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Long-lasting and widespread effects of fructose on behavior, metabolism, and cytokine gene expression: Insight from *in vivo* and *in vitro* studies

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Abstract

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A rise in fructose consumption has paralleled a similar rise in metabolic dysfunction, particularly in Western countries. Fructose, a large component of added sugars in processed foods and beverages, is not only differentially metabolized by the body compared to glucose, but has also been linked to increases in metabolic disorders, impaired HPA axis activity, and inflammation. Adolescents, some of the top consumers of diets high in fructose, may be more susceptible to the detrimental effects of fructose enriched foods and other environmental factors such as chronic stress. In the presented studies, I first evaluated the effects of a high fructose diet and chronic stress on behavior and HPA axis in adolescent male rats. In behavioral testing during adulthood, only fructose-fed animals demonstrated an increase in depressive-like and anxiety-like behaviors, as well as HPA axis hyperactivity. Notably, these animals were not more vulnerable to the consequences of chronic stress. In the second experiment, I further hypothesized that fructose-fed animals would demonstrate altered metabolic states. Our data indicated that a high-fructose diet fed during adolescence does in fact alter weight, food consumption, blood glucose, and visceral adiposity. In order to determine whether fructose was directly having an effect on neuronal and glial cell survival and gene expression, I used an in vitro model. I found that fructose exposure in neurons and co-cultures of neurons and microglia was detrimental to cell viability. Next, I evaluated inflammation as a potential mechanism for the changes observed in vivo and in vitro. Surprisingly, my data suggested that proinflammatory gene expression is decreased in neurons and co-cultured neurons and microglia. I propose that the cytokines TNF-α and IL-6 are downregulated in co-cultures due to the protective roles of these cytokines and cross-talk between neurons and microglia. These studies not only demonstrate the damaging effects of a high fructose diet given to adolescent rats, but they correspondingly illustrate the negative effects of fructose on cultured neurons and microglia.

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1. Introduction

Environment plays a critical role in shaping behaviors by altering the expression and function of proteins throughout the body and brain. Two key environmental factors are diet and stress exposure, both of which can have positive and negative effects. In these studies, I assessed the hypothesis that a high fructose diet and chronic stress would interact to alter metabolism and behavior. Further, I used an *in vitro* neuron and microglia co-culture model to determine the extent to which fructose directly influences these cells. This paper first reviews fructose consumption in US adults and adolescents and discusses the correlation between the rise in fructose consumption and the increased prevalence of metabolic dysfunction. In the second section, the HPA axis is presented as another factor that influences metabolism, as well as affective states. When chronically stimulated by prolonged exposure to stressors, the downstream actions of the HPA axis negatively alter metabolic and affective behaviors. A potential mechanism mediating this relationship between the HPA axis, metabolism, and affective states is the activation of inflammatory pathways. Evidence for this hypothesis is further discussed in light of our understanding about the role of inflammation in metabolic disorders and mood disorders. In the final section, I summarize the key experimental studies on the physiological and neurological effects of fructose consumption.

Fructose Consumption and the Metabolic Syndrome

Although the recommended amount of energy originating from carbohydrates is between 45% and 65%, the total amount of energy derived from added sugars should not exceed 25% (Institute of Medicine, 2005), and the World Health Organization recommends an even stricter

limit at 10% of total energy for both children and adults (World Health Organization, 2014). Nonetheless, the heaviest consumers of added sugars consume an average of 27% of calories from these added carbohydrates (Havel, 2005; Marriott, Olsho, Hadden, & Connor, 2010). Notably, adolescents make up a large portion of the top consumers (Havel, 2005). In one study of 8th graders, researchers found that approximately 32.4% of consumed calories were derived from added sugars in this sample (Havel, 2005; Dwyer et al., 2001). Added sugars typically contain 50-60% fructose, indicating that fructose is a significant component of consumption of added sugars. Consequently, per capita fructose consumption increased approximately 25% between 1975 and 2000, mainly through the consumption of sugars added to processed foods and sweetened beverages (Havel, 2005). Due to the continuing prevalence of added sugars in Western diets, we expect that fructose consumption has continued to increase to this date.

The significant increase in fructose consumption paralleled an increase in metabolic syndrome (MetS) and obesity among adults, children, and adolescents across the same time period (NIH, National Heart, Lung, and Blood Institute, 2011). MetS refers to a group of metabolic characteristics that are associated with an increased risk of developing metabolic disorders, such as type-2 diabetes and cardiovascular disease and subsequently, a higher risk of morbidity and mortality. MetS and cardiovascular disease are also associated with an increased risk for depression, and depressed individuals are more susceptible to metabolic dysfunction (Knol et al., 2006; Luppino et al., 2010). Consequently, detecting, treating, and preventing these risk factors is critically important for public health. A recent study compared the prevalence of MetS in US adults between 1988-1994 and 1999-2006. Researchers found that MetS had increased from 27% to 34% in US adults between these two time periods, with abdominal obesity and high blood pressure primarily driving the increase in both women and men

(Mozundar & Liguori, 2011). Approximately 4% of adolescents evaluated between 1988 and 1994 met the criteria for MetS, and obesity in this sample positively correlated with prevalence of MetS (Cook, Iitzman, Auinger, Nguyen, & Dietz, 2003). Rates of MetS among adolescents may be even higher in recent years due to the increase in overweight and obese children and adolescents since the early 2000s (Cook et al., 2003). The increasing rate of childhood and adolescent obesity and metabolic dysfunction is a significant problem due to the long-lasting and extensive effects that obesity and MetS can have later in life (Ebbeling, Pawlak, & Ludwig, 2002). Only recently has focused been directed towards researching and addressing this problem. One area of interest is the role of diet in promoting or preventing these metabolic disease states.

Fructose Metabolism

Although fructose and glucose have the same chemical formula, their distinctive structures result in key differences in how they are metabolized. When ingested, fructose is absorbed into intestinal absorptive cells called enterocytes located in the duodenum and jejunum. In the gut, some fructose is converted into lactate, glucose, and fatty acids, with approximately 25% converted into lactate (Sun & Empie, 2012; Tappy & Lê, 2010). Fructose is then transported to the portal bloodstream, which relocates the sugar to the liver via the portal vein. After reaching the liver, fructose can be converted into fructose-1-phosphate by fructokinase. Fructose-1-phosphate is then metabolized into triose phosphates, glyceraldehyde, and dihydroxyacetone phosphate, compounds that further increase circulating glucose and lactate levels by entering characteristic glycolytic pathways.

During classic metabolism, the enzyme phosphofructokinase is negatively regulated by downstream products of metabolism. Specifically, a hexose such as glucose is phosphorylated by hexokinase to become fructose 6-phosphate, which is subsequently phosphorylated to become

fructose 1,6-bisphosphate by the rate-limiting enzyme phosphofructokinase. Phosphofructokinase is regulated by citrate and ATP through allosteric inhibition and therefore serves as a signal to indicate that the cell does not need more energy produced or carbons transferred into the citric acid cycle. In contrast, fructose metabolism is not negatively regulated due to the actions of fructokinase. In tissues expressing fructokinase, fructose is rapidly converted into fructose-1-phosphate and is not inhibited by increasing levels of downstream products. Due to its separate regulatory pathway, fructose can be shunted into production of very low-density lipoprotein (VLDL) and triglycerides by providing substrates for acetyl-CoA, the central component of lipid metabolism. Fructose metabolism also stimulates further production of glucose from lactate and pyruvate (Elliott, Keim, Stern, Teff, & Havel, 2002). Elevations in lipid and glucose production result in glucose intolerance, insulin resistance, and overall increases in adiposity, through a variety of mechanisms that impair insulin receptor sensitivity and expression (Havel, 2005; Lyssiotis & Cantley, 2013). Insulin resistance and dyslipidemia are key components of the MetS, suggesting that the unique features of fructose metabolism may be some of the direct causes of metabolic dysfunction in the heaviest consumers of fructose.

Although fructose metabolism is thought to take place mainly in the liver, the selective fructose transporter, GLUT5, is expressed in muscle and the brain (Lane & Cha, 2009; Shepherd, Gibbs, Isslau, Gould, & Kahn, 1992). Therefore, extrahepatic metabolism of fructose may play a prominent role in disease states. In the CNS, fructose metabolism can be similar to hepatic metabolism in that fructose skips the rate-limiting step of glycolysis or fructose can be converted directly into fructose-6 phosphate and enter glycolysis like glucose (Lane & Cha, 2009). It is still unclear which metabolic pathway fructose enters in specific brain regions. Nonetheless, research

suggests that the hypothalamus, an important region in modulating food intake, metabolizes fructose by skipping the regulatory step. In the hypothalamus, this type of fructose metabolism results in ATP depletion and appetite stimulation (reviewed in Lane & Cha, 2009).

The relationship between food intake and metabolism encompasses carefully orchestrated processes that, when gone awry, can have powerful effects on health. Fructose-fed animals have consistently been shown to increase in body mass and adiposity. The main hypothesis for these results is that hormonal regulation of appetite is absent after fructose consumption. Glucose stimulates the secretion of appetite-suppressing hormones leptin and insulin, and suppresses the appetite-stimulating hormone ghrelin. Conversely, fructose does not stimulate leptin secretion, impairs leptin's ability to promote signals of satiety, fails to suppress ghrelin, and does not require insulin for its metabolism (Melanson et al., 2007; Tappy & Lê, 2010; Tillman, Morgan, Rahmouni, & Swoap, 2014; White, 2013). Specifically, insulin is released by pancreatic β-cells after feeding and results in appetite suppression and energy expenditure. Similarly, leptin secreted by adipocytes is responsible for long-term satiety after a meal. Because fructose consumption does not stimulate the release of these hormones, overeating can occur. Increases in caloric intake further lead to lipid production and storage, which can in turn impair insulin and leptin signaling (Elliott et al., 2002).

Chronic Stress, the Body and Brain

HPA Axis

In addition to diet, stress is an environmental factor that can have long-lasting and profound effects on an individual's brain and physiology. In order to maintain homeostasis, the body responds to stressful stimuli by altering biological systems, including the metabolic,

endocrine, and cardiovascular systems. The HPA axis can be activated by a variety of stressors, including environmental stimuli, as well as internal cues such as proinflammatory cytokines, key effectors of the innate immune system located in both the periphery and CNS. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system to elicit these biological changes. The HPA axis is especially important in producing the stress response due its release of glucocorticoids, the main stress hormones that act upon universally expressed receptors throughout the body to not only elicit metabolic and cardiovascular responses, but also shut off the stress response by means of a negative feedback loop. Briefly, in response to stressors, the HPA axis functions as follows: the paraventricular nucleus (PVN), found in the hypothalamus, releases corticotropin-releasing factor (CRF) into the portal system that then transports CRF to the anterior pituitary gland. CRF action in the pituitary further promotes the release of adrenocorticotropic hormone (ACTH) into circulation. ACTH travels to the adrenal cortex where it leads to the production and release of glucocorticoids (summarized in Smith & Vale, 2006). Glucocorticoids in the bloodstream, including cortisol in humans and corticosterone (CORT) in rodents, act on glucocorticoid receptors in both the periphery and central nervous system (CNS). In response to glucocorticoid binding, the cytoplasmic receptors are translocated into the nucleus where they enhance or suppress transcription of a wide range of genes, including the suppression of pro-inflammatory genes (Kadmiel & Cidlowski, 2013).The physiological effects of glucocorticoids, including changes in metabolic, cardiovascular, and immune function, allow the body to respond to stressors and aid in returning the body to homeostasis after the action of stressors (Neigh & Nemeroff, 2006; Pariante & Lightman, 2008).

The HPA axis is tightly regulated, mainly through the action of glucocorticoids and their receptors in the pituitary, PVN and other areas of the hypothalamus. The hippocampus and

prefrontal cortex are also important for shutting off the stress response. A large portion of research has been devoted to the hippocampus's role in negative feedback, in part due to the high expression of glucocorticoid receptors in this region, as well its other functions in learning, memory, and mood disorders (Herman & Cullinan, 1997). The hippocampus negatively regulates the release of CRF by responding to increased amounts of circulating glucocorticoids (Brown, Rush, & McEIn, 1999). Lesion studies illustrate this interaction, illustrating that hippocampal lesions result in hypersecretion of glucocorticoids whereas stimulation of the hippocampus inhibits the stress response (Brown et al., 1999).

Effects of Chronic Stress

Although physiological responses to transient stressors are typically adaptive, chronic levels of stress can be detrimental due to sustained activation of the HPA axis. In particular, chronic activation of the HPA axis considerably influences metabolism, eventually leading to accumulation of visceral adipose tissue, loss of muscle, and characteristics of the metabolic syndrome, including hypertension, type 2 diabetes, and dyslipidemia (reviewed in Pervanidou & Chrousos, 2012). The actions of glucocorticoids could explain some of the metabolic alterations resulting from chronic stress. Sustained elevations of glucocorticoids in the blood can result in insulin hypersecretion. Furthermore, due to the appetite stimulating properties of glucocorticoids, prolonged increases of the stress hormones lead to weight gain (Pervanidou & Chrousos, 2012). For example, in one study, mice given high concentrations of corticosterone in their drinking water gained more weight and secreted more insulin and leptin, demonstrating that glucocorticoids, apart from chronic stress, are able to alter rodent metabolism (Tamashiro, Sakai, Shively, Karatsoreos, & Reagan, 2011). Notably, the weight gain observed in many of these studies can be attributed to increased consumption of high-fat and high-sugar substances (Torres

& Nowson, 2007). Chronic activation of the HPA axis has also been associated with diabetes, suggesting that the a chronically active HPA axis affects multiple components of MetS (Tamashiro et al., 2011).

Chronic stress, and in particular glucocorticoid signaling, alters the CNS in numerous ways that have serious effects on behavior. In general, elevations of glucocorticoids are associated with atrophy of the cerebral cortex, ventricular dilation, cognitive deficits, and neurotoxicity (Phillips et al., 2006). Stress-related damage to the hippocampus and associated HPA-axis dysregulation is one hypothesis for depression and other mood disorders (Brown et al., 1999; Pariante & Lightman, 2008). A key piece of evidence for this hypothesis the observation that high levels of circulating cortisol are frequently detected in depressed individuals. Alternatively, dysregulation of negative feedback has been implicated in the pathophysiology of depression as well. For example, non-suppression of ACTH or cortisol in the dexamethasone suppression test (DST) is frequently characteristic of individuals with mood disorders. In the DST, the ability to suppress the HPA axis is evaluated in individuals by administering synthetic glucocorticoids that, in healthy individuals, bind to glucocorticoid receptors and inhibit the secretion of ACTH and therefore decrease circulating cortisol. In many depressed individuals, suppression of cortisol or ACTH is not observed after dexamethasone administration. One study reported that up to 43% of individuals with major depressive disorder (MDD) had non-suppression in the DST (Arana et al., 1985). Furthermore, smaller hippocampi are associated with individuals who have depression, implying that an inability to suppress the HPA axis through negative feedback may produce or exacerbate mood disorders (Brown et al., 1999). Alternatively, smaller hippocampi in this population may be the consequence of depression. Both hypotheses illustrate the importance of the hippocampus and HPA axis in the pathophysiology of

depression. In addition to glucocorticoids, the expression of glucocorticoid receptors has been implicated in depression in humans and animal studies (Bourke et al., 2013; Bourke, Harrell, & Neigh, 2012; Neigh & Nemeroff, 2006). Changes in receptor expression or sensitivity in the hippocampus and other regulatory regions result in impaired negative feedback of the stress response, possibly resulting in a depressed phenotype or increasing susceptibility to the detrimental effects of stress. Depression is associated with cognitive impairment, which is relevant given the central role of the hippocampus in learning and memory. Similarly, individuals with high cortisol irrespective of stress levels also have lower performance on cognitive and memory tests (Phillips et al., 2006).

Further evidence linking chronic elevations in glucocorticoids to neurophysiological changes comes from the adrenal disorder, Cushing's disease, which is characterized by excessive production of cortisol. These individuals have reduced hippocampal volumes, lower verbal memory skills, and high rates of depressive symptoms and obesity (Brown et al., 1999; McEIn, 2005). Overall, human research provides evidence for the hypothesis that some types of depression are associated with changes in HPA axis activity.

Animal studies have shed light onto some of the mechanisms that may underlie the relationship between stress and mood disorders. These models typically consist of psychological, social, or physical stressors or a combination of them and generally use rodents (Bourke & Neigh, 2011; Burgado et al., 2014; Pyter, Kelly, Harrell, & Neigh, 2013). Chronic stress in these models elicits depressive-like and anxiety-like behaviors as well as cognitive and memory impairments in rodents (Bourke & Neigh, 2011; Burgado et al., 2014; Pyter, Kelly, Harrell, & Neigh, 2013). Chronic stress results in neuronal damage, suppression of long-term potentiation, and decreases in neurogenesis in the hippocampus. Without stress, exposure to high levels of

corticosterone is associated with hippocampal damage, namely reduced neurogenesis and atrophy of neurons (McEIn, 2005; Phillips et al., 2006). Overall, stress, and in particular stress-induced production of glucocorticoids, results in significant behavioral changes associated with inhibition of neuroplasticity, most notably in the hippocampus. These neurological effects are detrimental to cognitive function and psychological health.

The type and severity of the consequences of chronic stress are dependent of many factors, including age. Early life stress is not only associated with an increased risk of developing mood and psychiatric disorders, but also with metabolic dysfunction. Retrospective studies in humans have demonstrated that individuals who undergo childhood or adolescent trauma are at risk of developing obesity and MetS (Pervanidou & Chrousos, 2012). Both physical and emotional stress are associated with dysregulation of eating patterns, possibly in response to changes in leptin/insulin levels and sleep patterns. One reason for this relationship is that the brain and body are developing during these sensitive periods. In particular, the gonadal, growth hormone, and thyroid axes, which regulate growth and development, are regulated by the HPA axis and therefore sensitive to the effects of chronic stress (Pervanidou & Chrousos, 2012). Moreover, chronic stress may have more permanent effects on the developing brain and body due to their plastic nature. Consequently, the harmful effects of persistent elevations of glucocorticoids, namely cell dysfunction and death, can place children and adolescents who experience these elevations during a critical period of development at an even higher risk for developing MetS, depression, anxiety disorders, and other behavioral outcomes (Cook et al., 2003).

Immune System: Role in Stress Response, Metabolic Dysfunction, and Mood Disorders

Due to the detrimental effects of chronic stress and metabolic dysfunction on health and cellular activity, an emerging hypothesis states that the immune system, in particular inflammatory pathways, play a significant part in promoting disease states. The immune system, composed of the innate and adaptive systems, is responsible for responding to infection and tissue damage (Barton, 2008). Coordinated responses from a variety of cells usually lead to the destruction of pathogens and infected or damaged cells. A key component of the innate immune system is the inflammatory response. In brief, the inflammatory pathway begins with recognition of pathogens through pattern recognition receptors and subsequent signaling to other inflammatory and immune cells. Proinflammatory cytokines and chemokines, such as the family of tumor necrosis factor (TNF) and interleukins (IL), are key in recruiting immune cells like neutrophils and macrophages which destroy the pathogens and infected or damaged cells. As previously mentioned, proinflammatory cytokines can also activate the HPA axis and consequently promote glucocorticoid signaling throughout the body and brain (Dunn, Swiergiel, & De Beaurepawere, 2005). When the body is successful in defeating the infection, the immune system works to restore the balance back to an anti-inflammatory state (reviewed in Barton, 2008). Inappropriate or over-stimulation of inflammatory responses may result in negative outcomes for peripheral and central tissues. A wide array of disorders, such as psychiatric illnesses, cancers, autoimmune disorders, and metabolic diseases, are associated with elevations in inflammatory signaling (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Hotamisligil, 2006; Miller, Maletic, & Raison, 2009). In subsequent sections, focus is placed on the role of inflammation in metabolic disorders and depression.

Inflammation and Metabolic Dysfunction

Peripherally, obesity is associated with increases in pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), in rodent and human adipose and liver tissues (Hotamisligil, 2005; Park et al, 2010; Kern et al., 2001). Increased cytokine production and general activation of the inflammatory pathways in the body are also associated with insulin resistance and type 2 diabetes (Hotamisligil, 2006). In particular, TNF-α plays a central role in the inflammatory response, insulin sensitivity, and glucose metabolism. Studies indicate that suppression of TNF-α in obese mice improves glucose homeostasis and insulin sensitivity whereas administration of TNF-α promotes insulin insensitivity, a key component of the MetS (reviewed in Hotamisligil, 2006). Importantly, adipose tissue in obese humans and mice is the primary location of inflammation, with morphological signs indicating the presence of immune cells such as macrophages (de Heredia, Gómez-Martínez, & Marcos, 2012).

In addition to peripheral inflammation, metabolic dysfunction has been associated with neuroinflammation (Purkayastha & Cai, 2013). Expression of proinflammatory markers is elevated in the hypothalamus in response to overeating. These markers, in particular NFkB/IKKB, correlate with progression of the metabolic disorders. Moreover, neurons and microglia are explicitly affected by diet-induced obesity and insulin insensitivity. In response to a high-fat diet, microglia upregulate production of cytokines such as IL-6. Insulin and leptin signaling are also disrupted in models of metabolic syndrome and are linked to proinflammatory mechanisms in the CNS. Notably, these changes, which further lead to metabolic disruptions in the body and an inflammatory state in the brain, can arise without an obese or overweight phenotype, indicating that obesity may be another symptom and not the cause of neuroinflammation (reviewed in Purkayastha & Cai, 2013)

Inflammation and Affective Disorders

The etiology and progression of depression and other affective disorders have been extensively studied in humans and animal models. One well-established hypothesis is that inflammation plays a key role in the psychopathology of affective disorders, in particular depression. Individuals who experience prolonged periods of sickness typically develop sickness syndrome, a phenotype that is consistent with depression even though these individuals do not have a history of mental illness (Dantzer et al., 2008). Increased in proinflammatory cytokines, chemokines, and adhesion molecule expression in the blood and cerebrospinal fluid are evident in patients with depression (Brown et al., 1999; Dantzer et al., 2008; Raison, Capuron, & Miller, 2006). The upregulation of adhesion molecules, such as macrophage chemoattractant protein-1 (MCP-1), are important for promoting the innate immune system (Chensue et al., 1996). Moreover, severity of depressive episodes correlates with concentrations of cytokines, namely IL-1, IL-6, and TNF-α, and susceptibility to depression is associated with some allelic variants of these proinflammatory cytokines (Lanquillon, Krieg, Bening-Abu-Shach, & Vedder, 2000; Levine et al., 1999; Miller et al., 2009). In animal models, exposure to high concentrations of the same cytokines systemically or in the hippocampus results in behavioral symptoms of depression, such as anhedonia and sleep impairments (Dunn et al., 2005). Furthermore, cytokine exposure affects neurotransmitter systems, including serotonin, norepinephrine, and dopamine systems, which are all implicated in the psychopathology of depression. The amygdala and hippocampus experience significant alterations in neurotransmitter metabolism after TNF-α and IL-6 administration (Raison et al., 2006). It is evident that the immune system plays a role in disease states beyond pathogenic infections, including obesity and affective disorders.

Fructose: Current Insights

Fructose and Metabolism. With the increase in fructose consumption and metabolic syndrome in the population, and the role of inflammation in promoting disease, an increasing number of studies are evaluating the consequences of fructose exposure in both humans and animal models.

Human Studies. The rise in added sugar and fructose consumption alongside the prevalence of MetS has encouraged researchers to study the acute and chronic effects of simple carbohydrate consumption. In one classic study, young men receiving a diet with 200 g of sucrose, a disaccharide composed of fructose and glucose, per day had increased triglycerides and hyperinsulinemia compared with men who had diets supplemented with starch, another carbohydrate that has similar effects on blood glucose levels (Akinyanju et al., 1968). Faeh and colleagues continued this early work by illustrating that diets high in fructose impaired glucose tolerance and promoted insulin resistance in men (Faeh et al., 2005). Other researchers have found similar results, with diets high in sucrose and fructose promoting the classic signs of MetS (reviewed in Johnson et al., 2007).

Animal Studies. Fructose-fed rats develop key components of the MetS whereas the same concentrations of glucose do not induce these metabolic changes (Nakagawa et al., 2006). In particular, results from high fructose diets in rodent models have demonstrated fructose-induced increases in triglycerides, resulting from triglyceride overproduction in conjunction with decreased clearance. Moreover, fructose-induced insulin insensitivity and impairments in leptin signaling are directly related to triglyceride production and accumulation (Basciano, Federico, & Adeli, 2005; Storlien, 1988).

Fructose and Behavior. Little research has been done evaluating the effects of a high-fructose diet on animal or human behavior, although a small body of literature on fructose-induced changes in learning and memory has recently evolved. For example, Ross and colleagues (2009) demonstrated the ability of a fructose diet to induce a spatial memory deficit in male rats. Other studies, also in rats, are consistent with these data (Wu et al., 2014).

Fructose, HPA Axis, and Inflammation. Recently, researchers demonstrated that a high fructose diet dysregulated glucocorticoid signaling in rat adipose tissue (Bursać et al., 2013; Kovačević, Nestorov, Matić, & Elaković, 2014). In one study, female rats fed a diet high in fructose had decreased expression and sensitivity of glucocorticoid receptors in adipocytes (Kovačević et al., 2014). Another group found that male rats on a high fructose diet had elevated Cort levels and receptor translocation into the nucleus (Bursać et al., 2013). As the main effectors of the stress response, glucocorticoids and their receptors are important regulators of physiological processes including energy metabolism and affective states. Therefore, a fructose diet's ability to dysregulate production and functionality of glucocorticoids and the receptors indicates a potential mechanism explaining the metabolic and behavioral alterations observed in fructose-fed animals and in the general population.

Fructose consumption has also been linked to increased inflammation in recent studies. In 2014 researchers in Mexico found that a high fat and high fructose diet reduced dendritic spine density and arborization in the hippocampus while increasing immune cell activation (Calvo-Ochoa, Hernández-Ortega, Ferrera, Morimoto, & Arias, 2014). Other groups have found increases in cytokine production and fructose toxicity in the brain in fructose-fed animals (Djordjevic et al., 2013; Farooqui, 2013). Interestingly, one study found hypothalamic gene remodeling in fructose-fed rats, with particular enrichment of immune and inflammatory pathways (Neigh, Johnson &

Harrell, 2013). In this study, fructose-fed rats had differentially expressed genes in the hypothalamus compared to chow-fed animals. Research into the potential of a high fructose diet to directly affect brain function may provide insight into the harms and benefits of certain diets and nutrients on the CNS.

Current Study

This study focuses on the effects of a high-fructose diet on behavior, metabolic outcomes, and HPA axis activity in male rats, in addition to the effects of fructose on cell survival and proinflammatory gene expression in cultured neurons and microglia. Importantly, this study differs from previous work in that it focuses on the effects of high-fructose consumption during adolescence and explores the potential interaction of the diet with chronic stress. Adolescence is a critical period characterized by significant changes in endocrine and neurological systems. Recent increases in fructose consumption and metabolic dysfunction in the adolescent population suggest that diets high in fructose can be especially detrimental to this age group. The chronic stress component serves to illustrate how a combination of environmental factors, like stress and diet, can interact to alter physiology and behavior. Furthermore, this study continues recent work to elucidate the effects of fructose on neural and glial cell survival and neuroinflammation.

In the first experiment, I hypothesized that male rats fed a high-fructose diet on a background of chronic stress during adolescence would demonstrate depressive-like and anxiety-like behaviors and altered HPA axis signaling later in adulthood. This hypothesis was based on current research that illustrates the ability of a high fructose-diet to alter metabolism, glucocorticoid signaling, and rodent behavior. In brief, I postulated that the high fructose diet would make male rats susceptible to chronic stress by altering HPA axis activity. In my second experiment, I sought to determine the metabolic effects of a high-fructose diet in adolescent

animals, irrespective of stress history. I predicted that the fructose diet would increase fat pad mass and significantly alter food intake, weight, and glucose metabolism. Previous studies have clearly illustrated the ability of a high fructose diet to elicit metabolic dysfunction in rodents, and also possibly in humans. In order to clarify the role of a high fructose diet on behavior, HPA axis activity, and metabolism, I determined the effects of fructose on neuronal and microglial cell survival, proliferation, and proinflammatory gene expression *in vitro*. It is unclear whether and how fructose can directly influence neuronal or glial gene expression and survival and whether the effects seen *in vivo* were driven by the metabolic changes, an increase in inflammation in the brain, or some other mechanism yet to be distinguished. I hypothesized that fructose alone, when replacing glucose in media, would directly affect neurons and microglia in culture conditions by decreasing survival and increasing inflammatory gene expression.

2. Methods

Animals

For the *in vivo* studies, timed pregnant Wistar rats (n=22) were obtained from Charles River (Wilmington, MA) on gestational day 12. Male pups (n=134) were culled on postnatal day (PND) 3 and weaned on PND 23. Animals were housed on a 14:10 reverse light:dark cycle in a facility controlled for humidity (60%) and temperature (20 °C-23 °C). All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Emory University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Diet

Rats were assigned to the Lab Rodent Diet 5001 (Chow Diet; n=59) or the high-fructose diet (HFD) two days post-weaning (PND 25; n=55). The fructose diet used (Research diets D05111802) is 55% fructose whereas the standard diet is 0.30% fructose. Both diets were supplemented with comparable levels of vitamins and minerals necessary for rodent health, and were reviewed by veterinary staff and approved by IACUC. Food consumption was measured daily and animal weights were measured weekly in both diet groups.

Experiment 1: Stress and Behavior

Mixed Modality Stress Paradigm

A mixed modality chronic stress paradigm that has been shown to produce behavioral changes in adolescent rats was used in this experiment (Bourke & Neigh, 2011). Animals

assigned to the stress cohort were individually housed, beginning at PND 35, until the end of the study (n=47). Non-stressed rats remained pair-housed throughout the study (n=51).

Stressed animals were further subjected to either social defeat or restraint for 12 days and the order of stressors was randomly determined (PND 37-49). In the social defeat portion of stress, experimental animals were placed in the home cage of a mature, territorial, Long-Evans rat during the light phase. After five minutes or after the intruder was attacked by the resident five times on the first day, three times on the second day, and once each day thereafter, a mesh barrier was placed in the cage, separating the experimental animal from the resident. This separation continued for 25 minutes, during which the experimental animal was exposed to the sights and smells of the resident. Pairings between experimental animal and resident were randomly assigned in order to prevent the formation of a stable dominance hierarchy. During restraint stress, animals were placed in a clear acrylic rat restraint (BrainTree Scientific, Braintree, MA, USA) for 60 minutes during the light phase. These restraints prevented head-to-tail turns but did not compress the rat.

Behavior

Testing began during adulthood after eight weeks on the diet and finished at ten weeks on the diet (n=98). Beginning on PND 76, the stress cohorts went through a series of behavioral tests: Open field (PND 76), Elevated plus maze (PND 92), and Forced swim test (PND 94). All behaviors were recorded using a video camera and analyzed using automated behavior analysis software (CleverSys, Inc., Reston, VA, USA). **Open field:** The Open field test is used to measure general locomotion and anxiety-like behaviors in rodents (Walsh & Cummings, 1976). During the light phase, rats were placed in the center of a square box (75 cm X 75 cm) and allowed to explore freely for 10 minutes. **Elevated plus maze**: The elevated plus maze is used to

assess anxiety-like behaviors (Pellow, 1986). The plus maze (San Diego Instruments) is 43.5 inches long, 4 inches wide, 19.5 inches high at the open arms, and 31.5 inches high at the closed arms. During the dark phase, animals are placed on the plus maze and allowed to explore for 5 minutes. **Forced swim test**: The forced swim test is utilized as a model of depressive-like behavior and learned helplessness in rodents (Borsini & Meli, 1988). The test was completed in a clear acrylic beaker (40 cm high X 18 cm diameter) filled with room temperature water. Rats were placed in the beaker for 10 minutes and immediately removed and rapidly decapitated. Rats will spend variable amounts of time struggling or floating in the water. Rats who spend more time immobile are considered more depressive-like than those who struggle for a greater amount of time. In my study, a rat was considered floating when its limbs were motionless for at least two seconds, and a struggling animal was one whose limbs were in motion and its head was above the water surface.

Corticosterone Analyses

Blood was collected at baseline and immediately after the ten-minute forced swim test in all animals. Plasma was separated by centrifugation and corticosterone levels analyzed using ELISA (sensitivity 27 pg/mL, Enzo Life Sciences, Farmingdale, NY, USA). All samples were run in duplicate.

Experiment 2: Metabolism

Diet and Metabolic Measurements

Male adolescent rats were assigned to either a standard diet or high fructose diet (n=36), as explained above, but no animals received chronic stress. Daily food consumption and weekly body mass were assessed beginning in adolescence and continuing for 9 weeks on the diet.

Fasting glucose was measured weeks using a *Freestyle* glucometer. For glucose measurements, rats were fasted overnight and blood from a tail prick was tested.

Fat Pad Collection

At PND 89-90, animals on the chow and fructose diets were euthanized and perfused with saline for two minutes (n=18) or perfused with saline followed by 4% paraformaldehyde for 10 minutes (n=18). Epididymal and retroperitoneal fat pats were collected from the saline perfused group. Collections were performed according to the methods described by Johnson and colleagues (P. R. Johnson & Hirsch, 1972). Each fat pad weight was then normalized to total body mass.

Experiment 3: Inflammation in vitro

Cell Lines

HT22 and BV2 cells were used in these experiments. HT22 cells are immortalized mouse hippocampal neurons and were obtained from the Salk Institute (La Jolla, CA). BV2 cells are immortalized mouse microglial cells (Tansey, Atlanta, GA). All control cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum and 5% penicillin streptomycin. Cells were incubated under standard conditions (37 ^{o}C, 5% CO_2). After reaching 90-95% confluency, cells were split at a 1:10 dilution (HT22s) or a 1:20 dilution (BV2s). In general, HT22 and BV2 cells used in the following experiments were obtained after 3-15 passages. Co-cultures were obtained by plating HT22 and BV2 cells at a 3:1 ratio. Specifically, $5.25x10^5$ HT22 cells were plated with $1.75x10^5$ BV2 cells in a T25 flask with a total of 5 ml of media. After the T-25 flask reached 90-95% confluency, cells were used for further experiments (co-culture group).

Sugar Manipulations

Normal DMEM contains 25mM D-glucose and is widely used for culturing both HT22 and BV2 cells (Li, Lee, Jeong, & Kim, 2012; Park, Jin, Kim, Kim, & Lee, 2012). In order to test the effects of fructose *in vitro,* I cultured neurons, microglia, and a co-culture of both with low (5mM) and high (25mM) concentrations of glucose and fructose relative to commonly used cell culture conditions. Microglia and co-cultures were also cultured with 12.5mM of glucose and fructose. DMEM without glucose was supplemented with the given glucose or fructose concentrations (Invitrogen, Carlsbad, CA). All other components of the experimental media were identical to control DMEM.

Cell Viability and Proliferation

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a water soluble salt that is used in assays to determine cell viability and proliferation (TIntyman & Luscombe, 1987). In short, the procedure followed was derived from Life Technologies Vybrant MTT Cell proliferation Assay Kits protocol. The MTT salt is converted by dehydrogenase enzymes of viable cells into a purple insoluble formazan. A solubilizing agent is then added and absorbance read using a spectrophotometer. Absorbance is linearly proportional to the number of viable cells in each well.

Five thousand cells from two cell group (HT22, Co-culture; n=8, 6 respectively) were plated onto each well in a 96-well plate. Designated amounts of glucose and fructose were added to media containing no sugar or phenol red. A total of 200 µl of media, sugar, and cells Were included in each well. Plates were placed in the incubator for 48-72 hours until cells were 90-100% confluent. For HT22 wells, media was aspirated off and fresh media with no sugar or

phenol red was added. For BV2 cells and co-cultures, plates were centrifuged at 1000 rpm in order to pellet cells and media was aspirated. Twelve mM MTT was then added to each well and plates were incubated for four hours under normal conditions. Sodium dodecyl sulfate (SDS) was dissolved into 0.01 M HCl and added to each well and mixed thoroughly. The plate was then incubated for four hours, after which time absorbance at 570 nm was read. Control wells contained media and reagents and were used as blanks. Six to nine samples from each condition were used in the analysis. **Dexamethasone manipulations:** DEX is a synthetic glucocorticoid used to model the effects of corticosterone in rodents. I hypothesized that fructose would exacerbate the effects of DEX on cell proliferation in media. A final concentration of 10^{-6} M of DEX dissolved in ethanol was added to HT22 cells on the 96-well plate, followed by the MTT assay.

Quantitative Real-Time Polymerase Chain Reaction

The three cell groups (neuronal, microglial, and cell culture; n=5 or 6) from low and high concentrations of glucose and fructose were plated on 12-well plates at a density of $3.1x10^4$ cells per well. Cells from three different passages were plated for a total of eight groups per cell type and condition. Plates were incubated under standard conditions for three to five days. Cells were pelleted by centrifugation and RNA extracted using the QiaShredder and RNEasy kit from Qiagen (Valencia, MA). The NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE) was used to assess RNA concentration and integrity. RNA standardization and reverse transcription was completed using High Capacity RNA to cDNA kit (Life Technologies, Grand Island, NY). PicoGreen assay (Invitrogen, Carlsbad, CA) was used to quantify cDNA. cDNA was then standardized to 10pg/µl. Quantitative real-time PCR (SYBR Green, Life Technologies) was used to amplify and quantify gene expression of TNF-α (forward: CTG AGG

TCA ATC TGC CCA AGT AC; reverse: CTT CAC AGA GCA ATG ACT CCA AAG), IL-6 (forward: GAG GAT ACC ACT CCC AAC AGA CC; reverse: AAG TGC ATC ATC GTT GTT CAT ACA), and HPRT1 (housekeeping gene; forward: GCC TAA GAT GAG CGC AAG TTG; reverse: TAC TAG GCA GAT GGC CAC AGG). SYBR primers were designed according to mouse genomics, and each gene was run in triplicate. The 7900HT Sequence Detection System (Applied Biosystems) was used to run each plate. Cycle threshold (Ct) values were averaged and ΔCt values calculated by normalizing to baseline, which was established as the 25mM glucose condition in each cell group. Changes in expression were reported as Fold Change, calculated using the following formula:

$$\text{Fold change} = 2^{-\Delta Ct}$$

The housekeeping gene was not used in these calculations due to differences in HPRT-1 gene expression in the co-culture groups (Figure 8).

Statistical Analysis

All analyses were done using Graphpad Prism 6.0 and IBM SPSS and all alpha values were set to 0.05. In experiments 1 and 2, Two-way ANOVAs were used to analyze the effects of diet (chow vs. high fructose) and chronic stress exposure (no stress vs. stress) on behavioral and metabolic outcomes and to evaluate the potential interaction of diet and stress. Holm Sidak post-hoc analyses were used when a main effect of diet or stress or an interaction was found. A Two-way repeated measures ANOVA was performed on caloric efficiency and blood glucose readings because consumption, weights, and blood glucose were each measured from all animals. In experiment 3, Two-way ANOVAs were utilized to evaluate the effects of sugar type (glucose vs. fructose) and concentration (25mM vs. 12.5mM [when present] vs. 5mM) Holm Sidak multiple

comparisons were performed when significant main effects or interactions were found. In order to assess the relationship among sugar type, concentration, and DEX administration, a Three-way ANOVA was employed. Further post-analyses were not completed in the DEX portion of experiment 3.

3. Results

Experiment 1

An adolescent high-fructose diet alone increases depressive-like and anxiety-like behaviors

In the forced swim test, rats fed a high-fructose beginning at weaning spent significantly less time struggling (Figure 1; Two-way ANOVA; main effect of diet $F_{1,22}$=4.501, p=0.02), had a reduction in latency to float (main effect of diet $F_{1,23}$=6.825, p=0.02), and spent more time immobile (main effect of diet $F_{1,22}$=6.745, p=0.02) compared with animals fed the standard chow. These behaviors are indicative of a depressive-like phenotype in rodents (Borsini & Meli, 1988). Chronic stress alone did not affect these behaviors or interact with diet (main effect of stress $F_{1,22}$=2.084, p=0.16; interaction $F_{1,22}$=0.007296, p=093).

In a measure of anxiety-like behavior, the EPM, fructose-fed male rats spent less time in the open arms of the maze (Figure 2; main effect of diet $F_{1,34}$=9.838, p=0.0035), demonstrating an anxiety-like phenotype that was not influenced by stress (main effect of stress $F_{1,34}$=1.459, p=0.23; interaction $F_{1,34}$=-.4743, p=0.50).

Notably, fructose-fed rats travelled farther (Figure 2; main effect of diet $F_{1,34}$=4.814, p=0.03) and faster (main effect of diet $F_{1,34}$=5.083, p=0.03) in the open field test when compared to chow animals, indicating that fructose did not impair motor function. Stress did not affect any measures in the open field test (main effect of stress $F_{1,34}$=0.8168, p=0.37; interaction $F_{1,34}$=0.7678, p=0.39).

A high-fructose diet increases baseline corticosterone

Plasma corticosterone levels were evaluated before and after the forced swim test. As expected, the acute stressor elevated corticosterone in all animals (Figure 3; main effect of diet F1,40=7.042; p=0.01). Post-hoc analyses revealed that fructose-fed rats had elevated baseline corticosterone (Holm Sidak mean difference 114.1 ng/ml).

Experiment 2

An adolescent high-fructose diet alters metabolism

Caloric efficiency, calculated as mg of weight gained per kCal consumed, was significantly increased in fructose-fed rats (Figure 4; Two-way Repeated Measures ANOVA; main effect of diet $F_{1,34}$=6.855, p=0.0131). Post hoc analyses indicated that caloric efficiency was significantly increased in fructose-fed animals at 6 and 8 weeks on the diet (Holm-Sidak; p<0.01, <0.01). In other words, rats on a high-fructose diet gained more weight per kCal consumed. Furthermore, an elevation in fasting glucose was observed in fructose-fed rats (Figure 4; $F_{1,34}$=18.21, p<0.01). Retroperitoneal and epididymal fat pad weights were also increased in the fructose-fed animals compared to standard chow animals (Figure 4; Unpaired t-test; t_{16}=4.270; p<0.01; t_{34}=3.034; p<0.01).

Experiment 3

Replacement of glucose with fructose *in vitro* decreases cell viability/proliferation in HT22 cells and HT22 + BV2 co-cultures

Both sugar type and concentration influenced cell viability in neuronal cultures (Figure 5; Two-way ANOVA, main effect of sugar $F_{1,25}=90.97$, $p<0.01$; main effect of concentration $F_{1,25}=42.15$, $p<0.01$). Furthermore, an interaction between type of sugar (glucose vs. fructose) and concentration (5mM vs. 25mM) was observed in HT22 cells (interaction $F_{1,25}=8.555$, $p<0.01$). At the 25mM concentration of glucose and fructose, cell viability was significantly lower compared to viability at the 5mM condition. Additionally, fructose exposure was detrimental to neuronal viability, with a more profound effect observed at the 25mM concentration. In BV2 cultures, a significant main effect of sugar type and interaction between type and concentration was observed (Figure 6; main effect of sugar $F_{1,24}=7.859$, $p=0.01$; main effect of concentration $F_{1,24}=1.777$, $p=0.19$; interaction $F_{2,24}=4.667$, $p=0.02$). In co-cultures of neurons and microglia, I observed a significant main effect of sugar type and concentration, in addition to an interaction between sugar and concentration (Figure 6; main effect of sugar $F_{1,23}=17.82$, $p<0.01$; main effect of concentration $F_{2,23}=10.91$, $p<0.01$; interaction $F_{2,23}=9.929$, $p<0.01$).

I also evaluated cell viability in neuronal cultures exposed to varying concentrations of fructose or glucose and DEX (10^{-6}M). There was a statistically significant three way interaction among sugar type, concentration, and DEX administration in viability of HT22 cells (Figure 7; Three-way ANOVA $F_{1,51}=5.722$, $p=0.02$).

Fructose differentially influences gene expression of proinflammatory cytokines in co-cultures vs. microglia

The proposed housekeeping gene, HPRT-1, was not affected by sugar type or concentration in BV2 cultures. However, HPRT-1 expression was not consistent in all groups in the HT22 and co-culture conditions. In HT22 cells, there was a main effect of sugar type and concentration

(Figure 8; main effect of sugar $F_{1,20}$=29.06, p<0.01; main effect of concentration $F_{1,20}$=14.78, p<0.01), in addition to an interaction between sugar type and concentration ($F_{1,20}$=5.157, p=0.03). There was a main effect of sugar type in co-cultures (Figure 8; main effect of sugar $F_{1,20}$=26.05, p<0.01; main effect of concentration $F_{1,20}$=2.474, p=0.13; interaction $F_{1,20}$=1.193, p=0.29). Based on these results, I did not use the housekeeping gene in further analyses. cDNA was standardized using PicoGreen as explained in the methods.

TNF-α gene expression was affected by both sugar type (Figure 9; Two-way ANOVA $F_{1,20}$=28.94, p<0.0001) and sugar concentration ($F_{1,20}$=5.253, p=0.0329) in the co-culture group (interaction $F_{1,20}$=.5922, p=0.4506). Co-cultures of neurons and microglia exposed to 5mM and 25mM fructose had decreased TNF-α gene expression. In contrast, gene expression of TNF-α (Figure 9; main effect of sugar $F_{1,20}$=0.007857, p=0.9302; main effect of concentration $F_{1,20}$=0.01747, p=0.90; interaction $F_{1,20}$=3.511, p=0.08) was unchanged in HT22 cells. Similarly, TNF-α (Figure 9; main effect of sugar $F_{1,20}$=0.3518, p=0.5597; main effect of concentration $F_{1,20}$=0.4695, p=0.5011; interaction $F_{1,20}$=0.7655, p=0.3920) gene expression was unaffected by differing amounts of glucose and fructose in in BV2 cultures.

Expression of IL-6 was significantly altered in co-cultures and HT22 cells, but not in BV2 cells. In neuronal cultures, cells exposed to fructose had lower IL-6 gene expression compared to those exposed to glucose (Figure 10; main effect of sugar $F_{1,19}$=52.54, p<0.01; main effect of concentration $F_{1,19}$ =0.03885, p=0.85). Sugar type and concentration interacted to affect IL-6 expression in these cells as well (interaction $F_{1,19}$=10.14, p<0.01). IL-6 gene expression was also significantly decreased in the co-cultures exposed to fructose (Figure 10; main effect of sugar $F_{1,20}$=36.96, p=0.0001). However, there was no significant main effect of concentration or interaction between sugar type and concentration in IL-6 expression in co-cultures (Figure 10;

main effect of concentration $F_{1,20}$=1.474, p=0.24; interaction $F_{1,20}$=1.304, p=0.27). In contrast, there was no difference in IL-6 expression in BV2 cells (Figure 10; main effect of sugar $F_{1,20}$=0.2569, p=0.62; main effect of concentration $F_{1,20}$=0.4677, p=0.50; interaction $F_{1,20}$=0.1807, p=0.68).

4. Discussion

The presence of added sugars and fructose in diets, specifically in Western countries, has significantly increased in the past three decades (Havel, 2005). Notably, the prevalence of metabolic dysfunction, including key disorders such as hypertension, obesity, and insulin insensitivity, has paralleled the increased consumption of sugar and fructose during this time period (Mozundar & Liguori, 2011). The increase in metabolic syndrome is significant because individuals with these dysfunctions are at a much higher risk of developing life-threatening conditions, such as heart disease and stroke. Both human and animal studies illustrate the negative effects of acute and chronic fructose administration, effects generally on metabolic factors, memory, brain morphology, and potentially on mood disorders (Basciano et al., 2005; Faeh et al., 2005; Lyssiotis & Cantley, 2013; Tappy & Lê, 2010). In particular, fructose consumption and the prevalence of metabolic dysfunctions have seen distinct rises in children and adolescents (Havel, 2005).

Chronic stress is also an important environmental factor that has profound consequences on mood and metabolism (Phillips et al., 2006). Developmental studies have emphasized the importance of the adolescent period in shaping both behaviors and metabolism (McCormick & Mathews, 2007). Because the HPA and related axes undergo significant changes during development, exposure to chronic stress during the adolescent period may have long-term consequences later in adulthood (Bourke & Neigh, 2011; McCormick & Mathews, 2007). One focus of these studies was the relationship between chronic stress and depressive-like and anxiety-like behaviors.

The consequences of both fructose consumption and chronic stress may be due to altered immune function, specifically an increase in inflammation in the periphery and CNS. Due to the

important effects that fructose consumption and chronic stress can have during adolescence, I hypothesized that chronic stress and fructose consumption would alter behavior and metabolism rats. I further hypothesized that fructose administration would have a direct effect on cell proliferation and proinflammatory cytokine expression in neurons and co-cultures of neurons and microglia *in vitro.* In the studies presented, a high-fructose diet fed to adolescent male rats significantly increased depressive-like and anxiety-like behaviors and altered metabolic components when evaluated in adulthood. Although I hypothesized that chronic stress would exacerbate the effects of a high fructose diet, I saw no effects of the stress alone and no interaction between stress and diet.

In the *in vitro* studies, my aim was to investigate the result of altering energy sources in brain-derived cell types in order to distinguish between physiological and neuroimmunological effects of fructose. I therefore evaluated the effects of varying glucose and fructose concentrations on cell viability and proliferation, as well as on gene expression of proinflammatory cytokines. I found that neurons and co-cultures of neurons and microglia had lower viability in the fructose media. In contrast, microglial viability was unaffected by fructose. Interestingly, cytokine gene expression was significantly decreased in neurons and co-cultures exposed to fructose, whereas this effect was not present in cultures of only microglia.

A high fructose-diet elicits depressive-like and anxiety-like behaviors in male rats

In the first experiment, adolescent rats were fed a high-fructose diet for 8-10 weeks beginning at weaning. A subset from each diet group was also exposed to mixed modality stress during the adolescent period, a chronic stress paradigm that has been shown to elicit depressive-like and anxiety-like behaviors in rats (Bourke & Neigh, 2011; Harrell, Hardy, Boss-Williams, Weiss, & Neigh, 2013). In adulthood, depressive-like and anxiety-like behaviors were evaluated

in these animals. Fructose-fed animals demonstrated significant increases in depressive-like and anxiety-like behaviors as assessed by the forced swim test and elevated plus maze. Both of these behavioral assays are established proxies for measuring these affective behaviors in rodents and are sensitive to antidepressant and anxiolytic activity (Borsini & Meli, 1988). The largest effect was seen in the forced swim test, in which fructose-fed animals spent more time immobile, less time struggling, and had a lower latency to first immobility than chow-fed animals. These results are noteworthy because fructose-induced depressive-like and anxiety-like behaviors have not been previously demonstrated. Contrary to my hypothesis, fructose-fed animals were not more susceptible to the effects of chronic stress. These results may indicate that male adolescent rats are resilient to chronic stress but not diet-induced metabolic and behavioral alterations. In addition, chronic stress did not alone have an effect on any behaviors analyzed. This result is consistent with previous work showing a sex difference in behavioral outcomes following chronic stress (Bourke & Neigh, 2011; Harrell et al., 2013). These authors concluded that female rats are more susceptible than males to behavioral changes following chronic stress. Because I used only male rats in these experiments, I do not know whether females are differentially affected by a high fructose diet alongside chronic adolescent stress.

Fructose-fed rats demonstrate altered HPA axis activity

Activity of the HPA axis is implicated in the neurobiology of depression in both human populations and animal studies (Neigh & Nemeroff, 2006; Pariante & Lightman, 2008). I therefore evaluated plasma corticosterone levels at baseline and after an acute stressor. Fructose-fed rats had elevated plasma corticosterone at baseline compared with chow animals. These results indicate that a high fructose diet given to adolescent rats impairs HPA axis signaling in adulthood.

The HPA axis is central to the physiological responses to stressors in the environment. The HPA axis is implicated in the pathophysiology of depression in both human and animal studies. More than 50% of patients with MDD have altered HPA axis activity as measured by the DST and baseline levels of glucocorticoids (Pariante & Lightman, 2008). Glucocorticoids, their receptors, and negative inhibition of the HPA axis by the hippocampus are key areas of interest when discussing depression and HPA axis activity. Depressed individuals express lower levels of glucocorticoid receptors in the periphery, suggesting that hyperactivity of the HPA axis may be driven by a decrease in negative inhibition in the CNS (Neigh & Nemeroff, 2006). In addition to human studies, animal research is consistent with this hypothesis. Administration of glucocorticoids to rats increases depressive-like behavior and experimental reduction of glucocorticoid receptor expression makes mice susceptible to depressive-like behaviors after an acute stressor (Neigh & Nemeroff, 2006).

The current study demonstrates the ability of a fructose diet to alter HPA axis activity, which may be part of the mechanism underlying the behavioral alterations observed. Importantly, the fructose diet had a profound effect on behavior and HPA axis activity that was not observed in the chronically stressed animals. These results suggest that a high fructose diet can function like a stressor by influencing activity of the HPA axis and behaviors. Finally, exposure to this dietary stressor during the adolescent period elicits long-lasting effects in male rats, indicating that adolescence is a sensitive period to dietary alterations. These data are consistent with previous work that indicates the ability of a high fructose diet to alter glucocorticoid signaling and the stress response in the periphery (Kelley, Allan, & Azhar, 2004; Kovačević et al., 2014).

A high fructose diet dysregulates metabolism in male rats

Studies using dietary manipulations such as increases in amounts of fructose and fat typically focus on metabolic changes. Therefore, I evaluated body mass, consumption, blood glucose levels, and fat distribution in fructose-fed rats. Adolescent rats were fed a diet high in fructose but with the same total calories as the standard chow fed to control animals. Throughout the study, body mass, consumption of diet, and glucose levels were analyzed. Although neither body mass nor consumption was significantly altered by the diet, fructose-fed rats consumed less for the amount of weight gained throughout the study, particularly in the later weeks of the diet. Fructose further increased circulating glucose levels in fasting rats and retroperitoneal and epididymal fad mass. These data are consistent with multiple studies that indicate an altered metabolic phenotype in fructose-fed animals (Basciano et al., 2005; Calvo-Ochoa et al., 2014; Storlien, 1988). Specifically, researchers have concluded that chronic fructose exposure results in the development of key metabolic dysfunctions in animal models, including increases in lipogenesis and adipose tissue, in addition to altered leptin and insulin signaling. Epidemiological and population-wide studies provide further evidence for a link between fructose consumption and metabolic syndrome in individuals, with emphasis on populations of top consumers such as children and adolescents (Cook et al., 2003; Havel, 2005; Sun & Empie, 2012; Tappy & Lê, 2010). Because rats were fructose-fed since weaning and into adulthood, my results illustrate the long-term metabolic consequences of diet. In combination with the behavioral data, the increased adiposity observed introduces a potential confounding variable. In other words, the decreased time spent struggling in the forced swim test may be a result of changes in body composition in the fructose-fed animals. Future studies can utilize other behavioral assessments, such as social interaction, to test for depressive-like behaviors in animals

with altered fat and muscle composition. Nonetheless, these studies provide strong evidence for changes in metabolism and depressive-like and anxiety-like behaviors in fructose-fed rats.

Fructose replacement of glucose *in vitro* directly effects neurons and microglia

In my final experiment, the goal was to elucidate the directly effect of altering energy sources on cells in the CNS. Utilizing immortalized hippocampal mouse neuronal (HT22) and mouse microglial (BV2) cell lines, I evaluated the effects of replacing glucose with fructose in the cell culture media. Using the MTT assay to assess cell viability, I found that fructose decreased cell proliferation and viability in HT22 cells and co-cultures of HT22 and BV2 cells. In HT22 cells, sugar type and concentration (25mM vs. 5mM) both influenced cell viability, where higher concentrations of both fructose and glucose resulted in lower viability of cultured neurons. A similar result was found in the co-culture condition. At the higher concentrations (25mM and 12.5mM), viability of cells exposed to fructose was significantly decreased. Conversely, no effect of sugar type or concentration was seen in the BV2 microglial cell line.

These data indicate that neurons and co-cultures of neurons and microglia are susceptible to high concentrations of fructose, whereas microglia alone tend to not be affected by the glucose replacement. I propose that the effect seen in the co-culture condition is mainly driven by the decrease in neuron viability because there does not seem to be a decrease in microglia viability at these same conditions. A potential mechanism for this is the ability of each cell type to take up and utilize fructose effectively. The selective fructose transporter, GLUT5, is expressed on microglia, but it is yet unknown whether neurons express the transporter. In these studies, I do not explicitly demonstrate the ability of neurons or microglia to take up fructose and metabolize it. Therefore, the differences observed in cell viability may be due to a difference in how effective these cell types are at using fructose as an energy source. Although future studies are

needed to investigate this problem, these studies show an ability for neurons to survive and proliferate during exposure to low concentrations of fructose, suggesting that neurons are able to use low concentrations of fructose safely and effectively.

I was further interested in whether fructose, instead of glucose, would make HT22 more susceptible to dexamethasone-induced cell death. Dexamethasone, a synthetic glucocorticoid, is harmful to neurons in culture and supplementation with added sugars is typically beneficial to neurons exposed to DEX (McIntosh & Sapolsky, 1996). I found that DEX interacted with sugar type and concentration, overall decreasing neuronal viability and proliferation in fructose-exposed cells. Further studies are important for determining how fructose makes neurons more susceptible to DEX-induced cell death and whether DEX has the same effects on microglia and co-cultures of neurons and microglia. Specifically, I could evaluate the expression of glucocorticoid receptors and downstream genes regulated by glucocorticoid action. These preliminary studies are novel in showing how fructose supplementation is not advantageous to neurons during DEX administration.

The immune system plays a central role in disease states, including obesity, metabolic dysfunction, and depression (Hotamisligil, 2006). Recent studies have shown that hypothalamic gene expression varies significantly between chow-fed and fructose-fed male rats, with genes in immune and inflammatory pathways differentially expressed between the animal groups (Neigh, Johnson & Harrell, 2013). Due to this potential role of immune and inflammatory pathways in fructose-mediated disease states, I hypothesized that the differences observed in cell viability would be mediated by increases in proinflammatory cytokines. Surprisingly, TNF-α and IL-6 gene expression was downregulated in co-cultures of neurons and microglia exposed to a fructose media at both the low and high concentrations and IL-6 expression was decreased in

neuronal cultures. In microglial cultures, cytokine expression was unaffected by fructose and concentration.

These data, which demonstrate that neurons and co-cultures respond differently to fructose than microglia, are unexpected. One hypothesis is that these cytokines are playing anti-inflammatory and neurotrophic roles in the cultures exposed to standard glucose conditions. Although these cytokines are typically considered inflammatory, their specific actions are tissue- and receptor-specific (Liu et al., 1998; Montgomery & Bowers, 2012; Van Zee et al., 1992). For example, both TNF-α and IL-6 have been shown to possess anti-inflammatory properties in autoimmune diseases that involve demyelination as well as in Parkinson's disease (Liu et al., 1998). Furthermore, these cytokines' ability to produce inflammatory states depends greatly on the expression of receptors. Most notably, the soluble receptor for TNF-α is protective against inflammatory insults (Montgomery & Bowers, 2012; Van Zee et al., 1992). Both TNF-α and IL-6 can also promote cell survival. TNF-α plays a central role in the developing nervous system, taking part in the pruning and selection of suitable neuronal connections (Montgomery & Bowers, 2012). IL-6 induced increases in cell survival in cultured cells during standard and neurotoxic conditions have been observed in cultured cholinergic and glutamatergic neurons, as well as in animal models (Gadient & Otten, 1997; Kushima, Hama, & Hatanaka, 1992; Toulmond, Vige, Fage, & Benavides, 1992). Because I observed decreased IL-6 gene expression in neurons and co-cultures, in addition to decreased cell viability in these same conditions, I hypothesize that IL-6 is important for maintaining viable cells and promoting growth and differentiation and that fructose decreases IL-6 expression, which leads to decreased cell viability. Moreover, I propose that neurons, and not microglia, are the primary targets of fructose-induced changes in cell viability and gene expression.

Microglia, due to their location in the CNS, are typical in a quiescent state. In order to maintain and alter this state, microglia are in constant communication with neurons. Research has shown that neurons release molecules that signal microglia to turn to an inflammatory state or to suppress this state (Biber, Neumann, Inoue, & Boddeke, 2007). Notably, the "off" signals can be secreted by neurons that are already in a compromised state (Tan, Town, & Mullan, 2000). I propose that the HT22 cells exposed to fructose are expressing the "off" signals due to the fact that they are in a comprised state, as seen in the cell viability assays. The "off" signal in turn decreases cytokine gene expression in the co-culture condition. Further studies are needed to evaluate the expression of the "off" signals and their receptors on microglia. Nonetheless, these studies shed light on the direct effects of fructose in neurons and microglia and, notably, demonstrate the profound consequences of fructose exposure on cell survival and expression of key genes.

There are several limitations of these *in vitro* studies. Primarily, I did not evaluate the effects of media containing both glucose and fructose. This aspect is important because fructose does not exist in the body without the presence of glucose. Therefore, it would be valuable to test how glucose in combination with fructose affects cell viability and gene expression. Moreover, neurons and microglia are not the only cells in the brain. Astrocytes may play a significant role in modulating the relationship between neurons and microglia in culture and have been shown to mediate the relationship between the immune system and neurons. Another limitation of these studies is that the decreases in cytokine gene expression may be due to overall decreases in gene expression due to the dying/unviable cells. Although I cannot make a conclusion about the gene expression levels in the co-cultured cells, I demonstrated that whereas neuronal cultures had decreased viability, they did not show decreased inflammatory gene expression. These data

suggest that decreases in gene expression are not the primary result of decreases in cell survival. Furthermore, more studies are needed to determine the mechanism of cell death in the neuron and co-culture conditions. It is clear that this cell death is not mediated by TNF-α, however there are many pathways involved in both apoptotic and necrotic death. Gene expression arrays that test a wide range of genes in these pathways are available and would be a suitable next step for these studies.

Significance

Taken together, these data provide strong evidence that fructose ingestion results in detrimental consequences on the body and the brain. In demonstrating the role of a high-fructose diet in altering depressive-like, anxiety-like behaviors, and metabolism in adult rats fed the diet since adolescence, these experiments are considerably novel. These studies are some of the first to consider an adolescent fructose diet and its effects on affective-like behaviors. Importantly, these studies may allow us to better understand the relationship among fructose ingestion, metabolic syndrome, and mood disorders, which are common in Western countries and are increasing in younger populations. Metabolic syndrome and mood disorders lead to life-altering conditions, including cardiovascular disease and an elevated risk of morbidity and mortality. For these reasons, understanding the physiological and neurological effects of fructose is imperative. Furthermore, these experiments add to a growing body of literature that illustrates the harmful effects of fructose and, more generally, the significant effect of diet on both behavior and the body. Understanding the effects of diet enhances potential for therapeutics and preventative measures, especially in susceptible individuals such as adolescents.

The *in vitro* studies are significant in that they are among the first to directly evaluate the effects of fructose in brain-derived cell lines. Culturing fructose with HT22 and BV2 cells alone

and in co-culture is a novel paradigm that can be used to study other effects of altering energy sources. The data presented here show significant consequences of fructose directly on cells, irrespective of changes in body composition and other metabolic factors. These studies also illustrate the ability of fructose to alter expression of key genes involved in immune responses in the brain. Overall, truly appreciating the role of fructose in the body will involve determining how fructose directly influences specific cell types. The rise in fructose consumption, especially among children and adolescents, and the considerable amount of evidence indicating detrimental effects of fructose on the body and brain make evaluating the mechanisms behind fructose-induced metabolic dysfunction and behavioral alterations critical for improving health. These studies are some of the first steps in the direction of a better understanding about fructose, MetS, mood disorders, and the immune system.

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Figures

Figure 1:

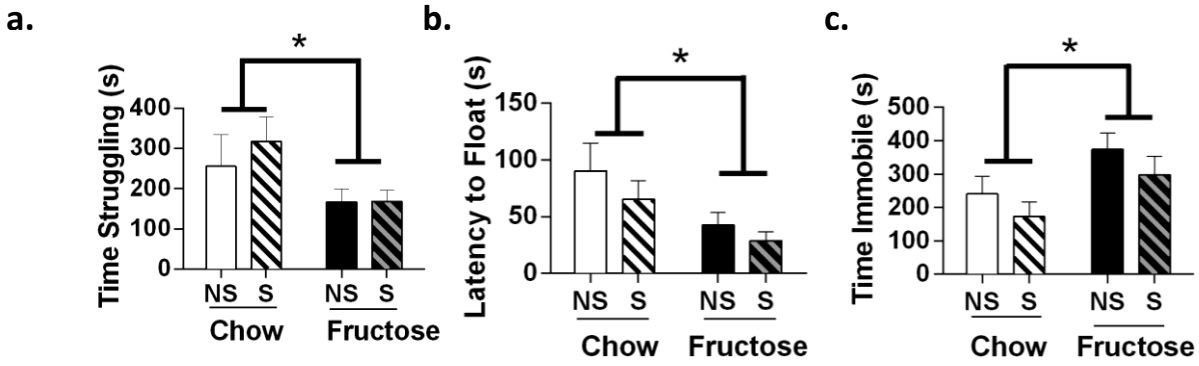

Fig 1. A high fructose diet elicits depressive-like behaviors in the forced swim test.

Adolescent male rats were fed a high fructose diet and then behaviorally tested in adulthood. a) Fructose-fed animals spent less time struggling and b) became immobile quicker compared to chow-fed animals. c) A high fructose diet also led to an increase in total time immobile in the forced swim test. * represents a main effect observed in a Two-way ANOVA. Data presented are mean ± SEM.

Figure 2:

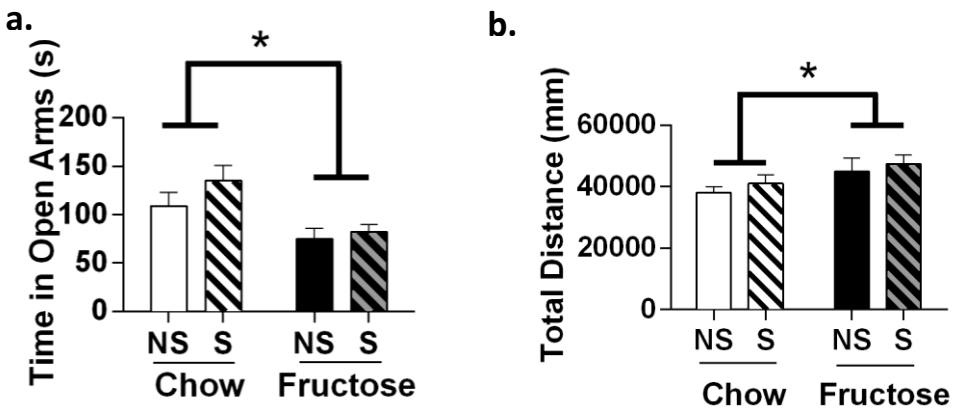

Fig 2. A high fructose diet elicits anxiety-like behaviors in the elevated plus maze.

a) Fructose-fed animals spent less time in the open arms of an elevated plus maze, suggesting an anxiety-like phenotype. b) These results were not due to locomotor differences, as illustrated by the increase in distance travelled by fructose-fed animals in the open field test. No effect of chronic stress was observed in any measures. * represents a main effect of diet in a Two-way ANOVA. Data presented are mean ± SEM.

Figure 3:

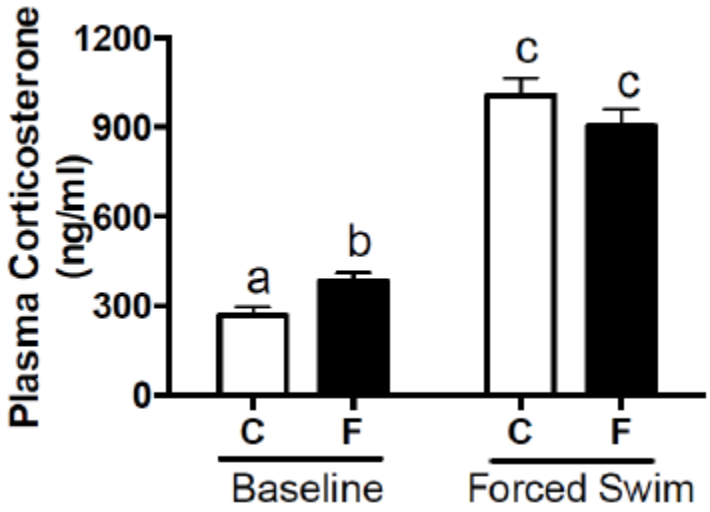

Fig 3. A high fructose diet increases baseline plasma corticosterone levels, indicating a hyperactive HPA axis

Fructose-fed animals had elevated plasma CORT levels at baseline. No difference was observed in CORT levels after exposure to an acute stressor (forced swim test). Groups with the same letter are not significantly different from each other, as demonstrated by post-hoc analyses. Data presented are mean ± SEM.

Figure 4:

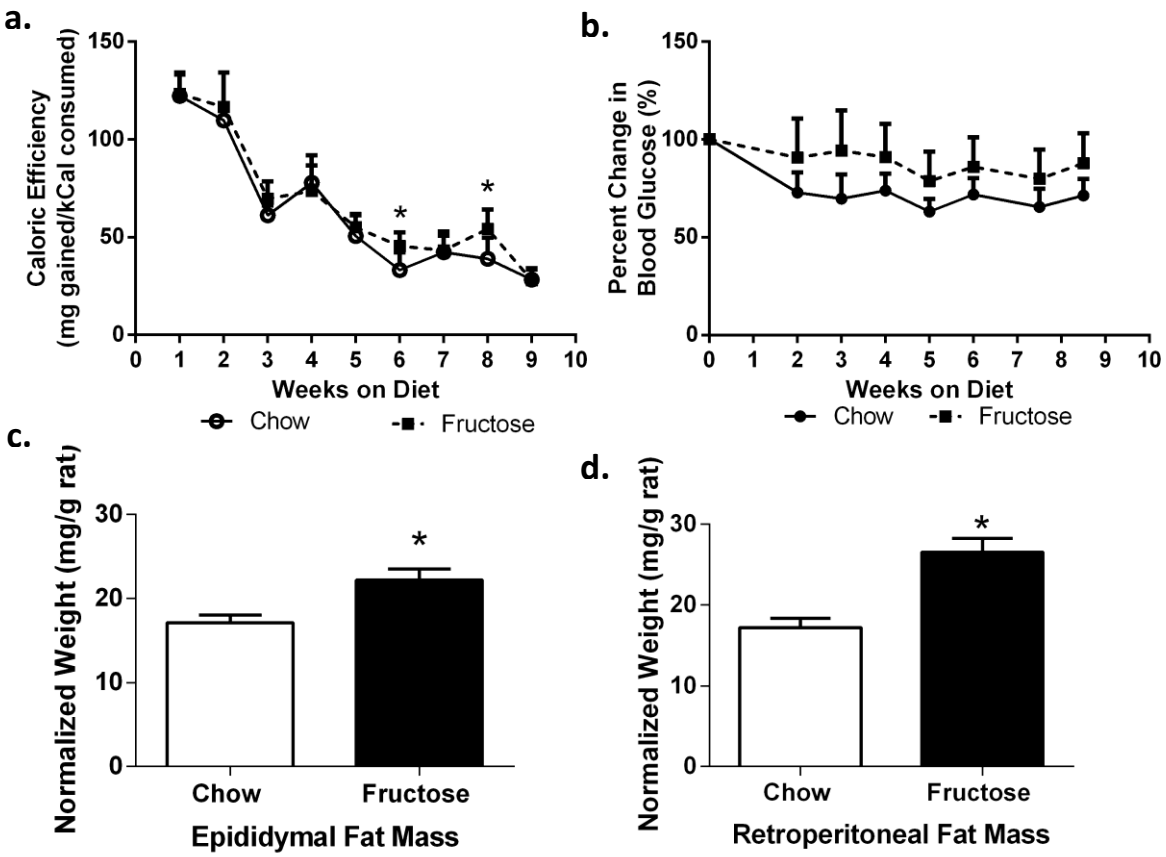

Fig 4. A high fructose diet alters metabolic parameters.

a) Caloric efficiency was increased in fructose-fed animas throughout the study. * represents a significant difference observed in post-hoc analyses. b) A high fructose diet also resulted in increased blood glucose levels throughout the study. c and d) Measures of visceral adiposity were significantly increased in fructose-fed animals. Data presented are mean ± SEM.

Figure 5:

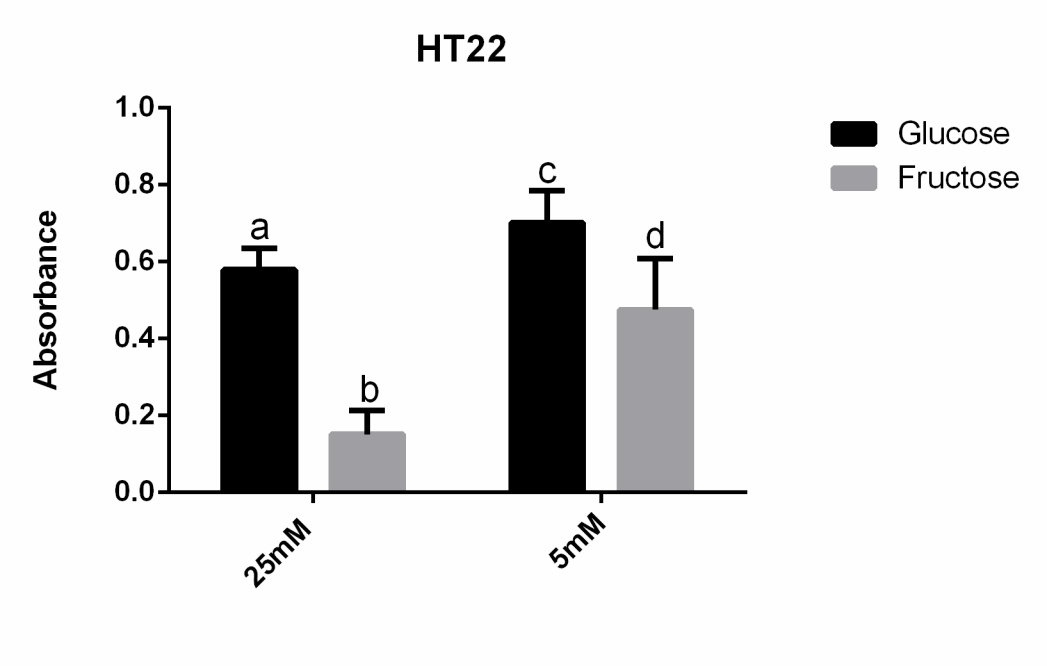

Fig 5. Sugar type and concentration influence neuronal viability in the MTT assay.

Absorbance amounts represent relative cell viability measured in the MTT assay. HT22 cells exposed to fructose had decreased cell viability compared to cells exposed to glucose, and a more profound effect was observed at the high concentration. Concentration also influenced viability in neurons exposed to glucose, in which lower amounts of glucose were beneficial to viability. a, b, c, and d represent the significant differences from post hoc analysis. Data presented are mean ± SEM.

Figure 6:

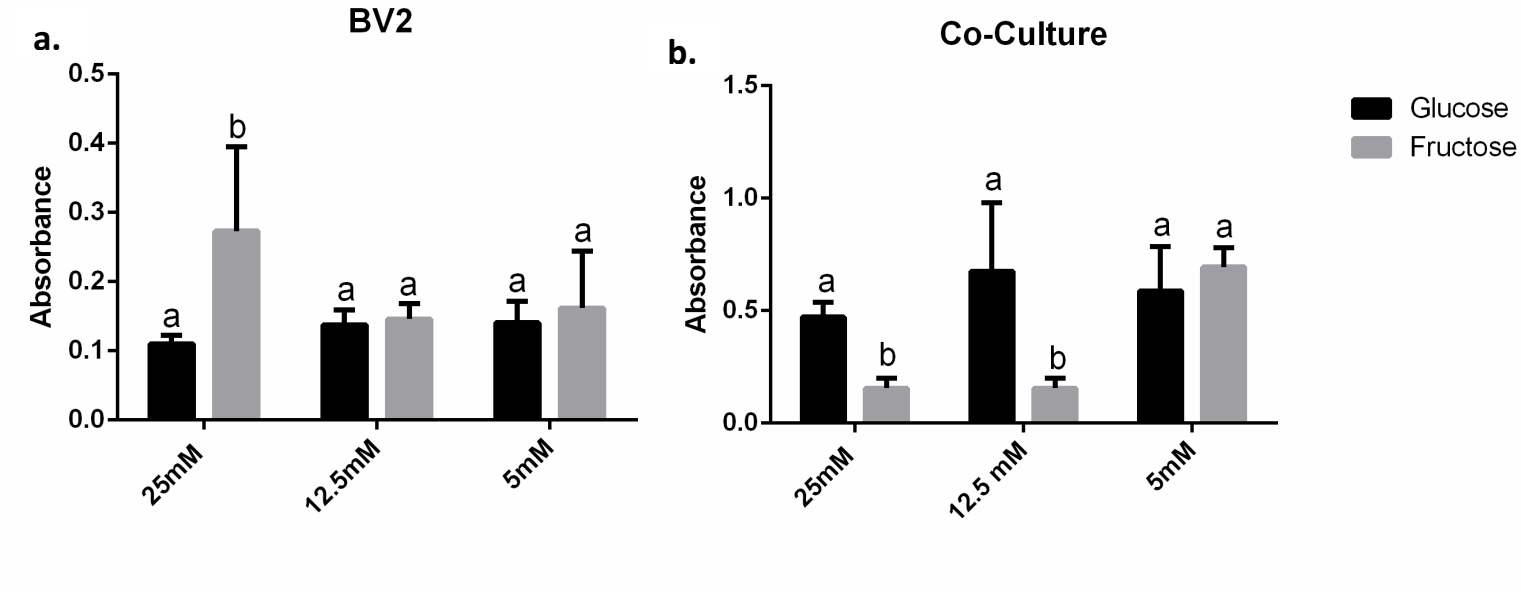

Fig 6. Sugar type and concentration influence microglial and co-culture viability in the MTT assay.

a) BV2 cells exposed to fructose were not affected except at the 25mM concentration, in which they demonstrate higher viability. b) Co-cultures of neurons and microglia were not affected at the 5mM fructose condition. However, cell viability was significantly decreased at the higher concentrations. a and b represent the significant differences observed in post hoc analysis. Data presented are mean ± SEM.

Figure 7:

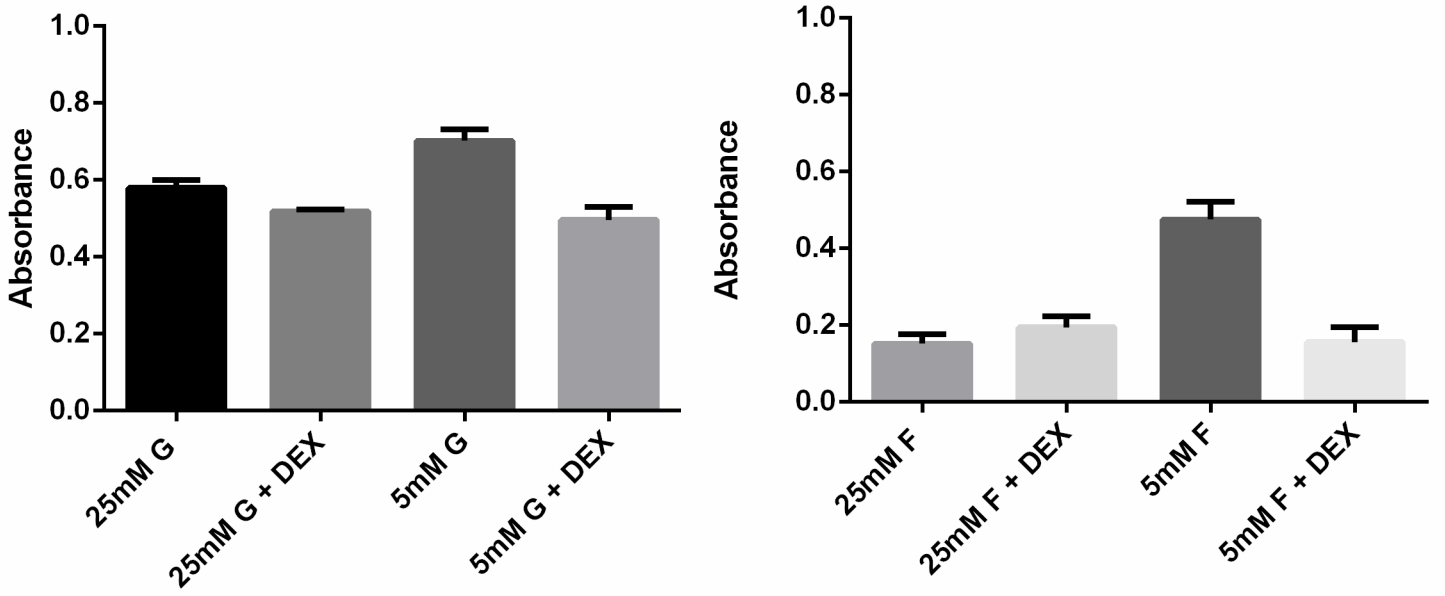

Fig 7. Sugar type, concentration, and DEX administration influence neuronal viability in the MTT assay.

Sugar type, concentration (5 vs. 25 mM) and DEX administration interacted to influence cell viability in HT22 cultures. Although illustrated separately, all data were analyzed together using a Three-way ANOVA. Data presented are mean ± SEM.

Figure 8:

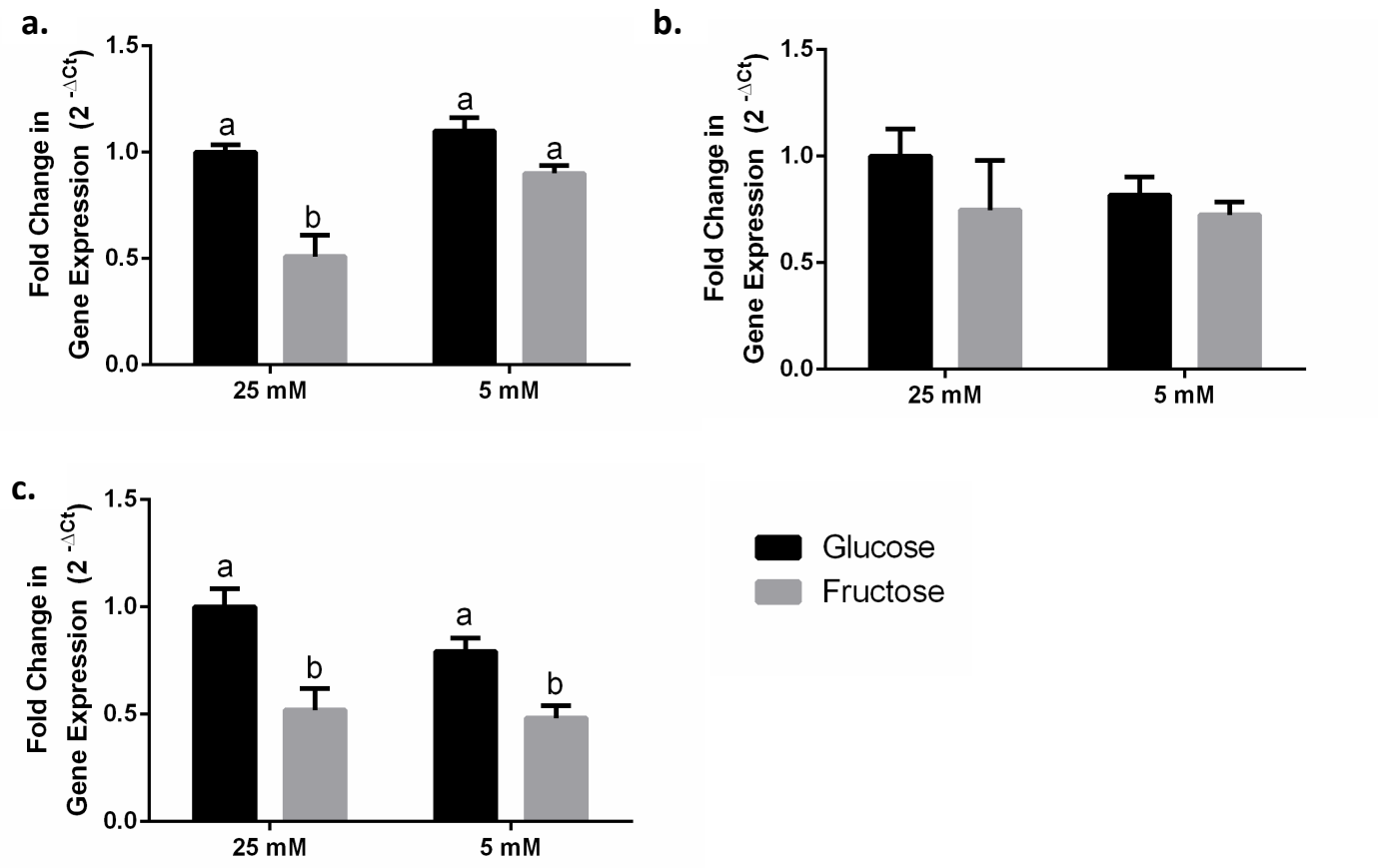

Fig 8. HPRT-1 gene expression in HT22 cells, BV2 cells, and Co-cultures

HPRT-1 is typically used as a housekeeping gene in both of these neuronal and microglial cell lines. a) In HT22 cells, sugar type and concentration interacted to decrease expression of HPRT-1. b) HPRT-1 gene expression was not altered in BV2 cells. c) In co-cultures, gene expression of HPRT-1 was significantly decreased in the 5mM and 25mM fructose conditions. Based on these results, I did not utilize the housekeeping gene in further data analyses. a and b represent the significant differences observed in post hoc analysis. Data presented are mean ± SEM.

Figure 9:

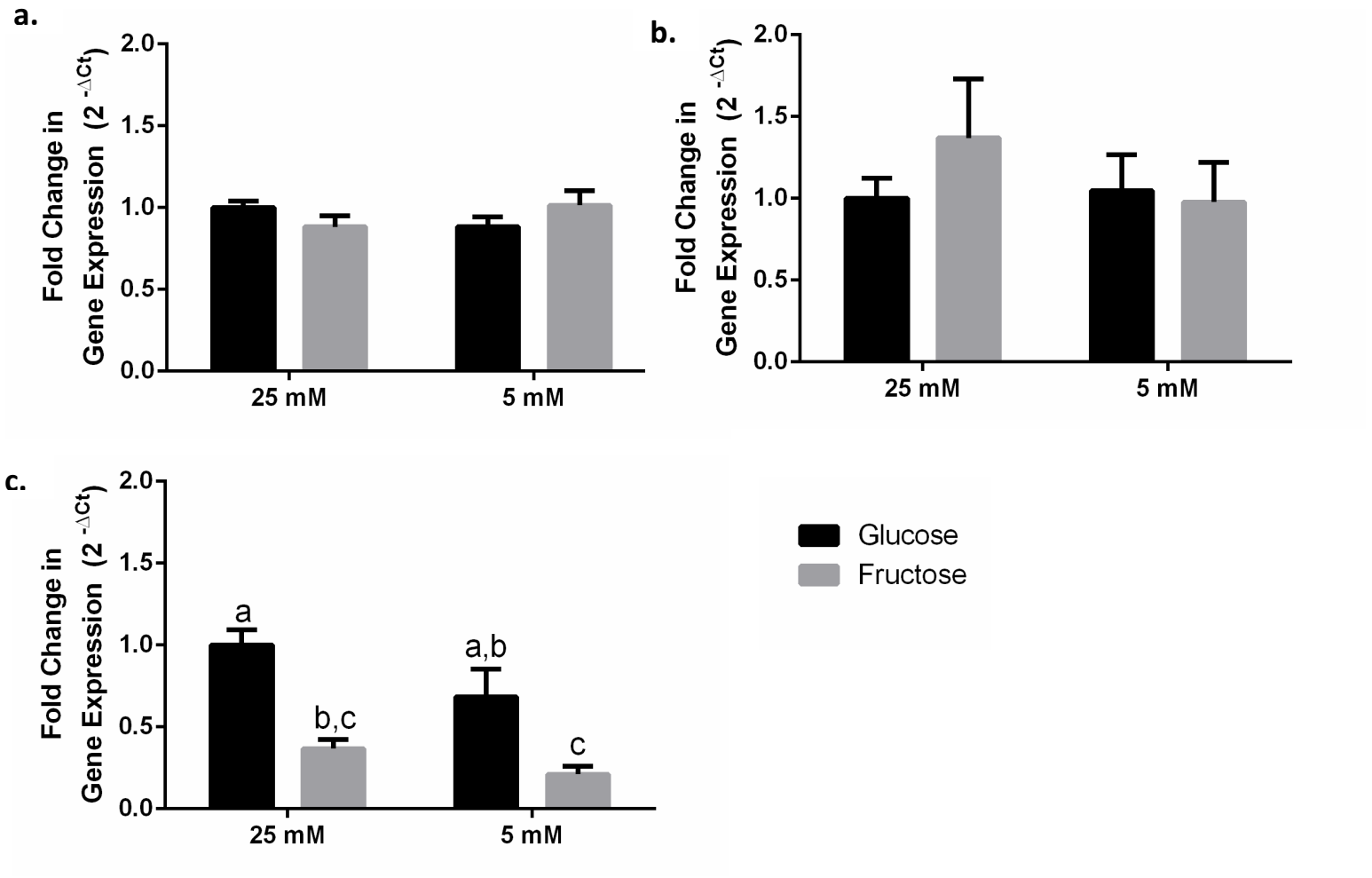

Fig 9. Gene expression of TNF-α in HT22 cells, BV2 cells, and Co-cultures

a and b) In HT22 and BV2 cultures, respectively, gene expression of TNF-α was not influenced by sugar type or concentration. c) In co-cultures, gene expression of TNF-α was significantly decreased in the 5mM and 25mM fructose conditions. a, b, c represent the significant differences observed in post hoc analysis. Data presented are mean ± SEM.

Figure 10:

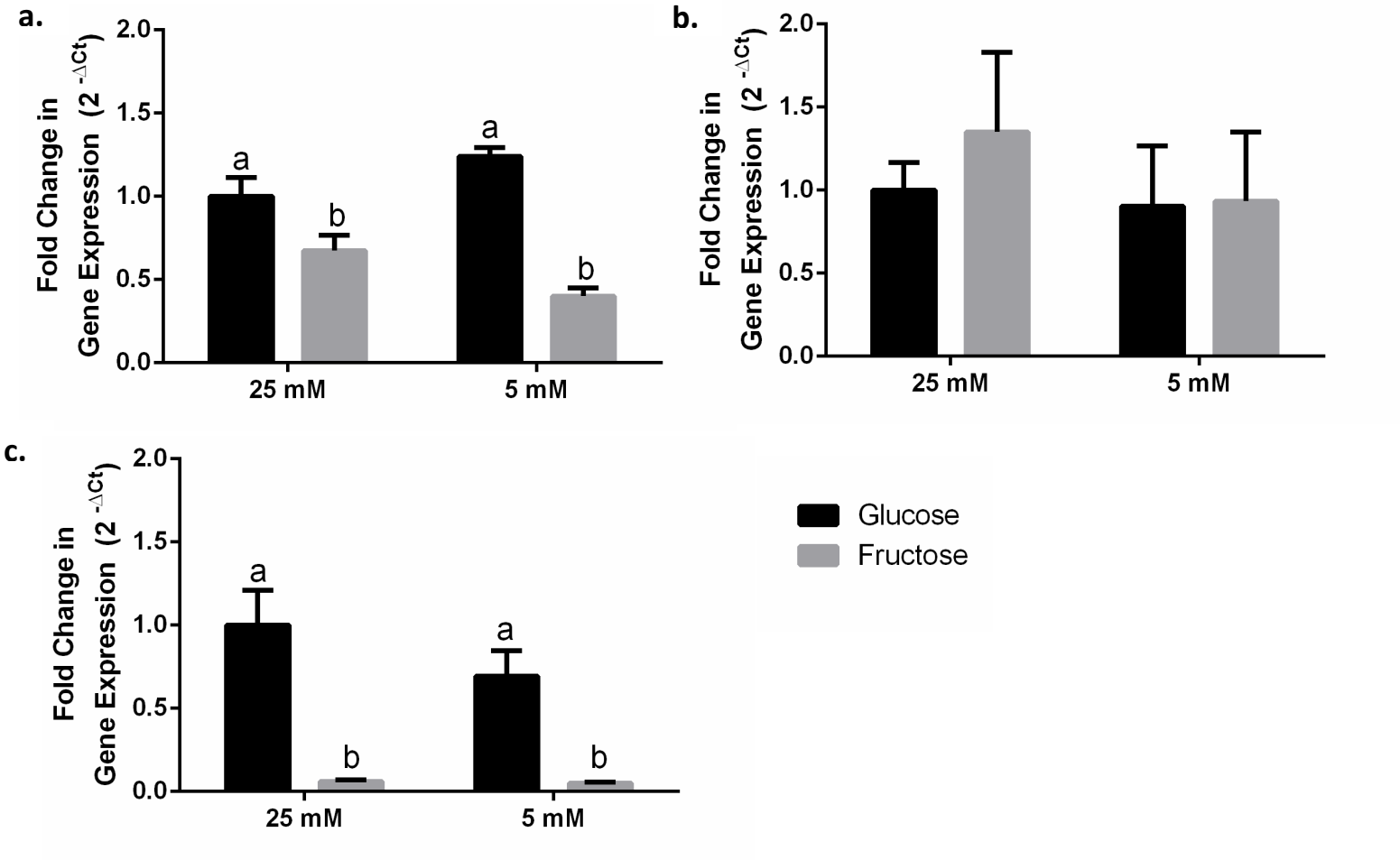

Fig 10. Gene expression of IL-6 in HT22 cells, BV2 cells, and Co-cultures

a) Exposure to fructose significantly decreases IL-6 gene expression in HT22 cells. b) In BV2 cultures, gene expression of IL-6 was not influenced by sugar type or concentration. c) In co-cultures, gene expression of IL-6 was significantly decreased in the 5mM and 25mM fructose conditions. a and b represent the significant differences observed in post hoc analysis. Data presented are mean ± SEM.