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Doctoral Dissertations, 2011-2026 Neuroscience

2013

The influence of obesity on structure, biochemistry and function of brain regions involved in cognition

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Princeton, NJ : Princeton University

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The influence of obesity on structure, biochemistry and function of brain regions involved in cognition

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A DISSERTATION PRESENTED TO THE FACULTY OF PRINCETON UNIVERSITY IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PSYCHOLOGY AND NEUROSCIENCE

RECOMMENDED FOR ACCEPTANCE BY THE DEPARTMENT OF PSYCHOLOGY

Adviser: Elizabeth Gould

JANUARY 2013

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Dedicated in loving memory of Dr. Bartley Hoebel, who was a mentor not only in science, but in all aspects of life.

Short Abstract

Obesity is a major public health problem, affecting more than one-third of the U.S. population. Several studies suggest deficits in cognition in obese people and several neuroimaging studies indicate reduced volume of certain brain regions, including the hippocampus and prefrontal cortex. These brain regions are important for cognition and anxiety regulation, and thus structural change in them may contribute to alterations in cognition and mood reported in overweight people. No studies have investigated the effects of weight gain on brain structure and function at a level of analysis that would permit identification of cellular mechanisms, which could lead to future treatment options. This dissertation uses a rat model of diet-induced obesity (DIO) to explore behavioral, structural and biochemical changes in three brain regions important in cognition: the medial prefrontal cortex (mPFC), perirhinal cortex (PRC), and hippocampus (HIP).

Obese rats performed poorly on cognitive tasks specific to the mPFC and PRC, but not the HIP, compared to normal weight controls. In order to begin to characterize the behavioral differences observed, the influence of obesity on brain volume, dendritic architecture and spine density, as well as on associated pre- and post-synaptic markers in the mPFC, PRC and HIP were determined. Deficits in mPFC and PRC-related tasks were accompanied by decreased dendritic spine density and decreased pre- and post-synaptic markers in the mPFC and PRC. Finally, to identify potential mechanisms that might be driving these results, hormones that have previously been linked to changes in brain structure, and/or metabolism and obesity, were surveyed. While there was no difference in testosterone, glucose or insulin levels between groups, leptin was

increased in the DIO model, providing a potential mechanism leading to changes in neurological structure and function. Further, obese rats had decreased peripheral corticosterone levels, a condition previously linked to decreased dendritic architecture, suggesting another potential involved mechanism.

The DIO animal model of obesity has allowed us to look into the cellular changes that underlie alterations in brain structure and function, and provided us with foundational research needed to identify mechanisms for future intervention.

Full Abstract

Obesity is a major public health problem, affecting more than a third of the U.S. population. Several neuroimaging studies suggest that obese humans exhibit reduced volume of certain brain regions, including the hippocampus and prefrontal cortex. These brain regions are important for cognition and anxiety regulation, and thus structural change in them may contribute to alterations in cognition and mood reported in overweight people. No studies have investigated the effects of weight gain on brain structure and function at a level of analysis that would permit identification of cellular mechanisms, which could lead to future treatment options.

This dissertation uses a rat model of diet-induced obesity (DIO) to explore behavioral, structural and biochemical changes in three brain regions important in cognition: the medial prefrontal cortex (mPFC), perirhinal cortex (PRC), and hippocampus (HIP).

Obese and non-obese rats were tested on a series of cognitive tasks that have been linked to the brain regions of interest, as well as a battery of tests to determine whether poor performance on cognitive tasks was confounded by deficits in basic sensory and motor abilities. While no deficits were found in visual, olfactory, somatosensory or motor functioning, obese rats did perform poorly on cognitive tasks specific to the mPFC and PRC, but not the HIP.

In order to further characterize the behavioral differences observed, the influence of obesity on brain volume, dendritic architecture and spine density, as well as on associated pre- and post-synaptic markers in the mPFC, PRC and HIP were

determined. While overall brain volume was not different between the obese and normal weight animals, the volume of the mPFC was smaller in the obese animals than the controls. Further, dendritic spine density was decreased on pyramidal neurons in layer II/III of the mPFC and layer II of the PRC, but not in the HIP (CA1 region). Obese rats also had decreased synaptic protein concentrations (spinophilin, synaptophysin, vesicular GABA transporter and vesicular glutamate transporter) in the mPFC and PRC, but not HIP.

Finally, to identify potential mechanisms that might be driving observed changes in cognition and brain structure in the DIO model, hormones that have previously been linked to changes in brain structure, as well as those that have been linked to metabolism and obesity, were surveyed. Peripheral levels of corticosterone, testosterone, insulin, and leptin, were determined in the diet-induced obesity model. Abnormal corticosterone levels, either decreased or elevated, have been associated with decreased dendritic architecture. In the current study, obese rats decreased peripheral corticosterone levels. There was also no difference in testosterone, glucose or insulin levels between groups, however, leptin was increased in the DIO model.

The DIO animal model of obesity has allowed us to look into the cellular changes that underlie alterations in brain structure and function, and provided us with foundational research needed to identify mechanisms for future intervention.

Acknowledgements

Over the past many years I have received support and encouragement from a great number of individuals. I would like to thank Dr. Elizabeth Gould, who adopted me into her research group and has graciously advised this dissertation. I would like to thank my reading and oral dissertation committees, comprised of Drs. Michael Graziano, Nicole Shelton, Nicholas Turk-Browne, Sam Wang and Ilana Witten, for their support and guidance in this research.

I am grateful to Drs. Julia Caponiti and Timothy Schoenfeld for their guidance in the laboratory, encouragement, friendship and comradely support. I also thank Maria Fasolino, Matthew Runkle and Greg Kirschen for their assistance with the research presented herein.

It is both a pleasure and an honor to call Dr. Nicole Avena my friend, advisor and mentor. Her guidance and friendship has been a source of encouragement and inspiration, which is by no means limited to the scope of this dissertation.

I have unceasing gratitude for the time I was able to spend with my advisor, Dr. Bart Hoebel. Bart's enthusiasm for science was infectious, his insight was invaluable and his dedication to teaching was admirable. He was a supreme mentor, and in my future career I hope to model his example. Not only was Bart zealous about science, but also about life. He taught me to engage with the world around me, love people and take advantage of each moment I am given. He encouraged me to pursue my passions without hesitation. I will carry his memory with me at all times.

My love and thanks are especially extended to my husband, Andrew. He has been a constant support throughout my graduate school experience, and without his love and faith, none of this would have been possible. My success in this adventure is as much his as it is mine. Equally important, I thank my parents, who must be credited for teaching me to think like a scientist and encouraging me to explore the world. They have been great supporters, true role models and unwavering mentors. From speaking encouragement into me when I needed it most to editing proposal drafts, their contributions to this dissertation, both intangible and concrete, have been invaluable and true labors of love. Finally, I thank my sister, Naomi, for her support, advice and constant encouragement to self-advocate, and my brother, Joshua, who lovingly provided digital design and formatting assistance, no matter how short a deadline I faced or how late at night it was. The work contained within this bound volume is truly the reflection of a team effort.

And most importantly, *Soli Deo gloria*.

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Abbreviations

ASST	Attentional set-shifting task
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 1
CD	Complex discrimination phase of ASST
DIO	Diet induced obesity
DR	Discrimination Ratio
EDS	Extradimensional attentional shift phase of ASST
HIP	Hippocampus
HPA axis	Hypothalamic-pituitary-adrenal axis
IDS	Intradimensional attentional shift phase of ASST
MAP-2	Microtubule-associated protein 2
mPFC	Medial prefrontal cortex
PFC	Prefrontal cortex
PRC	Perirhinal cortex
QUICKI	Quantitative insulin sensitivity check index
REV	Reversal phase of ASST
SD	Simple discrimination phase of ASST
vGAT	Vesicular GABA transporter
vGLUT	Vesicular glutamate transporter

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Chapter 1

General Introduction

Obesity and overweight are major public health concerns, with the combined affecting nearly 70 percent of the U.S. adult population (Flegal et al., 2012). Obesity increases the risk of a number of health conditions including hypertension, adverse lipid concentrations, type 2 diabetes, cancer and stroke, as well as leading to national annual medical costs estimated at $147 billion (Finkelstein et al., 2009). For these reasons, research surrounding obesity and weight gain are crucial.

Although most obesity research focuses on the deleterious metabolic-related effects of obesity, select studies have turned to the cognitive effects of excessive weight gain. Currently, most of that research focuses on the medically ill obese, such as those with diagnoses of metabolic syndrome and type II diabetes. Metabolic syndrome, a disease characterized by a clustering of central obesity, elevated blood pressure, dyslipidemia (elevated triglycerides and lowered high-density lipoprotein cholesterol), and insulin resistance, has been well associated with an increase risk for cognitive impairment or dementia (Crichton et al., 2011). Likewise, type II diabetes, characterized, predominantly, by insulin resistance (Alberti et al., 2009) has been suggested to effect cognitive functions such as intelligence, attention, psychomotor speed, cognitive flexibility, learning and memory (McCrimmon et al., 2012). Diabetes is also associated with an increased risk of developing dementia (Yaffe et al., 2004b; Biessels et al., 2006; Kloppenborg et al., 2008), and a 50-100% increase risk of Alzheimer's disease (Yaffe et al., 2004a; Biessels et al., 2008).

However, recent studies have reported correlations between obesity and cognitive performance in otherwise healthy subjects (Gunstad et al., 2007; Gunstad et al., 2008). While some studies have identified a correlation between higher body mass index (BMI, which is a reliable indicator of body "fatness" based on a manipulation of height and weight) during mid-life and development of Alzheimer's disease (Gustafson et al., 2003; Kivipelto et al., 2005; Whitmer et al., 2007) or dementia later in life (Kloppenborg et al., 2008; Fitzpatrick et al., 2009), recent studies indicate a role of obesity and cognitive decline earlier in life. Findings indicate that cognitive performance, both rudimentary and scholastic, declines with increases in body mass and energy consumption (Hillman et al., 2008; Tomporowski et al., 2008; Donnelly et al., 2009; Vadiveloo et al., 2009). This includes research in school-aged children, where an inverse relationship between obesity and cognition has been identified (Taras and Potts-Datema, 2005).

Accompanying these behavioral deficits, obesity has been associated with overall decreased brain volume (Ward et al., 2005), with further studies indicating reductions in the volume of certain brain regions, including the hippocampus (HIP) and prefrontal cortex (PFC) (Ward et al., 2005; Pannacciulli et al., 2006; Raji et al., 2010; Walther et al., 2010). Interestingly, no differences are seen in brain volume between the overweight and the obese, potentially indicating deleterious effects of any extra weight gain (Raji et al., 2010). In addition to these structural changes, functional changes have been noted. For example, recent proton magnetic resonance spectroscopy studies also showed that higher BMI was correlated with lower concentrations of *N*-acetyl-aspartate, a spectroscopic marker of neuronal integrity in frontal, parietal, and temporal lobes, with

the largest effects in the frontal lobe (Gazdzinski et al., 2008; Gazdzinski et al., 2010). Further, a negative correlation between BMI and metabolic activity in the PFC, as well as performance on tests of memory and executive function have been observed (Volkow et al., 2009). A decrease in blood flow, indicating decreased neural activity, has been shown in the prefrontal cortex of normal, healthy individuals who have elevated body mass (Willeumier et al., 2011).

Given that the PFC and HIP are important for cognition (Ergorul and Eichenbaum, 2006; Zald and Andreotti, 2010), it is possible that structural and functional changes in these specific areas may contribute to those observed alterations in cognition. Despite these compelling associations, few studies have investigated the effects of weight gain on brain structure and function at a level of analysis that would permit identification of cellular mechanisms. Implementing an animal model of obesity allows us to look into the cellular changes that underlie alterations in brain volume and function, and may set the stage for identifying future mechanisms of intervention.

Animal Models of Obesity

There are several animal models of obesity, including genetic, as well as environmental models. The *ob/ob* mouse (where “ob” stands for obesity) is one example of genetic obesity, where a spontaneous single-gene loss-of-function mutation generates decreased leptin expression and massive obesity. The *ob/ob* mutation is recessive, and mutant mice are normal at birth, compared to control littermates, but show uncontrolled food intake and the ensuing obesity (Kanasaki and Koya, 2011). The

db/db mouse (where “db” stands for diabetes) is a model where an autosomal recessive trait which encodes for a mutation in the leptin receptor gene, leads to defective leptin signaling, persistent hyperphagia and resulting obesity (Chen et al., 1996; Lee et al., 1996). Another autosomal recessive mutation leading to deficits in the leptin receptor gene, and the resulting obesity is seen in the Zucker Fatty Rat (Ogawa et al., 1995). While these models have been important in studies of obesity, spontaneous single-gene loss of function mutations are rare, and affect only a trivial portion of rodent and human populations (Farooqi and O'Rahilly, 2005). For example, all of the above models have mutations in the leptin gene or receptor; similar mutations in humans have only been reported in the clinical literature in 15 patients up until 2009 (Mazen et al., 2009).

Further, while monogenic models provide important information on the biology of obesity, human obesity is most likely mediated by multiple genes. Therefore, polygenic models could be much more relevant. The New Zealand Obese (NZO) mouse is one such polygenic model of obesity. NZO mice show the most profound obesity of all the phenotypes (Herberg and Coleman, 1977), combining hyperphagia and hypoactivity (Jurgens et al., 2006). However, only male NZO mice show obesity, which is inconsistent with the human condition. Most polygenic models of obesity are accompanied by diabetes, such as the Tsumura Suzuki Obese Diabetes Mouse (Suzuki et al., 1999), the Kuo Kondo mouse (Ikeda, 1994) and the Zucker Diabetic Fatty Rat (Friedman et al., 1991), making them irrelevant in studying the non-ill obese.

Genetically engineered models, either knockout or transgenic, can also be used in studying obesity. While several proteins, signaling molecules and hormones important in the development of obesity can be genetically removed in knockout

models, these animals tend to not live long enough to actually develop the obese phenotype. For example, insulin receptor-null mice tend to live no more than a matter of days (Bruning et al., 1998). Thus, these models are only effective in studying aspects of obesity, but not the condition holistically. Further, these knockout models don't mimic the human condition. Like spontaneous single-gene loss of function mutations, knockout models only represent disorders seen in the minority of clinical patients. For example, insulin receptor loss in humans has only been documented in four case reports in a recent 10 year period (Kitamura et al., 2003).

Transgenic models are another option for studying obesity, and the human obesity gene map noted at least 248 genes that, when mutated or expressed as transgenes in the mouse, result in aberrant body weight phenotypes (Perusse et al., 2005). But it is clear that in most circumstances, obesity is a polygenic trait, resulting from the combined actions of many genes (Speakman et al., 2007). Given so much variation, identifying which gene or combinations of genes are most important or clinically relevant when generating the obesogenic phenotype can be challenging. Further, while research advances have highlighted the importance of genetic factors in determining individual susceptibility to obesity, the dramatic rise in obesity in the US over such a short period of time strongly indicates this epidemic is driven by changes in environment rather than genetics (Hill and Peters, 1998; French et al., 2001). For that reason, the diet-induced obesity (DIO) model seems to be the most clinically relevant and will be implemented in the current studies.

Inducing obesity using hypercaloric diets (diet-induced obesity) has provided a means of precipitating obesity, and has often been used as a non-leptin-deficient model.

It is also most similar to the human condition of obesity, in that weight gain results from caloric overconsumption. Many common mouse and rat strains can be used in this model, such the C57BL/6J mice or the Sprague Dawley rats used in the current study. There is a great amount of variety in diets implemented in the DIO model, and while many diets, such as fructose or sucrose-rich diets, have been shown effective in inducing obesity, a combination of high-carbohydrate and high-fat most closely mimics the modern Western diet. Rodents fed high-sucrose, high-fat diets have increased body weight, and abdominal fat deposition, and eventually hyperinsulinaemia, hyperglycaemia and hyperleptinaemia. Increased body weight is significant after as little as 2 weeks, but the phenotype becomes more obvious given longer durations on the diet (Sutherland et al., 2008). This is beneficial in that researchers can study the development of obesity at several time points, including the pre- and post-diabetic or metabolic syndrome stages.

Even within the DIO literature implementing high-carbohydrate and high-fat diets, there is a great amount of variation in diet composition; fat can account for 20 to 60% of total energy, while sugar can account for up to 30%. The sources of these macronutrients can vary, with fats derived from animals or plants. A variety of sugars or sugar combinations can also be used, such as sucrose (which is most common), fructose or glucose. Further, high-fat, high-carbohydrate diets can be made available in addition to a lower fat diet (such as standard rodent chow) or as a nutritionally complete dietary alternative. The current study will use a commercially available, pelleted diet with 45% fat and 35% carbohydrate (Research Diets Inc). The fat sources are soybean oil

(12%) and lard (88%). Sucrose makes up 50% of the carbohydrates (17% of the overall caloric content).

Mimicking human consumption patterns, it is known that not all rats fed a high-fat diet will show the DIO phenotype, but the large majority do (~4% become morbidly obese, ~4% remain lean and the remainder represent a continuum between these extreme phenotypes (Tschop and Heiman, 2001)). Those animals susceptible to obesity are hyperphagic, while rats remaining lean on a high-fat diet consume about the same number of calories as rats fed standard rodent chow (Farley et al., 2003). While not implemented in the current study, this “obesity-resistant” group of animals that maintain normal levels of caloric intake, even while on a high-fat diet, might be an important group to investigate in light of identifying obesity treatments and prevention.

Given that the physiological aspects of the DIO model replicate many of the features observed with the human obesity, including a polygenic mode of inheritance (Levin et al., 1997), a persistence of the phenotype once it is established (Levin et al., 1989), a sub population that is resistant to weight gain (Tschop and Heiman, 2001), and eventual dysregulation of glucose homeostasis (Levin and Sullivan, 1987; Chang et al., 1990), the Sprague-Dawley DIO model is an appropriate tool for investigating human obesity, and the resulting effects on brain structure and function.

Brain areas identified in clinical studies of obesity

Given the clinical literature associating obesity with overall decreased brain volume (Ward et al., 2005), as well as decreases in volume of the HIP and prefrontal

cortex (Ward et al., 2005; Pannacciulli et al., 2006; Raji et al., 2010; Walther et al., 2010), and functioning requiring these areas, these are logical areas to initiate our analysis in the DIO model.

Obesity has been linked to executive dysfunction, including decreased performance in planning, problem solving and mental flexibility (Gunstad et al., 2007; Boeka and Lokken, 2008; Walther et al., 2010). The prefrontal cortex is the brain's executive processing center, allowing for cognitive flexibility, planning and forethought. It has been suggested that the prefrontal cortex, as a processing center, ultimately allows for the output of proper goal-oriented behavior. When this processing center, or its connectivity, becomes disrupted, deficits in cognitive flexibility, information processing speed, reasoning and attention are observed.

The hippocampus, a brain area involved in memory formation and spatial navigation, is linked closely to clinical accounts of obesity. Decreased hippocampus volume has been identified in the obese (Raji et al., 2010), and functional deficits in memory have been recorded in the diabetic obese (McCrimmon et al., 2012). The hippocampus is also closely linked to the medial prefrontal cortex (mPFC), with unidirectional projections from the ventral CA1 region of the hippocampus to the mPFC (Jay et al., 1989; Jay and Witter, 1991; Laroche et al., 2000). It is believed that the mPFC does project to the hippocampus, but not directly (Vertes et al., 2007).

The final brain area that we will explore in the current dissertation is the perirhinal cortex (PRC). This area has not been directly implicated in the clinical obesity literature, however, it is directly connected to both the mPFC and the HIP and has a well established role in memory (Brown and Aggleton, 2001). The PRC has a bidirectional

connection to the mPFC (Conde et al., 1995; Delatour and Witter, 2002), with strong, excitatory projections (Room et al., 1985; Room and Groenewegen, 1986; Sesack et al., 1989), as well as bidirectional connections with the CA1 region of the hippocampus. It has been suggested that the mPFC receives object information from the perirhinal cortex, which is integrated with spatial information received from the hippocampus; thus, an integrated network, formed by the mPFC, HIP and PRC, is necessary for fully functional recognition memory (Warburton and Brown, 2010).

The current study will explore behavioral and neurochemical assays of these three areas critical to memory and cognition.

Assays of Behavior

There is an elaborate literature disintegrating the roles of the mPFC, HIP and PRC in memory. In developing this literature, lesion studies have identified tasks that are specific to each area, or a specific combination of areas (Hannesson et al., 2004a; Hannesson et al., 2004b; Barker et al., 2007; Barker and Warburton, 2011). These tasks include the Novel Object Recognition Task, which is based on the premise that rodents will explore a novel object more than a familiar one, but only if they remember the familiar one. While previously many suggested that this task was hippocampal dependent, recent lesion studies indicate that this test does not require the HIP, but is in fact dependent on the PRC (Barker and Warburton, 2011). Meanwhile, lesion studies indicate that the Object Location Task is a hippocampus-dependent test, where the rat's ability to recognize an object it has experienced before in a changed location is

assessed. The Object-in-Place Task depends on both the HIP and either the PRC or mPFC (Barker and Warburton, 2011). This task can substantiate the results of the Novel Object Recognition and Object Location Tasks, while also alluding to a potential role of the mPFC. Finally, the Attentional Set-Shifting Task (ASST) will be used to specifically evaluate mPFC operation. As previously stated, one function of the mPFC is to modulate attentional processes and behavioral flexibility. The ASST is based on the premise that when attending to a perceptual feature of a stimulus (such as shape), learning to discriminate new complex stimuli is more rapid when the discrimination rule is based on the same perceptual dimension (Birrell and Brown, 2000). These tasks can each be performed in the DIO model, and will provide preliminary evidence for dysfunction in the previously mentioned brain regions. Behavioral differences on these tasks will then provide motivation to examine the involved brain regions on a more specific, neuronal and neurochemical level.

Brain Plasticity

Dendritic spines, which are the sites of most excitatory connections between neurons, are small membranous protrusions located on dendrites of many types of neurons (Sorra and Harris, 2000). In adult mammals, dendritic spines undergo changes in shape, size and number in response in neural activity, hormones and experiences (Alvarez and Sabatini, 2007). Our laboratory has shown that experience alters dendritic architecture and function within both the HIP and mPFC. Positive experiences, such as mating behavior, parenting, running or living in an enriched environment, increase

dendritic complexity (Kozorovitskiy et al., 2006; Stranahan et al., 2007; Leuner and Gould, 2010). Meanwhile, stress and the associated hormonal changes, decrease dendritic branching in hippocampal CA3 pyramidal cells (Gould et al., 1990; Woolley et al., 1990a; Watanabe et al., 1992), dendritic spine density on CA1 pyramidal neurons (Shors et al., 2001; Liston et al., 2006; Radley et al., 2006) and the mPFC (Radley et al., 2006; Radley et al., 2008). In addition to dendritic complexity, positive experiences enhance performance on cognitive tasks, such as the ones described previously, which require the HIP and mPFC (Leuner and Gould, 2010). Similarly, negative experiences, such as stress, impair cognitive performance (McEwen, 2000b; Liston et al., 2006). It has been suggested that these changes in dendritic architecture are therefore mediating the diminished cognitive function. Obesity can be categorized as a negative, or stressful biological state, and thus might similarly lead to diminished dendritic architecture and cognitive performance. In the current study, in order to examine dendritic architecture in obese versus control animals, dendritic spine density and branching of pyramidal cells in the brain regions of interest were determined. To further support data on dendritic architecture, this study looks at the presence of specific synaptic and dendritic markers Together, these methods allow us to begin to characterize neuronal differences in the obese state.

Hormone abnormalities in obesity

Several hormones have been implicated in dendritic architecture remodeling in the mPFC and HIP, with concurrent decrements in cognitive tasks that require the same

areas (Sousa et al., 2000; Liston et al., 2006; Radley et al., 2006). Further, several of these identified hormones are abnormally regulated in the obese state. For example, abnormal glucocorticoid levels have been associated with changes in dendritic architecture. Increased glucocorticoids have been associated with diminished dendritic architecture and spine density in the mPFC and HIP (Sousa et al., 2000; Wellman, 2001; Liston et al., 2006; Radley et al., 2006; Hajszan et al., 2009). Repeated injections of glucocorticoids not only produces dendritic shrinkage in both the HIP and mPFC, but also increase anxiety-like behavior and impaired cognitive function (Wellman, 2001). On the opposite end of the spectrum, abnormally low or absent glucocorticoids levels have also been associated with changes in dendritic morphology in the mPFC and HIP (Sousa et al., 1997; Cerqueira et al., 2007), as well as performance on mPFC and HIP dependent tasks, such as the behavioral flexibility task (Mizoguchi et al., 2004; Cerqueira et al., 2005) and the Morris water maze (Islam et al., 1995), respectively. While the literature agrees that obesity is associated with changes in the hypothalamic-pituitary-adrenal (HPA) axis, the directional implications are mixed. Some clinical accounts associate obesity with low basal cortisol levels (Jessop et al., 2001; Parra et al., 2006). However, several studies indicate that obesity is associated with elevations in glucocorticoid levels (Parente et al., 2008; Benson et al., 2009). Heightened glucocorticoids are typically indicative of a stressful biological state. This is consistent with the clinical literature, in that overweight has, not only, been linked to cognitive decline, but also to increased incidence of depression and anxiety (de Wit et al., 2010). Further, the mPFC and HIP are not only important for cognition (Ergorul and Eichenbaum, 2006; Zald and Andreotti, 2010) but also anxiety regulation (Bannerman et

al., 2003; Herry et al., 2010). This common link in brain region indicates that deficiencies in these brain areas might affect both cognition and mood, and might be triggered by a common underlying mechanism, such as heightened glucocorticoid levels.

Another example of a hormone that is associated with dendritic spine density and metabolism is testosterone. While typically identified as a sex hormone, testosterone has a much broader role, and is an important signaling molecule in regulating energy utilization and multiple cellular metabolic pathways (Traish et al., 2005; Singh et al., 2006; Traish et al., 2009). In males, obesity impairs testicular testosterone biosynthesis, and low levels of testosterone increases accumulation of fat depots, particularly in the abdomen (Saad et al., 2012). Low levels of testosterone, which are seen in obesity, have been linked to decreased dendritic spine density in the hippocampus (Leranth et al., 2003; Brand et al., 2011). This provides another hormonal mechanism that might potentially be driving functional and structural cognitive deficits.

Leptin is another hormone of interest. Leptin is most commonly known for its ability to signal fat storage reserves in the body, and mediate long-term appetitive control (Friedman and Halaas, 1998). However, recent studies indicate a role of leptin in memory. In the brain, leptin has also been shown to enhance hippocampal synaptic plasticity and improve learning and memory (Wickelgren, 1998; Farr et al., 2008). While a strong relationship been dendritic spine density and leptin has not been previously established, the effects of leptin on synaptic plasticity make it a contender for direct or indirect modulation of dendritic architecture.

It is important to examine and understand the role of these hormones in our animal model of DIO, in that hormones, such as the glucocorticoids, testosterone or leptin, that have been previously linked to both changes in the brain and obesity, are potential mechanisms for future intervention.

Summary

This dissertation uses a rat model of diet-induced obesity to explore behavioral, structural and biochemical changes in three brain regions important in memory: the mPFC, PRC, and HIP. Specifically I examine cognitive deficits using behavioral tests of cognition that have previously been linked to these brain areas (Chapter 2). I then examine the influence of obesity on the mPFC, PRC and HIP by looking at dendritic architecture, synaptic markers and brain volumes (Chapter 3). Finally, to further characterize the DIO model and identify potential mechanisms that might be driving observed changes in cognition and brain structure, I examine some hormones that have previously been linked to changes in brain structure, as well as those that have been linked to metabolism and obesity (Chapter 4).

Implementing the DIO animal model of obesity will allow us to look into the cellular changes that underlie alterations in brain structure and function, with the aim of identifying mechanisms for future intervention.

Chapter 2

Obesity is associated with diminished performance on cognitive tasks requiring the mPFC and PRC

Abstract

While there are many clinical findings associating obesity with cognitive deficits, this relationship has not been fully characterized in animal models. Using an animal model of diet induced obesity (DIO), we can assay cognitive behaviors in obese versus normal weight rats. In the current study, adult rats were maintained on a nutritionally complete, palatable, high-fat diet for 8 weeks. At this point, obesity, defined as elevated body weight, increased fat pad accrual and increased serum triglyceride levels, was obvious in the high-fat consuming rats compared to age and weight matched rats maintained on standard rodent chow. After obesity onset, behavioral tasks that are dependent on brain regions important in cognition and memory, including the medial prefrontal cortex (mPFC), perirhinal cortex (PRC), and hippocampus (HIP), were implemented. Rats were also tested for performance on standard sensory and locomotor tasks, to eliminate confounding behavioral deficits that might present as cognitive deficits. Obese rats showed deficits on behavioral tasks requiring the mPFC and PRC, but not HIP. Further, obese rats showed no deficits on sensory or locomotor tasks compared to controls. These results indicate a correlation between obesity and performance on cognitive functions requiring the mPFC and PRC. The data also provide a model, as well as motivation, to further explore potential cellular and structural

changes that could underlie alterations in brain function, with the aim of identifying mechanisms for future intervention.

Introduction

Although obesity has been correlated with cognitive deficits in clinical populations (Elias et al., 2003; Jeong et al., 2005; Li et al., 2008; Naderali et al., 2009), this relationship has not yet been fully characterized in an applicable animal model. In the current study, an environmental manipulation— access to a high-fat, calorically dense, palatable diet— is used to precipitate the obesogenic phenotype. This paradigm is referred to as diet-induced obesity (DIO) (Mercer and Archer, 2005) and is consistent with current thinking that that the obesity epidemic is driven, mainly, by the availability and consumption of high-fat, calorically dense, palatable foods, rather than genetic mutations (Hill and Peters, 1998).

Because clinical research indicates brain volume decreases in both the HIP and prefrontal cortex (Ward et al., 2005; Pannacciulli et al., 2006; Raji et al., 2010; Walther et al., 2010), it follows that tasks dependent on these areas might be effected in the DIO model. Using this model, we implemented validated behavioral tests of cognitive function, which have been shown to be dependent in these specific brain areas, to screen for behavioral evidence of dysregulation. We also selected tasks linked to the PRC, because of its direct connections to both the mPFC and the HIP and its role in memory (Brown and Aggleton, 2001). These tasks include the Novel Object Recognition Task, which is based on the premise that rodents will explore a novel object more than a familiar one, but only if they remember the familiar one. While previously many

suggestted that this task is hippocampal dependent, recent lesion studies indicate that this test does not require the HIP, but is in fact dependent on the PRC (Barker and Warburton, 2011). Meanwhile, lesion studies indicate that the Object Location Task, is a hippocampus-dependent test, where the rat's ability to recognize an object it has experienced before in a changed location is assessed. The Object-in-Place Task depends on both the HIP and either the PRC or mPFC (Barker and Warburton, 2011). This task can substantiate the results of the Novel Object Recognition and Object Location Tasks, while also alluding to a potential role of the mPFC. Finally, the Attentional Set-Shifting Task (ASST) can be used to specifically evaluate mPFC operation. One function of the mPFC is to modulate attentional processes and behavioral flexibility. The ASST is based on the premise that when attending to a perceptual feature of a stimulus (such as shape), learning to discriminate new complex stimuli is more rapid when the discrimination rule is based on the same perceptual dimension (Birrell and Brown, 2000).

In addition to the mentioned cognitive tasks, we examined basic processes, including basic locomotor activity, olfactory differentiating, vibrissae reflex, tactile response and vision. Deficits in any of these areas could confound the interpretation of performance on the cognitive tasks, leading to the conclusions that do not accurately reflect functioning in targeted brain regions. Therefore, in order to eliminate possible confounds, we must establish proper functioning in these basic sensory and motor paradigms.

Given the literature suggesting decreases in brain volume of both the HIP and PFC cortex, and the known connection between the PRC and the HIP and mPFC, we

hypothesize that cognitive function will be diminished on the Novel Object Recognition, Object Location, Object-in-Place and Attentional Set-Shifting tasks. Further, we anticipate that these deficits will not be accompanied by diminished performance on basic motor and sensory tasks.

Methods

Male Sprague–Dawley rats (275-315 g at the onset of the study) were obtained from Charles River (Germantown, NY) and housed individually in wire topped, plastic cages on a 12 h reverse light cycle (lights off at 6am). All animal procedures and protocols were approved by the Princeton University IACUC and follow the NIH Guide for the Care and Use of Laboratory Animals. Testing presented in this dissertation, in its entirety, utilized three separate cohorts of rats. **Supplementary Table 1** describes which aspects of experimentation were done in each cohort.

In the current chapter, two cohorts of animals were used; in each cohort, rats were weight-matched and divided into two groups (n=10/group), and given access to either a nutritionally complete, high-fat diet (4.7 kcal/g; Research Diets, New Brunswick, NJ, #12451; 45% fat, 20% protein, and 35% carbohydrate) or standard rodent chow (3.01 kcal/g; LabDiet #5001, PMI Nutrition International, Richmond, IN; 10% fat, 20% protein, 70% carbohydrate). All animals had ad libitum access to water. Chow intake and body weights were taken weekly. After 8 weeks on the described diets, testing began. Rats with body weights that overlapped between groups were removed from analysis, such that each cohort ended up with n=8-9/group. Rats were tested on a selection of the following behavioral tasks: Novel Object Recognition task, Object-in-

Place task and Object Location task or the ASST, as described below. The order of testing was counterbalanced across rats, but the ASST was always the final test, because it requires significant food deprivation prior to testing.

For the Novel Object Recognition, Object Location and Object-in-Place tasks, the testing apparatus consists of an open-field box (58 × 42 × 35 cm) and all testing was done between 4-6 h after dark onset, during the rats' active period. Animals were brought into the test room, where all phases of testing were done under full light. Each test consisted of a familiarization phase and a recognition phase, as described. The stimuli presented were objects constructed from Duplo blocks (Lego), and varied in shape, color, and size. The experimenter was not in the room during testing; trials were video recorded and analyzed later by an experimenter blind to the conditions. Object exploration was defined as directing the nose toward the object at 2 cm and/or touching the object with the nose or paws. A discrimination ratio was calculated by determining the difference between time spent interacting with the novel object and the known object, divided by the total interaction time with sample object and novel object.

Novel Object Recognition

The Novel Object Recognition task is based on the premise that rodents will explore a novel object more than a familiar one, but only if they remember the familiar one. The procedure was comprised of a familiarization phase and a recognition test, separated by a delay period (**Fig. 2.1 A**). In the acquisition phase, two identical objects were placed near the corners, on one wall in the arena (10 cm from each adjacent wall). During the familiarization phase, rats explored two identical objects (Duplo structures)

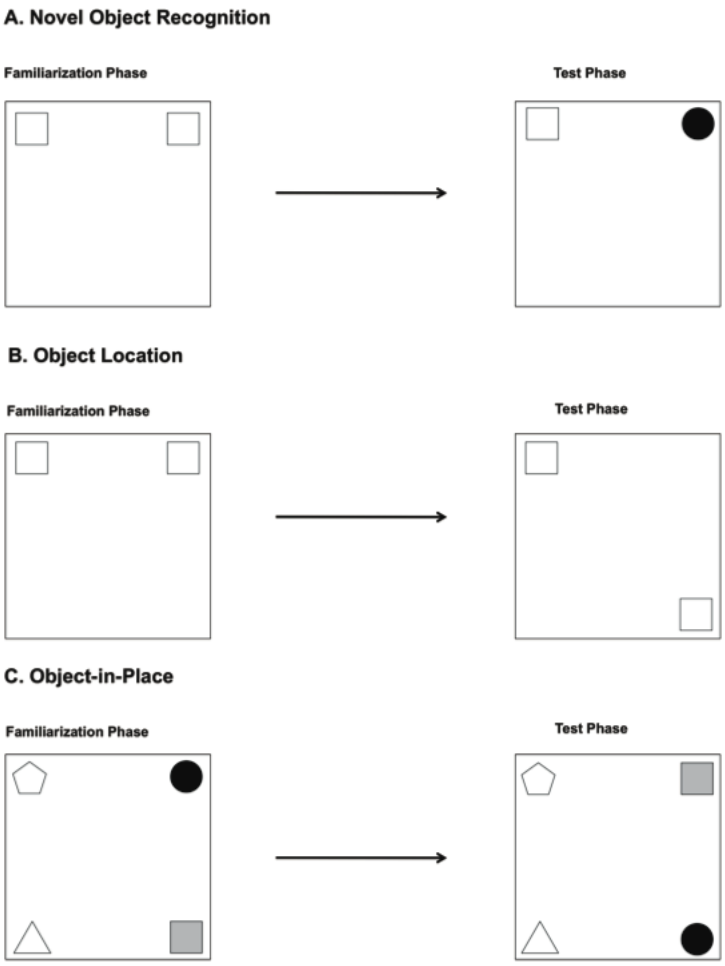


Figure 2.1. Diagrams of the three object recognition memory tasks: Novel Object Recognition Task (A), Object Location Task (B) and Object-in-Place Task (C).

for 3 min and were returned to their home cages for 3 hrs (Jessberger et al., 2009). During the recognition phase, rats were returned to the testing apparatus, presented with a third copy of the familiar object and a novel object, and allowed to explore them for 3 min. The left/right position of the novel object was counterbalanced between each rat.

Object Location

This test assessed the rat's ability to recognize that an object it had experienced before had changed location. In the familiarization phase, the rat was exposed to two identical objects, which were placed in the far corners of the arena (as in the Object Recognition task; **Fig. 2.1 B**). The animal was allowed to explore both objects during the familiarization phase of 3 min. Rats were returned to their home cage for 5 min, and then the recognition phase began in which an identical object was placed in the same position that had been occupied in the familiarization phase, while another identical object was placed in the corner adjacent to

the original position, such that the two objects were diagonal from each other (**Fig. 2.1 B**). Thus, both objects in the familiarization phase were equally familiar, but one was in a new location. The position of the moved object was counterbalanced between rats.

Object-in-Place

This task was comprised of a familiarization phase and a recognition phase separated by a 5 min delay, during which the animal was returned to the home cage. In the familiarization phase, the subjects were presented with four different objects. These objects were placed in the corners of the arena, 10 cm from the walls. Each rat was placed in the center of the arena and allowed to explore the objects for 5 min. In the recognition phase, two of the objects (which were both on the left or right of the arena), exchanged positions and the rat was allowed to explore the objects for 3 min (**Fig. 2.1 C**). The time spent exploring the two objects that had changed position was compared with the time spent exploring the two objects that had remained in the same position. The objects moved and the position of the objects in the recognition phase were counterbalanced between rats. If Object-in-Place memory is intact, the subject should spend more time exploring the two objects that are in different locations compared with the two objects that are in the same locations.

Attentional Set-Shifting

After 8 weeks of diets, obese rats and normal weight controls (n=8/group) were maintained on a restricted diet till reaching 85% body weight and then tested on the Attentional Set-Shifting Task (Fox et al., 2003; Liston et al., 2006). While 8 rats in the

control group completed the task, only 6 rats in the obese group completed the task; all rats that did not complete the task were removed from analysis.

In the task, rats were trained to dig for a food reward (1/3 FrootLoop) and make discriminations based on a texture covering a digging container or a digging medium in which the reward was buried (**Table 2.1**). The apparatus was an opaque Plexiglas box (50 × 40 × 30 cm) divided into three areas—a starting/holding area (16 × 40 × 30 cm) separated by a sliding opaque door from a choice area subdivided by a barrier into two (34 × 20 × 30 cm), each with a digging container (internal diameter and depth, 10 cm).

	Dimensions		Exemplar Combinations	
Discrimination	Relevant	Irrelevant	+	-
Simple Discrimination (SD)	Medium		M1	M2
Compound Discrimination (CD)	Medium	Texture	**M1**/T1 **M1**/T2	M2/T2 M2/T1
Intradimensional Shift (IDS)	Medium	Texture	**M3**/T3 **M3**/T4	M4/T4 M4/T3
Reversal (REV)	Medium	Texture	**M4**/T3 **M4**/T4	M3/T4 M3/T3
Extradimensional Shift (EDS)	Texture	Medium	**T5**/M5 **T5**/M6	T6/M6 T6/M5

Stimulus Pairs	
Medium pairs	**Texture pairs:**
Sawdust vs. Sand	Yellow fuzzy vs. Reversed yellow fuzzy
Gravel vs. Plastic beads	Waxed paper vs. Fine sandpaper
Ribbon vs. Crinkled paper strips	Velvet vs. Reversed velvet

Table. 2.1. Example of a typical sequence of discriminations, including the task-relevant dimension and all possible exemplar combinations. Half the rats are switched from medium to texture and half are switched from texture to medium. The correct exemplar is shown in bold for each discrimination and can be paired with either exemplar from the irrelevant dimension. In the IDS and EDS, the stimuli are novel exemplars of each dimension. The exemplars within a dimension are always used in pairs (listed in lower half of table). No two rats within a group receive the same combinations, but groups of obese and control rats are matched. The order of presentation of exemplars and the assignment of exemplars into positive (+) and negative (−) stimuli is randomized in advance.

Textures covering the digging containers were made from various materials (**Table 2.1**). The Attentional Set-Shifting procedure occurred over 3 d in the animal housing room, during the rats' active period, under red light. Rats were transferred to the starting area of the apparatus and the barrier lifted, allowing access to both containers. On the first day, each container was half filled with cob bedding and a reward was placed on top. After retrieving the reward from each container, rats were returned to the starting area and the containers rebaited. This procedure was repeated for three trials. If rats failed to retrieve both rewards within 5 min, the trial was repeated. This habituation procedure was performed on the 10th to 13th day of food restriction. The next day, rats were shaped to dig for rewards buried within both containers and then trained on both a texture and medium simple discrimination (SD). Shaping was done in four stages using containers filled with cob and the reward in each, as follows: (1) placed on top; (2) placed under a thin layer of cob; (3) buried beneath ~2 cm of cob; and (4) buried under ~4 cm of cob. The first three shaping stages consisted of three trials each. The last shaping stage consisted of six trials to ensure reliable digging. If rats failed to retrieve both rewards within 2 min, the trial was repeated until they did so. Immediately after shaping, rats were trained to locate the reward based on either digging medium (crumpled tissue paper vs shredded latex gloves) or texture (short white fuzzy material vs reversed short white fuzzy material) to a criterion of six consecutive correct trials. The order of the SDs and positive stimuli were determined randomly and represented equally across rats. These stimuli were not used again. On the last day, rats were tested on a series of five discriminations (**Table 2.1**). For all trials, rats had access to both containers, only one of which was baited with a reward. To eliminate the strategy

of using odor to find the reward, all media included a small amount of powdered cereal. The left–right positioning of the baited container across trials was randomized. In the first four trials for each discrimination, rats could recover the reward even if it initially dug in the incorrect container, an error still being recorded. This was done to maintain responding on the task and to ensure sampling of both stimuli. After the first four trials, following an incorrect choice, rats were not permitted to recover the reward and were returned to the starting/holding area. Trials were terminated after 2 min if rats failed to approach a container and dig, and marked as an error. If five consecutive no-dig trials occurred, the test was terminated and continued on the following day. Testing started with the simple discrimination (SD) in which rats discriminated between either two textures or two digging media, one of which predicted the food reward (positive stimulus). Containers were filled with a neutral medium (cob) if the SD involved texture and were untextured if the SD involved medium. Next, in a compound discrimination (CD), a new dimension was introduced, but the positive stimulus was the same as in the SD. This was followed by an intra dimensionalattentional shift (IDS) involving two new exemplars from each stimulus dimension with the task-relevant dimension remaining the same as in the SD and CD. The IDS was then reversed (REV), such that the formerly negative stimulus became the positive stimulus. Finally, in the extradimensional attentional shift (EDS), two new exemplars from each dimension were introduced, and the formerly task-irrelevant dimension became relevant. Testing for each discrimination continued until a criterion of six consecutive correct responses was reached.

Sensory and Locomotor Testing

Ambulatory Testing

Locomotor activity was measured in a computerized 43.2 × 43.2 × 30.5 cm open-field activity chamber with 16 infrared photocells on each of the three axes (MED Associates, Georgia, VT). Animals were placed in the chamber between 5-9 h into the dark period. Animals were allowed to acclimate for 15 min, and then total locomotor activity was measured for the next 15 min. Locomotor counts were quantified as the number of infrared beam breaks.

Olfactory Testing

This test was modified from a mouse paradigm to detect anosmia (Yang and Crawley, 2009). One non-social odor diluted in water (vanilla extract; McCormick; 1:100 dilution in water), and one social odor, (soiled cage of a female rat) were tested. Order of odors was counter balanced among rats. For testing, rats were moved into a clean cage, where they could interact with a cotton swab soaked in water for 3 min. The cotton swab is then replaced with one that has been saturated with the odorant, and the animal remained in the testing cage for an additional 3 min, during which it could interact with the odorant. Animals were then returned to their home cages and tested on the alternative odor the following day, using the same paradigm. Testing was video recorded, and the amount of time spent sniffing each cotton swab was be recorded by a blind observer. If olfactory senses are intact, the rat should spend more time interacting with the novel social and non-social odors than the water. The difference between time spent interacting with the water and the odorants was calculated.

Vibrissae Reflex

This test measures a reflex response to stimulation of the rat's whiskers (Crawley, 1999). Rats were tested in their home cages, 7 h into the dark cycle. A small paintbrush was used to stimulate the vibrissae. Two brush strokes were applied to the animals' vibrissae, approximately 10 sec apart. A whisker twitch on either of the two strokes was recorded as a response, while a lack of a twitch on both strokes was recorded as no response. Each rat was given a score of "1" for response or "2" for no response and total scores were compared between the obese and control rats.

Tactile Response

This test measures response to a tactile stimulation (Crawley, 1999). Rats were again tested in their home cages, 7 h into the dark cycle. An empty squirt bottle was be used to deliver a puff of air to the backs of the animals. Two puffs of air were applied to the animals' backs, approximately 10 sec apart. A whole body flinch on either of the two puffs was recorded as a response, while a lack of a whole body flinch on both puffs was recorded as no response. Each rat was given a score of "1" for response or "2" for no response and total scores were compared between the obese and control rats.

Vision

Vision was determined using object interaction time from the Novel Object Recognition task. The total amount of time interacting with, but not touching, the objects in the familiarization phase of the experiment was used to determine vision abilities.

Total time was compared between the obese and control rats.

Obesogenic Traits

At the end of the assigned behavioral tasks, animals were swiftly sacrificed via rapid decapitation. Bilateral body-fat pads from the abdomen and gonadal regions were collected and weighed individually and collectively by an observer blind to the experimental conditions. Trunk blood was collected and serum was processed for peripheral triglyceride levels as per the manufacturers' instructions, using enzymatic hydrolysis (Cayman Chemicals, Ann Arbor, Michigan).

Statistical Analysis

Rats whose body weights fell within the average body weight of the opposing group were removed from all analysis. Any rat that did not complete a behavioral task to the criteria set forth in the above methods was removed from analysis of that specific task. Body weight, chow intake and the Attentional Set-Shifting Task were analyzed using repeated measures ANOVAs, with post-hoc ANOVAs or Independent Students t-tests, when justified. Remaining data was analyzed using Independent Students t-tests, comparing the obese rats and control rats, with the exception of olfactory testing and vibrissae reflex, which were analyzed using a chi-square test for association.

Results

Indications of obesity and hyperphagia

Diet induced obesity (DIO), or obesity as the results of caloric overconsumption, is consistent with current thinking that that the obesity epidemic is driven mainly by the availability and consumption of high-fat, calorically dense, palatable foods, rather than genetic mutations (Hill and Peters, 1998). In rats, the DIO model reliably produces obesity, as indicated by increased weight gain, elevated triglyceride levels and increased abdominal fat (Hollister et al., 1967; Bengtsson et al., 1993; Bagnol et al., 2012). In the current study, rats fed a high-fat, palatable, nutritionally complete diet showed increased body weight over the 8 week access period compared to controls fed standard rodent chow, with an effect of week ($F_{8,112}$ = 1244.80, p < 0.0001; **Fig. 2.2**), diet group ($F_{1,14}$ = 32.34, p < 0.0001) and an interaction effect between diet group and week ($F_{8,112}$ = 33.84, p < 0.0001). Differences in body weight between groups were apparent as early as week 2 (t_{14} = 3.50, p = 0.004).

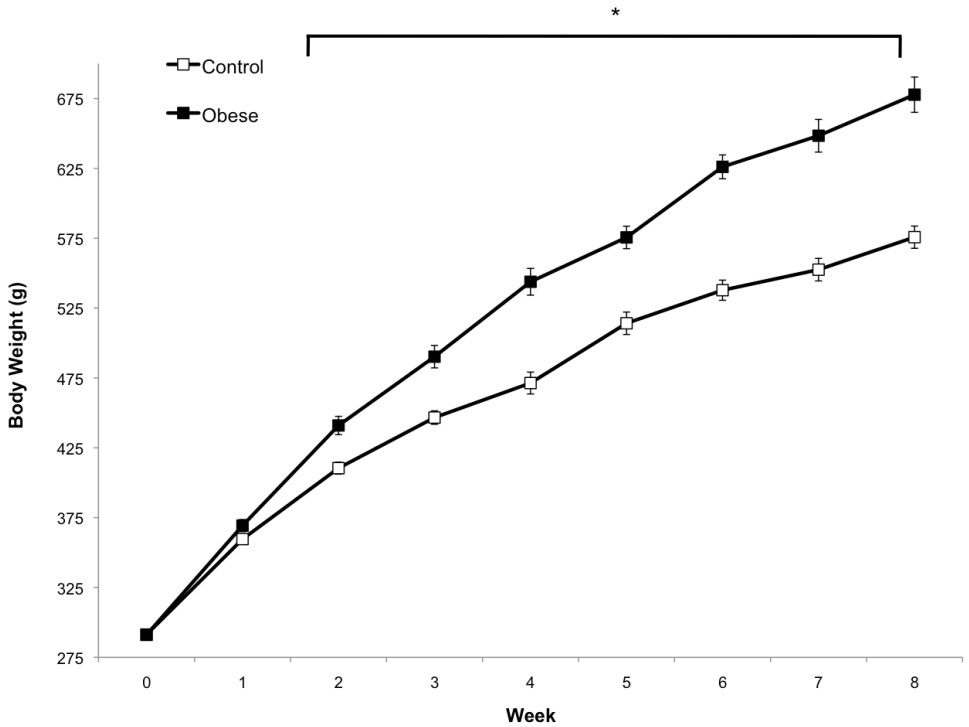


Figure 2.2. Differences are seen in body weight between animals consuming a high-fat diet versus a control diet as early as week 2. n=8/group, *p<0.05, error is SEM.

Obese rats were also hyperphagic, consuming more calories, on average, than the normal weight controls (interaction effect of week and group: $F_{7,98}$ = 5.44, p = 0.015; main effect of week: $F_{7,98}$ = 7.18, p = 0.006; main effect of group: $F_{1,14}$ = 9.30, p = 0.009; **Fig. 2.3**).

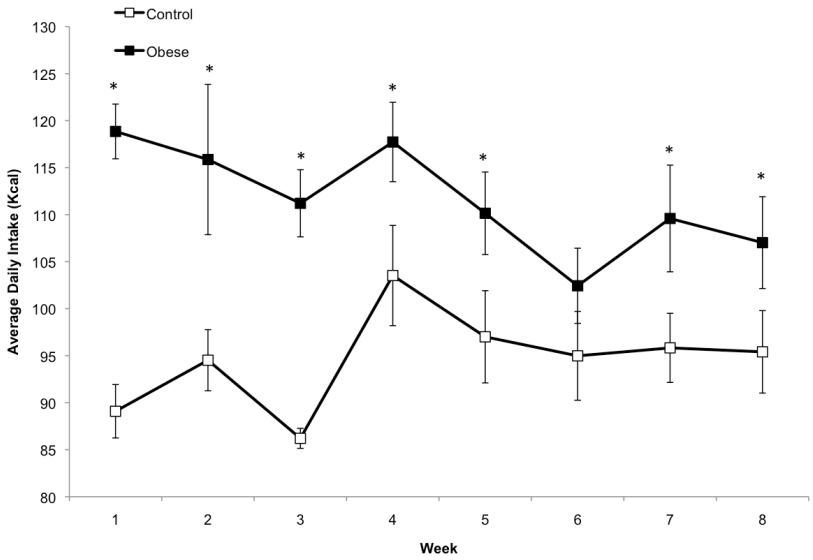


Figure 2.3. On average, rats consuming the high-fat diet were hyperphagic compared to rats consuming standard rodent chow. n=8/group, *p<0.05, error is SEM.

At the end of the study (week 8), rats in the obese group had elevated serum triglyceride levels compared to normal weight controls (t_{14} = 3.70, p = 0.002; **Fig. 2.4 A**) as well as increased abdominal (t_{14} = 4.84, p < 0.0001) and gonadal fat deposition (t_{14} = 6.01, p < 0.0001; Fig. **2.4 B**). Together these traits are used to characterize the obesogenic state.

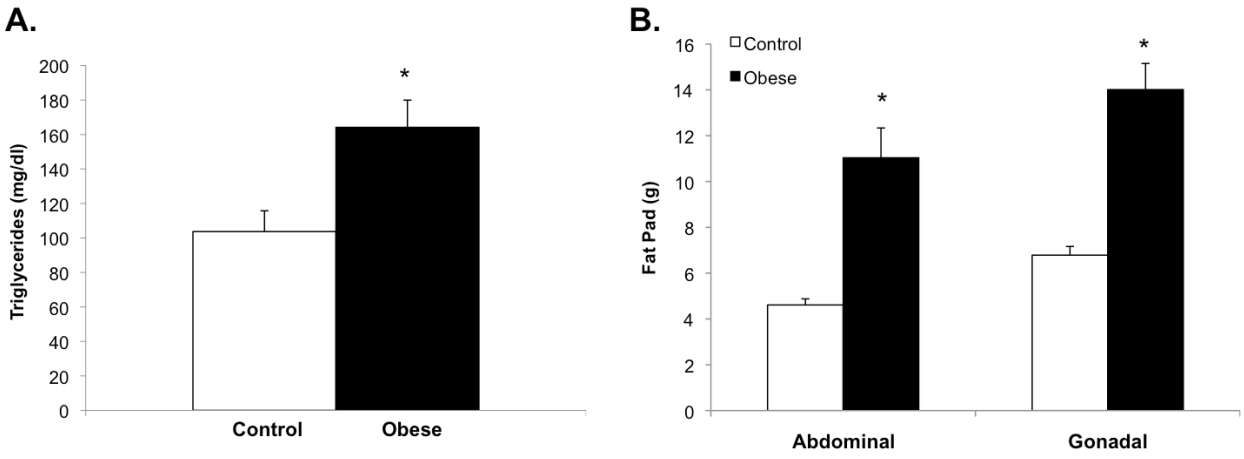


Figure 2.4. After 8 weeks, rats fed a high-fat diet had increased serum triglyceride levels (A) as well as increased abdominal and gonadal fat pads (B) compared to animals fed a standard chow diet. n=8/group, *p<0.05, error is SEM.

Obese rats perform poorly on Novel Object Recognition task

Several behavioral tests of memory were implemented in this obesogenic model to identify potential deficits in cognitive abilities. For the Novel Object Recognition task, the Object Location task, and the Object-in-Place task, discrimination between the known and the novel objects was calculated using a discrimination ratio (DR), which was calculated as the difference in the time spent exploring the novel and familiar objects divided by the total time spent exploring the objects, which takes into account individual differences in the total amount of exploration (Barker and Warburton, 2011). The Novel Object Recognition task is based on the premise that rodents will explore a novel object more than a

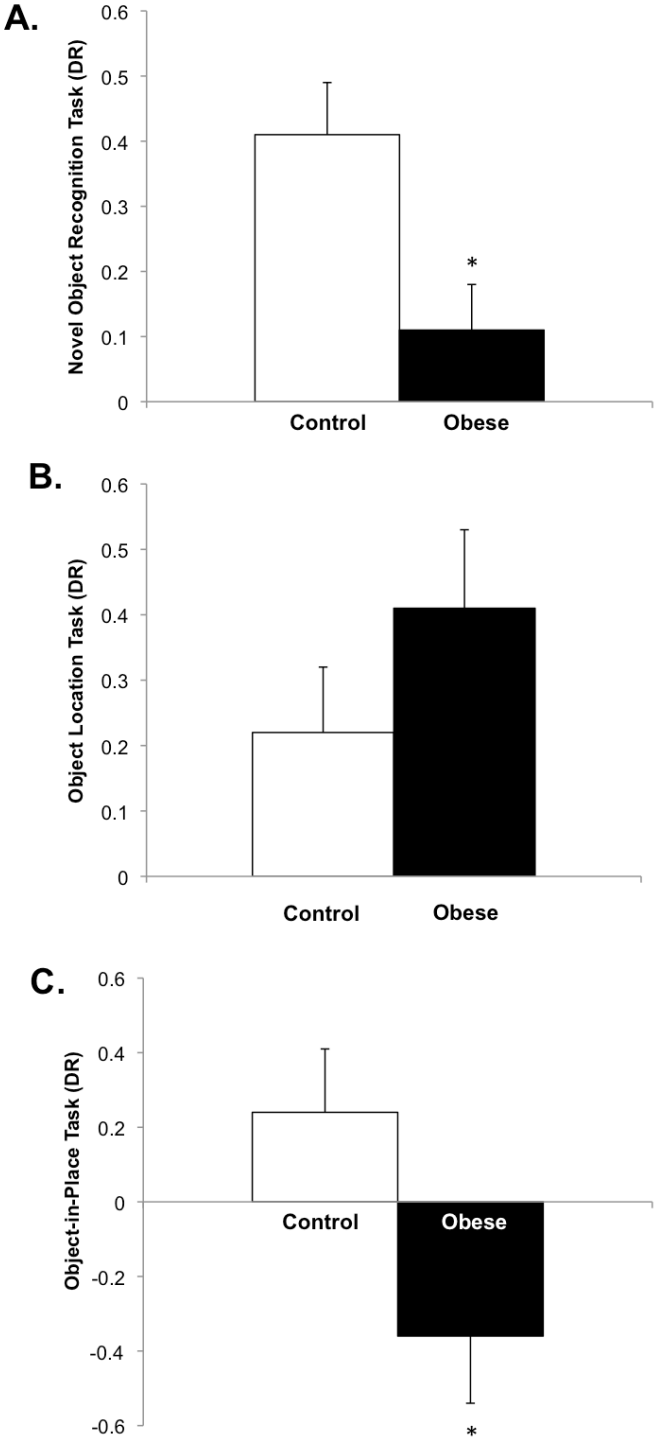


Figure 2.5. The above graphs show the discrimination ratios (DR) calculated as the difference in the time spent exploring the novel and familiar objects divided by the total time spent exploring the objects. Based on DR, obese animals performed poorly on the Novel Object Recognition Task (A) and the Object-in-Place Task (C), compared to controls. However, no differences were seen between groups on the Object Location Task (B). *p<0.05, error is SEM.

familiar one, but only if they remember the familiar one. During the familiarization phase, there was no difference between the amount of time each group of rats spent exploring the objects (Obese = 38.2 ± 3.8 sec, Control = 41.3 ± 3.1 sec; t_{12} = 0.64, p = 0.53). However, during the trial phase, obese rats had low discrimination ratio scores, indicating that they performed poorly on the Novel Object Recognition task, compared to normal weight controls (t_{12} = 2.34, p = 0.04; **Fig. 2.5 A**).

Obese and normal weight rats perform similarly on Object Location task

The Object Location task is a hippocampus-dependent test, where the rat's ability to recognize an object it has experienced before in a changed location is assessed. During the familiarization phase, there was no difference between the amount of time each group of rats spent exploring the objects (Obese = 37.8 ± 4.1 sec, Control = 48.5 ± 4.8 sec; t_{14} = 1.70, p = 0.11), nor was there a difference in the discrimination ratios between groups on the Object Location task (t_{14} = 1.03, p = 0.32; **Fig. 2.5 B**).

Obese rats perform poorly on Object-in-Place task

The Object-in-Place task depends on both the HIP and either the PRC or mPFC (Barker and Warburton, 2011), and can substantiate the results of the Novel Object Recognition and Object Location Tasks, while also alluding to a potential role of the mPFC. During the familiarization phase, there was no difference between the amount of time each group of rats spent exploring the objects (Obese = 113.3 ± 10.9 sec, Control = 124.0 ± 6.6 sec; t_{13} = 0.86, p = 0.40). Obese rats, however, performed poorly on the Object-in-Place task as evidenced by the lower discrimination ratio score compared to

the normal weight controls (t_{13} = 2.38, p = 0.03; **Fig. 2.5 C**), further indicating deficits in PRC functioning, and suggesting deficits in mPFC functioning.

Obese rats perform poorly on ASST

One function of the mPFC is to modulate attentional processes and behavioral flexibility. The Attentional Set-Shifting task (ASST) is based on the fact that when attending to a perceptual feature of a stimulus (such as shape), learning to discriminate new complex stimuli is more rapid when the discrimination rule is based on the same perceptual dimension (Birrell and Brown, 2000). Mastering this same-dimensional learning task is referred to as an intradimensional shift (IDS). By contrast, if the new discrimination requires attention to be paid to a different perceptual dimension (such as the color of the stimulus), requiring that the previously attended feature must be disregarded, learning is less rapid (Birrell and Brown, 2000). This is referred to an extradimensional shift (EDS). Lesion studies indicate that shifting attention from one sensory stimulus to another (the EDS) requires the mPFC.

Obese rats performed poorly on the ASST compared to normal weight controls ($F_{1,12}$ = 20.45, p = 0.001; **Fig. 2.6**). While there was no difference between the trials to criteria between groups on the SD ($F_{1,12}$ = -1.02, p = 0.33), CD ($F_{1,12}$ = 2.11, p = 0.56) and IDS tasks ($F_{1,12}$ = 2.16, p = 0.052), obese rats required more trials to reach criteria compared to normal weight controls on the REV ($F_{1,12}$ = 2.35, p = 0.037) and EDS tasks ($F_{1,12}$ = 2.74, p = 0.02). Obese rats also made more errors relative to normal weight controls on the CD ($F_{1,12}$ = 2.24, p = 0.045), IDS ($F_{1,\ 12}$ = 3.22, p = 0.007), REV ($F_{1,12}$ = 2.23, p = 0.046) and EDS tasks ($F_{1,12}$ = 3.10, p = 0.009).

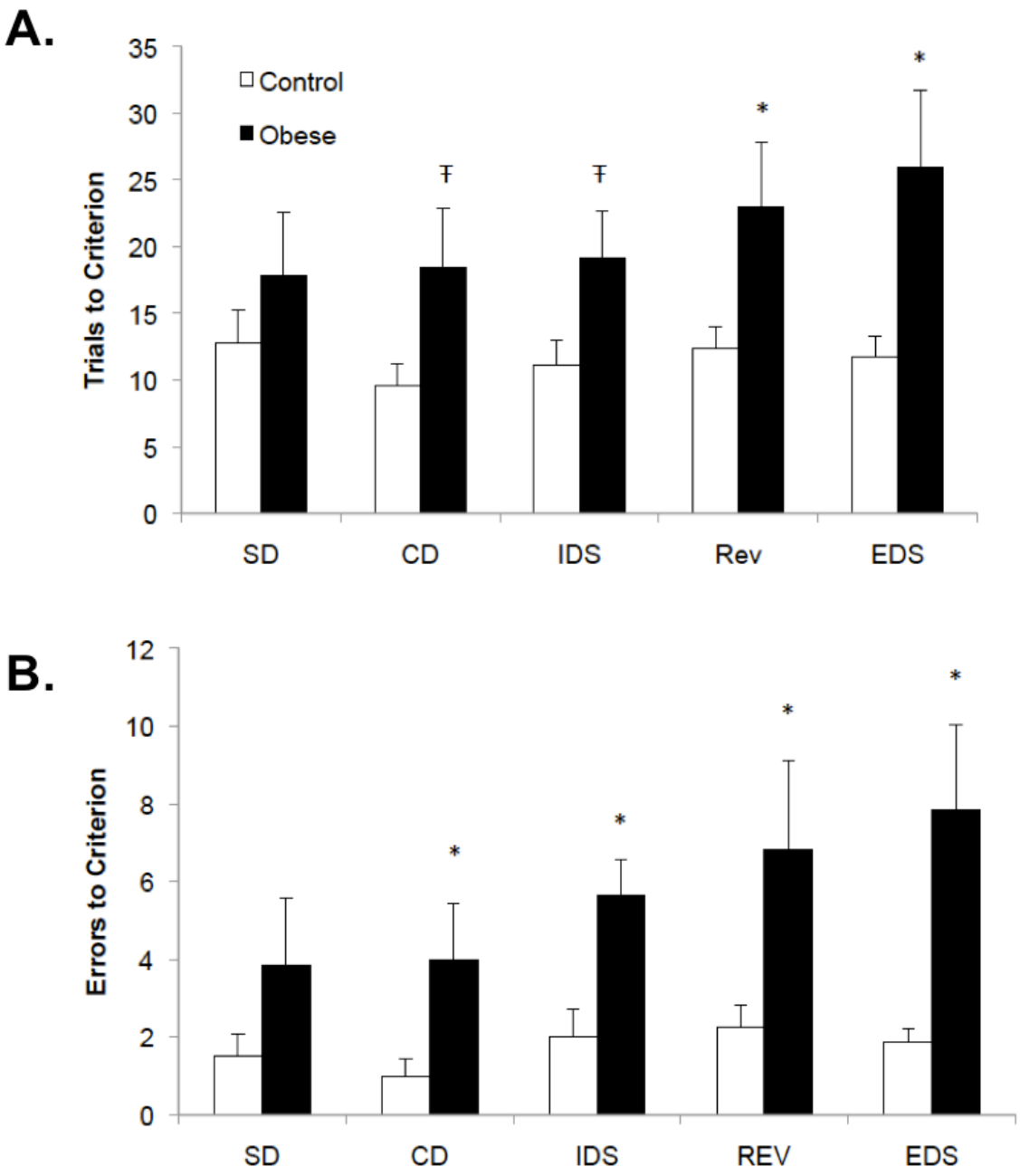


Figure 2.6. Obese animals performed poorly, compared to controls on the Attentional Set-Shifting Task. Obese animals reached criterion on the compound discrimination (CD), intradimensional shift (IDS) and reversal learning (REV) and extradimensional set shifting (EDS) in more trials than the control animals (A). Only the initial simple discrimination (SD) was not different between groups. They also made more errors before reaching criterion (B) on all phases, with the exception of the SD. *p<0.05, ∓p=0.05, n= 6-8/group, error is SEM.

No deficits were seen in basic sensory and locomor functioning in obese rats

The dramatic deficits in the ASST testing suggest that deficits might be the result of impairments in basic processes, not specifically in cognitive function. The following experiments examine sensory abilities needed to perform the tasks of cognition, specifically basic locomotor activity, olfactory differentiating, vibrissae reflex, tactile response and vision. In order to identify deficits in locomotor functioning, rats were tested for overall ambulatory activity in an open field, response to tactile and vibrissae stimulation (Crawley, 1999), olfactory response to two smells (Yang and Crawley, 2009) (vanilla and female rat) and vision (as indicated by time interacting with stationary object in open field). There were no differences between groups in any of these elements (**Table 2.2**; locomotion: $t_{14} = 0.52$, $p = 0.62$; tactile: Chi-square$_1 = 0.20$, $p = 0.65$; vibrissae: Chi-square$_1$ = n/a, because 100% response from both groups, no statistic is computed for a constant, $p > 0.05$; olfactory$_{vanilla}$: $t_{14} = 0.23$, $p = 0.82$; olfactory$_{female}$: $t_{14} = 0.56$, $p = 0.58$; vision: $t_{14} = 0.984$, $p = 0.34$). Together, these data indicate no deficits in basic motor or sensory processing.

	Test Summary	Obese	Control
Locomotion	beam breaks/15 min in locomotor chamber	1513±218 ambulatory counts	1731±321 ambulatory counts
Tactile	Response to air puff 1=response, 2=no response	1.4±0.2	1.6±0.2
Vibrissae	Response to whisker stimulation 1=response, 2=no response	1±0	1±0
Olfactory$_{vanilla}$	Difference in time spent exploring odorant vs. water	8.5±2.7 sec	6.5±4.4 sec
Olfactory$_{female\ rat}$	Difference in time spent exploring odorant vs. water	13.7±3.3 sec	9.4±4.8 sec
Vision	Time spent exploring object in open field	36.0±3.9 sec	40.8±2.8 sec

Table 2.2. Obese rats performed no differently than control rats on a series of basic sensory and motor tasks, including motor, somatosensory visual and olfactory testing, described above. Values represent mean ± SEM.

Discussion

The DIO model reliably produces obesity in rats, as indicated by increased weight gain, elevated triglyceride levels and increased abdominal fat (Hollister et al., 1967; Bengtsson et al., 1993; Bagnol et al., 2012). Our results show that as little as 2 weeks access to a nutritionally complete, high-fat, pelleted diet, rats show increased body weights compared to controls fed standard rodent chow (**Fig. 2.2**). After 8 weeks of access to the high-fat diet, this increase in body weight is accompanied by elevated serum triglycerides (**Fig. 2.4 A**) and abdominal fat pad weights (**Fig. 2.4 B**). It is important to note that not all rats fed a high-fat diet will show the DIO phenotype but the large majority do (~4% become morbidly obese, ~4% remain lean and the remainder represent a continuum between these extreme phenotypes (Tschop and Heiman, 2001)) making this a useful and reliable animal model of obesity in which to explore cognitive deficits.

In the current study, given the clinical literature suggesting decreased brain volume of the mPFC and HIP (Ward et al., 2005; Pannacciulli et al., 2006; Raji et al., 2010; Walther et al., 2010), we focused on these brain regions. One function of the mPFC is to modulate attentional processes and behavioral flexibility. Lesions of the mPFC have been shown to impair performance on Attentional Set-Shifting Task (ASST) (Birrell and Brown, 2000; Fox et al., 2003), a task that has been likened to the Wisconsin Card Sorting Task in humans. In the ASST, rats learn a series of associations between stimuli of certain sensory modalities and food rewards, and then switch these associations to other sensory modalities to complete the task. Shifting attention from one sensory stimulus to another, referred to as an extradimensional shift

(EDS), has been shown to require the mPFC (Birrell and Brown, 2000; Fox et al., 2003). In the current study, the obese animals preformed poorly on the EDS component of the ASST compared to normal weight controls, indicating impairments in the mPFC. This is consistent with a previous study that shows decreased performance on an operant test of delayed alternation performance with variable intervals between the trials (VIDA), which measures procedural rule learning, known to be sensitive to frontal lobe impairment (Winocur and Greenwood, 2005). However, in the current study, the obese animals also performed poorly on most aspects of the ASST testing, with the exception to the simple discrimination component, in which animals learn to discriminate container texture or digging medium to uncover a food reward. An example of diminished performance is the reversal component, which is dependent on the orbitofrontal cortex (OFC) (McAlonan and Brown, 2003). This expansive nature of ASST performance deficits could provide evidence of non-specific neurological deficits. However, normal performance in basic locomotor activity, olfactory differentiating, vibrissae reflex, tactile response and vision suggest deficits are not global. These data also allow us to eliminate confounds that might effect the interpretation of the cognitive testing.

Further, although performance on the ASST might suggest global performance deficits, no differences were seen on the Object Location task, indicating that disruptions in the hippocampus are unlikely (Barker and Warburton, 2011). Additionally, this demonstrates that obese animals are not experiencing global, non-specific neurological deficits (which might have been concluded strictly based on the performance on the ASST), but rather, deficits seem to be somewhat localized. These results, indicating normal functioning of the HIP, are also interesting, in that they were

not hypothesized based on the literature. Given the clinical literature suggesting decreased in HIP brain volume, as well as animal literature, in which obese rats fed saturated fat and refined sugar show impaired acquisition and retention of spatial memory in a water maze test (Molteni et al., 2002; Goldbart et al., 2006; Neves et al., 2008) and radial arm maze (Winocur and Greenwood, 2005), we initially hypothesized that in the current study, rats would show deficits on the HIP based task. There are several differences between the current study and those previous studies, which may account for the differences. The diets varied in fat content and source, as well as the duration. In all the previous studies, diets were maintained for 3 months to 2 years, while the current study used a shorter diet exposure duration (8 weeks), potentially leading to different results.

In addition to deficits in the mPFC task, the current study found deficits in the Novel Object Recognition task, which is dependent on the PRC (Barker and Warburton, 2011). These data are consistent with a study that found that obese rats fed a high sucrose diet for 8 weeks, perform poorly on the Object Recognition task (Jurdak and Kanarek, 2009).

The Object-in-Place task is dependent on a functional interaction between the hippocampus, mPFC and perirhinal cortex (Barker et al., 2007). Obese animals performed poorly on the Object-in-Place task compared to controls. Given the normal performance on the Object Location task, it is likely that performance on the Object-in-Place task as driven by deficits in the mPFC and PRC, but not the HIP.

Together, these behavioral data suggest that a model of diet-induced obesity in the rat is associated with deficits in tasks that require the mPFC and PRC, but not HIP.

Deficits in performance of the EDS phase of the ASST have been associated with diminished dendritic spine density and complexity of mPFC pyramidal neurons (Liston et al., 2006) and associated neuromarkers, which will be explored in the next chapter.

Chapter 3

Obesity alters the structure and synaptic biochemistry of the mPFC and PRC

Abstract

Using an animal model of obesity (diet-induced obesity model), we have found deficits among obese rats in behavioral tasks requiring the medial prefrontal cortex (mPFC) and perirhinal cortex (PRC), but not the hippocampus (HIP). To further understand the brain changes driving these functional aberrations, the current study explores dendritic architecture and synaptic protein concentrations in the mPFC, PRC and HIP, which are the three brain areas behaviorally profiled in Chapter 2. Specifically, we explored the pyramidal neurons in layer II/III of the mPFC, layer II of the PRC and the CA1 and CA3 regions of the HIP. In the current study, we assess dendritic architecture and dendritic spine density using Golgi staining and synaptic protein concentrations (including concentrations of synaptophysin, spinophilin, vGAT and vGLUT) using both immunofluorescence and Western blotting. Finally, mPRC and HIP volumes were assessed using Cavalieri's Principle in cresyl violet stained brain sections. Obese rats showed decreased spine density on the apical aspect of pyramidal neurons in the mPFC and PRC, but not HIP. Further, obese rats had decreased synaptic protein concentrations in the mPFC and PRC, but not HIP. Lastly, while there was no difference in the overall brain volume between the obese and normal weight

rats, obese rats did have decreased mPFC volume, but not HIP, volume compared to controls. These results begin to elucidate structural and protein differences between obese and normal weight rats.

Introduction

Chapter 2 describes deficits in behavioral tasks indicating decreased functioning in the mPFC and PRC, but not HIP. Given these deficits, this chapter explores structural and neurochemical factors that might begin to explain brain changes underlying the behavioral findings.

First, dendritic architecture and dendritic spine density were examined in the brain regions of interest. Research indicates that experience alters dendritic architecture and function within both the HIP and mPFC. Positive experiences, such as mating behavior, parenting and running, increase dendritic complexity (Kozorovitskiy et al., 2006; Stranahan et al., 2007; Leuner et al., 2010; Leuner and Gould, 2010), while negative experiences, such as chronic restraint stress, produce dendritic shrinkage (Liston et al., 2006; Radley et al., 2006). In addition to dendritic complexity, positive experiences enhance performance on cognitive tasks that require the HIP and mPFC (Leuner et al., 2010; Leuner and Gould, 2010). Similarly, negative experiences, such as stress, impair cognitive performance (McEwen, 2000b; Liston et al., 2006). It follows that obesity can be categorized as a negative, or stressful biological state, leading to the observed diminished cognitive performance, and thus might similarly lead to diminished dendritic architecture. In order to examine dendritic architecture in obese versus control animals, dendritic spine density and branching of pyramidal cells in both the mPFC and

PRC were determined. Further, we analyzed the HIP, despite the lack of behavioral abnormality in tasks known to be affected by deficits in this region, because of its known role in cognition and clinical evidence showing decreases in HIP volume in obese people (Ergorul and Eichenbaum, 2006; Zald and Andreotti, 2010).

To further support the dendritic spine density testing, this chapter looks at the concentrations of specific synaptic and dendritic markers using immunofluorescence. Specifically, we looked at concentrations of synaptophysin, a protein involved in synaptic vesicle trafficking (Thiel, 1993), spinophilin, a dendritic spine marker (Allen et al., 1997), vesicular GABA transporter (vGAT) and vesicular glutamate transporter (vGLUT), in the same brain areas that were analyzed for dendritic spine density. Microtubule-associated protein 2 (MAP-2), a protein that is important for dendritic structure, was also analyzed as a control protein and to eliminate the possibility that all proteins are down regulated in the DIO model. Though lacking the apical and basal specificity that can be determined using immunofluorescence, the above protein concentrations were confirmed using Western blot. These data allow us to further characterize and confirm dendritic spine density results.

Finally, given the fact that in humans, obesity has been associated with overall decreased brain volume (Ward et al., 2005), with further studies indicating reductions in the volume of the HIP and prefrontal cortex (Ward et al., 2005; Pannacciulli et al., 2006; Raji et al., 2010; Walther et al., 2010), we look at overall brain volume, as well as the volume of the mPFC and the HIP in the DIO model. We were unable to look at specific volumes of the PRC because of its small size and lack of observable boundaries.

Together, these studies will help to characterize those cognitive deficits seen in

Chapter 2.

Methods

As in Chapter 1, male Sprague–Dawley rats (275-315 g at the onset of the study) were obtained from Charles River (Germantown, NY) and housed individually on a 12 h reverse light cycle (lights off at 6am). Three cohorts of animals were used; in each cohort, rats were weight-matched and divided into two groups (n=10/group), and given access to either a nutritionally complete, high-fat diet (4.7 kcal/g; Research Diets, New Brunswick, NJ, #12451; 45% fat, 20% protein, and 35% carbohydrate) or standard rodent chow (3.01 kcal/g; LabDiet #5001, PMI Nutrition International, Richmond, IN; 10% fat, 20% protein, 70% carbohydrate). All animals had ad libitum access to water. After 8 weeks on the described diets, testing began. Rats with body weights that overlapped between groups were removed from analysis, such that each cohort ended up with n=8-9/group. The first cohort of animals was processed for Golgi impregnation, the second cohort was processed for immunofluorescence and cresyl violet and the final cohort was processed for Western blot.

Golgi Impregnation

For Golgi impregnation, rats were sacrificed via rapid decapitation, and the brains were removed from the skull and rinsed in dH_2O. Small blocks of tissue containing the PFC or HIP and PFC were immersed in a Golgi–Cox impregnation solution (Rapid GolgiStain kit, FD NeuroTechnologies), left undisturbed in the dark for 3 weeks, then transferred to RapidGolgi Stain solution for 2 d. Coronal sections (100 μm) were cut on a Vibratome and slide mounted and dried overnight. For development, slides were

rinsed 2 x 2 min in dH_2O and then incubated in RapidGolgi Stain developing solution for 10 min, rinsed in distilled water 2 x 4 min. Sections were then dehydrated in 50, 75 and 95% ethanol (4 min each) and 100% ethanol 4 x 4 min. Slides were cleared in Citrisolv for 5 min, and coverslipped with Permount.

All analyses were conducted blind to experimental conditions. Layer II/III pyramidal neurons in the mPFC, layer I/II pyramidal neurons in the CA1 region of the hippocampus and layer II of the PRC were examined for dendritic spine density and branching. Pyramidal neurons in the CA3 region of the hippocampus were also examined for branching.

On selected pyramidal neurons, five apical and five basal dendritic segments (each 10–25 μm long) were analyzed for dendritic spine density using a 100x oil objective on a BX-60 Olympus microscope equipped with a motorized stage and attached to a computer with Stereo Investigator software (Microbrightfield, Burlington, VT) (Leuner and Gould, 2010). For every cell, dendritic segments selected for analysis were as follows: (1) on secondary or tertiary dendrites; (2) ≥50 μm away from the soma for apical dendrites and 30 μm for basal dendrites; and (3) mostly in one focal plane. Only spines extending away from the shaft were counted. Randomly selected dendrites satisfying these criteria on six cells in each brain section, per animal were examined. Due to variations in staining quality, 7-8 brains were analyzed for each brain section for each dietary condition.

For dendritic length and branching analyses, cells were traced using the 40x objective providing an average length of apical and basal dendritic trees. Branch points

were counted when a dendrite exhibited a bifurcation or juncture whereby two distinct branches were detected.

Immunohistochemistry and Optical Intensity Analysis

Animals (n=8/group) were deeply anesthetized with Euthasol (1mL/kg, Virbac) and perfused transcardially with PBS followed by 4% paraformaldehyde. Following pefusions, brains were extracted and post-fixed for 24 h in 4% paraformaldehyde, and then coronal sections (40 μm) were cut in PBS using a Vibratome.

Sections were washed in Tris-buffered saline (TBS) (3x5 min). Tissue to be incubated in vGAT and vGLUT were blocked with 3% goat serum in TBS for 1 h. All samples were then incubated in either rabbit anti-spinophilin (1:500, abcam; 72 h at 4°C), rabbit anti-synaptophysin (1:500, Sigma-Aldrich; 48 h at 4°C), rabbit anti-vGAT (1:500; SySy; 24 h at 4°C), guinea pig anti-vGLUT-1 (1:5,000; Millipore; 72 h at 4°C), or mouse anti-MAP-2 (1:500, Sigma-Aldrich; 48 h at 4°C) with 0.3-0.5% Triton X-100 in TBS. After 3 x 5 min rinses in TBS, tissue was incubated for 1-2 h in the dark with goat anti-rabbit, goat anti-guinea pig or goat anti-mouse antibody conjugated to Alexa 488 (1:500, Molecular Probes). Tissue was again rinsed 3 x 5 min TBS and coverslipped with glycerol (3:1 in TBS).

Using a confocal microscope, average pixel intensity and cross-sectional area of staining were analyzed with settings for detector and amplitude gain, contrast and brightness optimized and held constant for a given marker/area combination, as described by Gazzaley and colleagues (Gazzaley et al., 1996). Pixel values representing the intensity of immunofluorescence staining were expressed as a gray

scale (0-255) and photometric offset was applied to each image to exclude the background. After offset was applied, average pixel intensity was multiplied by area of pixels falling over the threshold, and summed over all sampling sites, yielding an index of immunolabeling intensity. Ten 1-mm-thick sites (3.32 mm^2 in area each) were sampled in the mPFC and HIP for each marker in every region of interest in every animal included in the analysis. Because of its size, only 5 sites were sampled from the PRC in each animal.

Nissl Labeling and Brain Mass

Brains were removed from perfused animals, and immediately weighed. Forty-micrometer-thick sections from one hemisphere throughout the mPFC, PRC and HIP were stained for Nissl substance by using cresyl violet. Every 4^{th} slice was taken in the mPFC and every 6^{th} slice was taken in the PRC and HIP. Tissue was slide mounted and allowed to dry. It was then rinsed in dH_2O, placed in 0.5% cresyl violet (5-10 min), and rinsed again in dH_2O. Slides were then dehydrated in graded ethanol, starting with 70% (with glacial acetic acid) for 30-90 sec, then 95% for 2-3 min and 100% ethanol for 5 min. Slides were cleared in CitriSolv for 5 min, and then coverlipped with paramount. Under 4x objective on a BX-60 Olympus microscope equipped with a motorized stage and attached to a computer with Stereo Investigator software, mPFC and HIP areas were traced and area was calculated. Volumes were calculated using Cavalieri's principle. Areas of each region were summed, multiplied 2 (to account for two hemispheres), by 40 (to account for depth of each slice) and by the number of slices

between those analyzed (4 for the mPFC and 6 for the HIP) to arrive at the total estimate for the brain region of interest.

Western Blotting

After rapid decapitation, brains were immediately removed, and dissections were taken from the mPFC, PRC and HIP from 1-mm coronal sections using a stainless steel rat brain matrix and were rapidly frozen on dry ice and kept at −80°C until processed. Samples were later homogenized in 5μM Hepes lysis buffer (1% SDS) containing protease and phosphatase inhibitors. 30 μg (PRC) or 50 μg (mPFC and HIP) samples of total protein lysates were electrophoresed on 4–20% precast polyacrylamide gels (BioRad). Proteins were transferred to nitrocellulose membranes (BioRad) and blocked overnight in either 5% milk or Licor Blocking Buffer. Membranes were then incubated in one of the following: rabbit anti-spinophilin (1:1,000, Millipore), rabbit anti-synaptophysin (1:1,000, sigma), rabbit anti-vGAT (1:5,000, SySy), rabbit anti-vGLUT (1:5,000, SySy), mouse anti-MAP-2 (1:1,000, Sigma-Aldrich) or mouse anti-beta Tubulin antibodies (1:60,000, millipore) overnight at 4°C. Membranes were then incubated for 1-2 hours at room temperature with the appropriate IRDye secondary antibodies (Li-Cor, 1:10,000), and bands were visualized using Odyssey Imager (Li-Cor). National Institutes of Health Image J software was used to quantify bands, which were further normalized to tubulin to control for equal loading.

Statistical Analysis

Data was analyzed using independent Students t-tests, comparing measures

between the obese rats and control rats.

Results

Decreased dendritic spine density, but not branching is seen in DIO rats

It has been shown that experience alters dendritic architecture and function within both the HIP and mPFC, with negative experiences, or stress, producing dendritic shrinkage. This dendritic modification is also associated with decreased cognitive functioning in animal models (Sousa et al., 2000; Liston et al., 2006; Radley et al., 2006). In the current study, dendritic spine density and complexity were examined using single section Golgi-impregnation (**Fig. 3.1,** following page). Dendritic spine density on the apical dendrites of pyramidal neurons in layer II/III of the mPFC (t_{14} = 2.63, p = 0.02) and layer II of the PRC (t_{14} = 2.24, p = 0.04) were decreased compared to normal weight controls, while dendritic spine density on the apical dendrites of pyramidal neurons in the CA1 region of the HIP in obese rats were no different than controls (t_{14} = 1.21, p = 0.25). On the apical aspect of the dendritic tree, no differences in complexity (as indicated by number of branch points) were seen in layer II/III of the mPFC (t_{14} = 0.41, p = 0.69, **Table 3.1**), layer II of the PRC (t_{14} = 0.04, p = 0.97), CA1 (t_{14} = 0.43, p = 0.68) and CA3 (t_{14} = 0.72, p = 0.48) or the HIP. Further, in all three brain regions, no changes in spine density or complexity were observed in the basal dendritic tree of Golgi-impregnated cells (Spine density: mPFC t_{14} = 0.93, p = 0.37, PRC t_{14} = 0.96, p = 0.35, HIP_{CA1} t_{14} = 0.20, p = 0.85, **Table 3.2;** branch points: mPFC t_{14} = 1.17, p = 0.26, PRC t_{14} = 0.21, p = 0.84, HIP_{CA1} t_{14} = 1.12, p = 0.28, HIP_{CA3} t_{14} = 0.72, p = 0.48, **Table 3.1**).

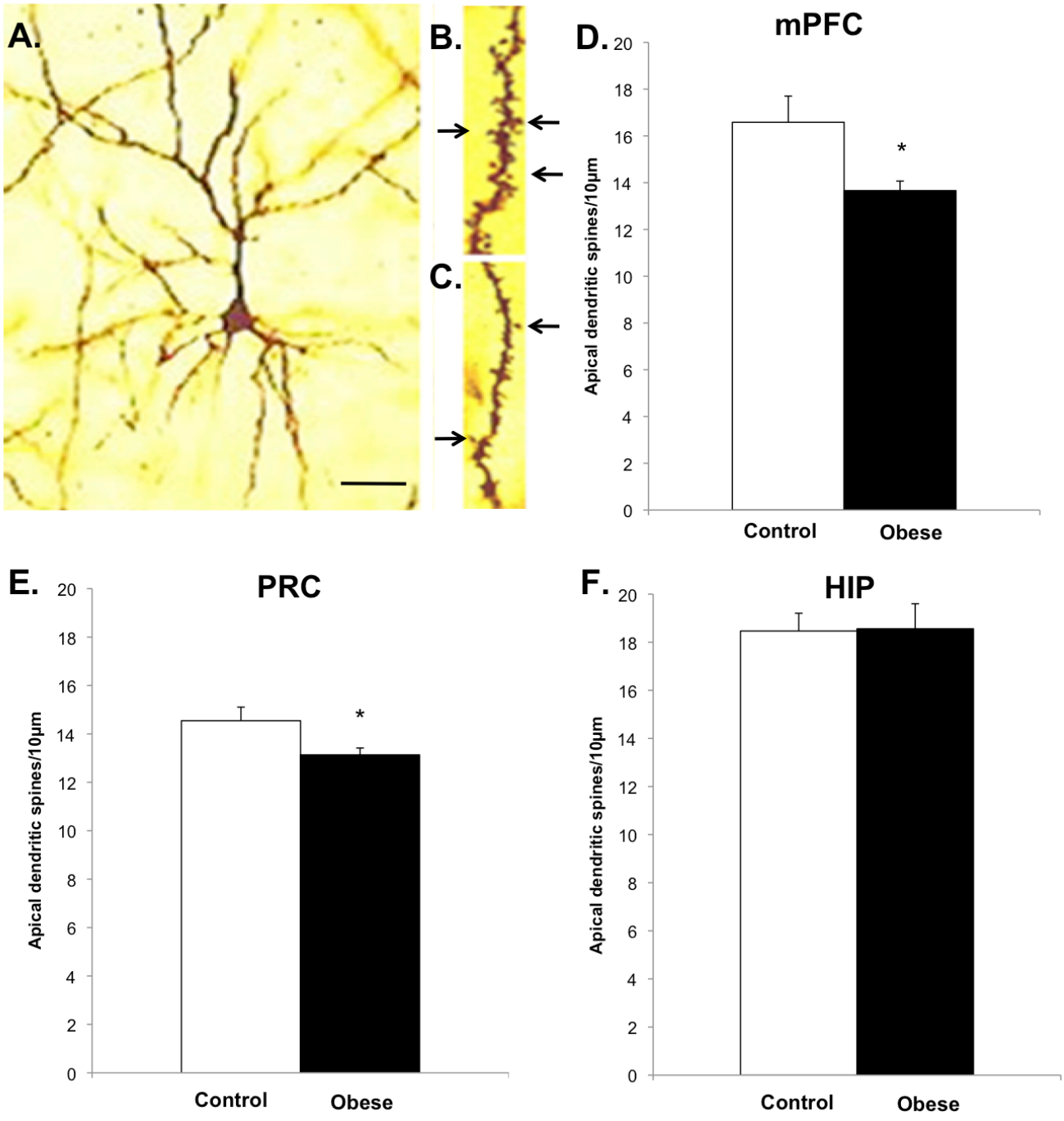


Figure 3.1. Photomicrograph of a Golgi impregnated layer II/III pyramidal cells in the mPFC pyramidal neuron (A), with magnified views of representative 10µm apical dendritic segments, from animals in control (B) and obese (C) groups. Arrows point to spines. Obese rats had decreased dendritic spine density on the apical aspect of the dendritic trees in layer II/III pyramidal neurons in the mPFC (D) and layer II of the PRC (E), but not the CA1 region of the HIP (F). Bars represent mean ± SEM. *p < 0.05. Scale bar in "A" is 30µm.

		Dendritic branching	
Brain Region		**Obese**	**Control**
mPFC	apical	8.2±1.2	8.6±1.0
	basal	9.8±0.7	8.6±0.8
PRC	apical	13.9±0.9	14.0±0.6
	basal	11.6±0.8	12.2±0.6
CA1 HIP	apical	23.3±5.2	26.3±5.7
	basal	13.1±2.0	13.8±2.0
CA3 HIP	apical	11.8 ±0.6	12.5±0.8
	basal	19.9±0.8	20.0±0.7

Table 3.1. No differences were found in dendritic spine branching between the obese and control rats in the apical or basal aspects of the dendritic tree in layer II/III mPFC, layer II PRC, CA1 or CA3 HIP pyramidal neurons. Values represent mean ± SEM.

	Spine density (spines/10µm)	
Brain Region	**Obese**	**Control**
mPFC	15.1±1.2	16.5±1.1
PRC	13.6±0.5	14.3±0.6
CA1 HIP	16.6±0.8	15.7±0.9

Table 3.2. Unlike the apical aspect of the pyramidal tree, no differences were found in dendritic spine density between the obese and control rats in the basal aspects of the dendritic tree in layer II/III mPFC pyramidal neurons, layer II PRC pyramidal neurons or CA1 HIP pyramidal neurons. Values represent mean ± SEM.

Pre- and post-synaptic markers were decreased in DIO rats

To further characterize the dendritic spine changes observed in obese rats, we examined the levels of several proteins concentrated at the synapse: spinophilin, synaptophysin, vGAT and vGLUT. MAP-2 was also analyzed as a control marker. Optical intensity levels for these markers were examined by using confocal microscopy in the locations that had been examined for changes in dendritic spine density: mPFC,

PRC and HIP. **Figure 3.2** shows photomicrograph representations of mPFC sections containing the apical dendritic tree of layer II/III pyramidal neurons labeled for spinophilin, synaptophysin, vGAT, vGLUT and MAP-2.

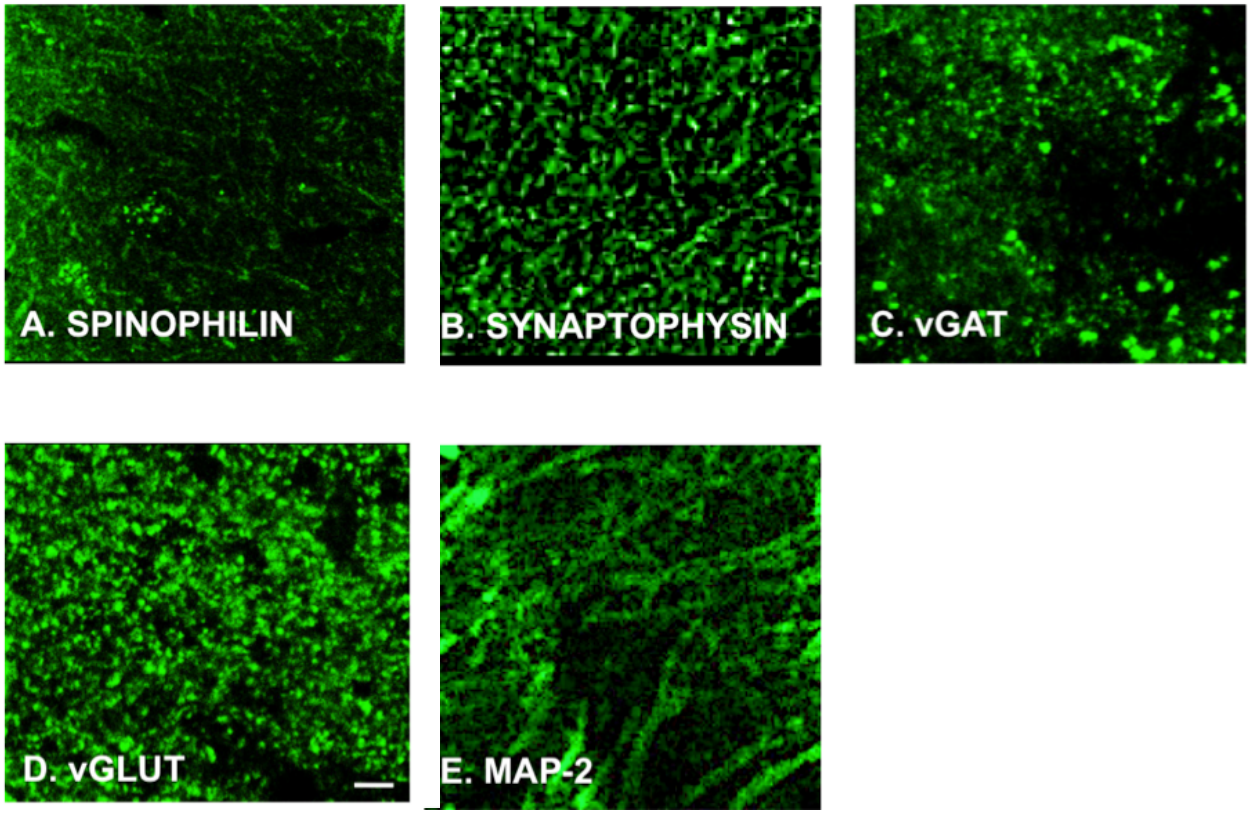


Figure 3.2. Photomicrograph representations of sections containing the apical dendritic tree of layer II/III pyramidal neurons mPFC, immunostained for pre- and post-synaptic markers, spinophilin (A), synaptophysin (B), vGAT (C), and vGLUT (D), as well as the control protein MAP-2 (E). Scale bar, in image "D", is 10μm, and can be applied to all images.

Obesity induced region-specific decreases in the levels of spinophilin, synaptophysin, vGAT and vGLUT, but not MAP-2 in the mPFC and PRC (**Fig. 3.3,** following page**).** No changes were seen for any of the markers in the HIP. (Spinophilin: mPFC, t_{17} = -2.17, p = 0.04; PRC, t_{16} = 3.70, p = 0.002; HIP, t_{18} = -1.52, p = 0.15; synaptophysin: mPFC, t_{18} = 2.30, p = 0.34; PRC, t_{14} = 4.74, p < 0.0001; HIP, t_{18} = 1.30, p = 0.21; vGAT: mPFC, t_{18} = 5.39, p < 0.0001; PRC, t_{15} = 2.92, p = 0.01; HIP, t_{17} = 0.77;

p = 0.45; vGLUT: mPFC, t_{18} = 3.29, p = 0.004; PRC, t_{15} = 2.82, p = 0.01; HIP, t_{17} = 0.02; p = 0.99; MAP-2: mPFC, t_{17} = 0.19, p = 0.85; PRC, t_{16} = 0.99, p = 0.34; HIP, t_{17} = 1.45, p = 0.16.)

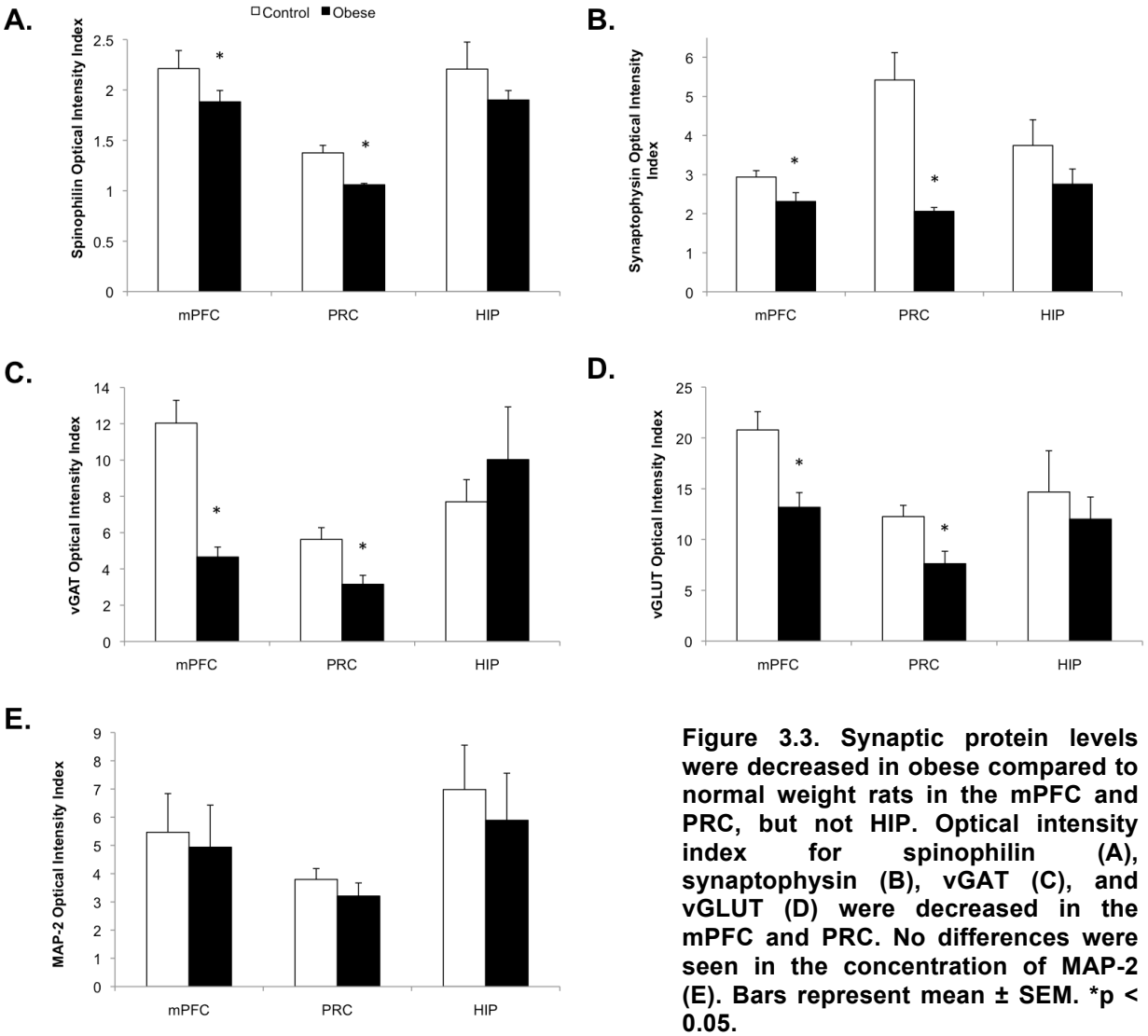


Figure 3.3. Synaptic protein levels were decreased in obese compared to normal weight rats in the mPFC and PRC, but not HIP. Optical intensity index for spinophilin (A), synaptophysin (B), vGAT (C), and vGLUT (D) were decreased in the mPFC and PRC. No differences were seen in the concentration of MAP-2 (E). Bars represent mean ± SEM. *p < 0.05.

Western blot analysis was used to validate the immunofluorescence data, and yielded the same results in the mPFC, PRC and HIP for spinophilin, synaptophysin, vGAT, vGLUT and MAP-2 as the optical intensity data. (**Fig. 3.4,** following page**;**

spinophilin: mPFC, t_{17} = 2.44, p = 0.03; PRC, t_{10} = 2.61, p = 0.03; HIP, t_{17} = 0.21, p = 0.84; synaptophysin: mPFC, t_{15} = 2.30, p = 0.04; PRC, t_{11} = 2.70, p = 0.02; HIP, t_{15} = 1.31, p = 0.21; vGAT: mPFC, t_{17} = 3.54, p = 0.003; PRC, t_{11} = 6.12, p < 0.0001; HIP, t_{17} = 0.48; p = 0.64; vGLUT: mPFC, t_{14} = 4.88, p < 0.0001; PRC, t_{11} = 3.05, p = 0.011; HIP, t_{15} = 0.17; p = 0.87; MAP-2: mPFC, t_{14} = 1.75, p = 0.10; PRC, t_{11} = 1.05, p = 0.32; HIP, t_{15} = 0.27, p = 0.79.)

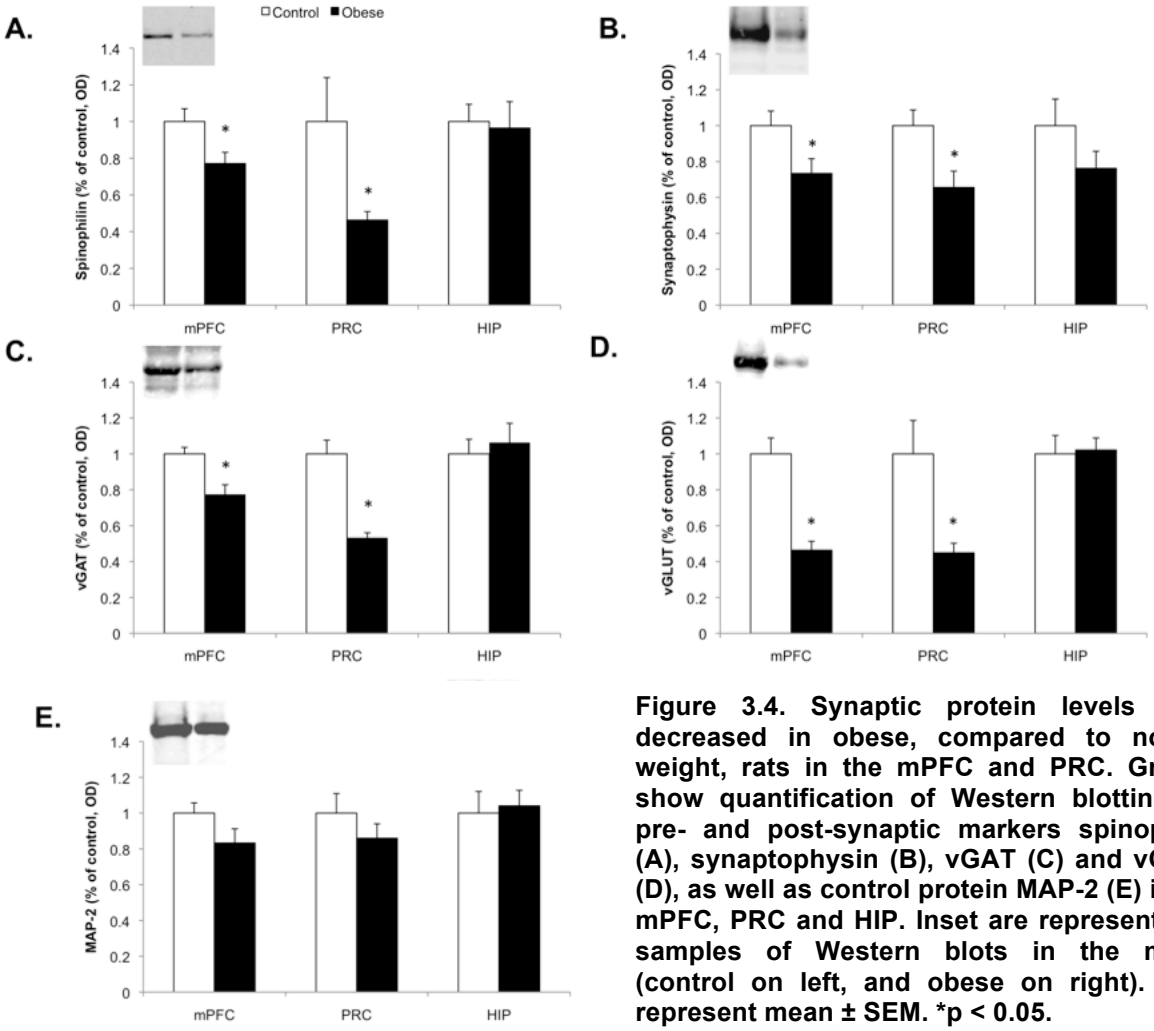


Figure 3.4. Synaptic protein levels were decreased in obese, compared to normal weight, rats in the mPFC and PRC. Graphs show quantification of Western blotting for pre- and post-synaptic markers spinophilin (A), synaptophysin (B), vGAT (C) and vGLUT (D), as well as control protein MAP-2 (E) in the mPFC, PRC and HIP. Inset are representative samples of Western blots in the mPFC (control on left, and obese on right). Bars represent mean ± SEM. *p < 0.05.

Recent evidence suggests that the ratio of VGLUT1/VGAT, which affects the excitatory/inhibitory neurotransmission balance, may be an important predictor of normal or abnormal cognitive functioning (Fung et al., 2011). In the current study, a difference was seen in the ratio between vGAT and vGLUT in the mPFC (optical intensity: t_{18} = 2.58, p = 0.02; obese: 0.38 ± 0.05 vs. control: 0.60 ± 0.07); Western blotting: t_{14} = 3.74, p = 0.002, obese: 1.69 ± 0.18 vs. control: 1.04 ± 0.07), but not in the PRC (optical intensity: t_{14} = 0.37, p = 0.72, obese: 0.56 ± 0.19 vs. control: 0.48 ± 0.06; Western blotting: t_{11} = 0.40, p = 0.69, obese: 1.26 ± 0.11 vs. control: 1.16 ± 0.23) or HIP (optical intensity: t_{16} = 1.02, p = 0.32, obese: 1.2 ± 0.57 vs. control: 0.62 ± 0.11); Western blotting: t_{15} = 0.84, p = 0.41, obese: 1.17 ± 0.14 vs. control: 1.02 ± 0.11).

Total brain mass was not altered in obese rats, but volume of mPFC was decreased

After perfusions, total brain mass was determined, with no differences between the obese rats and the normal weight rats (t_{18} = 1.25, p = 0.23, obese: 2.05 ± 0.07 g vs. control: 1.90 ± 0.09 g). However Cavalieri estimates of brain section volumes, determined in slices that were stained for Nissl substance by cresyl violet showed decreased brain volume in the mPFC of the obese rats compared to control rats (t_{15} = 2.50, p = 0.03), while no differences were seen in volume of the HIP between groups (t_{16} = 1.22, p = 0.24; **Fig. 3.5,** following page).

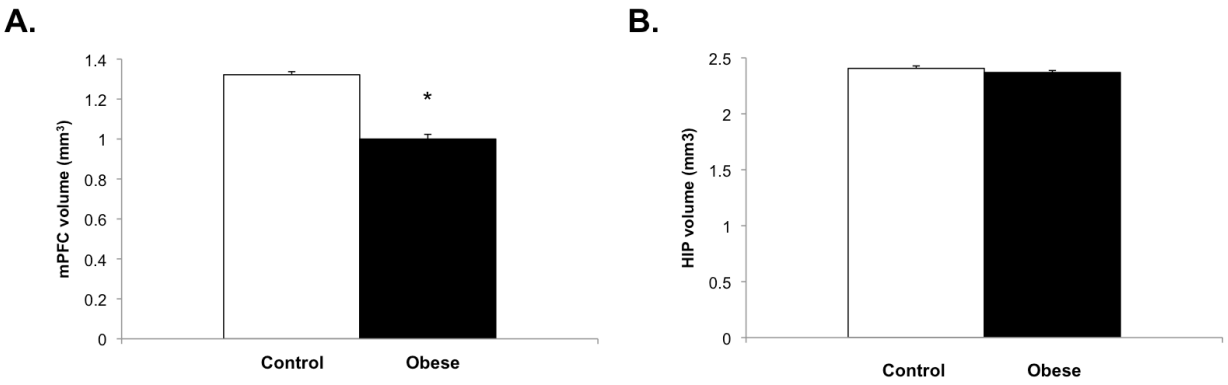


Figure 3.5. Using Cavalier's Principle, brain volumes were decreased in the mPFC of obese rats, compared to normal weight controls (A), but not in the HIP (B). Bars represent mean ± SEM. *p < 0.05.

Discussion

In this study we have shown that obesity influences the structure and biochemistry of rats' brains. After 8 weeks on a high-fat diet, decreased dendritic spine density on the apical aspect of pyramidal cells were found in layer II/III of the mPFC of obese rats compared to controls. And while a much smaller effect, decreased dendritic spine density was also found in obese rats, on the apical aspect of layer II PRC pyramidal cells. The decrease in spine density on mPFC and PRC pyramidal neurons occurred without concomitant changes in dendritic branching, indicating that changes in spine density reflect changes in the absolute number of spines. The decreases in spine density in the mPFC and PRC, seen here, also correlates with the impaired performance on the Novel Object Recognition task, Object-in-Place task and the Attentional Set-Shifting Task (Chapter 2), which are tasks dependent on the mPFC and/or PRC. It is of interest that the effect size of decreased dendritic spine density in the mPFC is much higher than that seen in the perirhinal cortex, potentially indicating that the mPFC is affected to a higher degree than the PRC. Further, given the clear

deficits in behavior on the PRC dependent task (Chapter 2), and the small effect of decreased dendritic spine density in PRC, it is possible that performance on the task was, in part, reflecting the strong, bidirectional connection between the mPFC and PRC. Given this connection, it could follow that decreased dendritic spine density in the mPFC could accentuate the mildly decreased dendritic spine density in the PRC, manifesting as apparent decreased performance on the PRC dependent task.

Golgi impregnation techniques assess, for the most part, spines emerging perpendicular to the dendritic shaft and parallel to the plane of the section. For this reason, estimates of spine densities using Golgi staining tend to be 3-4 times lower than those obtained with techniques such as confocal 3D reconstruction or serial electron microscopy imaging (Cerqueira et al., 2007). That said, the estimates in the current study are similar to those obtained by others, exploring the same brain regions, using similar approaches (Seib and Wellman, 2003; Silva-Gomez et al., 2003; Leuner and Gould, 2010).

While this dissertation discusses dendritic spines, as a single group, there are morphological diverse subgroups of spines. While classifications have been progressively refined, spines are generally assigned into three groups, based on relative sizes of the spine head and neck (Peters and Kaiserman-Abramof, 1970): mushroom spines, thin spines and stubby spines. Other identified spines include multi-head and filopodium spines (Nimchinsky et al., 2002). While these classifications are mostly qualitative, differences have emerged in the characteristics among each group. For example, the spine shape and volume is thought to be correlated with the strength and maturity of each spine-synapse, with mushroom spines being more likely to have

mature synapses, with larger, more complex post synaptic densities, than thin or filopodia-like spines (Bourne and Harris, 2008). Because we did not analyze the specific spine morphology, we do not know if differences among spine subgroups, and therefore spine maturity, exist in the brain regions of interest between obese and normal weight rats.

Since dendritic spines are a primary site of excitatory synapses (Holtmaat and Svoboda, 2009), obesity likely decreased the overall number of excitatory synapses in the mPFC and PRC. Detected decreases in levels of the pre-synaptic protein synaptophysin in the mPFC and PRC also suggest a decrease in the number of synapses. Changes in the levels of spinophilin and synaptophysin, may be directly related to degradation of synapses on spines, or they may also reflect decreased content of these proteins within synapses. The observations that MAP-2 was not altered by the obesity manipulation suggest that substantial global decrease in the levels of all neuronal proteins did not occur.

Further, dysregulation of spinophilin and synaptophysin, two dendritic spine-localizing proteins that have been implicated in long-term synaptic changes, may be directly associated with obesity-related impairments seen in Chapter 2. It has been found that spinophilin, a scaffolding protein, which was decreased in the mPFC of rats in the current study, affects glutamatergic synaptic transmission, specifically facilitating long-term depression. The protein also affects dendritic morphology, moderating spine density and neuronal migration (Allen et al., 1997; Feng et al., 2000; Sarrouilhe et al., 2006). Synaptophysin, an integral membrane glycoprotein found in pre-synaptic vesicles enabling exocytosis of neurotransmitter (Wiedenmann and Franke, 1985;

Edelmann et al., 1995), was also decreased in the mPFC of obese rats. While synaptophysin is thought to be a ubiquitous marker of synaptic integrity, it also determines synaptic strength and modulates efficacy of learning and memory. Synaptophysin knockout mice exhibit a wide variety of cognitive deficits, including impairments in spatial learning and object recognition (Schmitt et al., 2009). Rats maintained on a high-fat, high-sugar diet, and show insulin resistance, have reduced synaptophysin in the CA1 region of the hippocampus, as measured by optical intensity from immunofluorescence labeling (Stranahan et al., 2008).

vGAT and vGLUT are two other proteins whose potentially suboptimal concentrations in the relevant brain areas in obese animals could partially explain cognitive and memory deficits. vGAT is responsible for the uptake of GABA and glycine into synaptic vesicles, and since GABA concentrations have been shown to increase after inducing obesity via a high-fat diet and in type II diabetes from a homozygotic recessive mutation, this transporter may be down-regulated in the obese state (Chaudhry et al., 1998; Sickmann et al., 2012). Further, there is evidence that vGLUT, which regulates the amount of neurotransmitter packaged into vesicles at excitatory synapses, is important for long-term potentiation (LTP), a glutamate-dependent form of synaptic plasticity which is implicated in spatial learning and memory (Balschun et al., 2010). Obesity and type II diabetes may be associated with decreased glutamatergic activity in the cerebral cortex and the hippocampus, perhaps reflecting a down-regulation of vGLUT (Wojcik et al., 2004; Sickmann et al., 2010, 2012). Importantly, recent evidence suggests that the ratio of vGLUT/vGAT, which affects the excitatory/inhibitory neurotransmission balance, may be an important predictor of

normal or abnormal cognitive development (Fung et al., 2011). It is possible that the difference in ratio of vGAT to vGLUT between the obese rats and control rats in the current study could have profound effects on cognition.

It is of interest to note that both dendritic spine densities, as well as the concentrations of pre- and post-synaptic markers, were specifically altered in the apical, but not basal, aspect of the dendritic tree in both the mPFC and the PRC. Selective vulnerability of apical dendrites has previously been noted, specifically in the mPFC, when exploring the effects of stress on dendritic spine morphology (Wellman, 2001; Radley et al., 2004; Brown et al., 2005; Cerqueira et al., 2007). It has been suggested that this specification reflects the topographical distribution of inputs into layer II/III mPFC pyramidal cells. The basal and apical aspects of the dendritic tree receive afferents from distinctively different brain regions (Cerqueira et al., 2007), with, for example, the basal dendrites receiving thalamic projections (Shibata, 1993), and the apical dendrites receiving afferents from limbic structures, such as the hippocampus (Swanson and Cowan, 1977). These previous apical specific findings related to the effects of stress and glucocorticoids on brain structure and thus function, could allow us to begin to understand the apical specificity seen in the current study.

The extent to which the effects of obesity are specific to the mPFC and PRC circuitry remains unknown—our results leave open the question of the degree of regional specificity in the effects of obesity on the brain. However, interestingly, no effects were seen in the structure, biochemistry or functioning of the HIP, which could indicate some level of region specificity of deficits in obesity. The lack of disruption in HIP structure is also surprising given that previous research has indicated that negative

experiences, such as chronic restraint stress, produce dendritic shrinkage in the HIP (Liston et al., 2006; Radley et al., 2006) and impair cognitive performance on tasks requiring the same (McEwen, 2000b; Liston et al., 2006). Further, it has been established that chronic stress induces atrophy of apical dendrites of CA3 pyramidal cells, as determined by a decrease in the number of branch points. If we characterize obesity as a negative, or stressful biological state, we anticipate seeing structural and functional changes in the HIP. It is possible 8 weeks of an obesogenic state does not provide enough repeated biological stress, and that effects would be seen in the HIP after a longer duration of obesity. Others have also reported that long-term potentiation of the hippocampal CA1 region is markedly impaired in obese rats compared to lean rats (Gerges et al., 2003). And diminished performance on HIP tasks, such as the Morris water maze, has been shown in a mouse model of obesity (Wickelgren, 1998; Farr et al., 2008). It is important to note that in the previously mentioned study, animals were classified as leptin resistant (leptin has been shown to enhance hippocampal synaptic plasticity and improve learning and memory (Wickelgren, 1998; Farr et al., 2008)).

One role of leptin, a hormone produced predominantly in adipose tissue, is to signal fat storage reserves in the body, and mediates long-term appetitive controls (Friedman and Halaas, 1998), but centrally, it has also been shown to enhance hippocampal synaptic plasticity and improve learning and memory (Wickelgren, 1998; Farr et al., 2008). Leptin enhances cognition (Figlewicz et al., 2004; Farr et al., 2006), and specifically, leptin receptor-deficient animals have impaired hippocampal LTP and poor spatial memory (Li et al., 2002; Wayner et al., 2004). While obesity is associated

with central leptin resistance, it is often accompanied by increased peripheral leptin levels. Leptin resistance conjures different interpretations, and its complex nature gives way to several definitions. In the most general terms, leptin resistance has been identified as the inability of leptin, despite its increased levels in the periphery, to reduce obesity. While this resistance may be due to impaired cellular responses within specific brain regions (central leptin resistance), evidence indicates that, in models of diet-induced obesity, animals tend to maintain central leptin sensitivity, while developing peripheral resistance (Van Heek et al., 1997). For this reason, it is more likely that leptin deficiency, despite elevated peripheral levels, is caused by leptin's inability to reach target sites within the brain (peripheral leptin resistance). This could arise from triglyceride-mediated impairment of leptin transport across the blood-brain barrier, leading to defects in leptin receptor signaling, and blockades in downstream neuronal circuitries (Banks et al., 2004).

Another feeding-related peptide that might affect hippocampal memory, after longer-term obesity than that seen in the current study, is ghrelin. Ghrelin is produced by the stomach, with levels increasing before meals and decreasing after meals. It has been suggested to provide a peripheral signal to the brain that induces food intake and the associated weight gain (Wren et al., 2001). While increased ghrelin is associated with weight gain, obese individuals actually have decreased peripheral ghrelin levels (Tschop et al., 2001), which could have deleterious cognitive effects. In the brain it has also been suggested that ghrelin binds to neurons in the hippocampal formation, where it promotes dendritic spine synapse formation and generation of long-term potentiation, which is paralleled by enhanced spatial learning and memory. Targeted disruption of the

gene that encodes ghrelin resulted in decreased numbers of spine synapses in the CA1 region and impaired performance of mice in behavioral memory testing (Diano et al., 2006). In obese humans (Tschop et al., 2001) and mice (Perreault et al., 2004), ghrelin secretion is reduced and regulation is impaired. In the current study we do not examine ghrelin, it is possible that longer term obesity is needed to see these peripheral and central changes in ghrelin that could lead to hip dysregulation.

While I suggest that effects of obesity on hippocampus structure and function might be modulated by longer term obesity, and thus not apparent in the current study, it is important to note that some research indicates an effect of high-fat diet consumption, before the onset of obesity. For example, one study demonstrated that pre-obese male rats, but not female rats, fed a high-fat diet for four weeks, show decreased hippocampus neurogenesis along with elevated serum corticosterone levels, (Lindqvist et al., 2006) which are well known to inhibit hippocampal neurogenesis (Ambrogini et al., 2002; Heine et al., 2004), as well as decrease dendritic spine density (Sousa et al., 2000; Wellman, 2001; Liston et al., 2006; Radley et al., 2006; Hajszan et al., 2009). Further, diets rich in fat have been linked to changes in serum corticosterone levels. While some suggest serum levels of corticosterone increase in rats fed a high-fat diet as early as 7 days after initiation of the diet (Tannenbaum et al., 1997), potentially providing a mechanism for decreased neurogenesis, the literature on the effects of high-fat diet on corticosterone is mixed (Auvinen et al., 2011). The following chapter will examine corticosterone levels in the current DIO animal model after 8 weeks of diet access. We will also examine levels of other hormones, such as testosterone, which have also been associated with dendritic spine density.

Chapter 4

Diet induced obesity and changes in peripheral hormone levels

Abstract

Using an animal model of obesity (diet-induced obesity model), we have found deficits among obese rats in behavioral tasks requiring the medial prefrontal cortex (mPFC) and perirhinal cortex (PRC), but not the hippocampus (HIP). Accompanying these deficits, we have found decreased dendritic spine density of pyramidal neurons in layer II/III of the mPFC and layer II of the PRC, as well as decreased synaptic protein concentrations in the same regions. Deficits in behavior and function in brain regions involved in memory have previously been associated with abnormal hormone levels. In the current study, we examined peripheral levels of certain hormones, including corticosterone, testosterone, insulin and leptin, in the DIO model. Abnormal corticosterone levels (either high or low) are typically associated with decreased dendritic spine density, and the obese rats in the current study had increased peripheral corticosterone levels. There was also no difference in testosterone, glucose and insulin levels between groups. However, the obese rats did show hyperleptinemia compared to controls.

Introduction

There are several hormonal abnormalities associated with obesity, including

changes in glucocorticoid, testosterone, insulin and leptin levels (Kahn and Flier, 2000; Rosmond et al., 2000; Stanworth and Jones, 2009). Independently, dysregulation of each of these hormones has been associated with changes in dendritic architecture. Heightened, as well as diminished, glucocorticoid levels have been associated with diminished dendritic architecture and spine density in the mPFC and HIP (Sousa et al., 2000; Wellman, 2001; Liston et al., 2006; Radley et al., 2006; Cerqueira et al., 2007; Hajszan et al., 2009). Low levels of testosterone, such as those seen in obesity, have been linked to decreased dendritic spine density in the HIP (Leranth et al., 2003; Brand et al., 2011). Diabetes, a condition defined by insulin resistance and accompanied by leptin resistance, has been correlated with decreased spine density in the mPFC (Joghataie et al., 2007). As previously stated, reduced dendritic architecture and spine density (as seen in Chapter 3) in the mPFC and HIP occur in association with decreased performance on cognitive tasks that require these brain areas (as seen in Chapter 2) (Sousa et al., 2000; Liston et al., 2006; Radley et al., 2006). Thus, this chapter will examine hormones that have previously been linked to changes in brain structure and obesity, with the aim of identifying potential mechanisms for future intervention.

In addition to cognitive deficits, obese individuals also show increased instances of anxiety (de Wit et al., 2010). Further, exaggerated adrenocortical axis activity has been reported in several models of obesity (York, 1996; Parente et al., 2008), and it has been suggested that glucocorticoids may actually contribute to obesity by reducing the sensitivity of appetite to leptin regulation (Solano and Jacobson, 1999). Heightened glucocorticoid levels have also been associated with diminished dendritic architecture

and spine density in the mPFC and HIP (Sousa et al., 2000; Wellman, 2001; Liston et al., 2006; Radley et al., 2006; Hajszan et al., 2009), similar to those observed in the current study in the mPFC of obese animals. Further, reduction in dendritic architecture and spine density occur in association with decrements in cognitive tasks that require the effected brain regions (Sousa et al., 2000; Liston et al., 2006; Radley et al., 2006). Given the diminished cognitive abilities and dendritic architecture described in Chapters 2 and 3, it follows that elevated glucocorticoids might be playing a role in promoting these findings in obese animals. Again, some clinical reports conflict with this theory, indicating low corisol, which is typically associated with a low-anxiety state, in obese patients (Jessop et al., 2001; Parra et al., 2006). In order to further examine the role of the glucocorticoids, to supplement to serum measurement of corticosterone, behavioral assays of anxiety, which accompanies elevated corticosterone levels, were used to screen for the anxious phenotype in our animal model of obesity. Accompanying serum corticosterone levels were determined.

It should be noted that it is challenging to determine cause and effect of aberrant corticosterone levels. It is possible that obesity initially produces dysregulation of the HPA axis, which would in turn affect glucocorticoid levels (either raising or depressing them, as previously discussed), which could then impair dendritic architecture and function. An equally plausible model proposes that the metabolic disturbances of obesity initially compromise neurons, altering their functions. Since both the HIP and mPFC have been linked to feedback of the stress response (Herman et al., 1989; Jacobson and Sapolsky, 1991; Diorio et al., 1993; Herman and Mueller, 2006), impaired dendritic architecture in these areas might contribute to HPA axis dysfunction. Further,

changes in the regulation of the HPA axis might expose the HIP and mPFC to aberrant levels of glucocorticoids that would produce additional alterations in dendritic architecture and related behaviors, acting as a feedback mechanism. While it is difficult to determine the specific causal links among these events, data examining these changes provides an important first step.

In this chapter, we examined a few other peripheral hormones that have been linked to both reduced dendritic spine density and obesity. Testosterone is one such hormone that is not only dysregulated in obesity, but low levels of testosterone, such as those seen in obese males (Brand et al., 2011), have been linked to decreased dendritic spine density in the HIP (Leranth et al., 2003; Brand et al., 2011).

Diabetes, a condition defined by insulin resistance and accompanied by leptin resistance, has been correlated with decreased spine density in the mPFC (Joghataie et al., 2007). Because the animals in the current study have only had 8 weeks access to a high-fat diet, they should be in a pre-diabetic state. However, given the previous link between insulin resistance and dendritic morphology, it is important to verify that this does not account for the currently observed diminished spine density.

Methods

Testing was done in rats described in Chapters 1 and 2. Male Sprague–Dawley rats (275-315 g at the onset of the study) were obtained from Charles River (Germantown, NY) and housed individually on a 12 h reverse light cycle (lights off at 6am). Two cohorts of animals were used; in each cohort, rats were weight-matched and divided into two groups (n=10/group), and given access to either a nutritionally

complete, high-fat diet (4.7 kcal/g; Research Diets, New Brunswick, NJ, #12451; 45% fat, 20% protein, and 35% carbohydrate) or standard rodent chow (3.01 kcal/g; LabDiet #5001, PMI Nutrition International, Richmond, IN; 10% fat, 20% protein, 70% carbohydrate). All animals had ad libitum access to water. After 8 weeks on the described diets, testing began. Rats with body weights that overlapped between groups were removed from analysis, such that each cohort ended up with n=8-9/group.

Anxiety Testing

Open Field Maze

Time spent on the periphery versus the center of an open field was used as an indicator of anxiety (Belzung, 1999). Animals were placed in a computerized open-field activity chamber (43.2 × 43.2 cm × 30.5 cm) with 16 infrared photocells on each of the three axes (MED Associates, Georgia, VT). Animals were placed in the chamber under full light for 30 min. The first 10 minutes were analyzed for time spent in the center vs. surround.

Novelty Suppressed Feeding

Novelty supressed feeding implements a conflict that elicits competing motivations: the drive to eat and the fear of venturing into the center of the brightly lit arena, and can be used to identify behavioral anxiety (Shephard and Broadhurst, 1982). In this test, animals are food deprived overnight, and then put in a novel open arena (58 × 42 × 35 cm) with food placed in the center. The rat was placed in the open field, under full light, and the amount of time it took the animal to approach the food was

recoreded. If the rat did not approach the food after 6 min, the testing is terminating, and the animal is given a score of 360 sec.

Hormone Analysis

Upon the completion of behavioral testing, one cohort of animals was swiftly sacrificed via rapid decapitation, ~4 h into the dark period. Serum was processed and analyzed for corticosterone and testosterone, using commercially available solid-phase radioimmunoassay systems (Coat-A-Count kits, Diagnostic Products Corp., Los Angeles, CA).

Upon the completion of behavioral testing in a second cohort of animals, rats were fasted overnight, and then swiftly sacrificed via rapid decapitation, ~4 h into the dark period. Serum was processed and analyzed for blood glucose, insulin and leptin levels. Plasma glucose analysis was performed using the glucose oxidase method (glucose [trinder] kit, Sigma Chemical Co., St. Louis, MO). Insulin and leptin were measured using commercially available radioimmunoassays, according to manufacturers' instructions (Linco Research Inc., St. Charles, MO).

Statistical Analysis

Data was analyzed using independent Students t-tests, comparing serum hormone values between the obese rats and control rats.

Results

Obese rats had decreased serum corticosterone and no indications of anxiety

Because abnormal glucocorticoid levels have been associated with diminished dendritic architecture and spine density in the mPFC and HIP (Sousa et al., 2000; Wellman, 2001; Liston et al., 2006; Radley et al., 2006; Hajszan et al., 2009), serum corticosterone was analyzed. Obese rats had decreased corticosterone compared to the normal weight controls (t_{14} = 2.38, p = 0.03; obese = 46.52 ± 11.69 µg/dl, control = 124.03 ± 34.03 µg/dl). Behavioral tests for anxiety, including latency to feed and time spent exploring the center of an open field, showed no differences between groups (latency to feed: t_{14} = 0.19, p = 0.85, obese: 247.8 ± 41.0 sec vs. control: 247.4 ± 36.6 sec; open field: t_{14} = 0.71, p = 0.49, time in center of open field obese: 71.1 ± 16.2 sec vs. control: 87.6 ± 16.4 sec).

Obese rats had increased peripheral leptin levels

There was no difference in testosterone levels between groups (t_{14} = 1.21, p = 0.25; obese = 1.67 ± 0.26 ng/mL, control = 2.32 ± 0.47 ng/mL). Nor were there any differences between groups for fasting blood glucose levels (t_{14} = 1.44, p = 0.17; obese = 261.3 ± 7.5 mg/dL, control = 275.2 ± 6.1 mg/dL) or fasting insulin levels (t_{14} = 1.52, p = 0.15; obese = 2.68 ± 0.40 ng/mL, control = 1.95 ± 0.26 ng/mL). There was, however, a difference in serum leptin levels between groups (t_{14} = 6.71, p < 0.001; obese = 23.12 ± 2.26 ng/mL, control = 6.87 ± 0.86 ng/mL).

Serum Insulin and the Quantitative Insulin Sensitivity Check Index

Fasting serum glucose and insulin levels, reported above were used to determine the Quantitative Insulin Sensitivity Check Index (QUICKI). QUICKI is a mathematical model based on log-transformed fasting plasma glucose and insulin values, as follows: 1/(log [glucose] + log [insulin]), which predicts insulin sensitivity, with lower values representing more insulin resistance (Reungjui et al., 2007). In the current study, there was no difference in QUICK index between groups (t_{14} = 0.70, p = 0.50; obese = 0.36 ± 0.01, control = 0.37 ± 0.01), indicating

Discussion

Given the literature showing comorbidities in clinical populations between obesity and anxiety disorders (Becker et al., 2001; Ciechanowski et al., 2006), it was anticipated that the obese animals in the current study would show anxiety-like behavior, however, they did not. While contrary to the clinical literature on anxiety, these data are not wholly unexpected seeing that the animal literature on anxiety in obese models is limited and mixed (Chaouloff, 1994; Souza et al., 2007). For example, there are studies indicating that a high-fat diet can actually be protective against anxiety-like behavior. One week of access to a high-fat diet has been shown to decrease anxiety response on the elevated plus-maze (Prasad and Prasad, 1996). In the current study, we were careful to select two separate behavioral tests of anxiety, given that one was a food-motivated task, and could be confounded by differences in motivation salience. However, both of the anxiety-related tasks agreed, showing no differences in anxiety between the obese rats and the control rats, lending more credence to the data. Concurrent with the results on the anxiety testing, obese rats had decreased corticosterone levels compared to

controls. In the clinical literature, decreased cortisol has been observed in obese individuals (Jessop et al., 2001; Parra et al., 2006). Increased levels of circulating glucocorticoids are, however, often documented in obese with metabolic syndrome, which can develop with the progression of an obesogenic state (Bjorntorp and Rosmond, 2000b). It is possible that in the current DIO model, longer-term access to a high-fat diet, and the development of a metabolic syndrome state, could lead to elevated corticosterone levels. From this, we could propose that the metabolic disturbances of obesity initially compromise neurons, altering their function, and then initiating an HPA axis response. Since the mPFC has been linked to feedback of the stress response (Herman et al., 1989; Jacobson and Sapolsky, 1991; Diorio et al., 1993; Herman and Mueller, 2006), impaired dendritic architecture in this area might contribute to HPA axis dysfunction. Further, changes in the regulation of the HPA axis might then expose the mPFC to high levels of glucocorticoids that would produce additional alterations in dendritic architecture and related behaviors, acting as a feedback mechanism.

Alternatively, there is evidence in the literature that abnormally low corticosterone levels could lead to changes in dendritic morphology in the mPFC (Sousa et al., 1997; Cerqueira et al., 2007), effecting performance on mPFC mediated tasks, similarly to that seen in Chapter 2 (Mizoguchi et al., 2004; Cerqueira et al., 2005). Changes in dendritic morphology in the mPFC, which is important in anxiety regulation, could ultimately participate in initiating an HPA axis response, leading to the elevated corticosterone levels often associated with metabolic syndrome. This could then lead to further decrements in structure and functioning of the mPFC, as well as the HIP.

Also relevant, it has been suggested in the clinical literature that while glucocorticoids secretion might be elevated in the obese state, serum glucocorticoid concentrations are normal or low, due to enhanced peripheral uptake (Bjorntorp and Rosmond, 2000a; Salehi et al., 2005). In the animal literature, it has been shown that obese rats have normal levels of circulating glucocorticoids, but elevated levels in adipose tissue (Livingstone et al., 2000; Dallman et al., 2004).

It is important for future studies to further address the role of glucocorticoids in the early-obese state. Potentially, treating the hypocorticosteroid levels in early obesity could stall the development of further cognitive decline, and the potential future compromise of the HIP structure and functioning, and potentially avoid the elevated glucocorticoids levels seen in metabolic syndrome. While decreased glucocorticoids are linked to cognitive impairments, the link between excessive glucocorticoids and cognitive decline is more concretely established. Higher plasma cortisol levels are associated with poorer age-related cognitive ability in healthy male volunteers (MacLullich et al., 2005), and higher cortisol levels have also been associated with poorer cognitive function in domains including mental flexibility, non-verbal memory, immediate and delayed memory and general cognitive ability (Reynolds et al., 2010). In addition to cognitive decline, glucocorticoids have been linked to physiological changes. Glucocorticoids act at target tissues throughout the body to confer physiological changes that enable an organism to deal with an acute stressor and then return to a pre-stress level of functioning. However, while this response is advantageous for survival, repeated HPA axis activation can produce damaging physiological effects and exert a profound impact on brain function. For example, in the hippocampus, chronically

elevated glucocorticoids lead to dendritic remodeling of CA3 neurons, decreased neurogenesis, and even cell death (Sapolsky et al., 1985; Watanabe et al., 1992; Magarinos et al., 1998; McEwen, 2000a; Pham et al., 2003). The effects of chronic glucocorticoid exposure are not limited to the hippocampus; elevated corticosterone levels have been associated with diminished dendritic architecture and spine density in the mPFC (Wellman, 2001; Radley et al., 2006), potentially leading to even more pronounced deficits in the functioning of this area than seen in the current study.

While glucocorticoids have been shown to be key in diminishing dendritic architecture, and the associated functions, there have also been studies indicating that, in certain circumstances, elevated corticosterone can be associated with increased dendritic spine density and improved cognitive flexibility (Leuner and Gould, 2010). Therefore, corticosterone is not the only factor effecting dendritic spine changes. Another hormone that has been implicated in dendritic architecture is testosterone.

Clinical evidence indicates that testosterone levels are decreased in obesity (Stanworth and Jones, 2009). Low levels of testosterone have been linked to decreased dendritic spine density in the HIP (Leranth et al., 2003; Brand et al., 2011) and results from cell culture and animal studies provide convincing evidence that testosterone could have protective effects on brain function (Holland et al., 2011). In the current study, there was no difference between testosterone levels in the obese and control rats. Hyperinsulinemia, which was not seen in the current study, is shown to suppress serum testosterone levels (Pasquali et al., 1997). Again, this could indicate that the metabolic disturbances of obesity initially compromises neurons, altering their functions, which has

a downstream effect on testosterone levels. The decreased testosterone levels could then feedback to further effect dendritic spine density. However, more studies would be required to address this hypothesis.

Previous research has indicated diabetes effects cognitive functions such as intelligence, attention, psychomotor speed, cognitive flexibility, learning and memory (McCrimmon et al., 2012), and is associated with an increased risk of developing dementia (Yaffe et al., 2004b; Biessels et al., 2006; Kloppenborg et al., 2008), and Alzheimer's disease (Yaffe et al., 2004a; Biessels et al., 2008). In animal models of streptozotocin-induced diabetes, after no less than 8 weeks of disease induction, decreased dendritic spine densities in the prefrontal cortex, occipital cortex, and hippocampus have been observed (Martinez-Tellez et al., 2005; Joghataie et al., 2007; Malone et al., 2008). While each of these previous studies relies on streptozotocin-induced diabetes, maintaining rats on a high-fat, high-sugar diet for 8 months showed alterations in energy and lipid metabolism similar to clinical diabetes, including elevated fasting glucose levels and increased cholesterol and triglycerides. These rats also exhibited impaired spatial learning and reduced HIP dendritic spine density (Stranahan et al., 2008). While this relationship between diabetes and dendritic spine architecture is of interest, the current study focuses on a well, rather than sick, model of obesity. In order to confirm that insulin resistance, or a diabetic state, is not responsible for the changes in brain structure and function in Chapters 2 and 3, it was necessary to determine fasting blood glucose levels and fasting insulin levels, and from them estimate insulin resistance. The “gold standard” method of measuring insulin resistance

is the, rather invasive and time-consuming, euglycemic hyperinsulinemic clamp (DeFronzo et al., 1979). As an alternative, fasting serum insulin levels have been used as a crude estimate of insulin sensitivity (Ferrannini and Mari, 1998), but is not valid in conditions of impaired insulin secretion, such as type II diabetes. The QUICKI, implemented in the current study, provides a simple indirect index of insulin sensitivity, and allowed us to confirm no signs of insulin resistance or diabetes. It is possible that longer-term access to the high-fat diet (such as the 8 month access period used in Stranahan, 2008) would result in a diabetic-like state, and further cognitive and structural changes. However, the current results indicate that these cognitive deficits are not limited to the ill-obese, but can affect the well-obese. And further, cognitive deficits are not dependent on a preexisting state of insulin resistance, but rather, they might precede the onset of diabetes, and worsen as the disease develops and progresses.

Finally, there was a difference between the obese and control groups in serum leptin levels, with obese rats showing hyperleptinemia. This was anticipated given the literature that in the rat, as little as 3 days access to a high-fat diet can lead to elevated peripheral leptin levels (Wang et al., 2001). The relationship between hyperleptinemia and leptin resistance is difficult to untangle. Mice whose plasma leptin levels have been clamped, develop obesity in response to a high-fat diet, but do not develop leptin resistance (Knight et al., 2010). From examples such as this, it has been suggested that this initial increase in leptin level seen with intake of a high-fat diet and obesity is a causative factor in leptin resistance. The resulting leptin resistance increases susceptibility to further diet-induced obesity, creating an unregulated cycle of weight gain (Zhang and Scarpace, 2006). In the current study, we demonstrate

hyperleptinemia. However, because of the species and strain differences on the temporal development of diet-induced leptin resistance (Scarpace and Zhang, 2009), it is unclear if these rats are definitively experiencing leptin resistance, we have only the assumption that increased periphery and an inability to combat weight gain indicates leptin resistance, which conflicts with other studies, indicating it takes a longer duration of high-fat diet access for rodents to develop leptin resistance.

In rodents, leptin insensitivity is associated with cognitive deficits, especially in spatial memory (Li et al., 2002). Further, previous studies have implicated leptin in hippocampal synaptic plasticity (Wayner et al., 2004; Harvey et al., 2006). Leptin has also been shown to rapidly increase the density and motility of dendritic filopodia in hippocampal neurons and subsequently increases the density of HIP synapses (O'Malley et al., 2007). This provides evidence that leptin resistance might be involved in providing the sort of dendritic remodeling seen in the current study. While a relationship between leptin and the HIP functioning is established in the literature, the current study did not identify deficits in HIP structure or function. It is possible that in the current study we have an instance of hyperleptinemia, but it has not yet developed into leptin resistance. A longer duration of obesity could lead to definite central leptin deficiency, and pronounced effects in the HIP. While potential cognitive-related roles for leptin in the mPFC and PRC (directly or indirectly) are yet to be described, it is known that leptin receptors and projections are observed throughout the prefrontal cortex (Patterson et al., 2011), the role of leptin on cognitive function in these regions merits further investigation.

While these findings results are preliminary and superficial, one might suggest leptin as a potential therapeutic target in restoring cognition in obesity. That said, leptin therapy is extremely difficult. Peripheral levels of leptin are already elevated in the obese state, and it has been shown that the peripheral administration of additional leptin fails to reverse the obese state (Spiegelman and Flier, 2001). However, leptin injected directly into the brain suppress food intake in a mouse DIO model (Van Heek et al., 1997; El-Haschimi et al., 2000), indicating that the challenge in obesity is not a lack of leptin, but rather directing leptin to the appropriate brain targets. If, as previously suggested, elevated triglyceride levels block the transportation of leptin across the blood brain barrier, it has been suggested that administering a triglyceride-lowering drug, such as gemfibrozil, might lead to better cognitive function in the obese via increasing the ability of leptin to reach its necessary target locations. In fact, this theory supported by very limited evidence in rodents (Farr et al., 2008) and humans (Rogers et al., 1989) showing increased memory after gemfibrozil treatment. However, more research into the role of this hormone in obesity and cognition is necessary before making curative recommendations.

The list of hormones explored in the current chapter is not exhaustive, and there are other hormones that are linked to structural and functional deficits in cognitive-related brain areas, such as thyroid hormone, that might be more appropriate therapeutic targets.

Chapter 5

General Conclusions

Summary

This dissertation demonstrates that obesity influences the structure and biochemistry of rats' brains. After 8 weeks on a high-fat diet, decreased dendritic spine density on the apical aspect of pyramidal cells were found in the layers II/III of the mPFC and, to a lesser extent, in layer II of the PRC, but not the HIP. The observed decrease in spine density on mPFC and PRC pyramidal neurons occurred without a concomitant change in dendritic branching, indicating that changes in dendritic spine density reflect changes in the absolute number of spines. The concentrations of several pre- and post-synaptic markers were also decreased in these areas. The decreases in spine density and synaptic markers were accompanied by impaired performance on the Novel Object Recognition task, Object-in-Place task and the Attentional Set-shifting task, which are tasks requiring functioning mPFC and/or PRC. These deficits were accompanied by decreased peripheral corticosterone, but no differences in peripheral testosterone, as hypothesized. An increase in peripheral leptin was noted. While in the current study, behavioral and biochemical effects in the mPFC and PRC, but not HIP, could indicate region specificity, the extent to which the effects of obesity are specific to the mPFC and PRC circuitry remains unknown—our results leave open the question of the degree of regional specificity in the effects of obesity on the brain.

Possible functional implications of obesity brain changes

The decrease in dendritic spine density that accompanied obesity in the current studies has many potential implications for the functioning of the affected neurons. Individual synaptically connected dendritic spines can act as specialized compartments for rapid calcium signaling (Nimchinsky et al., 2002). This calcium signaling controls the induction of synaptic plasticity (Yang et al., 1999; Yuste and Bonhoeffer, 2001), via long-term potentiation and depression, which has been suggested as a mechanism for learning and memory (Malenka and Bear, 2004). It has been suggested that the presence of spines enhances synaptic efficacy and thereby the level of excitability of the network involved in the learning process. This would indicate that learning is not dependent on changes in spine density but that changes in spine density provide support in processing information used in memory formation (Leuner and Shors, 2004). However, it is debatable if changes in excitability results in changes in spine density or vice versa. Either way, the resulting changes in excitability can enhance future processing capabilities and have extensive effects on cell functioning. That said, decreased spine density, such as that seen in the present study, will lead to decreased anatomical support for learning, which would manifest itself as decreased performance on behavioral cognitive tasks.

The flexibility of dendritic spine density

While it is well documented that environmental influences can lead to spine pruning, which leads to decreased dendritic spine density such as that seen in the

current study, there is also evidence that this can be reversed. Mice raised in complete darkness show decreased spine density in layer V pyramidal neurons in the primary visual cortex (Valverde, 1967), however, spine density is recovered in some dendrites after only a few days of normal lighting (Valverde, 1971). Similarly, in the rat hippocampus, spine density of dentate granule cells is decreased after entorhinal cortex lesions, but then return to baseline levels with re-afferentation by the sprouting of nearby axons (Parnavelas et al., 1974). These previous studies illustrate the bidirectional nature of structural changes and indicate that dendritic spine density, and potentially the associated cognitive functioning, could be restored in the obese model. The plasticity of synaptic spines can also be seen in cases such as depression, where stress-induced reductions in hippocampal spine synapses and dendrites are reported, but these effects can be reversed by treatment with antidepressants (Bessa et al., 2009; Hajszan et al., 2009). In the current study, weight loss, or drug and hormone therapies may be effective in returning dendritic spine structure and function to pre-obese levels.

Increasing synaptic strength tends to produce new spines, as well as enlarge and stabilize existing spines, whereas synaptic depression causes spine shrinkage and retraction (Harms and Dunaevsky, 2007). Given the flexible nature of dendritic spine density in response to afferent input, while changes were not seen in the hippocampus in the current study, the direct connection between the PRC and the indirect connection between the mPFC and the hippocampus could lead to eventual decreased spine density in the hippocampus.

Sex differences

All of the studies in this dissertation were performed in male rats. Future studies will benefit from the exploration of the role of obesity in neurological architecture and function in female rats. In females, it has been suggested that estrogen is protective against food overconsumption and weight gain (van Seumeren, 2000; Roesch, 2006) (which explains, in part, why weight gain is seen during peri-menopausal years (van Seumeren, 2000)), as well as cognitive decline (Sherwin, 2006). Specific evidence shows that estrogen can increase dendritic spine density on hippocampus CA1 pyramidal neurons (Gould et al., 1990; Woolley et al., 1990b). Further, in females, central fat accrual leads to a predisposition to insulin resistance and hyperinsulinemia, which has been shown to stimulate testosterone synthesis, while simultaneously inhibiting binding functions, thereby enhancing levels of free testosterone. High levels of testosterone may then induce further accumulation of abdominal fat and insulin resistance (Hirschberg, 2009), providing a feedback mechanism that could exaggerate obesity and the associated cognitive effects. This is interesting given that testosterone levels are often decreased in obese males. Given the role of estrogen hormones in dendritic spine density, it would be interesting to examine brain structure and function in pre- and post-menopausal animal models of obesity.

Lack of deficits seen in structure and function of the hippocampus

One of the most surprising results seen in the current study is the lack of deficits in the behavioral and cellular analysis of the hippocampus. Given the clinical literature

showing decreased brain volume of the hippocampus (Walther et al., 2010), as well as previous animal studies that report that long-term potentiation (LTP) of the hippocampal CA1 region is markedly impaired in obese rats compared to lean rats (Gerges et al., 2003), as well as diminished performance on HIP tasks, such as the Morris water maze (Wickelgren, 1998; Farr et al., 2008). These results are also surprising given that previous research has indicated that negative experiences, such as chronic restraint stress, produce dendritic shrinkage in the HIP (Liston et al., 2006; Radley et al., 2006) and impair cognitive performance (McEwen, 2000b; Liston et al., 2006). It is possible that HIP deficits are not seen in the well-obese, and that some co-morbidity, such as insulin resistance is necessary to precipitate HIP deficits. These deficits may come after a longer duration of high-fat diet access. Further, this could provide evidence that changes in brain structure, biochemistry or functioning are the result of obesity, rather than a predisposing condition. Future studies could study multiple time points of obesity, in order to determine if deficits in function and structure of the HIP emerge later in the obesogenic state. Though the current set of studies implemented several tests of behavioral cognition, this set of tasks was not exhaustive. Future studies could implement other hippocampus-dependent tasks, such as trace eye blink conditioning and context fear conditioning, to enhance the current findings. It is possible that the Object-in-Place task is not sensitive enough to uncover emerging behavioral deficits, and alternative tasks are needed. However, the lack of changes in both the HIP CA1 and CA3 regions, as well as the consistency across all the behavioral and brain results lends support to the efficacy of the data.

Role for corticosterone in obesity

Another interesting finding in the current study was the role of the glucocorticoid, corticosterone. Both elevated and depressed corticosterone levels have been associated with diminished dendritic architecture in the mPFC (Wellman, 2001; Radley et al., 2006; Sousa et al., 1997; Cerqueira et al., 2007). Reduced dendritic architecture and dendritic spine density occur in association with decrements in cognitive tasks that require the mPFC (Liston et al., 2006; Radley et al., 2006). In the current study, corticosterone levels in the obese animals were lower than those seen in controls, and no anxiety-like behaviors were noted in the obese group. While the literature is mixed on the role of glucocorticoids in obesity, some report decreased cortisol in obesity. Some have even found that cortisol secretion is elevated but that circulatory concentrations are normal or low, suggesting that peripheral disappearance rate is elevated (Bjorntorp and Rosmond, 2000a). Other report hyper-adrenocortical axis activity in clinical findings and models of obesity (York, 1996; Parente et al., 2008), and it has been suggested that glucocorticoids may contribute to obesity by reducing the sensitivity of appetite to leptin regulation (Solano and Jacobson, 1999).

Previously we put forward a hypothesis that proposes that obesity initially produces dysregulation of the HPA axis, perhaps by altering the availability of energy stores, which, in turn, elevates glucocorticoid levels and leads to diminished dendritic architecture and function of the mPFC. However, another equally plausible model, that might account for the observed results, is that obesity initially produces dysregulation of the HPA axis, leading to *decreases* in glucocorticoid levels, which can also result in

diminished dendritic architecture and function of the mPFC. It is also possible that metabolic disturbances of obesity may initially compromise neurons in the mPFC, ultimately altering their functions, and since the mPFC has been linked to feedback of the stress response (Jacobson and Sapolsky, 1991; Diorio et al., 1993; Herman and Mueller, 2006), impaired architecture in these areas might contribute to HPA axis dysfunction (possibly leading to decreased glucocorticoids early in obesity, and elevated glucocorticoids later). Further, changes in the regulation of the HPA axis might expose the hippocampus and mPFC to aberrant levels of glucocorticoids that would produce additional alterations in dendritic architecture and related behaviors, potentially extending the effects from just the mPFC, to both the mPFC and HIP. While all these models are theoretically possible, it is difficult to determine the specific sequence of events and causal links among these changes, but future data examining the time course of these effects might be instructive.

As previously stated, in obese humans, it has been reported that cortisol secretion is elevated but that circulatory concentrations are normal or low, suggesting that peripheral disappearance rate is elevated (Bjorntorp and Rosmond, 2000a). Alternatively, it is possible that obesity changes other HPA axis indices, such as corticosteroid binding globulin levels or glucocorticoid receptor density in the brain. In the current study, only circulatory levels of corticosterone were examined, and future research might benefit in looking at other related measures, such as receptor density.

It would be further informative to examine corticosterone levels at baseline and after restraint stress and recovery from restraint stress, in order to further elucidate functioning. In addition, baseline measures of trunk blood could be obtained at different

time points in the diurnal cycle, specifically just after lights on (start of the inactive period) and just after lights off (start of the active period). If dysregulation of the glucocorticoids levels are observed, we can then design experiments to assess the effects of normalizing corticosterone regulation in obese rats, and examine consequential changes in dendritic architecture and function. For example, rats on different diets could be subjected to an adrenalectomy, followed by replacement of corticosterone at a low dose in the drinking water or sham operation. This would allow us to control corticosterone levels, while maintaining a relatively normal diurnal rhythm (rats drink more water, and hence have an increase in corticosterone at the start of their active period, during which endogenous corticosterone levels are naturally higher) (Stranahan et al., 2006). It would also be of interest to examine corticosterone at many time points from the onset of obesity to the development of metabolic syndrome, and determine factors (potentially, such as the development of insulin resistance) that might be driving a transition from decreased corticosterone levels in early obesity (such as those seen in the current study) and elevated corticosterone levels reported in metabolic syndrome. Understanding the events leading up to and accompanying this transition may lead to potential therapeutic targets.

Other potential mechanisms

In the current study, we did not see any difference in testosterone levels in obese animals. However, leptin levels were elevated, and could prove to be one potential therapeutic target in addressing cognitive deficits in obesity. That said, the current results, in the context of the available literature, are not complete enough to make such

a recommendation. Further research would benefit from elucidating the role of leptin in non-appetitive cognition. While hormones such as the glucocorticoids and leptin should be further investigated, it would also be beneficial to examine other potential mechanisms for structural and functional change, including additional hormone and growth factor influences, such as thyroid hormone. Identifying potential mechanisms responsible for deficits in cognitive function will also provide potential intervention targets.

Future directions

The experiments in this dissertation suggest a number of questions for future studies. One of those questions pertains to the breadth of brain dysfunction. We present data suggesting deficits in the mPFC and PRC. There are several other brain regions that may be affected in the obesogenic state; this set of studies does not inform how widespread these brain deficits are spread. However, the fact that we do no see effects in the HIP, or on sensory and motor tasks, indicates that the effects are not global. Future studies should determine how wide spread brain structure and function deficits span.

As previously stated, it is important to explore gender differences in brain structure and function in obesity. However, in addition to gender, age is another factor that could play a role in the effects of obesity. Especially with the rates of childhood obesity increasing (Ogden et al., 2010) it is important to understand age-specific effects of obesity. In addition to age, future experiments should determine the effects of the duration of diet exposure. For example, the current study could be repeated at the

following diet-duration time points: 1 day (a time point designed to assess the effects of novelty in the diet without substantial changes in body weight), 2 weeks (a time point when the diet is no longer novel and weight gain is not yet excessive), 8 weeks (a time point when excessive weight gain is statistically significant) and 20 weeks (after a substantial period of being overweight). It is possible that behavioral and neurochemical alterations in the HIP might be seen after a longer duration of a high-fat diet. Further, a decreases in testosterone and increase in corticosterone might also be apparent after longer durations high-fat diet exposure. With further understanding of the role of these hormones, we can begin to explore the possibility of treatment effects.

Supplementary Information

Cohort A	Cohort B	Cohort C
Novel Object Recognition task (chapter 2)	Brain volume (chapter 3)	Center vs. Surround (chapter 4)
Object Location task (chapter 2)	Immunoflorescence (chapter 3)	Latency to feed (chapter 4)
Object-in-Place task (chapter 2)	Cresyl violet (chapter 3)	Attentional Set-Shifting task (chapter 2)
Tactile testing (chapter 2)		Golgi analysis (chapter 3)
Vibrissa testing (chapter 2)		Fat pads (chapter 2)
Olfactory testing (chapter 2)		Serum triglycerides (chapter 2)
Locomotor testing (chapter 2)		Serum corticosterone (chapter 4)
Western blotting (chapter 3)		Serum leptin (chapter 4)
Serum glucose (chapter 4)		
Serum insulin (chapter 4)		

Supplementary Table 1. Summary of dissertation components that were completed in each of three individual cohorts of animals, and the chapter where data is presented.

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