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Purpose: Obesity is an adverse health condition characterized by excessive weight gain. Aside from the pathological conditions most commonly associated with obesity, recent epidemiological studies have suggested that obesity may be associated with impaired learning and memory. Previous research has linked inflammation and oxidative stress, cellular changes that occur consequent to obesity, to impaired cognitive function. Obese rodent models have been established and are commonly used for obesity-related research; however, with respect to the effect of obesity on cognitive function, the data remain inconclusive. In these studies, two obese mouse models, representing two obesity-inducing causes: genetic vs. environment, were behaviorally characterized in order to determine which model was best suited to study behavioral and cellular changes associated with obesity. Furthermore, a suitable model needed to be identified to carry on studies focusing on the relation between aging and obesity, as one would predict an exacerbation of the impairment in old obese models with age. The current studies were also based on the rationale that a dietary intervention at mid-life would ameliorate behavioral and biochemical changes observed with obesity.

Methods: In study I, separate groups of 6-month old male and female C57BL/6 and leptin-deficient (*ob/ob*) were subjected to a battery of behavioral tests for motor,

cognitive and visual function. In study II, separate groups of male C57BL/6 mice aged to 6- or 12-months were fed *ad libitum* either a control diet or a high-fat diet since 6-weeks of age. In a subset of the aged mice, a dietary intervention was introduced such that these mice were switched from the high-fat diet to the control diet at 6-months of age. Mice were subjected to a battery of behavioral tests at 6- or 12-months that required utilization of various component of memory, learning and visual function.

Results: In study I, cognitive impairments were observed in the obese mice, and were exacerbated in female mice. While overall spatial learning was unaffected, male and female *ob/ob* mice performed worse on an active avoidance paradigm, indicating frontal cortical impairment. The *ob/ob* mice also performed worse on vision-associated tests. The results from this study suggested that obesity impairs cognitive and visual function in a sex-dependent manner. In study II, increased anxiety was observed in diet-induced obese mice; however, spontaneous activity, spatial capacity and performance on the active avoidance paradigm were unaffected. The dietary intervention reversed the effect of obesity on anxiety-like behaviors, but failed to improve cognitive and motor function. Visual impairments were observed in diet-induced obese mice, and these impairments were exacerbated with age. The results from study II indicated that diet-induced obese mice could be used to study the effects of obesity on visual, but not cognitive function.

Conclusions: Overall, the hypothesis that obesity impairs cognitive function and exacerbates age-related impairments was not supported and the dietary intervention

had minor effects. However, in both models, obesity impaired visual function and it was exacerbated at older ages. These findings suggest that *ob/ob* and diet-induced obese mice are valid animal models for investigating obesity-induced visual disorders.

THE ASSOCIATION BETWEEN OBESITY, COGNITION AND VISUAL FUNCTION

DISSERTATION

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INTRODUCTION

Obesity

Obesity defines a metabolic state in which excess adipose tissue adversely affects the health status of an individual. The classification using the height-weight concept was originally developed by Metropolitan Life Insurance Company to estimate life insurance for policy holders (1983). Within this concept, body mass index (BMI) was introduced, reflecting a ratio of weight (kg) to height (m^2) and, for the past 200 years was the most commonly used tool to categorize weight status (Table 1). A person of normal weight has a BMI between 18.5-24.99, whereas an obese person has a BMI ≥30. Body adiposity index, calculated from hip circumference and height, was recently proposed as a more accurate parameter for the measurement of adiposity compared to BMI (Bergman et al., 2011).

In 2009, approximately $119 million was awarded to states and United States territories to support efforts to reduce obesity and promote overall health (Center for Disease Control (CDC); American Recovery and Reinvestment Act, 2009). Regardless of these government-sponsored programs, obesity remains prevalent in American

society. In 2004, the third National Health and Examination Survey (NHANES III) reported 65.7% of the adult population as overweight and half were classified as obese (Hedley et al., 2004). According to the Behavioral Risk Factor Surveillance System Survey conducted during 2006-2008, 26.7% of American adults (72 million) reported themselves as obese (BMI $\geq$ 30).

Of equal concern, the prevalence of childhood and adolescence obesity has continued to increase since 1980 (Dehghan et al., 2005; Hedley et al., 2004; Ogden et al., 2002). According to data from the National Health and Nutrition Examination Survey from 2007-2008, 9.5% of infants and toddlers (birth-2 years old) and 17% of children and adolescents (2-19 years old) were classified as obese (Dehghan et al., 2005; Hedley et al., 2004; Ogden et al., 2002). Additional studies suggested a tendency for overweight children to remain overweight in adulthood and to become obese compared to normal weight children (Field, Cook, & Gillman, 2005; Rugg, 2004; Serdula et al., 1993; Svensson et al., 2010; Whitaker et al., 1997). Children with a high risk BMI have an increased risk for the development of obesity-associated adverse health conditions (Field et al., 2005; Freedman et al., 2005; Dietz & Robinson, 1998).

The increased prevalence of obesity has resulted in an augmentation of conditions such as hypertension, metabolic syndrome, glucose intolerance, type II diabetes, cardiovascular disease, osteoarthritis, asthma, sleep apnea and stroke accounting for approximately 9.4% of national health care expenditures (Colditz, 1999; Freedman et al., 2007; Purnell et al., 2008; Rodriguez et al., 2002; Watson & Craft, 2003). In 1999, it was reported that an estimated 280,184 Americans died of obesity-

related conditions (Allison, Zannolli, & Narayan, 1999).

Obesity is a condition influenced by the interplay between a multitude of factors: genetic, environmental, behavioral and endocrine factors as described below.

Genetic Component of Obesity

According to the set-point theory, body weight and fat mass is genetically predetermined and defended throughout the life span (Wardlaw, Contemporary Nutrition, 5th ed, 2003). Factors associated with weight and energy balance are similar between family members, supporting the involvement of a genetic component to the development of obesity. Of those factors, weight, BMI, fat distribution, resting metabolic rate (RMR), thermic effect of food and energy cost of submaximal exercise show the greatest rates of heritability (Bouchard et al., May, 1990; Heitmann et al., 1999; MacDonald & Stunkard, 1990; Sorensen et al., 1989; Stunkard et al., 1986). Monozygotic twins reared together or apart, demonstrate 40-70% heritability for BMI (Stunkard et al., July, 1986).

Environmental and Behavioral Influences of Obesity

Genetic predisposition influences the development of obesity; however, it is unlikely to be the primary factor contributing to current obesity trends (Hill & Peters, 1998). Instead, it is more likely that a synergistic effect exists between genetic predisposition and lifestyle that dictates susceptibility to becoming obese. Environmental and behavioral changes within American society have provided for unlimited access of palatable, high fat foods coupled with a decrease in physical activity

(Hill & Peters, 1998).

Dietary contributions to obesity

Increased dietary fat intake promotes weight gain and obesity eventually leading to adverse health conditions (Hariri & Thibault, 2010; Hariri, Gougeon, & Thibault, 2010; Page et al., 1957). Dietary guidelines specific for overall fat intake have since been released in order to improve overall health and decrease the risk for development of detrimental health conditions (Page et al., 1957). The American Heart Association has recommended a diet consisting of less than 30% daily caloric intake from fat, including less than 10% saturated fat daily intake (Krauss et al., 1996). While optimal fat intake has yet to be established, decreased dietary fat intake promotes weight loss and improves blood lipid levels (Jeffery et al., 1995; Meksawan et al., 2004; Raeini-Sarjaz, et al., 2001; Schaefer, Gleason, & Dansinger, 2005).

Physical activity and obesity

Despite recommended dietary modifications, which reflect a decrease in overall energy intake and dietary fat intake, obesity rates continue to rise (Prentice & Jebb, 1995). Another factor contributing greatly to the development of obesity is an inadequate level of physical activity (Prentice & Jebb, 1995). The Surgeon General has established guidelines for physical activity, in an attempt to increase daily levels of physical activity among adults and children. Currently, it is recommended that adults exercise moderately for 150 minutes each week and that children exercise 1 hour, daily activity including vigorous activity (U.S. Department of Health and Human Services). Technological advances have allowed for an unlimited access to media sources acting

as a substitute for daily physical activity. It was recently reported that children (8-18 years) are exposed to 6-61/2 hours of media each day (Vandewater et al., 2007). According to the NHANES, increased BMI was observed in children who reported watching increased amounts of television (Andersen et al., 1998). The association between media exposure and body weight is influenced by parental media practices such that children of overweight parents watched more television compared to children of normal weight parents (Steffen et al., 2009).

Energy Balance and Endocrine Factors

Body weight is maintained through energy homeostasis, a balance between energy intake (food) and energy expenditure (physical activity) (Figure 1). A shift in either direction away from this established equilibrium affects weight status. Negative energy balance results from decreased energy intake coupled with either no change in energy expenditure or an increase in energy expenditure. Increased energy intake coupled with either no change in energy expenditure or a decrease in energy expenditure shifts the body towards a positive energy balance. Obesity results from a chronic status of positive energy balance.

Body weight is also influenced by the interplay between peripheral and central signaling. Hypothalamic involvement in energy homeostasis was originally suggested in the mid-twentieth century after lesioning of regions within the hypothalamus affected food intake (Nutrition classics. the anatomical record, volume 78, 1940: Hypothalamic lesions and adiposity in the rat.1983; Abizaid, Gao, & Horvath, 2006; Anand & Brobeck,

1951a; Anand & Brobeck, 1951b; Brobeck, 1946). Humoral signals have been identified to act on these neural circuits within the hypothalamus to produce orexigenic or anorexigenic responses (Abizaid et al., 2006).

Estrogen and obesity

One such hormone, estrogen, influences energy homeostasis through activation of estrogen receptors within the hypothalamus (Brown et al., 2010) and increased adiposity is observed when these receptors are silenced (Musatov et al., 2007). Estrogen is also accountable for the differences in body fat distribution between men and women (Shi, Seeley, & Clegg, 2009). Men and post-menopausal women tend to carry a majority of fat their fat mass centrally, putting them at an increased risk for the development of co-morbidities of obesity compared to pre-menopausal women (Demerath et al., 2008; Ford, 2005; Lee et al., 2009).

Leptin and obesity

Another hormone, leptin, acts on cells within the hypothalamus to control body weight through effects on food intake and energy expenditure (Abizaid et al., 2006; Ahima & Antwi, 2008). Leptin is an adipocyte-derived hormone encoded by the obese (*ob*) gene and influences homeostatic processes including food intake, thermogenesis and neuroendocrine function (Harvey et al.2005; Harvey, 2007a; Harvey, 2007b; Li et al., 2002). Activation of leptin receptors in the hypothalamus by leptin regulate these effects and abnormalities in leptin signaling (leptin-receptor deficient) leads to over-consumption of food, impaired thermogenesis, infertility, and ultimately results in morbid obesity in both humans and animals.

Leptin deficiency in humans results from a rare recessive missense mutation (c313C>TArg105Trp), which is homologous to the mutation in leptin deficient (*ob/ob)* mice, or a frameshift/premature stop mutation (c398delG ∆133G) in the *ob* gene (Baicy et al., 2007; Matochik et al., 2005). To date, four human cases of the missense mutation have been reported, all of which are leptin deficient and morbidly obese (Matochik et al., 2005). Circulating plasma concentrations of leptin are proportional to adipose tissue mass (Harvey, 2007a). Because obese individuals have increased adipose tissue, circulating concentrations of leptin are increased. Most obese individuals are classified as leptin-resistant, as they eventually become insensitive to the effects of leptin (Van Heek et al., 1997).

Five isoforms (Ob-Ra, b, c, d, and e) of the leptin receptor have been identified in body (Funahashi et al., 2003). A soluble form of the leptin receptor, Ob-Re, is expressed in the choroid plexus (Fei et al., 1997). Short forms of the receptor, Ob-Ra, c and d, are found in a variety of peripheral tissues including skeletal muscle, adipose tissue, kidney and liver (Fei et al., 1997). The long form of the receptor, Ob-Rb, is expressed predominately in nuclei of the hypothalamus (Fei et al., 1997; Funahashi et al., 2003) and is involved in energy regulation as *db/db* mice that lack functional forms of this receptor become severely obese (Chen et al., 1996). Recently, leptin receptors have been identified in several brain regions suggesting that they might have functions related to cognition (Harvey et al., 2005; Harvey, 2007a; Harvey, 2007b).

Cognitive Function and Obesity

Human Studies

In addition to adverse health conditions, results from recent studies have suggested that cognitive impairment is associated with obesity in animals and humans. In humans, a number of epidemiological studies suggested that BMI, obesity *per se*, is linked to adverse neurocognitive outcome. Obese subjects are at increased risk for impaired cognitive function, which is further exacerbated with increased abdominal obesity (Fergenbaum et al., 2009). Independent of age, a BMI over 25 is inversely related to performance on all cognitive tests, especially in tests related to executive function (Gunstad et al., 2006; Gunstad et al., 2007). As a subset to the Framingham Heart Study, Elias et al. (2003) examined the effect of obesity and hypertension on cognitive function in males and females aged 55-88. Obese men performed worse than any of the other groups studied in tasks associated with learning and memory (Elias et al., 2003). More recently, higher BMI was correlated to impaired cognitive performance in middle-aged (32-62 years) men and women (Cournot et al., 2006). Further, Miller et al. (2006) reported lower intellectual ability, lower achievement and more behavioral problems in children with early onset morbid obesity (Miller et al., 2006). Obesity-induced cognitive deficits in humans could be consequent to decreased brain volume or brain metabolism. Obese individuals have reduced whole brain and total gray matter volumes compared to normal and overweight individuals (Gunstad et al., 2008). More specifically, BMI in males negatively correlates with global loss and regional alterations in gray matter volume, both of which may affect cognitive function (Taki et al., 2008). In addition, BMI has been negatively correlated with brain metabolism in prefrontal cortex

and cingulate gyrus (Volkow et al., 2009).

Animal Studies

Rodent models of obesity include genetic models and environmental/behavioral models. Genetic models include rats and mice that have mutations in leptin gene, leading to leptin deficiency or leptin receptor mutants. The resulting literature is inconclusive as some studies report cognitive impairments and others have not. Zucker fatty rats have impaired spatial learning and memory when compared to controls (Li et al., 2002), whereas the *db/db* (Li et al., 2002; Ohta et al., 2003) and *ob/ob* (Finger et al., 2010) mice have marginal impairments on spatial function. Environmental rodent models, also called diet-induced obesity models have also been associated with poor cognitive performance. Diet-induced obesity via high-sucrose supplementation displayed impaired performance on tasks associated with spatial learning and memory (watermaze and novel object recognition) (Jurdak et al., 2008; Jurdak & Kanarek, 2009). Young rats fed high fat diets display impaired performance on tasks associated with hippocampal, frontal lobe and thalamic function (Winocur & Moscovitch, 1990). Further, Mielke et al (2006) reported impaired performance on tasks associated with procedural learning and memory consolidation in C57BL/6 mice fed a 45% fat diet (Mielke et al., 2006). While the diets are not the same in each of the above studies, studies suggested that diet-induced obesity may have detrimental effects on various aspects of brain function in animal models.

Biochemical Alterations associated with Obesity

Advanced Glycation End-Products (AGEs)

Various post-translational modifications, such as deamidation, nitration, oxidation, phosphorylation, ubiquitination, and glycation, occur throughout the lifespan (Harding et al., 1989). Glycation, also referred to as the Maillard reaction, is a non-enzymatic reaction initiated by addition of the carbonyl group from a sugar to the amino group of a protein leading to the formation of advanced glycation end-products (AGEs). Serum levels of AGEs are similar between obese and normal-weight adults (Gugliucci et al., 2009) and have been reported to be decreased in obese children (Sebekova, et al., 2009) but, AGE formation is augmented by hyperglycemia and insulin resistance, both of which are commonly observed with obesity (Riboulet-Chavey et al., 2006). Accumulation of AGEs within vessel walls contributes to the microvascular complications, vascular damage, generation of reactive oxygen species (ROS), and interference of insulin signaling pathways frequently observed in diabetic patients (Basta et al., 2005; Riboulet-Chavey et al., 2006; Schalkwijk, Brouwers, & Stehouwer, 2008).

Oxidative Stress

Oxidative stress is the result of an imbalance between pro-oxidants and antioxidants resulting in an accumulation of free radicals. Increased oxidative stress has been implicated in the pathogenesis of a number of diseases (Roberts et al., 2006; Vincent & Taylor, 2006; Vincent et al., 2007). Free radicals are by-products of normal metabolic reactions that are necessary for maintenance of cell redox state, cell function

and intracellular signaling (Vincent & Taylor, 2006; Vincent et al., 2007). Increased concentrations of free radicals, specifically reactive oxygen species, result in DNA, protein and lipid damage, compromise cellular integrity and may result in cytotoxicity.

Evidence for obesity-induced oxidative stress has recently been reviewed by Vincent and Taylor (2006). Obese men, women and children exhibit increases in markers of lipid peroxidation (thiobarbituric reactive acid substances, malondialdehyde, isoprostanes, low-density lipoprotein (LDL) oxidation lag time, 4-hydroxynonenal, lipid hydroperoxides, conjugated dienes), increases in protein and DNA damage (protein carbonyls, 8-hydroxy-oxyguanosine), altered redox status (glutathione peroxidase (GPx) activity, glutathione (GSH) concentration) in plasma and urine (Hernandez-Marco et al., 2009; Vincent et al., 2007).

Inflammation

Obese humans are under a state of chronic inflammation (Bastarrachea et al., 2007; Vincent & Taylor, 2006). Mature adipocytes, act as an endocrine and paracrine organs with the capability to produce and secrete various cytokines (adipokines) and bioactive mediators (Lyon, Law, & Hsueh, 2003; Van Gaal, Mertens, & De Block, 2006; Vincent & Taylor, 2006). These include pro-inflammatory adipokines (tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), leptin, plasminogen activator inhibitor-1 (PAI-1), resistin and angiotensinogen) and anti-inflammatory adipokines (adiponectin) (Lyon et al., 2003; Van Gaal et al., 2006; Vincent & Taylor, 2006). Some of these biomarkers have the capacity to influence secretion of systemic inflammatory markers. IL-6 is the major cytokine that induces the upregulation of C-reactive protein (CRP) in the liver. CRP is a

low-grade inflammatory protein that is a predictor for atherosclerosis (Lyon et al., 2003; Van Gaal et al., 2006). Chronic elevations in pro-inflammatory adipokines influence insulin signaling, endothelial function, coagulation, fibrinolysis which are risk factors for cardiovascular diseases (Van Gaal et al., 2006). Concentrations of adipose-derived cytokines correlate with the degree of adiposity (Lyon et al., 2003; Vincent & Taylor, 2006). Reduction in fat mass through dietary modifications and exercise interventions has led to decrease in the serum levels of various adipokines and has subsequently attenuated the degree of inflammation (Lyon et al., 2003; Vincent & Taylor, 2006).

Goals of the Current Research

The overall goal for the current study was to understand the association between obesity and cognitive function. The approach consisted of using two models of obesity to determine the difference between genetic and environmental influences on obesity-induced cognitive impairment. The study consisted of 2 separate projects, each of which targeted different aspects of the overall goal.

The goals for the first study were to (i) cognitively profile a genetically-engineered mouse model of obesity, (ii) determine whether obesity affected males and females differently and (iii) determine whether obesity-induced cognitive deficits could be attributed to increased oxidative stress, inflammation and AGEs in specific areas of the brain.

The goals for the second study were to (i) compare the cognitive profile of a genetically-engineered obese mouse model to that of a diet-induced obesity mouse model, (ii) determine whether age exacerbates obesity-induced cognitive impairments,

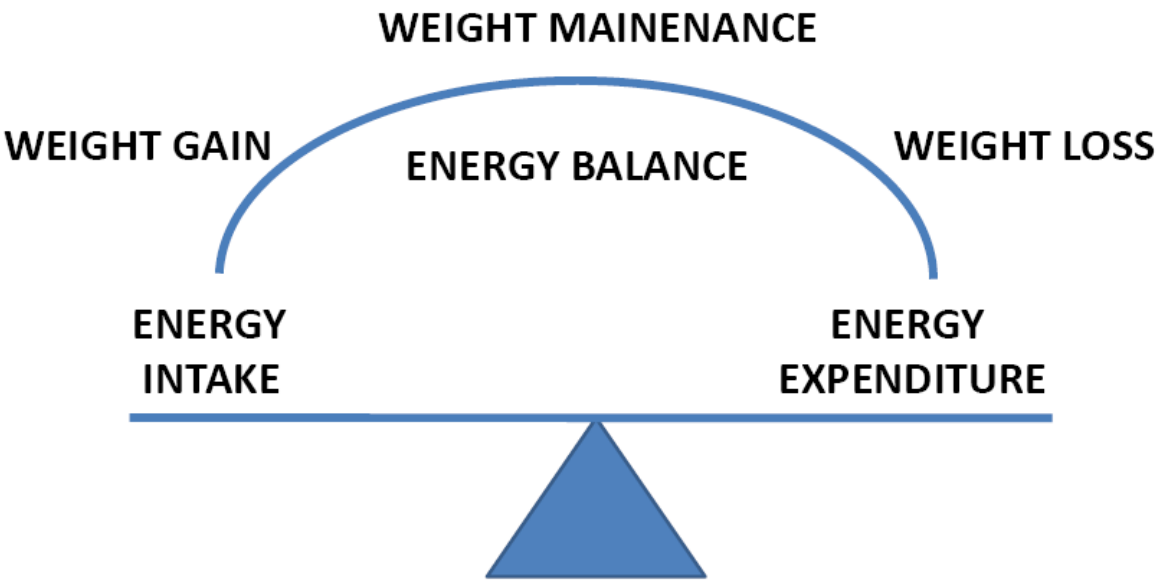
and (iii) determine if obesity-related cognitive deficits are permanent or reversible with a dietary intervention.

Table 1. BMI classification chart for adults adapted from WHO, 1995, WHO, 2000 and WHO 2004.

Classification	BMI(kg/m^2)	
	Principal cut-off points	Additional cut-off points
Underweight	**<18.50**	**<18.50**
Severe thinness	<16.00	<16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	**18.50 - 24.99**	**18.50 - 22.99**
		23.00 - 24.99
Overweight	**≥25.00**	**≥25.00**
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	**≥30.00**	**≥30.00**
Obese class I	30.00 - 34-99	30.00 - 32.49
		32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49
		37.50 - 39.99
Obese class III	≥40.00	≥40.00

Source: Adapted from WHO, 1995, WHO, 2000 and WHO 2004.

Figure 1. Energy balance (A), negative energy balance (B), and positive energy balance (C).


WEIGHT MAINENANCE
WEIGHT GAIN
ENERGY BALANCE
WEIGHT LOSS
ENERGY INTAKE
ENERGY EXPENDITURE

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CHAPTER II. COGNITIVE FUNCTION IS MODERATELY IMPAIRED IN 6-MONTH OLD MALE AND FEMALE *ob/ob* MICE

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CHAPTER II

COGNITIVE FUNCTION IS MODERATELY IMPAIRED IN 6-MONTH OLD MALE AND FEMALE *ob/ob* MICE

Summary

Obesity is a condition giving rise to a number of metabolic and cardiovascular complications, and has recently been linked to impaired cognitive function. Thus far, studies of genetically-induced obesity models have led to inconclusive data on the impact of obesity on cognitive function; furthermore the ob/ob model had not been extensively tested for motor and cognitive abilities. Accordingly, the current study assessed the effect of leptin-deficiency on motor activity and cognitive function and whether sex differences existed on functional tasks in response to leptin deficiency induced obesity. Furthermore, the study determined whether the obesity-associated impairments were linked to changes in inflammation and oxidative stress status. Six-month old male and female *ob/ob* and C57BL/6 mice were administered a behavioral test battery to assess locomotor activity and cognitive function. The *ob/ob* mice weighed and ate more, and were less active than controls, with an exacerbation of these observations in the female ob/ob group. No differences were observed in spatial learning and memory, as all mice were able to locate a hidden platform efficiently after 9 sessions. Impairments in fronto-cortical function were observed in male and female

ob/ob mice, but affected the genders differently. Males were affected with their learning, whereas females were impacted on learning and cognitive flexibility. Elevated blood levels of the pro-inflammatory cytokines, IL-6 and TNF-α, were observed in *ob/ob* mice. Carboxymethyl lysine, a marker of chemical glycation and oxidative stress, was decreased in *ob/ob* mice compared to controls, whereas no differences were found with protein damage levels. The findings from this study suggested that obesity impairs cognitive function in six-month old *ob/ob* mice in a sex-dependent manner; while exhibiting a low-grade systemic inflammation.

Introduction

Leptin, an adipose tissue-derived hormone, regulates metabolism and satiety by binding to neuronal receptors in the hypothalamus (1-5) influencing the expression of neuropeptides involved in energy regulation (proopiomelanocortin, agouti-related protein, neuropeptide Y, corticotrophin releasing hormone) (6-10), and its levels have been associated with adipocity (11-14). Leptin has also been involved in other mechanisms such as inflammation (15), reproduction (16), and lipolysis (17). Recently, leptin receptors have been identified in other brain regions such as hippocampus and cerebral cortex (18-21). Furthermore, and synaptic proteins and glial fibrillary acidic protein are decreased under leptin deficiency (22). These findings suggest a potential role of leptin on brain function, and more specifically cognition.

Epidemiological studies suggested a possible link between obesity and cognitive impairments (23-28) , that can be improved upon leptin replacement treatment (29). Decreased leptin concentration and uptake in the central nervous system has been associated with cognitive impairment in HIV infected men (Huang et al., 2007). In elderly individuals, high levels of leptin have led to contradictory reports as being associated with less cognitive decline assessed by the MMSE (30) or with impaired cognition as measured by the Trail Making Test B (31).

Altered leptin levels, observed with obesity, induce physiological changes that may contribute to cognitive dysfunction. Leptin stimulates oxidative stress in human endothelial cells (32) and mediates serum levels of inflammatory cytokines (15, 33, 34). Both oxidative stress (35, 36) and inflammatory markers (37) have been associated with

impaired cognition (38).

Rodent models such as leptin (*ob/ob*) and leptin receptor mutants (*fa/fa, db/db*) become severely obese and are commonly used to study the regulation of energy balance (39). Despite studies suggesting that disrupted leptin signaling and resultant morbid obesity impair cognitive function in leptin-receptor mutants (40-42), the extent to which obesity induced by leptin deficiency impairs cognitive function in a leptin deficient mouse model (*ob/ob* mice) has not been extensively reported. A number of studies have reported biochemical consequences to altered leptin signaling in the *ob/ob* mouse model, but their association to cognitive capacity has not been determined. The purpose of the current studies was to provide a motor, cognitive and biochemical evaluation of the leptin deficient obesity mouse model, ob/ob. Accordingly, six-month-old male and female ob/ob obese mice were tested on hippocampal and cortical dependent tasks to assess cognitive function, as well as spontaneous activity measurements. Furthermore, inflammation and oxidative stress markers were measured in serum and brain regions.

Materials and Methods

Animals. Thirty C57BL/6J and thirty B6.V-*Lep*^{ob}*/J* (ob/ob) mice (n=15/ sex/ genotype) were obtained from the Jackson Laboratory (Bar Harbor, Maine) at 3 months of age. The mice were housed in groups of 2 to 4 by sex and genotype in clear polycarbonate cages (28 x 17 x 12.5c m) in the University of North Texas Health Science Center vivarium. The mice were maintained at 23 $\pm$ 1°C, under a 12-hour light/dark cycle, with lights on at 7:00 a.m. and ad libitum access to food (Harlan Teklad

(2108) 8% Protein Rodent Maintenance Diet) and water. The mice were weighed on a weekly basis, and survival was monitored throughout the study. At 6-months of age and just prior to functional testing, food intake was measured daily for one week. Measurements of core body temperature were completed pre and post water exposure. Upon behavioral testing, the mice were euthanized and serum, cortex, hippocampus and cerebellum, and skeletal muscles were collected and saved at -80°C for biochemical assessments. All procedures involving the animals were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center.

Behavioral Measurements

At 6 months of age, male and female mice from both genotypes were subjected to as series of behavioral tests conducted in the following order: locomotor activity, spatial learning and memory, and discriminated avoidance.

Locomotor activity. Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16), as described previously (43, 44). Each mouse was placed in an acrylic chamber (40.5 x 40.5 x 30.5 cm) surrounded by a metal frame lined with photocells, inside a dimly lit, sound-attenuating chamber equipped with a fan to provide background noise. During a 16-min session, movements in the horizontal plane and vertical plane (7.6 cm above the floor) were detected by the automated photocell apparatus and by a software program to yield 14 different variables that describe horizontal, vertical, stereotypic and spatial components of the spontaneous activity in the apparatus.

Spatial learning and memory. Ability of the mice to learn and remember the location of a hidden platform was measured using a Morris water maze (MWM) as described previously (45, 46). On a given trial, each mouse was placed in a plastic tank (120 cm dia, 60 cm high) filled with opacified (nontoxic white paint) water maintained at 24 ± 1 °C. An escape was provided by means of a small 10 x 10-cm platform submerged 1.5 cm below the surface of the water. A computerized tracking system recorded the length of the path taken by the mouse to reach the platform, as well as the swimming speed (ANY-MAZE, Stoelting).

During a pre-training phase, the tank was covered by a black curtain to prevent pre-exposure of the mice to visual cues present outside the tank. On each trial, the mouse was placed at one end of a 10 x 65-cm (width x length) straight alley that had a platform at the other end, and allowed to swim until it reached the platform or a maximum latency of 60 s had elapsed. The mice received one session a day for 2 days.

After pre-training, the curtain was removed and the mice were tested for their ability to learn the location of the platform using spatial cues. Testing was divided into two phases: acquisition (nine sessions with the platform in a fixed location) and retention probe trial with a one-week delay. Each session consisted of five trials, at 90 s intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. Learning during the acquisition was determined by the average path length taken to the platform over sessions 2-4 (learning index). On the fifth trial of sessions 2, 4, 5, 7 and 9, a probe trial was given in which the platform was

submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 s, and the trial was ended when the mouse successfully located it. On these trials, spatial bias for the platform location was evaluated in terms of (i) the average percent of time spent in the platform quadrant and areas directly surrounding the platform location, and (ii) the number of entries into the platform zone.

A probe session (S10) was given 7 days after the last acquisition session (S9) and consisted of one trial lasting 60 s during which the platform was not available to the mice. Retention of acquired knowledge of platform location was expressed as percentage of time spent in areas surrounding the platform location.

Discriminated avoidance. A T-maze constructed of acrylic (black sides and clear top) and divided into 3 compartments: a start box (10 x 6.3 x 6 cm), a stem arm (17.5 x 6.3 x 6 cm) and 2 goal arms (14.5 x 6.3 x 6 cm) was utilized for discriminated avoidance task (47). The maze rested on a grid floor wired to deliver 0.69mA scrambled shock to the feet. Shock intensity (0.69mA) was determined as the intensity which provided 90% escape latency from the shock. The discriminated avoidance test consisted of 3 sessions separated by 1 h. On each training trial, the mouse was placed in the start box, and the start door was removed to signal the beginning of the trial. On the first trial of each session, the mouse received shock in the first arm entered and was permitted to escape shock by running to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5 s after the opening of the start door if the mouse had not entered the correct goal arm, or immediately upon entry into the incorrect arm. In either case, the shock continued until

the correct goal arm was entered or a maximum of 60 s had elapsed. Upon the mouse's entry into the correct arm, the door was closed and, after 10 s, the mouse was removed (by detaching the goal arm) and allowed to enter a holding cage for 1 min. Training continued at 1 min intervals until the mouse had met the criterion of a correct avoidance (running into correct arm under 5 s) on four of the last five training trials. The second and third sessions of avoidance training were reversals such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous session. Ability to learn the avoidance problem was considered inversely proportional to the number of trials required to reach criterion in each of the sessions. The latency to reach the goal on the last trial of the first session was assessed in order to determine if genotype affected the motivation provided by shock or limited the ability of the obese mice to perform the avoidance response.

Biochemical Measurements

Determination of protein carbonyls. The procedure to measure protein oxidation was modified from Levine et al. (1994) (48). Each brain region was homogenized in homgenizing buffer (10nM sodium phosphate, 0.9% sodium chloride, 200 μM DTPA and 1mM BHT, a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 0.1% Triton-X 100). Homogenates for cortex, cerebellum and hippocampus were diluted in homogenizing buffer to a protein concentration of 1 mg protein/mL. Diluted homogenate was added to 10mM dinitophenylhydrazine (DNPH) in 2N HCl (samples) or 2N HCl (blank) in a 1:5 ratio. After 1 hr incubation at room temperature in the dark, proteins were precipitated by addition of 10% (final concentration) trichloroacetic acid

(TCA). Then, samples were centrifuged at 5000 rpm for 5 min. The supernatants were discarded and the pellets were washed at least 3 times with 1mL ethanol/ethyl acetate (1:1) and then dissolved in 650 μL of denaturing buffer (100 μM of sodium phosphate buffer with 3% SDS at pH 6.8). The samples were read at 360 nm and protein concentration was determined using BCA protein detection system (Pierce, Rockford, IL). Using the extinction coefficient of DNPH (22.0 mmol/cm), total carbonylation (nmols of carbonyl/mg of protein) was calculated.

Determination of TNF-α and IL-6 concentrations. Plasma levels of IL-6 and TNF-α were measured using an enzyme immunoassay kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Absorbance of standards and samples was read at 450 nm. Cytokine concentration was determined by comparing absorbance of unknown samples to the standard curve prepared from the standards.

Determination of Nε-carboxymethyllysine *(CML) concentration*. CML concentration was determined using a kit from Cell BioLabs according to manufacturer's instructions (OxiSelect, Cell BioLabs, Inc., San Diego, CA). Absorbance of standards and samples was read at 450 nm. CML concentration was determined by comparing the absorbance of the sample to the standard curve prepared by the standard solutions.

Statistical analysis of data. The effects of Gender and Genotype on the behavioral tests and biochemical measurements were assessed using a two-way analyses of variance (ANOVA) with Sex and Genotype as between-group factors. Planned individual comparisons for each sex groups (male vs. female) and genotype groups (ob/ob vs. control) were performed using single degree of freedom F tests

involving the error term from the overall ANOVA. Weight, temperature, swim maze, discriminated avoidance, CML and protein carbonyls data were subjected to three-way ANOVAs, with either month, week, session, or region as the repeated measures. The alpha level for was set at 0.05 for all analyses.

Results

Body weight, food intake and body temperature

There was no change in body weight in the male or female control mice throughout the duration of the study; however the obese *ob/ob* male and female mice gained 13.3 and 16.9 g, respectively (Figure 1). Obese *ob/ob* mice weighed excessively more than the control ones, and sex affected the weights differentially in both genotypes. In the control group, the males were heavier than females, whereas in the *ob/ob* group, the females weighed more than the males. A two-way ANOVA with repeated measures on Week yielded significant main effects of Sex, Genotype and interactions (All *p*s<0.03). Obese *ob/ob* mice consumed 74% more food that the control ones regardless of sex (data not shown, $p<0.001$).

Basal body temperature was approximately 1°C higher in the control female mice than in any other groups (Figure 2). Following water exposure, the control mice had the same body temperature as before water exposure, whereas the temperature of the obese ob/ob mice decreased by about 5%. An Anova revealed a significant main effect of Genotype on body temperature post water exposure.

Locomotor Activity

All aspects of horizontal, vertical and spatial activity were considered in the analyses of spontaneous locomotor activity (Figure 3). Overall, *ob/ob* male and female mice were less active than control and male mice, with an exacerbation of the inactivity in the obese ob/ob female mice. A two-way ANOVA revealed significant main effects of Sex and Genotype for most measures of locomotor activity (and interaction (all *ps* < 0.005)

Water Maze

The length of the path taken to reach the hidden platform was determined to assess the efficiency with which the mice located the platform, independently of their speed of swimming (Figure 4). All groups learned to locate the platform efficiently by the last session of the water maze task. Analysis of the data confirmed the effect of testing session on path length (p<0.001) but also a significant interaction of Session * Genotype (p=0.007). The interaction was driven by the performance of the control groups on session 1. Accordingly, data from session 1 were analyzed separately (data not shown) and indicated that the control mice had longer initial path length supported by a main effect of genotype (p<0.001), but no interaction between genotype and trial (p>0.108). Regardless of genotype, female mice navigated a longer path length than their male counterparts from sessions 2 through 9 (p<0.05).

Swimming speed remained relatively steady throughout sessions, except for the male control mice (Figure 4). Obese mice swam significantly slower than control mice (p<0.001). While there was no difference in swimming speed between male and female in the obese *ob/ob* group, the control male group swam slower than the control

male group starting on session 4, supported by a significant interaction of Sex and Genotype (p <0.001).

Accuracy of spatial memory was measured by conducting a probe trial as the last trial for sessions 2, 4, 5, 7 and 9 (Figure 5) and expressed as the average percent of time spent in the platform location and in the area surrounding the platform (Probe index). Neither sex nor genotype affected the probe trial performance of the mice (p>0.548). However, the strength of the spatial bias for the platform was not as strong in the obese *ob/ob* mice, male and female, when compared to the control mice. Repeated measure ANOVA indicated only a significant main effect of Genotype for the number of target entries, but not for the probe index measure.

Discriminated Avoidance

Learning of a preemptive response (measured as number of training trials needed to reach a criterion for choosing the correct goal arm within 5 sec) is shown in Figure 6. The obese *ob/ob* mice took more trials to reach the establish criterion compared control mice, however the effect of genotype was dependent on the session of the test. These observations were supported by a main effect of Genotype and an interaction between Session, Genotype and Sex (all ps<0.046). For males, the effect of genotype was apparent in session 1 (acquisition) whereas for females the effect was seen for sessions but reached significance only on session 2 (reversal).

Inflammation measures

Peripheral inflammation was determined by measuring the concentration of TNF-α (Figure 8A) and IL-6 (Figure 8B) in serum samples. TNF-α concentration was higher

in the obese *ob/ob* male mice than in any other groups, which resulted in main effects of Genotype and Sex (all *p*s<0.04). Serum IL-6 concentration was increased in the obese *ob/ob* male mice compared to all other groups, yielding a significant main effect of Genotype ($p = 0.022$).

Glycation measure

Chemical glycation was determined by measuring carboxymethyllysine (CML) concentrations in the following tissues: cortex, hippocampus, cerebellum and skeletal muscle (Figure 9). Skeletal muscle had a higher concentration of CML than any of the brain regions, supported by a main effect of tissue ($p<0.001$). Concentrations of CML were decreased in the cortex and skeletal muscles of the obese *ob/ob* mice, yet reaching significance only in skeletal muscles ($p<0.001$; $p=0.062$ for cortex).

Protein oxidative damage

Protein oxidative damage was measured as concentration as carbonyls concentration in three brain regions: cortex, hippocampus and cerebellum (Table 1). Regional differences were observed in protein carbonyl concentrations with the hippocampus having the highest amount compared to other brain regions ($p < 0.001$). ANOVA indicated no significant effect of Sex or Genotype for protein carbonyl concentration within brain regions ($p>0.486$).

Discussion

The main findings of this study were that six-month-old hyperphagic obese mice have (i) impaired thermogenesis, (ii) decreased motor abilities, and (iii) impaired cortical

but not hippocampal function. Furthermore, the female ob/ob mice seemed more susceptible to the effect of leptin deficiency.

Hyperphagy and decreased spontaneous activity contributed to a significant increase in weight of the *ob/ob* obese mice compared to the controls, observations supported by previous studies. Hyperphagy has previously been described in ob/ob mice, male and female (49, 50). Daily physical activity level has been suggested to contribute to the development of obesity in humans and animals (51-53). The decrease in locomotor activity has been observed in previous studies in *ob/ob* mice of similar ages as the current study (52, 53). A sex comparison had not previously done and the current study reveals a higher weight increase in the female *ob/ob* mice compared to the males, and because food intake was identical, the discrepancy was attributed to a larger decrease in spontaneous activity.

Colonic temperature was measured following water exposure and resulted in an impaired thermogenesis in the *ob/ob* mice. Brown adipose tissue has a role in heat production and metabolic efficiency (54), and contains abnormalities in *ob/ob* mice (55-58). Zhang, et al. (2010) reported that *ob/ob* mice have different expression patterns of uncoupling proteins (UPCs), involved in energy balance. Decreased UPC 1 mRNA, exclusively found in brown adipose tissue, was observed in *ob/ob* mice and contributed to the temperature difference in *ob/ob* mice following cold water exposure (59). The colonic temperature difference in male and female *ob/ob* mice post water exposure was 1.54 ºC and 2.82 ºC, respectively. Consistent with these results, previous reports

suggested that impaired non-shivering thermogenesis accounts for a 1.5-2.5 ºC difference between adult lean and *ob/ob* mice (55, 60).

In the Morris water maze test, the *ob/ob* mice took similar path lengths than the controls indicating a lack of impairment caused by leptin-decifiency-induced obesity. However, the *ob/ob* mice did not develop as strong a spatial bias for the platform location as the controls, indicated by less target entries under probe testing. Notwithstanding, the *ob/ob* mice swam significantly slower than the controls, which could influence the number of target entries. Nevertheless, overall the mice did spend less time in the area of the platform location. Previous studies have indicated spatial memory deficits in Zucker fatty rats and db/db mice, both leptin receptor mutant models of obesity (40). In this study, the Zucker rats took longer path length and made less target entries than the controls; however the effect in the *db/db* mice on path length is minor as only one session was affected reflecting a decrease in maximal potential rather than an actual learning deficit. Nonetheless, the *db/db* mice exhibited less spatial bias for the platform similar to the *ob/ob* mice in the current study. Utilizing an appetitive spatial reference learning task (Y-maze), Finger et al. (2009) reported no differences in learning under stress in 12-week--old *ob/ob* mice (61) Furthermore, leptin administration did not improve spatial learning in Wistar rats (62). Altogether, these studies do not support a major effect of obesity in mouse models on spatial learning and memory.

The major difference between the studies utilizing Morris water maze is the swimming speed of the rodents. In the study by Li et al (2002), there was no effect of

genotype on swimming speed, whereas the *ob/ob* mice in the current study had speeds almost half of their controls. The mice were tested at different ages (2 vs. 6 months) reflecting major disparities in weights when compared to the controls (20g for controls vs. 40g for *db/db* mice and 70g *for ob/ob* mice) (63). The discrepancy in weights compared to controls was greatly increased in our study and could explain the differences in swimming speed.

In addition to spatial learning and memory, cognitive function of the mice was assessed by their ability to learn an active discriminative avoidance response and subsequently perform a reversal. The inability to perform a reversal of a task that has been previously well trained reflects cognitive inflexibility indicative of frontal cortical dysfunction (64-66). The performance of the male *ob/ob* mice was impaired during the learning session of the active avoidance task, whereas the female were mostly affected during the reversal though they had lower performance than the controls on all sessions. These results suggested a sex specific susceptibility to obesity impairing only learning in males but affecting learning and cognitive flexibility in females.

Biochemical alterations, including increased levels of pro-inflammatory cytokines (67, 68) oxidative damage (69, 70) and chemical glycation (71) have been associated with obesity in humans and rodents. Pro-inflammatory cytokines, like IL-6 and TNF-α, are commonly elevated in obese individuals and contribute to the low-grade inflammatory state commonly associated with obesity (67, 71). In the current study, the o*b/ob* mice had elevated serum levels of IL-6 and TNF- α compared to controls. Consistent with our results, Dube et al. (2008) found that *ob/ob* mice had increased

blood levels of IL-6 (39). In other tissues, like white adipose tissue, increased levels of TNF- α were observed in *ob/ob* mice (68).

Protein oxidative damage was not affected by obesity in the three brain regions tested in this study, cortex, hippocampus and cerebellum. To date, no other studies have determined the oxidative stress status in the brain of the ob/ob mice, however others have reported increased oxidative stress measured by oxidized to reduced glutathione ratio and/or protein damage in heart homogenates (72), cardiac cells (73) and thiobarbituric acid reactive substances were higher in serum and gastrocnemius (74) from *ob/ob* mice. Further studies are needed to examine the oxidative stress status of the brains of *ob/ob* mice, more specifically.

Nε-carboxymethyllysine (CML), a marker of chemical glycation and oxidative stress, is associated with characteristics of obesity and diabetes like hyperinsulinemia and hyperglycemia (75). Surprisingly, in the current study, CML levels did not differ between *ob/ob* mice and controls in any of the brain regions tested, and were actually decreased in skeletal muscle from *ob/ob* mice. The decrease in CML concentration in the skeletal muscle from the *ob/ob* mice could be attributed to morphological and biochemical differences in skeletal muscle observed in this mouse strain (76). Mean skeletal muscle mass, total protein content and muscle fiber size were consistently less in 18-22 week old *ob/ob* mice compared to their controls. These differences can be attributed partly to the increased intramuscular fat content in *ob/ob* mice (76). A similar trend was observed in serum levels of CML in obese children/adolescents (77). It can be speculated that obese children/adolescents have decreased muscle mass and thus

the effect on chemical glycation in these individuals may reflect a similar phenomenon observed in obese rodents.

In conclusion, leptin deficiency induced obesity leads to mild fronto-cortical impairments in young adult male and female *ob/ob* obese mice. This study is in accordance with the scarce literature on obese mouse models. Furthermore, female *ob/ob* mice exhibit exacerbated response to their condition compared to males. This discrepancy is certainly due to the weight gain and brown fat accumulation higher in female *ob/ob* mice. Finally, the *ob/ob* mice exhibited a low-grade systemic inflammation in accordance with other studies, but there was no reflection of increased oxidative stress in the brains of these mice. Further studies are required to determine the basis of the sex difference observed in the active avoidance task. Furthermore, age-related studies would determine whether the symptoms observed at 6-months continue to develop and age-associated brain dysfunction is accelerated in these mice. However, lifespan and overall health may hinder the possibility of such studies.

Figure 1. Body weight of male and female control and *ob/ob* mice as a function of time. Values reflect mean ± SE of 15 mice.

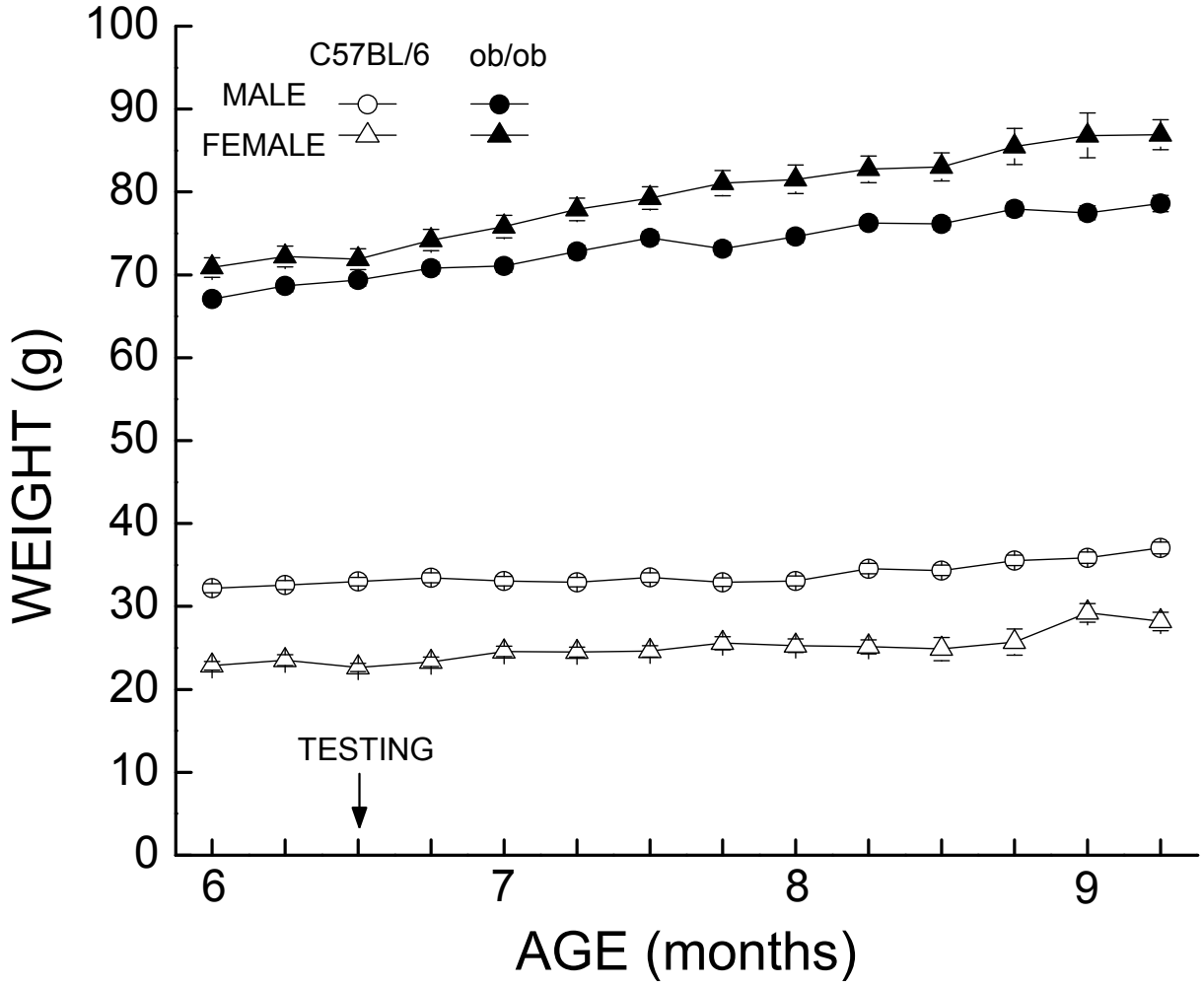
C57BL/6
ob/ob
MALE
FEMALE
WEIGHT (g)
AGE (months)
TESTING
0
10
20
30
40
50
60
70
80
90
100
6
7
8
9

Figure 2. Body temperature of 6-month- old male and female control and o*b/ob* mice pre and post water exposure. Values reflect mean ± SE of 15 mice. * denotes $p<0.05$ vs. sex-matched controls; # denotes $p<0.05$ vs. genotype-matched controls

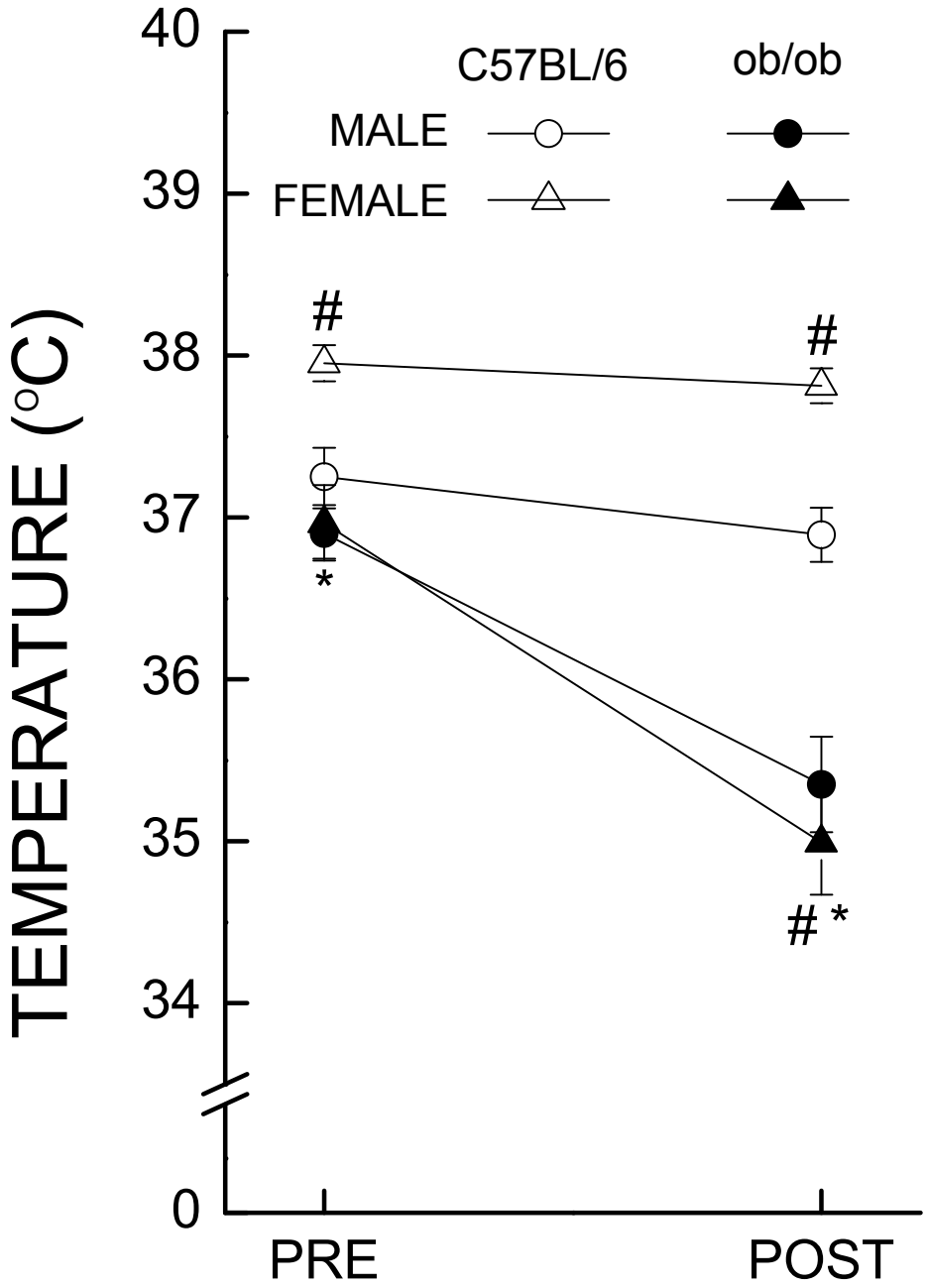
TEMPERATURE (°C)
40
39
38
37
36
35
34
0
C57BL/6
ob/ob
MALE
FEMALE
#
#
*
#*
PRE
POST

Figure 3. Horizontal, vertical, stereotypic, and spatial aspects of spontaneous activity in male and female *ob/ob* mice. Each value represents the mean ± SE of 15 mice expressed in units of standard deviation from the mean for the same measure of sex-matched controls. The components of locomotor activity were, HA, horizontal activity; HD, total distance traveled; HM, number of horizontal movements; AD, average distance per movement; HT, time making horizontal movements; AS, average speed of movement; RT, rest time; VA, vertical activity; AM, number of vertical movements; VT, time in vertical plane 7.6 cm above the floor; SC stereotypy counts; NS, average number of stereotypy; ST, time making stereotypic movements; MT, margin time. * denotes $p<0.05$ vs. sex-matched controls

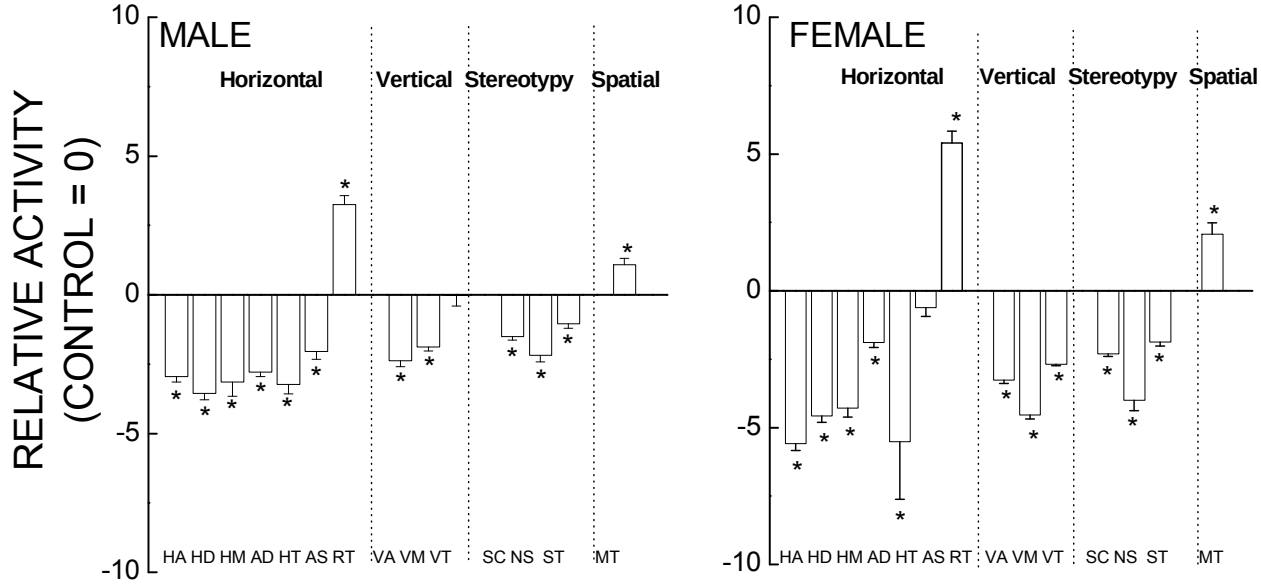
MALE
FEMALE
RELATIVE ACTIVITY
(CONTROL = 0)
10
5
0
-5
-10
Horizontal
Vertical
Stereotypy
Spatial
HA HD HM AD HT AS RT
VA VM VT
SC NS ST
MT

Figure 4. Morris water maze performance represented by path length (A) and swim speed (B) in 6-month- old control and *ob/ob* male and female mice. Values reflect mean ± SE of 15 mice.

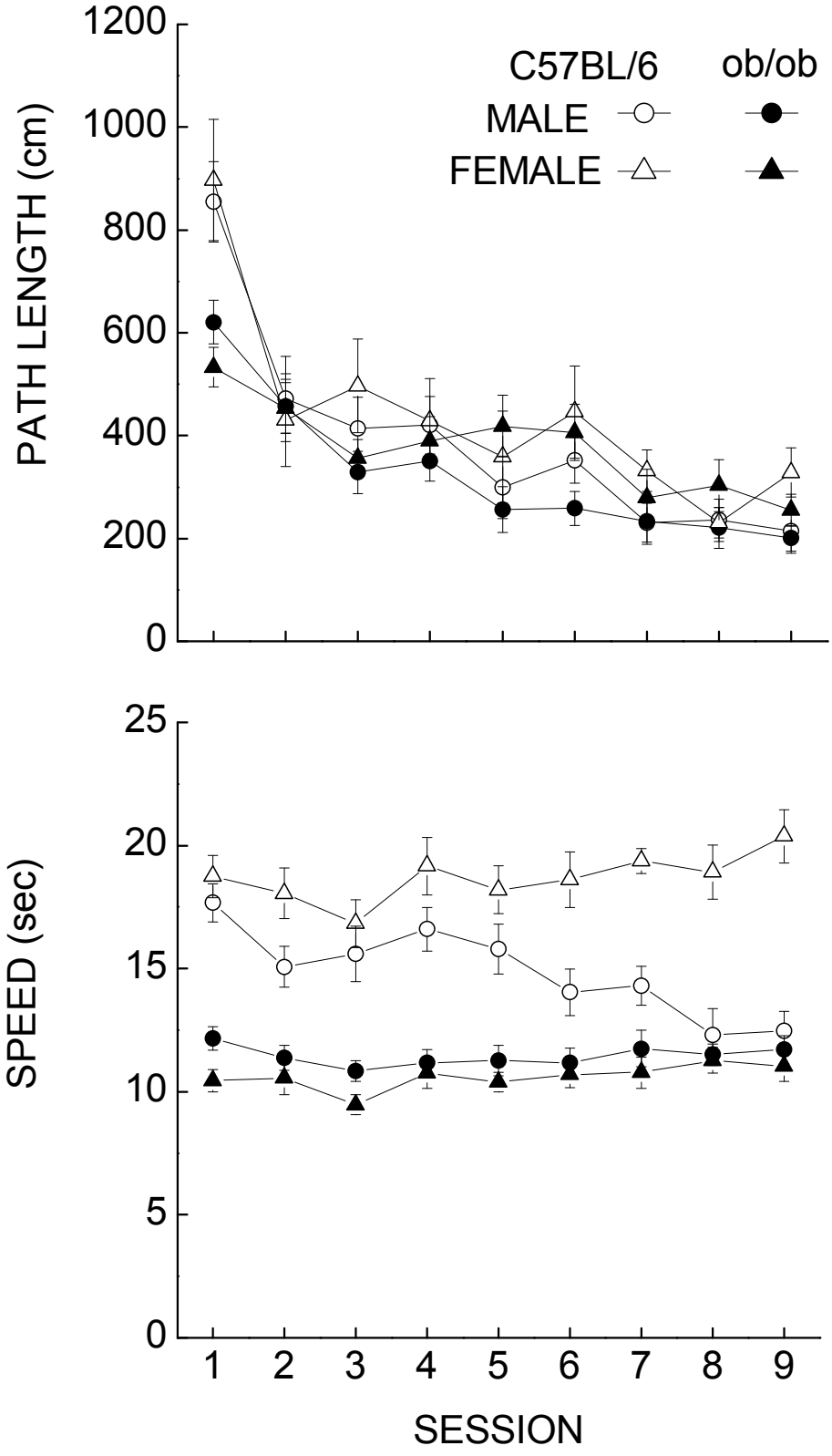
PATH LENGTH (cm)
1200
1000
800
600
400
200
0
C57BL/6
ob/ob
MALE
FEMALE
SPEED (sec)
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
SESSION

Figure 5. Morris water maze performance represented by percent of time spent in the areas directly surrounding the platform and number of entries over the platform location in 6-month- old control and *ob/ob* male and female mice. Values reflect mean ± SE of 15 mice. * denotes $p<0.05$ vs. sex-matched controls

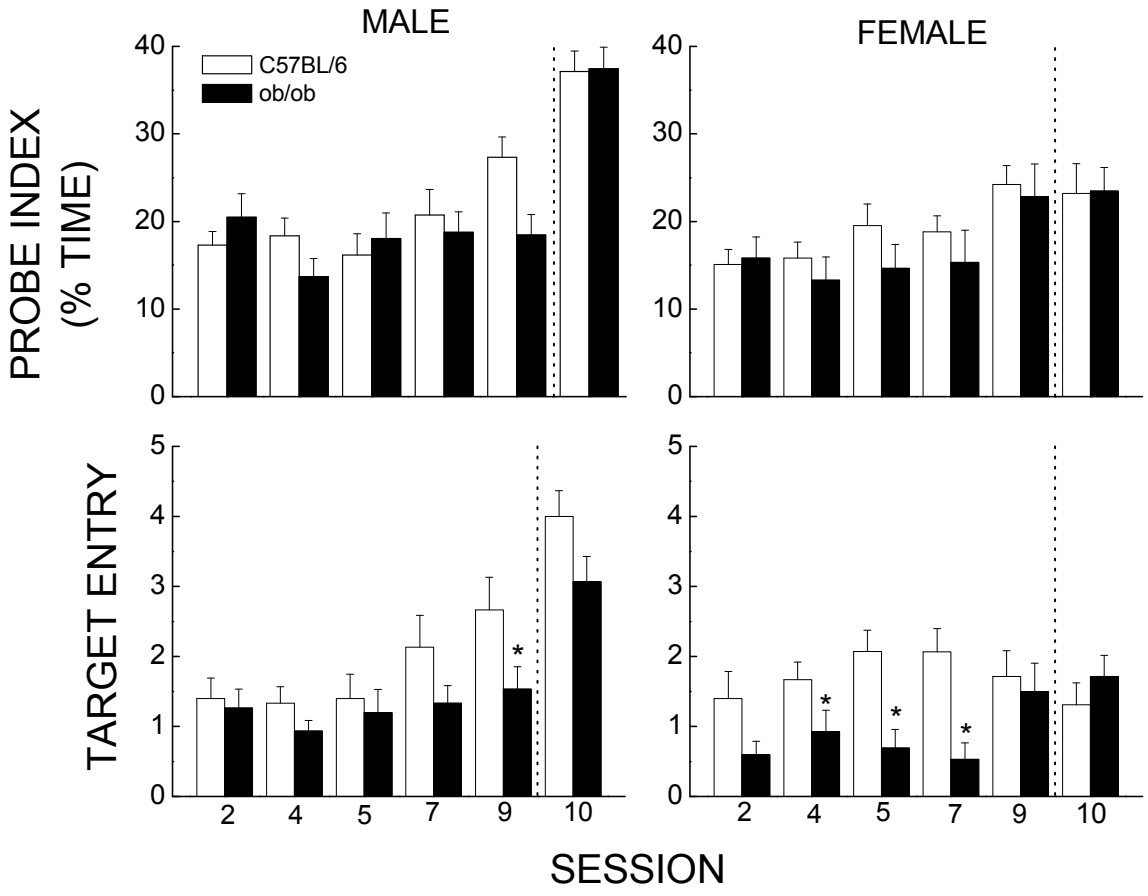
MALE
FEMALE
C57BL/6
ob/ob
PROBE INDEX
(% TIME)
TARGET ENTRY
SESSION
0
10
20
30
40
1
2
3
4
5
2
4
5
7
9
10
*

Figure 6. Discriminated avoidance performance expressed by the number of trials to reach criterion in 6-month- old male and female control and *ob/ob* mice. Values reflect mean ± SE of 11-15 mice. * denotes $p<0.05$ vs. sex-matched controls; # denotes $p<0.05$ vs. genotype-matched controls

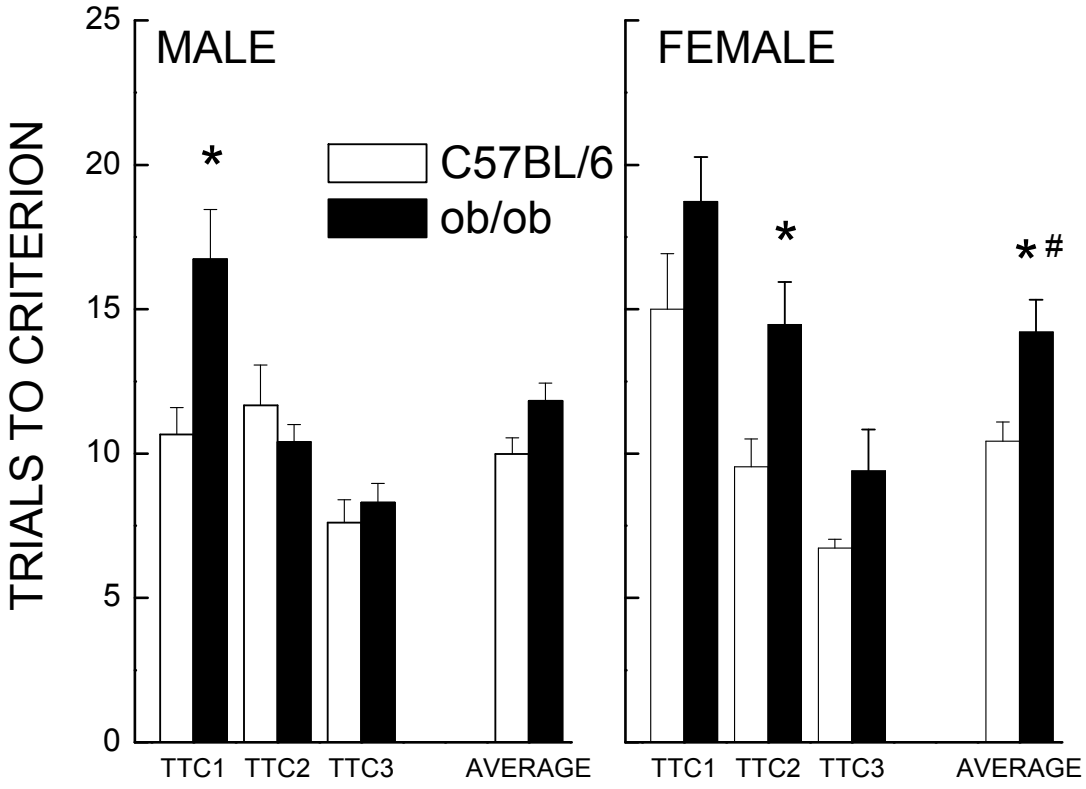
TRIALS TO CRITERION
25
20
15
10
5
0
MALE
FEMALE
C57BL/6
ob/ob
*
*
*#
TTC1
TTC2
TTC3
AVERAGE
TTC1
TTC2
TTC3
AVERAGE

Figure 7. Circulating pro-inflammatory cytokine concentration in 6-month- old male and female control and ob/ob mice. Values reflect mean ± SE of 3-5 mice. * denotes $p<0.05$ vs. sex-matched controls; # denotes $p<0.05$ vs. genotype-matched controls

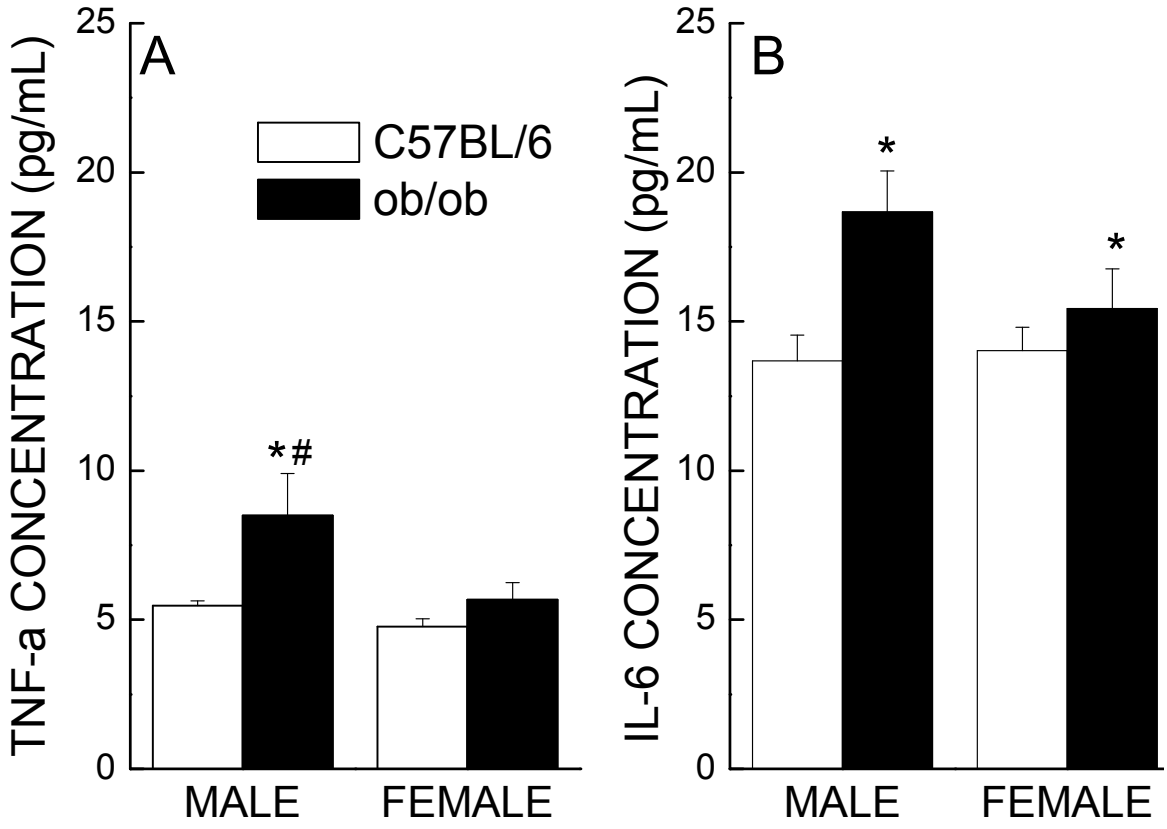
A
TNF-a CONCENTRATION (pg/mL)
25
20
15
10
5
0
C57BL/6
ob/ob
*#
MALE
FEMALE
B
IL-6 CONCENTRATION (pg/mL)
25
20
15
10
5
0
*
*
MALE
FEMALE

Figure 8. Carboxymethyllysine concentrations indifferent tissues from 6-month- old male and female control and ob/ob mice. Values reflect mean ± SE of 6-8 mice. * denotes $p<0.05$ vs. sex-matched controls

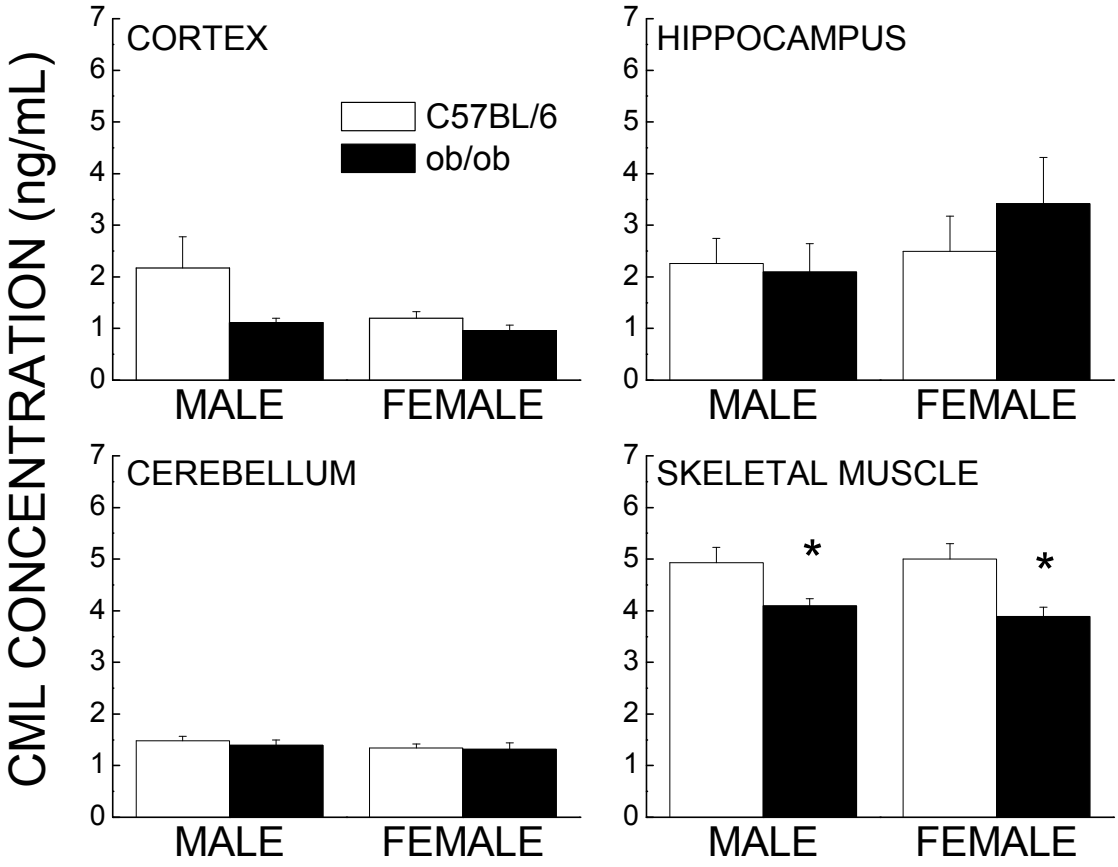
CML CONCENTRATION (ng/mL)
CORTEX
C57BL/6
ob/ob
HIPPOCAMPUS
CEREBELLUM
SKELETAL MUSCLE
*
*
MALE
FEMALE
0
1
2
3
4
5
6
7

Table 1. Protein carbonyl concentration in different brain regions from 6 month- old male and female control and *ob/ob* mice. Values reflect mean ± SE of 5-7 mice.

Table 1 Oxidative stress measures: Group means and Standard errors for protein carbonylation in different brain regions

	Male		Female	
Oxidative stress measures	C57BL/6	*ob/ob*	C57BL/6	*ob/ob*
Carbonylation (nmol CO/mg protein)				
Cortex	2.6 ± 0.2	2.1 ± 0.3	2.2 ± 0.2	2.2 ± 0.2
Hippocampus	3.1 ± 0.5#	2.8 ± 0.2#	2.7 ±0.4#	3.2 ± 0.3#
Cerebellum	2.6 ± 0.6	2.3 ± 1.5	2.2 ± 0.3	2.4 ± 0.1

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TRANSITION REMARKS

In Chapter II, the leptin-deficient mice (*ob/ob*) were used as a genetic model to test the effect of obesity on motor and cognitive function in 6-month old males and females. The data suggested that obesity, caused by leptin deficiency, affects spontaneous activity and moderately impairs cognitive function. Furthermore, the study indicated that the degree of impairment was sex dependent.

Obesity in our society is often caused by high energy intake not associated with appropriate energy expenditure. Therefore, Chapter III deals with an environmental model of obesity: diet-induce obesity, via a high fat diet implementation. The proposed study will determine if diet induce obesity leads to the same effect as the genetic model of obesity. Furthermore, this study introduces an aging factor to determine whether obesity will accelerate the effects of aging on cognitive function. Lastly, a dietary intervention, switch from a high fat to a low fat diet, will determine whether the obesity induced impairments are reversible.

CHAPTER III. DIET-INDUCED OBESITY DOES NOT IMPAIR COGNITIVE FUNCTION IN 6- OR 12-MONTH OLD MALE MICE

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Running title: The association between obesity, cognition and visual function

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CHAPTER III

DIET-INDUCED OBESITY DOES NOT IMPAIR COGNITIVE FUNCTION IN 6- OR 12- MONTH OLD MALE MICE.

Summary

Obesity is an underlying risk factor for cardiovascular disease and metabolic disorders, but also has recently been associated with impaired cognitive function. Genetic models are available, yet they do not reflect the major cause of obesity which is dietary fat intake. The objectives of this study were: (i) to determine the effect of a high fat diet on cognition in young and adult mice, (ii) to determine whether or not a low-fat intervention at mid-life would reverse observed impairments, (iii) to determine whether oxidative stress or peripheral inflammation contributed to obesity-related impairments. Six- and 12-month old male DIO mice were administered a behavioral test battery to assess locomotor activity and cognitive function. DIO mice weighed more, but consumed the same amount of food as control mice. Neither age nor diet affected the spontaneous activity of the mice or their performance in spatial learning and memory and on an active avoidance task. Mice on the high fat diet exhibited impaired thermogenesis and increase anxiety. Both effects were reversed with the low-fat diet intervention.Elevated blood levels of pro-inflammatory cytokines were observed in the

12-mo old mice, but not in the DIO mice. Chemical glycation was increased with age in the control mice; however it was decreased in the hippocampus and skeletal muscle of DIO mice. No differences in carbonyl content were found with age or diet. The findings from this study suggest that 6- and 12-mo old DIO mice are not cognitively impaired, yet low-grade inflammation is present. The lack of cognitive impairment is in accordance with the lack of biochemical changes.

Introduction

The consumption of high fat diets is a major contributor to the development of obesity and directly influences body composition [3, 46, 71, 72]. Epidemiological studies have demonstrated impaired learning and memory in middle-age and older obese individuals [15]. Obesity during adolescence and midlife increases by approximately 3 fold the risk for developing dementia and Alzheimer's disease later in life [10, 77-79]. Regardless of weight, the risk for dementia increases exponentially with age [52, 74]. Thus, it can be speculated that with age, obese individuals have an even greater risk than normal weight individuals to develop dementia.

While genetic variants (*ob/ob, db/db and fa/fa*) are frequently used for obesity studies, and have been associated with minor cognitive impairments and impaired LTP [37], they do not reflect the common cause of obesity in humans which is the consumption of high fat diets [27]. Mice fed high-fat diets develop conditions commonly associated with human obesity including weight gain, hypercholesterolemia, hyperglycemia and hyperinsulinemia [30, 84]. The resulting literature from studies of high fat diets and cognition reveals inconsistent conclusions due to variables such as sex, cognitive task, fat composition and length of diet consumption [30-32, 82]. Numerous studies on rats have reported a negative outcome of high fat intake on learning and memory [22, 24, 41, 80, 81], and a few have concluded no effect on similar tasks [17, 31, 32]. Studies in mice reflected a disparate outcome based on sex, with the males performing worse than the controls on a contextual fear conditioning task and step-down passive

avoidance task, while the females were unaffected [30]. In other studies, different cognitive tasks were used, novel object recognition or Morris water maze, and rats on the high fat diet were not impaired [31, 32].

Obesity is associated with low-grade systemic inflammation [59] and oxidative stress [33]. Obesity-induced increases in pro-inflammatory cytokines [12, 39] and markers of oxidative stress [4, 43, 45, 57] have been linked to insulin resistance [5, 29, 48, 73] and impaired cognition [16, 18, 23, 43, 86].

The purpose of the current study was to functionally assess a diet-induced obesity mouse model (DIO), and more specifically determine whether or not different aspects of cognition were affected. Furthermore, the interaction of obesity and aging will be determined by testing mice at different ages, as obesity may impair cognitive development and accelerate age-related cognitive dysfunction. Finally, the study will address whether a switch to a low fat diet in the young will reverse the obesity-induced impairments. Accordingly, six and twelve-month-old mice raised either on low or high fat diets, or switched from a high to a low fat diet will be tested for motor, emotional and cognitive status. Furthermore, inflammation and oxidative stress markers will be measured in serum and brain regions to determine their association with obesity-induced brain dysfunction.

Methods

Animals. A total of 75 6-month old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). All mice had been placed on specific diets since 6 weeks of age and remained on their respective diets throughout the experiment: 10%

kcal fat control diet (CAT # D12450B, control) or 60% kcal fat diet (CAT# D12492, DIO). A total of 45 mice were aged to 12 months in the UNTHSC vivarium prior to behavioral testing. A subset from the aging DIO group was gradually switched from the 60% fat diet to the control diet, and remained on the control diet for the duration of the experiment.

All mice were single-housed in clear polycarbonate cages (7.5" x 11.5" x 5") separated by a metal divider in the University of North Texas Health Science Center vivarium. The mice were maintained at 23 ± 1°C, under a 12-hour light/dark cycle, with lights on at 7:00 a.m. with ad libitum access to food and water. Mice were weighed weekly and food intake was measured monthly throughout the study. At 6- or 12-months of age, the mice underwent a battery of behavioral tests for motor and cognitive functions. Core body temperature was assessed prior to and following water entry during 2 phases of the water maze task. Following completion of the behavioral tests, the mice were euthanized and their brains harvested and dissected into 6 regions: cerebellum, cortex, hippocampus, striatum midbrain and hindbrain. The tissues were stored at -80°C until further processing. All procedures involving the animals were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center.

Behavioral Measurements

At 6 or 12 months of age, male DIO and control mice were subjected to as series of behavioral tests conducted in the following order: locomotor activity, spatial learning and memory, discriminated avoidance, and elevated plus maze.

Locomotor activity. Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16), as described previously [19, 47]. Each mouse was placed in an acrylic chamber (40.5 x 40.5 x 30.5 cm) surrounded by a metal frame lined with photocells, inside a dimly lit, sound-attenuating chamber equipped with a fan to provide background noise. During a 16-min session, movements in the horizontal plane and vertical plane (7.6 cm above the floor) were detected by the automated photocell apparatus and by a software program to yield 14 different variables that describe horizontal, vertical, stereotypic and spatial components of the spontaneous activity in the apparatus.

Spatial learning and memory. Ability of the mice to learn and remember the location of a hidden platform was measured using a Morris water maze (MWM) as described previously [42, 66]. On a given trial, each mouse was placed in a plastic tank (120 cm dia, 60 cm high) filled with opacified (nontoxic white paint) water maintained at 24 $\pm$ 1 °C. An escape was provided by means of a small 10 x 10-cm platform submerged 1.5 cm below the surface of the water. A computerized tracking system recorded the length of the path taken by the mouse to reach the platform, as well as the swimming speed (ANY-MAZE, Stoelting).

During a pre-training phase, the tank was covered by a black curtain to prevent pre-exposure of the mice to visual cues present outside the tank. On each trial, the mouse was placed at one end of a 10 x 65-cm (width x length) straight alley that had a platform at the other end, and allowed to swim until it reached the platform or a maximum latency of 60 s had elapsed. The mice received one session a day for 2

days.

After pre-training, the curtain was removed and the mice were tested for their ability to learn the location of the platform using spatial cues. Testing was divided into two phases: acquisition (nine sessions with the platform in a fixed location) and retention probe trial with a one-week delay. Each session consisted of five trials, at 90 s intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. Learning during the acquisition was determined by the average path length taken to the platform over sessions 2-4 (learning index). On the fifth trial of sessions 2, 4, 5, 7 and 9, a probe trial was given in which the platform was submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 s, and the trial was ended when the mouse successfully located it. On these trials, spatial bias for the platform location was evaluated in terms of (i) the average percent of time spent in the platform quadrant and areas directly surrounding the platform location, and (ii) the number of entries into the platform zone.

A probe session (10) was given 7 days after session 9 and consisted of one trial lasting 60 s during which the platform was not available to the mice. Retention of acquired knowledge of platform location was expressed as percentage of time spent in areas surrounding the platform location.

Discriminated avoidance. A T-maze constructed of acrylic (black sides and clear top) and divided into 3 compartments: a start box (10 x 6.3 x 6 cm), a stem arm (17.5 x 6.3 x 6 cm) and 2 goal arms (14.5 x 6.3 x 6 cm) was utilized for discriminated avoidance task [20]. The maze rested on a grid floor wired to deliver 0.69mA scrambled shock to

the feet. Shock intensity (0.69mA) was determined as the intensity which provided 90% escape latency from the shock. The discriminated avoidance test consisted of 3 sessions separated by 1 h. On each training trial, the mouse was placed in the start box, and the start door was removed to signal the beginning of the trial. On the first trial of each session, the mouse received shock in the first arm entered and was permitted to escape shock by running to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5 s after the opening of the start door if the mouse had not entered the correct goal arm, or immediately upon entry into the incorrect arm. In either case, the shock continued until the correct goal arm was entered or a maximum of 60 s had elapsed. Upon the mouse's entry into the correct arm, the door was closed and, after 10 s, the mouse was removed (by detaching the goal arm) and allowed to enter a holding cage for 1 min. Training continued at 1 min intervals until the mouse had met the criterion of a correct avoidance (running into correct arm under 5 s) on four of the last five training trials. The second and third sessions of avoidance training were reversals such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous session. Ability to learn the avoidance problem was considered inversely proportional to the number of trials required to reach criterion in each of the sessions. The latency to reach the goal on the last trial of the first session was assessed in order to determine if genotype affected the motivation provided by shock or limited the ability of the obese mice to perform the avoidance response.

Elevated Plus Maze. The elevated plus maze is used as a standard animal test of anxiety as described previously [49]. The elevated plus maze is constructed of plexiglass and is elevated three feet above the floor in a dimly illuminated test room. It consists of two arms that are open to the room and two that are closed such that the floor is not visible, with the same type of arms facing each other. A computerized tracking system (ANY-MAZE, Stoelting) was used to record the position of the mice. Shifts in preference for the closed versus open arms under different lighting conditions is used to detect anxiolytic and anxiogenic effects. The mice were tested under reduced lighting. All mice acclimated to the darkened room for 5 min prior to testing. Using the tracking system, the number of entries in open or closed arms as well as the time spent in each arm was recorded during a 5 min observation period. An anxiolytic response is defined as an increase in time spent in the open arms of the maze.

Biochemical Measurements

Determination of protein carbonyls. The procedure to measure protein oxidation was modified from Levine et al. (1994) [35]. Each brain region was homogenized in homgenizing buffer (10nM sodium phosphate, 0.9% sodium chloride, 200 μM DTPA and 1mM BHT, a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 0.1% Triton-X 100). Homogenates for cortex, cerebellum and hippocampus were diluted in homogenizing buffer to a protein concentration of 1 mg protein/mL. Diluted homogenate was added to 10mM dinitophenylhydrazine (DNPH) in 2N HCl (samples) or 2N HCl (blank) in a 1:5 ratio. After 1 hr incubation at room temperature in the dark, proteins were precipitated by addition of 10% (final concentration) trichloroacetic acid

(TCA). Then, samples were centrifuged at 5000 rpm for 5 min. The supernatants were discarded and the pellets were washed at least 3 times with 1mL ethanol/ethyl acetate (1:1) and then dissolved in 650 μL of denaturing buffer (100 μM of sodium phosphate buffer with 3% SDS at pH 6.8). The samples were read at 360 nm and protein concentration was determined using BCA protein detection system (Pierce, Rockford, IL). Using the extinction coefficient of DNPH (22.0 mmol/cm), total carbonylation (nmols of carbonyl/mg of protein) was calculated.

Determination of TNF-α and IL-6concentrations. Plasma levels of IL-6 and TNF-α were measured using an enzyme immunoassay kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Absorbance of standards and samples was read at 450 nm. Cytokine concentration was determined by comparing absorbance of unknown samples to the standard curve prepared from the standards.

Determination of Nε-carboxymethyllysine *(CML) concentration*. CML concentration was determined using a kit from Cell BioLabs according to manufacturer's instructions (OxiSelect, Cell BioLabs, Inc., San Diego, CA). Absorbance of standards and samples was read at 450 nm. CML concentration was determined by comparing the absorbance of the sample to the standard curve prepared by the standard solutions.

Statistical analysis of data. The effects of Age and Diet on performance on the behavioral tests were assessed using two-way analyses of variance (ANOVA) with Age and Diet as between-groups factors. The effect of the high fat diet was considered in a balanced ANOVA that did not include data from mice on the intervention (as this treatment was not administered to young mice in this study). Planned individual

comparisons between different age groups (6 mo vs 12 mo) and diet groups (10% vs. 60% fat diet) were performed using single degree of freedom F tests involving the error term from the overall ANOVA. For the weight, temperature, water maze, and discriminated avoidance data, three-way ANOVAs were performed for each dependent variable, with week, time point or session as the repeated measure. The effect of diet on IL-6 and TFN-α were considered in a two-way ANOVA, with diet and age as between group factors, whereas CML was considered in a three-way ANOVA, with Diet, Age and Region as between group factors. The alpha level was set at 0.05 for all analyses.

Results

Body weight, food intake and temperature

There was no change in body weight in the 6- and 12-month old control mice throughout the duration of the study (Figure 1), however the 6- and 12-month old DIO mice gained 10.1 and 2.8 g, respectively. The DIO mice weighed more than their age-matched controls and the difference increased with time on study from 8.4 to 17.3 g for the 6-month old mice and from 14.8 to 17.6 g for the 12-month old ones. A three –way ANOVA with repeated measures on Month yielded a significant interaction of Month, Age and Diet as well as main effects of Age and Diet (all *p*s<0.001).

Food intake was measured prior to the start of behavioral analyses and no effect of age or diet was found (all *p*s>0.319). Utilizing the specified kilocalories from the diets given to the mice, energy intake was calculated (Table 1). Because the old mice weighed more, yet ate the same amount of food, their energy intake per kg body weight was lower than the young mice. However, there was no difference in energy intake

between the control and the DIO mice. An ANOVA supported a main effect of Age (p<0.001).

Body temperature pre and post water exposure was approximately 0.5°C lower in 12-month old mice when compared to the 6-month old ones regardless of diet (Table1). Following water exposure, the body temperature of the 12-month-old DIO mice decreased by 0.7°C. A three-way ANOVA revealed a main effect of Age and an interaction of Time and Diet, which was due to the effect in the 12-month-old DIO mice (all ps<0.05).

Locomotor activity

Components of horizontal, vertical and spatial activity were considered in the analyses of spontaneous locomotor activity (Figure 2). There was no difference in distance traveled, rearing and resting time between the control and DIO groups (all ps>0.122). However, the intervention group had lower rearing activity and more resting time than the DIO mice (all ps<0.024). Overall, the DIO mice spent less time in the center of the locomotor activity box than the control or intervention groups. A two-way ANOVA yielded main effects of Age and Diet (all ps<0.034), with the effect of age mostly driven by the difference between 6 and 12-monh-old DIO mice (p=0.01).

Water Maze

The length of the path taken to reach the hidden platform was determined to assess the efficiency with which the mice located the platform, independent of their swimming speed (Figure 3A). All groups learned to locate the platform efficiently by the last session of the water maze task. Analysis of the data confirmed the effect of session

on path length (p<0.001). Across all sessions, there was no effect of diet or age on the distance travelled (all ps > 0.266). However, when considering sessions 2-4 (acquisition learning index), the 12-month-old mice took longer path length than the 6-month-old ones, however diet had no effect. An ANOVA resulted in a main effect of Age (p=0.02). Swimming speed was relatively variable across (Figure 3B). The DIO mice swam slower than the control and intervention mice, though the difference was more pronounced in the 12-month-old group. A three-way ANOVA revealed a significant main effect of Diet on swim speed (p<0.002) and an interaction between Age and Diet close to significance (p=0.058).

Accuracy of spatial memory was measured by conducting a probe trial on sessions 2, 4, 5, 7 and 9 (Figure 4) and expressed as the percent of time spent in the platform location and in the area surrounding the platform (Probe index). All mice developed a spatial bias for the location of the platform, but there was no influence of age or diet on their performance. A three-way ANOVA revealed a significant effect of Session (p=0.001), but no main effects of Age or Diet (all ps>0.317).

Elevated Plus Maze

Anxiety-related behavior was determined as a shift in preference in the elevated plus maze paradigm (Figure 6). The DIO mice spent more time in the closed arms (less time in the open arms) and made less entries into closed and open arms than the control mice. A two-way analysis of variance on all these dependent variables revealed main effect of Age on the number of entries in closed arms and main effects of Diet on

time spent in closed arms and number of entries in both closed and open arms (all *p*s <0.005). The DIO mice were also more immobile than any other groups, yet it had no bearing on the shift preference of the DIO mice. This observation was supported by an analysis of covariance with immobile time as the covariate revealing only a significant effect of Diet (all *p*s<0.005).

Discriminated Avoidance

Learning of a preemptive response (measured as number of training trials needed to reach a criterion for choosing the correct goal arm within 5 sec) is shown in Figure 7. On average, all mice took the same number of trials to reach the criterion regardless of age and diet. All mice took less and less trials to reach criterion across sessions. At 12 months of age, it seemed that the DIO and INT mice performed worse than the age-matched controls. However, a three-way analysis of variance yielded only a significant effect of Session (p<0.001).

Inflammation measures

Peripheral inflammation was determined by measuring serum TNF-α and IL-6 (Figure 8). The concentration of TNF-α was similar and 6- and 12- months old controls, whereas it was higher in the 6-month DIO than in the 12-month DIO. Furthermore, the DIO mice had higher TNF a concentration than the control mice. An ANOVA yielded only a significant interaction between Age and Diet (p=0.02), though the main effect of Diet was close to significance (p=0.058). The concentration of IL-6 in the serum was higher in the 12-month-old mice, but remained unaffected by diet. The ANOVA revealed a significant main effect of Age (p<0.001).

Glycation measure

Chemical glycation was determined by measuring Nε-carboxymethyllysine (CML) concentration in the following tissues: cortex, hippocampus, cerebellum and skeletal muscle (Figure 9). Skeletal muscle had a higher concentration of CML than any of the brain regions, supported by a main effect of tissue (p<0.001). In 12-month-old mice, skeletal muscles had higher concentrations of CML than the ones from the 6-month-old mice. This observation was supported by a main effect of Age (p=0.016). Across tissue, the concentration of CML was decreased in DIO mice regardless of age, with the most discernable effects observed in the hippocampus and skeletal muscle. Three- and two-way ANOVAs yielded main effects of Diet (all ps<0.02).

Oxidative stress measures

Oxidative stress was determined by protein carbonyl concentration in cerebral cortex, hippocampus and cerebellum (Figure 10). Regional differences were observed in protein carbonyl content with the hippocampus having the highest carbonyl content; however there were no effect of age or diet in any of the regions. A three-way analysis of variance yielded only a significant effect of Regions (p<0.001).

Discussion

The main findings of this study were that the mice,6- and 12-months-old, raised on a high fat diet had (i) increased weight and impaired thermogenesis, (ii) had normal motor function, spatial learning and memory and avoidance learning, (iii) increased anxiety-like behavior, (iv) increased pro-inflammatory cytokine levels ,and (v) decreased glycation levels in hippocampus and skeletal muscle.

Mice had been fed a diet containing 60% fat since 6 weeks of age, and were heavier than the control mice throughout the study. Within one week of consuming diets high in saturated fat, C57BL/6 mice develop increased body weight and display symptoms of metabolic dysfunction [40, 50, 83].. Data from other studies suggest that weight gain is associated with the fat composition of the diet and the energy density of the food consumed [84]. When energy intake is matched between mice fed a high-fat diet and mice fed a low-fat diet, increases in weight and adipose tissue mass are still observed [84]. In this study, increased body weight was still observed even though the mice consumed equal amount of food and the energy intake per kg body weight was similar between the groups. DIO mice ate ad libitum a diet containing 1.39 kcal more per g compared to the control diet suggesting that diet composition had a greater impact on weight than did food intake alone. Colonic temperatures were measured pre and post cold water exposure. Though all groups of mice have similar basal body temperature, we observed a decrease in temperature after water exposure indicating an impaired thermoregulation. This effect was however only detected in the 12-month-old DIO mice. Impairments in cold-induced thermogenesis have been observed in aged- [58, 68] and obese mice [70]. Furthermore, morphological abnormalities in brown adipose tissue have been observed in genetically obese mice which may account for the temperature difference observed with age and diet [7, 70]. While previous studies have indicated an improvement in cold tolerance with repeated exposure to cold temperatures [68], repeated exposure in the current study did not change the conclusion if impaired thermogenesis (data not shown).

All variables representing horizontal, vertical and stereotypical components of spontaneous activity remained unaffected by the high fat dietary intake in 6- and 12-month-old mice. Similarly, Hwang et al. (2010) observed no change in locomotor activity in C57BL/6 mice consuming a high-fat diet for 9-12 months.

The time spent in the center of the locomotor activity box was decreased in DIO mice at both ages. This observation speculates an effect of high fat diets on anxiety-like behavior. To confirm this determination, the mice were exposed to a shift preference paradigm, elevated plus maze, to measure anxiety levels in mice. The DIO mice indicated a preference for the closed arms suggesting an increased anxiety associated with high fat dietary intake [65]. Mice fed a 45% kcal fat diet since 9 weeks of age for 45 weeks also exhibited anxiety-like behaviors [25]. Furthermore, the DIO mice in this study gained less weight than the DIO mice in the current study, suggesting that increased fat intake produces anxiogenic effects independent of degree of weight gain.

Spatial learning and memory as well as active avoidance performance were not affected by age and diet. The mice ranged in age that were too close to see a significant effect of age [13]. The results from studies on the effects of diet manipulations are inconclusive, ranging from no effect to impairments. In accord with our data, studies using variations of the water maze paradigm for cognitive assessment reported that spatial learning and memory in high-fat fed, obese animals is not impaired [2, 31, 32, 40, 76]. On the other hand, learning deficits were observed when mice, similar in age, consuming a high-fat diet for ≥ 9 months were subjected to other cognitive tests such as contextual fear conditioning and step-down passive avoidance test [30, 40]. It is a

possibility that high fat diets and cognitive consequences are only specific to some tasks and not others.

Increased pro-inflammatory cytokines, AGEs and levels of protein oxidation are commonly observed with age and obesity [8, 9, 44, 53, 60, 77]. As expected, high fat feeding induced increases in serum TNF- α and IL-6 in DIO and 12-month old mice, respectively. Rats fed a 45% fat diet for 16 weeks also showed increases in these pro-inflammatory cytokines [14]. Increased production of TNF-α and IL-6 have been previously described as mediators involved in impaired insulin signaling, as seen in obesity [28, 29].

Interaction of AGE with its receptor (RAGE) alters cell signaling, mediates pro-inflammatory cytokines and can accelerate oxidative damage [1, 21, 51, 63]. CML accumulation has been observed in brain regions of aged mice [69], in the liver, heart, and serum of high-fat fed rats [36, 85] and mice [26] and in skeletal muscle of diabetic rats [61, 62]. Due to the small age difference, a lack of effect of age on CML levels was expected, however DIO mice had similar concentrations to controls in cortex and cerebellum and even decreased in hippocampus and skeletal muscles. The lack of accumulation of CML within brain regions of DIO paired to the decreased CML levels observed in the hippocampus of DIO mice, could be attributed to a compensatory response such that degradation and clearance rates of these end products is increased in DIO mice [56]. Compared to brain regions, levels of CML were greatest in skeletal muscle, but differences were observed with diet and age. CML levels were increased in skeletal muscle of 12-month old mice. As previously mentioned, diabetic rats have

increased CML accumulation in skeletal muscle [61, 62]. Furthermore, C57BL/6 mice are genetically predisposed to diabetes and, given an obesity-promoting environment will exude characteristics associated with obesity and diabetes [11, 54, 67, 75]. The increased CML levels observed in the skeletal muscle and elevated levels of IL-6 in 12-month old mice may suggest that these mice were diabetic, which could account for the observed impairments. As opposed to increased skeletal muscle CML in 12-month old mice, decreased levels were observed in the skeletal muscle of DIO mice. This observation has been previously reported in skeletal muscle of genetically obese mice (Chapter 2) and can be attributed to increased intramuscular fat content and decreased protein mass and content within the muscle fibers [34].

As mentioned, accelerated oxidative damage occurs with the accrual of AGEs [1, 51, 63] and is associated with performance on cognitive tests in aged mice [18] and in rats fed a high fat diet [76]. Considering the minor spatial learning deficits, increased pro-inflammatory cytokines and CML content observed in 12-month old mice, increased protein carbonyl content would be expected in the 12-month old mice. Despite reported increases in markers of oxidative stress in high fat fed C57BL/6J mice, only regional differences were observed in protein carbonyl concentration within brain regions [26].

An intervention approach was used to determine whether the obesity-associated changes are reversible. The high fat diet resulted only in increased anxiety, observed in two different emotional tasks, and the intervention diet produced a total or partial reversal of the anxiogenic behavior in the locomotor activity or elevated plus maze tasks, respectively. Exercise has been shown to decrease anxiety and could explain

that more active mice would be less anxious [6, 55]; however our data do not reflect that as the intervention mice were less active than the DIO mice. Additionally, biochemical measurements remained unaffected by the change back to low fat diet suggesting that they are not associated with affective behavior.

Even though only minor impairments were observed in 12-month old mice, the current study suggests that age, not diet, affects brain function. Surprisingly, DIO mice performed as well as controls on cognitive tests despite visual impairments, increased anxiety and impaired thermogenesis. The effect of diet on cognition remains controversial and seems to be dependent on source of dietary fat [80], species [38, 64, 80, 82], gender [30], and behavior task [30]. Because substantial cognitive deficits were not observed with diet or age, the lack of biochemical modifications observed within brain regions is not surprising.

Table 1. Food intake, energy intake and temperature of 6- and 12-mo old male mice. Values reflect mean ± SE 14-16 mice.

Table 1 General Assessment: Group means and Standard errors for food intake, energy intake and temperature

	6-month		12-month		
General Assessment	**Control**	**DIO**	**Control**	**DIO**	**Intervention**
Food Intake (g)					
Pre-behavior	3.5 ± 0.10	3.6 ± 0.24	3.4 ± 0.13	3.6 ± 0.19	4.04 ± 0.10
Energy Intake (kcal/kgbw)					
Pre-behavior	413.3 ± 11.2	480.8 ± 49.3*	310.4 ± 14.1#	326.6 ± 15.3#	351.8 ± 7.14
Temperature (VP)					
Pre	38.0 ± 0.15	38.0 ± 0.16	37.5 ± 0.18	37.5 ± 0.17	37.4 ± 0.16
Post	38.1 ± 0.10	37.9 ± 0.11	37.7 ± 0.08	#36.8 ± 0.26	37.6 ± 0.16

* denotes significant difference between diet ($p<0.05$)

# denotes significant difference between 6- and 12-month old mice ($p<0.05$)

Figure 1. Effect of diet and age on body weight as a function of time. Values reflect mean ± SE of 14-16 mice.

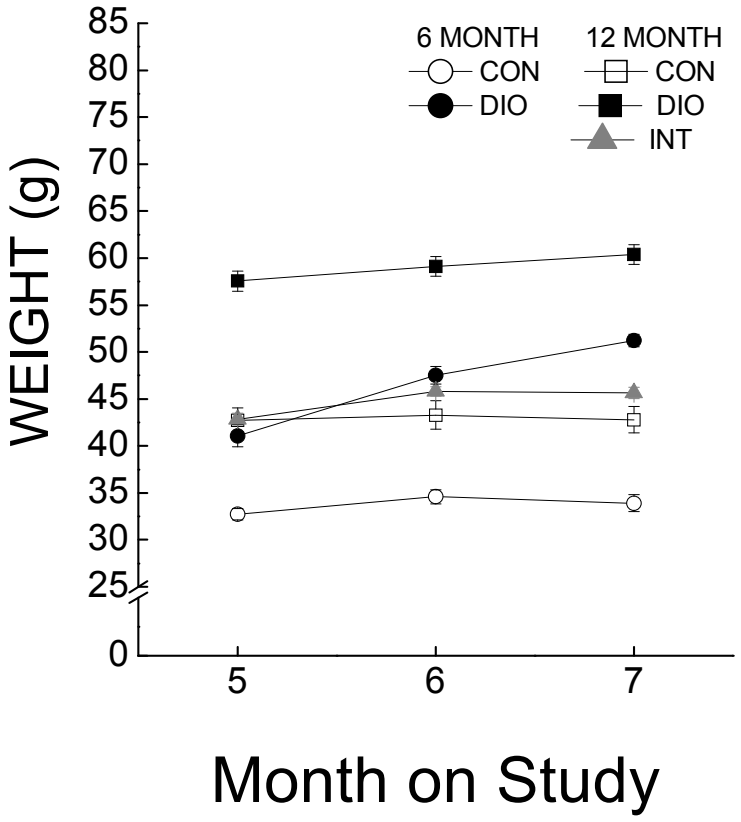

6 MONTH
CON
DIO
12 MONTH
CON
DIO
INT
WEIGHT (g)
85
80
75
70
65
60
55
50
45
40
35
30
25
0
5
6
7
Month on Study

Figure 2. Effect of a high fat diet on horizontal, vertical and spatial components of spontaneous locomotor activity in 6- and 12-month-old C57BL/6 mice. Values reflect mean ± SE of 14-16 mice.

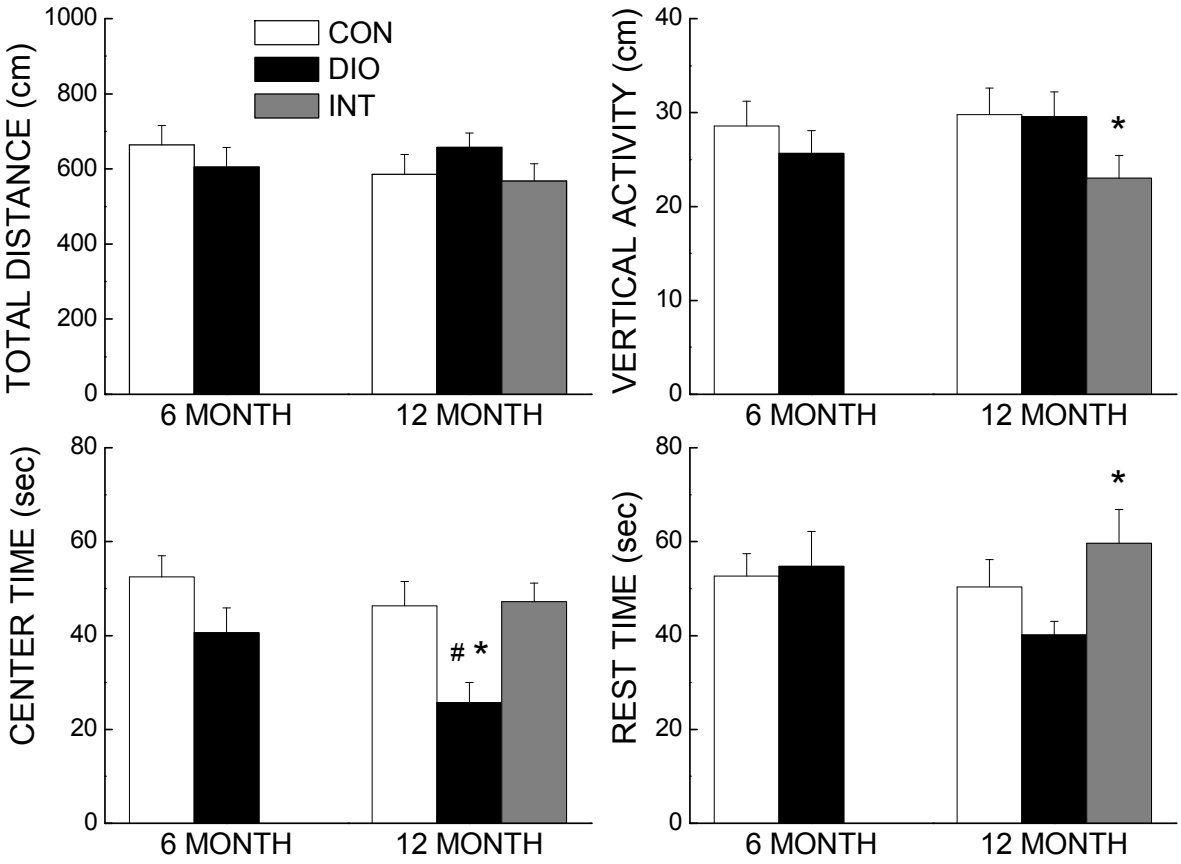

CON
DIO
INT
TOTAL DISTANCE (cm)
1000
800
600
400
200
0
6 MONTH
12 MONTH
VERTICAL ACTIVITY (cm)
40
30
20
10
0
*
CENTER TIME (sec)
80
60
40
20
0
*
REST TIME (sec)
*

Figure 3. Effect of age and high fat diet supplementation on water maze performance represented by path length (A) and swim speed (B). Values reflect mean ± SE of 14-16 mice.

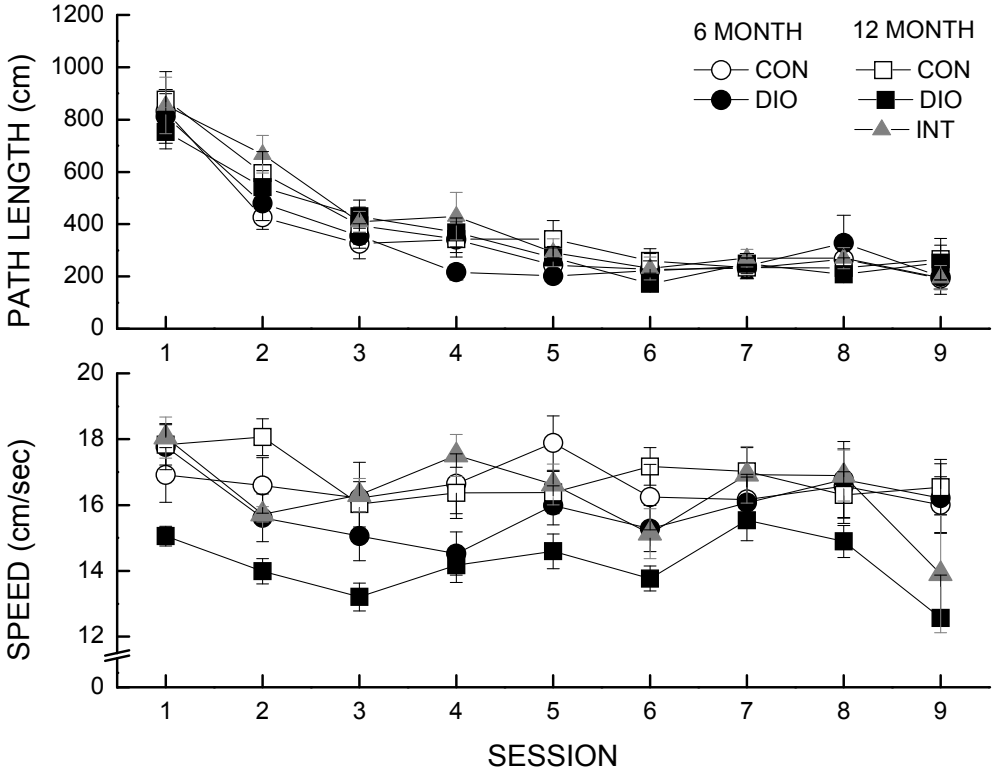

6 MONTH
CON
DIO
12 MONTH
CON
DIO
INT
PATH LENGTH (cm)
SPEED (cm/sec)
SESSION

Figure 4. Effect of age and high fat diet supplementation on probe performance in the water maze paradigm. Values reflect mean ± SE of 14-16 mice.

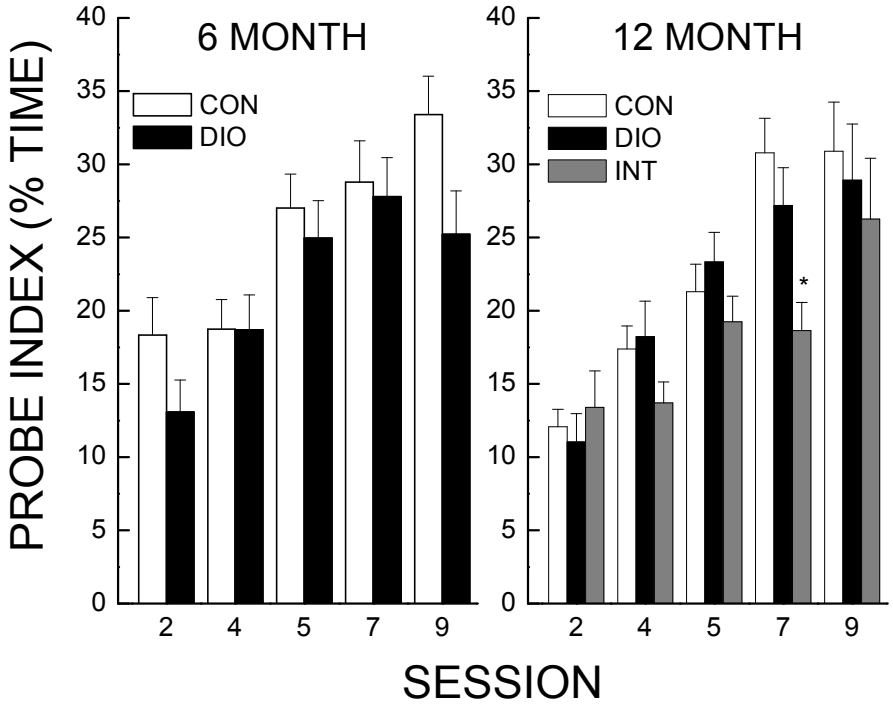

6 MONTH
12 MONTH
CON
DIO
INT
PROBE INDEX (% TIME)
SESSION
0
5
10
15
20
25
30
35
40
2
4
5
7
9
*

Figure 5. Effect of age and high fat diet supplementation on percent time spent in closed arms (top left), in open arms (top right), on number of entries in closed arms (bottom left) and open arms (bottom right) in the elevated plus maze. Values reflect mean ± SE of 14-16 mice.

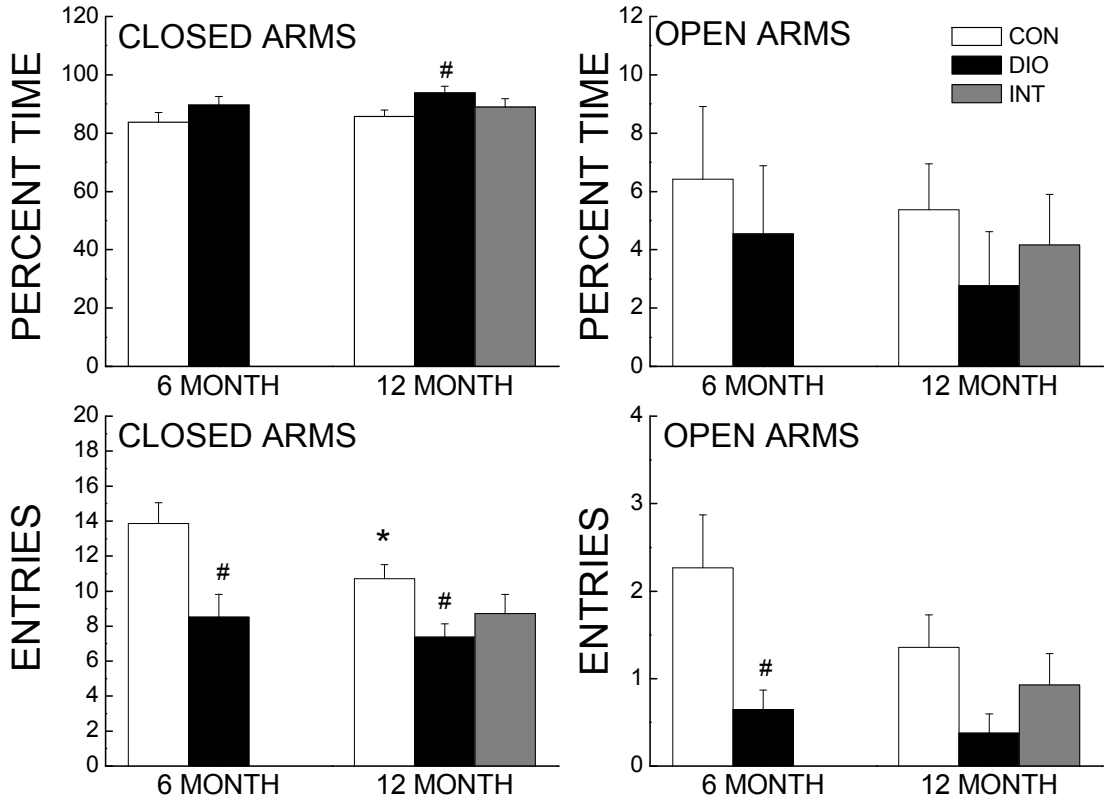

CLOSED ARMS
OPEN ARMS
CON
DIO
INT
PERCENT TIME
ENTRIES
6 MONTH
12 MONTH
#
*

Figure 6. Effect of diet and age on discriminated avoidance performance expressed by the number of trials to reach criteria by session (A) and overall performance (B). Values reflect mean ± SE of 14-16 mice.

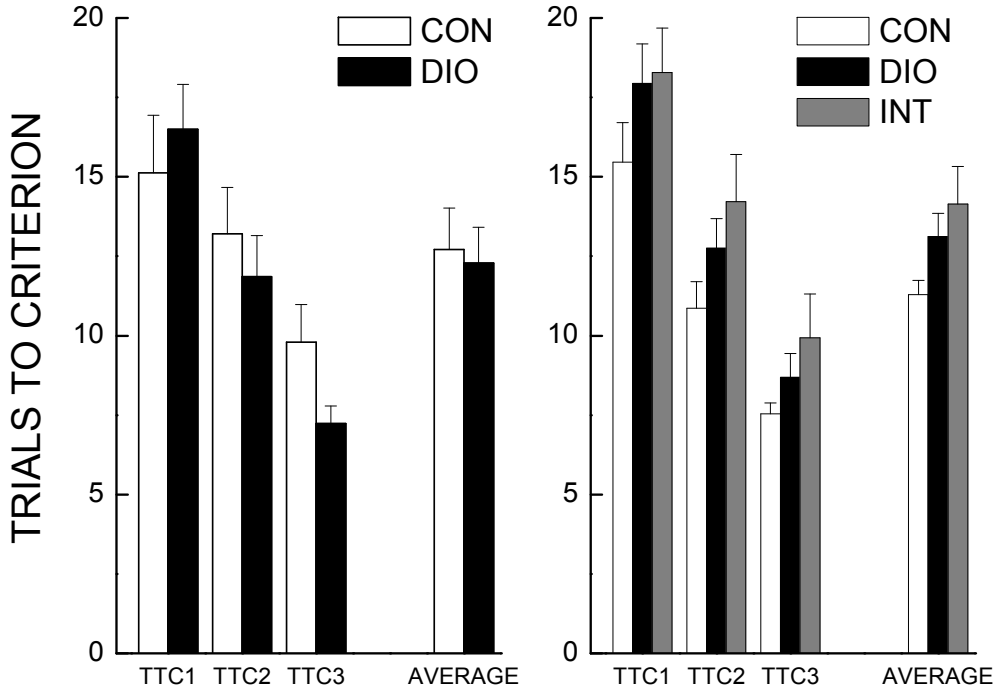

TRIALS TO CRITERION
20
15
10
5
0
CON
DIO
INT
TTC1
TTC2
TTC3
AVERAGE

Figure 7. Circulating pro-inflammatory cytokine concentration (TNF-α, N=4; IL-6, N=10-14). Values reflect mean $\pm$ SE of 14-16 mice.

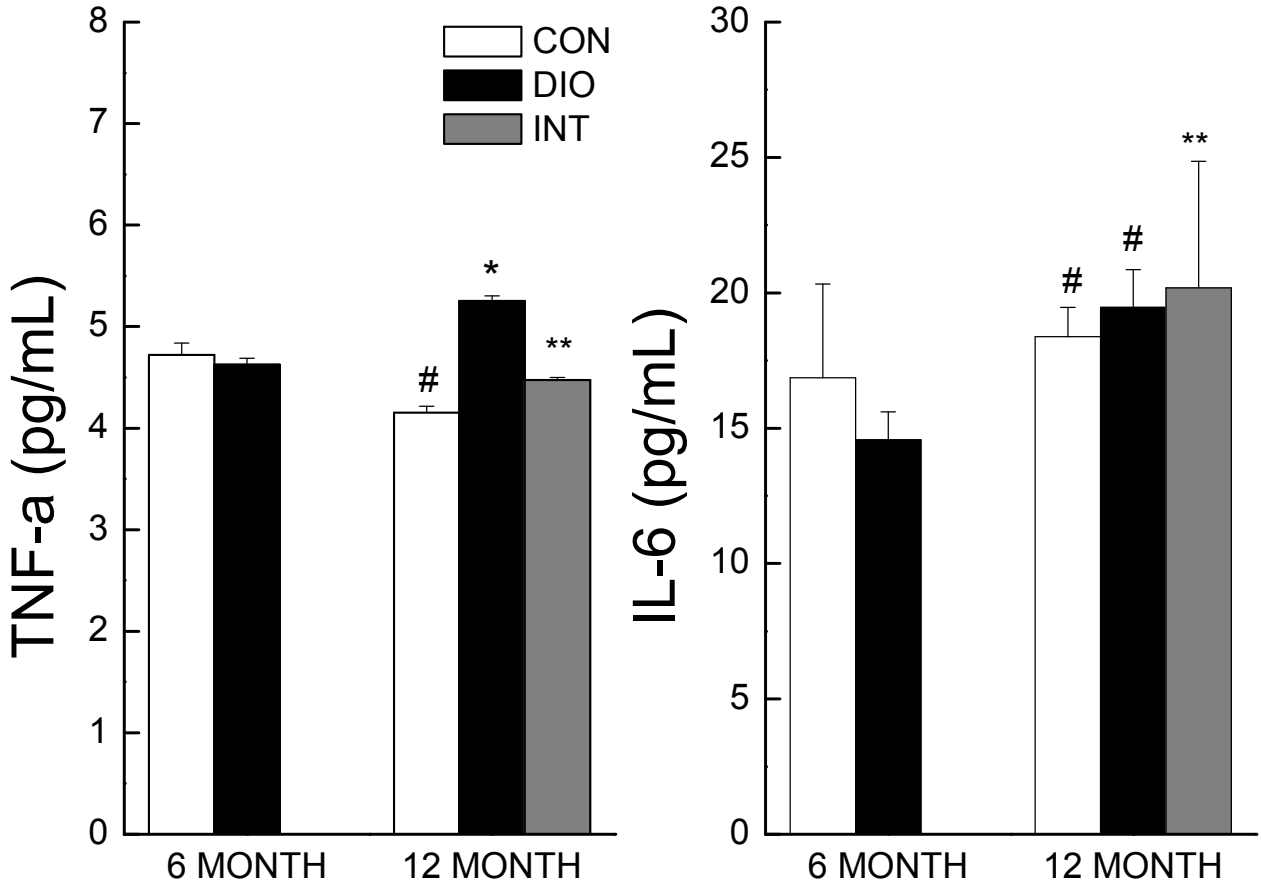

CON
DIO
INT
TNF-a (pg/mL)
IL-6 (pg/mL)
6 MONTH
12 MONTH
6 MONTH
12 MONTH
#
*
**
#
#
**

Figure 8. Carboxymethlyllysine content of cortex (A), hippocampus (B), cerebellum (C) and skeletal muscle (D) Values reflect mean ± SE of 14-16 mice.

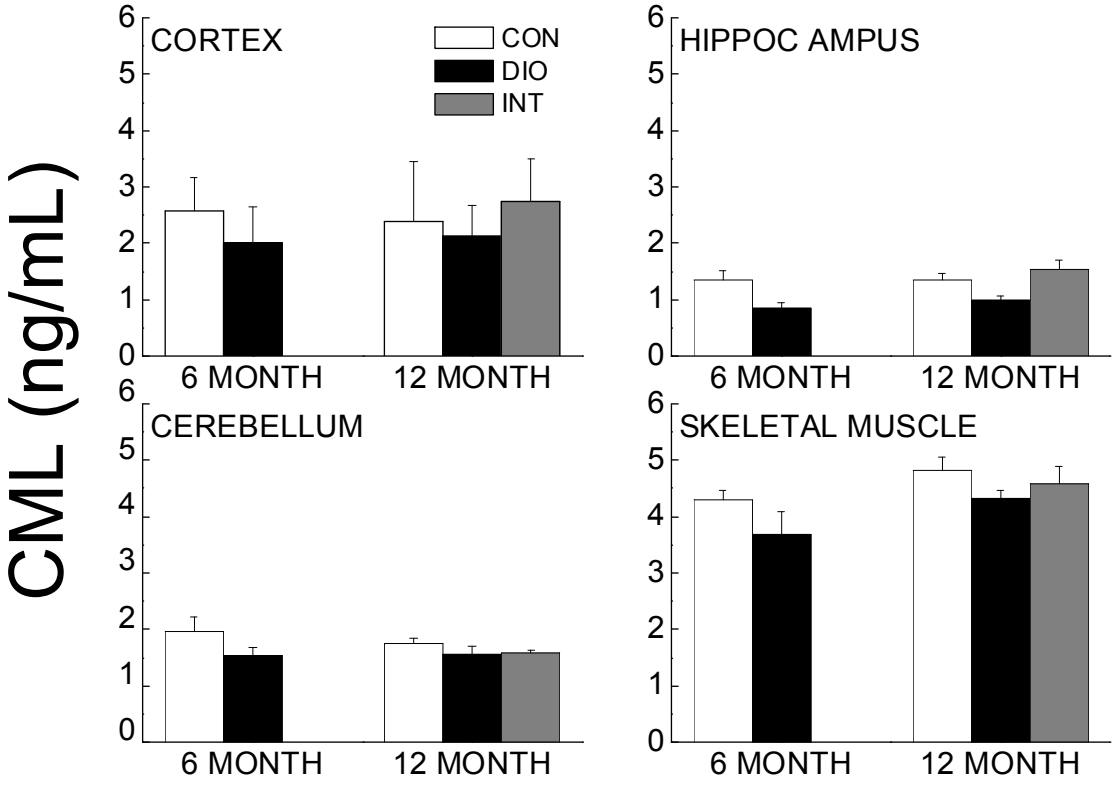
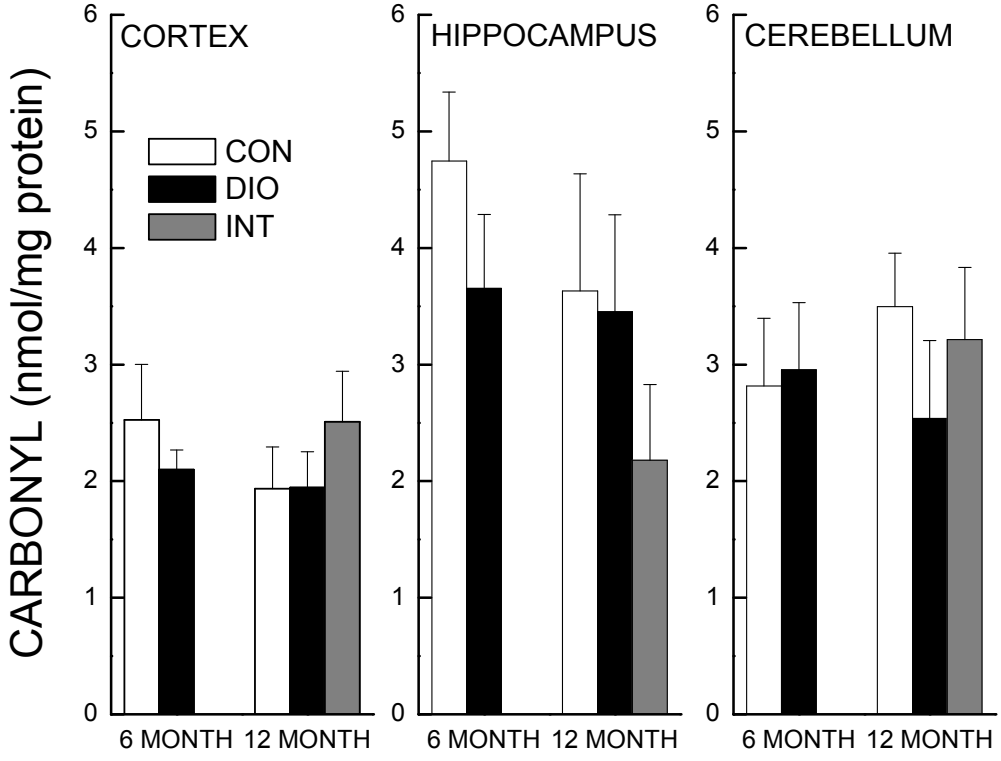

CML (ng/mL)
CORTEX
CON
DIO
INT
HIPPOC AMPUS
CEREBELLUM
SKELETAL MUSCLE
6 MONTH
12 MONTH
0
1
2
3
4
5
6

Figure 9. Protein carbonyl content of cortex, hippocampus and cerebellum. Values reflect mean ± SE of 14-16 mice.


CARBONYL (nmol/mg protein)
CORTEX
HIPPOCAMPUS
CEREBELLUM
CON
DIO
INT
0
1
2
3
4
5
6
6 MONTH
12 MONTH

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CHAPTER IV

ASSESSMENT OF VISUAL FUNCTION IN OBESE MICE

Introduction

Obesity is considered a risk factor for numerous eye diseases reviewed by (Cheung & Wong, 2007). This association was evaluated in a series of as yet unpublished investigations by this investigator and Dr. Margaret Rutledge. In separate experiments, visual function was assessed in two mouse models of obesity previously subjected to a behavioral test battery that evaluated motor, affective and cognitive function. Cognitive impairments were relatively minor and observed only in young male and female *ob/ob* mice (see Chapter II); however, significant visual impairments were observed in both models of obesity. The results of these studies are outlined in the following sections, and the outcomes are discussed in Chapter V in relation to the behavioral and biochemical results presented in Chapters II and III.

Experiment 1: *Visual function assessment in a genetic model of obesity*

Introduction

Obesity impacts overall health by contributing to the manifestation of a number of clinical conditions, including visual impairment (Bray, 1992; Cheung & Wong, 2007; Lean, 2000). Morphological alterations to the retina and overall progression of ocular

disease have been linked to obesity (Klein, Klein, Lee, & Jensen, 2001; Seddon, Cote, & Rosner, 2003).

Mice homozygous for the obese mutation Lep^{ob} (*ob/ob*) have been used to study obesity-induced health conditions (Bray & York, 1979). By 4-6 weeks of age, *ob/ob* mice exhibit phenotypic characteristics of obese humans, including increased adiposity, hyperglycemia, and hyperinsulinemia (Dubuc, 1976; Lindstrom, 2007; Tomita et al., 1992). The purpose of this experiment was to assess the degree of visual impairment in *ob/ob* mice as an initial step toward examining underlying mechanisms in obesity-induced visual dysfunction.

Methods

Animals: A total of thirty 10-11 week old mice, C57BL/6J and B6.V-Lep <ob>/J (ob/ob) (*n*=15/sex/genotype), were obtained from The Jackson Laboratory (Bar Harbor, ME). Beginning at 6 months of age, mice were administered a series of behavioral tests, assessing spontaneous motor activity and cognitive capacity (refer to Chapter II). Included in the battery were two approaches to assess visual function of the mice: i) visible platform task and ii) tests of visual acuity and contrast sensitivity.

Visible platform. The visible platform test required the mice to swim in an open tank, filled with opacified water, and locate a hidden platform identified only with a small triangular flag (5 cm each side, 11 cm^2) that was raised above the surface of the water (6 cm from the water surface to the bottom of the flag). Eight sessions were administered, 2 per day separated by 2 h, each consisting of five trials at 10 min

intervals. On each trial, the platform was moved to a different location, and the mouse was lowered into the water from a different starting location. Thus, the mouse had to learn to associate the location of the flag with the location of the platform. A learning index was calculated as the average path length taken on sessions 2-4. Similarly, speed index was calculated as the average swimming speed of the mouse during sessions 2-4. A computerized tracking system recorded the length of the path taken by the mouse to reach the platform, as well as the swimming speed (ANY-MAZE, Stoelting).

Assessment of visual acuity and contrast sensitivity. Visual capacity was measured as described previously by Prusky and colleagues (Prusky, Alam, Beekman, & Douglas, 2004). The acuity testing apparatus consists of an acrylic box (39 x 39 x 32.5 cm) affixed with mirrored floors and ceilings. Attached to each of the four walls was a 20-in computer monitor (Dell) facing inwards. A computer program (OptoMotry, Cerebral Mechanics, Lethbridge, Alberta, Canada) was used to project visual stimuli onto the monitors such that a virtual cylinder with vertical gratings was produced. When rotated, the gratings moved at 12 degrees/second. An elevated platform (15 cm from the floor; 7 cm diameter) was positioned in the center of the apparatus. A mouse was placed on top of the platform and allowed to acclimate until it was no longer active. A video camera, located in the ceiling of the apparatus, enabled the behaviors of the mouse to be clearly visible during testing and transmitted information to the Power Macintosh computer. Visual acuity threshold was determined with contrast set at 100% (i.e., the bars of the gratings were maximally black and the background was maximally

white). On the first trial, a grating of low spatial frequency (0.042 cycles/degree) was projected onto the walls, rotating at 12 degrees/second in a clockwise direction (thus effectively testing the left eye). If the mouse could visually detect the moving stimuli, it would indicate this by following the movements with its eyes and head. When this tracking behavior was observed, the same stimuli were rotated in the counterclockwise direction (thus effectively testing the right eye). A series of gratings of increasingly higher spatial frequencies was presented (rotating in one, then the alternative direction) as long as the mouse indicated that it could detect the grating movements. When the mouse ceased to respond to a particular spatial frequency, a lower frequency grating was presented; when the mouse responded to a frequency, the frequency was increased. The acuity threshold for each mouse was set at the highest spatial frequency to which the animal responded. A visual acuity threshold was determined (for each eye). These two numbers were averaged to achieve a mean visual acuity for each mouse. During a separate test session, a contrast threshold was decided for six spatial frequencies (0.031, 0.064, 0.092, 0.103, 0.192, 0.272 c/d). This test was meant to demonstrate the ability of an animal to visually detect a stimulus even when the brightness of the stimulus is lowered. The initial contrast level was set at 100% for each of the above spatial frequencies. Contrast was lowered until the mouse ceased to respond to the particular grating. The lowest contrast setting which elicited a tracking response was determined for each of the six spatial frequencies, and from these numbers a contrast-sensitivity function was calculated with the formula: 100/C where C is the lowest contrast that elicited a response at a particular frequency. This data

transform means that when an animal can see at a very low contrast setting, the sensitivity number will be large, indicating better visual performance at a particular spatial frequency.

Results and Discussion

Body weight was measured prior to visual testing, when mice were approximately 7 months of age (Figure 1). Male and female *ob/ob* mice weighed more than same-sex control mice and the weight difference was greater in females than in males. An analysis of variance yielded significant main effect of Genotype and interaction between Genotype and Sex (all *ps*<0.001).

Performance on the visible platform test was assessed by the learning index (Figure 2). All mice were able to locate the visible platform efficiently after eight sessions. However, obese mice took significantly longer path lengths, represented by the learning index, when compared to control mice. Regardless of genotype, female mice took a longer path length than male mice. These observations were supported by main effects of Sessions, Genotype and Sex (all *ps* <0.011).

Swimming speed of the mice was assessed by speed index (Figure 2). Male and female obese mice swam slower than control mice and this difference was greater in female mice than in male mice. An analysis of variance indicated a significant main effect of Genotype and Interaction between Genotype and Sex (all *p*s <0.007).

As noted earlier, visual acuity threshold was defined as the highest spatial frequency at which the mouse responded to gratings (Figure 3). Overall, acuity threshold for female mice was less than for male mice. More importantly, acuity

threshold was less for *ob/ob* mice than for controls, and this effect was observed for female mice and for male mice. An ANOVA revealed main effects of Sex and Genotype (all *ps*<0.03).

The ability of the mice to distinguish between the gratings (of 6 spatial frequencies) and background when contrast was less than optimal provided an estimate of contrast sensitivity (Figure 4). As is apparent in the figure, mice were able to detect some frequencies better than others when contrast conditions were degraded. Although female mice were less able than males to detect stimuli under more degraded conditions, the peak of the contrast curve centered around 0.10 c/d for males and females of both genotypes. Of more interest, *ob/ob* mice responded less to rotating gratings than controls when contrast settings were low. A two-way ANOVA yielded significant main effects of Sex and Genotype (all *ps*<0.001).

Noteworthy, both female mice and *ob/ob* mice performed worse on the visible platform test than did males and controls. This reflected the results for the acuity and contrast sensitivity assessments. The degree to which obesity impairs visual function seems to be gender specific in these mice. Similarly, Klein et al. (2001) reported that obesity in humans was more strongly associated with eye disease in females than in males. Taken together, these studies suggest that obesity impairs visual function, and that the observed impairments are more pronounced in females.

Experiment 2: *Visual function in a dietary model of obesity.*

Introduction

Visual impairments were observed in obese *ob/ob* mice (see Experiment 1). The intent of the current study was to determine if environmentally-induced obesity also leads to visual impairments. Furthermore, this study will introduce an aging component, which has also been associated with visual dysfunction. The study of aging in the *ob/ob* obese mouse model was not feasible because overall heath status of these mice declined rapidly with age. Mice fed high-fat diets gain weight rapidly and eventually become obese (Hwang et al., 2010). Nutritional factors are associated with the progression of eye disease. A high saturated fat diet is associated with increased progression of eye disease (Mitchell et al., 2003; Parekh et al., 2009; Smith, Mitchell, & Leeder, 2000), Similar to *ob/ob* mice, diet-induced obese (DIO) mice also exhibited visual impairments in water maze testing. The purpose of the current study was to assess the degree of visual impairment in 12-month-old DIO mice and determine whether the observed impairments were influenced by age.

Methods

Animals: A total of 30, 6-month-old male C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME) where mice were fed one of two specific diets beginning at 6 weeks of age. They remained on their respective diets until 12 months of age: 10% kcal fat diet (CAT # D12450B, considered the control diet, CON) or 60% kcal fat diet (CAT# D12492, DIO). Furthermore, a subgroup of the DIO mice were switched to the 10% diet at 6 months and were tested at 12 months (INT). All mice were housed as described in Chapter III and had *ad libitum* access to food and water. Visible platform task was performed on 6- and 12-month-old mice, controls and DIO; whereas

the tests for acuity and contrast sensitivity utilized only the 12-month-old controls and DIO(with the DIO being a subgroup untested on the visible platform). Mice were weighed prior to assessment of visual function.

Protocols for visible platform, visual acuity, and contrast sensitivity in Experiment 1 were used to assess visual function in Experiment 2.

Results and Discussion

Body weight was measured prior to visual testing (Figure 6), at which time 12-mo-old DIO mice weighed more than control mice, supported by a main effect of Diet (p<0.001).

DIO mice took a longer path length to find a visible platform compared to control mice, and the INT mice seemed to perform midway between the controls and the DIO mice (Figure 5). A two-way analysis of variance revealed a significant effect of Diet (p=0.004) and a trend for Age (p=0.051), but individual comparisons did not yield a significant effect of the intervention (p>0.169). Overall the 12-month-old mice swam slower than the young ones and the DIO mice were also slower when compared to their age matched controls. An ANOVA revealing significant main effects of Age and Diet supported these observations (all ps <0.002).

Acuity threshold was lower for DIO mice than for control mice, supported by a main effect of Diet (p<0.001). As was also observed in Experiment 1, mice detected some frequencies better than others when contrast conditions were not optimal. The peak of the contrast curve centered around 0.10 c/d for mice maintained on either diet, and contrast sensitivity was equivalent for the two dietary conditions (p=0.773).

It is apparent that diet-induced obesity impairs visual function in adult male mice, and it seemed to be exacerbated with age inferred from the visible platform data.

Visual impairments were observed in both mouse model of obesity (genetic vs. environment). Although the DIO mice were perhaps not impaired to the same degree as the ob/ob obese mice, we believe that they provide for a more feasible model to further study obesity-induced impairments and its interaction with age.

Figure 1. Mean body weight (g) of male and female ob/ob and control mice. All values represent the mean ± SE of 15 mice per condition. * $p<0.0001$ compared to sex-matched control

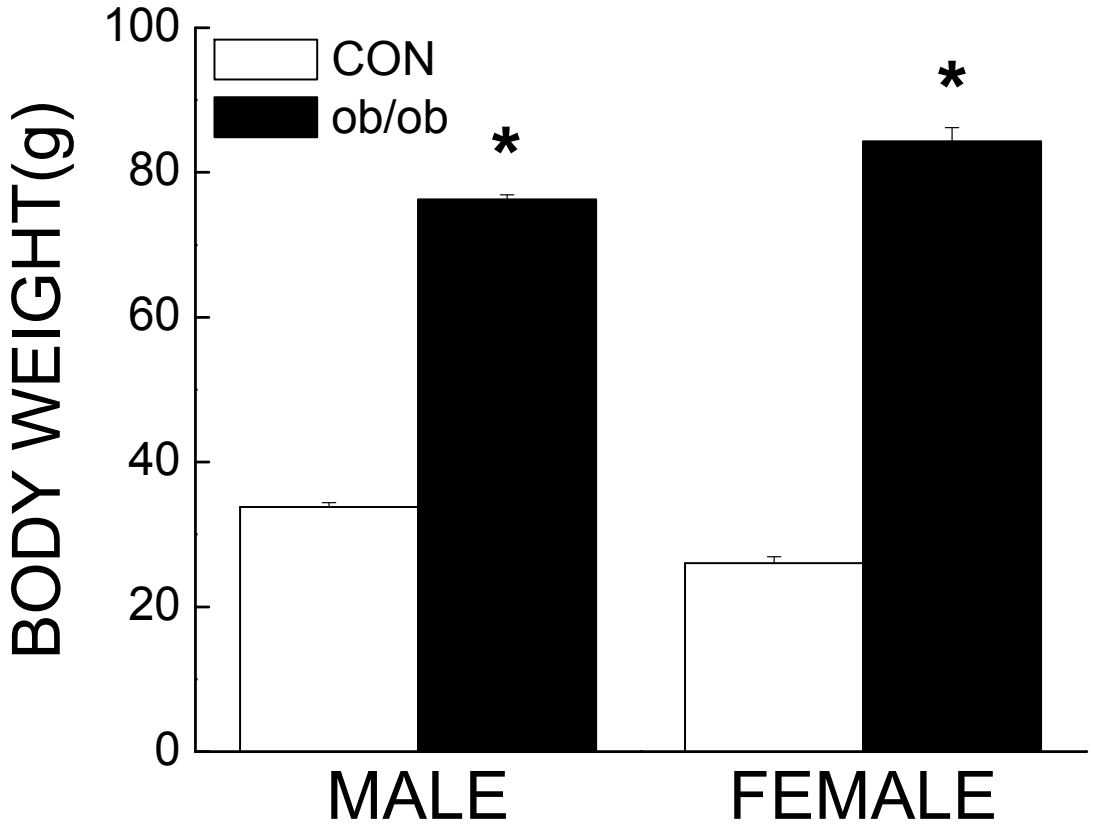

BODY WEIGHT(g)
100
80
60
40
20
0
CON
ob/ob
*
*
MALE
FEMALE

Figure 2. Learning index (acquisition phase) of 6-month old male and female ob/ob and control mice when tested in the visible platform test. All values represent the mean ± SE of 15 mice per condition. * $p<0.001$ compared to sex-matched control

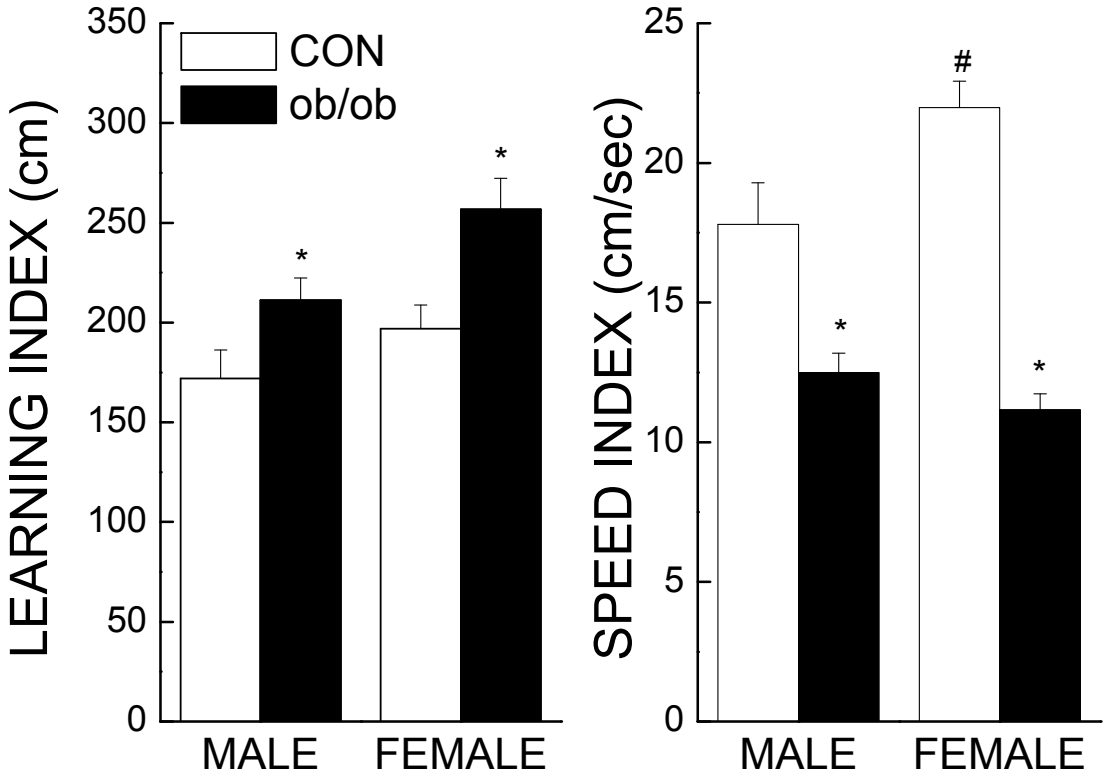

LEARNING INDEX (cm)
350
300
250
200
150
100
50
0
CON
ob/ob
*
*
MALE
FEMALE
SPEED INDEX (cm/sec)
25
20
15
10
5
0
*
#
*
MALE
FEMALE

Figure 3. Mean acuity threshold for male and female *ob/ob* and control mice. All values represent the mean ± SE of 15 mice. * $p<0.05$ compared to sex-matched control

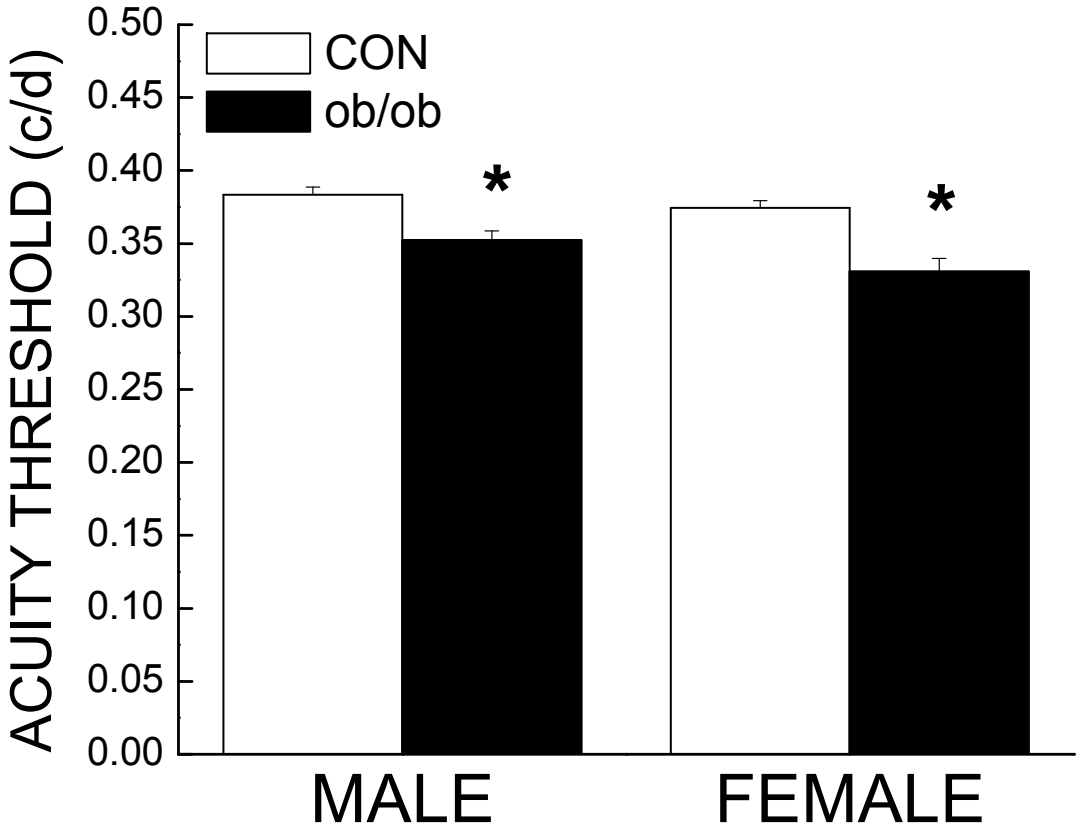

ACUITY THRESHOLD (c/d)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
CON
ob/ob
*
*
MALE
FEMALE

Figure 4. Contrast sensitivity function across six spatial frequencies of male and female *ob/ob* and control mice. All values represent the mean ± SE of 15 mice. * $p<0.001$ compared to sex-matched control

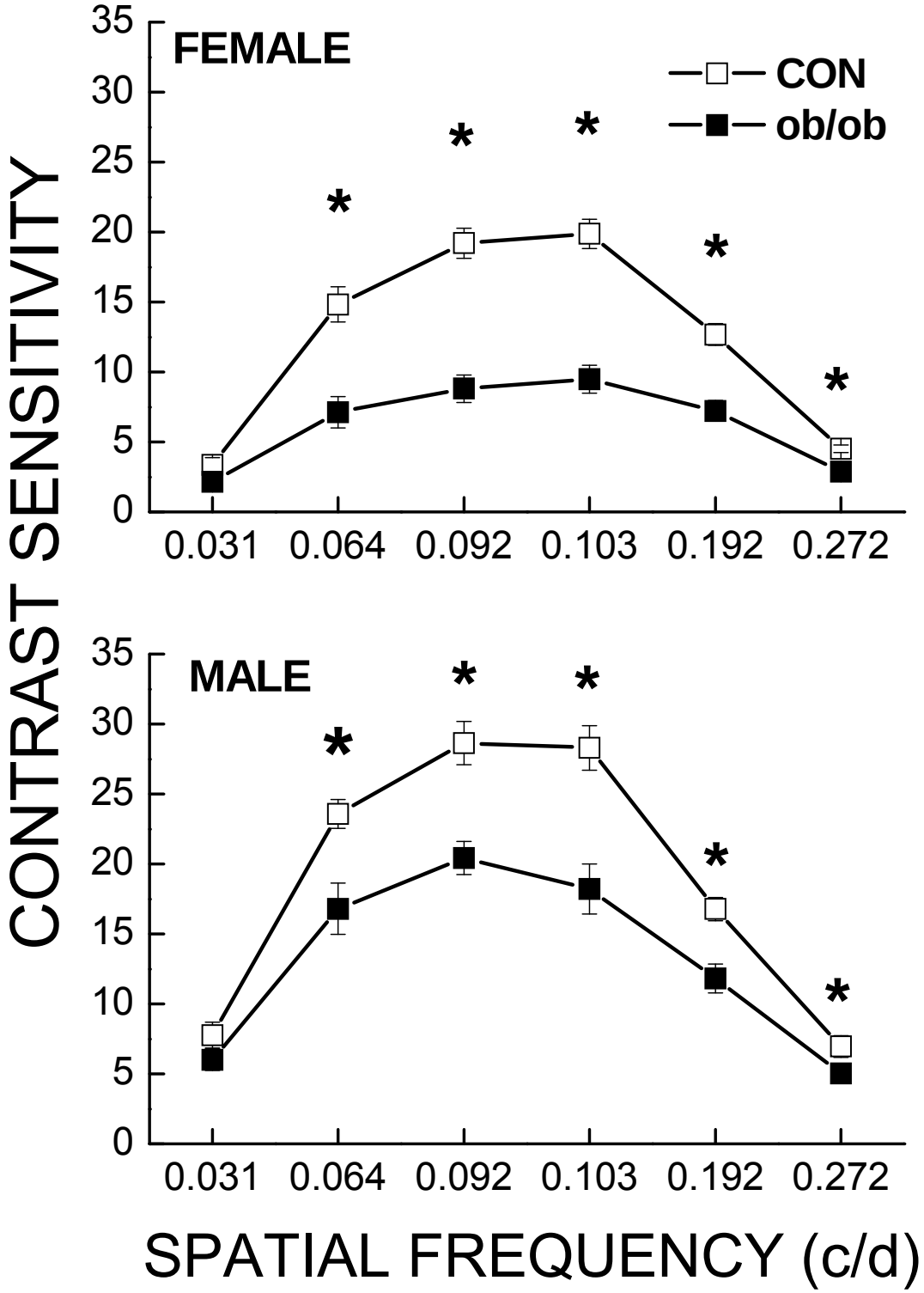

FEMALE
CON
ob/ob
MALE
CONTRAST SENSITIVITY
SPATIAL FREQUENCY (c/d)
0
5
10
15
20
25
30
35
0.031
0.064
0.092
0.103
0.192
0.272
*

Figure 5. Mean body weight of DIO and control mice. All values represent mean ± SE

* $p < 0.001$ compared to age-matched control

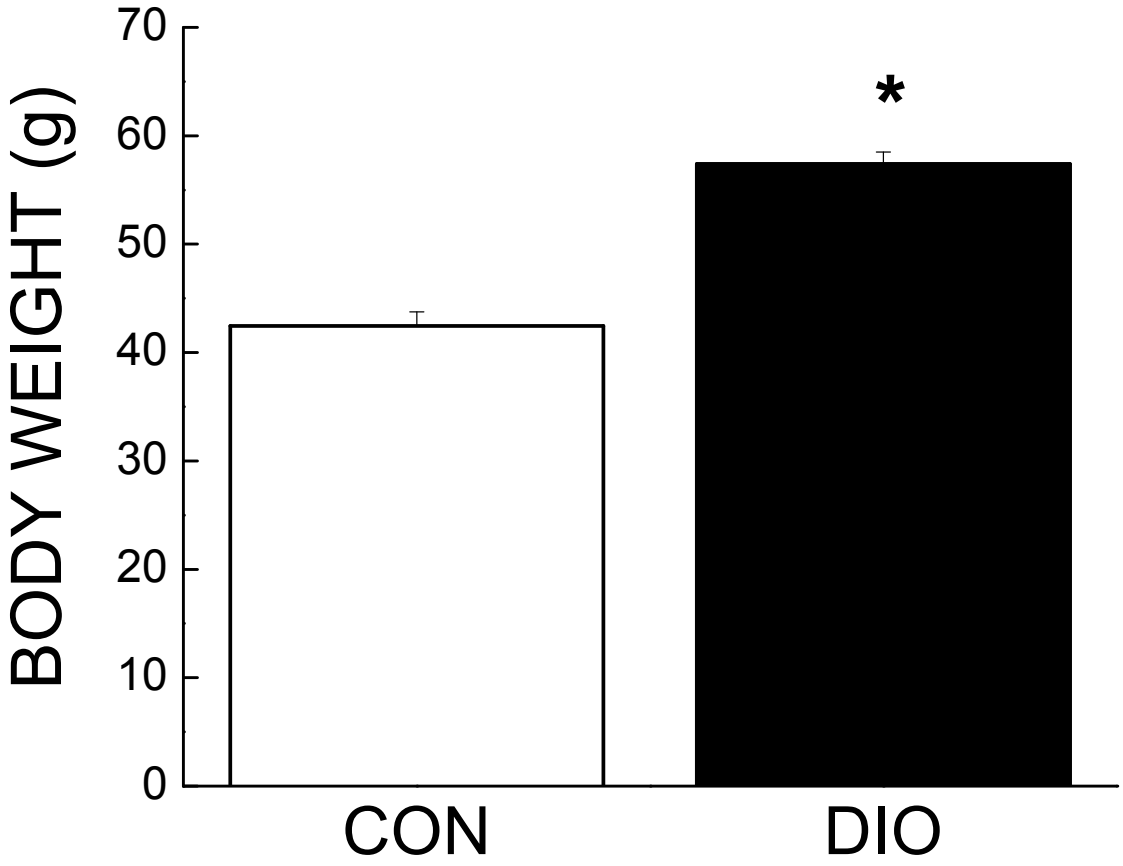

BODY WEIGHT (g)
70
60
50
40
30
20
10
0
*
CON
DIO

Figure 6. Learning index (acquisition phase) of DIO and control mice on the visible platform test. (All values represent the mean ± SE of 14-16 mice per condition. * $p<0.05$ compared to age-matched control; #$p<0.05$ compared to diet-matched control

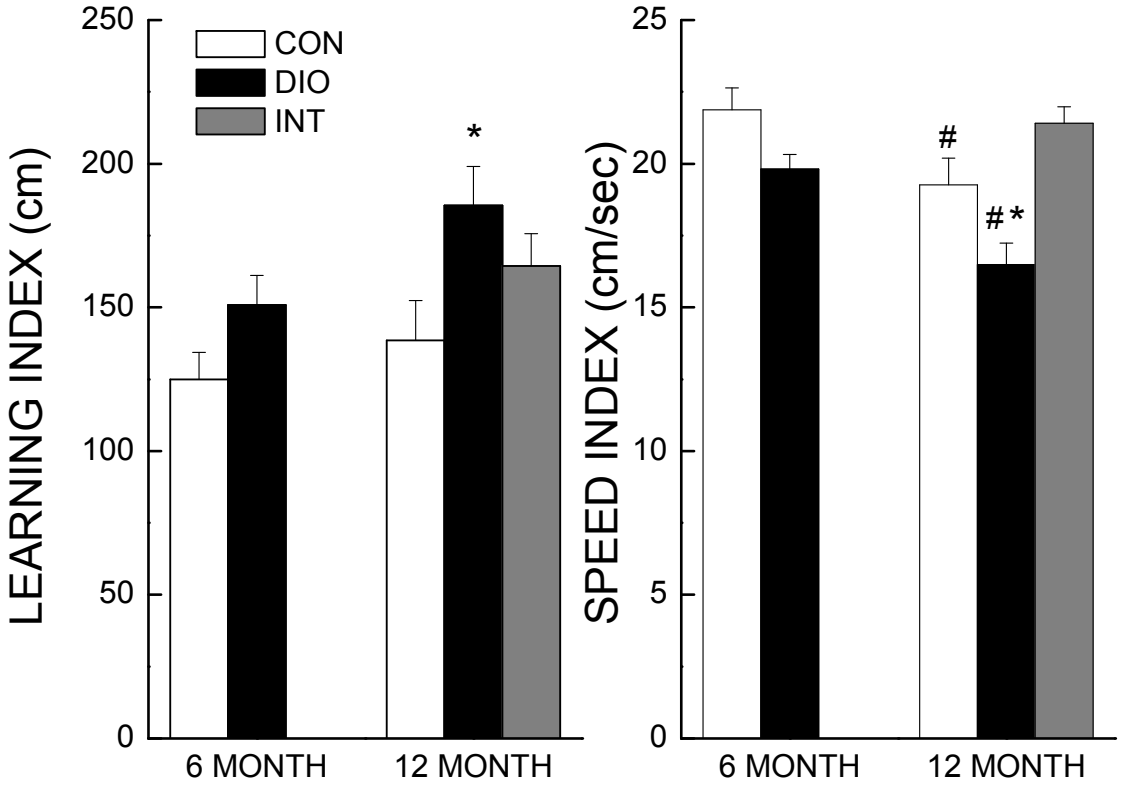

LEARNING INDEX (cm)
250
200
150
100
50
0
CON
DIO
INT
*
6 MONTH
12 MONTH
SPEED INDEX (cm/sec)
25
20
15
10
5
0
#
#*
6 MONTH
12 MONTH

Figure 7. Mean acuity threshold for DIO and control mice. All values represent the mean ± SE of 15 mice. * $p<0.001$ compared to age-matched control

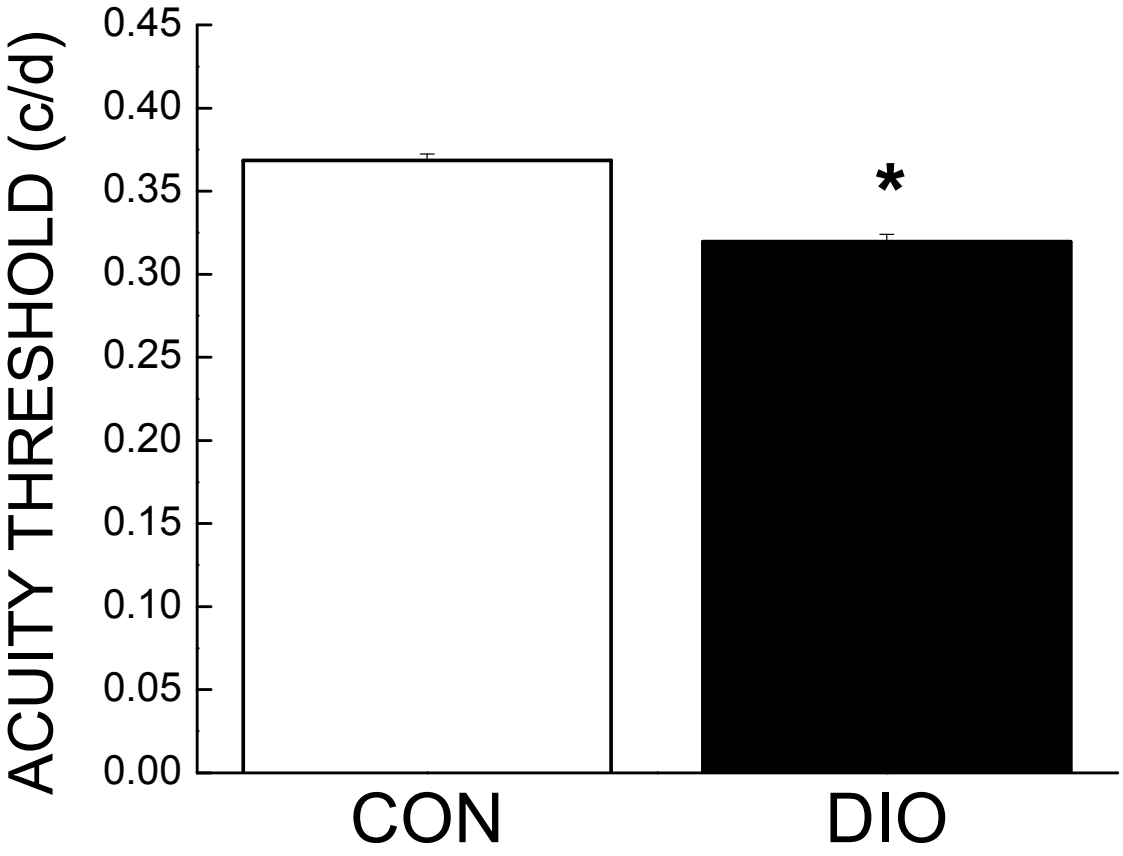
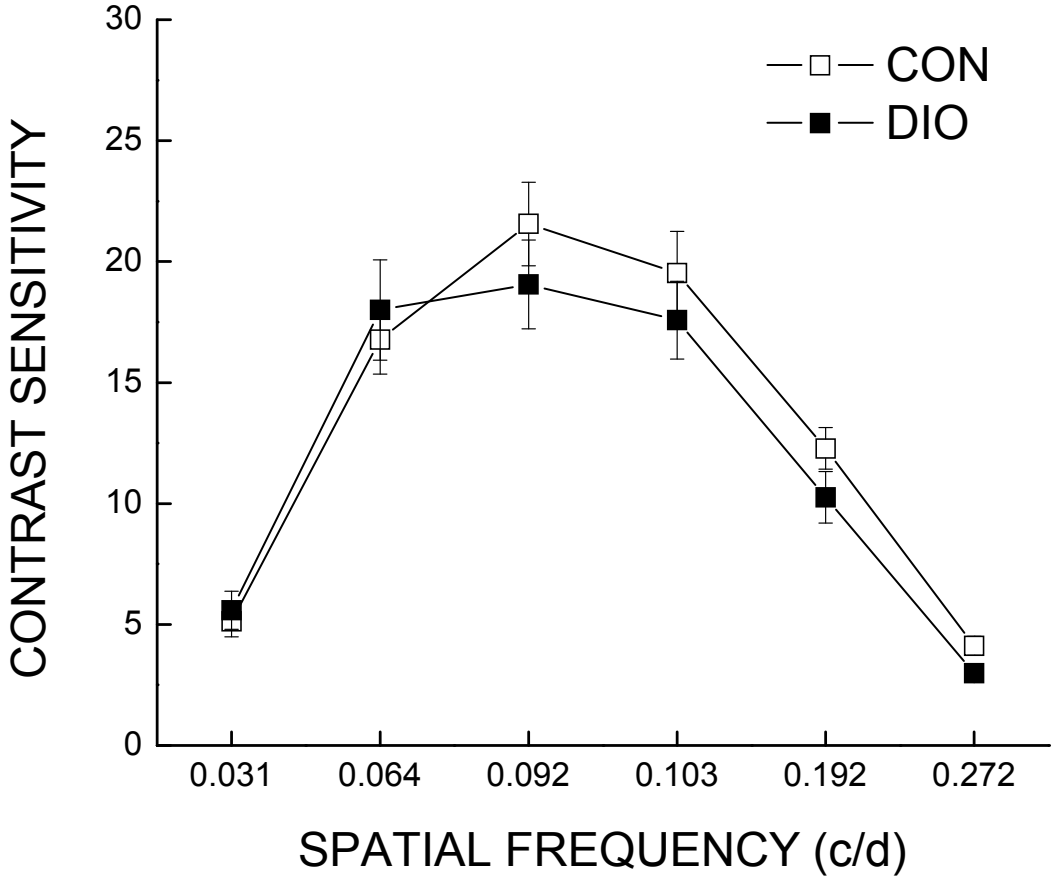

ACUITY THRESHOLD (c/d)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
CON
DIO

Figure 8. Contrast sensitivity function across six spatial frequencies of DIO and control mice. All values represent the mean ± SE of 15 mice.


CONTRAST SENSITIVITY
30
25
20
15
10
5
0
CON
DIO
0.031
0.064
0.092
0.103
0.192
0.272
SPATIAL FREQUENCY (c/d)

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CHAPTER V

DISCUSSION

Obesity, an adverse health condition, has recently been associated with decreased cognitive function in epidemiological studies. More specifically, obese individual regardless of age and gender perform worse than normal weight people on learning and memory tasks (Elias et al., 2003; Fergenbaum et al., 2009; Gatto et al., 2008; Gunstad et al., 2006; Gunstad et al., 2007; Li et al., 2008).

To date, the mechanisms underlying the effect of obesity on cognitive function remain unclear. However, the cellular changes commonly associated with obesity have also been associated with age-related cognitive decline and thus, are likely contributing factors (Bastarrachea et al., 2007; Carney et al., 1991; Forster et al., 1996; Rebrin, Forster, & Sohal, 2007; Smith et al., 1991; Unoki & Yamagishi, 2008; Vincent & Taylor, 2006; Yudkin et al., 2000). Furthermore, the interaction between aging and obesity remains undetermined. It has not been established whether obese individuals display an accelerated rate of brain aging or whether they start with impaired function yet the rate of cognitive dysfunction is identical to normal weight individuals. Most studies have looked at young or adult or aged individuals but there are no studies comparing all age groups. Different models of obesity have been developed to explore potential

mechanistic targets contributing to adverse physiological outcomes that occur with obesity (Augustine & Rossi, 1999; Tschop & Heiman, 2002). The models support either genetic predisposition to obesity or environmentally-induced gain weight. With regards to cognitive function, data from these animal models, regardless of the cause of obesity, are inconsistent (Hwang et al., 2010; Jurdak, Lichtenstein, & Kanarek, 2008; Jurdak & Kanarek, 2009; McNeilly, Williamson et al., 2011; Winocur et al., 2005). The literature is fairly scarce and the discrepancy amongst the studies may explain the various outcomes of obesity on cognitive function ranging from no effect to impairments. In order to investigate the interactive effects of age and obesity, we first had to identify and select a suitable model for both factors. Therefore, we set out to compare a genetic and environment model of obesity on their influence of cognitive and visual function at a young age. We then studied the interactive effect of age and obesity on cognitive function, and determined whether the obesity-induced behavioral and biochemical changes were reversible.

Investigations included within this report provided a behavior profile of 2 obese mouse models in an attempt to determine the mouse model best suited for obesity-related studies. Mouse models selected for cognitive assessment in the current studies reflect genetic and environmental extremes, such that impairments would be apparent. It was hypothesized that obesity would impair cognitive function and that the observed impairments would be exacerbated with age. A reversal of obesity-induced cellular changes can occur with weight loss (Gugliucci et al., 2009; Murri et al., 2010; Rached-Amrouche et al., 2007), therefore, it was further hypothesized that obesity-induced

cognitive impairments would be ameliorated following a dietary intervention at mid-life. The results of the two research papers included in this document contribute substantially in evaluating the correctness of this hypothesis.

Table 1 summarizes studies conducted in our laboratory and their effects on behavioral function and biochemical markers in *ob/ob* and DIO mice. Despite use of different models, obesity had only marginal/or no effect on biochemical markers and cognitive function in adult mice.

Our first study (Chapter II) tested motor, cognitive and visual function in 6-month old male and female obese, leptin-deficient mice (*ob/ob*). This is the first comprehensive behavioral study of this model. The findings in this study suggested that obesity only moderately impairs cognitive function. Results indicated that the degree to which obesity influences cognition is dependent on sex, with females being more sensitive to obesity-induced impairments. Obesity in male mice impaired cortex-associated learning, whereas it impaired learning and cognitive flexibility in the female mice. Furthermore, in a spatial learning task, obese female performed worse on the first session compared to obese males, yet all were able to learn the task to the same extent as the lean controls. The overall lack of obesity-induced spatial learning impairments observed in this study is in accordance with a previous study conducted by Finger et al. (2009) in which young *ob/ob* mice performed as well as control mice on a spatial reference learning task (Finger, Dinan, & Cryan, 2010).

It is worth noting that *ob/ob* mice moved less, swam slower and took a longer path length to find a visible platform, suggesting both motor and visual impairments.

Decreased visual acuity and contrast sensitivity further indicated (Chapter IV) that obese mice were visually impaired. Both motor and visual impairments observed in *ob/ob* mice were exacerbated in females, which could account for the observed gender differences. Collectively, these data suggest that the impairments observed in the female mice may not be due solely on impaired cognitive function, but may be attributed to visual deficits in these mice.

The *ob/ob* mice become extremely obese at an early age (Lindstrom, 2007); yet, this strain did not seem suitable for aging studies due to a rapid decline in quality of life (Harrison, Archer, & Astle, 1984) after 6-months of age even though other studies do not support a shortening of the lifespan. Therefore, in order to observe whether or not obesity-induced impairments were exacerbated with age and, to more accurately reflect human obesity, a diet-induced obese model was used in place of the *ob/ob* model in a second study.

Cognitive, visual and motor function were tested in 6- and 12-month old male C57BL/6 mice that had been maintained on either a control (10% kcal from fat) or high-fat (60% kcal from fat) diet since 6-weeks of age (Chapter III). A subset of the 12-month-old mice fed the high fat diet were gradually switched to the control diet at 6-months of age to assess the effects of a dietary intervention on the predicted obesity-induced impairments. Interestingly, results from this study indicated a lack of cognitive impairments in obese mice. The finding that high fat fed obese mice were not spatially impaired are in accordance with additional studies suggesting a lack of cognitive impairments in high-fat fed animals (Alzoubi et al., 2009; Jurdak et al., 2008; Jurdak &

Kanarek, 2009; Mielke et al., 2006; White et al., 2009). On the contrary, cognitive impairments have been reported in high-fat fed mice in studies that employed different methods to measure cognition other than those used in the current studies (Hwang et al., 2010). Learning acquisition index of the water maze test was impaired in 12-month old mice, suggesting that cognitive dysfunction may be more strongly associated with age than obesity. Additional findings from this study indicated increased anxiety, and visual impairments (Chapter IV) in high-fat fed mice. The anxiogenic effect was exacerbated in 12-month-old mice.

Despite only moderate effects on cognition, impairments were observed in visible function in both *ob/ob* mice and DIO mice. Typical metabolic changes associated with obesity include hyperglycemia, hyperinsulinemia, hypercholesterolemia and dyslipidemia (Keller & Lemberg, March 2003). Chemical glycation measures are commonly associated with these metabolic changes (Ansari & Rasheed, 2010; Nawale, Mourya, & Bhise, 2006) and, even though our study did not support this association, it is likely that *ob/ob* mice and DIO mice displayed some of these as these changes are observed within 4 weeks in *ob/ob* mice (Dubuc, 1976; Lindstrom, 2007; Tomita et al., 1992) and one-week in high fat fed, obese animal models (Petro et al., 2004; Winzell and Ahren, 2004; Mielke et al., 2006a). Long-term changes to these metabolic markers can lead to diabetic retinopathy, the leading cause of blindness in developed countries (Rodriguez-Fontal, 2009; Hann, 2009). Eventually, *ob/ob* and DIO mice learned to associate a visual cue with a hidden platform and perform as well as control mice on

other behavior tasks suggesting that these mice were not blind, but had mild visual problems likely resulting from altered metabolic markers.

From this study, it can be concluded that diet-induced obesity impairs visual, but not cognitive function in an age-dependent manner.

Together, the current findings are not in accord with the aforementioned epidemiological studies that report cognitive dysfunction in obese humans and, therefore, do not support the hypothesis that obesity impairs cognitive function. Biochemical analyses revealed increased serum levels of pro-inflammatory cytokines of *ob/ob* mice, but levels of protein oxidation and chemical glycation measured in brain and skeletal muscle homogenates of *ob/ob* and DIO mice indicated that there was no significant increase in carbonyl or CML content with obesity. In fact, levels of CML were decreased in skeletal muscle homogenates of *ob/ob* and DIO mice and in the hippocampus of DIO mice. While not expected, these results are in accordance with a human study in which plasma levels of CML were decreased in obese children (Sebekova et al., 2009). The absence in cellular changes likely account for the overall lack of cognitive impairments observed in these studies.

A schematic summary of the hypothesized mechanism by which obesity impairs cognitive and visual function is shown in Fig 1. Although the current studies do not provide direct evidence in support of the proposed association between obesity, functional loss and biochemical markers, aforementioned literature introduced in chapter 1 indicates a strong correlation between these factors and provide a possible explanation to obesity-induced loss of function. As depicted in Fig 1, obesity results in

the increased oxidative damage, AGEs and inflammation. Increases in these biochemical markers are observed with age and have been linked to age-related cognitive decline. The interplay between these markers contributes to impaired cell signaling or metabolic dysregulation, and ultimately results in functional loss.

In conclusion, the degree to which obesity affected cognitive function was limited. Even though marked differences in performance were observed between male and female *ob/ob* mice on an active avoidance paradigm, the overall effect on cognition in both studies was marginal. Increased inflammation was observed in ob/ob mice, but additional cellular changes were not observed. However, visual impairments were observed in obese mice, suggesting that *ob/ob* and DIO mice may be valid animal models for investigating obesity-induced visual, but not cognitive impairments.

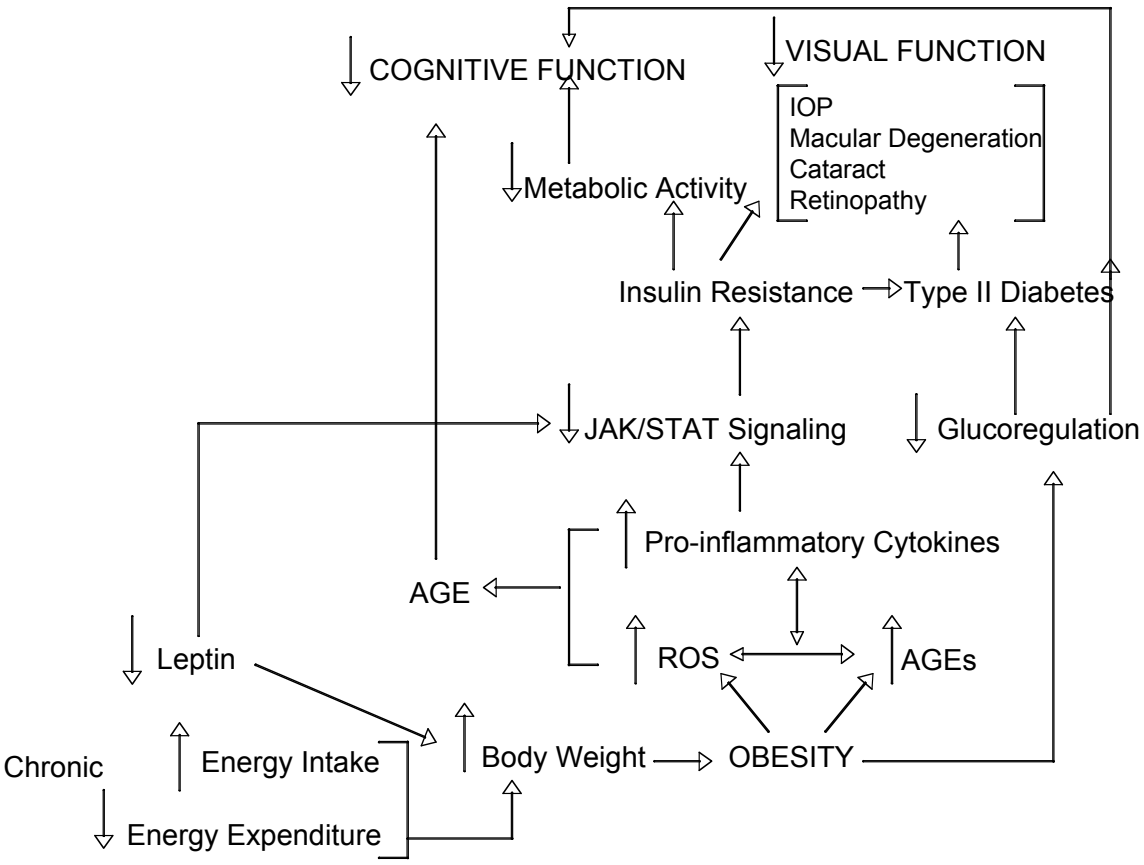
Even though high fat diets contribute to obesity and, in some cases affect cognitive function, (Hwang et al., 2010) a recent study suggested that consumption of a high fat and high sugar diet, more accurately reflects human food intake and has a greater impact on behavioral performance (Molteni et al., 2002). Future studies should determine the effect of a high fat/high sugar diet on cognitive function in aged male and female mice and determine whether or not a dietary intervention is capable of reversing observed impairments.

Table 1. Summary of biochemical and behavioral changes that occur with obesity.

	ob/ob		6-month	12-month	
Biochemical Analyses	Male	Female	DIO	DIO	INT
Pro-inflammatory Cytokine					
TNF-a (SERUM)	↑	↑	0	0	0
IL-6 (SERUM)	↑	↑	0	↑	↑
Chemical Glycation (CML)					
CX	0	0	0	0	0
HP	0	0	↓	↓	0
CB	0	0	0	0	0
SK	↓	↓	↓	↓	0
Protein Oxidation (CO)					
CX	0	0	0	0	0
HP	0	0	0	0	0
CB	0	0	0	0	0
Behavior Profile					
Motor Function					
Locomotor Activity	↓	↓	0	0	0
Swim Speed	↓	↓	↓	↓	↓
Cognitive Function					
Water Maze	0	↓	0	↓	↓
Active Avoidance	↓	↓	0	0	0
Visual Function					
Visible Platform	↓	↓	↓	↓	↓
Visual Acuity	↓	↓	n/a	↓	↓
Contrast Sensitivity	↓	↓	n/a	0	0
Anxiousness					
Elevated Plus Maze	n/a	n/a	↑	↑	↓

Relative to control

Figure 1. Proposed mechanism describing obesity-induced loss of function.


COGNITIVE FUNCTION
VISUAL FUNCTION
IOP
Macular Degeneration
Cataract
Retinopathy
Metabolic Activity
Insulin Resistance
Type II Diabetes
JAK/STAT Signaling
Glucoregulation
Pro-inflammatory Cytokines
AGE
Leptin
ROS
AGEs
Chronic
Energy Intake
Body Weight
OBESITY
Energy Expenditure

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