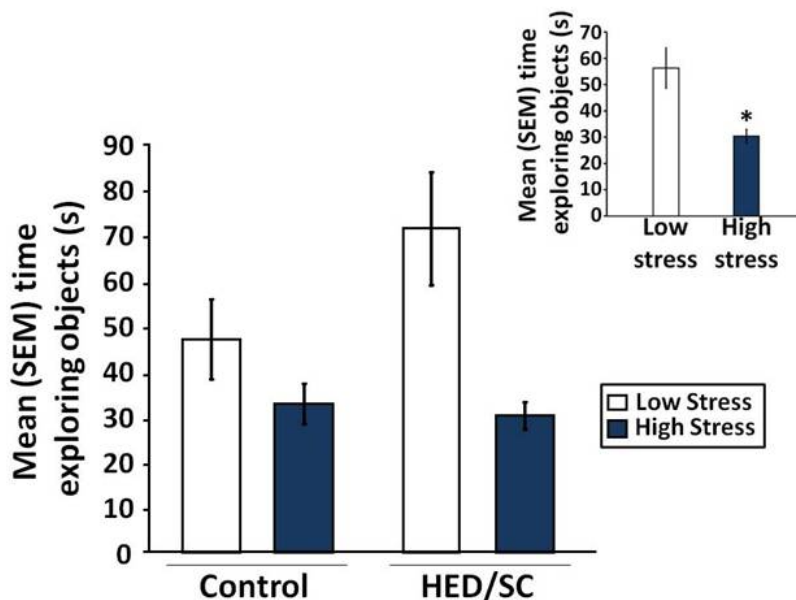
**Figure 4.9: Spatial object recognition training in rats injected with metyrapone.**

The effects of metyrapone (50 mg/kg) and/or consumption of a HED on mean ( $\pm$  SEM) **(A)** time spent exploring objects (control diet vehicle:  $n = 15$ ; control diet metyrapone:  $n = 9$ ; HED vehicle:  $n = 14$ ; HED metyrapone:  $n = 11$ ), **(inset)** time spent exploring objects (vehicle:  $n = 29$ ; metyrapone:  $n = 20$ ;  $*p < 0.05$  vs. vehicle), **(B)** number of quadrant crossings, and **(inset)** number of quadrant crossings ( $*p < 0.05$  vs. vehicle) during spatial object recognition training.

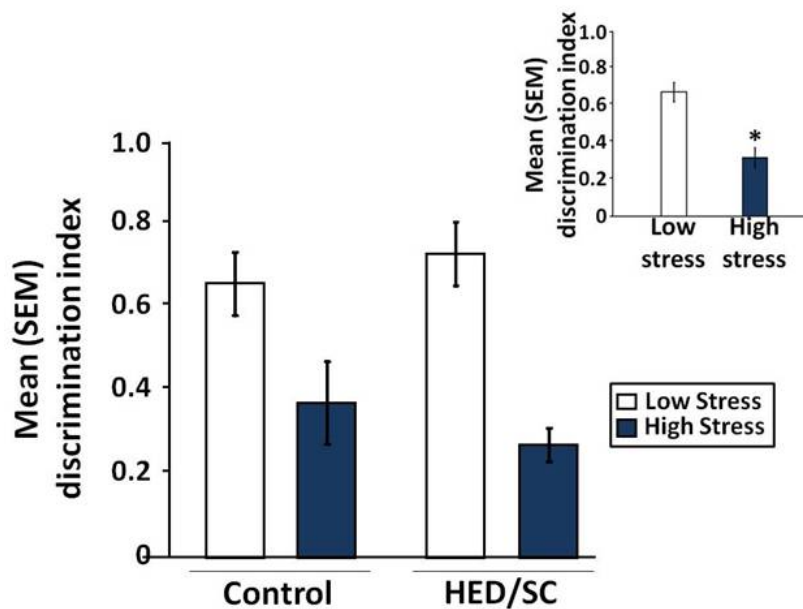


**Figure 4.10: Spatial object recognition testing in rats injected with metyrapone.**

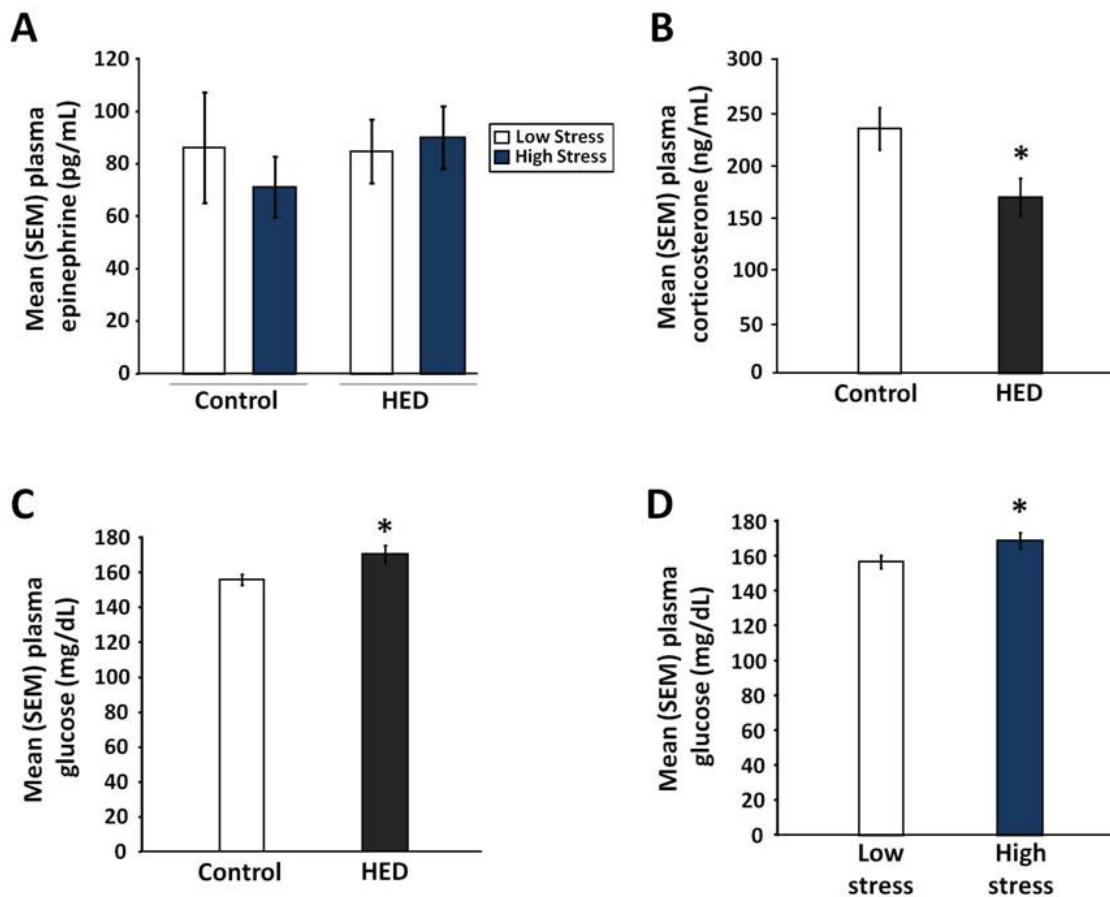
The effects of metyrapone (50 mg/kg) and/or consumption of a HED on mean ( $\pm$  SEM) discrimination index scores and (**inset**) discrimination index scores (control:  $n = 24$ ; HED:  $n = 25$ ;  $*p < 0.05$  vs. control) during the spatial object recognition memory test.



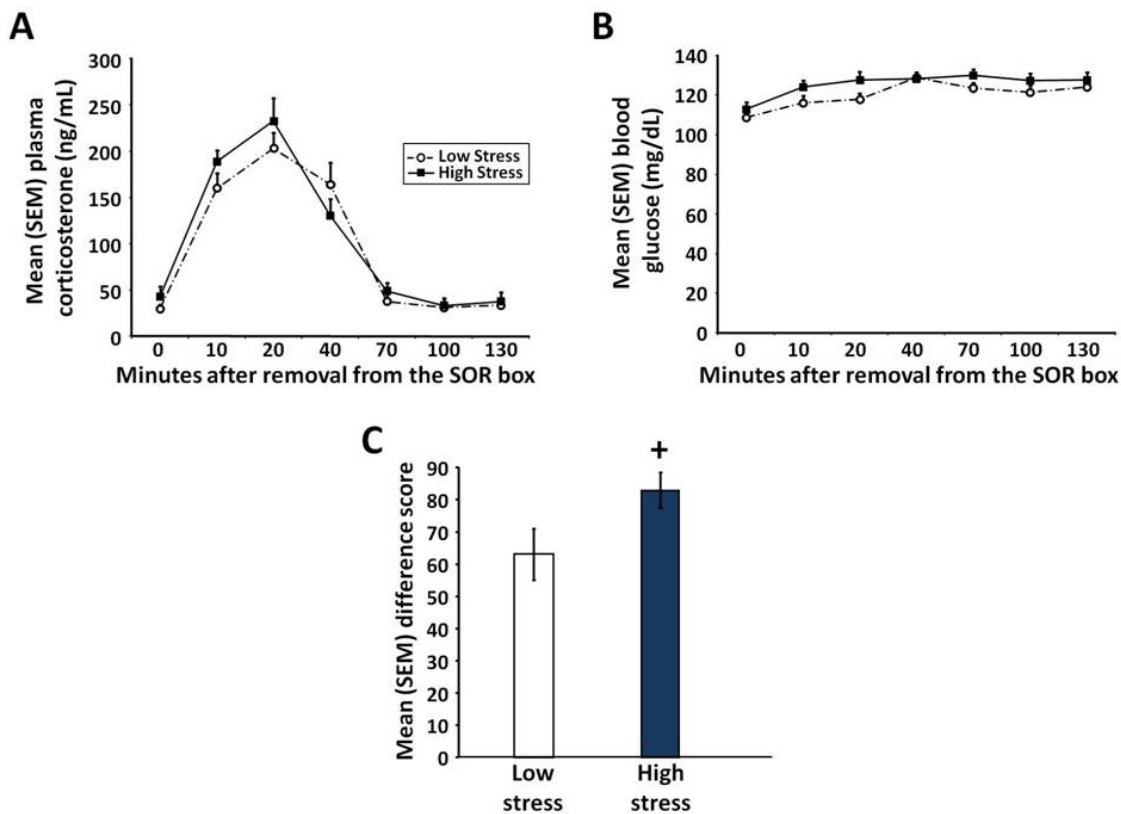
**Figure 4.11: Spatial object recognition training in rats fed a high energy diet then standard chow.** The effects of stress and/or consumption of a HED followed by 4 weeks of standard chow on mean ( $\pm$  SEM) time spent exploring objects (control diet low stress:  $n = 7$ ; control diet high stress:  $n = 7$ ; HED/SC low stress:  $n = 6$ ; HED/SC high stress:  $n = 6$ ;  $*p < 0.05$  vs. all groups) and (**inset**) time spent exploring objects (low stress:  $n = 13$ ; high stress:  $n = 13$ ;  $*p < 0.05$  vs. low stress) during spatial object recognition training.



**Figure 4.12: Spatial object recognition testing in rats fed a high energy diet then standard chow.** The effects of stress and/or consumption of a HED followed by 4 weeks of standard chow on mean ( $\pm$  SEM) discrimination index and (**inset**) discrimination index scores ( $*p < 0.05$  vs. low stress) during the spatial object recognition memory test.



**Figure 4.13: Plasma stress hormone concentrations.** The effects of stress and/or consumption of a HED on mean ( $\pm$  SEM) (A) plasma epinephrine concentrations (control diet low stress:  $n = 5$ ; control diet high stress:  $n = 6$ ; HED low stress:  $n = 5$ ; HED high stress:  $n = 7$ ), (B) plasma corticosterone concentrations (control:  $n = 14$ ; HED:  $n = 14$ ; \* $p < 0.05$  vs. control), (C) plasma glucose concentrations (\* $p < 0.05$  vs. control) and (D) plasma glucose concentrations (low stress:  $n = 14$ ; high stress:  $n = 14$ ; \* $p < 0.05$  vs. low stress).



**Figure 4.14: Plasma stress hormone concentrations and open field difference scores.** The effects of stress on mean ( $\pm$  SEM) (A) plasma corticosterone concentrations (low stress:  $n = 8$ ; high stress:  $n = 9$ ), (B) blood glucose concentrations and (C) open field difference scores (low stress:  $n = 8$ ; high stress:  $n = 7$ ;  $^+p = 0.07$  vs. low stress).

## CHAPTER 5: GENERAL DISCUSSION

High energy diets, such as the “Western” diet consumed by most Americans, are detrimental to physical as well as cognitive health. The goal of the current experiments was to determine if certain peripheral and central consequences of high energy diets contribute to the diet-induced cognitive impairments. These experiments confirmed that high energy diets induce fatty liver, potentiate epinephrine-induced increases in blood glucose concentrations and negatively impact cognition. The results also demonstrate novel findings that diet-induced non-alcoholic fatty liver disease (NAFLD) impairs memory and high energy diets augment the ability of epinephrine to increase blood glucose concentrations and disrupt the emotional modulation of memory. Taken together, these results indicate that high energy diet-induced impairments in liver function contribute to the diet-induced cognitive disruptions.

To our knowledge, the current experiments were the first to demonstrate that high energy diets may not necessarily impair memory but instead prevent the memory-enhancing effects of acute stress (see Chapter 4). Including stress as an independent variable allowed us to detect the interaction between stress and memory. This is an important finding because stress was not manipulated in previous studies, and by testing hippocampal-dependent memory in only stressful or nonstressful tasks, the interaction between high energy diets and stress on memory was overlooked (Molteni, Barnard et al. 2002; Ross 2008; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009; Darling et al., unpublished). In fact, the diet-induced effects on memory in the water maze task observed in Chapter 2 may not actually be memory impairments but instead a prevention of stress-induced memory enhancement.

The results of Chapter 3, in which the high energy diet potentiated the ability of epinephrine to increase blood glucose concentrations, suggest that the high energy diet shifted the dose-response properties of epinephrine. This is important because epinephrine and glucose impact hippocampal-dependent memory in a dose-dependent manner such that moderate doses enhance memory, and high doses of either hormone have no effect or impair memory (Gold and Van Buskirk 1975; Gold, van Buskirk et al. 1977; Gold 1986; Gold, Vogt et al. 1986). Therefore, a shift in the dose-response curve could have important consequences for memory performance. To be specific, in control rats, a dose of 0.1 mg/kg epinephrine enhances memory (Gold and Van Buskirk 1975; Gold and van Buskirk 1978; Talley, Kahn et al. 2000), most likely by increasing blood glucose concentrations to those that are optimal for memory performance (Gold 1986; Hall and Gold 1986; Hall and Gold 1992). If the high energy diet shifted the dose response curve to the left, this same dose of epinephrine would result in blood glucose concentrations that were within the range that impairs or does not affect memory (Gold 1986; Hall and Gold 1986; Hall and Gold 1992). The results of Chapter 4 support this interpretation. To be specific, stress did not enhance or impair memory in rats fed the high energy diet. Although we did not measure *blood* glucose concentrations in this particular experiment, we did measure *plasma* glucose concentrations in a separate group of rats after training in the high or low stress version of the spatial object recognition task. Given that plasma glucose concentrations are approximately 10% higher than blood glucose concentrations (Kuwa, Nakayama et al. 2001), the 0.1 mg/kg epinephrine dose and novelty-induced stress resulted in comparable circulating glucose levels (see Chapters 3 and 4). This raises the possibility that we did not observe a memory enhancement or impair-

ment because novelty-induced stress increased blood glucose in the high energy fed rats to concentrations that were outside the range for influencing memory. Additional experiments are required to test the hypothesis that the high energy diet disturbs the emotional modulation of memory through the potentiated ability of epinephrine to release hepatic glucose.

The results of the current experiments indicate that the diet-induced effects of cognition are caused by diet-induced impaired liver function. The high energy diets caused non-alcoholic fatty liver disease (NAFLD; see Chapters 2 and 3). Non-alcoholic fatty liver disease impaired memory (or prevented stress from enhancing memory) in the water maze task, but the underlying mechanism was not identified in this experiment. The next set of experiments found that the diet-induced NAFLD potentiated epinephrine-induced glucose release (Chapter 3). Finally, the findings from the last set of experiments suggest that this increase in the acute stress response results in an inability of stress to enhance memory in high energy fed rats.

Another important finding from these experiments is that it is possible a diet intervention could reverse the negative effects of high energy diets on memory. Removing the lard and sucrose sources from the diet restored the ability of epinephrine to increase blood glucose concentrations to control levels, indicating the diet-induced impairments in liver function are not permanent (Chapter 3). This finding also suggests that after the diet intervention, circulating blood glucose concentrations during a stressful task may remain within the range of optimal memory performance. The results from the diet intervention experiment in Chapter 4 were inconclusive, because stress impaired memory in both diet groups. Additional experiments are needed to confirm that the cognitive effects

of high energy diets can be reversed by simply removing the high energy dietary components.

There are a few important experimental procedures that differ between the current studies. First, there were two kinds of high energy diets employed in these studies. The first was a 60% fructose diet (Chapter 2). This diet is used as a model of NAFLD (Ackerman, Oron-Herman et al. 2005; Svegliati-Baroni, Candelaresi et al. 2006; Ahmed, Redgrave et al. 2009) and causes memory impairments in adult rats (Ross, Bartness et al. 2009). For these reasons, the fructose diet was chosen to study the whether NAFLD caused diet-induced memory impairments in rats fed during adolescence. There were a few disadvantages to using the high fructose diet. For example, this amount of fructose is not clinically relevant, as humans consume about 10% of their daily caloric intake as fructose (Vos, Kimmons et al. 2008). In addition, this diet was offered to the rats in a single pellet, which prevented the rat from choosing how much fructose to consume. This could be an important difference when investigating the interaction between diet and stress, because previous research indicates that involuntary consumption of high energy dietary components increases corticosterone release in response to a stressor (la Fleur, Houshyar et al. 2005). Finally, the control diet also induced NAFLD in control rats; therefore, if NAFLD contributes to the diet-induced memory impairments, then the control diet may have impaired memory. Therefore, the fructose diet was replaced with the high energy cafeteria-style diet for Chapters 3 and 4. This cafeteria-style diet is more clinically relevant than the 60% fructose diet. It contains excess fat in addition to sugar, and it offers these high energy foods in the context of healthy alternatives. In addition, the diet components are not all in one pellet like the fructose diet, which allows

the rat to choose how much of the high energy foods to consume. Of importance, this high energy cafeteria-style diet also causes NAFLD (Aoun, Michel et al. 2010; see Chapter 3) and hippocampal-dependent memory impairments that are similar to those observed in rats fed a 60% fructose diet (Ross, Bartness et al. 2009; Darling et al., unpublished). Of importance, both the high fructose and cafeteria-style high energy diets induced hippocampal-dependent memory impairments during stressful tasks (see Chapters 2 and 4). These results support previous findings showing that diets high in fat, sugar, or both impair hippocampal-dependent memory (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009; Darling, et al., unpublished).

In addition to diet, the age of the rats varied between experiments. In Chapter 2, the rats were fed a high energy diet beginning at weaning and throughout adolescence (post-natal days 21-60) into adulthood. Hippocampal-dependent memory was tested and presence of NAFLD was verified in adulthood. This timeline allowed for the investigation into the effects of a high energy diet fed during development on NAFLD and memory in adulthood. This is important because human adolescent males consume the most fructose (Vos, Kimmons et al. 2008; Marriott, Cole et al. 2009). In Chapters 3 and 4, the rats were fed the high energy diet beginning in adulthood, and liver function and hippocampal-dependent memory were tested in adult rats. These experiments employed adult rats because the effects of high energy diets on the ability of epinephrine to increase blood glucose concentrations and the emotional modulation of memory had not been established in adult rats. This was the first step before beginning to investigate the effects of these factors on development. Given the extensive plasticity that takes place in the brains of developing animals (Eayrs and Goodhead 1959; Aghajanian and

Bloom 1967; McIlwain and Bachelard 1971) and that the hippocampus is sensitive to dietary conditions during adolescence in rats (Granados-Rojas, Aguilar et al. 2004; Feoli, Siqueira et al. 2006; Jiang, Yu et al. 2012), it is possible that rats fed a high energy diet during adolescence may be more susceptible to diet-induced cognitive deficits when compared with rats fed a high energy diet during adulthood. The difference in age does not appear to be an issue when investigating the effects of high energy diets on NAFLD and hippocampal-dependent memory. To be specific, the high energy diets produce similar memory impairments in the water maze task in rats fed during adolescence and rats fed only during adulthood (see Chapter 2, (Ross, Bartness et al. 2009), and these impairments are similar to those observed in rats fed the cafeteria-style diet during adulthood (Darling et al., unpublished).

A third difference between these experiments is the amount of time the rats were fed the high energy diets in each experiment. The rats in Chapter 2 were fed the fructose diet for 12 weeks because control and fructose-fed rats tested at an earlier time point were unable to learn the water maze task (unpublished observation). The rats in Chapter 3 were fed the high energy diet for 9 weeks because it had been previously established that the cafeteria-style diet induced fatty liver and impaired memory in the water maze task in rats fed for this time period (Darling et al., unpublished). Finally, rats in Chapter 4 were fed the cafeteria-style diet for 4 weeks, because the high energy diet impaired water maze performance (Darling et al., unpublished) and potentiated the ability of epinephrine to increase blood glucose concentrations (unpublished observation) at this time. The difference in the amount of time spent consuming the high energy diets does not appear to be a concern. In each experiment, the high energy diet induced he-

patic lipid accumulation, impaired liver function and/or affected hippocampal-dependent memory. These results support findings from previous studies demonstrating that high energy diets impair hippocampal-dependent memory when rats are fed for at least 4 weeks (Molteni, Barnard et al. 2002; Stranahan, Norman et al. 2008; Ross, Bartness et al. 2009).

The behavioral task was also different between Chapters 2 and 4. In Chapter 2, rats were tested in a spatial water maze task, and in Chapter 4, rats were tested in a spatial object recognition task. It is important to note that both tasks are hippocampal-dependent (Morris, Garrud et al. 1982; Mumby, Gaskin et al. 2002; Broadbent, Squire et al. 2004). In Chapter 2, using the water maze task allowed for the comparison of diet-induced memory impairments observed in previous experiments (Ross, Bartness et al. 2009). In order to test the hypothesis that high energy diets impair the emotional modulation of memory, the experiments in Chapter 4 required a task in which the stressfulness could be manipulated. Using the spatial object recognition task permitted the use of a low stress or high stress version of the same task.

The results of the current experiments indicate that high energy diets interact with stress to impact hippocampal-dependent memory. These effects on memory may be the result of an increased amount of circulating glucose. To be specific, high energy diets increase the amount of circulating epinephrine (Kaufman, Li et al. 1993), which increases the amount of circulating glucose (Sacca, Vigorito et al. 1983). In addition, epinephrine-stimulated glucose release was potentiated by high energy diets (see Chapter 3). Therefore, there was a significant increase in the amount of glucose and available to the brain (see Chapter 3), and the concentration of glucose was beyond the mid-range

concentration that enhances memory (Gold 1986; Gold, Vogt et al. 1986). In fact, it is possible that the amount of glucose in the brain exceeds the amount that influences memory. Therefore, stress may not be able to enhance memory in high energy-fed rats as it can in control rats. The results of the first experiment in Chapter 4 support this interpretation.

The current experiments set the stage for future experiments. For example, additional experiments are needed to confirm that glucose is increased in the hippocampus during a stressful task in rats fed a high energy diet. These future experiments could employ microdialysis and spectrophotometry to measure the amount of glucose present in the hippocampus of these rats. In addition, there may be other brain areas involved in the interaction between high energy diets and stress on memory. For example, epinephrine stimulates the vagus nerve (Miyashita and Williams 2006), and the vagus nerve sends connections to the amygdala (Fallon, Koziell et al. 1978; Riche, De Pommery et al. 1990; Petrov, Krukoff et al. 1993). Previous research indicates that the amygdala is important for the emotional modulation of memory (Gold and van Buskirk 1978; Roozendaal, Hui et al. 2006; Roozendaal, Okuda et al. 2006). The current studies do not investigate this pathway, but it is possible that high energy diets could impact epinephrine-stimulated vagus nerve activity. Finally, the present experiments do not address how high energy diets directly impact the liver in order to potentiate the ability of epinephrine to increase blood glucose concentrations (see Chapter 3). These experiments do suggest that insulin resistance (IR) may be responsible for this effect, but additional studies are needed to confirm the presence of IR. An alternative hypothesis is that there is an increase in the number of epinephrine receptors in the liver. Previous research has

shown that epinephrine receptors are increased in hepatic tissue in obese mice (Begin-Heick 1994). In addition, future experiments could measure the amount of glycogen, or stored glucose, in the liver of high energy-fed rats. High energy diets increase hepatic glycogen stores (Hoefel, Hansen et al. 2011), and this increase in a glucose precursor could result in more glucose available for epinephrine to release into the bloodstream in high energy-fed rats.

In summary, the present experiments add to the growing body of literature demonstrating that high energy diets are harmful to both physiology and cognition. These studies demonstrate that a diet that closely resembles the “Western” diet impairs liver function and negatively impacts memory. Most importantly, the current experiments identify an interaction between high energy diets and stress in which the memory-enhancing effects of stress are eliminated.

## 5.1 References

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- Zavaroni, I., S. Sander, et al. (1980). "Effect of fructose feeding on insulin secretion and insulin action in the rat." Metabolism **29**(10): 970-3.

## APPENDICES

### Appendix A: Curriculum Vitae

#### **Amy Patricia Ross**

Neuroscience Institute  
Georgia State University  
920 Petit Science Center  
100 Piedmont Ave SE  
Atlanta, GA 30303

Laboratory: 404-413-6345  
Fax: 404-413-5471  
Cell: 412-849-8292  
email: aross17@student.gsu.edu

#### **EDUCATION:**

DEGREE: Bachelor of Science

MAJOR and ADVISOR: Psychology, John Mullinnex

MINOR: Biology

INSTITUTION: University of Pittsburgh at Johnstown, Johnstown, PA (2001-2005)

DEGREE: Master of Arts

MAJOR and ADVISOR: Neuropsychology and Behavioral Neuroscience, Marise Parent

INSTITUTION: Georgia State University, Atlanta, GA (2005-2008)

DEGREE (Currently seeking): Doctor of Philosophy in Neuroscience

MAJOR and ADVISOR: Neuroscience, Marise Parent

INSTITUTION: Georgia State University, Atlanta, GA (2008 – present)

#### **RESEARCH EXPERIENCE:**

Fall 2003-Spring 2005, Undergraduate Research Assistant with Dr. John Mullennix,  
University of Pittsburgh at Johnstown - Voice Perception Research

Summer 2005, Research Assistant with Dr. Marise Parent, Georgia State University –  
Neurobiology of Learning and Memory Laboratory

Fall 2005-present, Graduate Student with Dr. Marise Parent, Georgia State University –  
Neurobiology of Learning and Memory Laboratory

#### **PUBLICATIONS:**

**Ross, A.P.**, Bartness, T.J., Mielke, J.G., & Parent, M.B. (2009). A high fructose diet impairs spatial memory in male rats. *Neurobiology of Learning and Memory*, *92*, 410-416.

Mullennix, J.W., **Ross, A.**, Smith, C., Kuykendall, K., Conard, J., & Barb, S. (2011). Typicality effects on memory for voice: Implications for eyewitness testimony. *Applied Cognitive Psychology*, *25*, 29-34.

Bruggeman, E.C., Li, C., **Ross, A.P.**, Doherty, J.M., Williams, B.F., Frantz, K.J., & Parent, M.B. (2011). A high fructose diet does not affect amphetamine self-administration or spatial water maze learning and memory in female rats. *Pharmacology, Biochemistry and Behavior*, *99*, 356-364.

**Ross, A.P.**, Bruggeman, E.C., Kasumu, A.W., Mielke, J.G., & Parent, M.B. (2012). Non-alcoholic fatty liver disease impairs hippocampal-dependent memory in male rats. *Physiology and Behavior*, 106, 133-141.

#### **POSTERS AND PRESENTATIONS:**

- Ross, A.**, Socha, J., & Shawley, N. (2004). Typicality, familiarity, and distinctiveness voice ratings for classification in human cognition. Poster presented at the 7<sup>th</sup> Annual Laurel Highlands Undergraduate Psychology Conference, Johnstown, PA, March, 2004.
- Mullennix, J.W., **Ross, A.**, Socha, J., Kocsis, J.J., & Shawley, N. (2004). Evidence for male and female voice prototypes. Poster presented at the 16<sup>th</sup> Annual American Psychological Society Meeting, Chicago, IL, May, 2004.
- Ross, A.**, Smith, C., & Mullennix, J.W. (2005). Do voice typicality ratings predict confusions for voice memory? Poster presented at the annual meeting of the Midwestern Psychological Association Conference, Chicago, IL, May, 2005.
- Silva, M.M., **Ross, A.P.**, Bartness, T.J., & Parent, M.B. (2006). The effects of a high fructose diet on body mass, fat pad distribution, body lipids, and blood plasma measurements. Poster presented at the Behavioral Research Advancements in Neuroscience Summer Symposium, Emory University, August, 2006.
- Ross, A.P.**, Brito, M.N., Brito, N.A., Harris, R.B., Bartness, T.J., & Parent, M.B. (2006). A high fructose diet may impair memory in rats. Poster presented at the annual meeting of the Society for Neuroscience, Atlanta, GA, October, 2006.
- Krench, M., **Ross, A.**, & Parent, M. (2007). Sustaining live hippocampal tissue in artificial cerebral spinal fluid as indicated by the reduction of 2,3,5-triphenyl tetrazolium chloride to formazan. Poster presented at the Behavioral Research Advancements in Neuroscience Summer Symposium, Emory University, August, 2007.
- Muldrow, A.D., **Ross, A.P.**, & Parent, M.B. (2007). The effects of a high fructose diet on body functioning. Poster presented at the Ronald E. McNair Summer Research Poster Session, Georgia State University, August, 2007.
- Ross, A.P.**, Bartness, T.J., Mielke, J., Fernander, K., & Parent, M.B. (2007). Effects of a high fructose diet on hippocampal-dependent learning and memory. Poster presented at the annual meeting of the Society for Neuroscience, San Diego, CA, November, 2007.
- Koire, A.M., **Ross, A.P.**, & Parent, M.B. (2008). High fructose diet may affect hippocampal-dependent memory in juvenile rats. Poster presented at the Behavioral Research Advancements in Neuroscience Summer Symposium, Emory University, July, 2008.
- Ross, A.P.**, Kasumu, A., Bruggeman, E., Kimmons, J., Blanck, H.M., & Parent, M.B. (2008). The effects of consuming a high fructose diet beginning in adolescence on adult rat hippocampal-dependent memory. Poster presented at the annual meeting of the Society for Neuroscience, Washington, D.C., November, 2008.
- Bagley, B.J., **Ross, A.P.**, Kimmons, J., Blanck, H.M., & Parent, M.B. (2008). Effects of feeding adolescent rats a high fructose diet on learning and memory. Poster presented at the Psychology Undergraduate Research Conference, Georgia State University, December, 2008. **\*\*Best Neuroscience Poster Award**

- Yee, E.F., **Ross, A.P.**, & Parent, M.B. (2009). Insulin resistance in adolescent fructose-fed rats. Poster presented at the Behavioral Research Advancements in Neuroscience Summer Symposium, Emory University, July, 2009.
- Frantz, K.J., Lee, A., Doherty, J., Li, C., Williams, B., **Ross, A.P.**, Bruggeman, E., & Parent, M.B. A high fructose diet does not affect amphetamine self-administration in female rats. Poster presented at the annual meeting of the Society for Neuroscience, Chicago, IL, November 2009.
- Young, T.B., **Ross, A.P.**, Kimmons, J., Blanck, H.M., & Parent, M.B. (2009). The effect of high fructose consumption beginning in adolescence on adult hippocampal-dependent memory. Poster presented at the Psychology Undergraduate Research Conference, Georgia State University, October, 2009. **\*\*Best Neuroscience Poster Award**
- Ross, A.P.** (2010). Sugar on the brain: A high fructose diet impairs memory in male rats. Talk given at the W.M. Keck Center for Collaborative Neuroscience Student Presentation Day, February, 2010. **\*\*Best Oral Presentation Award**
- Ross, A.P.** (2010). Forget the fructose. Talk given at the Brains and Behavior Annual Retreat, April 2010.
- Ross, A.P.**, Darling, J.N., & Parent, M.P. (2010). Non-alcoholic fatty liver disease potentiates the effects of epinephrine on blood glucose levels. Poster presented at the annual meeting of the Society for Neuroscience, San Diego, CA, November, 2010.
- Darling, J.N., **Ross, A.P.**, Bartness, T.J., & Parent, M.P. (2010). Voluntary consumption of a high calorie diet impairs hippocampal-dependent memory in male rats. Poster presented at the annual meeting of the Society for Neuroscience, San Diego, CA, November 2010.
- Balakrishnan, L.J., **Ross, A.P.**, & Parent, M.P. (2011). Western Diet + Stress = Memory Impairment. Poster presented at the Psychology Undergraduate Research Conference, Georgia State University, October, 2011.

#### **PROFESSIONAL SOCIETIES:**

Society for Neuroscience, Spring 2006 - present  
Center for Behavioral Neuroscience, Spring 2006 - present

#### **HONORS, AWARDS and FELLOWSHIPS:**

Center for Behavioral Neuroscience Graduate Scholar, Fall 2005 – present  
Brains and Behavior Fellow, Fall 2009 – present  
Center for Behavioral Neuroscience Travel Award, Spring 2010  
Best Oral Presentation, W.M. Keck Center for Collaborative Neuroscience Student Presentation Day, February 2010  
Georgia State University Dissertation Award, Spring 2011  
Honeycutt Fellow, Fall 2011 - present

#### **TECHNIQUES, SKILLS and BEHAVIORAL ASSAYS:**

Brain cannulation surgeries  
Intracranial injections

Perfusions  
 Cryostat tissue sectioning  
 Thionin brain tissue staining  
 Oil red-o liver tissue staining  
 Brain dissection  
 Hippocampal slice incubation  
 SDS page and western blotting  
 ELISAs  
 Water maze  
 Spontaneous alternation  
 Object recognition  
 Inhibitory avoidance

**SERVICE:**

Graduate Student Representative, Neuroscience Institute Graduate Program  
 Committee, Fall 2008 – Fall 2009  
 Graduate Recruitment Activities – Spring 2006 – present  
 Brain Awareness Volunteer: Classroom visits, Brain Expos at Zoo Atlanta –  
 Spring 2007 – present  
 Mentor for BRAIN, ION, and McNair programs (see below)

**STUDENTS MENTORED:**

Nathan Waldron, Georgia State University, Spring 2006  
 Mariana Silva, North Carolina State University, BRAIN Intern, Summer 2006  
 Dorothy Bota, Decatur High School, ION Student, Summer 2006  
 Ade Kasumu, Spellman College, Spring 2007,  
 Georgia State University, Fall 2007-Spring 2008  
 Kevin Fernander, Georgia State University, Spring 2007-Summer 2007  
 Megan Krench, Penn State University, BRAIN Intern, Summer 2007  
 Aja Muldrow, Georgia State University, McNair Scholar, Summer 2007  
 Walid Radwan, Georgia State University, Fall 2007-Spring 2008  
 Bethany Bagley, Georgia State University, Summer 2008-Fall 2008  
 Amanda Koire, Pomona College, BRAIN Intern, Summer 2008  
 Thomas Brett Young, Georgia State University, Spring 2009-Spring 2010  
 Christopher Mylenbusch, Georgia State University, Spring 2009-Spring 2011  
 Estella Yee, Willamette University, BRAIN Intern, Summer 2009  
 Lalita Balakrishnan, Georgia State University, Fall 2010-present  
 Amanda Arnold, Georgia State University, Spring 2011-Summer 2011  
 Jenine Ampudia, Georgia State University, Spring 2011-present  
 Eseosaserea Igbini, Georgia State University, Summer 2011-present  
 Saima Masud, Georgia State University, Fall 2011-present