The effects of diet induced obesity and metabolic irregularities on hippocampal-based cognition and neuroplasticity in young female and male rat

A dissertation

Submitted by

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In partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

In

Psychology

TUFTS UNIVERSITY

Graduate School of Arts and Sciences

February 2017

ADVISER: ROBIN KANAREK

Abstract

Recent research has associated obesity with impairments in cognitive behavior and alterations in brain-derived neurotrophic factor (BDNF), with the majority of studies examining this in older adult or aging animals. To expand upon these efforts, two experiments were performed to examine the effects of diet-induced obesity (DIO) on spatial performance and hippocampal BDNF expression in young adult female and male rats. To investigate these issues, female rats (Experiment 1 and male rats (Experiment 2) were fed either a standard chow diet or the standard diet and a 32% sucrose solution which has been associated with diet-induced obesity (DIO). Rats remained on these diets for 4-, 8- or 12-weeks. Compared to their chow-fed counterparts, female DIO rats consumed significantly more calories, weighed significantly more, and exhibited significant alterations in glucose metabolism. However, these obesity-related physiological changes were not associated with concurrent impairments in spatial ability as measured using the Morris water maze, and only the 4-week DIO dietary intervention demonstrated a difference in hippocampal BDNF mRNA expression. Compared to their chow-fed counterparts, male DIO rats consumed significantly more calories than their chow-fed counterparts, weighed significantly more, and exhibited significant alterations in glucose metabolism. However, obesity-related physiological alterations were not associated with concurrent impairments in spatial ability or differences in BDNF mRNA expression, with the exception of the 12-week DIO animals performing significantly better than their chow-fed counterparts during the reversal probe trial on the final day of training. These findings were unexpected and will be discussed further later in the thesis.

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Introduction

Obesity prevalence and Association with Cognitive Dysfunction

Obesity, as defined by the Centers for Disease Control (CDC), as a range in weight that is considered unhealthy and is associated with secondary disease (CDC, 2012). As put forth by the CDC, the adult criterion for obesity is having a body mass index (BMI) over 30 (CDC, 2010). While BMI is a standard set forth by the government, its validity has been debated because it does not take into account factors like muscle mass and overall body type. Nevertheless, it is the standard used at present. Currently, the United States obesity prevalence is high and has generally thought to be due to a behavioral trend towards excess ingestion of calories combined with a decrease in active lifestyles (CDC, 2010). Currently, 34% of adults and 17% of children and adolescents ages 2-19 in the U.S. are obese (CDC, 2010). In addition, nearly 75% of baby boomers are either obese or overweight (American Heart Association, Fact Sheet, 2010), which is an epidemiological factor that has the potential to cause a considerable increased prevalence and severity of age-related cognitive decline in coming decades. Indeed, the degree of obesity-mediated cognitive impairment seems to occur in an age-dependent manner, with older individuals often exhibiting more obvious forms of cognitive impairment than their younger counterparts (Fitzpatrick et al., 2009). This propensity towards exhibiting the most severe cognitive deficits is likely related to general age-related cognitive decline. Scientists have suggested that severe overweight may accelerate the rate of normal age-related cognitive decline (Whitmer et al., 2005; Bruce-Keller et al., 2009). Based on accumulated data, obesity-related cognitive decline is a health concern that is most urgent for aging populations (reviewed in Desai et al., 2010; Luchsinger, 2010). While the need for obesity-cognition

research in the elderly is well-established, innovative and expanded research in younger populations is just recently underway.

The prevalence of obesity in individuals under the age of 20 in the United States has tripled over the past 25 years (Skelton et al., 2009). This is noteworthy statistic for several reasons. Perhaps most relevant here is the uniqueness of this population: reports have shown that a large percentage of obese children will become obese adults or have diseases associated with obesity (Freedman et al., 2001; Wright et al., 2001; Baker et al., 2007). Instances of lifelong obesity could promote an accumulative exacerbation of adverse neural outcomes, with one possible outcome being a future epidemic of premature age-related cognitive decline and neurodegenerative disease. This relationship may be even more apparent in those who have other risk factors for neurodegenerative disease. These types of neural outcomes are most likely if the harmful the effects of obesity on the brain develop in a manner similar to its metabolic complications, with exacerbation of symptoms over the long-term (Jung, 1997; Collins et al., 2009). The potential for a public health outcome of this nature, as well as its neurobiological substrates, is speculative at this time but, nonetheless, deserves attention. However, less severe disturbances in mental capacity are quite likely as the trend in obesity continues in younger individuals of the U.S. and other Western populations. Regardless of the severity of cognitive outcome, lifelong obesity is unlikely to support optimal cognitive aging or overall health.

While the United States leads the way in obesity prevalence, the global spread of excess weight is increasingly significant. Based on data collected in 2008, there are over 600 million obese individuals globally (World Health Organization, 2015). Moreover, compared to past trends when obesity occurred primarily in developed nations, an increase in obesity is now

evident in both developing and developed nations as globalization and Western eating trends spread (WHO, 2010).

Causes of Obesity

Obesity has several different and potentially associated causes, which include genetic and environmental factors and their interactions. Genetic causes of obesity have been well documented (for review see Waalen, 2014). Briefly, though, studies have found that obesity is associated with heritability with twin studies demonstrating high concordance rate of 70% with body weight (Clark, 1956). More recent research suggests that the heritability of obesity is between 40 and 70% (Barsh et al., 2000). Other factors that influence the development of obesity have been developed through monogenic models of obesity, which are models of obesity that demonstrate how single gene mutations can affect obesity (Waalen, 2014). Although most scientists would agree that obesity usually involves gene/gene or gene/environment interactions, monogenic models provided science with important information regarding how genes affect hormonal control, adiposity networks, and appetite control. The first study to demonstrate how single mutations can affect body weight came out of Jackson Laboratories in 1949. Their ob/ob mouse grew up to 3x the size of other mouse strains (Ingalls et al., 1950). Since this early work, other single gene models of obesity have been developed and used to study obesity (Tartaglia et al., 1995). Genome-wide association studies have also yielded much data on the genetic determinants of obesity. Currently, there are 32 established loci for body mass index (Frayling et al., 2007; Loos et al., 2008; Speliotes et al., 2010). In addition, there are 14 loci associated with waist to hip ratio that are related to adiposity and overall weight (Heid et al., 2010).

Another determinant of obesity is environmental factors. Two of the most important factors in the environment when obesity is concerned are diet and exercise. Several studies have demonstrated that diet and exercise are critical to the maintenance of body weight and dietary excess combined with little to no exercise is associated with obesity (Barte et al., 2014; Drenowatz et al., 2014). In fact, Hermoso and colleagues (2014) evaluated the effectiveness of an exercise and an exercise/diet intervention on obesity and parameters of metabolic syndrome in boys. They found that the interventions were both effective at reducing obesity, with the exercise only intervention showing the highest efficacy at reducing metabolic syndrome. Further, the authors found that the interventions had lasting effects during periods called detraining—a post intervention measurement. Continued study of these very important factors that influence obesity will be important in reducing overall numbers of obese individuals.

Another cause of weight gain and obesity involves prescription drugs. Increasingly prescription drugs are being prescribed and are causing weight gain among many individuals. The reasons for weight gain with prescription drugs are thought to be varied and may include changes in metabolism and increases in appetite. Specific classes of medications are known to be related to weight gain and obesity, particularly psychiatric medications (Chowdhury et al., 2014; Jacobowitz et al., 2014).

Obesity and Disease

Obesity is associated with the development of several severe secondary health conditions and diseases. Peripherally occurring metabolic disruptions are very common in

obese individuals and early manifestations are collectively referred to as metabolic syndrome (reviewed in Grundy, 2012). The primary symptoms of metabolic syndrome include hyperglycemia, hyperlipidemia, hypertension, and insulin resistance (Grundy, 2012). Although the symptoms of metabolic syndrome can be reversed or moderated through careful dietary modification and/or the initiation of moderate exercise programs (Kirwan et al., 2009; Roberts et al., 2011), metabolic disruptions can also produce more chronic metabolic conditions such as type 2 diabetes if not monitored and treated (Emanuela et al., 2012; Garber, 2012). Type 2 diabetes is a very common obesity-related metabolic disease, affecting millions around the world (Garber, 2012). Physiologically, type 2 diabetes is a progression of the metabolic abnormalities present in metabolic syndrome and is often more resistant to behavioral based interventions—its most effective treatment often involves pharmacological interventions (Negri et al., 2010). However, it should be noted that part of the ineffectiveness of behaviorally based interventions in individuals with type 2 diabetes is possibly due to patient non-compliance with diet and/or exercise programs, indicating that disease resistance has a behavioral component (incidentally, behavioral non-compliance is possibly also related to the metabolic syndrome to type 2 diabetes progression). Regardless of the contributing factors, the most severe cases of type 2 diabetes seem to be better managed pharmacologically.

Although once medically defined as a disease of adult onset, physicians are presently diagnosing type 2 diabetes with increasing frequency in individuals under the age of twenty (Zeitler et al., 2010). According to the American Diabetic Association's most recent estimates SEARCH, a diabetes investigative committee, found that, overall, the prevalence of type 2 diabetes had increased 21 percent among American youth from 2001-2009, with current estimates of well over 20,000 individuals under 20 with the disorder. This number may be a

drastic underestimate, as some believe that there are up to two million cases of diagnosed prediabetes in the pediatric population (Duncan, 2006). The present number of pre-diabetic and diabetic children and adolescents mirrors a continued global trend towards an overall increase in prevalence of diabetic pathology (Atkins and Zimmet, 2010). Combined, type 2 diabetes and prediabetes affect an estimated 104 million American adults (National diabetes Fact Sheet, 2011). The increasing prevalence of prediabetic youth is a particularly concerning trend given the high prediabetes- to- diabetes conversion rate that occurs with aging (The et al., 2010).

Obesity has other long-term health complications that affect multiple biological systems. In addition to disruption of peripheral metabolism, obesity predisposes one to other systemic pathologies such as heart disease (Janero et al., 2011) and certain forms of cancer (Calle and Thun, 2004; Li et al., 2012). Cardiovascular disease remains the leading cause of death nationally (CDC, 2015) and the now the most frequent cause globally as well (WHO, 2012). Undoubtedly, the continued global increased prevalence in obesity will negatively affect the prospective health of millions (CDC, 2015).

Not only does obesity profoundly affect peripheral physical heath, but also in the next sections, it will become apparent that obesity has been recently associated with measures of decreased brain health. Cognitive and brain health are other important indicators of the effects of obesity, and the remainder of the thesis will consider factors involved in the relationship between obesity, brain health, and cognition. Among all of the factors and elements that will be considered throughout this thesis, the first factor that will be considered is the neurotrophin brain derived neurotrophic factor and its interactions/involvement with obesity.

Brain Derived Neurotrophic Factor: Historical Perspective and Functional Properties

Brain derived neurotrophic factor is an important indicator of brain health. Brain derived neurotrophic factor (BDNF) is a member of the small protein, multi-factor neurotrophin family (nerve related substances) and an integral factor in learning and memory processes. Therefore, alterations in the expression and function of this protein may underlie some of the cognitive deficits reported in obese individuals.

BDNF was the second neurotrophin to be characterized following nerve growth factor (NGF). Its discovery, in 1982, resulted from an effort to provide an alternative survival factor for several neuronal populations, specifically dorsal route ganglion cellular populations that were unresponsive to NGF. Scientists first purified BDNF from a pig brain (Barde et al., 1982) and, early on, it was characterized as a factor supporting neural fiber outgrowth of chick sensory neurons (Barde et al., 1982). BDNF was distinguished from NGF, a related member of the neurotrophin family, because of its different antigenic properties and specified functional characteristics (Barde et al., 1982).

BDNF protein is encoded by the *Bdnf* gene, which in humans is located on chromosome 11 (Jones and Reichardt, 1990; Maisonpierre et al., 1991). The human *Bdnf* gene consists of several untranslated 52exons with independent promoters. These can be spliced to a 32single coding exon to generate several splice variants of BDNF mRNA (Pruunsild et al., 2007). The 32coding exon (exon IX) contains the sequence that codes for the pro-BDNF protein, an immature form of the neurotrophin. In addition, the 32untranslated region of exon IX is composed of two alternative polyadenylation (polyA) sites, that allow for the generation of one short splice variant and one long splice variant (Timmusk et al., 1993). The use of distinct BDNF mRNA splice variants allows for temporal and spatial regulation of BDNF protein expression, which appears to

be critical in the modulation of synaptic plasticity and spine development in dendrites (Timmusk et al., 1993), which are integral components of cellular processes driving learning and memory. Rodent *Bdnf* gene characterizations have revealed homologous structural and functional properties to that of human *Bdnf* gene. BDNF in the rat brain guides neuroplasticity, which is related to learning and memory in several areas of the brain, but research has placed much emphasis on the hippocampus because of its essential role in specific types of learning and memory (Gomez-Pinilla and Tyagi, 2013).

Considering that BDNF is a multifunctional growth factor playing essential functions in the developing and mature brain, identified *Bdnf* genetic variants likely contribute to neural and behavioral alterations. The Bdnf Val66Met single nucleotide polymorphism (SNP) is highly prevalent in humans and has been associated with cognitive deficits. It consists of a valine substitution for methionine (Spelitoes et al., 2010) in the 66th position on the amino acid sequence of the protein (Egan et al., 2003). This SNP has functional consequences, impeding activity-dependent secretion of BDNF and signaling (Chen et al., 2004). It was reported that obese children and adolescents were more likely than non-obese children and adolescents to be Val66Met polymorphism carriers (Skledar et al., 2012). While this study did not examine cognition as it relates to BDNF and the valine, methionine substitution, a small number of other studies have examined these factors in combination and their results suggest this relationship (Beckers et al., 2008; Skeldar et al., 2012). Although most studies did not test cognitive functioning in obese individuals, there was decreased serum BDNF, which could be a factor that suggests decreased cognitive functioning.

BDNF is widely expressed in the developing and mature brain , and promotes neuronal survival and differentiation and synaptic plasticity (Phillips et al., 1990). It interacts with two receptors, the low-affinity nerve growth factor receptor p75, and the tyrosine kinase receptor B (TrkB), its primary receptor. TrkB has been well characterized in terms of its interactions with BDNF, and has been shown to regulate neuronal survival and differentiation (Bothwell, 1995). Mature forms of BDNF activate TrkB and their interactions regulate dendritic structure, synaptic formation, and synaptic plasticity (Lee et al., 2000). Within cellular populations, BDNF is transported both retrogradely and anterogradely to synapses, interacts with TrkB receptors, and affects neurogenesis, plasticity, and NT release (Reichardt, 2006). One particularly pertinent function of BDNF-TrkB interactions is activity-dependent neuroplasticity, a cellular process associated with learning and memory (Waterhouse and Xu, 2009). Generally, synaptic plasticity involves activity-dependent specific strengthening or weakening of individual synapses that result in the representation, processing, and storage of information in intricate neural networks (Waterhouse and Xu, 2009). Several studies have found that diseases of the metabolism, such as obesity and diabetes can impair BDNF-TrkB interactions and, subsequently, activity-dependent neuroplasticity (Grillo et al., 2011a; Grillo et al., 2011b; Porter et al., 2011; Porter et al., 2012; Gupta et al., 2013; Sharma and Fulton, 2013). A more detailed description of these and similar studies will be provided in later sections of this thesis—the basic idea to note here is that the neurotrophin BDNF interacts with TrkB to affect neuroplasticity, which can, in turn, be affected by different disease states.

P75 receptor has also been well characterized in terms of its signaling interactions and diverse set of functions (Reichardt, 2006). One of the important functions of P75 is interacting with BDNF and producing hippocampal long-term depression (Woo et al., 2005; Sakuraqi et al.,

2013; Yang et al., 2014), which is the functional opposite to the mediation of long-term potentiation that occurs with BDNF-TrkB interactions (Zakharenko et al., 2003; Zukuraqui et al., 2013; Yang et al., 2014). Specifically relevant to the interaction between BDNF and P75 is that P75 is bound with high affinity by the precursor for BDNF called proBDNF (Yang et al., 2014). Zakuraqui and colleagues (2013) examined the two distinct signaling pathways associated with BDNF and P75 and TrkB and reported that the two pathways are essential for the development/enhancement of (TrkB) and elimination of (P75) synapses in the brain. The researchers also confirmed this functional specificity through masking of the function of the specific signaling interactions (Zakaraqui et al., 2013). Research has reported that its biological functions may involve pro-apoptotic activity when bound by neurotrophins such as BDNF (Ichim et al., 2012). Others have reported the opposite, that P75 receptors that are unbound will be pro-apoptotic (Ribizadeh et al., 1993). It is very likely that proapoptotic function participates in re-structuring of synapses and eventual elimination of synapses that are no longer functionally useful as was suggested in previously cited studies (Zakaraqui et al., 2013; Yang et al., 2014). Indeed, apoptosis has been reported to be an important part of long term depression (Egashira et al., 2010).

Brain-derived neurotrophic factor serves several essential biological functions, including facilitating neuroprotection and promoting neuroplasticity in several regions of the brain, which is relevant to this thesis because of its cognitive focus (reviewed in Hennigan et al., 2007). Additionally, there is accumulating evidence to suggest that BDNF is involved with the activation of molecular memory pathways and other processes that support memory formation (Rendeiro et al., 2012). Many studies have demonstrated that alterations in BDNF protein levels can lead to cognitive deficits (Suri et al., 2012; Kwon et al., 2013). Specifically, obesity and

obesity promoting diets can cause fluctuations in brain BDNF content in rodents that result in cognitive deficits some of which have been demonstrated to be reversible (Yamada-Goto et al., 2012; Kaczmarczyk et al., 2013; Moy and McNay, 2013).

BDNF and Learning Properties

Given its molecular and functional properties BDNF is an ideal candidate to study when investigating mechanisms underlying learning and memory deficits in obese individuals. Increases in BDNF are typically associated with improved learning and preserved cognitive functioning, whereas decreases in this neurotrophin generally have the opposite effect. For instance, resistance exercise in mice increased BDNF levels in the hippocampus and performance on the Morris water maze, a standard test of spatial cognition (Suijo et al., 2012). Along with increases in BDNF, mice exhibited elevated content of other plasticity molecules such as CREB, indicating that signaling pathways relevant to cognition had been activated, particularly in the hippocampus (the brain region activated during maze training) (Suijo et al., 2012). In another exercise study, rats that had sustained stroke or healthy rats were exercised on a treadmill for 30min/day, 7 days a week for a predetermined time (Quirie et al., 2012). The results demonstrated that exercise had therapeutic effects in rats that had experienced a stroke. Specifically, stroke rats had increased levels of mature BDNF in frontal regions after the exercise intervention (Quirie et al., 2012). One limitation of this study was that the researchers did not attempt to detect any functional or behavioral effect of the exercise treatment. It would be interesting to determine the relationship between changes in BDNF and behavior. In addition, Noble and colleagues (2015) found that exercise reduced diet-induced cognitive impairment and increased BDNF levels in rats. Similar findings were reported in several other studies (Gomez-

Pinilla et al., 2008; Liu et al., 2009; Griesbach et al., 2010; Sun et al., 2015; Sleiman et al., 2016).

The effects of obesity on BDNF levels has been investigated. Yu and colleagues (2009) explored how different dietary conditions and propensity to develop obesity affected BDNF expression in the mouse hippocampus. When diet-induced (DIO), diet-resistant (DR) (mice that are resistant to gaining weight on obesogenic diets), energy restricted pair-fed, and low fat (LF) diet mice were compared, BDNF and TrkB mRNA expression were decreased in the hippocampus of DIO mice fed a high-fat diet. There were no differences between DIO and DR mice on the pair-feeding or LF diets. These findings suggest that the DIO mice, or only those mice most susceptible to gaining weight, were susceptible to reduced expression of BDNF and TrkB in their hippocampi when fed a high-fat diet. This effect supports an interaction between obesity and dietary composition that produces an overall susceptibility to decreases or alterations in neuroplasticity factors.

Human studies have also examined potential links between obesity and BDNF levels. Araya and colleagues (2008) completed such a correlative study, which examined how calorie restriction (CR) affected serum BDNF concentrations in obese individuals, and in turn, how that was related to insulin function (as measured by oral glucose tolerance test). Results indicated significant improvement in several measures of general health as well as increases in insulin sensitivity in obese individuals who remained on the CR diet for 3 months. In addition to these improvements, there were significant increases in serum BDNF concentrations that were negatively correlated with weight and positively correlated with insulin sensitivity. The scientists indicated that the changes in BDNF serum concentrations in CR obese individuals may have been due to the changes in dietary composition, and that BDNF serum concentration may be

affected by dietary composition. However, it is unclear whether the total weight loss due to the change in diet may also play a substantial role. Further research is needed to determine the nature of this effect.

In a more recent study of a related subject, Karczewska-Kupczewska and colleagues (2011) reported a positive relationship between serum BDNF concentration and insulin sensitivity in healthy, non-obese women, with women with lower insulin sensitivity having lower serum BDNF concentrations. These findings suggest that, independent of obesity, decreased insulin sensitivity is associated with reductions in serum BDNF concentrations. Additionally, it is an important finding because insulin insensitivity affects millions of obese individuals and the possibility that we can detect changes in insulin sensitivity early, could have important implications for a growing obese population. Finally, we must consider the potential cognitive consequences of lowered concentrations of BDNF as an ongoing health concern for individuals who suffer from decreased insulin sensitivity, such as obese individuals. Indeed, obesity has been associated with disturbances from mild cognitive deficits (Lokken et al., 2009; Roberts et al., 2010) to neurodegeneration (Cereda et al., 2007; Whitmer et al., 2008; Fitzpatrick et al., 2009) and any effort at prevention of these conditions is worthwhile.

Obesity is a physiological stressor, and investigating the effects of substances that act as protective factors against other physiological insults might be a relevant and important line of research. Of note, BDNF provides neuroprotection and promotes neuroplasticity in several regions of the brain and is an integral molecular mediator of a host of essential neural processes, such as neural differentiation, survival, proliferation, and plasticity (Gomez-Pinilla and Vaynman, 2005). There is also evidence to support BDNF's involvement in the potentiation of molecular processes underlying learning and memory (Lu et al., 2011; Rendeiro

et al., 2012). Indeed, BDNF supports essential functions during induction of both early and late phase long-term potentiation (L-LTP) in the hippocampus. One study found that BDNF supported LTP by stimulating neuronal inputs by acting as a mediator of NMDAR and AMPA receptor insertion on the postsynaptic membrane (Meis et al., 2012). Another study examining the protective effects of exercise found that exercise preserved cellular cascades that regulate early long term potentiation and positively modulated P-CaMKII and BDNF in the hippocampus (Zagaar et al., 2013). This study suggests that exercise protects processes that are essential to learning and memory, such as P-CaMKII and BDNF-mediated LTP. While we have discussed different findings from various studies, the cellular process has not been described in depth. More precisely, BDNF mediates cell surface expression of NMDAR, which are required and sufficient for LTP induction. BDNF also mediates neurotransmitter release and structural changes such as spine formation and synaptogenesis. Far less is known regarding BDNF-mediated L-LTP as it relates to obesogenic states, as a means of identifying additional putative mechanisms for changes in long-term memory. The cumulative evidence predicts that obesity-related decreases in levels of hippocampal BDNF would manifest cognitively as a diminished potential for long-term memory formation.

There is evidence to support the hypothesis that expression of BDNF is sensitive to and altered by obesity-promoted metabolic complications. Rodent models have shown that obesity, whether it is diet-induced or genetically determined, is associated with decreases in BDNF levels in several regions of the brain and cognitive impairment (Rios et al., 2001; Molteni et al., 2002; Wu et al., 2004; Stranahan et al., 2008). Among all brain regions, obesity has been shown to disproportionately decrease BDNF levels (Stranahan et al, 2008). Additional observations of a similar kind may prove particularly useful in advancing our understanding of obesity's role in

pathogenic processes that result in dementias and Alzheimer's disease. Indeed, early aberrations of hippocampal tissue often occur in neurodegenerative disease (Rekart et al., 2004; Donix et al., 2013; Yeung et al., 2013). Given this observation regarding possible neurotrophic involvement in AD etiology, it is possible that aberrations in BDNF signaling represent the start of an anomalous downstream molecular cascade that precedes general neuropathy and neurodegeneration. The exact biological mechanisms responsible for any such neuromolecular events remain to be elucidated.

Additional evidence from rodent models suggests that obesity-induced cognitive decline is proportional to decreases in levels of BDNF in the hippocampus, with larger reductions in BDNF associated with greater cognitive deficits (Stranahan et al., 2008; Yamada-Goto et al., 2012). One limitation of previous research regarding obesity-promoted reductions in BDNF levels is that it had provided data limited to neuromolecular alterations. However, research demonstrating concurrent obesity-related molecular and structural/morphological in the hippocampus has begun to provide evidence to challenge this limitation (Stranahan et al., 2008; Park et al., 2010; Tozuka et al., 2010). Stranahan and colleagues (2008) found that, in addition to reductions in hippocampal BDNF, rats maintained on a high calorie diet (HCD) for 8 months had decreased dendritic spine density and impaired LTP in the CA1 region. These data suggest that long-term high calorie feeding can result in neurochemical and morphological changes in the hippocampus, lending support to the hypothesis that alterations in hippocampal BDNF levels may be associated with changes in hippocampal function. Similarly, Park and collaborators (2010) found that a high-fat diet that contributed to obesity lead to lipid peroxidation, decreased BDNF levels, and impaired neurogenesis in the hippocampus. As early as 2002, Molteni and colleagues demonstrated that a diet high in saturated fat and refined

sugars lead to reductions in hippocampal BDNF protein. Molteni and collaborators (2002) also observed reductions of other molecules associated with BDNF mediated synaptic transmission, such as Synapsin I and GAP-43. Although structural changes were not measured directly in this study, changes in molecules that direct activities at the synapse may indicate structural alterations in the hippocampus. Notably, Moltenti et al. (2002) and Stranahan et al. (2008) found that deceases in BDNF levels, hippocampal structural integrity, and cognitive functioning were associated with the severity of obesity and length of dietary treatment. Together, these data suggest that the severity of cognitive impairment in obesity may be related to the degree to which neurotrophic support is limited in the hippocampus; consequently, the degree of cognitive impairment may be predicted based on decreases in BDNF.

The structural and cellular basis for memory formation in the hippocampus

The hippocampus plays a pivotal role in learning and memory processes and BDNF is highly expressed in this brain region relative to other areas. In general, hippocampal structure is conducive to learning, with its molecular circuitry being organized to promote processes such as long-term potentiation, the cellular process thought to underlie learning and memory. Two C-shaped structures, the cornu ammonis (CA) and the dentate gyrus form an interlocking C shaped structure that comprises most of the overall structure of the hippocampus. The CA is further subdivided into 4 different, abutting structures, termed CA1-CA4. The specific regions and subregions that occupy the different parts of the hippocampus serve both distinct and overlapping functions. Aside from sending neural signals to the CA 3 region of the hippocampus, the dentate gyrus is involved in spatial function and is the site of adult neurogenesis (progenitor cells are also important here during developmental periods) (Vivar and van Praag, 2013). For the most part, the cornu ammonis is dedicated to neuroplasticity and long term potentiation, with the

CA3 region containing abundant synaptic input and producing robust LTP **(**Johnstone and Raymond, 2013**)**. Several studies have investigated the effect of diet and obesity on molecules expressed in the hippocampus and known to mediate memory processes. For example, a study examined the effects of calorie restriction on glutamate N-methyl-D-aspartate receptors (NMDARs) in CA1. They observed a decrease in the NR2A and NR2B subunits of NMDA receptors in obese non-calorie restricted animals, which was normalized by calorie restriction (Yilmaz et al., 2011). These findings are relevant because NMDARs are essential participants in several aspects of hippocampal-dependent learning (Yilmaz et al., 2011) and, as evidenced by their study, obesity may disrupt these essential processes. The authors suggest that one mechanism by which calorie restriction may promote restoration of NMDARs is through reducing oxidative stress (Yilmaz et al., 2011). If this is, indeed, the mechanism at play, then there are potentially many benefits of calorie restriction beyond just reducing obesity, such as those reported by Yilmaz and colleagues (2011). The full extent of such benefits will be determined through rigorous continued study of the molecular and cognitive benefits of reducing oxidative stress. In a more recent study, investigators examined the effects of Liraglutide on LTP and associated proteins in CA1 of obese and non-obese mice (Porter et al., 2013). Liraglutide is a glucagon-like peptide-1 (GLP-1) agonist used for the treatment of diabetes that can reverse the negative obesity-brain interactions such as cognitive disruptions. Data showed that, whereas LTP was entirely abolished in obese mice compared to lean controls, Liraglutide treatment resulted in a restoration of LTP in obese mice to levels comparable to those of lean controls. Concomitant with the restoration of LTP in obese mice, there were improvements in multiple metrics of insulin function and a significant increase in the expression of Mash 1, a protein thought to promote cell survival and neurogenesis (Kim et al., 2011). The cumulative

data inform cellular and molecular events that might underlie the reversibility of obesity-brain interactions impairing cognitive function.

Finally, it is important to discuss the function of the entorhinal cortex. The entorhinal cortex has aptly been described as a “hub” for memory processes, serving as the primary neural interface for the hippocampus and the neocortex (Lipton and Eichenbaum, 2008). In addition, it has been demonstrated that high frequency coupling occurs between the entorhinal cortex and the hippocampus during the encoding or retrieval of spatial learning in rats (Igarashi et al., 2014). These data suggest that the entorhinal cortex is involved in the process of learning. The importance of the entorhinal cortex can be determined in several ways. One group of researchers approached the question using a dietary manipulation. Arsenault and colleagues (2012) varied the amount of unsaturated fatty acid in the diets of mice to determine what would happen to the physiological response of neurons in the entorhinal cortex of these animals. They found that the animals with the highest levels of unsaturated fatty acids in their diets had the greatest modulation, or increased action potentials in neurons of their entorhinal cortex. Diets high in saturated fats have been linked to obesity in humans and rodents in several studies (Lin et al., 2013; Pranprawit et al., 2013), which suggests that obesity might decrease the responsiveness of neurons in the entorhinal cortex. While this could be a promising avenue of research, the effects of diet and obesity on entorhinal cortex function remain vastly under studied. Nevertheless, these findings constitute only a small number among a host of studies that suggest that obesogenic diets and obesity can adversely affect hippocampal physiology, structure, and function in general. A recent study demonstrated that high fat induced hippocampal apoptosis and gliosis were reversed by pharmacological administration of an isoflavone (Rivera et al., 2013).

Isoflavones, known for their antioxidant properties, can be potent anti-obesity and anti-high-fat diet substances, protecting the brain and body from the negative effects of obesity and obesogenic diets (Babu et al., 2012; Praisain et al., 2012). Investigations of this kind are useful in determining effective therapeutic strategies for treating obesity and related metabolic disturbances, and concomitant cognitive deficits.

The cellular basis for memory processes is an important mechanistic aspect of these processes to consider because of the hippocampal role in obesity-related cognitive decrements. In 1949, Hebb first discovered that memories could be stored by forming associative connections between neurons. At the time, this was an impressive study and it would be the beginning of research that led to some of the most important discoveries in memory science. Several decades later, scientists would extend Hebb's theories to demonstrate that electrically induced increases in neural firing could increase synaptic strength, essentially arriving at the neural equivalent of Hebb's original findings (Jeffery, 1997). This neural equivalent of Hebb's work became known as the process of long-term potentiation (LTP), and excitement surrounding exactly what it represents on the neural level and beyond has grown over the years. Potentially among the most important of its functions is that LTP is thought to function as a cellular mechanism of learning and memory whereby the strengthening and association of synapses promotes learning and memory. Though considered by many in science a valid theory, and supported by much research (Martin and Clark., 2007; Ahmed et al., 2012; Nabavi et al., 2014), there is still debate surrounding the exact role of LTP in learning and memory processes. Another form of cellular learning is termed long term depression (LTD), and is sometimes referred to as the functional opposite of LTP (Mills et al., 2014). Additionally, if LTD is deficient during learning one of its functions, extinction of previously learned functions, will not occur properly (Mills et al., 2014).

Mills and colleagues (2014) found that the stabilization of a learning complex in vivo was associated with impaired cognitive functioning and decreased LTD, suggesting that LTD was integral to said cognitive functions. As suggested by the previous study, LTD is an important form of plasticity and thought by many to be involved in the process of unlearning (Dong et al., 2013; Mukherjee et al., 2014). Given its unique properties, LTD can participate in many properties of neuroplasticity and interact with LTP.

Years of research on LTP as a substrate for learning and memory have revealed that the relationship between LTP and memory are much more complex and intricate than once thought. Studies have revealed that LTP is not always the cellular mediator for consolidation of memories. For example, one study demonstrated that blocking LTP did not have any effect on spatial performance, whereas inhibiting LTD did impair spatial consolidation (Ge et al., 2010). Results of this study suggest that in some instances LTP may not participate in memory processes like consolidation; in fact, in this case it inhibited long-term depression, which usually produces an activity dependent reduction in neural response—the neuro-functional opposite to LTD. These results demonstrate that some neurobiological processes are preferentially mediated by LTD— processes such as consolidation may be regulated by decreased neural activity and LTD in certain regions of the brain. Additionally, it seems there are multiple layers to this relationship, between LTP and consolidation, and it is not clear which factors actually influence learning and memory (Raymond, 2007). As described by Raymond (2007), there are several factors that can influence the successfulness of LTP induction including the cell type, developmental stage, and the actual induction protocol used. Raymond (2007) also described how different types of LTP influenced results of studies, with his primary comparison being between early (E)-LTP and late phase LTP (L)-LTP and their associated stages (E)-LTP (stage 1)

and (L)-LTP (stages 2 and 3). Over the years, the different stages of LTP have been defined in order of increasing persistence as, LTP 1, LTP 2, and LTP 3. Additionally, Raymond and Redmand found evidence suggesting that the different phases of LTP are maintained by different sources of Ca++ (Raymond and Redman, 2000; Raymond and Redman, 2006). This specificity of the processes of the phases of LTP make them difficult to replicate experimentally. This factor will be discussed more shortly. As stated above, LTP1 is equivalent to E-LTP, a rapidly decaying, protein synthesis-independent and probably involves post-translational modifications of various synaptic proteins. LTP2 is an intermediate phase of L-LTP that involves dendritic protein synthesis but is independent of gene transcription. Finally, LTP3 represents the robust, translation- and transcription-dependent component of L-LTP. It is the various biological characteristics of these distinct stages of LTP that makes them difficult to replicate across experiments. If experimental conditions are not held constant and experimental comparisons are not well informed, then induction success will ultimately vary. It is these types of observations that will continue to fuel the debate. Generally speaking, however, current thinking suggests that it is likely a combination of molecular cascades that result in the memory process—a process that is probably often the result of LTP, but is also moderately influenced by several or different mechanisms and biological circumstances. While there is still some debate as to the many nuances of LTP, its importance in the field of neuroscience remains very strong. Neuroplasticity or activity dependent changes in neural structure and function that can promote long-term changes in neural populations are an important property of hippocampal neural cells.

Another, scientifically groundbreaking, property of the hippocampus is that it has the capacity to give birth to new, adult-born neurons, or AHN. Adult hippocampal neurogenesis is a

process that involves the generation of newborn neurons in two privileged areas of the adult brain, the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles (Kempermann and Gage, 2000). Adult neurogenesis has been discovered in all mammals studied to date, including humans (Eriksson et al., 1998), which is important in light of all of the research that has been dedicated to examining hippocampal function in obese organisms. The detailed process of adult neurogenesis encompasses the proliferation of resident neural progenitor cells and their consequent differentiation, migration, and functional integration into the pre-existing neural network (Kupperman et al., 2003). Of these newly integrated cells, many die within two weeks while the surviving neurons, located in the subgranular zone, send out axonal projection to CA3 neurons and the hilus to form functional synapses within three weeks (Kupperman et al., 2003). After connections are functional, the new neurons start to also receive input from the cortex and are able to start firing action potentials, which represents neural maturity and full integration into the neural circuitry (van Praag et al., 2002). Molecular control of regulating AHN is very complex and remains to be fully described. Some of the mechanisms that are currently under investigation include growth hormones, cytokines, neurotransmitters, DNA methylation, histone acetylation, and non-coding RNAs (Mu et al., 2010). Evidence suggests that these factors intervene at different stages of proliferation, differentiation, survival, migration, and integration of adult neurogenesis (Syahrul et al., 2013). Interestingly, BDNF has also been implicated in several of these processes, which demonstrates how integrated many neural events are (Gomez-Pinilla and Vaynman, 2005).

As suggested earlier, AHN has some potential implications for learning and memory, which has been supported by some studies of ablation/blockade of neurogenesis (reviewed in Koehl et al.,2011). There also has been some more recent work with dietary supplements, such as

antioxidants and high-fat diets, and their role in promoting/inhibiting neurogenesis. The focus here will be on the blockade and dietary supplementation studies because they can tell us more about how AHN might affect behavior or memory. The first study explored blocking the growth of new neurons in the hippocampus of animals in an effort to determine whether hippocampal neurogenesis was required for hippocampal-dependent learning tasks. These studies revealed that adult-born hippocampal neurons are required for some forms of hippocampal-dependent learning. Petrus and colleagues (2009) found that NMDA and benzodiazepines acted as agonists, promoting neurogenesis, or antagonists depending on which cell precursor stage was active. Specifically, GABAergic activity blocked AHN when the cells were in initial stages of development and glutamatergic activity promoted AHN during advanced stages of development. The effect of dietary supplementation on neurogenesis is an emerging area of research. Fan and colleagues (2012) investigated the protective and neurogenic properties of licorice flavonoids in adult rats. They found that rats supplemented with the flavonoid substance exhibited reduced serum corticosterone and increased neuronal progenitor cell number in the dentate gyrus of the hippocampus. Additionally, these rats displayed reduced depressive behavior, a measure that somewhat departs from the behavior discussed thus far but, nevertheless, demonstrates that neurogenesis can be associated with changes in behavior. Additional studies that have investigated the subject of obesity and/or nutrition and their impact on adult hippocampal neurogenesis. For example, Park and colleagues (2010) investigated whether a high-fat diet would induce changes in neurogenesis in the hippocampi of male mice. They found that a seven week high-fat diet, which promotes oxidative stress, significantly decreased the number of newly born cells in the dentate gyrus of the hippocampus without overall neuronal loss. HFD also increased the level of malondialdehyde (MDA), a marker for oxidative stress, and decreased the

level of brain-derived neurotrophic factor (BDNF) in the hippocampus relative to control animals. One limitation of this study is that they did not measure behavior. It would be useful to know whether the decreased neurogenesis and decreases in other neuroplasticity factors were associated with behavioral deficits, learning and memory in particular.

Just a year before the work of Park and colleagues (2010) was published, researchers were interested in determining whether the detriments of a high-energy diet and/or obesity could be passed to one's offspring. In this study, adult female mice were fed either a normal diet (ND, 4% fat) or a high-fat diet (HFD, 32% fat) six weeks before mating and throughout pregnancy and the majority of lactation. They found that HFD offspring showed obesity and hyperlipidemia during suckling (Tozuka et al., 2009). Additionally, the results demonstrated that malondialdehyde, a product of peroxidized lipids, reduced the proliferation of hippocampal progenitor cells in vitro and that neurogenesis in HFD offspring during postnatal development was similarly lowered relative to the ND animals. This was an important study because it showed that obesity-related brain changes, specifically neurogenesis, could be passed to offspring, potentially through metabolic and oxidative changes that occur with obesity. Not only are there direct effects of diet and obesogenic factors on neurogenesis on the affected organism, but these factors seem to be robust enough to affect brain development of later generations. The translational possibilities to humans have yet to be explored.

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While there are several studies that suggest that a high-fat diet and/or obesity can compromise hippocampal neurogenesis, some studies have only partially supported these findings. Interested in the potential effects of adolescent obesity on brain functioning, Boitard

and colleagues (2012) compared the effects of a high-fat diet in juvenile and adult mice. What they found was that the juvenile mice seemed to show sensitivity to the diets, displaying several cognitive deficits and decreased neurogenesis, relative to the adult animals fed the same diet (Boitard et al., 2012). An interesting approach to the subject and certainly novel in findings, this study seems to be in contrast with the work of Park and colleagues (2010) where a high-fat diet did adversely affect neurogenesis in adult mice. There could be a number of explanations for the differences, but one that usually is apparent right away is diet differences. Boitard and colleagues (2012) used a 24% fat/HFD and Park and colleagues (2010) used a 45% fat/HFD in their studies. The difference in fat percentage alone is a significant one in the sense that it may explain why Boitard and colleagues findings diverge from the literature. Various factors and mechanisms involved in obesity related changes in cognition have been established, and I will proceed to discuss the brain and cognitive evidence in the next sections of the thesis.

Effects of Obesity on Brain Structure, Function, and Cognitive Capacity: Human Evidence

Developing interest in the effects of obesity on brain and behavior has revealed a compelling body of work. Research examining brain structure has revealed morphological changes in various regions in brains of obese individuals (Jagust et al., 2005; Pannacciulli et al., 2007; Gazdzinski et al., 2008; Yau et al., 2010). Imaging studies have provided measures of brain structural integrity and brain activity responses to appetitive stimuli and food intake in obese individuals (reviewed in Neary and Batterham, 2010). For example, abnormal cholesterol levels have been associated with prefrontal white matter abnormalities in obese individuals relative to healthy controls (Cohen et al., 2011). Moreover, abdominal obesity was has been associated with decreased gray matter (Debette et al., 2014). Together, these studies suggest

that obesity can compromise the integrity of gray and white matter of the brain, leaving both neurons and astrocytes vulnerable to the effects of the disease. In another study with adults, gray matter volume in essential regions for the control of food intake was different in obesity-prone versus obesity-resistant individuals (Smucney et al., 2012). Obesity-resistant and obesity prone participants were defined by the following criteria, obesity-resistant participants were selected by their natural tendency to be thin and they had lower or normal BMI. Obesity-prone participants were selected because they struggled with their weight on a regular basis and were overweight or obese. The specific brain regions in this study that were found to be decreased in obesity-prone participants included the orbitofrontal cortex, left insula, and cerebellum, all of which have been related to food intake (Smucney et al., 2012). Functional imaging has also reported differences in brains of obese individuals as compared to normal weight individuals or different groups of obese individuals. In one study, the default mode network (DMN), a functionally related network that includes the posterior cingulate cortex, cuneus/precuneus, medial frontal cortex, parietal cortices, anterior cingulate cortex, and insula was normalized in obese individuals after a 6 month exercise intervention (McFadden et al., 2013). Normalization of the network was associated with decreased activation of the DMN, decreased hunger ratings, and decreased weight in obese individuals (McFadden et al., 2013). The authors suggested that the normal overactivity of the DMN in obese individuals may be related to overeating and behaviors that help maintain the obese state. In another investigation of brain activity in obese individuals who had been in a 6-week dietary intervention (reduction of 400 cals per day), successful weight loss was linked to increased activation in right temporal areas including hippocampus and fusiform gyrus (Hege et al., 2013). The authors attributed this increased activation in those particular brain regions to altered control over food intake and a

better ability to eat less and successfully lose weight. On the other hand, activation was distinctly different in those obese individuals who gained weight. Together, these findings suggest there is a neural network that helps determine individuals who may be better at regulating their food intake.

Behavior is also a component of many of these imaging investigations. Vainik and colleagues (2013) reviewed studies that found that there are different neurobehavioral correlates in obese individuals relative to normal weight individuals that involve neuroticism, motivation, and self-control. These behavioral tendencies were related to differences in critical prefrontal structures, and manifested in obese individuals as increased neuroticism, decreased motivation, and decreased self-control. The scientists attributed the brain and behavioral differences in obese individuals as being part of the side effects of obesity. In other work that examined behavioral aspects of obesity, children with obesity tended to demonstrate mild impairments in memory and other measures of cognitive functioning (Lokken et al., 2009; Roberts et al., 2010). However, a recent study argues that it is actually the evaluation of an obese child's cognitive function that is essential to determining cognitive outcomes (Zavodny, 2013). This finding implies that stigma surrounding being obese might be detrimental to an obese child's cognitive functioning equally as much or more than actual physiological processes. Researchers have yet to determine what the contribution of each component is in relation to cognitive performance in obese children and adolescents. Relative to younger individuals, older adults often have an increased propensity for cognitive deficits that can resemble, or actually mimic, neurodegenerative mental decline (Cereda et al., 2007; Whitmer et al., 2008; Fitzpatrick et al., 2009), and these deficits have a clearer neurobiological basis. Cognitive effects resembling

neurodegenerative decline are particularly concerning because they have the potential to cause a significant escalation of obesity-related healthcare costs as the baby boomer generation ages.

Among the literature on the effects of obesity on cognitive performance, there is a growing body of epidemiological research that is recent and important. An imaging study found that obese Mexican children had reduced executive functioning relative to their normal weight counterparts (Bauer et al., 2014). In addition, the brain imaging revealed that there was a positive correlation between left globulus pallidus and BMI. The authors hypothesized that this difference in brain structure in obese individuals contributed to a dysfunctional network underlying the development of obesity. In another imaging study, the authors were interested in determining whether obesity without complications from insulin resistance would be associated with cognitive impairment and changes in brain structure (Yau et al., 2014). Relative to lean counterparts, obese children had significantly lower academic achievement (i.e., arithmetic and spelling) and tended to score lower on working memory, attention, psychomotor efficiency, and mental flexibility. Additionally, obese, non-insulin resistant children had reductions in the thickness of the orbitofrontal and anterior cingulate cortices as well as reductions of microstructural integrity in major white matter tracts without gross volume changes. The authors concluded that the cognitive and brain changes were likely a less severe or more subtle forms of what occurred in previous studies with obese individuals with insulin complications. Several other studies have reported cognitive or brain changes in obese individuals (Li et al., 2008; Mayaan et al., 2011; Hawkins et al., 2014; Liang et al., 2014). Interestingly, when you examine the nature of this literature you find that the cognitive deficits in obese individuals fall into one of two categories—impairments on specific cognitive tasks or impairments in realms of cognition that are important in the control of food intake. It is important to recognize this

distinction in the literature so that you can accurately understand the data being presented. Overall, though, there is evidence to suggest that obesity is related to changes in different aspects of cognitive functioning and to changes in areas of the brain the control these functions.

Obesity and Brain Effects: Evidence from Animal Behavior

Research on the effects of obesity in experimental rodent models has provided support for observations made in humans suggesting that obesity produces cognitive deficits (Molteni et al., 2002; Wu et al., 2003; Jurdak et al., 2008; Stranahan et al., 2008; Jurdak and Kanarek., 2009; Sharma et al., 2012; Davidson et al., 2013). In obese humans, obesity-related cognitive impairments are typically more apparent in older individuals. Similarly, the extent of cognitive decline in animal models is influenced by the age of the animal and the severity of derangement of glucose and/or lipid metabolism at time of testing, which is, in turn, related to dietary composition. Younger animals tend to exhibit neurocognitive resiliency to the effects of obesity and cognitive deficits are less acute when metabolic function is only moderately impaired (Jurdak et al., 2008; Jurdak and Kanarek., 2009). Early developmental periods tend to promote preserved cognitive functioning and fewer metabolic symptoms whereas aging generally has an adverse effect on these outcomes (Roberts et al., 2010). It is worth noting that human investigations have confirmed that aging adversely affects cognitive functioning in general, independent of the presence of metabolic abnormalities (reviewed in Salthouse, 2010; Smith et al., 2013). Smith and colleagues (2013) discovered, however, that mitigating factors such as exercise could slow or reverse aging effects on cognition in older individuals. Similar findings have been reported with aging animal models (Yu et al., 2013; Flores et al., 2014; Gu et al., 2014; Merkely et al., 2014; Sumiyoshi et al., 2014). In all of the studies cited, exercise, or the

combination of exercise and another factor, moderated the effects of aging on brain functioning. While the effects of aging on the brain and obesity have been well documented, the possibility of blatant cognitive effects or extreme perturbations in metabolic function in younger organisms is unclear. One reason for this is that severe metabolic disturbance is less common in younger organisms but not unheard of in this population. As obesity and the treatment of obesity becomes better identified among young people, it will be worth exploring the possibility that it contributes negatively to brain integrity. In addition, determining the extent to which these moderating factors, age, physical health/exercise, and others, separately influence obesity-related cognitive decline will be an important next step in disentangling their intricate relationship. At the same time, we will better be able to comprehend how their neurobiological collaboration influences cognitive decline.

Physiological Hypotheses for Cognitive Deficits

Peripheral-Central Metabolic Contribution

Several hypotheses have been put forth to explain the relationship between obesity and cognitive impairment. One suggestion based on human and animal models posits that obesity-related cognitive deficits involve, at least in part, a reciprocal and aberrant interaction of peripheral and central metabolisms (Zhang et al., 2009 et al.; Gunstad et al., 2010). Indeed, these changes have been described as a sophisticated neurophysiological interaction that represents a central recapitulation of systemic metabolic functioning (Zhang et al., 2009). More specifically, scientists have suggested that obesity related systemic metabolic perturbations, such as those occurring with insulin resistance, are accompanied by similar changes in brain insulin regulatory factors (Zhang et al., 2009; Gunstad et al., 2010). Converging lines of

research suggest that proper brain insulin signaling may be integral to the maintenance of cognitive functioning in obese states (and in general), as insulin resistance produces impairments in cognitive abilities (McNay et al., 2010; reviewed in McNay and Recknagel, 2011). Among recent findings, McNay and colleagues (2010) reported that obesity and insulin resistance were associated with decrements in spatial ability that were partially reversed with intrahippocampal insulin administration. McNay and colleagues (2010) reported that the blockade of endogenous hippocampal insulin signaling disrupted cognitive functioning, suggesting that insulin is an essential mediator of brain energetics and hippocampal-dependent cognitive processes under normal metabolic circumstances. Taken together, these data provide support to the idea that disrupted hippocampal insulin signaling may be a neurochemical mechanism underlying obesity-associated cognitive impairment, and that targeted treatments of insulin signaling/function may be effective at reversing or lessening metabolically induced cognitive deficits. Researchers have suggested that specific factors, such as AMP-activated protein kinase (AMPK), which is involved in regulating the response to insulin, energy metabolism, and energy levels, could potentially provide therapeutic applications for obesity and the metabolic syndrome (Lee et al., 2013). Therefore, it is possible that AMPK could be used to successfully treat cognitive symptoms of obesity as well. Several additional factors including glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), and oxyntomodulin (OXM) that modulate the activity of insulin may also function as successful therapeutics for obesity and diabetic pathology are currently being investigated (Irwin and Flatt, 2013). Based on these data, it is apparent that insulin plays a critical role in sustaining proper cognitive functioning.

Disruption of lipid metabolism can also affect cognition, with intrahippocampal delivery of triglycerides impairing rodent spatial ability (Farr et al., 2008). Farr and colleagues (2008) injected triglycerides into the hippocampi of mice, which impaired their performance on the Morris water maze. Similar effects were observed when mice were fed a high-fat diet and became obese. The complete mechanism of this impairment of rodent spatial ability has not yet been elucidated, but the authors suggest that triglycerides impair NMDA receptor (NMDAR) dependent maintenance of long-term potentiation through the inhibition of normal Ca++ entry/signal cascades at synapses (Farr et al., 2008). Though Ca++ activity was suggested as being involved, the authors did not measure this suggestion directly. When obesity and its inherent complexity are considered, researchers have suggested that a combination of abnormalities peripheral and central glucose and lipid regulation contribute to reported cognitive deficits (Bruce-Keller et al., 2008; Zhang et al., 2009; Uranga et al., 2010). Taken together, these findings converge on the idea that peripheral and central metabolic changes accompanying obesity are linked and potentially related to cognitive deficits.

Additional Physiological Hypotheses

Cerebral Vascularization

Based on the hypothesis presented above, the interplay between the brain and peripheral metabolism are likely associated with the development of the obesity related cognitive deficits. associated with obesity. However, several additional hypotheses have been proposed that may explain obesity-related cognitive deficits. One proposal is that disruption in neurological function is the result of changes in cerebral vascularization and cerebral blood flow in obese individuals. Generally, obesity is associated with a decrease in cerebral blood flow (Selim et al., 2008; Bruce-

Keller et al., 2009; Uranga et al., 2010). Aging is also characterized by reduced elongation in endothelial cells, fewer endothelial mitochondria, and progressive impairment in endothelium dependent vasodilation (Taddei et al., 1995; d'Alessio, 2004; Finch, 2005), all characteristics of decreases in blood flow. With the progression of some of these vascular abnormalities described above is the development of conditions such as vascular dementia. Vascular dementia, a condition of reduced cognitive capacity related to the degeneration of cerebral vascularization and reduced blood flow in the brain, has been related to obesity (Debette, 2013; Xu et al., 2013; Exalto et al., 2014; Niedowicz et al., 2014). The results of these studies are important because they suggest—both indirectly and directly—that early life obesity could lead to different forms of dementia later in life. Overall, the effects of obesity on cerebral vascularization and related conditions suggest that there may be an interaction between aging, obesity, and cognitive deficits.

Oxidative Stress

Oxidative stress is another factor that may influence obesity related cognitive deficits. Oxidative stress occurs when oxidative products reach a level where they promote cellular and/or tissue dysfunction (Ding et al., 2006; Al Ghouleh et al., 2011). As was the case for the cerebral blood flow hypothesis, the cytotoxic effects of oxidative stress are increased by obesogenic diets; high-fat diets that promote obesity and metabolic abnormalities have been demonstrated to promote increased levels of oxidative stress (Urakawa et al. 2003; Furukawa et al, 2004.; reviewed in Shah et al., 2007; Matsuzawa-Nagata *et al.* 2008; Mantena et al.2009; Sinha-Hikim et al., 2011; Ma et al., 2014; Tucsek et al., 2014). A recent investigation by Ma and colleagues

(2014) were interested in measuring changes in markers of oxidative stress in the brains of rats fed a high-fat diet for 10 weeks. The markers of oxidative stress in the brain included, lipid peroxidation, mitochondrial reactive oxygen species. and mitochondrial membrane potential. The authors found that the high-fat diet led to increased lipid peroixidation, increased mitochondrial reactive oxygen species, and decreased mitochondrial membrane potential relative to animals fed a standard diet. While Ma et al. (2014) did not assess cognitive functioning, another study did take both factors into account (Tucsek et al., 2014). Considering molecular changes specifically between obesity and oxidative functioning, some organisms with obesity and metabolic syndrome exhibit altered functionality of critical pathway proteins, such as AMPK an integral enzyme in cellular energy homeostasis and a learning molecule, which can be partially reversed with natural substances containing antioxidants (Mor and Unnikrishnan, 2011). This finding is relevant because AMPK is critically involved in learning processes (and other important functions) and is mutually regulated with neurotrophins such as brain-derived neurotrophic factor (BDNF) (Gomez-Pinilla et al., 2008), which will be a major subject of this thesis and will be revisited several times throughout the document. Considered with the investigations described above, these findings suggest that oxidative stress may be one mechanism, among a complex array, of obesity promoted cognitive deficits.

Inflammation

Obesity is characterized by low-level inflammation in peripheral tissues. Once thought to be an inert vehicle for energy storage, over the past 20 years, adipose tissue has been recognized as a metabolically dynamic endocrine organ that produces several bioactive

substances. Some of these substances—or proinflammatory factors—increase during obesity or obesogenic /high-fat diets, and can have a negative effect on functioning. Tumor necrosis factor (TNF-alpha), Interleukin (IL)-1, IL-6, and C reactive protein are all proinflammatory factors that increase during obesity (Lee et al., 2013). TNF-alpha, IL-1, and IL-6 are all related to obesity directly and produced by adipocytes (Omran et al., 1991), while C-reactive protein is produced by the liver and is a more universal inflammatory factor, one that rises in response to levels of IL-6 (Danesh et al., 2004). Each proinflammatory factor serves a specific function. For instance, TNF-alpha exerts its negative effects by antagonizing insulin signaling (Lee et al., 2013). This is an important functional consideration because obesity and type 2 diabetes are associated with decreases in insulin sensitivity (Bocca et al., 2013), and the mechanism underlying this likely involves the actions of TNF-alpha (Liu et al., 2013). Lui and colleagues (2013) fed rats a diet that was high in the ratio of n3/n6 fatty acids, which resulted in decreases in levels of inflammatory factors, including TNF-alpha. This finding suggests that TNF-alpha played a role in promoting insulin resistance because when its levels were lowered, insulin sensitivity improved.

Not only does inflammation/proinflammatory factors affect peripheral functions, there are several studies that have demonstrated that brain functioning is impaired or diminished (Thaler and Swartz, 2010; Cai and Liu, 2012: reviewed in Cai, 2013), which may help elucidate the causes of cognitive dysfunction in obese individuals who have perturbed levels of these substances. While the entire brain is susceptible to inflammation related to obesity, the hypothalamus has been researched extensively because its role in energy regulation. In their minireview of the topic, Thaler and Swartz (2010) described how peripheral inflammation due to

obesity or high fat diets was mimicked in the hypothalamus of the brain. This process in the hypothalamus promotes further weight gain because leptin and ghrelin signaling is altered. Moreover, Jeon et al. (2012) showed that peripheral and central inflammation due to high fat feeding was reversed through treatment with resveratrol, an AMP-activated protein kinase activator, and potent anti-inflammatory substance. When the inflammation was reversed with the medication, cognitive performance improved as measured with the Morris water maze. The improvement in cognitive performance was attributed to the reduction of tumor necrosis factor-± and Iba-1 expression. This finding suggests that part of the mechanism underlying cognitive impairment involves proinflammatory substances and their regulation. In another study, the researchers were interested in determining whether diabetic mice would have impaired abnormal anxiety like behaviors, hippocampal-dependent performance, and how that might be linked to central inflammation (Dinel et al., 2011). They found that that diabetic mice demonstrated increased anxiety-like behaviors and impaired hippocampus-dependent cognitive behaviors, which the authors attributed to increased inflammatory cytokine production and decreased BDNF levels (Dinel et al., 2011) These studies demonstrate clearly the link between peripheral and central functioning. This will be an underlying theme throughout the thesis.

Our Work

Our present series of studies extend much of the existing literature on the subject of obesity, metabolism, and brain effects. We were particularly interested in determining whether the brain effects of obesity are developmentally sensitive. This question may have serious implications for a considerable population of obese youth that may suffer accelerated

neurological decline due to chronic obesity. Previous experiments in our lab that attempted to address these questions suggested that obesity can negatively affect both spatial and non-spatial cognition in relatively young animals (Jurdak et al., 2008; Jurdak and Kanarek., 2009). More specifically, young adult rats fed a diet supplemented with sucrose for several weeks exhibited obesity, elevated fasting blood glucose, elevated triglycerides, and demonstrated cognitive impairments on Morris water maze training (Jurdak et al., 2008; Jurdak and Kanarek, 2009). The objective of the current series of experiments was to extend this aspect of our original studies by attempting to elucidate some of the neurological mechanisms underlying the observed cognitive deficits associated with chronic, diet-induced obesity. As a continuation of previous work, we examined potential neuromolecular effects of diet-induced obesity through analysis of hippocampal BDNF expression. This was an important broadening of our work because brain-derived neurotrophic factor has been identified as an important putative molecular marker of alterations in neural plasticity.

In the present experiments, we used a model of diet-induced obesity (DIO) that had been designed in our lab and used in previous investigations. In this particular model, in addition to their standard diet, rats are fed highly palatable sucrose solution, which leads to a 10% to 15% per day increase in calorie consumption compared to animals fed a standard diet. Rats chronically consuming this sucrose-supplemented diet readily gain weight and often exhibit metabolic disturbances similar to those seen in humans with prediabetes or type 2 diabetes (Kanarek and Hirsch, 1977; Kanarek and Orthen-Gambill, 1982; Kanarek et al., 1987). We have distinguished this particular model of DIO from other commonly employed models because the primary obesogenic component is sucrose. Most DIO paradigms use diets that are either composed of a combination of saturated fat/trans fat and refined sugars or saturated/trans

fat alone. However, preliminary studies that examined fat-induced versus sucrose-induced obesity indicated that adverse metabolic and cognitive effects were most apparent in rats fed a sucrose-supplemented diet when the diet period was relatively short-term (Jurdak et al., 2008; Jurdak and Kanarek., 2009). Consequently, the effects of sucrose feeding became the interest of the present investigations. Further motivating our interest in the biological effects of this particular macronutrient was evidence that high fructose diets have potent metabolic effects; rodent models have shown perturbations of lipid metabolism in just two weeks of feeding (Kelly et al., 2004). Given similarities in metabolic response to sucrose in our lab and fructose feeding previously (Kelly et al., 2004; Fukuchi et al; 2004), we determined that sucrose may have potent metabolism-perturbing dietary characteristics that should be investigated further. Specifically, we aimed to determine whether sucrose-promoted shifts in metabolism would be accompanied by neural alterations and cognitive deficits. In addition, because this interesting relationship had received minimal attention in the literature, we investigated sucrose-induced obesity as it pertains to the expression of BDNF in hippocampal neurons. While there has been considerable interest in determining the neurophysiological effects of trans/saturated fat diets (Winocur and Greenwood, 1999; Greenwood and Winocur, 2001; Wu et al., 2004; Pathan et al., 2008; Kosari et al., 2012; Park et al., 2012), the effects of refined sugars are less well characterized. Furthermore, an investigation of this nature was considered timely with recent scientific research reporting that the negative consequences of diets high in refined carbohydrates may exceed those of diets high in saturated fats (Hu, 2010; Agrawal and Gomez-Pinilla, 2012). Our decision about the high sugar diet was made with the knowledge that a high fat/high sugar combination probably best represents a typical Western style diet; however, our primary interest was isolating the effects of sugar in the present studies.

Another unique aspect of our investigation was our examination of the early effects and time course effects of DIO on neurocognitive function. To address these research goals, we conducted two primary experiments using model of diet-induced obesity described above. In both experiments, either rats were fed a standard diet or a standard diet supplemented with a highly palatable sucrose solution (DIO) for four, eight, or twelve weeks. The shortest period of the dietary treatment was selected because it was at least one week shorter than the diet period we had previously demonstrated would produce cognitive impairment (Jurdak et al., 2008), and had not been investigated using our model of DIO. The other dietary durations were selected because they were regular 4-week intervals (for consistency), with which we could investigate DIO associated cognitive deficits and attendant changes in BDNF expression. Additionally, previous investigations had not examined these variables at relatively short diet durations or at varying developmental time points (i.e., 8 versus 12 weeks), as was our goal.

Female rats were used in Experiment 1 and male rats were used in Experiment 2. Although our experimental design did not permit a direct sex-based comparison, it provided a starting point for identifying potential sex-specific differences in the effects of diet-induced obesity on metabolism, cognition, or neuromolecular indicators of brain function. We feel that this was an important metric to use because the literature is disproportionately based on investigations of these effects in male rats, and there is increasing evidence to suggest that obese female rodents are less susceptible to the metabolic and cognitive effects of over nutrition than males (Gomez-Perez et al., 2008; Hwang et al., 2010). Additionally, and importantly, these sex-specific differences in susceptibility have been found in humans with obesity as well (for a comprehensive review see Sugiyama and Agellon, 2012). Briefly, though, premenopausal women have been found to exhibit resilience to obesity-associated metabolic conditions and

related cardiovascular complications, with nearly a ten-year delay between the onset of these conditions in men and women (Carr, 2003; Llyod-Jones et al., 2010). Furthermore, metabolic disease occurs much more frequently in postmenopausal women and this is thought to be associated with fluctuations or reductions in estrogen levels (Llyod-Jones et al., 2010). In his review of the subject, Alemany (2012) proposed that the interactions between sex-hormones and glucocorticoids regulate metabolic syndrome. Age is a third variable that might impact this interaction. For example, levels of androgens and estrogens decrease with age, though the extent of decrease in androgens is less pronounced (Gould et al., 2007), a process that occurs in parallel to increased corticosteriod production (Blouin et al., 2005). The decrease in androgens and estrogens concomitant with age-related increases in corticosteroid production leads to increased susceptibility to metabolic disease for both males and females, but the onset of metabolic disease is more common in menopausal or postmenopausal females because of the marked decrease of estrogen that occurs during this developmental period (Blouin et al., 2005). With the exception of the use of male rats in Experiment 2, the experiments were similar in basic methodology. Minor differences are specified in the subsequent sections.

Following the dietary interventions, cognitive testing was performed. Consistent with previously described research, we were interested in assessing spatially based cognitive function as it related to assigned dietary intervention and duration of the specified diet. The behavioral test selected for this purpose was the Morris water maze, a classic test of spatial ability that allows for inferences regarding hippocampal functioning to be made (Morris et al., 1982; Morris, 1989; Morris et al., 1989). We selected for this test because behavioral response is easily motivated without food reward. While rats are efficient swimmers, the task induces moderate to high stress, which is an effective motivator for escape from the pool. Using spatial

paradigms where food motivates the behavioral response would be potentially confounding in studies such as ours that attempt to assess behavioral or neuromolecular effects of diet-induced obesity and accompanying metabolic states. Consequently, we elected not to use tests of spatial ability that require the use of food (e.g., radial arm maze, delayed alternation paradigms). Additionally, food dependent paradigms could be asymmetrically motivating depending on the dietary condition. Obese animals may be quickly satiated, while their chow-fed counterparts may find the food highly motivating—both in terms of appetite and novelty of the reward—for the duration of the test.

We used a version of the water maze that allows for the examination of both standard procedural/motor aspects of memory (swimming to the hidden platform) and spatially based aspects of memory, as well as flexibility and adaptability of behavior through the use of a reversal trial which requires spatial reorientation to a new platform location. As suggested earlier, this specific version of the water maze is more complicated than a standard paradigm in that it requires learning and consolidation spatial cues relevant to an initial platform location, followed by re-application and reconsolidation of these spatial cues when learning the alternate platform location during reversal learning (Quan et al., 2010). In the version of the water maze-reversal protocol used, rats are required to learn the initial platform location during standard learning trials over several days, and then had to learn the location of a platform positioned opposite to its original location with an equivalent number of trials, but with only one day to acquire new information. This particular use of the reversal trial requires that the animal at least partially inhibits spatially relevant cues that initially allowed for navigation of the maze—information that is well learned—in order successfully learn the new platform location. Although the complexity of this particular task may require activation of regions of the brain

beyond the hippocampus, it remains a validated test of hippocampal function (Quan et al., 2010). Moreover, successful water maze performance generally relies on the coordinated action of various brain regions (Quan et al., 2010). However, given that the spatial aspects of the water maze still require disproportionate involvement of the hippocampus (Brandeis et al., 1989; Poucet and Benhamou , 1997; Poucet et al., 2000) we deemed it appropriate for the objectives of the present series of studies. Our application of the reversal paradigm was an essential element of the spatial testing because, since we were examining the effects of a relatively short dietary treatment in younger animals, it was possible that behavioral effects would be somewhat subtle and more easily discerned with a relatively challenging learning task. In addition to behavioral indicators of hippocampal integrity, we determined the effects of obesity-induced shifts in peripheral metabolism on BDNF mRNA expression.

Our specific hypotheses were that diet-induced obesity would produce abnormalities in glucose metabolism that would be associated with cognitive impairments and differences in BDNF expression. The extent of the changes in cognition and BDNF expression would be relative to the length of the dietary intervention, with longer interventions producing more pronounced outcomes. Finally, the cognitive and metabolic consequences of diet-induced obesity may be less pronounced in female rats due to a reported estrogen-mediated neuroprotective effect (Lebesgue et al., 2010).

Experimental Materials and Methods

Animals and Dietary Composition

Sixty female (Experiment 1) and sixty male (Experiment 2), experimentally naïve, Long-Evans rats (Charles River Laboratories, Portage, MI) were used. Rats were six weeks old (200-225g) when they arrived in the laboratory. The animals were individually housed in hanging stainless steel cages, in a temperature 22 ± 1°C and humidity-controlled room with a 12:12 reverse light cycle (lights on at 7 pm). All experimental and animal care procedures were performed with the approval of the Tufts
University Institutional Animal Care and Use Committee and in accordance with the NIH Guidelines for the Care and Use of Animals in Research.

Dietary Composition

All rats had *ad libitum* access to ground Purina® chow (3.4 kcal/g) and water throughout the experiment. Additionally, animals in the DIO condition were provided *ad libitum* access to a 32% sucrose solution (1.28 kcal/g). The ground chow diet was supplied in Wahmann LC306 food cups with lids. The cups were secured to the floor of each cage to prevent spillage. Water and sucrose were presented in glass bottles with leak proof spouts. Diet intake was measured three times weekly and body weights were measured once weekly during this time.

Procedures

Animal Assignment

Following a one-week acclimation, half of the animals in each experiment were assigned to a diet-induced obesity (DIO) condition or a standard diet condition for 4-, 8-, or 12- weeks (10 animals per condition, in each treatment duration).

Fasting Blood Glucose and Oral Glucose Tolerance

Fasting blood glucose was assessed one week prior to behavioral testing in each group (during weeks 3, 7, and 11). After an 18-hour fast, blood samples were obtained from a small incision site at the tip of each animal's tail. Immediately following determination of fasting blood glucose levels, animals were given a 50% sucrose solution (2 ml/kg body weight) using oral gavage. Blood glucose levels were measured at 15, 30, 60, 90 and 120 minutes following the sucrose load.

Morris Water Maze

Behavioral testing was performed during the dark portion of the daily lighting cycle (beginning at 10 am, which was three hours after the onset of the dark cycle). Hippocampal-dependent learning and memory were measured using the Morris water maze. The water maze used was a circular pool (182.9 cm) where the water was rendered opaque with non-toxic paint. The pool was divided into 4 equal quadrants that were labeled arbitrarily as north, south, east, and west. The pool was located in a room that contained extramaze spatial cues, which remained in a fixed position for the duration of testing. Rats were trained in the maze over 4 consecutive days with 5 trials per day. The platform (2 cm beneath the surface) remained in the same location for the first 3 days. On the fourth day reversal trails, the platform was relocated to the quadrant opposite to its initial location. Probe trials were conducted prior to the reversal trials and 24 hours after the reversal training day. After each training trial, rats were gently removed from the water, towel dried, and returned to their home cages. During the training and reversal trials, the latency to reach the platform, swim speed, and path efficiency were measured, and the probe trial measurement of interest was total time spent in each of the 4 quadrants. Images were digitized

through the use of a ceiling-mounted camera and behavioral data were acquired using Any Maze behavioral tracking software (Stoelting Inc, Wood Dale, IL).

Hippocampal Dissections

Upon completion of the water maze testing, rats were anesthetized with isoflurane, decapitated, and bilateral hippocampi were dissected and rapidly frozen at -80°C.

Quantitative Reverse Transcription-PCR

RNA was extracted using Tri Reagent® (as per manufacturer's specifications) from whole hippocampi, treated with DNase, and tested for genomic contamination with PCR. Reverse transcription (RT) to generate cDNA was performed with 2µg RNA and using 200 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and 150 ng of random hexamers (Invitrogen) in a 20µl reaction. Real-time PCR amplification was conducted using a MX-3000P Stratagene (La, Jolla, CA) cycler and SYBR green PCR master mix (Qiagen, Valencia, CA). For each primer set, product amplification specificity was confirmed by dissociation curve analysis and agarose gel electrophoresis. Additionally, dissociation curves were created using serial dilutions and the efficiencies for each primer set was calculated. The amplification efficiency for all the primers utilized in this study was between 90 and 100%. A validation experiment was performed for each primer set to show that the PCR efficiencies were approximately equal to those of the reference gene. A two-step protocol was used: annealing temp: 55°C. GAPHDH and Actin were used as normalizer genes. The following primer sequences were used for detecting total BDNF transcript: forward: 5'GAAAGTCCCGGTATCCAAAG3', reverse: 5' CCAGCCAATTCTCTTTTT 3'. Primer sequences for the reference genes used were obtained

from http://pga.mgh.harvard.edu/primerbank/index.html Actin (ID# 6671509a1) and GAPDH as detailed in Fan, Jing, and Zang (2004). All samples were analyzed in triplicates, and non-template controls were included to determine the presence of any level of contamination. Amplification products ranged from 154 to 201 bp. Data gathered were analyzed using the comparative Ct method (reviewed in Schmittgen and Livak, 2008).

Data Analysis

Dietary intake, body weight, metabolism, and BDNF mRNA relative expression were analyzed using unpaired t-tests. Water maze learning trials were analyzed as a repeated-measures ANVOVA, with trial as the within subjects factor and treatment as the between subjects factor. Probe trial data were analyzed using an un-paired t-test.

Results Experiment 1-Females

Average Daily Caloric Intake

Averaged across the week prior to behavioral testing, DIO animals consistently consumed significantly more calories than their chow-fed counterparts in the 4-week (chow = 68.5±2.1 kcals, DIO = 99.1±3.7 kcals, $t(15) = 6.8$, $p<.01$), 8-week (chow = 77.8±2.5 kcals, DIO = 124.3±6.1 kcals, $t(16)= 7.0$, $p<.01$), and 12-week(chow=78.8±2.5 kcals, DIO= 92.9±3.4 kcals, $t(18)=3.5$, $p<.01$) dietary interventions.

Body Weight

Body weight of chow-fed and DIO animals were equivalent at the beginning of the experiment. However, DIO rats weighed significantly more than chow-fed rats when tested on

the Morris water maze after the 4-week (chow=268.25±3.1 g, DIO=300.71±5.6 g, $t(18) = 5.1$, $p<.05$), 8-week (chow = 294.7±6.2 g, DIO = 378.68±3.7 g, $t(18)=5.5$, $p<.01$) and 12-week (Chow = 302.5±7.9 g, DIO = 398.6±18.1 g, $t(18) = 4.8$, $p<.01$) dietary interventions. Average body weights reported were obtained during the final week of the diet periods. Refer to Figures 1-3 for additional data on weekly body weight measurements over the course of dietary the interventions.

Fasting Blood Glucose and Oral Glucose Tolerance

As measured the week prior to behavioral testing, there were no differences in fasting blood glucose after only three weeks on diets (135.4 mg/dL in chow-fed rats) and (144.2 mg/dL in DIO rats). However, diet-dependent significant differences emerged in the 8-week (chow = 125±5.8 mg/dL, DIO = 144.8±6.7 mg/dL, $t(18) = 2.2$, $p<.05$) and12-week (chow = 131.4±3.8 mg/dL, DIO = 151.8±2.9 mg/dL, $t(18) = 5.7$, $p<.01$) diet interventions.

When submitted to an area under the curve (AUC) analysis, blood glucose levels were significantly elevated following glucose administration in the 8-week DIO female animals as compared to their chow-fed counterparts, $t(18) = 2.2$, $p<.05$. Blood glucose levels were also higher in the 12-week DIO animals compared to their chow-fed counterparts, $t(18) = 4.2$, $p<.01$) There were no other diet-dependent differences observed. Refer to figures 4-6 for additional data on fasting blood glucose and glucose tolerance.

Morris Water Maze

Using repeated measures ANOVA there were no diet or time sensitive differences in any of the behavioral measures observed in Morris water maze testing. This was in contrast to

previous findings in our lab, and is inconsistent with investigations of high fat and high sucrose diets or high fat alone where spatial impairment did occur in rats (Molteni et al., 2002; Pancani et al., 2013; Soares et al., 2013 White et al., 2013). Given the limited nature of these findings we have chosen to present the results as graphs at the end of the document.

Hippocampal BDNF mRNA

Relative total hippocampal BDNF expression was significantly increased in the 4-week DIO dietary intervention when compared to their chow-fed counterparts with actin and GAPDH as references genes, (chow = 1.22±.21, DIO = 3.11±.69, $t(17) = 2.5$, $p<.05$) and (chow = 1.19±.25, DIO = 2.93±.57, $t(18) = 2.46$, $p<.05$), respectively. There were no further diet-dependent differences in BDNF expression. Refer to figures 15-17 for data on BDNF mRNA.

Results Experiment 2-Males

Average Daily Caloric Intake

Averaged across the week prior to behavioral testing, 4-week DIO animals consumed significantly more calories than their chow fed counterparts, (chow=93.1±5.7 kcals, DIO=113.0±4.7 kcals, $t(18) = p<.01$). However, averaged across the week prior to behavioral testing DIO rats were not consuming significantly more calories than their chow-fed counterparts in the 8-week (chow = 104.3±2.5 kcals, DIO = 100.0±4.8 kcals, $t(18) = 2.7$, $p>.05$) and 12-week (chow=125.8±3.9 kcals, DIO = 127.6±5.6 kcals, $t(22) =2.9$, $p>.05$) dietary interventions. Although intake did not differ in a diet-dependent manner in the 8-and 12-weeks groups during the week prior to behavioral testing, further analysis revealed that there was a significant difference in weekly intake over the course of the experiment. Averaged over the entire 8-week

(chow = 122.6±4.6 kcals, DIO = 136.4±7.2 kcals, $t(14) = 2.7$, $p<.05$) and entire 12-week (chow =128.1±5.8 kcals, DIO = 143.1±6.4 kcals, $t(22) =2.9$, $p<.01$) dietary interventions the DIO animals did consume significantly more calories than their chow-fed counterparts.

Body Weight

Body weights were equivalent across dietary conditions at the beginning of the experiment. DIO animals weighed significantly more than chow-fed rats in the 4-week (chow = 422.21±10.05 g, DIO = 471.70±10.68 g, $t(18) = 3.4.1$, $p<.01$), 8-week (chow = 543.10±14.20 g, DIO = 603.11±14.18 g, $t(17) = 3.1$, $p<.01$) and 12-week (Chow = 656.1±22.15 g, DIO = 719.5±11.8 g, $t(18) = 2.5$, $p<.05$.. Reported body weights were obtained once during the final week of the dietary intervention. Refer to figures 21-23 for additional data on body weight over the course of dietary interventions.

Fasting Blood Glucose and Oral Glucose Tolerance

Fasting blood glucose levels differed according to dietary condition and length of intervention. Interestingly, a difference in fasting blood glucose was only apparent after 4-weeks of dietary intervention, DIO (176.4±3.5 mg/dL) and chow (161.0±6.5 mg/dL), approaching significance, $t(18) = 2.08$, $p = .052$. No other differences approached significance.

There were no significant differences in blood glucose levels following the glucose administration in the 4-week and 8-week dietary conditions, and tests were not performed in the 12-week males due to equipment failure. Refer to figures 24-26 for glucose metabolism data.

Correlational Analyses

For the 4-week animals, there was a significant positive correlation between body weight and fasting blood glucose, r(0.595), p>.05 across all animals. However, there were no significant correlations between body weight or BDNF mRNA levels and average latency per trial or average daily latency to platform, probe, or reversal trials.

For the 8- and 12-week animals, body weight and fasting blood glucose were significantly positively correlated, r(0.663), p<.01 across all animals. There were no significant correlations between body weight or BDNF mRNA levels and average latency per trial, average daily latency to platform, probe, or reversal trials.

Morris Water Maze

There were no significant findings regarding Morris water maze performance. This was in contrast to previous findings in our lab, and is inconsistent with investigations of high fat and high sucrose diets or high fat alone where spatial impairment did occur in rats (Molteni et al., 2002; Pancani et al., 2013; Soares et al., 2013 White et al., 2013). Alternate reasons for these findings will be presented in the discussion of this paper. Given the limited nature of these findings we have chosen to present the results as graphs at the end of the document.

Hippocampal BDNF mRNA

There were no diet-dependent differences in hippocampal BDNF expression at any of the dietary time points. This was in part due to inconsistencies and technical difficulties with data analysis. These data are not presented.

Correlational Analyses

There were no significant correlations between body weight, fasting blood glucose, and BDNF mRNA levels, or any of the spatial behaviors as measured by the MWM.

Discussion

The behavioral outcomes were in contrast to previous studies conducted in our laboratory as well as studies published in the field on similar subjects. Relative to standard fed animals, DIO animals displayed no significant behavioral difference in their performance on the water maze. Previously, our data indicated that young adult rats consuming a high sucrose diet were impaired on both spatial and non-spatial tasks (Jurdak et al., 2008; Jurdak and Kanarek, 2009). One factor that may account for these contrasting results is that we used female rats in Experiment 1, whereas our research in 2008 and 2009 was conducted with male rats. Research in other laboratories has found that female rodents are less prone to the metabolic and, in turn, cognitive effects of obesity (Gomez-Perez et al., 2008; Hwang et al., 2010). In fact, some research has reported that estrogen provides a neuroprotective effect in female rodents (Sharma and Mehra, 2008; Lebesgue et al., 2010). Although this sex difference was part of our reasoning for studying male rats as a follow-up experiment in the current series of experiments, it may also help explain why we did not obtain significant behavioral results with the female rats in general. Based on our data, however, it is likely not the primary explanation for our female behavioral

data because the female DIO rats in our study did exhibit metabolic abnormalities at eight and twelve weeks. These data will be revisited when molecular findings are discussed.

It is more challenging to explain why we failed to obtain significant behavioral results with the male DIO rats in the second experiment. Compared to their chow-fed counterparts, DIO rats were not impaired on any aspect of the Morris water maze. However, in our 2008 study, rats in the DIO condition performed worse than chow-fed animals in acquisition and retention of the Morris water maze (Jurdak et al., 2008). There were methodological differences that may have affected the outcomes of the present study and that conducted in 2008. For example, the MWM paradigm used in initial study did not use reversal trials and the probe trial followed acquisition training by several days. Regardless of methodological differences, the occurrence of spatial deficits in our initial study of DIO, but not in our most recent observations may have been due to male DIO rats not consistently exhibiting elevated fasting blood glucose compared to chow-fed rats. Unexpectedly, the only finding to approach significance with regard to glucose metabolism was the 4-week DIO males' fasting blood glucose levels. Interestingly, at the 4-week time point both groups had elevated fasting blood glucose levels, but the DIO group was clearly higher. However, at 8 and 12 weeks, average fasting blood glucose levels were only slightly higher in DIO rats, and were nearly equivalent in the chow-fed animals, indicating elevated blood glucose levels in both dietary conditions. This poses an issue because it seems that blood glucose levels of control animals were elevated when this had not been an issue in past investigations (Jurdak et al., 2008; Jurdak and Kanarek, 2009). The most likely reason that we observed elevated blood glucose levels in the control animals is that they underwent some form of stress, whether that be during the actual blood testing period, or sometime previous to that, which artificially raised their blood glucose levels. This is a distinct possibility because the chow diet alone should not have caused elevations

in blood glucose levels. In addition, studies have shown that stress can raise blood glucose levels in rats (Feng et al., 2012; Neves et al., 2012). Given that we have used the same tail vein method of obtaining blood in previous studies (Jurdak et al., 2008; Jurdak and Kanarek, 2009) with no stress effects in the control group, it is possible that this group of rats was metabolically more vulnerable to the effects of stress. Another possible explanation for the increase in fasting blood glucose levels in the control male rats would be that they gained more weight than previously recorded in our studies. This, however, was not the case as they gained a standard amount of weight that was comparable with previous studies. With these factors in mind, both the control and DIO rats may have demonstrated impairments on the Morris water maze, masking any difference that would have otherwise manifested (i.e., the rats in the standard diet group may have performed less well than would be expected). Although it may not have been a significant problem in our study, it may be worth investigating the use of alternate and less stressful methods of obtaining blood levels. Inherent differences in vulnerability to stress, however, cannot be anticipated.

There may have been factors outside of stress that affected our results. While not examined directly in our study, the effects of different diets on cognition could be worth investigating in future studies. Many studies have employed high fat or a combined high fat and high sugar diets and successfully induced obesity, metabolic syndrome, and cognitive dysfunction (Beilharz et al., 2013; Moy and McNay, ; 2013;Woo et al., 2013; McNeilly et al., 2012; Porter et al., 2012; Yamada-Goto et al., 2012; McNeilly et al., 2011; Morris et al., 2010). In all of the studies cited, the dietary interventions seemed to be potent inducers of obesity, metabolic syndrome, and cognitive impairments. However, this was not the case in my studies. It is possible that a high fat diet or a high fat diet combined with sugar is a more effective dietary

intervention than sugar alone. The evidence for this supposition is strong and building in the literature. Future studies should address this issue in more depth.

The molecular findings of these studies were limited to the first experiment (with female rats) and the 4-week time point. Interestingly, the 4-week DIO group had significantly up-regulated BDNF mRNA with no concurrent change in behavior when compared to their chow-fed counterparts. This is an unusual pattern of findings because usually with a decrease in BDNF expression there are decrements in cognitive functioning when compared to groups with no change in BDNF expression (Stranahan et al., 2008; Yu et al., 2009; Tozuka et al., 2010). Given these findings, one would anticipate that there would be some accompanying behavioral response to alterations in BDNF mRNA expression in the opposite direction. Indeed, some investigators suggest that increased BDNF expression is associated with enhanced memory and cognitive functioning (Vaynman et al., 2007; Uysal et al., 2011; Bolognin et al., 2012). Although the studies cited here explicitly used manipulations that were intended to increase the levels of BDNF (i.e., running or antioxidants), our experiment inadvertently revealed increased BDNF expression in DIO animals after four weeks dietary intervention. Given that published studies have only reported changes in BDNF expression as they relate to memory or cognition, our results require a more preliminary interpretation. Although there can be no conclusions regarding improved or impaired spatial cognition as it is associated with BDNF expression, it is possible that the upregulation in BDNF that we observed at the 4-week time point potentially represented a compensatory and transient response to the high sugar feeding. The observed upregulation of BDNF may have then been a compensatory mechanism in the sense that its increase accounted for any changes in peripheral metabolism. In this sense, the response would represent recapitulation of peripheral metabolism as discussed previously. Although there is no

way to directly associate these two events, there is evidence that this type of recapitulation occurs. In addition, some studies have shown that central and peripheral BDNF levels are associated (Rothman et al., 2012). It is this ability for BDNF to act simultaneously systemically and centrally that is the rationale for my hypothesis here. The overall result in my study based on my hypothesis would be that the interaction described above would prevent cognitive impairment in the female rats. One way to test this hypothesis is to interfere with BDNF pathway signaling by partially blocking TrkB receptors. Using a partial antagonist may be effective in blocking the increase in viable BDNF as was observed in 4-week female rats. Using a complete antagonist would affect other functions too much, so this should be a sufficient test to determine any link between central and peripheral metabolisms in this paradigm.

The proposed transient nature of BDNF up-regulation requires a more intricate explanation. The first part of the explanation is that upregulation of BDNF levels was only observed after four weeks of DIO intervention. Consequently, animals in the 8-and 12-week interventions demonstrated elevated blood glucose levels, which may have been normalized by increased BDNF expression in the hippocampus at those time points. However, despite having elevated fasting blood glucose levels, female DIO rats exhibited no impairments in spatial ability. I will propose two explanations for this particular finding. The first is that, although the increased expression in BDNF seemed to be transient, it is possible that at or around four weeks the ‘transient’ effect actually prompts a more lasting compensatory/preventative molecular cascade. The second proposal revisits the data that indicate that female rodents are less susceptible to obesity’s metabolic and cognitive effects (Gomez-Perez et al., 2008; Hwang et al., 2010). These two proposals are not entirely independent; the neuroprotective effects of estrogen receptor alpha agonists (Lebesgue et al., 2010) may interact with a proposed lasting

compensatory effect of up-regulated BDNF.This effect of estrogen receptor alpha agonists on BDNF may be one manner in how BDNF acts to affect cognition. However, the effect of BDNF is likely multifaceted. Another area that would be important in terms of learning and memory is that BDNF's upregulation probably supports NMDAR-mediated long-term potentiation. In this way, BDNF acts to potentiate learning and memory processes when there is probable dysregulation of central systems, particularly in the hippocampus where much of this learning occurs. Those are two powerful mechanisms by which BDNF might potentiate neuroprotection and learning.

There were several limitations in these studies. The first limitation, and something that was supposed to set our studies apart from the literature, was that we employed young adult rats instead of older/aging rats, as is more common. Our initial intention with using younger rats to both model childhood obesity and to develop a model of the neurodevelopmental effects of obesity, but based on our results additional research is required. The younger rats in our study seemed to be more resistant to obesity related cognitive deficits and BDNF down-regulation than older rats used in previous work (Molteni et al., 2002; Stranahan et al., 208; Park et al., 2010). Although we have had contrasting results using young adult rats to model obesity in children, it is an effort that should continue. Children may have more subtle cognitive and molecular changes that occur as the result of obesity and further research with animals should explore this idea—such studies would include more sensitive measures in molecular or metabolic changes and more challenging paradigms measuring hippocampal-dependent learning and memory.

Another factor that was present, but unlikely to affect our study is that when animals increase their sugar intake, it is usually accompanied by a decrease of vitamins, minerals, and protein, a potential factor in any observed cognitive deficits. However, in the present study this was not an issue because we did not see any cognitive deficits—issues that are generally obvious with protein and vitamin or minerals deficits. In future studies, it may be worthwhile to examine something the effect of low protein or vitamin diets on cognition and BDNF expression.

There are also a set of potential limitations surrounding the use of the Morris water maze that should be addressed. The first has to do with the particular model used in the MWM testing. Given that our DIO rats and chow-fed rats have different body densities, they may exhibit different swim speeds and tolerance to water temperature (which was held constant). While differences in swim speed was ruled out, determining how the animal tolerates the water temperature is more challenging—though ostensibly it would be related to motivation/swim speed. Flotation is another issue that could affect, particularly, DIO rats' ability to swim. Based on swim speed, even if there were differences in buoyancy, it was not relevant to overall test results. Another limitation with regard to the use of the MWM is that we only measured changes in one area of the brain, the hippocampus, and there other regions that are participating during MWM testing. In particular, the amygdala is active during moderately stressful tests like the MWM, and so it would worth investigating amygdalar activity/changes in addition to hippocampal measures.

Estrogen-BDNF interactions

Given that BDNF was up-regulated in response to the obesogenic diet in female rats, it is important to consider the potential role of estrogen in potentiating or mediating this neuromolecular response. Generally, estrogen interacts with BDNF through the colocalization of estrogen receptors to BDNF cells and its receptor TrkB (Miranda et al., 1993). As further evidence for estrogen's regulation of BDNF, studies have demonstrated that estrogen replacement in ovariectomized rats is associated with increased BDNF mRNA expression in the olfactory bulb (Singh et al., 1995; Jezierski and Sohrabji, 2000; Jezzierski and Sorabji, 2001), hippocampus (Singh et al., 1995; Gibbs, 1998; Gibbs, 1999; Liu et al., 2001; Zhou et al., 2005), cortex (Sohrabji and Miranda, 1995) and the amygdala (Liu et al., 2001; Zhou et al., 2005) . As one would anticipate, then, studies have found the opposite; ovariectomy is associated with decreases in BDNF mRNA expression (Takuma et al., 2007; Hou et al., 2011;Kramar et al., 2012). Together, these studies suggest that estrogen is a modulator of neurotrophin activity, and that perturbed estrogen levels can lead to dysregulation of BDNF expression. Several lines of evidence have converged to suggest that estrogen-BDNF interactions can influence cellular functions that support learning and memory through changes in synaptic spine density in the prefrontal cortex and hippocampus (Luine et al., 2011; Luine and Frankfurt, 2012a; Luine and Frankfurt, 2012b). As these brain areas are both integral to supporting learning and memory functions, changes in either estrogen or BDNF levels in these regions may affect high-level cognitive functions. In fact, several studies have demonstrated that decreases in estrogen levels are associated with morphological changes at the level of the dendrite, which were accompanied by perturbed cognitive function (Gonzalez et al., 2012; Ingaki et al., 2012; Luine and Frankfurt, 2012; Phan et al). Given the neural colocalization of estrogen and BDNF, and estrogen's modulatory actions on BDNF, their integrated role in maintaining neuroplastic functions has

been well established. However, this essential neuro-hormonal interplay can be perturbed to produce brain pathologies. Several studies have demonstrated that estrogen deficiency is associated with small increased risks for neurodegenerative conditions such as Alzheimer's disease (Pike, 1999; Singh et al., 1999; O'Hagen et al., 2012;) and Parkinson's disease (Quesada et al., 2008; Al Sweidi et al., 2012; Bourque et al., 2012), both conditions that affect cognitive functioning to some extent.

Obesity is another condition reported to affect the brain and cognitive functioning in animal and human studies (Pedersen et al., 2012; Benito-Leon et al., 2013; Moy and McNay, 2013; Pancani et al., 2013). Perturbation of estrogen and BDNF levels has been associated with obesity and its metabolic symptoms indirectly through a study that assessed the beneficial effects of exercise on estrogen and BDNF (Gligolorska and Manchevska, 2012). These results suggest that obesity may perturb estrogen and BDNF, an event that might be reversible through exercise. Further study on the precise mechanisms suggested in this scenario should be established. Given these studies, the findings of my work become interesting because there was an upregulation of BDNF mRNA in female rats, which may be related to altered estrogen signaling, and BDNF-estrogen interactions. If this is the mechanism underlying the increase in BDNF mRNA expression noted, then it suggests a potentially neuroprotective response to the obesogenic diet, and increased protection in female animals. Alternately, increased BDNF mRNA expression could represent a compensatory mechanism that was activated in response to the diet. Unfortunately, we did not measure changes in brain estrogen levels or estrogen receptor expression in the hippocampus. This limits the interpretation of an estrogen-BDNF interaction. The authors suggest a more comprehensive examination of both estrogen and BDNF in response to obesity in future investigations as BDNF-estrogen interactions could be key to understanding

obesity-related neuropathology. In addition, parallel investigations of factors such as food intake motor activity and spatial activity would be worthwhile.

Another limitation of these studies was that we only measured one putative molecular marker of neuroplasticity, BDNF. Due to technical issues with the processing of tissue for mRNA analyses, a host of potential genes was not explored. The tyrosine kinase B receptor (TrkB) was the intended initial starting point. TrkB is the primary receptor for BDNF, which when bound by BDNF is rendered active, initiating downstream neural activity that such as interactions with plasticity cascades (Cunha et al., 2010). Actions of this binding include promoting an increased availability of glutamate (Martin and Finsterwald, 2011), which is the primary ligand of the *N*-methyl D-aspartate receptor, another essential cellular participant in long-term potentiation. The examination of TrkB expression in animal models of obesity has been quite limited (Tsao et al., 2007; Xu et al., 2010), and has focused primarily on the obesity ameliorating effects of TrkB agonists. To date, there are limited investigations examining hippocampal TrkB receptor density or mRNA expression as it relates to cognition and alterations in BDNF levels in obesity. Given that BDNF and TrkB are so intricately linked, research into their mechanistic role in obesity-related cognitive dysfunction is suggested.

Two other hormones that scientists have linked to obesity, food intake, and more recently, to learning—leptin and ghrelin—were initially of interest in this investigation. Leptin is well known for being involved in the regulation of food intake, body weight, thermogenesis, and other neuroendocrine functions (Oomuora et al., 2010). With respect to the brain, leptin receptors have been shown to be essential mediators of long-term potentiation and other molecular-mediators of learning (Oomura et al., 2006; Harvey et al., 2006; Oomura et al.,

2010). Leptin receptor deficient diabetic rodents have been shown to be long-term potentiation deficient, with accompanying deficits in spatially memory (Li et al., 2002). Because the rats were both diabetic and leptin receptor deficient it is difficult to ascertain whether it was the leptin deficiency or the diabetes or a combination of both that led to the cognitive dysfunction. Generally, leptin has also been demonstrated to facilitate neurobiological learning and memory processes (Oomura et al., 2006).These data suggest that hippocampal leptin receptors are essential participants in the neurobiological processes that underlie memory formation. Further work should specifically target how variations in hippocampal leptin levels are related to obesity and memory. With the following information about leptin, there is a paradox in obese humans where they have increased plasma leptin levels (Ozcelik et al., 2004), but humans with obesity also have cognitive deficits (Lamport et al., 2014). It is possible that this discrepancy can be explained by a difference in peripheral and central levels of leptin where central levels are lowered. Another explanation for this discrepancy is that there is possible leptin resistance or insensitivity occurring where high levels of leptin in the obese have less effect on cells (Hoisoi et al., 2014).

As with leptin, ghrelin plays a key role in appetite regulation and neuroendocrine functions (Lim et al., 2010). Ghrelin is secreted from the stomach and is found throughout the body (Horvath et al., 2001). It acts not only in the periphery but centrally on its natural receptor, growth hormone secretagogue receptors, (GSH-R). GSH receptors are localized in specific brain regions such as the hippocampus and hypothalamus (Atcha et al., 2009; reviewed in Cong et al., 2010). Ghrelin sensing neurobiological pathways regulate appetite (Lim et al., 2010) and certain cognitive functions that demonstrate both cross- communication and interrelated functions (Carlini et al., 2004). While some brain regions containing GSH-R are more closely

associated with appetite regulation (e.g., hypothalamus), others have emerged as being related to the potentiation of memory (reviewed in McNay, 2007).

As with studies of rodents lacking leptin receptors, animals lacking GSH-R demonstrate impaired hippocampal learning (Carlini et al., 2004; Davis et al., 2011). One study noted that animals demonstrated enhanced spatial memory, among other types of non-spatial memory (e.g., object recognition) when ghrelin was administered systematically (Diano et al., 2006). Together, these data suggest GSH-R mediated hippocampal memory formation and function. Given that these peptides are integral in appetitive processes and, in turn, disorders of the appetite, it is worthwhile to examine their role in associated processes; what remains to be elucidated is how obesity may alter their receptor expression in the brain.

Avenues for Further Research

Many potential avenues for future research are suggested by the literature and findings from my own work. My dissertation research was first proposed with the goal of determining the consequences of obesity on brain functioning using a rodent model. This work was intended to be distinguished from other studies in the field because I attempted to examine how obesity affects the brain using developing organisms; however, the success of this effort was limited to data that were preliminary.

My initial interest in using younger rats to study this issue was the potential scientific importance of gaining knowledge that would translate well to the human population and the increasing prevalence of obesity and related metabolic disorders such as metabolic syndrome in children and adolescents (Al-Agha et al., 2012). Gaining insight on the potential adverse effects

of obesity in children and adolescents is essential because they may endure a lifetime of the adverse effects of obesity—if it becomes a chronic health issue. Although my research was preliminary in identifying some of the factors that may be involved in obesogenic pathology, it suggests additional work in this area. This, however, will not be the basis for my future research proposed here. Instead, the focus of the research will delve into an area that is also intriguing when it comes to the topics of obesity and cognitive functioning. This focus, and the subject of ongoing research, is omega-3 supplementation as a protective factor against the adverse effects of obesity. In the same way that youth may protect the brain from metabolic insult, research has suggested that antioxidants, essential fatty acids, and various nutritional extracts that have demonstrated protective properties. The following proposed research will still focus on obesity and BDNF, but will use omega-3 fatty acids as an adjuvant to the typical DIO diet fed to the animals. Preceding the research will be a short literature review, providing some basic information on omega-3 fatty acids.

Potential Beneficial and Protective Effects of Omega-3 Fatty Acids

Omega-3 (n-3) and omega-6 (n-6) fatty acids are the primary constituents of the polyunsaturated fatty acid (PUFA) family. n-6 PUFAs include linoleic acid (LA) and arachidonic acid (ARA) and n-3 PUFAs include alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) (Parker et al., 2006). PUFAs are found in the lipid bilayer of cell membranes, and are essential to the maintenance of membrane fluidity (Heinrichs, 2010). *n*-3 and *n*-6 PUFAs are incorporated into phospholipids, sphingolipids and plasmalogens (Arteburn et al., 2010). *n*-6 PUFAs are produced in the body from linoleic acid (LA). *n*-3 fatty-acids, including eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) are synthesized from alpha

linolenic acid (ALA) (Parker et al., 2006). PUFAs are released via neurotransmitter stimulation and metabolized to active compounds including prostaglandins, thromoxone, leukotrienes, and others. These compounds act as neuronal second messengers, interact with G-protein coupled receptors on glial cells, thereby affecting neuromodulation and synaptic output, influence cell migration, moderate neurogenesis, and synaptogensis and increase adenylate cyclase and protein kinase A, which mediate serotonin, norepinephrine, and dopamine receptors (Fontari et al., 2005). While both *n*-3 and *n*-6 PUFAs are necessary for cells to maintain normal structure, function, and signal transduction, the ratio of *n*-3 to *n*-6, rather than the absolute levels, is most important to these cellular processes (Simopoulos, 2011).

Present Western diet consists of significantly greater n-6 to n-3 ratio than that of our ancestors (between 17:1 to 10:1, n-3 to n-6, compared to 1:1 of today). This is due to increased intake of n-6 PUFAs (e.g., vegetable oils, including corn, safflower, sunflower and soybean oil) and a decreased intake of n-3 PUFAs (e.g., fish, fish oil, wild game, wheat germ, walnuts and plants) (Heinrichs, 2010, Parker et al., 2006). Both n-3 and n-6 PUFAs are necessary for cells to maintain normal structure, function, and signal transduction. However, the ratio of n-6 to n-3 PUFAs, rather than their absolute levels is most important to these cellular processes (Simopoulos, 2011). For example, the ratio of n-6 to n-3 PUFA determines whether the fatty acids will have inflammatory or anti-inflammatory actions. n-6 PUFA intake increases inflammation, whereas n- 3 PUFAs reduce n-6 PUFA activity, and thus reduces inflammation (Calder, 2009; Calder, 2010).

Long-chain omega-3 polyunsaturated fatty acids, EPA (eicosapentaenoic acid) DHA (docosahexaenoic acid), and ALA (alpha linolenic acid), are known for their favorable effects on

cardiovascular conditions and cancer (Abeywardena and Belobrajdic, 2016). A recent study of the effects of EPA and DHA supplementation on risk factors for cardiovascular disease demonstrated that EPA on CVD risk reduction may relate in part to the lowering of Lp-PLA_2 without adversely affecting LDL-C (Asztalos et al., 2016). This study demonstrates that EPA is associated with lowering of CVD risk factors. In general, omega-3 fatty acids are recognized for their favorable health effects due to their high functionality, with the position of double bond closest to the methyl end of the molecule and having important roles in cell membranes (Valentine and Valentine, 2004) and as precursors of bioactive lipid mediators (Calder, 2010). Despite their important biological roles, the body does not readily synthesize omega-3s and dietary intake of these substances is needed.

Beyond their favorable outcomes when used to supplement treatments for cancer (Eltweri et al., 2016) and heart disease (Saboori et al., 2015), new research has suggested that they are useful for the treatment of obesity and related conditions (Cavaliere et al., 2016; Huerta et al., 2016; Jangale et al., 2016; Simopoulous, 2016), In a recent study, dietary omega-3 fatty acids attenuated diet-induced obesity and insulin resistance in rats by producing anti-inflammatory effects (Cavaliere et al., 2016). In another study, the effects of omega-3 fatty acids were reviewed to demonstrate that the overall ratio of omega-3 to omeg-6 fatty acids is important in the development of obesity, with a lower ratio of omega n-3 to n-6 being related to increased risk for developing obesity (Simopoulos, 2016). This review was based on animal and human research. Based on these findings, omega-3 fatty acids are protective against obesity. Other researchers have had similar findings, with n-3 fatty acids being protective or beneficial in obesity in children and adults (Kabir et al., 2007; Lopez-Alarcon et al., 2011; Vasicova et al., 2011). In addition, some researchers have found that n-3 fatty acids combined with aerobic exercise improves body

composition and cardiovascular risk factors (Hill et al,, 2007). Several other studies have demonstrated that n-3 fatty acids improve satiety in obese individuals (Parra et al., 2008), leptin and ghrelin concentrations in obese adults (Ramel et al., 2009), and in overall health in obesity (Kunesova et al., 2006).

While the above-cited research supports the protective effects of n-3 fatty acids against cardiovascular disease and obesity, the National Institutes of Health (NIH) has taken a much more cautious approach to their recommendations on omega-3 supplementation. In their 2016 fact sheet, the NIH describes several studies that did not find a protective effect of n-3 fatty acids or just did not find enough evidence to support this claim. They also state that omega 3s as part of a healthy lifestyle (eating fish) may help determine their protective effects; the healthy lifestyle being the most essential determinant. These negative findings should also be considered as the following studies are proposed.

Given the present research, it is possible that n-3 fatty acids are beneficial to obesity and related health states. In other research, obesity has been associated with cognitive deficits as well as general declines in health. Different interventions have been employed to try to prevent the obesity-related cognitive declines such as antioxidants, exercise, and several other variables that either reduce obesity or antagonize its negative health effects. Omega-3 fatty acids have not been used in this type of neutraceutical capacity with obesity, and it would be interesting to determine whether they would help minimize or prevent obesity-related cognitive dysfunction. There is some research to suggest that omega-3s will help prevent cognitive deficits associated with obesity. This area of research has utilized omega-3s in depressed individuals to determine whether the intake or supplementation would alleviate depressive symptoms. Research has found that omega-3 supplementation and/or intake helps minimize symptoms of depression. Specifically, the intake of

fish has been inversely related to depressive symptoms (Tanskanen et al., 2001; Timonen et al., 2004; Sanchez-Villegas et al., 2008; Asborg et al., 2009; Colangelo et al., 2009). Higher intakes of fish were associated with lower incidences of depression. Interestingly, these correlations were stronger for women than they were for men. Other researchers examined three different doses of EPA (1, 2, and 4/g per day) on symptoms of depression (Peet and Horrobin, 2002). They found that 1g/day for 4 weeks improved all measures of depression (Peet and Horrobin, 2002). In another supplementation study, 2 g/EPA/day relative to placebo improved depressive symptoms after 2 weeks (Nemets et al., 2002). Not only have n-3 fatty acids been demonstrated to affect depressive symptoms, but also research has shown that they can influence cognition. This research is discussed in the next section.

The purpose of the proposed research is to use animal models of obesity to determine the effects of n-3 fatty acids on obesity and metabolically related declines in cognition. The first study will involve the administration of alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) to obese rats to determine which of the n-3 fatty acids is the most potent regulator of cognitive dysfunction in obese animals. The second study will use the most effective fatty acid against obesity related cognitive dysfunction, and combine it with exercise. This study will extend those that have found that exercise and n-3 fatty acids are effective against obesity. Another aspect of both studies is that brain derived neurotrophic factor will be measured; it is a common molecule assessed for learning and memory processes.

Omega-3 fatty acids are major components of neuronal membranes and have a wide array of functions, from modulating synaptic plasticity to neuroimmune-modulation and neuroprotection (Luchtman and Song, 2013). Given these findings, cognitive processes will be examined in both studies. Previously, n-3 fatty acids have been shown to improve cognition in healthy populations

(and even in instances of dementia) (Kidd, 2007; Mazereeuw et al., 2012; Denis et al., 2013; Luchtman and Song, 2013). In one review of this literature, brain aging and stress is hypothesized to be minimized by optimal n-3 fatty acid intake through the modulation of endogenous brain mechanisms (Denis et al., 2013). Other research suggests that n-3 fatty acid intake is associated with protective effects from childhood to aging, and even in demented states, research that is supported by animal and clinical studies (Luchtman and Song, 2013). Provided this evidence, it is thought that n-3 fatty acids will also have protective effects against the cognitive deficits associated with obesity.

Study 1: Omega-3 Fatty Acids and Obesity Related Cognitive Deficits

The first study is designed to test the effects of omega-3 supplementation on cognitive functioning in obese rats. In addition to tests of cognitive functioning, measures of metabolic functioning will be measured. These are important tests to administer because obesity can often disrupt glucose and insulin metabolism (Stranahan et al., 2008; Jurdak et al., 2009), and it is important to see what effect (if any) n-3 supplementation can have on these changes in metabolic functioning. Additionally, corticosterone is often elevated in obesity, so it will be interesting to observe the effect of omega-3 fatty acid supplementation on corticosterone levels. Finally, measurements of BDNF expression in different areas of the brain will be made. Generally, obesity decreases BDNF expression (Stranahan et al., 2008); it remains to be seen what effect n-3 fatty acids will have on brain derived neurotrophic factor. A few studies suggest that omega-3 fatty acids modulate BDNF gene expression in different regions of the brain in obese rats (Abdel-Maksoud et al., 2016). Additional research is needed.

Experimental Materials and Methods

Animals

Eight groups (160 rats total, 80 males and 80 females) of experimentally naïve, Long-Evans rats (Charles River Laboratories, Portage, MI) will be used. Rats will be 30 days old when they arrive in the laboratory. Dietary intervention will continue for 12 weeks for all four groups of rats.

The animals will be individually housed in hanging stainless steel cages, in a temperature 22 ± 1°C and humidity-controlled room with a 12:12 reverse light cycle (lights on at 7 pm). Individual housing, while sometimes considered a stressor, is necessary to successfully control/monitor the dietary intake of each animal. The animals will be acclimated to the environment for one week before the dietary interventions. All experimental and animal care procedures that will be in accordance with the NIH Guidelines for the Care and Use of Animals in Research and approved by Tufts IACUC.

Dietary Composition

Animals in the diet-induced obesity (DIO) conditions will be provided *ad libitum* access to a high saturated fat, high sugar diet , 58% kcal fat, 25.5% kcal sucrose, 16.4% kcal protein, D12331; Research Diets Inc., New Brunswick, NJ, USA . The specific obesogenic diet was chosen because of its efficacy in inducing obesity and metabolic syndrome (Yuan et al., 2001). Ad libitum access to a control diet, 10.5% fat kcal, 73.1% carbohydrate kcal, 16.4% protein kcal will be given to control animals, D12328; Research Diets Inc, New Brunswick, NJ, USA. All diets will be supplied in Wahmann LC306 food cups with lids. The cups will be secured to the floor of each cage to prevent spillage. Water will be presented in glass bottles with leak proof spouts ad libitum. Diet consumption will every three days and body weight will be measured weekly. The addition of fat to the high sugar diet will be a strategic move in terms of ensuring

that the diet will have the most potent effects on weight gain and changes in metabolism. Previous studies have shown these effects of a high fat, high sugar diet (Stranahan et al., 2008; Beilharz et al, 2013). Because there was evidence suggesting the reliable effects of a Western type diet (high fat and high sugar), this will be an objective pursued in the present study. In addition, the adoption of a Western type diet is one factor implicated in the rise of obesity worldwide. Lastly, given that the sucrose-supplemented diet did not reliably produce cognitive deficits it will be a good idea to try a different approach.

In addition to the high-fat diet, obesogenic animals will be supplemented with n-3 fatty acids. Each of the three fatty acid types will be given at 400 mg/kg/day orally (by gavage) (Abdel-Maksoud et al., 2016) to the respective dietary groups.

Procedures

Animal Assignment

Animals will be assigned to one of eight groups; a control group, an ALA/obesogenic group, a DHA/obesogenic group, an EPA/obesogenic group, an ALA group, a DHA group, an EPA group, and an obesogenic group. Each group will be fed their respective diet for 12 weeks. Equal numbers of male and female rats will be used in this study. The rationale for using both sexes in this study is that female rats/organisms are not well represented in obesity studies and there are reported sex differences in female organisms in susceptibility to obesity (Hwang et al., 2010). In addition, women are underrepresented in clinical trials (Johnson et al., 2014). Based on previous studies, it is predicted that female rats will have a less severe response to the obesogenic diet due to a reported estrogen-mediated protective effect (Lebesque et al., 2010).

Fasting Blood Glucose and Glucose Tolerance

Fasting blood glucose will be assessed one week prior to behavioral testing in each group. After an 18-hour fast, blood samples will be obtained from a small incision site at the tip of each animal's tail. Immediately following determination of fasting blood glucose levels, animals will be given a 50% glucose solution (2 ml/kg body weight) using IP injection. Blood glucose levels will be measured at 15, 30, 60, 90 and 120 minutes following the glucose load to obtain a measure of glucose tolerance.

Insulin Measurements

Trunk blood obtained during decapitation will be used to measure insulin levels (non-fasting). Serum will be separated by centrifugation (10,000 rpm, 10 min) and placed in Eppendorf tubes. Serum insulin will be measured using rat insulin ELISA kits (Alpco Diagnostics, Salem, USA). This is a method utilized by Kumar and colleagues (2013). The full protocol found at (Alpco Diagnostics, Salem, USA) will be followed to determine blood insulin levels and whether or not there are any group differences. Studies have shown that decreased and increased insulin levels are associated with insulin resistance (Choi et al., 2014; Seo et al., 2014).

Corticosterone Measurements

Trunk blood obtained during decapitation will be used to measure corticosterone levels. Blood will then be centrifuged at 3,000 g for 15 min at 4°C. Corticosterone level will be determined by radioimmunoassay using a specific kit (Coat-A-Count Rat Corticosterone – Diagnostic Products Corporation). The full protocol will be followed to determine corticosterone levels and whether or not there are group differences. Corticosterone is an important to measure because it has been demonstrated to be elevated in instances of obesity (Vrabcova et al., 2014).

Estrous Cycle Monitoring

For female rats, phases of the estrous cycle will be ascertained by vaginal swabbing for 10 days prior to the Morris water maze testing. Information obtained about estrous cycle phase will be used when determining whether phase of estrous cycle influenced behavior on the water maze. Previously, there have been reported sex differences in water maze acquisition in humans (Snieder et al., 2015) and cycle differences in animals (Freye, 1995; Galeo, 1995). However, others have reported no sex differences on water maze learning in animals (Healy et al., 1999).

Morris Water Maze

Behavioral testing will be performed during the dark portion of the daily lighting cycle (beginning at 10 am, which will be three hours after the onset of the dark cycle). Hippocampal-dependent learning and memory will be measured using the Morris water maze. The water maze used will be a circular pool (182.9 cm) where the water will be rendered opaque with non-toxic paint. The pool will be divided into 4 equal quadrants that will be labeled arbitrarily as north, south, east, and west. The pool will be located in a room that contains extramaze spatial cues, which will remain in a fixed position for the duration of testing. Rats will be trained in the maze over 5 consecutive days with 5 trials per day. The platform (2 cm beneath the surface) will remain in the same location for the first 3 days. On the fifth day reversal trails, the platform will be relocated to the quadrant opposite to its initial location. Probe trials will be conducted prior to the reversal trials and 24 hours after the reversal training day. After each training trial, rats will be gently removed from the water, towel dried, and returned to their home cages. During the training and reversal trials, the latency to reach the platform, swim speed, and path efficiency will be measured, and the probe trial measurement of interest will be will be total time spent in each of the 4 quadrants. Images will be digitized through the use of a ceiling-mounted camera and

behavioral data will be acquired using Any Maze behavioral tracking software (Stoelting Inc, Wood Dale, IL).

Brain Dissections

Upon completion of the water maze testing, rats will be anesthetized with isoflurane, decapitated, and the hippocampus and hypothalamus, will be dissected and rapidly frozen at -80°C. Brain structures from a few behaviorally naïve animals in each group will be reserved to examine the any differences due to behavioral testing.

Quantitative Reverse Transcription-PCR—BDNF

RNA will be extracted using Tri Reagent® (as per manufacturer's specifications) from the hippocampus and hypothalamus Each brain section will then be treated with DNase, and tested for genomic contamination with PCR. Reverse transcription (RT) to generate cDNA will be performed with 2µg RNA and using 200 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and 150 ng of random hexamers (Invitrogen) in a 20µl reaction. Real-time PCR amplification will be conducted using a MX-3000P Stratagene (La, Jolla, CA) cycler and SYBR green PCR master mix (Qiagen, Valencia, CA). For each primer set, product amplification specificity will be confirmed by dissociation curve analysis and agarose gel electrophoresis. Additionally, dissociation curves will be created using serial dilutions and the efficiencies for each primer set will be calculated. The amplification efficiency for all the primers utilized in this study will be between 90 and 100%. A validation experiment will be performed for each primer set to show that the PCR efficiencies will be approximately equal to those of the reference gene.

A two-step protocol will be used: annealing temp: 55°C. GAPHDH and Actin will be used as normalizer genes. The following primer sequences will be used for detecting total BDNF transcript: forward: 5'GAAAGTCCCGGTATCCAAAG3', reverse: 5' CCAGCCAATTCTCTTTTT 3'. Primer sequences for the reference genes used will be obtained from http://pga.mgh.harvard.edu/primerbank/index.html Actin (ID# 6671509a1) and GAPDH as detailed in Fan, Jing, and Zang (2004). All samples will be analyzed in triplicates, and non-template controls will be included to determine the presence of any level of contamination. Amplification products should range from 154 to 201 bp. Data gathered will be analyzed using the comparative Ct method (reviewed in Schmittgen and Livak, 2008).

Data Analysis

Dietary intake, body weight, and metabolism will be analyzed using SPSS. BDNF transcripts will be analyzed using the CT method as used in the original studies.

Water maze learning trials will be analyzed as a repeated-measures ANOVA, with trial as the within subjects factor and dietary treatment as the between subjects factor. Sex and age will also be analyzed separately for each between subjects factor.

Probe trial data will be analyzed using an ANOVA as well. The final data analysis will involve correlations between behavioral and brain findings to determine whether there is a relationship between the two factors.

Data Predictions and Interpretation-Study 1

Fasting Blood Glucose and Oral Glucose Tolerance

Fasting blood glucose will be elevated in the animals fed the obesogenic diet. Additionally, oral glucose tolerance will be abnormally high in the animals fed the high sugar, high fat conditions when compared to chow fed animals. The changes in glucose metabolism will be due to increases in body weight and the obesogenic diet, which are associated with metabolic syndrome. This anticipated result is supported by the literature (Stranahan et al., 2008; Jurdak et al., 2009), which has found that glucose metabolism is disrupted by high-energy diets. Omega-3 supplementation will likely attenuate the elevations in glucose metabolism, normalizing them as suggested by research (Hill et al., 2007).

Insulin Measurements

Insulin is anticipated to be increased in obesity because of insulin insensitivity in rats fed the high sugar, high fat diet. The increase in insulin levels will be due to developing hyperinsulinemia. The rationale for these predictions is based on several studies demonstrating that obesity and high-energy diets are associated with elevated insulin levels (Leisgang et al., 2014; Seo et al., 2014). Omega-3 supplementation is hypothesized to improve insulin sensitivity, as it is effective at improving many health factors associated with obesity (Kabir et al., 2007).

Corticosterone Measurements

Corticosterone levels will be elevated due to high energy feeding compared to those animals fed a standard chow diet. Elevated corticosterone levels will represent abnormalities in metabolism and will be associated with decreased cognitive functioning. The reasoning behind these predictions is that corticosterone has been demonstrated to be elevated in obesity and in response to high-energy feeding (Swierczynska et al., 2014; Wosiki-Kuhn et al., 2014). Omega-3

supplementation is hypothesized to lower corticosterone levels because it is related to better body composition (Hill et al., 2007), which is also related to reductions in corticosterone.

Estrous Cycle Monitoring

There will be no diet-dependent difference in estrous cycle. A Pub Med search of the literature yielded no studies to support a diet-induced difference in estrous cycles. This lack of difference between diet groups will eliminate potential estrous cycle effects on cognition.

Morris Water Maze

Animals fed the high energy diet will show progressively pronounced impairments in learning and retention of the Morris water maze as a function of age (with older animals having more pronounced impairment. These learning impairments will represent dysfunction that is predominantly hippocampally based, and related to obesity and disruption of glucose metabolism. The reasoning behind these predictions is based on literature that has demonstrated that obesity and high-energy diets impair spatial learning and memory (Stranahan et al., 2008; Jurdak et al., 2009; Neha et al., 2014). Omega-3 supplementation should improve cognitive functioning because it has been hypothesized to improve health factors associated with obesity (Kunesova et al., 2006).

Quantitative Reverse Transcription-PCR—BDNF

There will be a relative decrease in expression of BDNF, in the hippocampus of animals fed the high energy diet, and this decrease in expression will represent and be related to cognitive impairments in the Morris water maze. There are several previous findings that support this prediction (Martins et al., 2013;Woo et al., 2013;Wosiki-Kuhn et al., 2014). There will also be a

relative decrease of BDNF mRNA in the hippocampus and hypothalamus suggested by previous reports (Abdel-Maksoud et al., 2016). Omega-3 supplementation is hypothesized to attenuate decreased expression of BDNF because of its ability to improve physiologic factors associated with obesity (Kunesova et al., 2006).

Study 2- Omega-3s and Exercise as an Intervention for Obesity and Related Cognitive Deficits

In the second study, the combination of exercise and n-3 fatty acid supplementation will be used to determine its effectiveness against cognitive deficits and decreases in BDNF associated with obesity. Generally, research has found that exercise is effective against cognitive decline and decreases in BDNF in obese animals (Lee et al., 2014; Noble et al., 2014; Cai et al., 2016; Kim et al., 2016; Woo et al., 2016). In fact, a recent study found that exercise reduced memory impairment associated with a high fat diet and increased BDNF production in hippocampal neurons (Noble et al., 2014). In another study, treadmill exercise alleviated cognitive impairments by enhancing neuroplasticity in the hippocampi of diet-induced obese mice (Kim et al., 2016). Similarly, Cai and colleagues (2016) reported that treadmill exercise increased levels of BDNF. There are also some human studies to support the positive effects of exercise on cognition and/or BDNF (for comprehensive review see Szuhany et al., 2015; Elnier et al., 2016;Herting et al., 2016; Hwang et al., 2016; van Dongen et al., 2016) With an abundant literature (briefly reviewed here) to suggest that exercise improves factors associated with obesity, including neurocognitive ones, it was selected as a variable in the second study.

Together, exercise and n-3 fatty acid supplementation should produce a strong effect in Experiment 2.

Experimental Materials and Methods

Animals and Dietary Composition

Eight groups (160 rats total, 80M and 80F) of experimentally naïve, Long-Evans rats (Charles River Laboratories, Portage, MI) will be used. Rats will be 30 days old when they arrive in the laboratory. The animals will be individually housed in hanging stainless steel cages or, animals in the exercise condition, will be housed in cages with running wheels attached. Animals in the exercise condition will have free access to running wheels (amount of exercise will be tracked). The animals will be housed in a temperature 22 ± 1°C and humidity-controlled room with a 12:12 reverse light cycle (lights on at 7 pm). The animals will be acclimated to the environment for one week before the dietary interventions. All experimental and animal care procedures that will be performed will be in accordance with the NIH Guidelines for the Care and Use of Animals in Research and the IACUC at Tufts University.

Dietary Composition

Animals in the diet-induced obesity (DIO) conditions will be provided *ad libitum* access to a high saturated fat, high sugar diet , 58% kcal fat, 25.5% kcal sucrose, and 16.4% kcal protein, D12331; Research Diets Inc., New Brunswick, NJ, USA . The particular diet was chosen because of its demonstrated efficacy in promoting obesity (Petro et al., 2004) For control animals, a standard ground chow diet will be supplied in Wahmann LC306 food cups with lids. The cups will be secured to the floor of each cage to prevent spillage. Water a will be presented

in glass bottles with leak proof spouts *ad libitum*. Food intake will be monitored every other day and body weight will be measured weekly.

In addition to the high-fat diet, obesogenic/exercise animals and n-3 exercise/no exercise will be supplemented with n-3 fatty acids. The most potent fatty acid from Experiment 1 will be given at 400 mg/kg/day orally (by gavage) (Abdel-Maksoud et al., 2016) to the obesity/exercise group.

Procedures

Animal Assignment

Following a one-week acclimation, 160 rats will be distributed between the obesogenic/n-3 exercise condition, obesogenic/n-3 no exercise condition, n-3 exercise condition, n-3 no exercise condition, an obesogenic condition, an obesogenic exercise condition, a control exercise condition, or a control condition. This will yield 20 rats per condition, with 10 males and 10 females per group. Based on previous studies, it is predicted that female rats will have a less severe response to the obesogenic diet due to a reported estrogen-mediated protective effect (Lebesque et al., 2010).

Fasting Blood Glucose and Oral Glucose Tolerance

Fasting blood glucose will be assessed two weeks prior to behavioral testing in each group. After an 18-hour fast, blood samples will be obtained from a small incision site at the tip of each animal's tail. Immediately following determination of fasting blood glucose levels, animals will be

given a 50% sucrose solution (2 ml/kg body weight) using oral gavage. Blood glucose levels will be measured at 15, 30, 60, 90 and 120 minutes following the sucrose load.

Insulin Measurements

Blood will be obtained during decapitation and used for insulin measurements (non-fasting). Serum will be separated by centrifugation (10,000 rpm, 10 min) and placed in Eppendorf tubes. Serum insulin will be measured using rat insulin ELISA kits (Alpco Diagnostics, Salem, USA). This is a method utilized in Kumar and colleagues (2013). The full protocol found at (Alpco Diagnostics, Salem, USA) will be followed to determine blood insulin levels and whether or not there are any group differences.

Corticosterone Measurements

Blood will be obtained during decapitation and used for corticosterone measurements., It will then be centrifuged at 3,000 g for 15 min at 4°C. Corticosterone level will be determined by radioimmunoassay using a specific kit (Coat-A-Count Rat Corticosterone – Diagnostic Products Corporation). The full protocol will be followed to determine corticosterone levels and whether or not there are group differences.

Brain Dissections

Upon completion of the water maze testing, rats will be anesthetized with isoflurane, decapitated, and the hippocampus and hypothalamus, will be dissected and rapidly frozen at -80°C. Brain structures from a few behaviorally naïve animals in each group will be reserved to examine the any differences due to behavioral testing.

Quantitative Reverse Transcription-PCR—BDNF

RNA will be extracted using Tri Reagent® (as per manufacturer's specifications) from the hippocampus and hypothalamus Each brain section will then be treated with DNase, and tested for genomic contamination with PCR. Reverse transcription (RT) to generate cDNA will be performed with 2µg RNA and using 200 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and 150 ng of random hexamers (Invitrogen) in a 20µl reaction. Real-time PCR amplification will be conducted using a MX-3000P Stratagene (La, Jolla, CA) cycler and SYBR green PCR master mix (Qiagen, Valencia, CA). For each primer set, product amplification specificity will be confirmed by dissociation curve analysis and agarose gel electrophoresis. Additionally, dissociation curves will be created using serial dilutions and the efficiencies for each primer set will be calculated. The amplification efficiency for all the primers utilized in this study will be between 90 and 100%. A validation experiment will be performed for each primer set to show that the PCR efficiencies will be approximately equal to those of the reference gene. A two-step protocol will be used: annealing temp: 55°C. GAPHDH and Actin will be used as normalizer genes. The following primer sequences will be used for detecting total BDNF transcript: forward: 5'GAAAGTCCCGGTATCCAAAG3', reverse: 5' CCAGCCAATTCTCTTTTT 3'. Primer sequences for the reference genes used will be obtained from http://pga.mgh.harvard.edu/primerbank/index.html Actin (ID# 6671509a1) and GAPDH as detailed in Fan, Jing, and Zang (2004). All samples will be analyzed in triplicates, and non-template controls will be included to determine the presence of any level of contamination. Amplification products should range from 154 to 201 bp. Data gathered will be analyzed using the comparative Ct method (reviewed in Schmittgen and Livak, 2008).

Estrous Cycle Monitoring

For female rats, the estrous cycle will be ascertained by vaginal swabbing for 10 days prior to the Morris water maze testing. Information obtained about estrous cycle will be used when considering any differences in behavior obtained during either the water maze.

Exercise Protocol

Animals will have voluntary running wheel access and will be singly housed. Running wheels, which animals could access at will, will be externally fixed to standard shoebox cages via a short tunnel. Running will be monitored using Activity Wheel Monitoring Software (Lafayette Instrument, Lafayette, IN).

Morris Water Maze

Behavioral testing will be performed during the dark portion of the daily lighting cycle (beginning at 10 am, which will be three hours after the onset of the dark cycle). Hippocampal-dependent learning and memory will be measured using the Morris water maze. The water maze used will be a circular pool (182.9 cm) where the water will be rendered opaque with non-toxic paint. The pool will be divided into 4 equal quadrants that will be labeled arbitrarily as north, south, east, and west. The pool will be located in a room that contains extramaze spatial cues, which will remain in a fixed position for the duration of testing. Rats will be trained in the maze over 5 consecutive days with 5 trials per day. The platform (2 cm beneath the surface) will remain in the same location for the first 5 days. On the sixth day reversal trails, the platform will be relocated to the quadrant opposite to its initial location. Probe trials will be conducted prior to the

reversal trials and 24 hours after the reversal training day. After each training trial, rats will be gently removed from the water, towel dried, and returned to their home cages. During the training and reversal trials, the latency to reach the platform, swim speed, and path efficiency will be measured, and the probe trial measurement of interest will be will be total time spent in each of the 4 quadrants. Images will be digitized through the use of a ceiling-mounted camera and behavioral data will be acquired using Any Maze behavioral tracking software (Stoelting Inc, Wood Dale, IL).

Data Analysis

Dietary intake, body weight, metabolism will be analyzed using SPSS. Water maze learning trials will be analyzed as a repeated-measures ANOVA, with trial as the within subjects factor and treatment as the between subjects factor. Probe trial data will be analyzed using an ANOVA as well. Finally, correlations between behavior, metabolic, and neuronal findings will be conducted.

Data Predictions and Interpretation-Study 2

Fasting Blood Glucose and Oral Glucose Tolerance

Fasting blood glucose will be elevated in the animals fed the obesogenic diet. For the obesogenic/exercise/n-3 group, fasting blood glucose will be normalized because of the exercise and n-3 combination. Blood glucose levels should also be attenuated in the obesogenic exercise group. Exercise alone has potent effects on blood glucose levels, so combined with n-3 administration there should be a significant effect. These anticipated results are supported by the

literature (Stranahan et al., 2008; Jurdak et al., 2009), which has found that glucose metabolism is disrupted by high-energy diets.

Insulin Measurements

Insulin is anticipated to be increased in obesity because of insulin insensitivity in rats fed the high sugar, high fat diet. The increase in insulin levels will be due to developing hyperinsulinemia and metabolic syndrome. The rationale for these predictions is based on several studies demonstrating that obesity and high-energy diets are associated with elevated insulin levels (Leisgang et al., 2014; Seo et al., 2014). The obesity/exercise/n-3 group should demonstrate a moderated effect of insulin, a normalization of the effect. Insulin levels are predicted to be attenuated in the obesogenic exercise group as well. Exercise has been demonstrated to improve insulin signaling (Carter et al., 2015) and n-3 supplementation has been shown to improve insulin function (Hirabara et al., 2013).

Corticosterone Measurements

Corticosterone levels will be elevated due to high energy feeding compared to those animals fed a standard chow diet. The obesity/exercise/n-3 group will demonstrate moderation of corticosterone elevations due to a decrease in obesity. The obesogenic exercise group will also show improvements in corticosterone. The reasoning behind these predictions is that corticosterone has been demonstrated to be elevated in obesity and in response to high-energy feeding (Swierczynska et al., 2014; Wosiki-Kuhn et al., 2014), and thus, a reversal of obesity should attenuate elevations in corticosterone.

Estrous Cycle Monitoring

There will be no diet-dependent difference in estrous cycle. A Pub Med search of the literature yielded no studies to support a diet-induced difference in estrous cycles. This lack of difference between diet groups will eliminate potential estrous cycle effects on cognition.

Morris Water Maze

Animals fed the high energy diet will show pronounced impairments in learning and retention of the Morris water maze. The animals in the obesogenic/exercise/n-3 group will demonstrate normal learning on the Morris water maze due to reduced obesity and improved metabolic functioning. The exercise obesogenic group will demonstrate close to normal learning, but the improvements will not be as pronounced as in the obesogenic/exercise/n-3 group. These learning impairments will represent dysfunction that is predominantly based in the hippocampus, and related to obesity and disruption of glucose metabolism. The reasoning behind these predictions is based on literature that has demonstrated that obesity and high-energy diets impair spatial learning and memory (Stranahan et al., 2008; Jurdak et al., 2009; Neha et al., 2014).

Quantitative Reverse Transcription-PCR—BDNF

There will be a relative decrease in expression of BDNF, in the hippocampus of animals fed the high energy diet, and this decrease in expression will represent and be related to cognitive impairments in the Morris water maze. There are several previous findings that support this prediction (Martins et al., 2013;Woo et al., 2013;Wosiki-Kuhn et al., 2014). Omega-3 supplementation and exercise are hypothesized to attenuate decreased expression of BDNF because of their ability to improve physiologic factors associated with obesity (Kunesova et al.,

2006). The obesogenic exercise group will also have attenuated decreases in BDNF expression, but it will not be as pronounced as the group also receiving n-3 supplementation.

Limitations

There are a few limitations of the proposed experiments. Using only the Morris Water Maze is potentially limiting because you cannot assess non-spatially based learning and memory. Adding a task such as the T-maze might be suggested for future studies. However, for this preliminary group of studies a simpler paradigm was chosen to avoid potential effects of multiple behavioral tests on BDNF levels.

The measurement of BDNF expression in only one brain region is another limitation of the studies. The amygdala and frontal lobes could have been interesting areas to investigate as well, but for simplicity's sake, and because there was a spatially dependent focus, the hippocampus was the primary region selected. Further investigation of other brain regions is suggested in future research. Related to brain region, substrate of brain function is another area that was limited in these studies. BDNF was the only substrate of brain function measured. In future studies, other substrates of brain function should be measured. One key factor that should be measured is Trk B. Other memory molecules are also good candidates for such an investigation.

Summary and Conclusions

Considered together, the findings of my work and the proposed research are efforts in the right direction to model the effects of obesity on the brain under different circumstances. With

continued research, we may find that younger organisms/children are indeed susceptible to the effects of obesity on the brain and cognition. At this time, research is mixed on this issue and further examination of the variables is needed. Several research questions could be posed related to obesity in children. At what age is the brain susceptible to the effects of obesity? This would be important when trying to implement interventions or preventative programs in obese children. What are the exact effects of obesity on the young brain? This is a relevant question when it comes to potential treatments for any cognitive deficits associated with obesity; will exercise suffice or is a more pronounced treatment necessary? These are just two of the several issues that can be examined in future research, whether it be using animal models or human participants. That said, such studies could have great translational potential. Animal studies could inform human research and vice versa.

The two omega-3 studies also have great translational potential. Starting with the exercise/omega-3 study, reversal of obesity is frequently a topic of intense interest for many obvious therapeutic uses. Several animal and human studies have examined the effect of dietary-based or exercise induced reversal of obesity (Lee et al. 2014; Noble et al., 2014;Savoye et al., 2014; Cai et al., 2016; Kim et al., 2016), with overwhelmingly positive results. Some results of these studies show a reduction of weight as an index of improvement or reversal of obesity while others demonstrate decreases in biochemical parameters associated with obesity or over nutrition. In addition, omega-3s have been demonstrated to mitigate the effects of obesity independent of other factors (Cavaliere et al., 2016; Huerta et al., 2016; Jangale et al., 2016; Simopoulos, 2016) The proposed exercise/omega 3 study is an extension of the current literature as it examines not only what is occurring in the periphery during weight loss, but any central changes that might occur, namely changes in BDNF levels in the hippocampus. As a

translational piece, the hippocampal and other brain regions could be explored using fMRI on obese individuals who have undergone various dietary interventions, including omega-3 supplementation. In fact, this method provides more information than the animal study as you can collect information before and after the dietary intervention from the same participant.

In summary, translation of basic research is important in the field of obesity research. Once we get an idea of where the animal and human studies overlap and diverge in their findings, we can really start to make progress as good research in one area may aggregate to create exciting findings in another. It is up to the scientific community to take this approach to research to maximize the meaning of their findings. It will be through collaboration that we gain the deepest insight into the obesity-brain relationship.

Conclusions

As was reiterated throughout this paper, the neurological effects of obesity are becoming pervasive and complex, and any attempt at modeling childhood obesity as it affects the brain is particularly challenging because of the inherent resiliency of the developing brain, and ethical issues that arise (i.e., with reversal paradigms). Despite these challenges, modeling obesity's effects at every developmental milieu is important to not only childhood research, but to aging research. Given that so many young obese individuals will remain obese for their entire lives, it is essential to determine whether there is a cumulative neuropathological presence of obesity. In addition, it is necessary that we determine whether or not the brain is protected in younger obese individuals and when this protection is finally overwhelmed by the metabolic pathology accompanying obesity. To accomplish this would be an immense contribution to understanding the developmental course of obesity. Conversely, and equally important, is identifying any subtle differences that occur in the brains of younger obese individuals. While

young brains may be inherently resilient, and the present series of studies was very preliminary in achieving any of these objectives regarding obesity in young organisms, there are endless possibilities and interesting approaches to answering these questions. Some of these avenues of research I proposed in the previous sections, but beyond that, research in this area should definitely remain an important part of future endeavors.

In addition to continuing research such as I've described earlier in this section, it is equally important to make translational research a major objective in the future. Different research groups should collaborate closely and compare findings from animal studies with those from human studies. Identifying areas where they overlap will be important to the progress of translational research. Also important will be indentifying areas where the research differs, which will allow additional information to be collected on obesity and its effects on the brain. This research could include large longitudinal studies that follow organisms from a young age to older age when they are suffering from obesity and the metabolic consequences. Some of the outcomes of such studies could reveal cognitive and other brain effects of obesity over time. Some of the research questions that would be interesting to answer are when is obesity most harmful to the brain and are there measurable effects in younger organisms?

The possibilities for research on the young topic of obesity and brain effects are almost limitless. It is an interesting field to pursue because obesity affects so many people worldwide, and it is particularly prevalent in the U.S. The practical impact of obesity research is large for a couple of reasons. First, as evidence has already suggested obesity is not healthy for the body or the brain. Second, as it becomes more common knowledge that obesity is damaging to the brain it may be easier for medical professionals to encourage obese individuals to change their health

behaviors in an attempt to reduce body weight. Lastly, on a more global level programs aimed at reducing obesity will incorporate more information about obesity's affect on the brain. Overall, research on obesity will provide insights into many disease processes and it should help people on a more practical level as well. Maybe in the future, obesity's metabolic and brain effects will become a molecular type marker for diagnostic purposes, and for predicting longer-term outcomes such as neurodegeneration.

Figures

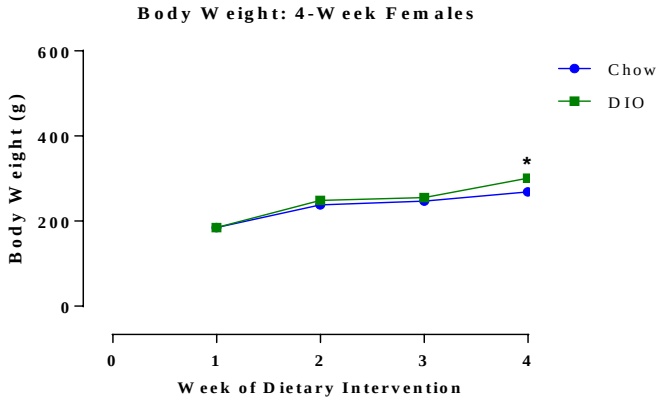

Figure 1. DIO animals weighed significantly more than their chow-fed counterparts by week four of the dietary intervention.

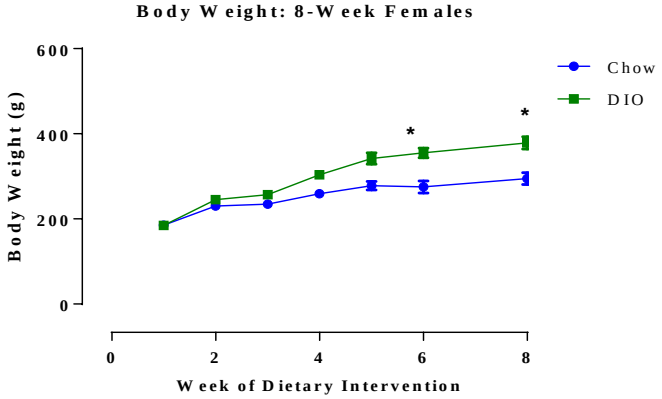

Figure 2. DIO animals weighed significantly more than their chow-fed counterparts from week six through week eight of the dietary intervention.

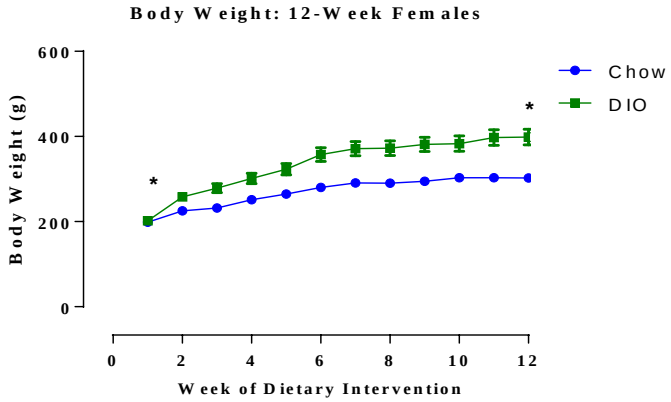

Figure 3. DIO animals weighed significantly more than their chow-fed counterparts from week two through week twelve of the dietary intervention.

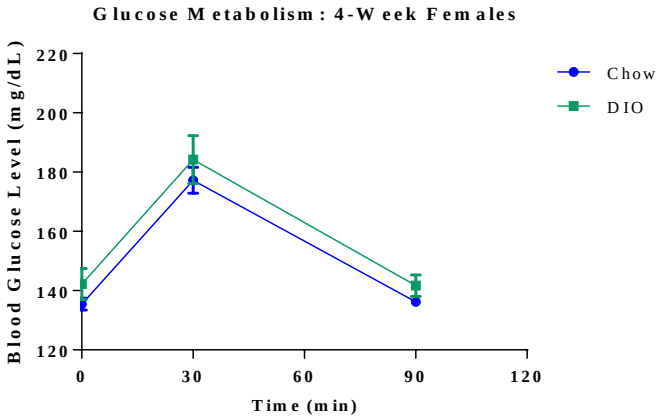

Figure 4. There were no diet-dependent differences in fasting blood glucose or blood glucose levels post-glucose load.

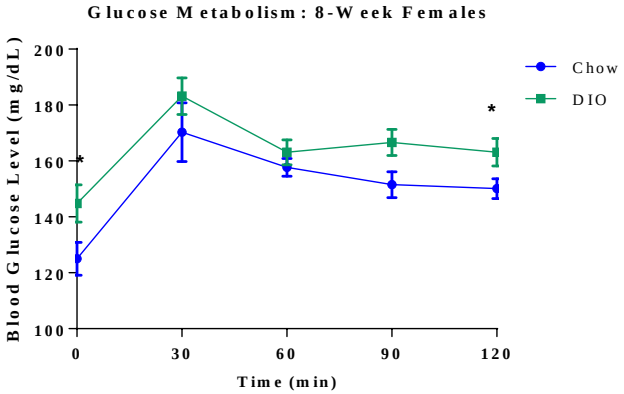

Figure 5. The DIO animals demonstrated significantly elevated fasting blood glucose and, when submitted to an area under the curve analysis, there were significant differences in blood glucose levels post-glucose load.

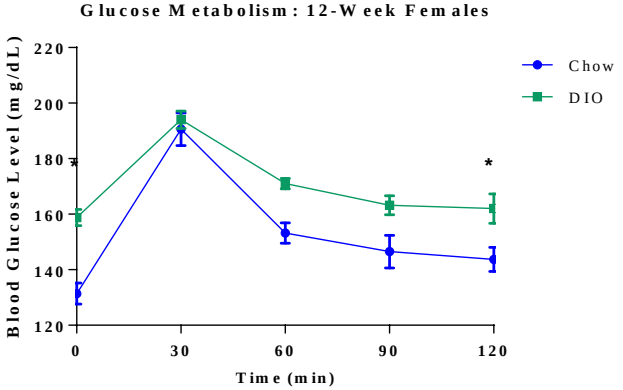

Figure 6. The DIO animals demonstrated significantly elevated fasting blood glucose and, when submitted to an area under the curve analysis, there were significant differences blood glucose levels post-glucose load.

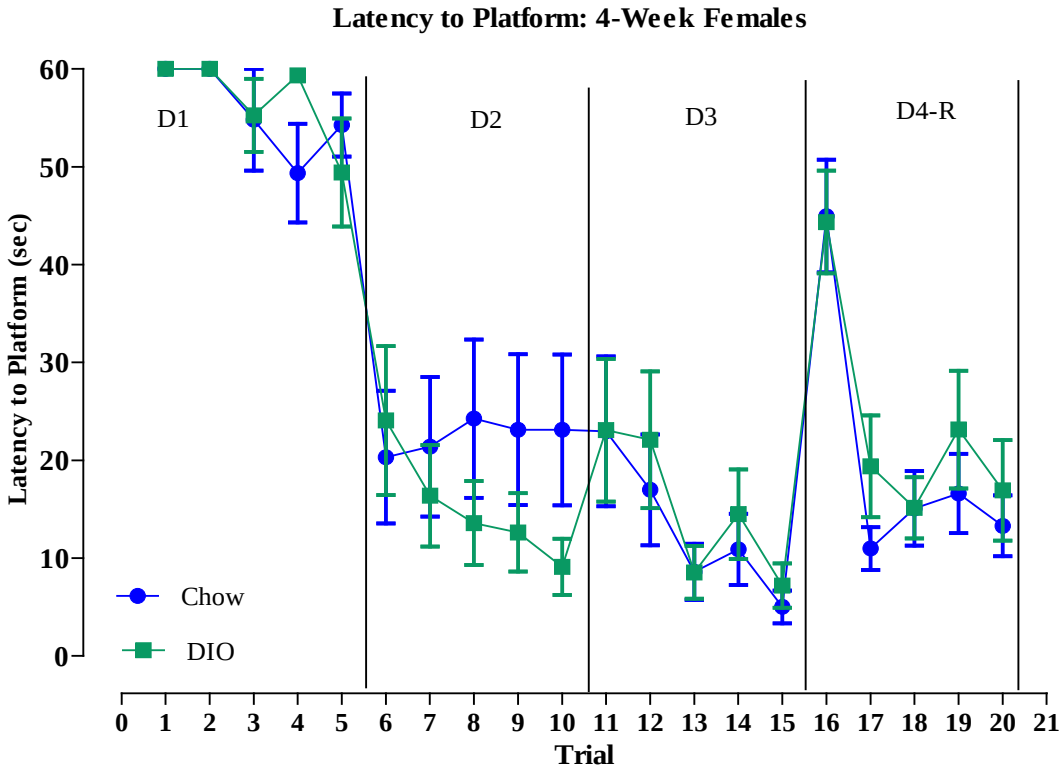

Figure 7. There were no diet-dependent differences on acquisition or reversal learning trials in the 4-week female rats.

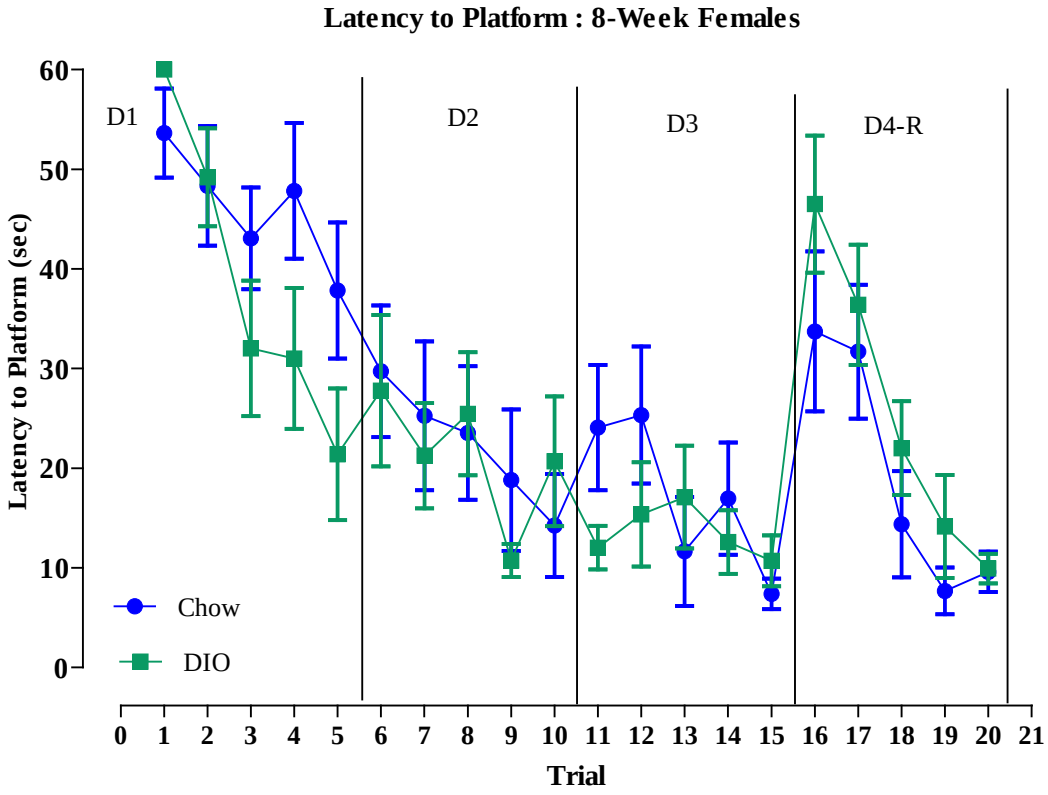

Figure 8. There were no diet-dependent differences on acquisition or reversal learning trials in the 8-week female rats.

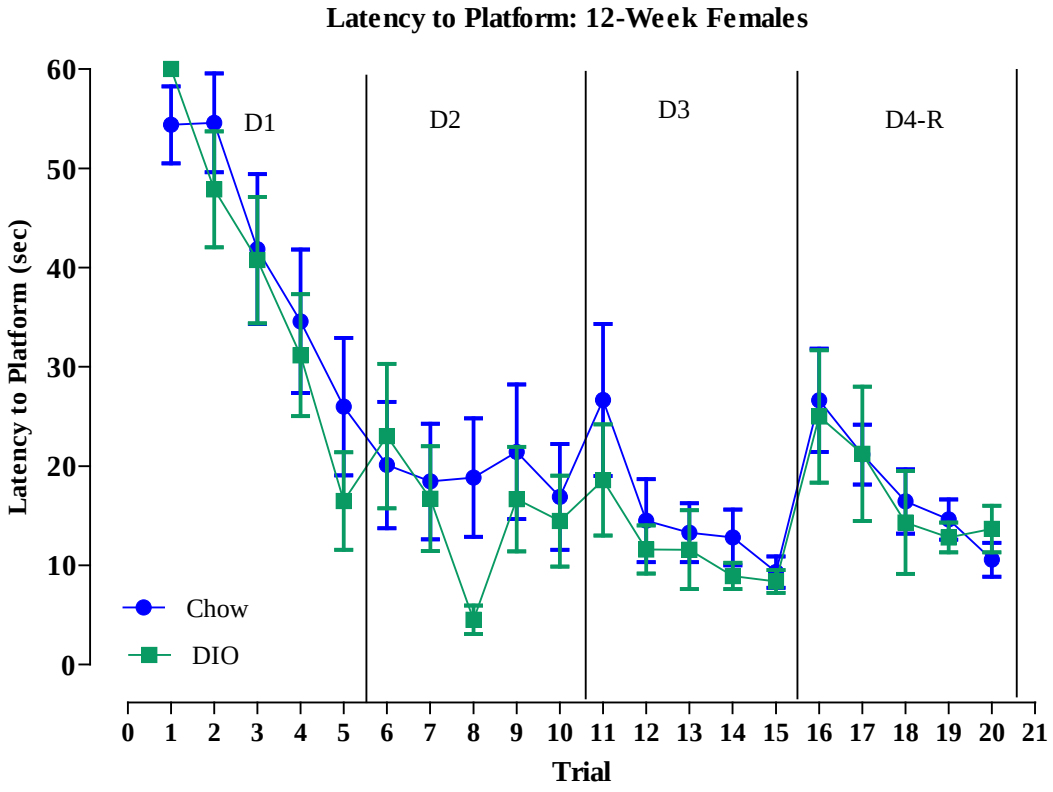

Figure 9. There were no diet-dependent differences on acquisition or reversal learning trials in the 12-week female rats.

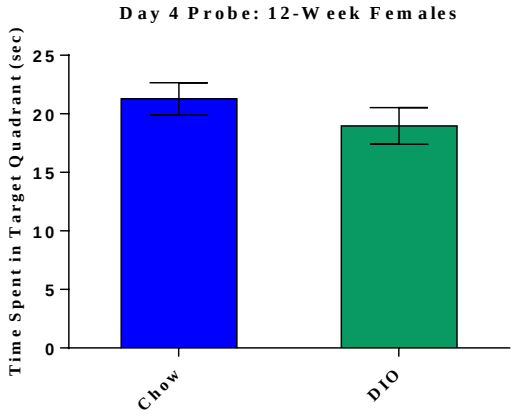

Figure 10. Time spent swimming in target quadrant did not differ significantly by diet group during the probe trial conducted on day four of training.

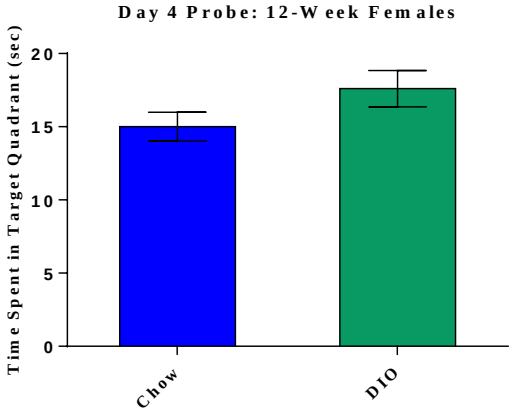

Figure 11. Time spent swimming in target quadrant did not differ significantly by diet group during the probe trial conducted on day four of training.

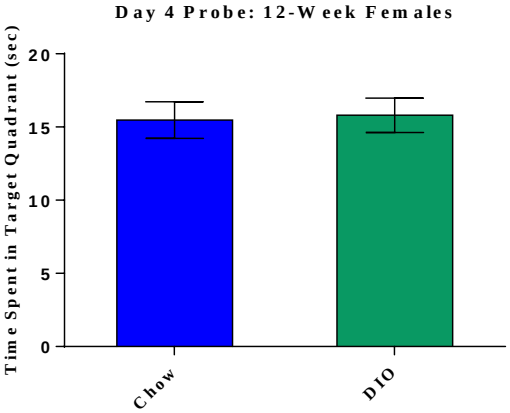

Figure 12. Time spent swimming in target quadrant did not differ significantly by diet group during the probe trial conducted on day four of training.

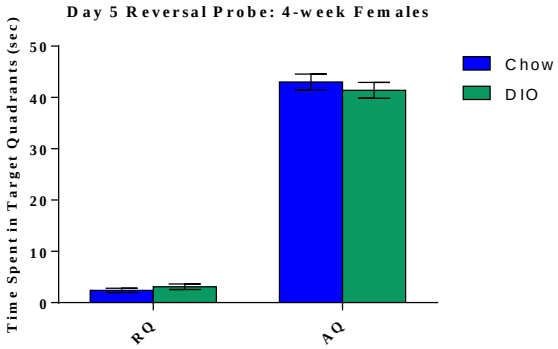

Figure 13. A comparison of time spent swimming in the reversal quadrant (RQ) and the acquisition quadrant (AQ). While there were no diet-dependent differences in performance during the reversal probe, both groups of animals demonstrated a spatial preference for the quadrant learned during acquisition training.

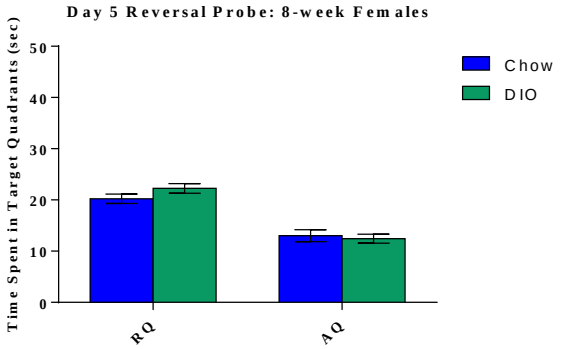

Figure 14. A comparison of time spent swimming in the reversal quadrant (RQ) and the acquisition quadrant (AQ). While there were no diet-dependent differences in performance during the reversal probe, both groups of animals demonstrated a slight spatial preference for the quadrant learned during reversal training, which is to be expected because it is the most recently learned information.

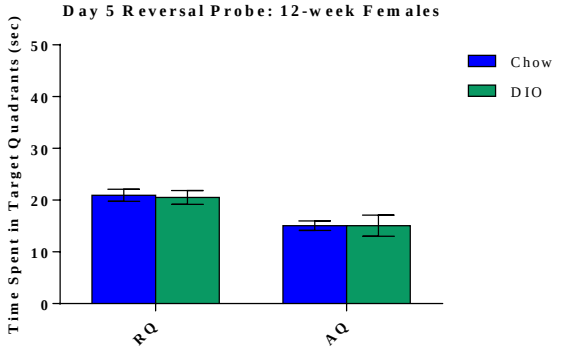

Figure 15. A comparison of time spent swimming in the reversal quadrant (RQ) and the acquisition quadrant (AQ). While there were no diet-dependent differences in performance during the reversal probe, both groups of animals demonstrated a slight spatial preference for the quadrant learned during reversal training, which is to be expected because it is the most recently learned information.

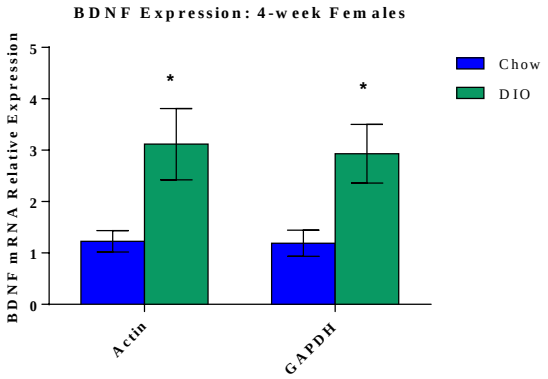

Figure 13. Total relative expression of brain-derived neurotrophic factor mRNA as compared to actin and GAPDH as normalizer genes. At 4-weeks dietary intervention, there was a significant increase in BDNF mRNA expression in whole hippocampal samples of DIO animals using actin and GAPDH as normalizers.

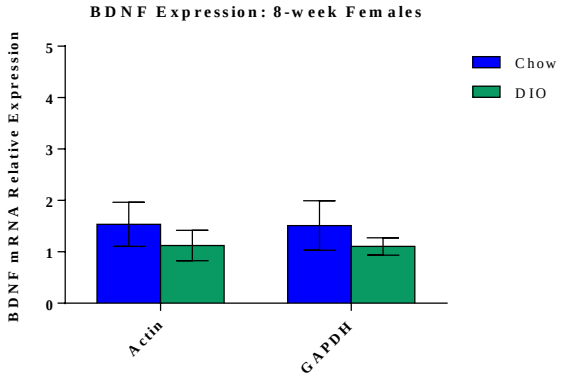

Figure 14. Total relative expression of brain-derived neurotrophic factor mRNA as compared to actin and GAPDH as normalizer genes. At 8-weeks dietary intervention, there was a decrease in BDNF mRNA expression in whole hippocampal samples of DIO animals, but this difference did not reach significance.

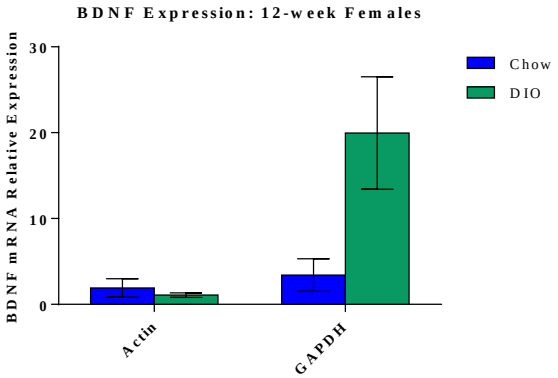

Figure 15. Total relative expression of brain-derived neurotrophic factor mRNA as compared to actin and GAPDH as normalizer genes. At 12-weeks dietary intervention, there were no significant differences in BDNF mRNA expression.

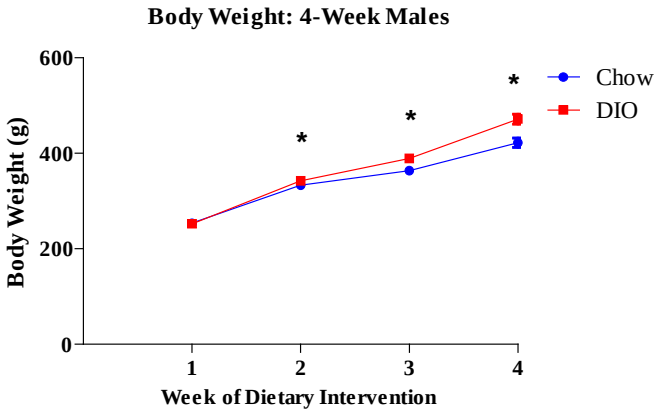

Figure 19. DIO animals weighed significantly more than their chow -fed counterparts from week two through week four of the dietary intervention.

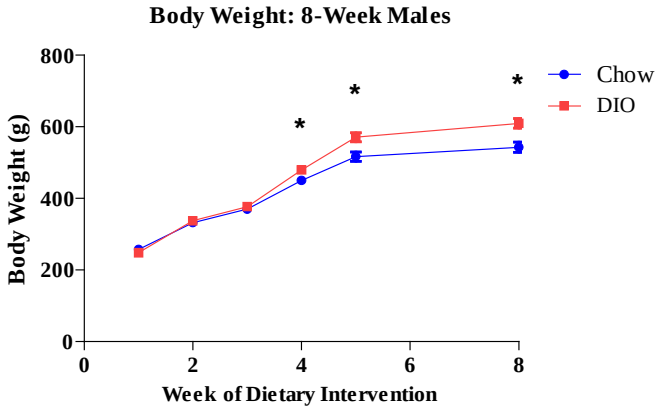

Figure 20. DIO animals weighed significantly more than their cho w-fed counterparts from week four through week eight of the dietary intervention.

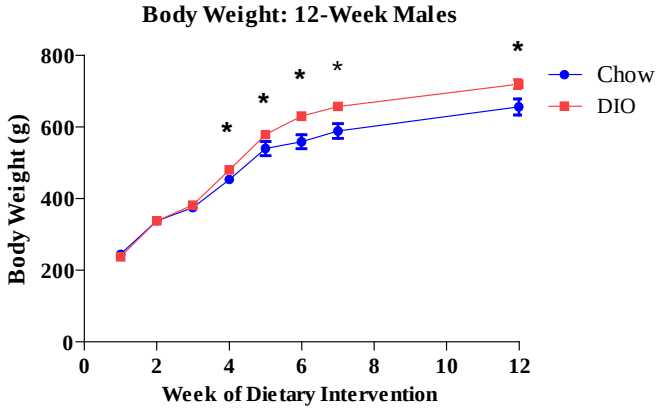

Figure 21. DIO animals weighed significantly more than their cho w-fed counterparts from week four through week twelve of the dietary intervention.

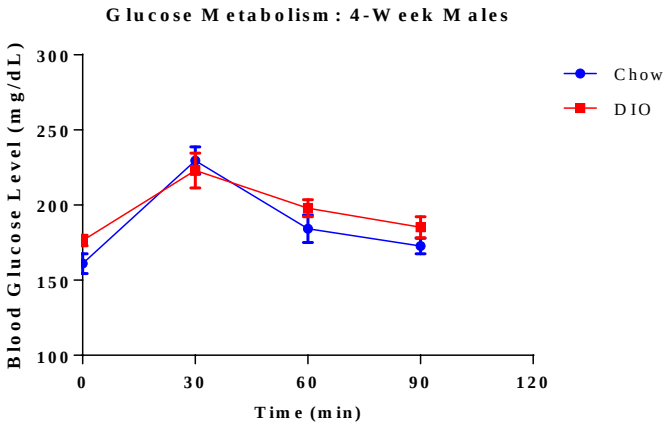

Figure 22. There were no diet-dependent differences in fasting blood glucose levels or glucose levels post-glucose load.

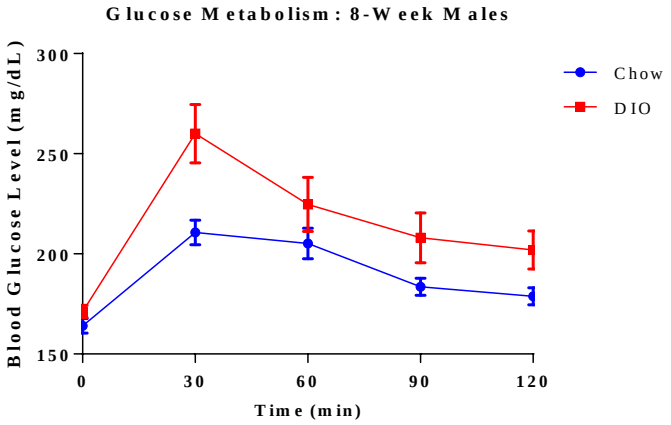

Figure 23. There were no diet-dependent differences in fasting blood glucose levels or blood glucose levels post-glucose load.

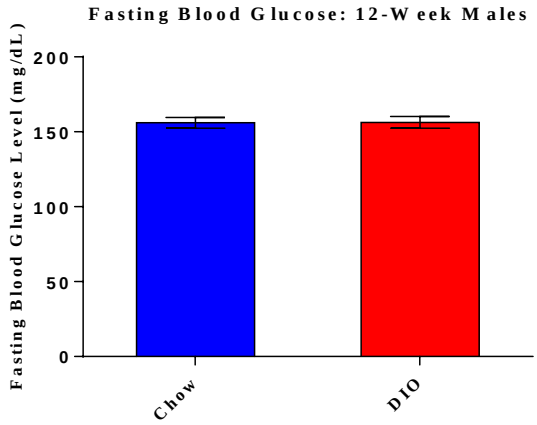

Figure 24. There were no diet-dependent differences in fasting blood glucose.

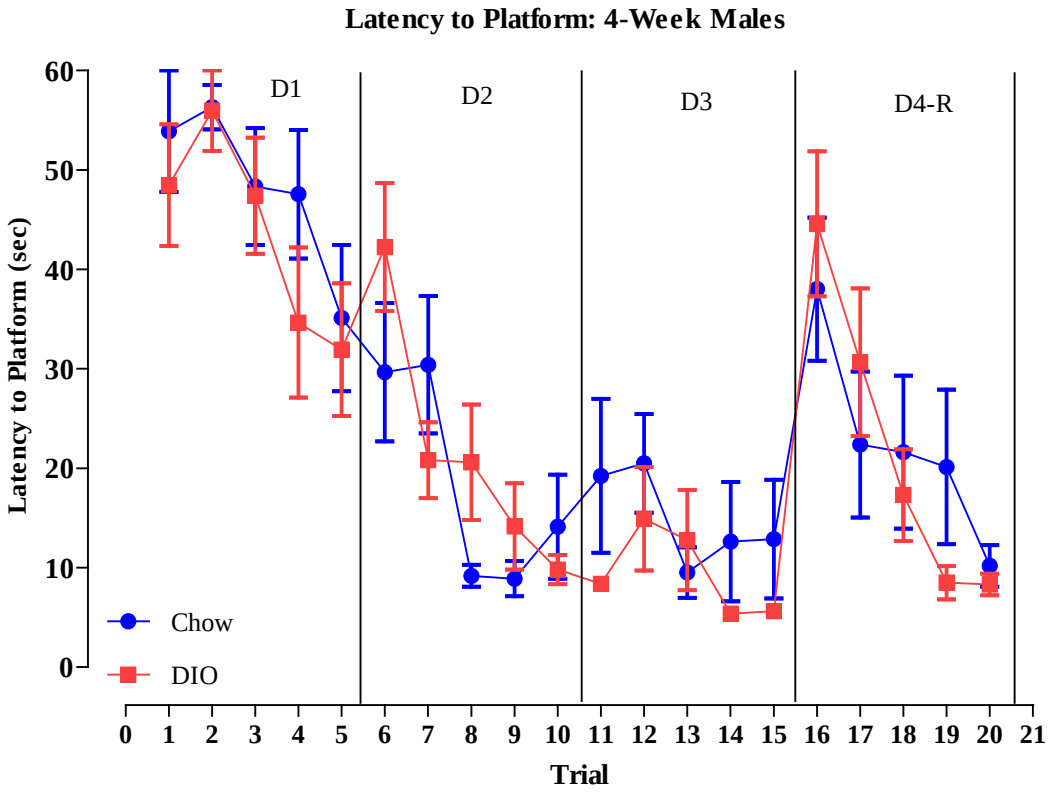

Figure 25. There were no diet-dependent differences on acquisition or reversal learning trials in the 4-week male rats.

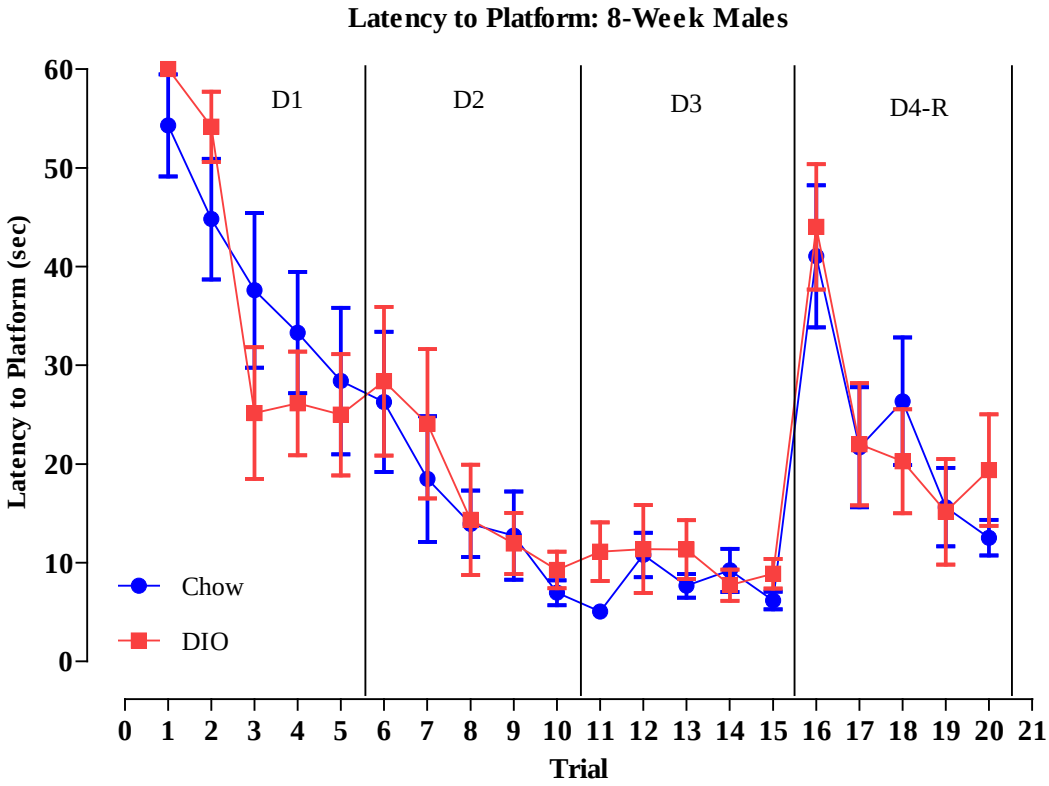

Figure 26. There were no diet-dependent differences on acquisition or reversal learning trials in the 8-week male rats.

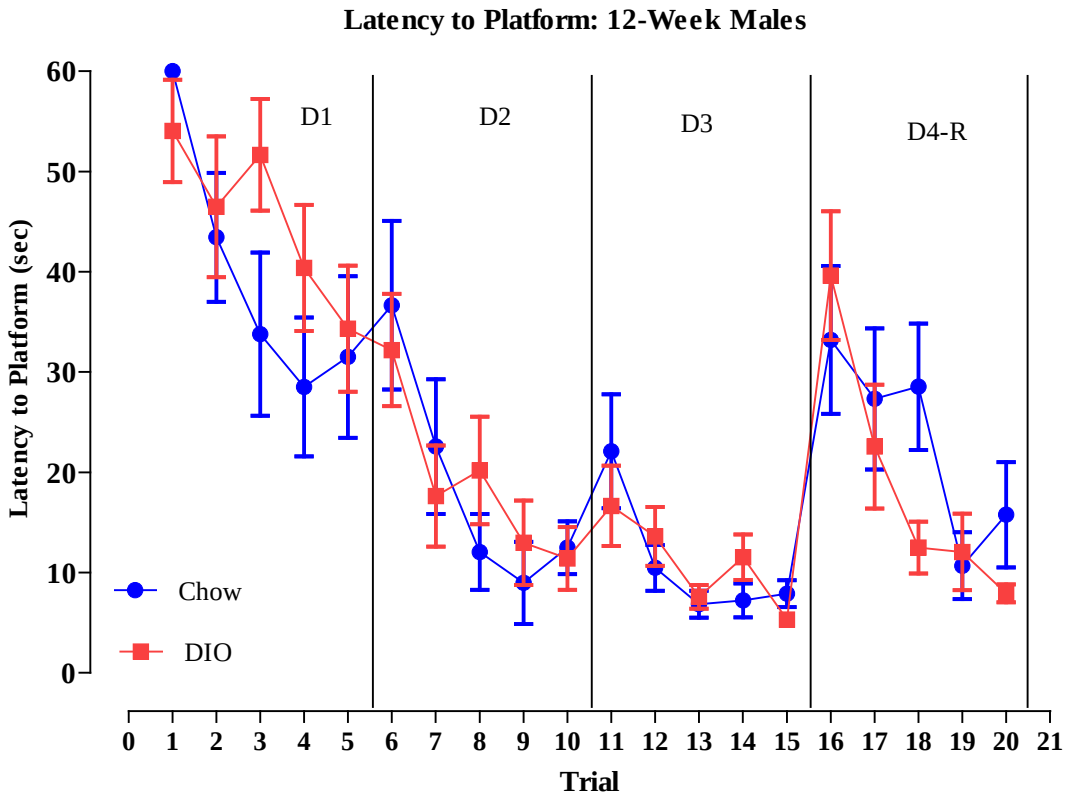

Figure 27. There were no diet-dependent differences on acquisition or reversal learning trials in the 12-week male rats.

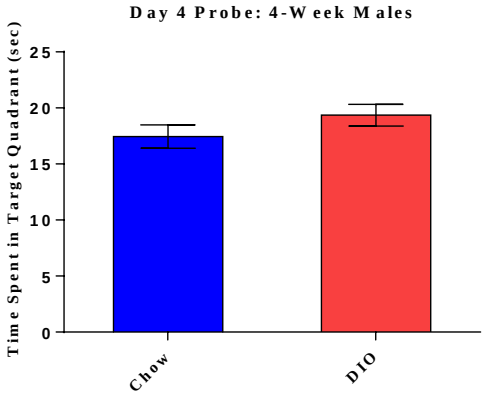

Figure 28. Time spent swimming in target quadrant did not differ significantly by diet group during the probe trial conducted on day four of training.

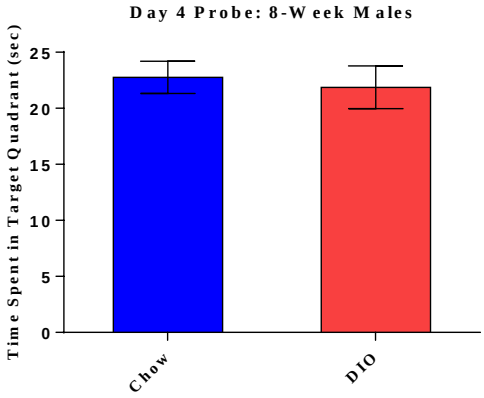

Figure 27. Time spent swimming in target quadrant did not differ significantly by diet group during the probe trial conducted on day four of training.

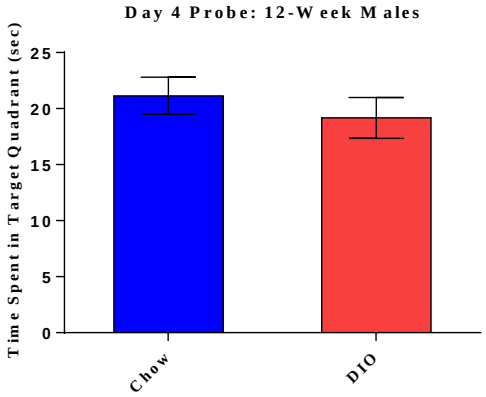

Figure 28. Time spent swimming in target quadrant did not differ significantly by diet group during the probe trial conducted on day four of training.

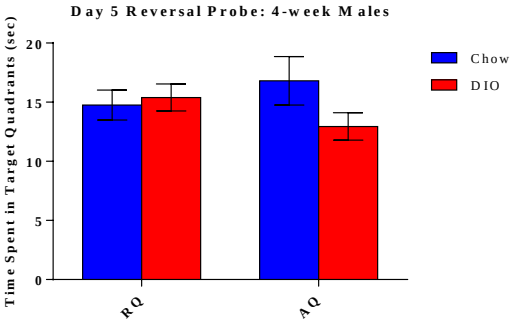

Figure 29. A comparison of time spent swimming in the reversal quadrant (RQ) and the acquisition quadrant (AQ). While there were no diet-dependent differences in performance during the reversal probe, both groups of animals demonstrated a nearly equivalent preference for each learned quadrant locations.

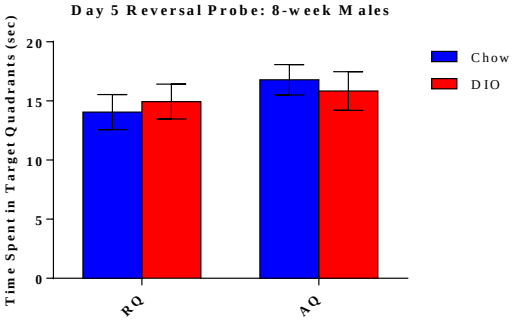

Figure 30. A comparison of time spent swimming in the reversal quadrant (RQ) and the acquisition quadrant (AQ). While there were no diet-dependent differences in performance during the reversal probe, both groups of animals demonstrated a slight preference for the acquisition quadrant, which was the location learned initially.

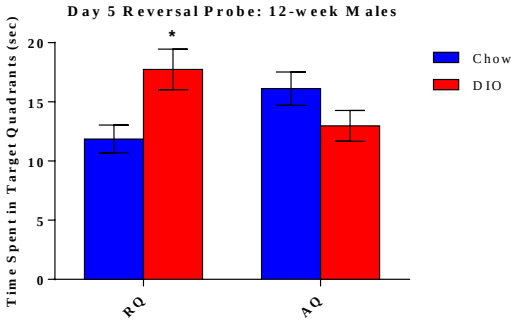

Figure 30. A comparison of time spent swimming in the reversal quadrant (RQ) and the acquisition quadrant (AQ). Unexpectedly, there was a significant differences in performance during the reversal probe, with the DIO animals spending more time in the reversal quadrant than their chow-fed counterparts. This may have been, in part, due to the chow-fed animals' preference for the acquisition quadrant.

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