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Dopamine and Leptin Interactions in Rodent Models of Obesity and Cocaine Abuse

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Abstract of the Dissertation

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Dopamine (DA) is a catecholamine neurotransmitter and along with its receptors plays a well-documented role in motivation and goal-directed behavior. Clinical studies have previously documented striatal DA D2 receptor (D2R) deficits in obese individuals and chronic cocaine users. These studies suggest that impaired DA-D2R signaling in the striatum may underlie deficits in goal-directed behaviors that can ultimately lead to obesity and drug addiction. However, such studies have not addressed the question of whether D2R deficits precede obesity and cocaine abuse or whether D2R decline in response to repeated overeating and cocaine use.

This thesis aims to answer this question by further investigating the involvement of D2R in obesity and cocaine abuse in rodents. In particular, this thesis documents striatal D2R deficits in genetic and environmentally-induced obese as well as cocaine-exposed rodents using small animal positron emission tomography (μPET) and receptor autoradiography. Furthermore, a functional role for D2R in mediating cocaine use is demonstrated by genetically upregulating D2R in the striatum by way of an adenoviral viral vector expressing the D2R gene, which attenuated operant responding for intravenous cocaine in rats. Using μPET, this thesis also shows that D2R can serve as a biomarker that predicts susceptibility to weight gain and cocaine abuse since D2R levels negatively correlated with both future weight gain and cocaine preference in rats. Finally, using μPET evidence is presented that peripheral leptin signaling affects DA-D2R interactions in the striatum and that these effects are dependent upon individual susceptibility to weight gain and cocaine preference.

The central premise is that leptin is an adiposity signal whose effects are not limited to homeostatic mechanisms as originally thought, but also to goal-directed behavior and that this behavior is modulated in part via leptin's action on striatal D2R. This work leads to a better understanding of peripheral and central nervous system interactions involved in energy regulation and motivation and highlights the involvement of such interactions in obesity and cocaine abuse.

To

my fiancée Margaret, for motivation
my sister Olga, for admiration
my mother Christina, for dedication
my father Euthymios, for inspiration

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List of Abbreviations

CNS - Central Nervous System
DA - Dopamine
D2R - Dopamine D2 Receptors
ANS - Autonomic Nervous System
CCK - Cholecystokinin
GLP-1 - Glucagon-like Peptide 1
GRP - Gastrin-releasing Peptide
BBB - Blood Brain Barrier
LH - Lateral Hypothalamus
ARC - Arcuate Nucleus
PVN - Paraventricular Nucleus
CPP - Conditioned Place Preference
WHO -World Health Organization
SAMHSA - Substance Abuse and Mental Health Services Administration
kDa - Kilodalton
Ob(lep) - Leptin gene
LepR - Leptin receptor
Jak-Stat - Janus Kinase- Signal Transducer and Activator of Transcription
ERK - Extracellular Signal-regulated Kinase
PI3K - Phosphoinositide 3-kinase
cAMP/PDE3B - cyclic Adenosine Monophosphate/phosphodiesterase 3B
NPY - Neuropeptide Y
AgRP - Agouti-related Peptide
POMC - Proopiomelanocortin
VMN - Ventromedial Nucleus
NTS - Nucleus of the Solitary Tract
SN - Substantia Nigra
VTA - Ventral Tegmental Area
NAc - Nucleus Accumbens
DOPA - Dihydroxyphenylalanine
VMAT - Vesicular Monoamine Transporter
DAT - Dopamine Transporter
D1R - Dopamine D1 Receptors
D3R - Dopamine D3 Receptors
D4R - Dopamine D4 Receptors
D2L - D2 Long
D2S - D2 Short
CSA - Cocaine Self-Administration
PET - Positron Emission Tomography
ARG - Autoradiography
NAS - National Academy of Sciences
NRC - National Research Council
FR1 - Fixed Ratio 1
ROI - Region of Interest
CPu - Caudate Putamen

CB - Cerebellum
ANOVA - Analysis of Variance
KO - Knockout
MAP - Maximum a Posteriori
PMOD - Pixel-wise Modeling
BMI - Body Mass Index
Ob U - Obese Unrestricted
Ob R - Obese Restricted
Le U - Lean Unrestricted
Le R - Lean Restricted
APO - Apomorphine
ST - Striatum
CG - Cingulate Cortex
FC - Frontal Cortex
EIA - Enzyme Immunoassay
MI - Mutual Information
MRTM0 - Multi-reference Tissue Model 0
BP - Binding Potential
NF - Non-fasted
F - Fasted
CTX - Cortex
Acb - Accumbens
Bmax - Maximum Binding
Kd - Receptor Affinity
ICV - Intracerebroventricular
DOPAC - 3,4-dihydroxyphenylacetic Acid
OM - Osborne Mendel
S5B - S5B/PL
AL - Ad-libitum
R - Restricted
HF - High-fat Fed
IP - Intraperitoneal

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Chapter 1

Introduction and Background Review

1.1 Introduction

Chronic drug use and overeating are two behaviors that result in devastating health consequences since they predispose the individual to potentially fatal disorders such as drug addiction and obesity. Addiction and obesity are unique cases that result from disturbances in ingestive behavior (Volkow & Wise, 2005). Each disorder is selective to certain individuals, and each is subject to genetic predispositions (differences in peripheral and central nervous system (CNS) mechanisms), environmental factors (availability of drugs or palatable food) (Erlanson-Albertsson & Zetterstrom, 2005; Volkow & Wise, 2005) as well as periods of developmental vulnerability (Buka et al., 2003; Mennella et al., 2004). Furthermore, drug addiction and obesity share striking similarities in specific brain mechanisms that regulate the motivational aspects leading to each disease and are also characterized by similar behavioral manifestations such as impulsivity and compulsiveness (Volkow & Wise, 2005). Finally, treatment strategies for both disorders are particularly similar and many prospective medications aimed at regulating drug abuse also regulate obesity and vice-versa (Volkow & Wise, 2005). Since we are currently facing an obesity epidemic and there is a desperate need for medications aimed at treating drug addiction, it is imperative to study these disorders in parallel, since potential discoveries of how they overlap may produce significant and specific treatment interventions for both.

Dopamine (DA) is a catecholamine neurotransmitter with a well-documented role in motivation and goal-directed behavior. Clinical studies have previously documented low DA D2 receptor (D2R) binding availability in the striatum of obese individuals and chronic cocaine users (Wang et al., 2004). These studies have suggested that low concentrations of D2R or impaired DA-D2R signaling in the striatum may underlie deficits in goal-directed behaviors that can lead to excessive food and cocaine seeking/consumption and eventually to obesity and drug addiction (Wang et al., 2004). The goal of this thesis is to examine the relationship between striatal DA-D2R and leptin signaling in rodents, and how this relationship may affect behaviors relevant to obesity and cocaine abuse. The main idea is that the periphery provides energy-store information to motivational brain circuits, which in turn can initiate the appropriate goal-directed behaviors to increase or decrease energy intake

and that deficits in this signal may lead to behavioral disorders like overeating and cocaine abuse.

1.2 Peripheral and Central Regulation of Ingestive Behavior

1.2.1 Ingestive Behavior

The definition of the term "ingestive behavior" is critical for the thematic stance of this thesis. The English term "ingest" means "to consume regularly", or "to take up, as of knowledge or beliefs" and is derived from the Latin "ingenium" which is the past participle of "ingerere". In English, "ingerere" translates as "to put in" (Websters-Merriam). Based on these definitions and etymology, "ingestive behavior" can be interpreted as any act that involves putting something into one's body, whether by eating, drinking, injecting, snorting, or even learning. Within this context, the acts of seeking and/or consuming food and drugs of abuse can be thought of as similar processes. Likewise, disorders like obesity and drug addiction that result from repeated consumption of food and drugs can be thought of as originating from disturbances in ingestive behavior (Volkow & Wise, 2005). This approach, although crude at first sight is also beneficial in that it facilitates the overlapping of insights and constructs from obesity and drug addiction and therefore may lead to new discoveries for each disorder.

1.2.2 Homeostatic Mechanisms and Ingestive Behavior

Energy homeostasis consists of various interdependent processes that are regulated by the CNS to maintain energy stores such as glycogen in the liver and muscles, glucose in the blood, and fat in adipose tissue at appropriate levels for given environmental conditions (Woods & D'Alessio, 2008). The CNS accomplishes this by regulating the secretion of specific metabolic hormones primarily via the autonomic nervous system (ANS) (Woods & D'Alessio, 2008). Such signals are broadly separated into two main categories: satiety and adiposity signals. Satiety signals include ghrelin, cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and gastrin-releasing peptide (GRP). Such signals are phasic in nature. They are synthesized and act directly on the brain, and also secreted in the periphery, particularly in the digestive tract where they act on vagal afferents to the CNS (Figure 1.2.1) (Woods & D'Alessio, 2008). Adiposity signals are characterized by tonic secretion and include insulin and leptin which enter the CNS by active transport via the blood brain barrier (BBB) and act on specific sites within key areas of the brain such as the lateral hypothalamus (LH), the arcuate (ARC), and paraventricular nuclei of the hypothalamus (PVN) (Woods & D'Alessio, 2008).

While involvement of homeostatic mechanisms in feeding cannot be understated, the involvement of such mechanisms in drug use is less clear. The principle of homeostasis in drug use may be exemplified by features of drug abuse and addiction such as withdrawal (i.e. the brain responds to an imbalance in the system which drug use reverts to normal). Indeed, recent studies have examined the contribution of feeding-

related homeostatic mechanisms to drug abuse. More recent studies have shown that the administration of the anorexigenic peptide leptin facilitates the extinction of cocaine self-administration (Gray et al; unpublished results), while administration of the orexigenic peptide ghrelin augments cocaine conditioned place preference (CPP) (Davis et al., 2007).

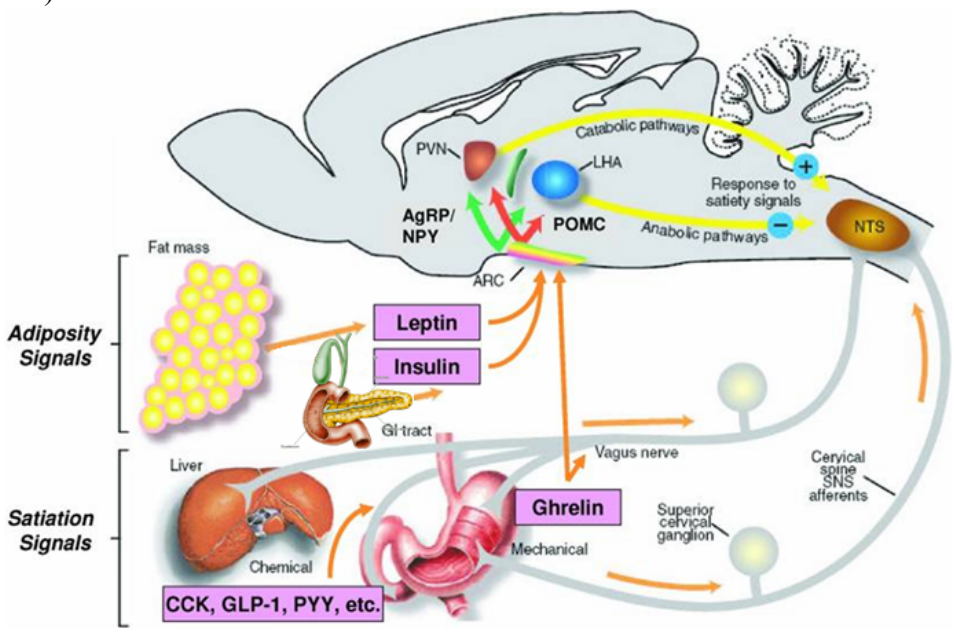

Figure 1.2.1 Central regulation of energy balance. Adapted from (Woods & D'Alessio, 2008).

1.2.3 Non-homeostatic mechanisms and Ingestive Behavior

Along with the above homeostatic mechanisms, body weight and appetite regulation are also affected via non-homeostatic mechanisms that are influenced by taste, pleasure, habits, social interactions, convenience, availability, and stress (Strubbe & Woods, 2004). Drug use is thought to be affected by similar non-homeostatic mechanisms (Corwin & Hajnal, 2005). All of the aforementioned factors (with the exception of taste) increase the propensity to drug use and can facilitate the development and persistence of addiction (Corwin & Hajnal, 2005).

1.3 Ingestive Behavior Disorders

1.3.1 Overeating and Obesity

Feeding involves one of the most widely studied reward survival mechanisms (Fetissov et al., 2002) and along with the need to reproduce, it can be considered as the underlying foundation to all other reward-related behaviors. It is believed that this

fundamental role of feeding in survival, coupled with excessive food availability and the type of food consumed (high-fat, high-sugar, high-salt), has led to food over-consumption, thus leading to a major obesity epidemic in the United States (Isasi et al., 2006). Since 1980, the prevalence of obesity has increased dramatically. The percentage of overweight adolescents has increased by more than 300 percent and the latest data from the National Center for Health Statistics show that in the United States, 60 million people (roughly 30 percent of adults 20 years of age and older) are obese (Ogden et al., 2010). Although one of the national health objectives for the year 2010 is to reduce the prevalence of obesity among adults to less than 15%, current data indicate that the situation is worsening rather than improving (Flegal et al., 2010; Ogden et al., 2010). According to the World Health Organization (WHO) in 2005, there were about 1.6 billion overweight adults (15 years and above) and at least 400 million obese adults worldwide (WHO, 2008).

Obesity can originate from one or multiple underlying causes. The two main causes of obesity are attributed to genetic and environmental factors. In turn these may be further affected by physical, psychological, and cultural factors. In general, obesity arises from a chronic dysregulation of energy balance, that is, the proportion of energy intake compared to energy expenditure. Energy intake is modulated by the amount and type of food consumed. Energy expenditure is modulated by metabolism, physical activity and lifestyle. For both energy intake and expenditure, this modulation can be offset by multiple factors, including imbalances in or tolerance to endogenous peptides and neurotransmitters whose primary or secondary role is normal food intake and/or metabolic regulation

1.3.2 Cocaine Abuse and Addiction

Drug addiction is a chronic disorder that is characterized by the urge to use one or more drugs of abuse and the inability to control drug intake and continued use regardless of the destructive consequences involved (Pierce & Kumaresan, 2006). The major factor that contributes to this disorder is the powerful reinforcing effect that characterizes drugs of abuse (Pierce & Kumaresan, 2006). This overwhelming power leads to compulsive drug taking, which in turn constitutes an essential requirement for the neuronal adaptations that produce addiction (Pierce & Kumaresan, 2006). The focus of this thesis is on the psychostimulant cocaine. In 2004, 34.2 million Americans aged 12 and over reported lifetime use of cocaine and 7.8 million reported using crack (cocaine analog) (SAMHSA, 2005). There were an estimated 2.1 million new users of cocaine in 2007, and most were aged 18 or older although the average age of first use was 20 years (SAMHSA, 2008).

1.4 Leptin

1.4.1 Leptin Signaling

The adiposity signal leptin is a 16 kilodalton (kDa) peptide, identified in 1994, that is secreted primarily from white adipocytes as well as the stomach and other tissues (skeletal muscle, liver, brain) in direct proportion to body fat (Zhang et al., 1995; Margetic et al., 2002). In humans, the gene that encodes leptin (Ob(lep)) is located on chromosome 7 (Maffei et al., 1995). There are three distinct types of leptin receptors; the long form (LepRb), the short forms (LepRa and LepRc) and the soluble form. In the brain, leptin receptors are primarily expressed in the ARC. In the periphery, they have been localized in the ovary, prostate and testis (Li & Friedman, 1999; Ur et al., 2002; Bates & Myers, 2003). Peripheral circulating leptin is found in two forms, the free active form of leptin and leptin bound to the soluble form of the leptin receptor which is thought to serve as a compensatory feedback system to decrease active circulating leptin levels. Leptin receptors signal through the Janus Kinase- Signal Transducer and Activator of Transcription (Jak-Stat) (via Stat3 activation) (Hakansson & Meister, 1998; Elias et al., 1999; Munzberg et al., 2003) as well as the Extracellular Signal-regulated Kinase (ERK), Phosphoinositide 3-kinase (PI3K), and the cyclic Adenosine Monophosphate/phosphodiesterase 3B (cAMP/PDE3B) pathways (Niswender et al., 2001; Zhao et al., 2002).

Leptin deficiency is a condition where leptin's effects on energy balance are compromised. Leptin deficiency can arise due to random mutations in the Ob(lep), the LepR, or from constant overeating resulting in leptin resistance. Impairment in normal leptin signaling can lead to a variety of symptoms, some of which include overeating, type II diabetes, weight gain, reproductive problems, bone formation deficiency and cardiovascular complications (Sahu, 2003). Obese humans and diet-induced obese animals have been shown to exhibit a resistance to leptin transport and/or action (Sahu, 2003). In humans, mutations resulting in leptin deficiency are documented but they are extremely rare (Farooqi, 2008). There are several animal models of leptin deficiency. The ob/ob mouse is a naturally occurring spontaneous point mutation in the Ob(lep) that prevents leptin from being produced. The db/db mouse and the fa/fa rat are naturally occurring mutations in the LepR, which prevents proper leptin signaling. Such animal models develop obesity-related symptoms as early as three weeks of age which include hyperphagia, hyperglycemia, hyperinsulinemia, hyperleptinemia, glucose intolerance, infertility, hypothermia, impaired wound healing and cardiovascular problems. Ob/ob and db/db mice and fa/fa rats show excessive weight gain and adiposity (Zhang et al., 1995).

1.4.2 Leptin and Adiposity

Leptin is secreted from adipose tissue and this secretion depends on both the status of long-term fat stores of the organism as well as its short-term metabolic needs (Campfield et al., 1995; Halaas et al., 1995; Considine et al., 1996; Ostlund et al., 1996). Upon secretion, leptin is transported through the BBB (Banks et al., 2006) and acts

directly on the ARC via its receptors that are located on neurons expressing the orexigenic peptides Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) and the anorexigenic peptide Pro-opiomelanocortin (POMC) (Hakansson & Meister, 1998; Elias et al., 1999; Munzberg et al., 2003). Leptin also acts on other brain regions within and outside the hypothalamus including the ventromedial nucleus (VMN) and the nucleus of the solitary tract (NTS).

Leptin's effects are not purely anorexigenic. Leptin also regulates sympathetic nervous system activity and metabolism specifically by increasing energy expenditure and fat oxidation (Hwa et al., 1997; Shimabukuro et al., 1997; Wang et al., 1999). Finally leptin regulates adiposity by altering neuroendocrine hormone secretion (Ahima & Flier, 2000) and reproduction (Barash et al., 1996; Wade et al., 1997; Gruaz et al., 1998).

1.4.3 Leptin and Ingestive Behavior

The effect of nutritional status on motivation has been well-documented (Bell et al., 1997; Carr, 2007; Figlewicz et al., 2007). These studies have shown that negative energy balance states (via food deprivation) are associated with increased motivation towards rewarding stimuli (i.e. food, drugs). Food deprivation decreases peripheral leptin concentrations (Thanos et al., 2008) while feeding increases its secretion (Baskin et al., 1999). Exogenous leptin that is administered peripherally or centrally leads to a reduction in food intake and body weight while low leptin concentrations in the periphery or brain cause food intake and body weight to increase (Woods & D'Alessio, 2008). More importantly, leptin administration decreases the effects of negative energy balance on motivated behavior towards food and drugs (Fulton et al., 2000; Figlewicz et al., 2001; Shalev et al., 2001) that is independent of food consumption (Figlewicz et al., 2001; Getchell et al., 2006). Recent reviews (DiLeone, 2009; Morrison, 2009) further describe these consummatory-independent effects of leptin as being mediated by brain areas that lie both within and outside the hypothalamus (i.e. LH and the mesolimbic DA system) and that have traditionally been involved in reward seeking. In fact, in one of these reviews (Morrison, 2009) leptin is described as playing an important role in regulating synaptic function, neuronal structure and plasticity, neuroprotection and also cognition. This view further implicates leptin's role in regulating motivated behaviors relevant to reward seeking via effects on learning and memory.

1.5 Dopamine

1.5.1 Dopamine Signaling

DA is the most abundant catecholamine neurotransmitter in the brain. DA neurons are classified into three main classes based on their projection length:

1) Ultra-short projection class: this class includes amacrine-like neurons in the retina and the olfactory bulb characterized by very short intralaminar dendritic extensions.

2) Short projection class: this class comprises three distinct systems: a) DA neurons located in the ARC, which project to the pituitary and median eminence b) DA neurons located in the posterior hypothalamus, the zona incerta and PVN, c) DA neurons located in the NTS and in the periaqueductal gray.

3) Long projection class: this class includes DA neurons that originate in the retrorubal field, the subtantia nigra (SN) and the ventral tegmental area (VTA) and project to the neostriatum, the limbic cortex, and other limbic areas such as the olfactory tubercle, septal areas, the nucleus accumbens (NAc) and the amygdaloid complex.

DA is not capable of crossing the BBB and is synthesized in CNS nerve terminals as a two step process from tyrosine. The first step involves catalysis of tyrosine hydroxylase and results in the production of dihydroxyphenylalanine (DOPA). The second step involves catalysis of DOPA from DOPA decarboxylase and results in the production of DA.

DA synthesis originates in the cytosol and is then transported into presynaptic vesicles by the vesicular monoamine transporter (VMAT). When DA vesicles reach the presynaptic terminal, DA is released into the synaptic cleft by action potentials through a calcium-dependent mechanism. Voltage-gated calcium channels activation causes the fusion of the DA-containing vesicles with the presynaptic membrane. A pore forms in the presynaptic membrane from which DA is released into the synaptic cleft and then diffuses across the synapse and binds to postsynaptic DA receptors. DA binding to postsynaptic receptors can be either excitatory or inhibitory. Any excess unbound DA is removed from the synapse into the presynaptic terminal for storage and to be reused by use of a specific reuptake mechanism, the DA transporter (DAT). In addition to the DAT, there are specific DA receptors located at the presynaptic membrane that are called autoreceptors. These receptors monitor and modulate the release and synthesis of DA from the presynaptic terminal and are found at nerve terminals, the soma and dendrites of DA neurons.

The diverse anatomy and distribution of DA neurons suggests that DA is involved in diverse brain functions. Indeed, DA regulates a broad range of functions such as arterial blood-flow, motor activity, cognition, learning, anxiety, hormone secretion (Pijl, 2003), feeding, and reinforcement of addictive substances and behaviors (Volkow et al., 2002a). These functions have been ascribed to the different DA neuron projections. For instance, DA neurons in the nigrostriatal system are primarily involved in the initiation and maintenance of motor behavior while DA neurons in mesolimbic and mesocortical systems are involved in rewarding, goal-directed and motivation-dependent behavior. DA neurons in the tuberoinfundibular system are primarily involved in regulating pituitary and hypothalamic peptide release which in turn influence homeostatic behaviors such as feeding, drinking and blood-flow regulation.

DA receptors are classified into two main families and each family contains several receptor subtypes. The D1-like receptor family contains the D1 (D1R) and D5 receptor (D5R) subtypes. The D2-like family contains the D2R, D3 (D3R) and D4 receptor (D4R) subtypes. DA autoreceptors also belong to the D2-like receptor

family. All DA receptors are metabotropic and are characterized by a seven transmembrane domain.

The D2R was first cloned in 1988 in the rat (Bunzow et al., 1988). D2R show a widespread distribution in the brain with highest to lowest concentrations in the striatum, midbrain, spinal cord, hypothalamus and hippocampus. There are two isoforms of the D2R that are termed "long" (D2L) and "short" (D2S). Both isoforms inhibit adenylate cyclase activity and are susceptible to agonist-induced desensitization. D2S are show greater distribution than D2L in the posterior cerebral cortex, the amygdala, the hypothalamus and the brain stem. On the other hand, D2L show greater distribution than D2S in the extrapyramidal basal ganglia.

1.5.2 Mesolimbic Dopamine and Adiposity

Previous studies have shown that the D2R has been associated with obesity. Studies conducted in animal models of obesity (ob/ob mice, fa/fa rats, obesity-prone Sprague–Dawley rats and seasonally obese animals) have documented that treatment with DA agonists reverses the obesity, in part by activation of D2R (Pijl, 2003). Furthermore, evidence of the D2R's involvement in obesity is given by the higher risk of weight gain observed in patients chronically treated with antipsychotic drugs (D2R antagonists) (Friedman, 2004). Genetic studies have reported decreased D2R efficacy and increased incidence of obesity in humans (Noble et al., 1994). Finally, morbidly obese humans (Wang et al., 2001) and obese rats (Hajnal et al., 2008) and mice (Huang et al., 2006) show deficits in mesolimbic D2R levels.

1.5.3 Mesolimbic Dopamine and Ingestive Behavior

It has previously been shown that DA mediates food intake through goal-directed mechanisms (McQuade et al., 2004) and that normal DA levels in the brain are indispensable for feeding and survival (Fetissov et al., 2002). For instance, DA signal impairment in DA-deficient mice causes them to die of starvation by 3 weeks of age if not treated with DA agonists (Hnasko et al., 2004). Indeed DA cells increase their firing rate (increasing DA release in the NAc) when expecting food and their firing in turn is influenced by food deprivation (Meguid et al., 2000). While DA has been classically described as a neurotransmitter involved with locomotor activity and reward, more recent work has shown DA to be associated with the prediction of reward (Schultz et al., 2000), motivation to procure the reward (McClure et al., 2003) and facilitation of conditioned learning (Fenu & Di Chiara, 2003). Thus, a possible mechanism of DA involvement in food intake and obesity could be that DA signaling is increased in response to food as a stimulus predicting reward, leading to the motivation to procure the food (Fenu & Di Chiara, 2003). In this respect, since eating is a highly rewarding behavior, DA may be responsible for regulating the reward or reinforcement (Epstein et al., 2007) necessary to maintain, accentuate, or abolish feeding behavior (Berridge, 1996; Berridge & Robinson, 1998). There is evidence from microdialysis studies of lower DA levels in the NAc of ob/ob when compared to controls (Fulton et al., 2006) and of attenuated striatal DArgic responses to insulin in

fa/fa rats (Orosco et al., 1992). It has also been shown that fa/fa rats have high DAT mRNA levels, (Figlewicz et al., 1998) as well as increased sensitivity of DAT for DA uptake (Owens et al., 2005). Furthermore, decreases in striatal DA levels have been reported in fa/fa rats (Shimizu et al., 1991). Accordingly, subchronic food restriction (1 week) has been shown to lead to a decrease in extracellular DA in the NAc (Pothos et al., 1995a; Pothos et al., 1995b). Also chronic food deprivation and diet-induced obesity, two conditions that are characterized by differences in feeding have both been related to decreases in extracellular DA levels in the ventral striatum (Pothos et al., 1998b). Finally, fa/fa ad-libitum fed rats had significantly lower DA metabolites in the striatum compared to lean rats (Orosco et al., 1986).

The role of D2R in drug reward has been documented by studies showing that D2-like receptor agonists are self-administered by rodents and non-human primates (Ranaldi et al., 2001) and D2-like receptor agonists decrease cocaine self administration (CSA) (Barrett et al., 2004). Antagonists produce the opposite effect (Caine et al., 2002) and mice lacking the D2R also show increased CSA compared to respective wild-types (Caine et al., 2002).

Genetic studies have found that individuals carrying the Taq 1 A1 allele of the D2R gene have decreased D2R levels (Thompson et al., 1997) (Noble, 2000) though one study did not find this association (Laruelle et al., 1998). Individuals with this allele are more vulnerable to addictive behaviors, compulsive food intake (Noble et al., 1993; Volkow et al., 1999a) and are more likely to be obese (Noble et al., 1994) (Comings & Blum, 2000). It is believed that low D2R levels predispose subjects to search for reinforcers; in the case of drug-addicted subjects for the drug and in the case of obese subjects for food as a means to temporarily compensate for a decreased sensitivity of D2R regulated reward circuits (Wang et al., 2004). Treatment strategies aimed at restricting drug use and pathological eating in drug addicts and obese subjects would thus restrict the compensatory effects that excessive drug and food intake have on reward in these individuals. Behaviorally, this could either lead to relapse towards drug use and pathological eating or, when this is not possible (i.e. rehabilitation center, diet clinic, etc.), to the adoption of alternative reinforcing behaviors. Specifically, drug addicts in rehabilitation may take on pathological eating, while obese subjects on a diet may take on drug use in an effort to increase feelings of reward. Furthermore, when drugs are not available to cocaine addicts and food is not available to obese individuals, the DA system may compensate by increasing D2R levels in an effort to increase reward and therefore alleviate symptoms associated with decreased reward sensitivity. Interestingly, inasmuch as cocaine addicts and obese individuals are characterized by low D2R levels, abstinence from cocaine and chronic food deprivation are both associated with high D2R levels (Volkow et al., 2002c).

Imaging studies have recently corroborated the involvement of D2R in both drug abuse and obesity by documenting reductions in D2R levels in the striatum of cocaine addicts (Volkow et al., 1997), cocaine-exposed non-human primates (Nader et al., 2006), pathologically obese individuals (Wang et al., 2001) and hyperphagic obese rodents (Hamdi et al., 1992; Hajnal et al., 2008; Thanos et al., 2008), while patients with anorexia nervosa have reported higher than normal striatal D2R availability (Frank et al., 2005). Furthermore, positron emission tomography (PET) imaging

studies in humans have corroborated an involvement of D2R both in food reward (Small et al., 2003) as well as in the motivation to procure food (Volkow et al., 2002b). What remains to be elucidated is whether cocaine addicts and obese subjects are born with inherently lower levels of D2R, or whether the addiction and obesity result from drug use and pathological eating behaviors.

1.6 Leptin and Dopamine Interactions

Feeding can be affected by homeostatic mechanisms such as the "need" to eat, or non-homeostatic mechanisms such as taste or pleasure (Berthoud & Morrison, 2008). In particular, mesolimbic DA has been described as having a central role in the behavior associated with non-homeostatic feeding (Berridge & Robinson, 1998). Activation of this pathway has been associated with the motivating and rewarding properties of food as well as drugs (Berridge & Robinson, 1998). Tasty or pleasurable foods such as a diet high in fat have been shown to interact with and modulate the effectiveness of the leptin-CNS feedback loop (Lin et al., 2001). Specifically, this finding suggests that constant high-fat feeding may result in a loss of adipose signal effectiveness, which can also affect specific neural circuits like mesolimbic DA, which mediate motivation and reward. This effect of the environment on specific neural circuits is thought to have contributed to the continuously escalating obesity trends of the 1990s and 2000s (Hill et al., 2003).

Caloric intake modulates cocaine intake. Specifically, food restriction (which leads to decreases in leptin) augments CSA (for review please see (Carr, 2007)), while diet-induced obese rats show impaired acquisition of CSA (Wellman et al., 2007). This latter finding agrees with epidemiological studies which report a 25% decrease in the odds of developing substance abuse disorders in obese individuals (Simon et al., 2006). The effects of caloric intake on drug intake are thought to be mediated by leptin (Trinko et al., 2007). Food intake results in a temporary increase in blood leptin levels (Baskin et al., 1999) and obese patients and rodents have been shown to exhibit a resistance to leptin transport and/or action (Sahu, 2003), a condition reversed by chronic food restriction (Thanos et al., 2008). In turn, low levels of circulating leptin are thought to underlie increased responding to obtain rewards (Figlewicz & Benoit, 2009). Leptin signaling directly modulates mesolimbic DA neuron activity and impacts both food intake and drug addiction-related behaviors (for review please see (Trinko et al., 2007) and (Figlewicz & Benoit, 2009). DA concentration decreases as blood leptin increases (Hagan et al., 1999) and short term leptin treatment decreases both DA release and concentrations in the NAc (Krugel et al., 2003) and the hypothalamus in a dose-dependent manner (Brunetti et al., 1999). Leptin's involvement in drug reward has been documented by studies showing that it attenuates the propensity to heroin relapse caused by food restriction (Shalev et al., 2001) and that ob/ob mice show attenuated amphetamine sensitization, which is reversed by leptin treatment (Fulton et al., 2006). Also, when given cocaine, ob/ob mice show reduced D2R-mediated synaptic current activity, an indication of decreased cocaine sensitivity, which leptin also reverses to normal levels (Roseberry et al., 2007).

Chapter 2

Dopamine D2 Receptors and Cocaine Abuse

2.1 CSA decreases D2R binding in mice

2.1.1 Introduction

Cocaine is an indirect-acting monoamine agonist, binding with approximately equal affinity at the DA, serotonin and norepinephrine transporters. The downstream effects of DA mediate the subjective and reinforcing effects of cocaine leading to its high abuse potential (Nader et al., 2006). Cocaine has the common ability to increase extracellular DA in the NAc (Di Chiara & Imperato, 1988). In fact, a single exposure to cocaine or other drugs of abuse induces long-lasting effects on electrophysiological and behavioral responses, revealing their powerful ability to control brain plasticity (Vanderschuren et al., 1999; Ungless et al., 2001; Faleiro et al., 2003; Saal et al., 2003; Fourgeaud et al., 2004; Valjent et al., 2005). The aim of this study was to evaluate the time-course changes of D2R binding in mice in response to different lengths of CSA exposure by using in vitro autoradiography (ARG). In so doing, we wanted to see whether acute and/or prolonged exposure to cocaine decreases D2R binding.

2.1.2 Methods

Animals

Male 3-4 week old C57/BL6 mice (Charles River, Wilmington, MA, USA) were individually housed in a 12-hr reverse light-dark cycle (lights off at 0800hr) in a controlled room (72 ± 2°F and 40-60% humidity). Experiments were conducted during the dark cycle from 1200hr to 1500hr. During CSA sessions both water and Purina laboratory mouse chow was available on an ad-libitum basis. During the food training experiments, water was available ad-libitum but mouse chow was restricted (5 g/day) to encourage operant responding for food pellets. All experiments were conducted in conformity with the National Academy of Sciences Guide for Care and Use of Laboratory Animals (NAS & NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

Drugs

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving it in saline for a dose of 1 mg/kg.

Apparatus

Operant chambers (30 x 25 x 30 cm; Coulbourn Instruments, Allentown, PA, USA) were placed inside sound attenuation cubicles. One side wall contained two levers and a food receptacle in the center. The left lever was designated as the active (reinforced) lever while the right lever was inactive (non-reinforced) lever. Both levers were situated directly under their respective cue lights. The infusion pump was set at a fixed rate of 0.025ml/s for 2s. All CSA experimental variables were programmed using Graphic State v3.02 software (Coulbourn Instruments, Allentown, PA, USA).

Food Training

Mice were trained daily for at least 3 days (60min/session) using a fixed-ratio 1 (FR1) schedule. By pressing the active lever, one food pellet (20mg) was released into the receptacle as the cue light was illuminated for a 30s timeout period. Pressing the inactive lever had no programmed consequence. Successful lever discrimination was achieved when mice met previously described criteria of an active/inactive lever press ratio $\geq$ 2:1 over three consecutive days (Larson & Carroll, 2005). All mice went through one additional session of food retraining to ensure conditioning met criteria after surgery.

Surgery

Surgery was performed prior to operant training on animals at 4-5 weeks of age. Mice were anesthetized with 100/10 mg/kg Ketamine/Xylazine (Fort Dodge, TX/Lloyd Laboratories, IA). A four-centimeter (4cm) lateral incision was made along the chest and the right jugular vein was dissected out and isolated from the surrounding tissue. A 20-gauge heparin tipped, silicon catheter (Instech Solomon, PA) was implanted into the jugular vein and anchored to the surrounding fascia with a 7–0 silk suture (Biosurgery, MA). The venous line was flushed with normal saline to test patency and to compensate for volumetric blood loss. The distal catheter tubing was subcutaneously routed to the dorsal region of the animal, where it was attached to a backmounted 22-G cannula (Plastics One, VA). The line was flushed daily with saline and filled with heparin (80 IU/ml) /cefazolin (200 mg/ml) locking solution (Sigma, USA/G.C. Hanford, NY) to prevent clotting and thrombus formation. Animals were given a one-week recovery period before they are started on the operant conditioning task. Catheter patency was tested every three (3) days with a 0.1 ml infusion of 100/10 mg/kg ketamine/xylazine solution. The catheter was determined to be patent only if the mouse lost the righting reflex within 3s of the injection. If the catheter was not patent, the mouse was immediately taken out of the study and only data from patent days was used.

CSA

After recovery, animals were put on a fixed-ratio food training schedule using operant boxes (Coulbourn Instruments, PA) until animals reached a stable FR4 lever pressing for food baseline (achieved a criteria of <20% variation in the mean number of food pellets for 3 consecutive days) before being started on cocaine SA.

After food training, CSA sessions (60 min/day) were started for 2, 10 and 15 days. At the start of the first session, mice received one priming infusion of cocaine. A single press of the active lever resulted in an immediate delivery of cocaine (1 mg/kg) followed by a 30s timeout period. During the timeout period, the cue light above the active lever was illuminated and the drug was not available. The inactive lever did not have a programmed consequence but responses were recorded. A total of 4 groups were examined: (1) Sham, (2) 2 days of CSA, (3) 10days of CSA, and (4) 15 days of CSA. Cumulative lever-press and drug infusion data were recorded for each session. All data were acquired using Graphic State software (Coulbourn Instruments, PA).

Tissue Preparation

Each animal was deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The brain was rapidly removed and frozen in an isopentane and dry ice bath and stored in a -80^0C freezer. The brain was then transferred to a cryostat (Leica CM3050S), and sections were cut 14 μm thick at -18^0C. Sections were mounted on Fisherbrand, Colorfrost */Plus microscope slides and stored at -80^0C until binding was conducted.

D2R ARG

Before binding began slides were gradually brought back to room temperature and then pre-incubated at room temperature for ten (10) minutes in 50mM Tris-HCl buffer (pH 7.4). The slides were then incubated in total binding buffer (50mM Tris-HCl, 0.4 nM [^{3}H]spiperone (Perkin Elmer), 10 μM ketanserin tartrate (Tocris Bioscience)) at room temperature for one (1) hour. To determine non-specific binding some slides in parallel were incubated for one (1) hour in specific binding buffer in the presence of 10 μM haloperidol (Tocris Bioscience). Next, the slides were washed 2 x 10 minutes in ice-cold 50mM Tris-HCl and a 2s dip into ice-cold dH_2O.

Data Analysis

After binding was completed, one (1) ml aliquots of incubation solution were taken and added with five (5) ml of Ultima Gold XR (scintillation solution) and the amount of radioactivity was measured with a liquid scintillation counter. Slides were dried overnight at room temperature in a dessicator and placed in a glass slide cassette for qualitative and quantitative analysis using a β-Imager 2000 (Biospace, Paris, France/Biospace Lab, USA).

Using βvision+ software (Biospace, Paris, France/Biospace Lab, USA), Regions of Interest (ROIs) were drawn on the left and right: Caudate Putamen (CPu), NAc and Cerebellum (CB) for each brain slice. The data was then calibrated using a tissue

homogenate standard as μCi/g and then expressed as a ratio of receptor rich/receptor poor binding (CPu/CB and NAc/CB).

2.1.3 Results

Active Lever Presses

One-way analysis of variance (ANOVA) showed a significant main effect between days of CSA in active lever responses with ($F(2, 77)=5.75$; $p=0.005$). Pair-wise comparisons (Holm-Sidak: $\alpha=0.05$) revealed significantly lower active lever presses at 10 day ($t=3.16$) and 15 days ($t= 2.65$) compared to 1 day of CSA (Figure 2.1.1a).

Inactive Lever Presses

One-way ANOVA showed no significant main effect between days of CSA in active lever responses with ($F(2, 77)=1.14$; $p=0.325$).

Infusions

One-way ANOVA showed a significant main effect between days of CSA in infusions with ($F(2, 77)=3.35$; $p=0.04$). Pair-wise comparisons (Holm-Sidak: $\alpha=0.05$) revealed significantly lower infusions at day 10 ($t=2.56$) compared to day 1 of CSA (Figure 2.1.1b).

Effects of Cocaine Exposure on D2R Binding in the CPu

One-way ANOVA showed a significant main effect in CPu D2R binding [$F(3,297) = 28.71$; $p <0.001$]. Pair-wise comparisons (Holm-Sidak: $\alpha=0.05$) revealed that D2R binding levels in mice exposed to 10 ($t=8.11$) and 15 days ($t=5.95$) of CSA were significantly lower than D2R binding in naïve mice. Also, D2R binding levels for mice exposed to 10 ($t=6.72$) and 15 days ($t=4.5$) of CSA were significantly lower than D2R binding in mice exposed to 1 day of CSA. Finally, D2R binding in mice exposed to 15 days of CSA was significantly greater than D2R binding in mice exposed to 10 days of CSA ($t=2.72$) (Figure 2.1.1c).

Effects of Cocaine Exposure on D2R Binding in the NAc

One-way ANOVA showed a significant main effect in NAc D2R binding [$F(3,297) = 63.06$; $p <0.001$]. Pair-wise comparisons (Holm-Sidak: $\alpha=0.05$) revealed that D2R binding levels in mice exposed to 10 ($t=9.96$) and 15 days ($t=10.4$) of CSA were significantly lower than D2R binding in naïve mice. Also, D2R binding levels for mice exposed to 10 ($t=8.99$) and 15 days ($t=9.25$) of CSA were significantly lower than D2R binding in mice exposed to 1 day of CSA (Figure 2.1.1d).

Figure 2.1.1a

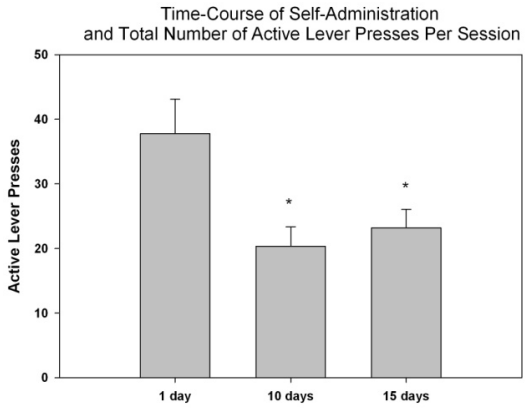

Figure 2.1.1b

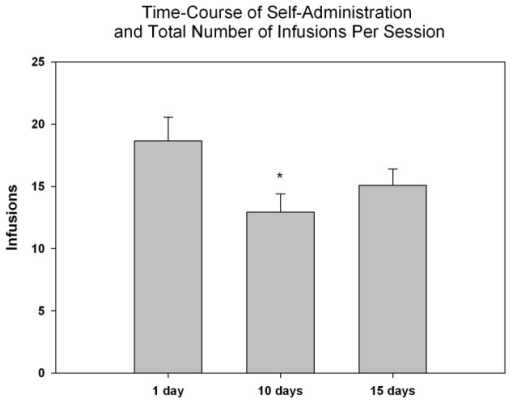

Figure 2.1.1c

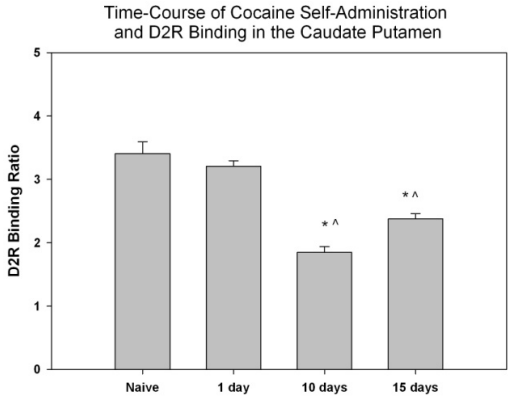

Figure 2.1.1d

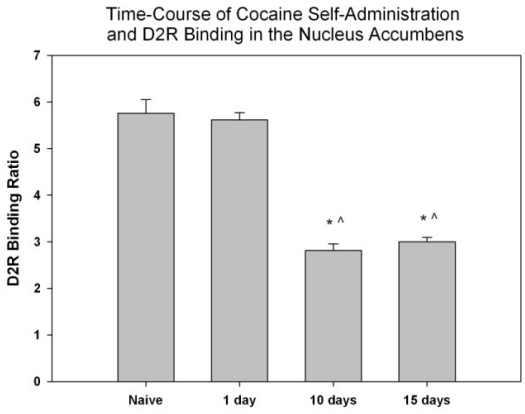

Figure 2.1.1. (a) Total active lever presses during the last day of CSA for each group. (b) Total infusion during the last day of CSA for each group. (c) [^{3}H]spiperone D2R binding by experimental group in the CPu. (d) [^{3}H]spiperone D2R binding by experimental group in the NAc.

2.1.4 Discussion

Here we found that prolonged (10 and 15 days) cocaine exposure decreased lever pressing and infusions for cocaine in mice as compared to acute 1 day exposure. We saw a similar decline in D2R binding in both the CPu and NAc after 10 and 15 days of CSA but not after 1 day. This suggests that D2R deficits observed in human cocaine abusers may arise from continued stimulation of D2R by excess synaptic DA. The constantly elevated levels of DA that remain in the synapse (since cocaine blocks DA reuptake) after 10 and 15 days of repeated cocaine exposure would lead to

downregulation of D2R, as we observe. In turn, low D2R levels may lead to increases in motivation to seek and consume more cocaine.

2.2 D2R DNA transfer decreases CSA in rats

2.2.1 Introduction

The involvement of the mesolimbic DA pathway in the initiation and maintenance of substance abuse including cocaine, has been generally established (Steketee, 2005). Specifically, among the structures in the mesolimbic reward circuit the NAc is believed to play a critical role in cocaine's reinforcing effects (Chang et al., 1994; Laurier et al., 1994; Carelli et al., 1999; Nicola & Deadwyler, 2000).

Cocaine's primary mechanism of action on reward is believed to be its ability to block DAT, thus rapidly increasing the availability of DA in the synaptic cleft (Ritz et al., 1987). Increased levels of striatal DAT occupancy by cocaine correlate with subjective reports of "highs" in human subjects (Fowler et al., 2001) , and μdialysis studies show that both active and passive administration of cocaine lead to elevated levels of extracellular DA in the NAc of rats and mice (Rouge-Pont et al., 2002). In addition, DA receptor agonists and antagonists can modulate or disrupt self-administration behavior (Koob et al., 1987; Corrigall & Coen, 1991; Hubner & Moreton, 1991), CPP (Baker et al., 1998; Sora et al., 1998; Vorel et al., 2002), and locomotor responses to cocaine (Baker et al., 1998; Chausmer & Katz, 2001).

The D2R subtype in particular has been suggested as an important component of the reinforcing effects of cocaine and other drugs (Rouge-Pont et al., 2002). Low D2R levels in ventral striatum (where the NAc is located) have been found in the brains of cocaine addicted humans, including cocaine abusers (Volkow et al., 1996), and in strains of rats bred to self-administer large quantities of ethanol (McBride et al., 1993; Thanos et al., 2004), whereas in these same rats the density of D1R and D3R receptor subtypes does not appear to differ from levels found in wild type animals (McBride et al., 1997). It has also recently been found that mutant mouse strains which do not express the D2R self-administer cocaine at a far greater rate when compared to wild-type animals (Caine et al., 2002). These lines of evidence suggest that the D2R plays a significant role in the reinforcing effects of cocaine and other abused substances, and that D2R levels may be one of the neurobiological variables that modulate the vulnerability of individuals to drug abuse.

In humans, the A1 allele of the D2R gene is associated with alcoholism, cocaine abuse, smoking, methamphetamine abuse, opioid abuse, gambling, obesity, schizophrenia and depression (for review see (Noble, 2003)) and lower D2R in key structures in mesolimbic reward pathways such as the NAc and amygdala (Pohjalainen et al., 1998) . However, some have failed to document such associations (Gelernter et al., 1993; Sery et al., 2001) and therefore the involvement of the A1 allele of the D2R gene remains controversial.

The previous section reports a downregulation of D2R binding in response to chronic cocaine intake. Previous studies have demonstrated that D2R gene-transfer into the NAc of the brain significantly attenuated alcohol intake and preference in Sprague Dawley rats (Thanos et al., 2001; Thanos et al., 2004); inbred alcohol preferring P rats (Thanos et al., 2004) and in mice (Thanos et al., 2005). Inasmuch as ethanol and cocaine show overlap in the circuits involved in their rewarding effects (Koob et al., 1987; Di Chiara et al., 1992), we hypothesized that upregulation of D2R in the NAc of rats self-administering cocaine would reduce their cocaine intake.

2.2.2 Methods

Animals

Sixteen adult male Sprague-Dawley rats (300-400 g) were individually housed in a controlled environment (22±2 °C, 50±10% relative humidity) and subjected to a 24-hour reverse 12 hr light / 12 hr dark cycle (lights off at 0800h) to maintain activity during the daytime. Weights were obtained daily and food and water was provided ad libitum. Experiments were conducted in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NAS & NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

Drugs

Cocaine hydrochloride (0.3 mg/kg; Sigma, St. Louis, MO) was dissolved in saline (0.9% NaCl).

Procedures

Rats were trained to lever press for 45 mg food pellets (Bio-Serv, Frenchtown NJ) in operant chambers (Coulbourn Instruments, Allentown PA) on a 2-hour fixed ratio 1 (FR-1) schedule daily. Data retrieval and processing was completed using Graphic State software (Coulbourn Instruments, PA). After reaching a criterion of at least 50 pellets per session for three consecutive days, rats were operated and jugular catheterization performed as previously described (Thanos et al., 2007). While rats were anesthetized [ketamine and xylazine (100 mg/kg, 10 mg/kg)] for jugular catheter implantation, they were also placed in a Kopf stereotaxic apparatus and were implanted unilaterally with a 22-gauge guide cannula (Plastics One, Roanoke, VA) into the NAc shell (+1.2 mm AP, ±1.4 mm ML, -6.8 mm DV (Paxinos & Watson, 1986). Laterality of cannula placement was randomly assigned so that half the rats received left NAc implants while the other half received implants into the right NAc. The guide cannula was then secured to the skull with four small stainless steel screws and dental cement.

Following a 1-week recovery from surgery, the rats were re-introduced to the operant –conditioning apparatus and the food FR1 protocol for 3-4 days before being started on the cocaine protocol. In the cocaine protocol, lever responses were followed by intravenous cocaine infusions. Each daily CSA session lasted two hours and was conducted at approximately the same time of day (1000 - 1200hrs). Approximately 100 µl of cocaine solution (0.3 mg/kg) was delivered intravenously following a single press

of the reinforced (active) lever, followed by a thirty second timeout period during which presses of the active lever were recorded but not reinforced. A second or "dummy" lever was also provided in the conditioning box, and responses on this lever were recorded but had no programmed consequences.

After 7 days of CSA, all animals were treated with a microinfusion into the NAc of the control, replication-deficient adenovirus or Null Vector (AdCMV.Null), as previously described (Thanos et al., 2001; Thanos et al., 2004), and then returned to the daily CSA sessions for 1-week. Subsequently, all animals were similarly microinfused once into the NAc with the D2R vector [(AdCMV.DRD2), [see previous studies for details (Ikari et al., 1995; Umegaki et al., 1997; Ingram et al., 1998; Thanos et al., 2001; Thanos et al., 2004)] and then returned to the CSA for 14 days.

Microinfusion of the vectors was carried out using an automated syringe pump (Razel, Stamford, CT) and a 26-gauge 5 μl Hamilton microsyringe connected to a 28-gauge internal cannula. Each microinfusion administered unilaterally into the NAc shell 2 μl of vector [adenoviral vector containing the cDNA for the D2R receptor (AdCMV.D2R) (10^{10} pfu/ml)] over 10 minutes so as to reduce the risk of procedure-induced lesions. During the control treatment with the Null vector, the same procedure was followed, except that the solution infused was 0.2M phosphate buffered saline.

Following completion of the behavioral experiments, brains were harvested from all rats for histological confirmation of the cannula placements. Briefly, each animal was deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and the brain was rapidly removed and frozen in an isopentane /dry ice bath, and stored in a freezer at -80^{o} C. Next, 20 μm thick coronal sections were cut on a cryostat (Leica CM3050S, Leica Microsystems, Nussloch, Germany). Sections were stained with 1% cresyl violet, coverslipped with Permount and allowed to air dry before verification of cannula placement in the NAc under light microscopy.

2.2.3 Results

Rats did not show any signs of malaise or weight loss after treatment with the vector and no alterations in general behavior were noted. This was consistent with previous studies (Thanos et al., 2001; Thanos et al., 2004). Treatment with the D2R vector produced a significant albeit transient reduction in the number of cocaine infusions and reinforced lever responses in cocaine-experienced rats. In both the infusion and reinforced lever response data, by post-treatment day 11, most rats had returned to baseline levels of CSA.

Null & D2R Vector Treatment: Infusions and Lever Responses

Two statistical analyses were conducted. I. A one-way ANOVA compared the number of infusions and active lever presses during the three time phases a) days 1 to 7 (pre Null Vector treatment); b) days 8-15 (post Null vector treatment) and c) days 16-22 (post D2R vector treatment). II. The second analysis, utilized a one-way repeated measures ANOVA to compare infusions and lever presses on individual days.

One-way ANOVA

The one-way ANOVA showed a significant main effect between the three phases of CSA ($F(2, 21) = 20.776$; $p<.001$; Figure 2.2.1a). Multiple comparisons (Holm-Sidak) showed that the average number of infusions was not statistically different between pre and post null vector treatment (days 1-7 vs. days 8-15; $t=1.072$; $p>0.05$; Figure 2.2.1a). On the other hand, the average number of infusions post D2R vector (on days 16-22) was significantly lower to both pre Null vector treatment; (days 1-7; $t=4.889$; $p<.001$) and post Null vector (days 8-15; $t=6.122$; $p<.001$; Figure 2.2.1a).

Similarly with respect to active lever presses, a one-way ANOVA showed a significant main effect between the three groups ($F(2, 21) = 21.600$; $p<.001$). Multiple comparisons (Holm-Sidak) showed that the average number of lever presses prior to D2R vector treatment (days 1-7 vs. days 8-15) did not statistically differ ($t=.802$; $p>0.05$). On the other hand, the average number of lever presses post D2R vector (days 16-22) was significantly lower as compared to both pre Null vector (days 1-7; $t=5.172$; $p<.001$) and post Null vector (days 8-15; $t=6.144$; $p<.001$) (Figure 2.2.1b).

One-way Repeated Measures ANOVA

A one-way repeated measures ANOVA showed a significant main effect over time for cocaine infusions ($F(28, 202) = 2.418$; $p<0.001$). Multiple pairwise comparisons (Holm-Sidak method) showed that treatment with the D2R vector significantly attenuated cocaine infusions (Figure 2.2.1a). We did not find any statistically significant difference in the number of cocaine infusions prior to D2R vector (days 1-15; Figure 2.2.1a).

A one-way repeated measures ANOVA showed a significant main effect over time for active lever responses ($F(28, 202) = 2.387$; $p<0.001$). Multiple pairwise comparisons (Holm-Sidak method) showed that treatment with the D2R vector significantly attenuated active lever responses to cocaine (Figure 2.2.1b). Since again we did not find any statistical significance for any day prior to D2R vector (days 1-15).

Figure 2.2.1a

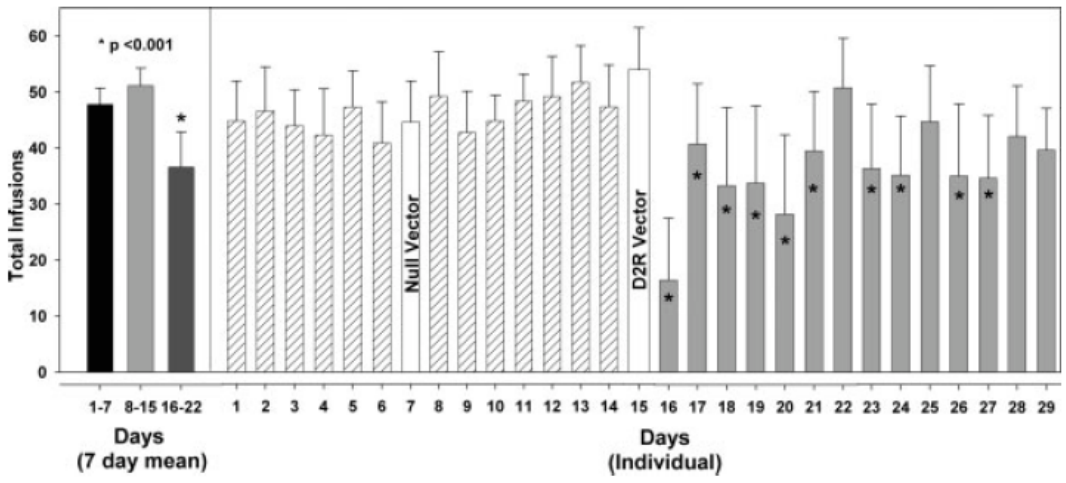

Figure 2.2.1b

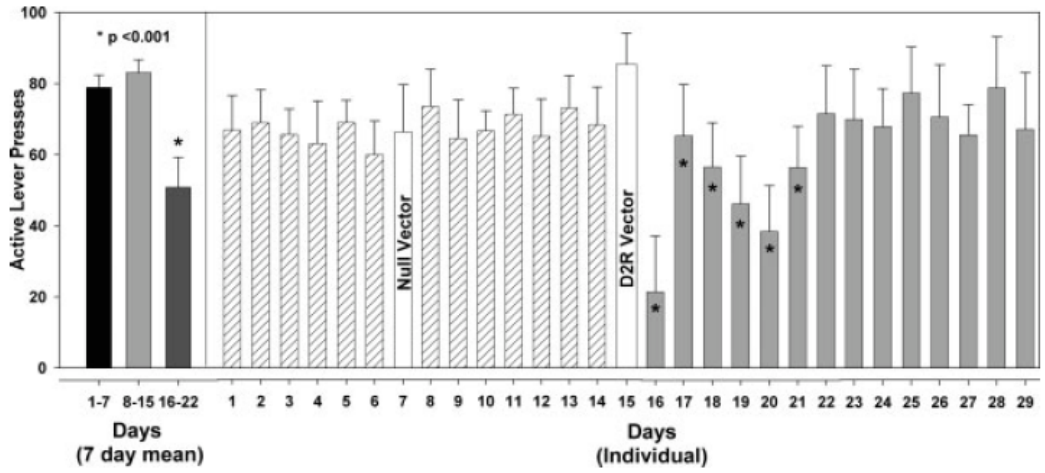

Figure 2.2.1. (a) Mean (+SEM) infusions of 0.3 mg/kg cocaine before and after treatment with the D2R vector. Mean Days: (*) The mean number of infusions on days 16-22 (after D2R vector treatment) was significantly lower ($p<.001$) than mean infusions on all other days prior to D2R vector treatment. Individual Days: (*) Infusions on days 16-21, 23, 24, 26 and 27 were significantly lower compared to infusions on day 15 ($p<.001$). (b) Mean (+SEM) reinforced (active) lever responses to 0.3 mg/kg cocaine before and after treatment with the D2R vector. Mean Days: (*) The mean number of active lever presses on days 16-22 (after D2R vector treatment) was significantly lower ($p<0.001$) than mean infusions on all other days prior to D2R vector treatment. Individual Days: (*) Lever Presses on days 16-21 were significantly lower compared to infusions on day 15 ($p<.001$).

2.2.4 Discussion

D2R adenoviral upregulation caused a significant decrease in CSA (infusions and lever responses) for up to 6 days post treatment. More specifically, the D2R vector had a robust (75% decrease) effect in the number of cocaine infusions. This effect was of short duration and was observed for the first 6 days following D2R vector treatment and then returned to baseline levels with the exception of a few sporadic days during the next 6 days. The attenuation of cocaine consumption by D2R upregulation was consistent with previous studies on ethanol consumption, where alcohol consumption was significantly attenuated. In previous alcohol studies, D2R upregulation attenuated ethanol intake for 8 days in Sprague Dawley rats (Thanos et al., 2001; Thanos et al., 2004) and 20 days for the inbred alcohol preferring P rats (Thanos et al., 2001; Thanos et al., 2004). It has been previously shown that this D2R vector produced significant D2R upregulation for 8 days before returning to baseline by day 10 (Ingram et al., 1998; Ogawa et al. 2000; (Thanos et al., 2001; Thanos et al., 2004).

Several factors may be involved in explaining the difference in the duration of this effect of D2R upregulation in cocaine versus alcohol administration. To start with

the role of D2R is different in the mechanisms of cocaine and alcohol reward. Previous work has suggested that ethanol-induced DA release in the NAc is insensitive to DA autoreceptor (D2R and/or D3R) regulation (Adam-Vizi, 1992; Levi & Raiteri, 1993; Yan, 2003) whereas, cocaine-induced DA release in the same region is thought to depend on regulation by D2R and D3R (Yan, 2003).

Furthermore, it has been shown that D2R knockout (KO) mice can still be trained to self-administer cocaine, and do so often at higher rates than wild type mice (Caine & Ralph-Williams, 2002), suggesting that D2R did not represent the only route through which the pharmacologically rewarding effects of cocaine took place. It is not clear however what specific route may be involved in CSA in these mice since like all transgenic animals, D2R KO mice may show compensatory changes in other receptors and neurotransmitter systems (Bolon, 2004) involved in CSA. In contrast it is much harder to compensate for the inhibition of the rewarding effects of alcohol on D2R KO mice (Risinger et al., 2000; Thanos et al., 2005).

The findings from this study are consistent with our work and that of others proposing that D2R play an important role in modulating drug self-administration behavior. By increasing the absolute number of D2R in the NAc, we have successfully, if transiently, disrupted CSA. Since the main effect of upregulating D2R in the NAc is likely to be increased transmission along the included DA pathways, analogies related to the other determinants of synaptic DA transmission in the NAc can be made. For example, studies showing that CSA may be triggered by low levels of accumbal DA in cocaine-experienced rats (Gerrits et al., 2002) suggest one way that our treatment might be decreasing the desire to self-administer cocaine. D2R upregulation could increase the probability of an interaction between transient DA increases and receptor activation, which would increase the sensitivity to the DA enhancing effects of drugs of abuse. As a result the amount of drug required to activate reward pathways would be lowered explaining the decrease in the total amount of cocaine ingested. Alternatively D2R upregulation may increase the number of constitutively active D2R receptors. However, the mechanism(s) underlying how D2R upregulation decreases CSA requires further investigation.

2.3 PET imaging of D2R with [^{11}C]raclopride predicts cocaine preference in rats

2.3.1 Introduction

PET imaging provides a non-invasive way of assessing brain function. Through the development of μPET scanner, the benefits of this technology have been extended to the study of rodents. Using PET and μPET imaging, studies have previously reported deficits in D2R binding availability in drug-addicted humans (Wang et al., 2004) and cocaine-exposed primates (Nader et al., 2008). In the first two sections, this thesis reports downregulated D2R binding in response to chronic cocaine intake in mice and that upregulation of D2R via gene-transfer techniques can attenuate cocaine intake. A fundamental question that has emerged from these studies is whether D2R deficits are

caused exclusively by repeated drug exposure, or whether individuals and animals are genetically prone to such deficits. If the answer to this question is the latter, then the notion of using D2R binding availability in conjunction with μPET as a predictive biomarker for assessing vulnerability to drug abuse becomes apparent. In this paper, we attempted to answer this question by measuring D2R binding availability using μPET in 10 drug-naïve adult Sprague-Dawley rats and then correlating these measures with cocaine preference 2 months after scanning. We hypothesized that D2R binding availability would negatively correlate with future measures of cocaine preference in rats and thus could serve as a predictive non-invasive measurement of cocaine abuse susceptibility.

2.3.2 Methods

Animals

Rats between 12-14 weeks of age (n=10) were obtained from Taconic (Taconic, NY) and housed on a reverse 12 hour light/dark cycle with lights off at 7 am. Rats were fed a standard (Purina) laboratory rat chow. Food intake was monitored daily at approximately 1500h and all rats were weighed every other day immediately after food monitoring. All experiments were conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (NAS & NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

In-vivo [^{11}C]raclopride D2R μPET

μPET assessment of D2R in these rats was performed using a μPET R4 Scanner (CTI Concorde Microsystems, Knoxville, TN). Each rat was briefly anesthetized with isoflurane (~2-3 min) and its lateral tail vein was catheterized. Once awake, each rat was injected intravenously with [^{11}C]raclopride (547 ± 72 μCi); specific activity, 10.4 ± 4.3 mCi/nmol). Injected volumes were approximately 400μl. 30 minutes later each rat was anesthetized with isoflurane and placed in a stereotaxic head holder (David Kopf Instruments; CA, USA) in a prone position on the bed of the scanner and scanning commenced. Total acquisition time was 30 min (17 frames: 6 frames, 10 s; 3 frames, 20 s; 8 frames, 60 s; 4 frames, 300 s) and data were acquired in fully 3-dimensional mode with maximum axial acceptance angle (+-28 degrees). Images were reconstructed using the optimization algorithm *maximum a posteriori* (MAP) with 30 iterations and a smoothing value of 0.01 mm. Qualitative and quantitative assessment of μPET images was performed as described previously using the Pixel-wise Modeling (PMOD) software environment (PMOD Technologies, Zurich Switzerland) (Schiffer et al., 2009).

Cocaine CPP

CPP was assessed two months after μPET scanning. The CPP apparatus (Coulbourn Instruments) contained three compartments and has been previously described (Thanos et al., 2009). The CPP procedure was modified from (Thanos et al., 2009) and consisted of the following four phases: i) habituation, (ii) pretest, (iii)

conditioning, and (iv) test. The habituation phase was administered on the first day. This consisted of transporting the animals to the room where the CPP apparatus was located. 30 minutes later the animals were transported back to the animal facility. On the second day, rats were transported to the CPP procedure room and placed in the middle chamber of the apparatus and were allowed 10 minute access to all chambers. Time spent in each chamber was recorded. For the next 8 days rats were injected with either cocaine (10 mg/kg (IP)) (Sigma-Aldrich (St. Louis, MO) or saline on alternate days. Using preference measurements from the pretest phase, rats were administered cocaine in the non-preferred chamber as observed during pretest. Rats were only allowed access to one of the two chambers each day during the conditioning phase. Daily sessions during this phase lasted for 30 minutes. The CPP test phase was conducted on day 11 with procedures identical to the pretest phase. The cocaine preference score was calculated as the percent change in time spent in the cocaine-paired chamber on test day vs. that on pretest day.

2.3.3 Results

Linear regressions analysis (Figures 2.3.1a & 1b) showed that D2R binding availability significantly correlated with the CPP score in the ventral but not the dorsal striatum. The regression was significant at the p=0.003 level with a correlation coefficient of r=0.83. A z-test showed that the correlations between the dorsal and ventral striatum were significantly different (z=2.94; $p_{2\text{-tail}}$=0.003).

Figure 2.3.1a

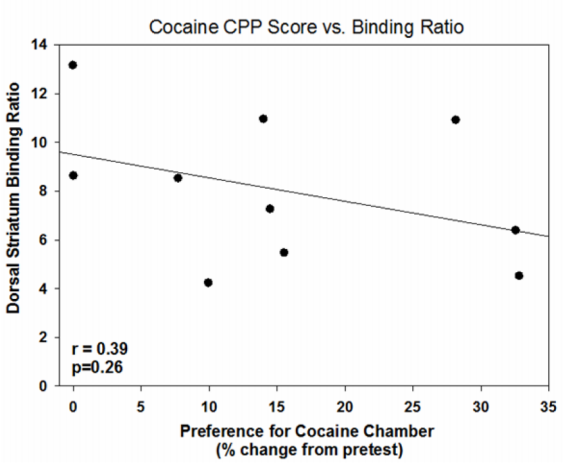

Figure 2.3.1b

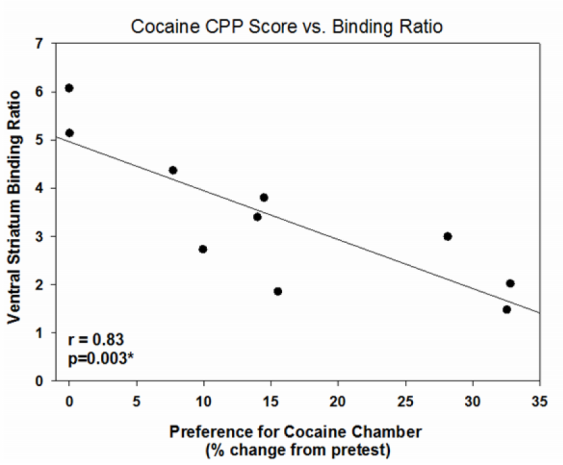

Figure 2.3.1. (a) D2R binding ratio expressed as receptor rich/receptor poor ratio (dorsal striatum/cerebellum) and plotted against cocaine preference 2 months post scan. (b) D2R binding ratio expressed as receptor rich/receptor poor ratio (ventral striatum/cerebellum) and plotted against cocaine preference 2 months post scan.

2.3.4 Discussion

We found that rats with the highest binding levels showed the lowest cocaine preference and vice versa. Specifically, we found a significant and strong negative correlation between D2R binding availability and future cocaine preference in the ventral but not the dorsal striatum. These findings suggest that inherent deficits in D2R may lead to cocaine abuse predisposition. Furthermore, since the two correlations were significantly different, these results suggest that the ventral striatum plays a primary role in mediating susceptibility to cocaine abuse. These results suggest the potential for using D2R binding availability to predict future susceptibility to cocaine abuse in rodents and potentially non-human primates. Using such a measurement, one can classify rodents and primates into those that express susceptibility to drug abuse and those that do not. One can then use molecular-genetic approaches to investigate gene-expression that may be differentially expressed in these animals before and after the drug exposure. Findings from such studies can lead to the development of new treatments for drug addiction. Finally, since PET imaging using [^{11}C]raclopride is a non-invasive research paradigm that is routinely employed in clinical research, this approach may have the potential to be translated to humans in order to determine individual susceptibility to cocaine abuse.

Chapter 3

Dopamine D2 Receptors and Obesity

3.1 Age- and diet-related changes in D2R binding in leptin receptor-deficient obese rats

3.1.1 Introduction

Obesity is one of the major and fastest growing public health problems worldwide. It is estimated that in the USA approximately one quarter of adults are obese (Flegal, 2002). The increases in obesity are likely to reflect the interplay between susceptibility genes in a changing environment where food is widely available, diverse and highly palatable (Friedman, 2004). Because food intake is regulated not only by nutrient and caloric requirements but also by its reinforcing properties (Carr, 2007) the wide availability and high palatability of food in our environment facilitates overeating.

DA is one of the neurotransmitters modulating the reinforcing properties of food (Carr, 2007). Indeed DA cells fire (increasing DA release in the NAc) when expecting food and their firing in turn is influenced by food deprivation (Meguid et al., 2000). While DA has been classically described as a neurotransmitter involved with locomotor activity and reward, more recent work has shown DA to be associated with the prediction of reward (Schultz et al., 2000; Phillips et al., 2007), with the motivation to procure the reward (McClure et al., 2003; Phillips et al., 2007) and with the facilitation of conditioned learning (Fenu & Di Chiara, 2003). Thus, a possible mechanism of DA involvement in food intake and obesity could be that DA signaling is increased in response to food as a stimulus predicting reward, leading to the motivation to procure the food while at the same time facilitating conditioned learning (Fenu & Di Chiara, 2003). PET imaging studies in humans have recently corroborated an involvement of DA both in food reward (Small et al., 2003) as well as in the motivation to procure the food (Volkow et al., 2002b).

The importance of DA's role in obesity has been supported both by preclinical and clinical studies (Hamdi et al., 1992; Meguid et al., 2000; Wang et al., 2001). Studies done in animal models of obesity (ob/ob mice, obese Zucker rats, obesity-prone Sprague–Dawley rats and seasonally obese animals) have documented reduced DA activity in the

tuberoinfundibular pathway that projects to the hypothalamus (Friedman, 2004). In these animal models, treatment with DA agonists reverses the obesity; presumably by activation of D2R and D1R (Pijl, 2003). Evidence of DA's involvement in obesity, particularly through its interactions with D2R, is given by the higher risk of weight gain and obesity observed in patients chronically treated with antipsychotic drugs (D2R antagonists) (Friedman, 2004). Also PET brain imaging studies have documented reductions in D2R availability in the striatum of obese individuals (Wang et al., 2001). In the obese subjects but not in the controls, D2R availability levels were inversely related to the body mass index (BMI), suggesting an involvement of the DA system in excessive weight gain. In contrast PET studies done in patients with anorexia nervosa have reported the opposite; higher than normal striatal D2R availability (Frank et al., 2005).

The hormone leptin has been shown to be an important signal in the regulation of energy balance (Hagan et al., 1999) and is secreted by adipocytes (Hagan et al., 1999). It has been reported that brain leptin exerts its effects at neurons in the ARC of the hypothalamus and stimulates the LepR (Ahima & Flier, 2000). Rodents and humans with homozygous mutations in the leptin or leptin receptor genes manifest hyperphagia and obesity (Hagan et al., 1999). When sensitivity for leptin signaling to the brain diminishes, numerous adaptive changes of the central regulatory systems occur, which are developed and characterized in the Zucker phenotype (Fetissov et al., 2000).

It has previously been shown that anorectic concentrations of leptin in the brain reduced the activity of mesolimbic DAergic neurons in vivo and also suppressed DA-related motivational aspects of feeding (Krugel et al., 2003). Also, in DA and leptin deficient mice, DA was shown to be required for the initiation of food intake in the absence of leptin (Szczypka et al., 2000). Furthermore, leptin receptors have been found to be extensively co-expressed within DA neurons of the VTA and SN (Figlewicz et al., 2003) suggesting that DA release in the striatum in relation to food intake is modulated by leptin.

The fa/fa rat is a popular model of obesity in which a mutation in the leptin receptor (Chua et al., 1996) causes obesity, diabetes and hyperphagia (Fetissov et al., 2000). fa/fa rats with this mutation are obese while their littermates without the mutation are lean. It is true that the majority of obesity cases are attributed to environmental factors. We utilized the fa/fa rat based on the severe hyperphagia and weight gain that the model shows. These characteristics, coupled with the leptin receptor mutation would facilitate the examination of potential changes in D2R brought upon by leptin-DA interactions within the context of obesity and hyperphagia. We hypothesized that obese rats given unrestricted access to food would have lower D2R levels than their lean counterparts and that food restriction would attenuate such differences.

3.1.2 Methods

Animals

Male 1 month old Zucker Obese fa/fa (Ob; N=20; and Lean (Fa/Fa) or (Fa/fa) (Le; N=20) rats were used. Rats were divided into 4 groups of 10 rats in each group. Specifically: i) Ob rats with unrestricted (U) food access, ii) Ob rats with restricted (R) food access, iii) Le U rats and iv) Le R rats. At 1 month of age half the rats were placed

on restricted food access (70 % of ad-libitum fed animals) and the rest on free access to food (ad-libitum). Rats were obtained from Harlan (Indianapolis, IN) and housed on a reverse 12 hour light/dark cycle with lights off at 7 am. All experiments were conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (NAS & NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

Behavior Assessment: Food Intake and Weight

Rats were fed a standard (Purina) laboratory rat chow. Food intake was monitored daily at 1500h and all rats were weighed every other day. Restricted diet rats were fed daily at 1500h and the amount of food given was continuously adjusted to 70% of food intake of the ad-lib rats.

Behavior Assessment: Effects of Apomorphine

Rats were placed in an Optical Sensor Plexiglas arena (Minimitter Corporation, Oregon, USA) which consisted of an optical sensor that was securely attached to the wire top of the animal home cage. Locomotor activity data was collected every two weeks over a period of 4 months. After an initial one-time 60 minute habituation session, each animal was given two 60-minute sessions in the same boxes: i) saline and ii) apomorphine (APO) (1 mg/kg) on two consecutive days. Saline injections always preceded APO injections. Optical beam breaks were recorded every minute and the total number of beam breaks for each group of animals was summed into 60 minute sessions.

D2R ARG

Each animal was deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The brain was rapidly removed and frozen in an isopentane and dry ice bath and stored in a -80^0C freezer. The brain was then transferred to a cryostat (Leica CM3050), and sections were cut 14 μm thick at -18^0C. Sections were mounted on glass microscope slides and stored at -80^0C until binding was conducted. Slides were gradually brought back to room temperature and then pre-incubated at room temperature for ten minutes in 50 mM Tris-HCl buffer (pH 7.4). The slides were then incubated in specific binding buffer (50 mM Tris-HCl, 0.4 nM [^{3}H]spiperone, 10 μM ketanserin tartrate) at room temperature for one hour. To determine non-specific binding some slides in parallel were incubated for one hour in specific binding buffer in the presence of 10 μM haloperidol. Next, the slides were washed 2 x 10 minutes in ice-cold 50 mM Tris-HCl and ice-cold dH_2O. Afterwards, 20 μl aliquots of incubation solution were taken and added with 3 ml of Ultima Gold XR (scintillation solution) and the amount of radioactivity was measured with a liquid scintillation counter. Slides were then dried overnight in a dessicator and placed in a glass slide cassette for qualitative and quantitative analysis using a β-Imager (Biospace, Paris, France/Capintec, USA). Using Betavision+ software (Biospace, Paris, France/Capintec, USA), ROI's were drawn on the left and right striatum (ST), CB, cingulate (CG) and frontal cortices (FC) of each brain slice. The data was then calibrated using a tissue homogenate standard. Mean values (μCi/g) of the left and right receptor rich regions (ST, CG and FC) for each group were

later normalized for receptor poor regions (CB) and the data are reported in respective ratios (receptor rich/receptor poor).

MRI Imaging

For accurate qualitative and quantitative image analysis using μMRI-μPET image coregistration, MR templates from 1 and 4 month old lean Zucker rats were acquired on a 4T Siemens scanner using the 2d proton density sequence with the following optimized parameters: TR/TE =200/20ms, a 256X256 matrix, a field of view of 32x32mm and a slice thickness of 1mm with an in plane spatial resolution 194mm.

D2R μPET Imaging

μPET assessment of D2R in these rats was performed using a μPET R4 Scanner (Concorde Microsystems). Each animal was fasted for 24h before being scanned in the μPET. Next each rat was anesthetized and injected intravenously with [^{11}C] raclopride, a DA D2R-specific ligand, and dynamic PET scanning followed for 60 minutes. Raclopride was purchased from Sigma and [^{11}C]raclopride was synthesized as described previously (Volkow et al., 1999b)

Rats were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (10mg/kg) and placed in a stereotaxic head holder (David Kopf Instruments; CA, USA) in a prone position on the bed of the scanner. Next, the lateral tail vein was catheterized and 250 μl of blood were collected from the rats in $EDTA_K$ coated Multivette (Sarstedt, USA) tubes prior to [C^{11}]raclopride administration. Blood samples were centrifuged at 3,000 rpm for 10 minutes; plasma was collected and immediately placed on dry ice. Plasma insulin and leptin concentrations were measured in duplicate with commercially available insulin and leptin enzyme immunoassay (EIA) kits obtained from Linco Research (Linco Research, Missouri, USA).

Animals were then injected via the same tail vein catheter with [^{11}C]raclopride (424 +/- 49 μCi); specific activity, 1.7-12.3 mCi/nmol). Injected volumes were approximately 400 μl. Total acquisition time was 60 min (24 frames: 6 frames, 10 s; 3 frames, 20 s; 8 frames, 60 s; 4 frames, 300 s; 3 frames, 600 s), and data were acquired in fully 3-dimensional mode with maximum axial acceptance angle (+-28 degrees). Images were constructed using Fourier rebinning (Matej et al., 1998) followed by 2-dimensional filtered back-projection with a ramp filter cutoff at Nyquist frequency.

In order to control for the effect of 24h fasting of unrestricted rats we scanned these rats two times separated by 2 weeks. One scan consisted of unrestricted rats without any fasting (food always available right up to scan) and the second scan consisted of the same rats with 24h fasting prior to scan.

μPET Analysis

Using the PMOD software suite (PMOD Technologies, Switzerland), μPET images was co-registered with the previously generated MRI templates. Initially the two images were co-registered using the method of Mutual Information (MI) (Woods et al., 1992); (Woods et al., 1993) through a MI algorithm implemented within the PMOD environment, followed by a manual adjustment of the μPET image in all three planes

(sagittal, coronal, transversal) so that the Harderian glands and ST from both imaging modalities matched. ROI's for left and right ST and CB were selected using the μMRI-μPET coregistered Harderian glands as a reference point. Specifically the ST and CB for each animal were identified as 8 and 12 slices respectively, caudal to the Harderian glands (slice thickness, 1.2 mm). It has been shown previously that the Harderian glands (located just rostral of the brain), because of their uptake of radioactivity, are routinely used as markers in rodent PET studies (Hume et al., 1996; Kuge et al., 1997; Fukuyama et al., 1998).

Qualitative assessment of μPET images was performed, and representative μPET images of each rat were acquired. The image analysis was performed using the Fusion, PxMOD and Kinetic programs included in the PMOD v. 2.55 software suite. Quantitative analysis of the μPET images consisted of the MRTM0 Ichise Binding Potential (BP). This multi-reference tissue model provides an accurate noninvasive method of estimating D2R binding potential without having to sample blood (Ichise et al., 2003).

3.1.3 Results

Body weight

Body weight was examined in each group over the 4-month period. A multifactor three-way ANOVA revealed significant main effects in body weight with respect to Strain ($F=461.683$; $df=1, 79$; $p<0.001$, Figure 3.1.1), Diet ($F=320.027$; $df=1, 79$; $p<0.001$, Figure 3.1.1), and Age ($F=5288.733$; $df=1, 79$; $p<0.001$; Figure 3.1.1). At 1 month, there were no significant differences in weight between any of the groups. At 4 months, Ob U rats showed significantly higher weight gain than Le R ($t=23.873$; $p<0.05$), Le U ($t=26.545$; $p<0.05$) and Ob R ($t=28.154$; $p<0.05$). Similarly, at 4 months, Le U and Ob R showed significantly higher weight gain compared to Le R, ($t=7.441$;$p<0.05$) and ($t=5.832$; $p<0.05$) respectively. There were no differences between the Ob R and the Le U rats.

Food Intake

Throughout the study food intake was monitored in the two unrestricted rat groups. A two-way ANOVA showed significant main effects with respect to Strain ($F=39.044$; $df=1, 39$; $p<0.001$; Figure 3.1.2) and Age ($F=272.048$; $df=1, 39$; $p<0.001$; Figure 3.1.2). At 1 month, there were no differences in food intake between Ob and Le rats. By the fifth week, the rats began to show significant differences in food intake levels (Figure 3.1.2) with the Le rats reaching at peak at 7 weeks while Ob rats reached a peak at around 9 weeks. At 4 months Ob rats had higher food intake levels compared to Le ($t=6.477$; $p<0.05$; Figure 3.1.2). Specifically, Ob rats consumed on average an additional 10 g of food than Le U rats (30% more). Finally, food intake levels were significantly higher at 4 compared to 1 month for both Ob ($t=13.722$; $p<0.05$; Figure 3.1.2) and Le rats ($t=9.604$; $p<0.05$; Figure 3.1.2).

Behavior Assessment: Effects of APO

This behavioral paradigm focuses on revealing behavioral differences in locomotor activity that are associated with APO administration rather than examining changes in general locomotor activity. APO is a nonselective DA agonist thought to bind with slightly higher affinity to D2-like than D1-like receptors (Jenner, 2003; Dias et al., 2006) and therefore this paradigm provides an additional measure of DA and D2R function in response to experimental treatment. For this reason the data were reported as the relative percent difference between saline and APO treated sessions for each group at 1 and 4 months.

A Three-way ANOVA revealed significant differences in the percent change of locomotor response to APO (Figure 3.1.3) with respect to Strain ($F=13.958$; $df=1, 95$; $p<0.001$) and Age ($F=4.713$; $df=1, 95$; $p= 0.033$) but not with respect to Diet. Subsequent multiple comparisons (Holm-Sidak) revealed several key differences. At 1 month, there were no differences in the percent change of locomotor activity in response to APO between Ob and Le rats. At 4 months, APO caused a significant percent decrease in locomotor activity as compared to 1 month levels for all groups. Both unrestricted and restricted rats were equally affected, but the decline was more pronounced in the Ob. Multiple comparison procedures (Holm-Sidak method) showed that percent changes in APO compared to saline-paired locomotor activity was lower in the Ob U ($t=4.821$; $p<0.05$), Ob R ($t=4.712$; $p<0.05$), Le U ($t= 3.467$; $p<0.05$) and Le R ($t=3.479$; $p<0.05$) rats at 4 compared to 1 month of age and that this decline was more significant for the Ob (37 %) than for the Le (16 %) (see Figure 3.1.3).

D2R ARG: ST

Brain sections were assessed with ARG for striatal D2R binding at 1 and 4 months (Figure 3.1.4a). A Three-way ANOVA showed significant differences with respect to Strain ($F= 45.341$; $df=1, 63$; $p<0.001$, Figure 3.1.4b), Diet ($F=38.915$; $df=1, 63$; $p<0.001$, Figure 3.1.4b), and Age ($F=41.748$; $df=1, 63$; $p<0.001$, Figure 3.1.4b). Subsequent multiple pairwise comparison tests (Holm-Sidak method) were used to measure the differences of striatal D2R binding data between and within all groups. At 1 month, Le U rats had higher D2R binding levels as compared to Ob U rats ($t=4.945$; $p<0.05$). All four groups of rats at 4 months of age showed lower D2R binding levels than at 1 month. At 4 months, Le U rats had higher D2R binding levels than Ob U rats ($t= 4.719$, $p<0.05$). Also, Ob R rats had significantly higher D2R levels than Ob U ($t=12.237$, $p<0.05$) and Le U ($t=9.790$; $p<0.05$). Furthermore, at 4 months Le R rats had higher D2R levels than Le U rats ($t=12.315$, $p<0.05$) and Ob U rats ($t=14.528$; $p<0.05$). Food restriction had a significant effect on age related D2R loss for both groups of rats; whereas D2R levels tended to decrease between 1 and 4 months in the unrestricted rats, this loss was attenuated in the restricted rats.

D2R ARG: CG

A one-way ANOVA ($F=7.389$; $df=5, 39$; $p<0.001$) was used to assess differences in D2R binding in the CG (Figure 3.1.4c). Multiple pai-rwise comparisons (Holm-Sidak) revealed no significant differences at 1 month of age. At 4 months we found significantly

lower D2R binding in Ob U compared to 1 month old Ob U ($t=4.148$; $p<0.05$) and Le U ($t=4.377$; $p>0.05$) rats. Similarly, 4 month old Le U rats showed lower D2R binding compared to 1 month old Ob U (4.167; $p<0.05$) and Le U ($t=4.410$; $p<0.05$) rats. Unlike the unrestricted rats, D2R binding levels did not differ between 1 and 4 months in the restricted fed groups. At 4 months, Ob R and Le R rats showed greater D2R binding compared to Ob U ($t=2.114$; $p<0.05$) and Le U ($t=2.874$; $p<0.05$) rats respectively. Le R rats also showed greater binding compared to Ob U rats ($t=2.973$; $p<0.05$).

D2R ARG: FC

A one-way ANOVA ($F=5.608$; $df=5, 39$; $p<0.001$) was used to assess differences in D2R binding in the FC (Figure 3.1.4c). Multiple pair-wise comparisons (Holm-Sidak) revealed no significant differences at 1 month of age. At 4 months we found significantly lower D2R binding in Ob U compared to 1 month old Ob U ($t=3.876$; $p<0.05$) and Le U ($t=3.633$;$p<0.05$) rats. Similarly, 4 month old Le U rats showed lower D2R binding compared to 1 month old Ob U ($t=3.573$; $p<0.05$) and Le U ($t=3.310$; $p<0.05$) rats. Unlike the unrestricted rats, D2R binding levels did not differ between 1 and 4 months in the restricted fed groups. At 4 months, Le R rats showed greater D2R binding compared to Le U ($t=3.078$; $p<0.05$) and Ob U (3.403;$p<0.05$). Finally, at 4 months Le U rats showed greater D2R binding compared to Ob U ($t=2.044$; $p<0.05$).

Weight vs. D2R binding levels

The plot showing the relationship between striatal ARG D2R binding and weight was fitted with a linear regression model (Figure 3.1.5). The regression model showed significant relationship between predicting D2R from weight. Specifically, using the D2R binding levels as the dependent variable and weight as the independent, we found a highly significant regression across groups ($r = -0.90$; $p < 0.0001$; Figure 3.1.5).

Insulin

Analysis of the blood samples for insulin levels using a two-way ANOVA revealed significant main effects (Figure 3.1.6a) with respect to Strain ($F=279.17$; $df=1, 39$; $p<0.001$), Diet ($F=81.64$; $df=1, 39$; $p<0.001$) and their interaction ($F=96.48$; $df=1, 39$; $p<0.001$). Subsequent pairwise multiple comparisons revealed significantly greater plasma insulin concentrations in Ob U compared to Ob R ($t=13.33$; $p<0.001$) and Le U ($t=18.76$; $p<0.001$). Within the two restricted groups, Ob R had higher plasma insulin concentrations than Le R rats ($t=4.87$; $p<0.001$) but there was no significant difference in insulin concentration between Le U and Le R rats ($t=0.56$; $p=0.58$).

Leptin

Analysis of the blood samples for leptin using a two-way ANOVA revealed significant main effects (Figure 3.1.6b) with respect to Strain ($F=284.26$; $df=1, 39$; $p<0.001$), Diet ($F=77.49$; $df=1, 39$; $p<0.001$) and their interaction ($F=87.67$; $df=1, 39$; $p<0.001$). Subsequent pairwise multiple comparisons revealed significantly greater plasma leptin concentrations in Ob U compared to Ob R ($t=9.47$; $p<0.001$) and Le U ($t=17.24$; $p<0.001$). Within the two restricted groups, Ob R had higher plasma leptin

concentrations than Le R rats (t=3.36; p<0.001) but there was no significant difference in leptin concentrations between Le U and Le R rats (t=0.64; p=0.69).

D2R μPET Imaging

Non-invasive μPET D2R brain imaging was examined at two time points for each group of rats: 1 and 4 months (Figure 3.1.7a and 7b). A 2-way ANOVA (Figure 3.1.7b) showed a main effect for Age (F=199.086; df= 1, 359; p<0.001) and Group (F=18.481; df=3, 359; p<0.001). Since we could not differentiate between Strain and Diet as two independent factors within the 2-way ANOVA design, the subsequent pairwise multiple comparisons (Holm-Sidak method) assessed a generalized comparison for all data at 1 and 4 months. This showed significantly greater D2R availability at 1 versus 4 months (t=14.762; p<0.001) for all the data irrespective of groups. In order to assess individual group differences at 1 versus 4 months of age a 1-way ANOVA was conducted (F=48.250; df=5, 359; p<0.001; Figure 3.1.7b) and this yielded greater D2R availability in the 1 month old Ob U rats (Figure 3.1.7b) compared to 4 month old Ob U (t=8.503; p<0.001), Le U rats (t=13.534; p<0.001), Ob R (t=7.003; p<0.001) and Le R (t=7.121; p<0.001). The 1-way ANOVA showed greater D2R availability in 1 month old Le U rats (Figure 7b) compared to 4 month old Le U (t=11.851; p<0.001), Le R (t=5.752; p<0.001), Ob U (t=7.258; p<0.001) and Ob R (t=5.790; p<0.001). At 4 months of age, Ob U rats showed significantly greater D2R binding than Le U rats (t=4.593; p<0.001) and showed a trend towards lower D2R binding values than Ob R rats (t=1.861 p=0.06). Also, Le R had significantly greater D2R binding than Le U rats (t=7.618; p<0.001). Finally, 4 month old Ob R rats had greater D2R availability than 4 month old Le U rats (t=6.702; p<0.001; Figure 3.1.7b).

In order to determine whether D2R binding levels in the μPET experiment were altered in response to non-fasted (NF) or 24h fasted (F) conditions in the 4 month old Ob and Le ad-lib fed animals, a 2-way multifactor ANOVA (Figure 3.1.7c) showed a main effect for Group (F=34.77; df= 3; 335; p<0.001) and fed or fasting State (F=14.65; df=1, 335; p<0.001). Since we could not differentiate between Strain and Diet as two independent factors within the 2-way ANOVA design, the subsequent pairwise multiple comparisons (Holm-Sidak method) assessed a generalized comparison for all data between the NF and F state. This showed significantly greater D2R availability in the NF versus F states (t=3.99; p<0.001; Figure 3.1.7c) for all the data irrespective of groups. In order to assess individual group differences in the NF versus F state, a 1-way ANOVA was conducted (F=20.85; df=5, 335; p<0.001; Figure 3.1.7c) and this yielded greater D2R availability in the NF Ob U rats compared to F Ob U (t=2.47; p<0.01); NF Le U (t=4.67; p<0.001) and F Le U rats (t=7.61; p<0.001). F Ob U rats showed greater D2R availability than F Le U rats as well (t=5.15; p<0.001). Also, the F Ob R rats had significantly greater D2R binding availability than F Le U (t=7.51; p<0.001) and NF Le U (t=4.41; p<0.001).

The 1-way ANOVA also showed greater D2R availability in NF Le U vs. F Le U rats (t=2.94; p<0.01). Finally, F Le R rats had higher D2R binding than F Le U (t=8.54; p<0.001), NF Le U (t=5.22; p<0.001) and F Ob U (t=2.73; p<0.01).

Figure 3.1.1

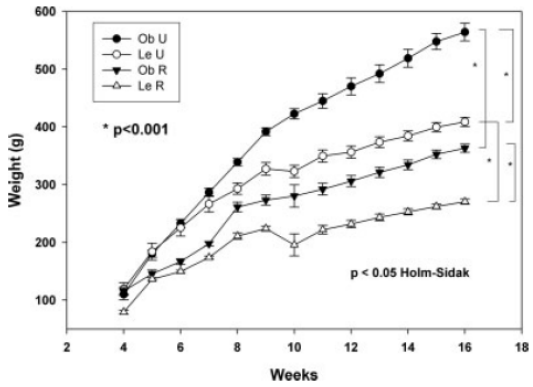

Figure 3.1.2

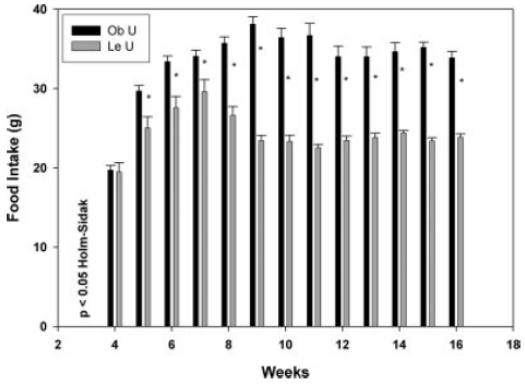

Figure 3.1.1. Mean (± SEM) weight over development across groups. (*) $p<0.05$
Figure 3.1.2. Mean (+SEM) food intake of unrestricted Zucker rats over development. (*) $p<0.05$

Figure 3.1.3

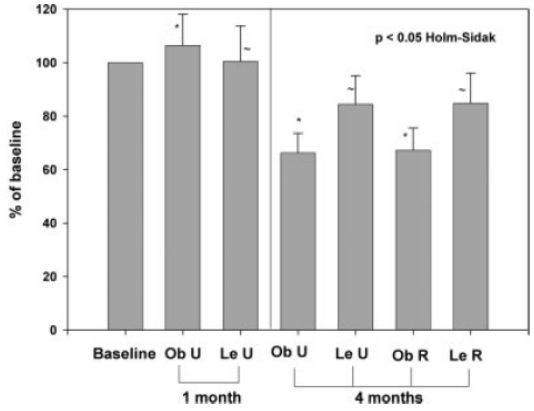

Figure 3.1.3. Mean locomotor activity expressed as a percentage from baseline (+ SEM) at 1 and 4 months of age as assessed after intraperitoneal administration of 1mg/kg APO. (*) Ob U and Ob R rats show decreased APO-induced locomotor activity between 1 and 4 months ($p<0.05$), (~) Le U and Le R rats show decreased APO-induced locomotor activity between 1 and 4 months ($p<0.05$).

Figure 3.1.4

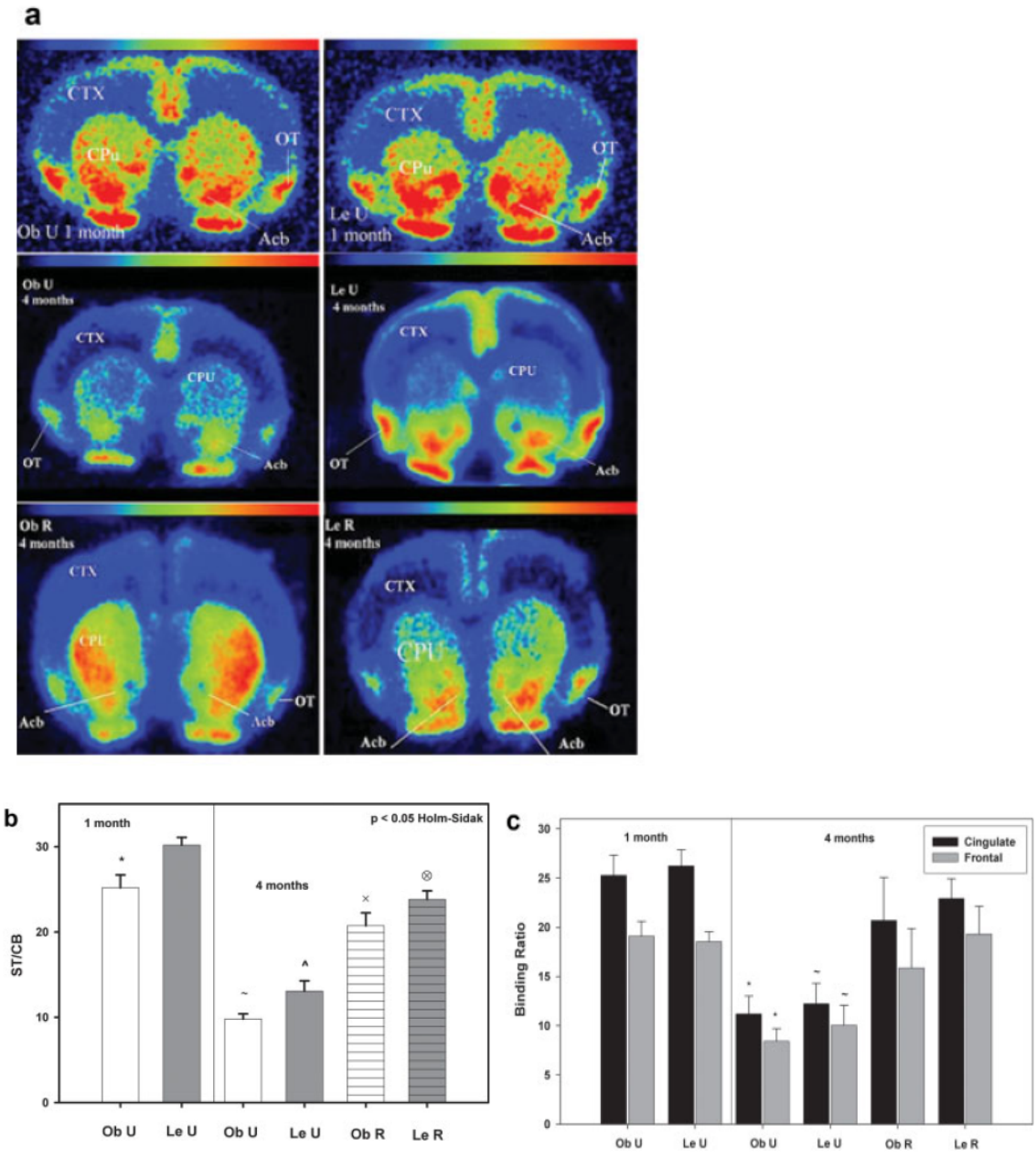

Figure 3.1.4. (a) Digital autoradiograms of [^{3}H]spiperone ARG at 4 months in Ob and Le Zucker rats. CTX = cerebral cortex; Acb = accumbens, OT = olfactory tubercle. (b) Mean (+SEM) [^{3}H]spiperone binding ratio (receptor rich/receptor poor) in Ob and Le Zucker rats at 1 and 4 months in the striatum. (*) 1 month old Ob U rats show decreased D2R binding compared to 1 month old Le U rats ($p<0.05$). (~) 4 month old Ob U rats show decreased D2R binding compared to 1 month old Ob U and 4 month old Ob R rats ($p<0.05$), (^) Le U show decreased D2R binding compared to 1 month old Le U and 4 month old Le R rats ($p<0.05$). ($\times$) Ob R rats show decreased D2R binding compared to 1 month old Ob U rats ($p<0.05$) ($\otimes$) Le R rats show decreased D2R binding compared to 1

month old Le U rats ($p<0.05$). (c) Mean (+SEM) [^{3}H]spiperone binding ratio (receptor rich/receptor poor) in Ob and Le Zucker rats at 1 and 4 months in the cingulate and frontal cortices. (*) 4 month old Ob U rats show decreased D2R binding compared to 1 month old Ob U and 4 month old Ob R rats (~) 4 month old Le U rats show decreased D2R binding compared to 1 month old Le U and 4 month old Le R rats.

Figure 3.1.5

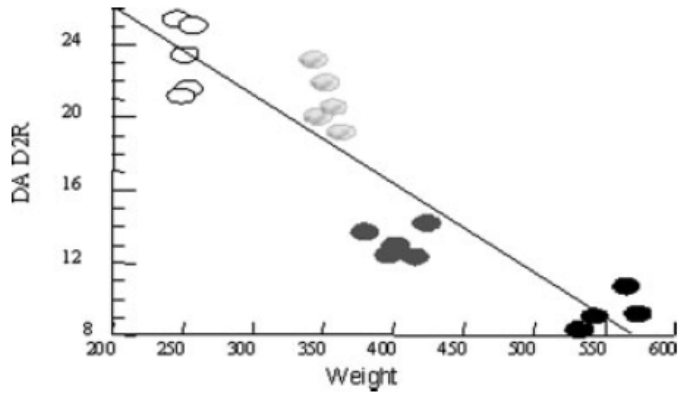

Figure 3.1.5. Regression plot for the relationship between weight (grams) and D2R ARG binding levels (ST/CB) for the Le R (white circles), Ob R (light gray circles), Le U (dark gray circles) and Ob U (black circles). The regression is highly significant ($r = 0.90$, $p < 0.0001$).

Figure 3.1.6

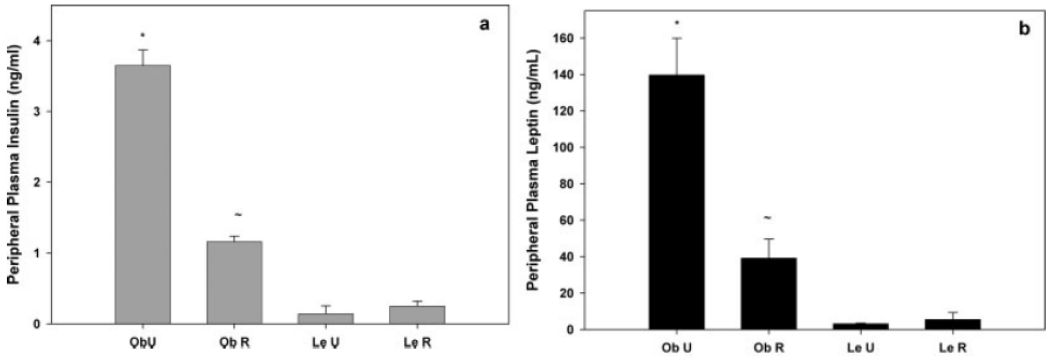

Figure 3.1.6. (a) Mean (+SEM) Fasted (24 hour) plasma insulin concentrations in 4 month old Obese and Lean Zucker rats (*) Ob U rats show greater insulin levels compared to Ob R, Le U and Le R rats ($p<0.05$) (~) Ob R rats show greater insulin levels compared to Le U and Le R rats ($p<0.05$). (b) Mean (+SEM) fasted (24 hour) plasma leptin concentrations in 4 month old Obese and Lean Zucker rats (*) Ob U rats show greater leptin levels compared to Ob R, Le U and Le R rats ($p<0.05$). (~) Ob R rats show greater leptin levels compared to Le U and Le R rats ($p<0.05$).

Figure 3.1.7

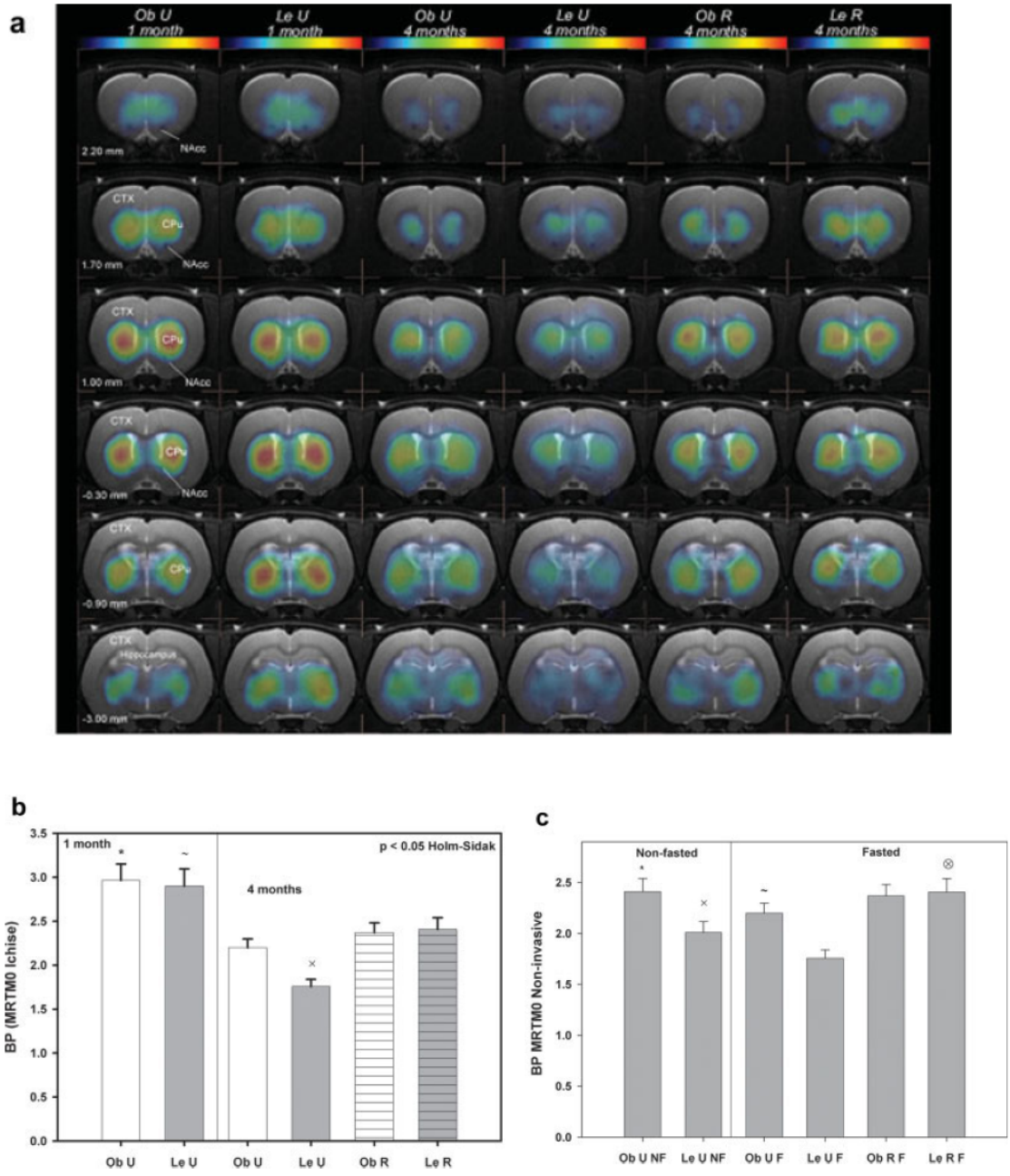

Figure 3.1.7. (a) [^{11}C]raclopride µPET imaging of Ob and Le Zucker rats at 1 and 4 months of age. (b) Mean (+ SEM) [^{11}C]raclopride µPET MRTM0 Ichise BP of Ob and Le Zucker rats at 1 and 4 months of age. (*) 1 month old Ob U rats show greater D2R availability compared to 4 month old Ob U and Ob R rats ($p<0.05$). (~) 1 month old Le U rats show greater D2R binding availability compared to 4 month old Le U and Le R rats ($p<0.05$). (×) 4 month old Le U rats show lower D2R availability compared to 4 month old Le R rats ($p<0.05$). (c) Mean (+SEM) MRTM0 [^{11}C] raclopride BP in fasted (F) and non-fasted (NF) 4 month old Obese and Lean Zucker rats. (*) NF Ob U rats show greater

D2R availability compared to NF Le U and F Ob U rats ($p<0.05$). (~) F Ob U rats show greater D2R availability compared to F Le U rats ($p<0.05$). ($^{\times}$) NF Le U rats showed greater D2R availability compared to F Le U rats ($p<0.05$). ($^{\otimes}$) F Le R rats show greater D2R availability compared to F Le U rats ($p<0.05$).

3.1.4 Discussion

Food Intake & Weight gain

The alteration of the leptin signaling pathway, as observed in the Ob Zucker rat, has a profound effect on food intake, weight and locomotor activity. Although at 1 month we show no significant difference in weight and food intake between Ob and Le rats, at 4 month the differences were very apparent; Ob rats weighed 39 % more and consumed 42 % more than their lean counterparts. Since Ob and Le rats differ in a mutation in the leptin gene the differences between Ob and Le highlight the regulatory role of leptin in maintaining food consumption within a fixed range regardless of food availability. As expected food restriction significantly reduced weight at 4 months in the Ob R (compared to Ob U; 55 % difference) and Le R rats (compared to Le U; 51 % difference), which was consistent with previous results (Meguid et al., 2000).

Behavioral Assessment: APO-paired Locomotor Activity

At 1 month, we did not observe differences in the percent change of locomotor response to APO administration in Ob and Le unrestricted rats, suggesting that Ob and Le rats at this age may be characterized by similar DA and D2R profiles. On the other hand, weight and food intake in the Ob U rats went up between 1 and 4 months of age to 512 % and 172 % respectively while the percent change in APO-paired locomotor activity decreased by 38 %. Similarly, Le U rats showed a 343 % and 23 % increase in weight and food intake respectively, while the percent change in APO-paired locomotor activity declined by 16 %. Finally, Ob R rats showed a 315 % increase in weight while percent changes in APO-paired locomotor activity declined by 38 % and Le R rats showed a 226 % increase in weight followed by a 16 % percent change decline in APO-paired locomotor activity. The difference in the above percent changes in locomotor activity in response to APO between the Ob and Le strains (38 % vs. 16 %) regardless of food restriction suggests an involvement of LepR deficiency not only in food intake and weight gain but also in locomotor activity tested under administration of the non-selective DA agonist APO. This suggests that Ob and Le rats at this age share different DA profiles and possibly differences in specific DA receptor concentrations. Nevertheless, we do not know if this is attributed to direct effects of leptin, weight or to secondary effects of other regulatory messengers (i.e. insulin, glucose, etc.) that differ in these rats.

D2R characterization using μPET and β-Imager ARG

μPET and ARG yielded different results in the characterization of the effects of food restriction on D2R. This is likely to reflect the fact that the two procedures examine differently D2R levels. Specifically, μPET is conducted under in-vivo conditions where

the animal is alive and therefore D2R binding is subject to several factors beyond our control. One major factor is competition of endogenous DA with raclopride. Raclopride is in fact highly displaceable by DA (Morris & Yoder, 2007) and therefore any increases in endogenous DA would likely show up as decreases in D2R binding and vice-versa. Similarly, other endogenous messengers, behavioral status (fed vs. fasted, aroused, etc.) and even anesthesia may compromise binding levels. On the other hand, ARG is done under more controlled experimental conditions where, endogenous messengers like DA are removed by pre-washing the slides with specific buffers, thereby reducing variability in D2R binding due to endogenous competition. Another major factor that plays a role in the different results each technique generates is the use of different radioligands. For μPET imaging we use raclopride whereas for ARG we use spiperone. Unlike raclopride, DA displaces spiperone to a much less extent (Morris & Yoder, 2007). Also, it has been suggested that the two tracers may bind to D2R differentially (raclopride binds to cells in the membrane while spiperone may penetrate the cell membrane and bind to internalized receptors as well) (Laruelle, 2000). Fasting has been shown to increase endogenous DA (Miklya et al., 2003). For these reasons, binding of [^{11}C]raclopride to striatum is expected to be sensitive to changes in DA that ensue secondary to fasting whereas ARG would not. By using these two measures in concert, we may be able to use these differences to our advantage so that we can obtain a stable measure of D2R binding concentrations using spiperone and ARG and then using these results go on to hypothesize what happens to DA concentrations using raclopride and μPET. Since D2R binding with ARG has a much higher resolution compared to μPET, it allows for greater sensitivity in localization of the D2R within the brain. For these reasons, the discussion that follows regarding the effects of strain and food restriction on D2R is based primarily on the ARG results and that of food restriction on DA release is based on μPET.

It should be noted that the values for D2R binding availability that we report reflect the average activity throughout the entire striatum (Figures 3.1.7a) as well as partial volume and spillover effects which are a limitation of small-animal PET imaging (Thanos et al., 2002) (Laforest et al., 2005) and therefore the reader should take caution in relying on the μPET images for the accurate representation of D2R binding availability. Furthermore, our interpretation is limited by the inability of separating auto and postsynaptic D2R and is primarily based on a static model of the relationship between DAT availability and regulation of D2R by the extracellular DA levels. Finally, the relative difference in tonic vs. phasic release of DA in response to food consumption in the two strains would further complicate our interpretation. In light of this, the discussion about the relationship between the observed effects on D2R availability and the actual release of DA remains speculative and further validation studies are required for the proper characterization of this methodology. Nevertheless, it remains a valuable approach since it provides a longitudinal approach to the study of DA system function without having to deal with complicated issues like tissue integrity and survival which are both significant limitations of other approaches (i.e. microdialysis, dissections) to DA system function.

D2R as a function of strain

It has previously been shown that Zucker rats display a unique DA profile (Meguid et al., 2000) in particular, with regards to D2R levels (Hamdi et al., 1992). These previous findings showed that neither D2R density (Bmax) nor affinity (Kd) were significantly different at 1 month of age between Ob and Le Zucker rats (Hamdi et al., 1992). Our results at 1 month of age were not in agreement with these findings, since we found significantly higher (~20 %) D2R binding in Le U compared to Ob U rats.

The effect of age on D2R has also been previously characterized with somewhat inconsistent findings (Tarazi et al., 1999). Relevant to our findings is a longitudinal study, that reported a 51 % increase in D2R levels in Le U rats between 1 and 6 months, while D2R levels remained unchanged for Ob U rats (Hamdi et al., 1992). In contrast, here we show a 56% decline in striatal D2R in Le U rats and a 60% decline in Ob U between 1 and 4 months of age.

At 4 months Le U rats had significantly higher (35 %) striatal D2R binding levels when compared to Ob U rats, which is consistent with Hamdi's findings of higher D2R levels in Le U than Ob U rats at 6 months of age (Hamdi et al., 1992). The above discrepancies may be explained both by differences in sensitivity (500% higher for ARG using a β-imager compared to film) (Langlois et al., 2001) as well as differences in the D2R radioligands used. Specifically, in the Hamdi, 1992 article [^{3}H]nemonapride and not [^{3}H]spiperone was used for D2R binding. These two ligands differ significantly in the labeling of D2R (Vivo et al., 2006). Also, it appears that Hamdi did not utilize a pre-wash in his binding protocol to remove D2R bound DA and therefore binding in that experiment may have been done in the presence of DA while in our study it was not.

Our findings of low D2R levels in Ob U rats corroborate the findings from recent clinical imaging data showing that obese individuals have reduced D2R binding levels compared to lean controls (Wang et al., 2001; Wang et al., 2004). Note that human obesity resembles the unrestricted food condition in the animal model except for the fact that while the Ob Zucker rats have LepR inactivation most obese subjects do not. Similar to the human data (inverse correlation between the D2R striatal levels and the BMI in the obese subjects), we also documented an inverse correlation in the Ob rats between the D2R levels and their weight (Figure 3.1.5). Since we found more D2R binding in Le U relative to Ob U rats, we may assume that Ob rats experience a greater loss of D2R as they age, perhaps due to increased weight gain, food intake as well as changes in regulatory messengers like insulin and leptin. The fact that the D2R decline was decreased in the Ob R may suggest that the greater D2R loss with age in the Ob U reflects increased food intake and weight gain as was shown in the imaging study of morbidly obese subjects (Wang et al., 2001; Wang et al., 2004)) as well as decreases in leptin and insulin as shown in our experiment (Figures 3.1.6a & 6b). Moreover, food restriction also decreased the age-related losses in D2R in Le R rats but had no significant effect on insulin and leptin. Thus the present findings suggest an effect of food intake on the age related D2R losses. This is consistent with prior studies showing that food restriction alters the effects of aging (Ingram et al., 2001) on motor activity and reward (Carr et al., 2003), and thus an attenuation of age-induced loss of D2R could underlie the benefits that food restriction has on these behaviors .

Previous studies have shown lower DA levels in the hypothalamus of 9 week old Zucker Ob as compared to Le rats in 24 h fasted or non-fasted conditions (Meguid et al., 2000). This is relevant since the lower DA levels in the Ob Zucker rats would result in greater D2R availability to bind to the [^{11}C]raclopride during the μPET scan. While no effect was reported from 24h fasting on the DA levels, it is important to note that the Meguid (Meguid et al., 2000) findings were from the hypothalamus and not in the striatum. It is unclear how the decreased DAergic signaling in Ob rats relates to their increased food intake. It has been suggested that weak DAergic signaling, which is involved with satiety would result in greater food consumption to bring about satiety in Ob than in Le rats (Orosco et al., 1995). However the later interpretation mostly pertains to the DAergic tuberoinfundibular hypothalamic pathway and not the mesolimbic and striatonigral DA pathways that target the ventral and the dorsal striatum respectively. Since these pathways have been implicated in reward (and prediction of reward) and motivation; an alternative suggestion is that deficiency in these pathways may lead to pathological eating as a means to compensate for low motivation, reward or saliency. Thus, one of the limitations to interpreting the present finding is that we don't have microdialysis DA data from the striatum of fasted and non-fasted Ob and Le Zucker rats. Future studies will provide us with these answers to whether Ob R rats had higher striatal DA levels than Ob U rats.

<u>DA differences due to 24h fasting for all ad-libitum fed animals (Acute food restriction in chronically ad-lib animals)</u>

In this study, we provide evidence of DA displacement (altered in-vivo D2R binding availability) due to feeding. In order to control for changes in D2R binding availability attributed to differences in feeding status, the animals were all fasted prior to being scanned.

The reason we chose a 24 hour fasting period, is because restricted rats were fed once every 24 hours and usually consumed all of the food within an hour of being fed. In this way, it is as if they were consistently fasted for a period of approximately 24 hours. Fasting both the restricted and ad-libitum fed rats for this amount of time provides a basis for the direct comparison of changes in D2R binding availability in the four experimental groups during a fasting period (much the same way that glucose sensitivity is tested in diabetics). That is, it shows how D2R binding availability is affected in each group, under normalized conditions, and after food restriction or no food restriction. The caveat here is that the restricted rats are conditioned to the food restriction while the ad-libitum fed rats are not. This raises the question of how D2R binding availability of a rodent who is on a chronically restricted feeding regiment differs from that of a rodent who is not when both are in a state of negative energy balance that is brought upon by reduced food intake. Fasting has been shown to increase DA release in the striatum in ad-lib fed Wistar Rats (Miklya et al., 2003). Furthermore, 24h fasting has been shown to decrease the DAT mRNA levels (Patterson et al., 1998). DAT is known to regulate the DA signal and in DAT KO mice DA signaling is chronically increased (Gainetdinov et al., 1999). Both of these findings support the notion that fasting increases DA. Our data supported this as well since ad-lib 24h-fasted groups had slightly lower [^{11}C]raclopride binding potential in striatum than the non-fasted (Figure 3.1.7c). Obese Zucker rats are hyperinsulinemic and

have high insulin levels (both in Ob Zucker rats and rats receiving insulin intracerebroventricularly (ICV)). Insulin has been associated with increased DAT mRNA (Figlewicz et al., 1998) as well as with increased sensitivity of DAT for DA uptake (Owens et al., 2005). So, the high DAT mRNA and the more efficient DA uptake by DAT associated with hyperinsulinemia in Ob U Zucker rats could translate to lower DA signaling. Indeed decreases in both striatal DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels have been reported in Ob Zucker rats (Shimizu et al., 1991). This would explain the higher D2R binding potential that we observed in the unrestricted (Ob U and Le U) than in the fasted condition.

In conclusion, these findings may help shed light on how the DA system is functioning during fasted states in animals that are on restricted feeding and animals that are not. In a clinical setting this may translate to an examination of the effects of dieting in obese and lean individuals and the respective DA system responses.

DA differences between chronic food restriction in Ob and Le Zucker rats and acute (24h fasting) food restriction

"Subchronic" food restriction (1 week) has been shown to lead to a decrease in extracellular DA in the NAc (Pothos et al., 1995a; Pothos et al., 1995b). However, more "chronic" food restriction (4 weeks) lead to 32% lower DAT binding levels in the striatum (Zhen et al., 2006) which would translate to an increase in DA signaling. In our model of chronic (4 months) food restriction, we found significantly greater D2R binding potential in Le R compared to Le U rats. While we saw the same effect in D2R in the ARG data (lower D2R in Ob U versus Ob R), we failed to see such an association (with μPET) between Ob U and Ob R rats. This may be due to a higher DA signal in the Ob R than the Le rats so that it would have a greater effect on the competition with [^{11}C]raclopride for D2R binding, which could account for the lack of difference in μPET data between Ob U and Ob R even though μPET was able to detect the Le R > Le U seen with the ARG. Moreover, even though the Ob Zucker rats are characterized by very high insulin levels (Bray, 1977; Beck et al., 2004), we showed that Ob R rats have lower insulin and leptin levels due to the chronic food restriction (~30%) which would also serve as potential causes for their greater DA signal.

Previous research has shown that chronic food deprivation and diet-induced obesity, two conditions that are characterized by differences in feeding, have both been associated with decreases in extracellular DA levels in the ventral striatum (Pothos et al., 1998a). Furthermore, in both food-deprived and obese rats, amphetamine-induced DA release is higher than in controls (Pothos et al., 1998a). Similarly, obese and lean female ad-libitum fed Zucker rats did not differ in striatal DA metabolites at 5 weeks of age but by 16 weeks the obese had significantly lower DA metabolites in the striatum compared to lean rats (Orosco et al., 1986). Even though this experiment was conducted in female rats, the ages are almost identical to those examined in our experiment (4 and 16 weeks) and therefore may provide a closely related depiction of what we see in our results. The above studies point to a low basal release of DA in chronically food restricted and obese rats and suggest that the low basal DA levels may be due in part to increased storage of DA in synaptic vesicles. These results are further supported by findings of elevated basal DAT mRNA levels in the VTA (Figlewicz et al., 1998) and striatum (Mortensen &

Amara, 2003) in obese Zucker rats compared to lean controls, which suggest that these rats exhibit increased DA clearance and hence, decreased DA signaling. Therefore, elevated DAT in obese rats could serve as a compensatory mechanism to decrease excess synaptic DA due to feeding and in turn would reduce D2 receptor stimulation (Giros et al., 1996), eventually contributing to the decreased D2R levels we observe. Contrary to obesity, there is evidence to support that chronic food restriction does not lead to DAT elevations (Zhen et al., 2006). In this case, food-restricted rats may show the same low basal DA release and increased DA storage as obese rats, as well as the increased DA release from synaptic vesicles in response to feeding but DA clearance would not be facilitated. As opposed to obese rats, DA clearance and possibly D2R stimulation would not be compromised in food-restricted rats. This could potentially account for the D2R elevation we observe in food-restricted rats. The literature points to DA as a reward/satiety signal and it has been theorized that the larger the net change of DA release, the larger the feelings of reward that are associated with the perceived stimulus. It is hypothesized that obese rats have lower DA levels compared to lean rats and therefore the change in DA signal brought upon by the presence of food or feeding in these animals may elicit a larger increase (net change) in the DA signal than in controls, thereby increasing the reward attributed to food and would in turn increase food seeking and/or consumption.

Leptin and Dopamine

Our study further supports the above findings, since the significant attenuation of D2R loss as a response to food restriction is greater in the Ob R than in the Le R group. Specifically, Ob R rats showed that the attenuation of D2R loss by food restriction was 40 % greater than for the Le R group. Furthermore, food restriction led to a significant decrease in plasma leptin concentrations in Ob R compared to Ob U rats, thereby suggesting that leptin inhibits the response of the DA system to chronic food restriction. Under normal circumstances, chronic food deprivation is associated with decreased circulating leptin and obesity is associated with leptin resistance. In both cases leptin signaling is decreased. Findings showing that ob/ob have lower DA levels in the ventral striatum compared to controls (Fulton et al., 2006) points to an association between decreases in leptin-signaling and decreases in DA release. Our results in leptin receptor-deficient Zucker rats which like the ob/ob are characterized by impaired leptin signaling support this.

Contrary to this however, an increase in leptin signaling is also associated with decreases in DA release. Specifically, leptin receptors have been localized on VTA DA neurons (Figlewicz et al., 2003) and peripheral or central leptin administration inhibits VTA DA neuron firing (Figlewicz et al., 2003; Hommel et al., 2006). This finding is in agreement with evidence that leptin injections into the lateral ventricle were shown to decrease basal and feeding-induced DA release in the ventral striatum (Krugel et al., 2003). It is not clear why normal leptin signaling as well as impairments in leptin signaling have been associated with the same decreases in DA. Perhaps the decreases in the DA signal observed in the above studies have to do with secondary or compensatory effects of leptin inhibition. In relation to our findings, the above evidence suggests that leptin-receptor deficient Zucker rats would be resistant to the inhibitory effects of leptin

on VTA DA neuron firing. This does not necessarily mean that obese Zucker rats would show increases in DA release in areas downstream of the VTA like the ventral striatum. Alternatively, this scenario correlates with findings of increases in vesicular DA observed in obese rodents (Pothos et al., 1998a). This would in turn explain the lower D2R levels we observe in the obese Zucker rats.

Here we show behavioral and imaging data that support the notion of lower D2R levels in the CNS of Ob than in Le rats and in food unrestricted than food restricted conditions indicating that both genotype as well as food availability affect D2R levels. We also show attenuation by food restriction of the D2R decline with aging that was most accentuated in the Ob than in the Le rats. This finding provides evidence for a role of leptin in modulating adaptation responses of the DA system with respect to food restriction. Finally we provide indirect evidence that fasting enhances DA extracellular levels which we demonstrate as a relative decrease in [^{11}C]raclopride binding to D2R (more competition from the increase in endogenous DA). Inasmuch as food restriction attenuates the D2R losses associated with aging this may underlie the salutary effects that food restriction has on locomotor activity and on sensitivity to reward in aged animals.

Other factors acting both peripherally and centrally may be involved in the feeding profile of obese Zucker rats. It would be logical to assume that the leptin receptor mutation in these rats may be affecting other peripheral or central signals that are independent of leptin's actions. In our experiment we observe that the obese restricted rats still become obese, even after they were fed 70%. These rats weigh less than Ob U rats but are still obese compared to lean ad-libitum fed controls. In this respect, the changes in D2R we observe may constitute secondary compensatory effects brought upon by differences in other metabolic signals. This may be further supported by the finding that food restriction affects D2R in both obese and lean rats, thus pointing to DA changes independent of obesity.

3.2 Effects of diet on D2R binding in rats susceptible or resistant to diet-induced obesity

3.2.1 Introduction

Obesity is a complex metabolic state that is associated with increased adiposity, type 2 diabetes mellitus, hyperlipidemia, coronary artery disease, arthritis, gallstones, cancer and psychosocial disability. Obesity is currently the second leading cause of preventable death (after smoking) in the United States (Mokdad et al., 2004) and is predominantly brought on by increased food intake, poor dietary habits and decreased physical activity and is modulated by both genetic and/or environmental factors (Mutch & Clement, 2006). Given the soaring percentage of Americans classified as overweight or obese (Mokdad et al., 2000), identifying neurobiological factors associated with behaviors that results in obesity is of paramount importance.

DA is a neurotransmitter that has been implicated in the motivational and hedonic aspects of eating (Berthoud & Morrison, 2008). Recent evidence suggests that obesity is

associated with a hypofunctioning central DA system (Wang et al., 2001; Geiger et al., 2008; Thanos et al., 2008; Davis et al., 2009). Some of these studies have reported low D2R availability in obese humans (Wang et al., 2001) and rodents (Hamdi et al., 1992; Huang et al., 2006; Hajnal et al., 2008; Thanos et al., 2008; Davis et al., 2009) in brain structures that are part of the mesocorticolimbic DA system. A more recent study reported that administration of bromocriptine, a D2R agonist, was associated with increased D2R binding and decreased obesity-related symptoms (Davis et al., 2009). Similar to obese individuals, alcoholics and drug-addicts also show low D2R availability and in rodents low striatal D2R availability is associated with impulsivity (Dalley et al., 2007), which in turn is linked with compulsive behaviors (Belin et al., 2008). Based on this interpretation, lower levels of D2R in striatum may serve as a potential risk-factor for dietary obesity and eating disorders (Wang et al., 2001), but whether inherently low D2R levels lead to compulsive food intake and obesity remains unclear. Accordingly, since feeding increases DA levels (Bassareo & Di Chiara, 1999; Gambarana et al., 2003), constantly elevated DA levels may lead to compensatory decreases in D2R, however, whether obesity or compulsive food intake leads to decreased D2R still remains unclear.

When placed on a high-fat diet, Osborne-Mendel (OM) rats consumed the most calories and gained the most weight out of six other rat strains including Sprague-Dawley, Hoppert, Wistar-Lewis, Hooded, Gray and S5B/PL (S5B) rats (Schemmel et al., 1970). In the same study, S5B/PL rats consumed the least amount of calories and gained the least amount of weight (Schemmel et al., 1970). When given a three macronutrient taste-preference test, OM rats consumed more calories from a high-fat diet than a low-fat/high-carbohydrate or low-fat/high-protein diet (Nagase et al., 1996; Schaffhauser et al., 2002). When given the same taste-preference test, S5B rats consumed more calories from a low-fat/high-carbohydrate diet (Nagase et al., 1996; Schaffhauser et al., 2002). In fact, S5B rats decrease the amount of food they consume in order to receive the same caloric amount as if they were consuming a regular chow diet and thus experience only a slight increase in body weight when placed on a high-fat diet (Bray et al., 1987; Fisler et al., 1993).

The aim of this study was to characterize striatal D2R levels in two rat strains that differ in their propensity to gain-weight and consume a high-fat diet (OM and S5B/PL rats) in three different environmental conditions: regular (low-fat) chow diet, high-fat diet and food restriction. We hypothesized that striatal D2R levels would be lower in OM rats than in S5B/PL rats before access to a high-fat diet and that both the high-fat diet and food restriction would differentially affect D2R levels in the two strains.

3.2.2 Methods

Animals

Male 2 month old Osborne-Mendel (OM; N=12; and S5B/PL (S5B; N=12) rats were used. Rats from each strain were divided into 3 groups of 4 rats in each group. Specifically OM and S5B rats were put in the following groups for 8 weeks: i) unrestricted (AL) food access, ii) restricted (R) food access (70 % of AL fed animals) and iii) high-fat (HF) food access. Rats were bred at Pennington Biomedical Research Laboratory and housed on a reverse 12 hour light/dark cycle with lights off at 7 am. All

experiments were conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (NAS & NRC, 1996) and Pennington Biomedical Research Laboratory Institutional Animal Care and Use Committee protocols.

Behavior Assessment – Food Intake and Weight

AL and R rats were fed a standard (Purina) low fat (LF) rat chow. HF rats were fed a diet of chow consisting of 30% fat. Food intake and body weight was monitored three times a week at 0900h. Rats were fed daily at 0900h and the amount of food given was continuously adjusted to 70% of food intake consumed by AL rats.

Plasma Insulin and Leptin

Using retro-orbital punctures, approximately 100 µl of blood was collected in glass capillary tubes. Blood samples were kept on ice until they were centrifuged at 3,000 rpm for 10 minutes; plasma was collected and immediately stored at -80C. Plasma insulin and leptin concentrations were measured in duplicate with commercially available insulin and leptin EIA kits obtained from Linco Research (Linco Research, Missouri, USA).

In-vitro [^{3}H]spiperone ARG

Each animal was deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The brain was rapidly removed and frozen in an isopentane and dry ice bath and stored in a -80^0C freezer. The brain was then transferred to a cryostat (Leica CM3050S), and sections were cut 14µm thick at -18^0C. Sections were mounted on Fisherbrand, Colorfrost */Plus microscope slides and stored at -80^0C until binding was conducted. Slides were gradually brought back to room temperature and then pre-incubated at room temperature for ten (10) minutes in 50 mM Tris-HCl buffer (pH 7.4). The slides were then incubated in specific binding buffer (50 mM Tris-HCl, 0.4 nM [^{3}H]Spiperone (Perkin Elmer), 10 µM ketanserin tartrate (Tocris Bioscience) at room temperature for one (1) hour. To determine non-specific binding some slides in parallel were incubated for one (1) hour in specific binding buffer in the presence of 10 µM haloperidol (Tocris Bioscience). Next, the slides were washed 2 x 10 minutes in ice-cold 50 mM Tris-HCl and ice-cold dH_2O. Afterwards, one (1) ml aliquots of incubation solution were taken and added with five (5) ml of Ultima Gold XR (scintillation solution) and the amount of radioactivity was measured with a liquid scintillation counter. Slides were then dried overnight at room temperature in a dessicator and placed in a glass slide cassette for qualitative and quantitative analysis using a β-Imager 2000 (Biospace, Paris, France). Using Betavision+ software (Biospace, Paris, France), Region of Interests (ROI's) were drawn on the left and right caudate putamen (CPu) and NAc and of each brain slice. The data was then calibrated using a tissue homogenate standard. Mean values (µCi/g) of the left and right receptor rich regions (CPu, NAc) for each group are reported.

3.2.3 Results

Food Intake

A 2-way repeated measures ANOVA showed significant main effects for the factors Group ($F=57.18$;$dF=5,191$; $p<.001$), Time ($F=3.80$;$dF=7,191$; $p<.001$) and their interaction Group x Time ($F=2.85$;$dF=35,191$; $p<.001$). Pair-wise comparisons (Holm-Sidak: $p<.05$) (Figure 3.2.1a) showed that at the end of the study period OM-HF rats had significantly greater food intake compared to S5B-R ($t=7.69$), OM-R ($t=6.36$) and S5B-HF ($t=3.26$). OM-AL rats ate significantly more than S5B-R ($t=8.24$), OM-R ($t=6.91$) and S5B-HF ($t=3.81$) rats. Also S5B-AL rats ate significantly more compared to S5B-R ($t=7.84$), OM-R ($t=6.51$) and S5B-HF ($t=3.41$) rats. Finally, S5B-HF rats ate significantly more than S5B-R rats ($t=4.43$) and OM-R ($t=3.10$) rats.

Caloric Intake

A 2-way repeated measures ANOVA showed significant main effects for the factors Group ($F=79.94$;$dF=5,191$; $p<.001$), Time ($F=3.65$;$dF=7,191$; $p=.001$) and their interaction Group x Time ($F=2.63$;$dF=35,191$; $p<.001$). Pair-wise comparisons (Holm-Sidak: $p<.05$) (Figure 3.2.1b) showed that at the end of the treatment period OM-HF rats had greater caloric intake compared to S5B-R ($t=12.36$), OM-R ($t=11.27$), OM-AL ($t=5.59$) and S5B-HF ($t=5.91$) rats. OM-AL rats ate significantly more calories than S5B-R ($t=6.78$) and OM-R ($t=5.69$) rats. Also S5B-AL rats ate significantly more calories compared to S5B-R ($t=6.45$) and OM-R ($t=5.36$) rats. Finally, S5B-HF rats ate significantly more calories than S5B-R rats ($t=8.87$) and OM-R rats ($t=7.77$), and significantly less calories than OM-HF ($t=3.49$) rats.

Body weight

A 2-way repeated measures ANOVA showed significant main effects for the factors Group ($F=58.14$;$dF=5,191$; $p<.001$), Time ($F=1222.19$;$dF=7,191$; $p<.001$) and their interaction Group x Time ($F=31.34$;$dF=35,191$; $p<.001$). Pair-wise comparisons (Holm-Sidak: $p<.05$) (Figure 3.2.2) showed that at the end of the study period OM-HF rats had significantly greater body weight compared to OM-AL ($t=2.36$), S5B-AL ($t=9.29$), S5B-R ($t=17.59$), OM-R ($t=10.79$) and S5B-HF ($t=10.27$). OM-AL rats weighed significantly more than S5B-R ($t=15.23$), OM-R ($t=8.44$), S5B-HF ($t=7.92$) and S5B-AL ($t=6.93$) rats. Also S5B-AL rats weighed significantly more compared to S5B-R rats ($t=8.3$), S5B-HF weighed significantly more than S5B-R rats ($t=7.31$) and OM-R rats weighed significantly more than S5B-R rats ($t=6.79$).

Leptin

A 2-way ANOVA showed significant main effects for the factors Strain ($F=24.29$;$dF=1,71$; $p<.001$), Diet ($F=15.42$;$dF=2,71$; $p<.001$) and their interaction Strain x Diet ($F=17.97$;$dF=2,71$; $p<.001$). Pair-wise comparisons (Holm-Sidak: $p<.05$) (Figure 3.2.3) showed that OM-HF rats had significantly greater leptin levels compared to OM-R ($t=7.99$), OM-AL ($t=3.84$) and S5B-HF rats ($t=6.44$). Also OM-AL rats had significantly greater leptin levels than OM-R rats ($t=4.15$) and S5B-AL rats ($t=3.93$).

Insulin

A 2-way ANOVA showed significant main effects for the factors Strain ($F=34.72;dF=1,71$; $p<.001$), Diet ($F=27.04;dF=2,71$; $p<.001$) but not their interaction Strain x Diet ($F=5.40;dF=2,71$; $p=.007$). Pair-wise comparisons (Holm-Sidak: $p<.05$) (Figure 3.2.3) showed that S5B-HF rats had significantly greater insulin levels than S5B-R rats ($t=6.69$) and S5B-AL ($t=5.89$) rats. OM-HF rats had significantly greater insulin levels compared to OM-R ($t=3.38$) rats. OM-AL rats had significantly greater insulin levels than S5B-AL ($t=5.29$) rats. Finally, OM-R rats had significantly greater insulin levels than S5B-R ($t=4.11$) rats.

D2R ARG

A 2-way ANOVA showed significant main effects for the factors Group ($F=317.01;dF=5,1861$; $p<.001$), Brain Region ($F=202.45;dF=6,1861$; $p<.001$) and their interaction Group x Brain Region ($F=8.58;dF=30,1861$; $p<.001$).

Differences between groups

Pair-wise comparisons (Holm-Sidak: $p<.05$) between groups were performed (Figure 3.2.4). CPu: S5B-AL rats had greater D2R binding compared to S5B-R ($t=6.58$), S5B-HF ($t=10.79$), OM-AL ($t=1.972$), OM-R ($t=4.87$) and OM-HF ($t=13.53$) rats. S5B-R rats had greater D2R binding compared to S5B-HF ($t=3.39$) and OM-HF ($t=6.57$) rats. Also S5B-HF rats had greater D2R binding compared to OM-HF rats ($t=3.52$). OM-AL rats had greater D2R binding compared to OM-R ($t=2.83$), OM-HF ($t=11.84$) and S5B-HF ($t=8.92$) rats. OM-R rats had greater D2R binding compared to OM-HF ($t=9.59$) and S5B-HF ($t=6.42$) rats. NAc: S5B-AL rats had greater D2R binding compared to S5B-R ($t=9.37$), S5B-HF ($t=14.28$), OM-AL ($t=6.90$), OM-R ($t=12.47$) and OM-HF ($t=17.33$) rats. S5B-R rats had greater D2R binding compared to S5B-HF rats ($t=3.92$) and OM-HF rats ($t=7.68$). OM-AL rats had greater D2R binding compared to OM-R ($t=5.51$), OM-HF ($t=12.11$), S5B-R ($t=3.77$) and S5B-HF ($t=8.41$) rats. OM-R rats had greater D2R binding compared to OM-HF ($t=7.88$) and S5B-HF rats ($t=3.70$). Finally, S5B-HF had significantly greater D2R binding compared to OM-HF rats ($t=4.16$).

Differences within groups

S5B-AL rats showed greater D2R binding in the NAc compared CPu ($t=9.46$). S5B-R rats showed greater D2R binding in the NAc compared to CPu ($t=3.14$). S5B-HF rats showed no significant differences between the two brain regions.

OM-AL rats showed greater D2R binding in the NAc compared to CPu ($t=4.96$). OM-R rats showed greater D2R binding in the NAc compared CPu ($t=3.73$). OM-HF rats showed no significant differences between any of the two brain regions.

Figure 3.2.1a

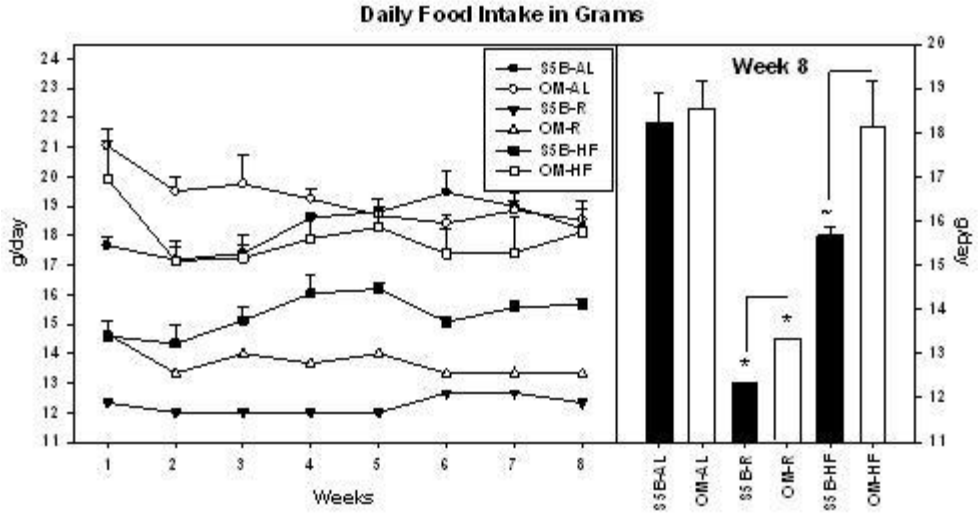

Figure 3.2.1b

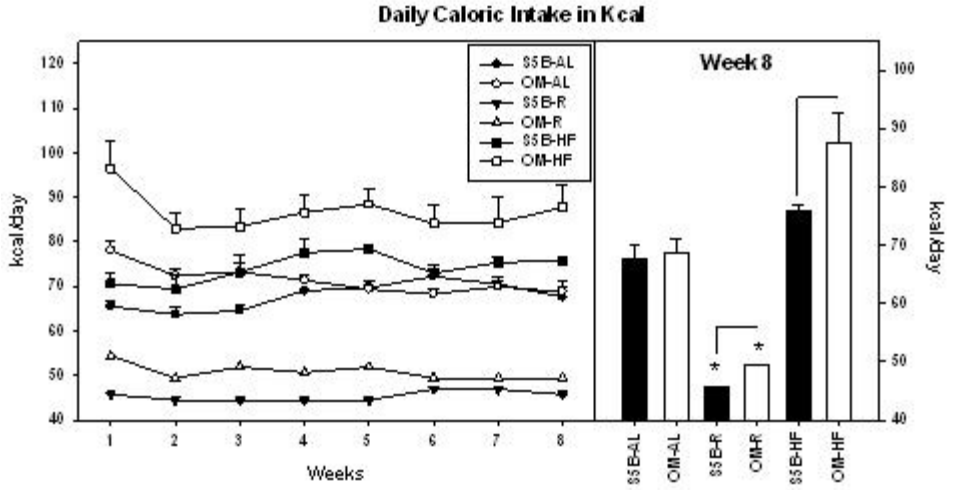

Figure 3.2.1. (a) Mean (+SEM) daily food intake in OM and S5B/PL ad-libitum, restricted and high-fat diet fed rats. (b) Mean (+SEM) daily caloric intake in OM and S5B/PL ad-libitum, restricted and high-fat diet fed rats.

Figure 3.2.2

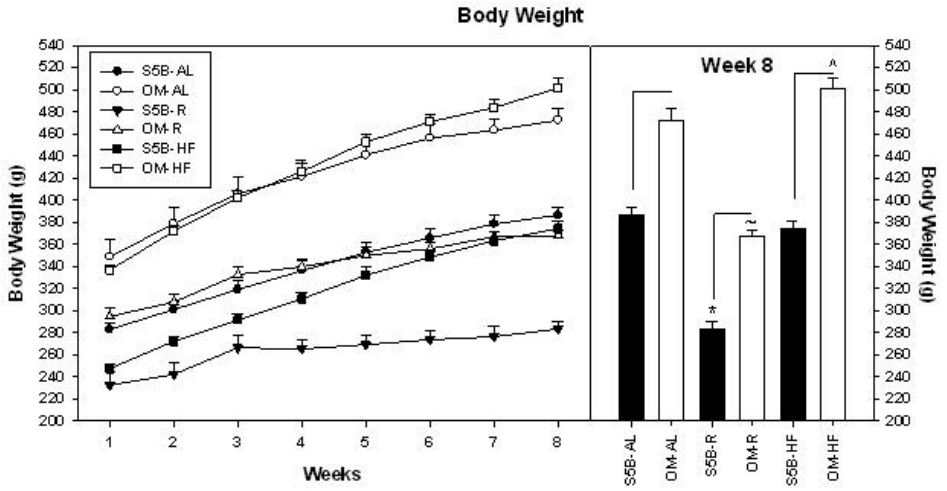

Figure 3.2.2. Mean (+SEM) daily body weight in OM and S5B/PL ad-libitum, restricted and high-fat diet fed rats.

Figure 3.2.3

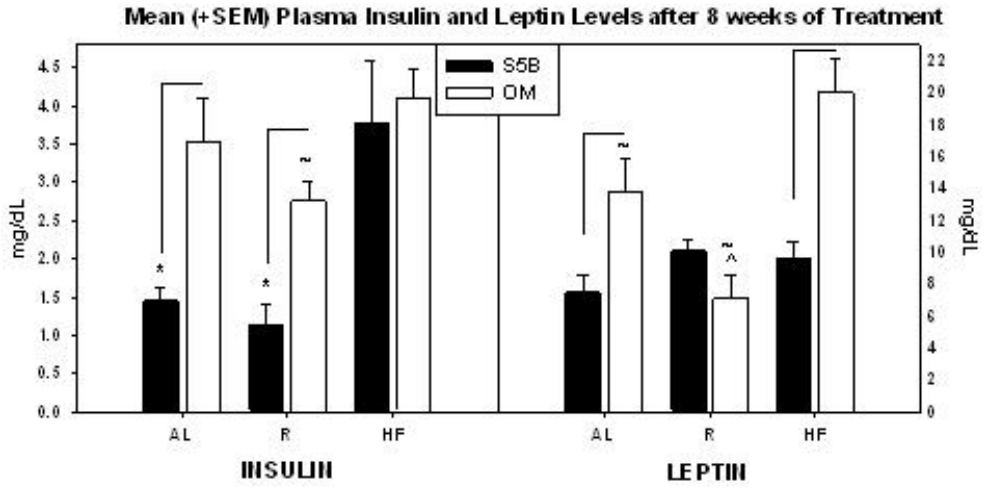

Figure 3.2.3. Mean (+SEM) insulin and leptin plasma concentrations in OM and S5B/PL ad-libitum, restricted and high-fat diet fed rats.

Figure 3.2.4

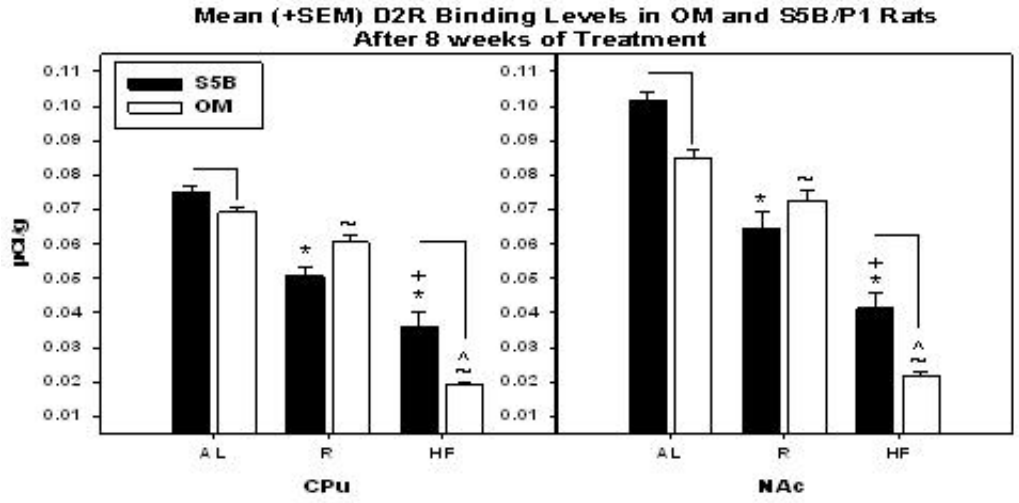

Figure 3.2.4. Mean (+SEM) D2R binding in OM and S5B/PL ad-libitum, restricted and high-fat diet fed rats.

3.2.4 Discussion

Food Intake and Body Weight

When exposed to a low-fat diet, OM and S5B/PL rats showed no difference in the amount of food consumed at 8 weeks even though OM rats weighed approximately 23% more than S5B/PL rats. When fed a high-fat diet OM rats consumed approximately 16% more food, 16% more calories and weighed 34% more than S5B/PL rats. S5B/PL rats fed a high-fat diet also showed about 16% greater food and 12% more calorie intake than similarly aged S5B/PL rats placed on a low-fat diet. On the other hand, calories and the amount of low-fat and high-fat food consumed by OM rats was approximately the same. Food restriction led to a significant decrease in body weight in both OM and S5B/PL rats (approximately 29% and 36% from low-fat diet fed rats respectively). At 8 weeks, food restricted OM rats had similar body weight levels as low-fat and high-fat diet fed S5B/PL rats.

The increased caloric intake in OM-HF compared to OM-AL rats and the fact that low-fat diet fed OM rats weighed 23% more than S5B/PL rats, while both groups consumed the same amount of food and calories, suggests that OM rats are characterized by both a susceptibility to consume high-fat food as well as metabolic disturbances that lead to a predisposition in gaining weight outside the context of caloric intake. On the other hand, the decreased food intake of high-fat diet exposed S5B/PL rats suggests that they are selectively resistant to consuming a high-fat diet.

OM rats have abnormal insulin levels that don't normalize with food restriction whereas they also have high levels of leptin but these high leptin levels decrease when rats are placed on the restricted diet. The high insulin levels observed in the OM rats for all conditions or the S5B for the fat diet are consistent with insensitivity to insulin. In the case of the OM rats the fact that they don't decrease insulin levels even with food

restriction suggests a fundamental deficit in insulin regulation. Indeed early onset of insulin resistance in OM rats has been previously suggested (Petrescu et al., 2008). The response to leptin in the OM rats is also unique in that whereas food restriction results in leptin increases in the case of the OM rats it led to a decrease in concentration.

Low D2R binding after exposure to low-fat food is associated with increased body weight and elevated circulating leptin but not feeding

The susceptibility to weight gain in OM-AL rats was associated with the lower D2R binding as compared to S5B-AL rats in the CPu and NAc. This finding agrees with previous studies reporting associations between reduced striatal D2R binding and increased body weight in both humans (Wang et al., 2001) and rodents (Hamdi et al., 1992; Huang et al., 2006; Hajnal et al., 2008; Thanos et al., 2008; Davis et al., 2009). However the small sample sizes (4 per cell) precludes us to make a generalization of these findings and require confirmation in a larger sample. Nonetheless the fact that for both strains the high fat diet was associated with lower D2 receptors suggest that metabolic responses triggered by high fat consumption such as insulin insensitivity of high leptin levels may contribute to the D2R downregulation.

Since the insulin levels don't differ from the OM in restricted versus high fat diet whereas high fat food does. One could speculate that the downregulation of D2R with fat diets is in part mediated by high leptin levels (note that leptin is high despite heavy weight which is likely to reflect insensitivity). Note also that the high leptin levels could also explain the lower D2R in food restriction for the S5B, though it wouldn't explain the lower D2R in food restriction for the S5B. Though it wouldn't explain the lower D2R in OM with food restriction since leptin values are lower than with all. Thus based on these findings the association between leptin and D2R needs further evaluation.

Indeed previous studies have reported that OM rats are less sensitive to leptin signaling (Ishihara et al., 2004) and show reduced leptin receptor (LepR) mRNA levels in the hypothalamus (Madiehe et al., 2000). The potential role of leptin in D2R regulation is consistent with our findings and that of others showing reduced striatal D2R binding and DA signaling in hyperinsulinemic and hyperleptinemic LepR deficient (fa/fa) rats (Hamdi et al., 1992; Thanos et al., 2008). A regulatory role for peripheral leptin is further supported by studies demonstrating reduced extracellular NAc DA levels after leptin infusion (ICV) (Krugel et al., 2003), reduced firing of DA neurons in response to intravenous leptin (Hommel et al., 2006) and decreases in D2R binding in response to peripherally administered leptin in mice (Pfaffly et al., 2010). LepR are co-expressed on DA neurons of the VTA (Figlewicz et al., 2003; Fulton et al., 2006; Hommel et al., 2006) and these receptors are behaviorally relevant to feeding (Hommel et al., 2006). Like leptin, insulin has also been reported to play a modulatory role on striatal DA. Insulin receptors are also co-expressed on VTA DA neurons (Figlewicz et al., 2003). Also, hypoinsulinemic rats are characterized by decreased DAT cell-surface expression (Williams et al., 2007) and reduced amphetamine-induced striatal DA release (Sevak et al., 2007), while insulin infusion (ICV) increased DAT mRNA and functional activity (Figlewicz et al., 2003).

Previous studies have also documented involvement of other neurotransmitters in the susceptibility to weight gain in OM rats such as increases in μ-opioid receptors

(Barnes et al., 2006), NPY, and corticotropin releasing hormone (CRH) mRNA (Schaffhauser et al., 2002) in the hypothalamus. Lower levels of extracellular norepinephrine and serotonin in the hypothalamus have also been reported in OM rats (Shimizu et al., 1994).

Within the above context, our data suggest that the OM rat is an example of how low striatal D2R levels (including NAc) can serve as risk factors for predisposition towards weight gain that is independent of caloric intake. In support of this view, a recent study reported that postnatally derived (P0-P1) VTA neurons from inbred obesity-prone rats showed reduced DA exocytosis (decreased DA quantal size) (Geiger et al., 2008) suggesting that the reduced D2R binding we observe in OM-AL rats may be a consequence of impaired presynaptic DA signaling that originates from the VTA, possibly due to elevated circulating leptin levels and thus inhibitory effects of leptin on midbrain DA neurons. D2R regulate and are regulated by DA signaling and therefore our results, taken together with the above findings suggest that OM rats may also be characterized by reduced activity of mesolimbic DA. Since, acute feeding increases DA release and turnover in mesolimbic DA areas (Hernandez & Hoebel, 1988; 1990; Yoshida et al., 1992), one can hypothesize that such disturbances may lead to a predisposition towards hyperphagia as a compensatory mechanism to increase DA levels and reach satiety. This view is supported by findings demonstrating that 15-week old inbred obesity-prone rats showed significantly reduced basal NAc DA levels and reduced evoked DA release in mesolimbic DA regions, and these findings were coupled with increased low-fat food intake (~14%) compared to obesity-resistant rats (Geiger et al., 2008). Our findings in 16 week old rats do not support this view since we found reduced D2R binding in OM rats that was not associated with respective differences in food or caloric intake. Our data in low-fat diet fed rats support the notion that leptin and insulin are involved in regulating D2R and that this regulation happens outside the context of feeding.

Reductions in D2R binding after exposure to high-fat food are associated with elevated circulating leptin: potential effects of palatability

A previous study showed reduced striatal D2R binding in obesity-prone mice after exposure to a high-fat diet (40% fat) for 6 weeks (Huang et al., 2006). Another study showed that mice exposed for twenty days of high-fat diet consumption significantly increased D2R and decreased DAT density in the dorsal and ventral parts of the caudal caudate putamen (D2R: 32% and 35% respectively, DAT: 33.3% and 28.8% respectively) compared with low-fat diet (South & Huang, 2008). In addition, in this study it was reported that high-fat feeding also increased D2 binding in the NAc shell (36%) and that D2R and DAT density remained unchanged following reversal of the diets from high-fat to low-fat diet (South and Huang, 2008). It is unclear whether this change in D2R levels was accompanied by elevated or decreased leptin or insulin levels since these were not reported. At 8 weeks of treatment, S5B-HF rats showed lower D2R binding (60% decrease in NAc), and consumed less food (16%) but more calories (12%) than S5B-AL rats. OM-HF rats showed lower D2R binding (74% decrease in NAc), an insignificant difference in food intake (~3% greater), but greater caloric intake (28%) than OM-AL rats. The fact that exposure to high-fat food decreased D2R binding for both

OM and S5B/PL rats and that these differences were paralleled by increases in calories of fat (not grams of food) consumed suggests that the decreases in D2R may have been mediated by the type of calories consumed, taste and/or changes in leptin and insulin brought upon by exposure to high-fat food. Although OM-HF and S5B-HF rats had 45% and 29% greater leptin and 14% and 160% greater insulin levels than OM-AL and S5B-AL rats respectively, the increases in leptin in S5B-HF rats and insulin in OM-HF rats were not significant. The fact that we observed a more pronounced decrease in D2R binding in OM-HF compared to S5B-HF rats and from low-fat diet fed rats suggests that OM rats may be more susceptible to decreases in D2R binding brought upon by exposure to a high-fat diet. It is unclear whether this susceptibility is guided by the greater caloric intake, type of calories, palatability of the high-fat diet or further elevated leptin and insulin levels. Insulin is unlikely since OM-HF and S5B-HF rats showed similar levels of circulating insulin. Leptin is more likely since the difference in circulating leptin between OM and S5B rats increased after a high-fat diet. Along with leptin, high-fat food intake was also different between OM-HF and S5B-HF rats and this is another factor that may be associated with greater reduction of D2R in OM-HF rats. However, increased high-fat food intake is expected to lead to elevated leptin circulating levels and since leptin signals fat stores from the periphery to the brain, it is likely that effects of high-fat food on D2R are mediated in part by leptin. Finally palatability may be a factor affecting D2R differently in the two strains. In support of palatability as a potential modulator of D2R, OM and S5B/PL rats have been shown to differ in sensitivity to taste responses towards fat (Gilbertson et al., 2005) and as described earlier, OM rats readily prefer a high-fat diet over a low-fat/high-carbohydrate and low-fat/high-protein diet while S5B/PL rats do not (Nagase et al., 1996; Schaffhauser et al., 2002). Furthermore, a recent study showed that sham-feeding of fat (100% corn oil) increases NAc DA levels in rats (Liang et al., 2006). It is therefore likely that constant increases in DA levels in response to fat taste may lead to the downregulation in D2R we observed. Such DA increases have been interpreted as being indicative of associating the palatability of food with its post-ingestive consequences (Fenu et al., 2001; Di Chiara & Bassareo, 2007) and therefore interactions between the gut and the brain. More specifically the relationship of taste, energy-related messengers like leptin, and mesocorticolimbic DA may be instrumental in the development of eating patterns and habits, and therefore disturbances in these factors may lead to eating disorders and obesity.

Food restriction normalizes D2R differences in OM and S5B/OL rats and these changes are associated with decreases in circulating leptin

Eight weeks of food restriction decreased D2R binding in the CPu and NAc compared to ad-libitum feeding in both OM and S5B/PL rats. Previous studies have reported reduced basal NAc DA levels in food restricted rats characterized by a 20% reduction in weight (Pothos et al., 1995a; Pothos et al., 1995b) which was similar to the weight reduction seen in our study (OM-R and S5B-R rats showed a 29% and 36% reduction in body weight respectively). However this differs from our prior finding in zucker rats in which we showed that food restriction led to a decrease in D2R downregualtion. The differences between both findings are unclear and merit further evaluation. Although food restriction decreased D2R in both OM-R and S5B-R rats, the

decrease was more pronounced in S5B-R rats. This finding was expected since these rats are less susceptible to the effects of starvation than their obesity-prone counterparts (Sakaguchi et al., 1988). Unlike OM-AL and S5B-AL rats that differed in D2R binding, we found no differences in D2R binding between OM-R and S5B-R rats. Since OM-R and S5B-R rats were exposed to the same, albeit less, low-fat diet as OM-AL and S5B-AL rats, and we did not find an association between food intake and D2R binding in AL rats, the 8% greater food intake in OM-R compared to S5B-R rats is unlikely to be the cause for their D2R profiles. Our results suggest the more likely cause for the D2R binding levels we report in OM-R and S5B-R rats is leptin. Specifically, food restriction decreased leptin levels in OM-R rats to comparable levels of S5B-R rats. Therefore, lower circulating leptin levels in OM-R, or increased efficiency of leptin signaling may account for the respective changes in D2R we observed.

Our findings suggest that-low striatal D2R levels in OM and S5B rats potentially reflected elevated circulating leptin levels. Inasmuch as OM rats had higher leptin and insulin levels the latter of which did not normalize with food restriction suggest that these may serve as risk factor towards weight gain irrespective of feeding. Conversely, higher striatal D2R levels in S5B/PL rats may play a protective role against excessive feeding, weight-gain and high-fat food consumption. This resistance in S5B/PL rats to consume the high-fat diet was also coupled with lower circulating leptin levels, which suggests a role for peripheral leptin in striatal D2R regulation. Food restriction, which decreased circulating leptin in OM rats to those comparable of S5B/PL rats, also reversed the difference in D2R binding between the two strains. High-fat fed OM rats showed significantly lower D2R binding and higher circulating leptin concentrations compared to S5B/PL rats, suggesting that the difference in D2R binding between high-fat fed OM and S5B/PL rats may also be mediated via leptin and /or insulin. Compared to low-fat fed rats, S5B/PL rats exposed to a high-fat diet showed increases in caloric intake and decreased D2R binding while leptin did not change, suggesting that these rats may be more sensitive to taste or post-ingestive effects of fat.

A limitation of this study however is that the focus was on the striatum and we did not assess D2R binding in other areas of the mesocorticolimbic DA system (i.e. VTA, SN, prefrontal cortex) as well as regions involved in food-related homeostasis (i.e. hypothalamus). Future studies are aimed at examining D2R binding in these regions and that the samples for any one group were small and thus further studies are warranted to replicate these findings. Our findings support a role for peripheral messengers like leptin in regulating D2R levels.

3.3 PET imaging of D2R with [^{11}C]raclopride predicts weight gain in rats

3.3.1 Introduction

Using PET and μPET imaging, studies have previously reported deficits in D2R binding availability in obese humans (Wang et al., 2001) and genetically obese rats

(Thanos et al., 2008). A fundamental question that has emerged from these studies is whether such deficits are caused by constant overeating in obesity, or whether the specific individuals and animals are genetically prone to such deficits. If the answer to this question is the latter, then the notion of using D2R binding availability as a predictive biomarker for assessing vulnerability to obesity becomes apparent. In this paper, we attempted to answer this question by measuring D2R binding availability using μPET in 10 non-obese adult Sprague-Dawley rats and then correlating these measures with body weight 2 months after scanning. We hypothesized that D2R binding availability would negatively correlate with future measures of body weight in rats and thus could serve as a predictive non-invasive measurement of weight gain susceptibility.

3.3.2 Methods

Animals

Rats between 12-14 weeks of age (n=10) were obtained from Taconic (Taconic, NY) and housed on a reverse 12 hour light/dark cycle with lights off at 7 am. Rats were fed a standard (Purina) laboratory rat chow. Food intake was monitored daily at approximately 1500h and all rats were weighed every other day immediately after food monitoring. All experiments were conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (NAS & NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

In-vivo [^{11}C]raclopride D2R μPET

μPET assessment of D2R in these rats was performed using a μPET R4 Scanner (CTI Concorde Microsystems, Knoxville, TN). Each rat was briefly anesthetized with isoflurane (~2-3 min) and its lateral tail vein was catheterized. Once awake, each rat was injected intravenously with [^{11}C]raclopride (547 ± 72 μCi); specific activity, 10.4 ± 4.3 mCi/nmol). Injected volumes were approximately 400 μl. 30 minutes later each rat was anesthetized with isoflurane and placed in a stereotaxic head holder (David Kopf Instruments; CA, USA) in a prone position on the bed of the scanner and scanning commenced. Total acquisition time was 30 min (17 frames: 6 frames, 10 s; 3 frames, 20 s; 8 frames, 60 s; 4 frames, 300 s) and data were acquired in fully 3-dimensional mode with maximum axial acceptance angle (+-28 degrees). Images were reconstructed using the optimization algorithm MAP with 30 iterations and a smoothing value of 0.01 mm. Qualitative and quantitative assessment of μPET images was performed as described previously using the PMOD software environment (PMOD Technologies, Zurich Switzerland) (Schiffer et al., 2009). Food intake and body weight was measured up to 2 months after scanning.

3.3.3 Results

A linear regression analysis comparing D2R binding availability (binding ratio) and body weight in the dorsal and ventral striatum 2 months after scanning was

performed. The regression was significant at the p=0.01 (dorsal striatum) and p=0.03 (ventral striatum) level with correlation coefficients of r=0.74 and r=0.67 respectively (Figures 3.3.1a & 1b). A z-test showed that the correlations between the dorsal and ventral striatum were significantly different (z=2.32; $p_{2\text{-tail}}$=0.02).

Figure 3.3.1a

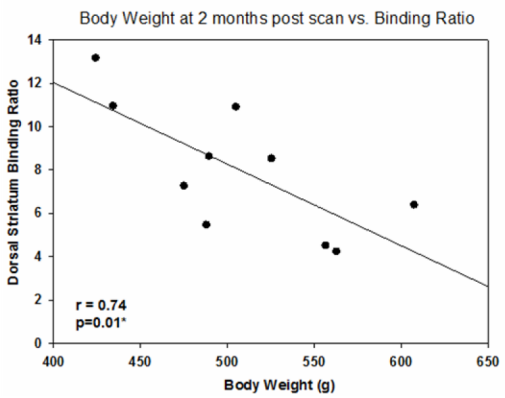

Figure 3.3.1b

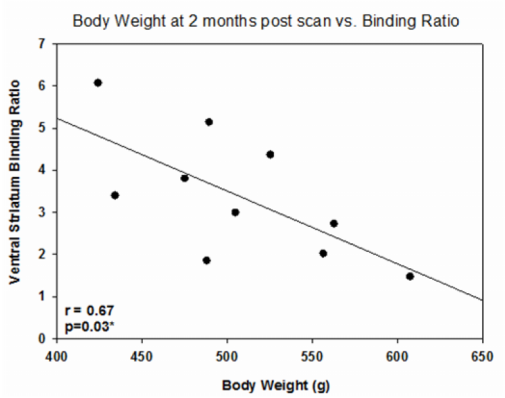

Figure 3.3.1. (a) D2R Binding ratio expressed as receptor rich/receptor poor ratio (dorsal striatum/cerebellum) and plotted against body weight 2 months post scan. (b) D2R Binding ratio expressed as receptor rich/receptor poor ratio (ventral striatum/cerebellum) and plotted against body weight 2 months post scan.

3.3.4 Discussion

We found that rats with the highest binding levels showed the lowest body weight and vice versa. Specifically, D2R binding availability in both the dorsal (Figure 3.3.1a) and ventral striatum (Figure 3.3.1b) significantly and negatively correlated with future body weight. These findings suggest that both the dorsal and ventral striatum may be involved in body weight regulation. However, since the correlations significantly differed, our results suggest that the dorsal striatum plays a stronger role in weight gain susceptibility compared to the ventral striatum. These results suggest the potential for using D2R binding availability to predict future body weight in rodents and potentially non-human primates. Using such a measurement, one can classify rodents and primates into those that express susceptibility to weight gain and those that do not. One can then identify specific genes and alleles that may be differentially expressed in these animals before and after the onset of weight gain. Findings from such studies can lead to the development of new treatments for obesity. Finally, since PET imaging using [^{11}C]raclopride is a non-invasive research paradigm that is routinely employed in clinical research, this approach may have the potential to be translated to humans in order to determine individual susceptibility to weight gain.

Chapter 4

Dopamine, Dopamine D2 Receptor, and Leptin Interactions

4.1 Displacement of [^{11}C]raclopride by leptin predicts weight gain and cocaine preference in rats

4.1.1 Introduction

PET studies in humans have corroborated an involvement of D2R both in food reward (Small et al., 2003) as well as in the motivation to procure food (Volkow et al., 2002b). Such studies have also corroborated the involvement of D2R in obesity by documenting reductions in striatal D2R availability in the striatum of pathologically obese individuals (Wang et al., 2001), while patients with anorexia nervosa have reported higher than normal striatal D2R availability (Frank et al., 2005). Using μPET, section 3.1 reported that obese Zucker fa/fa rats show reduced striatal D2R availability compared to lean rats. Since fa/fa rats are leptin-resistant and morbidly obese, and since the morbidly obese humans from the (Wang et al., 2001) study were more than likely to have been leptin-resistant, it is currently unknown whether it is the obesity itself or diminished leptin signaling that leads to decreased D2R availability. The aim of the experiments in this chapter was to document a role for leptin in modulating striatal DA-D2R interactions in-vivo. The main hypothesis was that changes in leptin signaling affect DA-D2R interactions in the striatum and thereby can increase motivation and sensitivity to food seeking, since leptin signals peripheral energy stores to the brain. The central premise is that leptin is an adiposity signal whose effects are not only limited to homeostatic mechanisms, but also to goal-directed behavior such as food seeking.

4.1.2 Methods

Animals

Rats were obtained from Taconic (Taconic, NY) and housed on a reverse 12 hour light/dark cycle with lights off at 7 am. Rats were fed a standard (Purina) laboratory rat chow. Food intake was monitored daily at approximately 1500h and all rats were weighed every other day immediately after food monitoring. All experiments were

conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (NAS & NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

In-vivo [^{11}C]raclopride D2R μPET

Raclopride was purchased from Sigma and [^{11}C]raclopride was synthesized as described previously (Volkow et al., 1999b). μPET assessment of D2R in these rats was performed using a μPET R4 Scanner (CTI Concorde Microsystems, Knoxville, TN). Each rat was scanned twice. Scan 1 (baseline): Each rat was briefly anesthetized with isoflurane (~2-3 min) and its lateral tail vein was catheterized. Once awake, each rat was injected intravenously with (565 ± 54 μCi); specific activity, 12.7 ± 1.8 mCi/nmol) [^{11}C]raclopride. Injected volumes were approximately 400 μl. 30 minutes later each rat was anesthetized with isoflurane and placed in a stereotaxic head holder (David Kopf Instruments; CA, USA) in a prone position on the bed of the scanner and scanning commenced. Total acquisition time was 30 min (17 frames: 6 frames, 10 s; 3 frames, 20 s; 8 frames, 60 s; 4 frames, 300 s) and data were acquired in fully 3-dimensional mode with maximum axial acceptance angle (+-28 degrees). Images were reconstructed using a MAP algorithm (as described in 2.3). Scan 2 (challenge): These procedures were identical to Scan 1 with the exception of an intraperitoneal (IP) injection of either saline or rat recombinant leptin (Sigma-Aldrich, St.Louis, MO) at doses of 20, 40 and 80 μg/kg immediately prior to [^{11}C]raclopride administration. Since raclopride is highly sensitive to displacement by endogenous DA, we measured the percent change in D2R binding between the two scans (as an indicative measure of D2R binding displacement in response to leptin). Qualitative and quantitative assessment of μPET images was performed as described previously (Thanos et al., 2008).

4.1.3 Results

Effects of Leptin on D2R Binding Displacement in the Dorsal Striatum

One-way ANOVA showed a significant main effect [($F(3, 19)=3.28$; $p=0.048$)]. Pair-wise comparisons (Holm-Sidak; $\alpha=0.05$) showed that compared to saline, 20 ($t=2.52$; $p=0.023$) and 40 μg/kg ($t=2.53$; $p=0.022$) of leptin significantly decreased [^{11}C]raclopride displacement by ~30% in the dorsal striatum: 80 μg/kg leptin did not affect [^{11}C]raclopride displacement in the dorsal striatum ($t=0.72$; $p=0.48$) (Figure 4.1.1a).

Effects of Leptin on D2R Binding Displacement in the Ventral Striatum

One-way ANOVA showed a significant main effect [($F(3, 19)=1.39$; $p=0.284$)]. No significant effects among any groups were observed in the ventral striatum (Figure 4.1.1b).

Correlation between Leptin-induced alterations in D2R Binding Displacement and Propensity to Gain Weight

Linear regression analyses comparing D2R binding displacement (% change in binding ratio between the 80 µg/kg leptin challenge scan and the baseline scan) and body weight in the dorsal and ventral striatum 2 months after scanning were performed. The regressions were significant at the p=0.03 (dorsal striatum) and p=0.03 (ventral striatum) level with correlation coefficients of r=0.91 and r=0.91 respectively (Figures 4.1.2a & 2b).

Correlation between Leptin-induced alterations in D2R Binding Displacement and Cocaine Preference

Linear regression analyses comparing D2R binding displacement (% change in binding ratio between the 80 µg/kg leptin challenge scan and the baseline scan) and cocaine preference in the dorsal and ventral striatum 2 months after scanning were performed. The regressions were not significant with p=0.18 (dorsal striatum) and p=0.22 (ventral striatum) correlation coefficients of r=0.70 and r=0.66 respectively (Figures 4.1.2c & 2d).

Figure 4.1.1a

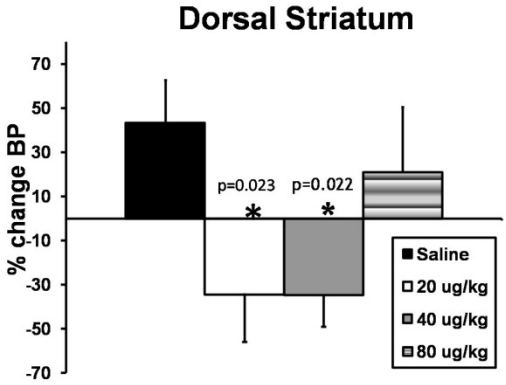

Figure 4.1.1b

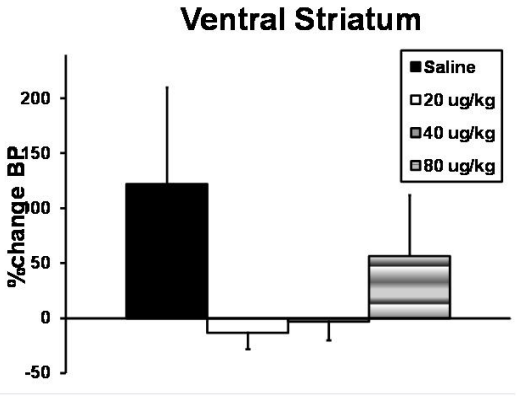

Figure 4.1.1. (a) Percent change in D2R BP between challenge (leptin or saline) and baseline scans in the dorsal striatum. (b) Percent change in D2R BP between challenge (leptin or saline) and baseline scans in the ventral striatum.

Figure 4.1.2a

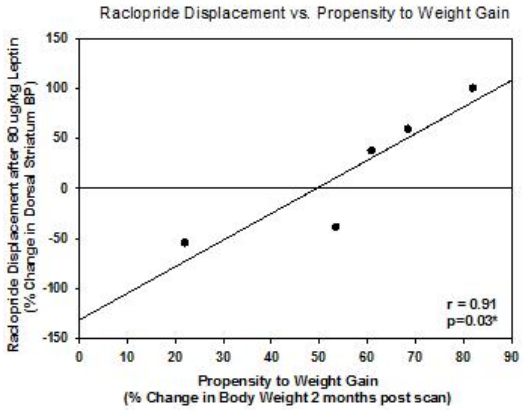

Figure 4.1.2b

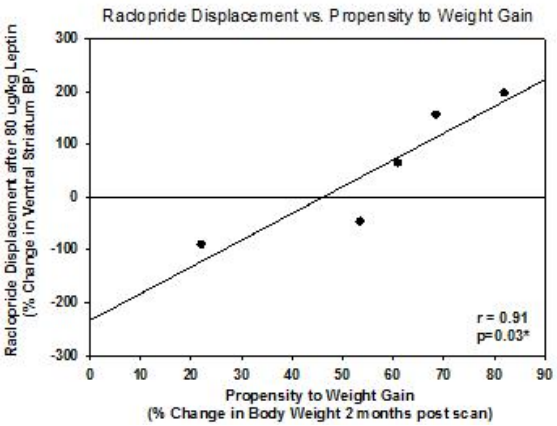

Figure 4.1.2c

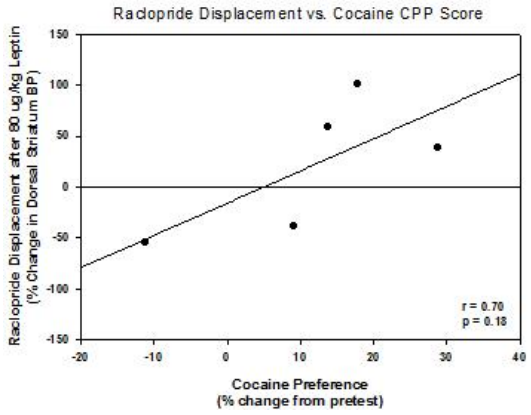

Figure 4.1.2d

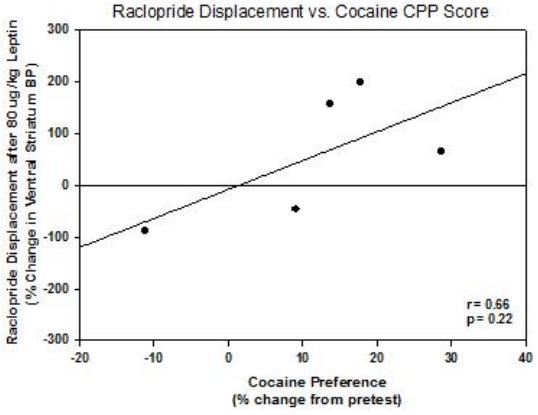

Figure 4.1.2. Percent change in D2R BP between challenge (leptin or saline) and baseline scans in the dorsal striatum (a) and ventral striatum (b) plotted against total weight gain 2 months post scan. (b) Percent change in D2R BP between challenge (leptin or saline) and baseline scans in the dorsal striatum (c) and ventral striatum (d) plotted against cocaine preference 2 months post scan.

4.1.4 Discussion

The above results suggest that low peripheral leptin concentrations lead to increases in striatal DA-D2R stimulation while higher doses produce variable effects on DA-D2R stimulation and that these effects depend on individual susceptibility to weight gain but not cocaine preference.

Repeated Imaging with [^{11}C]raclopride using a Test/Retest Paradigm Decreases DA-D2R Binding

Saline-treated rats showed a ~45% and ~120% increase in BP in the dorsal and ventral striatum respectively (Figure 4.1.1a & b). [^{11}C]raclopride is highly displaceable by DA (Morris & Yoder, 2007) and therefore test-retest decreases/increases in BP are

thought to reflect increases/decreases in D2R stimulation by DA (via DA-raclopride competition for D2R binding sites). Therefore, outside the context of leptin, this specific test-retest imaging paradigm decreases DA-D2R binding in both the dorsal and ventral striatum, possibly due to repeated exposure to anesthesia. Future studies aimed at using similar techniques should take potential anesthesia issues into account in the imaging design.

Effects of Leptin on DA-D2R Binding in the Dorsal and Ventral Striatum are Dependent on Dose and Individual Susceptibility to Weight Gain but not Cocaine Preference

In contrast, 20 and 40 μg/kg leptin significantly decreased BP by ~30% in the dorsal striatum but did not affect BP in the ventral striatum. These findings are directly opposite to the binding response seen when rats were challenged with saline and suggest that leptin, at these doses, produces an increase in DA-D2R binding in both the dorsal and ventral striatum, (albeit this effect is more pronounced in the dorsal striatum). Interestingly, similar leptin doses decrease feeding in mice (Harris et al., 1998). In accordance with this, increased D2R stimulation is considered to have anorexigenic effects as D2R agonists decrease feeding and adiposity (Davis et al., 2009). Taking both of these into account, we conclude that the anorectic effects of DA (via D2R stimulation) are mediated in part by peripheral leptin. This is a compelling argument as it highlights the crosstalk between the CNS and the periphery and provides a potential mechanism relating ingestive behavior to energy homeostasis. Furthermore, since the increase in DA-D2R stimulation was more pronounced in the dorsal striatum, it is possible that the dorsal striatum is more involved in leptin-DA interactions that mediate ingestive behavior than the ventral striatum.

The uniform effect of 20 and 40 μg/kg leptin at decreasing D2R BP was not seen at the highest dose. In fact, 80 μg/kg leptin produced variable results in both the dorsal and ventral striatum with some rats showing increases and others decreases in BP. Linear regression analysis was carried out in an attempt to explain this variability (Figure 4.1.2) and showed that leptin was able to decrease BP in rats that showed the least amount of weight gain at 1 and 2 months after scanning but increased BP in rats that showed the greatest weight gain at these same time points. It is possible that at this dose, leptin produces differential effects on D2R binding and that the direction of the effect is determined by individual susceptibility to weight gain. Interestingly, 80 μg/kg leptin approximates the plasma leptin concentrations of obese rats (Michaelides et al., 2006), concentrations which are also characteristic of leptin resistance. Rodents with such elevated leptin concentrations are characterized by increased feeding and adiposity. These results suggest that during leptin resistance or situations where there are highly elevated blood leptin concentrations, leptin signaling works to decrease D2R binding is rats that have some type of resistance to weight gain, while in others such leptin plasma concentrations may induce opposite effects on striatal DA, that lead to increased D2R binding and hence decreases in D2R stimulation by DA. This is a very compelling argument since it describes a potential mechanism that may explain in part individual differences in weight gain and food reward. Accordingly, striatal DA-D2R deficits previously observed in obese individuals may be influenced by individual differences in

leptin metabolism. Furthermore, D2R BP changes in response to leptin may be used to predict susceptibility to weight gain in normal subjects.

The adopted imaging paradigm allows for indirect measures of synaptic DA levels using a within-subject *in-vivo* design. This approach allows for an attenuation of variability attributed to comparisons between different animals. Also, unlike μdialysis, which provides indirect measures of extracellular DA levels, this approach is completely non-invasive. Furthermore, since μPET imaging is reproducible animals can be scanned repeatedly over a longitudinal timeframe in order to examine changes in synaptic DA levels due to chronic effects of leptin administration. Another notable strength of this approach is the fact that we were able to discriminate between dorsal and ventral regions of the striatum by combining the use of a sophisticated iterative reconstruction algorithm with analysis procedures where μPET images were coregistered to a rodent MRI template set in stereotaxic coordinates. This way we were able to approximate pixels to specific coordinates that are routinely used for targeting these brain structures. Other notable strengths have to do with the doses of leptin administered. Most published studies have utilized supraphysiological leptin doses (in mg range), directly administering these to the CNS (this is a requirement for sufficient diffusion of the peptide through the brain to the target of interest). On the other hand, we used an administration approach that more closely resembles physiological circulating leptin concentrations (in μg range) as well as its action in the periphery (it is thought to be released in the periphery and transported to the CNS). However, although this specific imaging paradigm has significant strengths, there are also some limitations of the approach that should be mentioned. First, there is no direct quantification of DA using this approach. Alternatively, changes in DA are inferred based upon [^{11}C]raclopride dynamics. Although the short time-course of the specific paradigm (imaging window of ~2 hours) accounts for discrepancies in [^{11}C]raclopride due to leptin-induced changes in receptor density, we cannot exclude the notion that [^{11}C]raclopride dynamics may be influenced by other factors such as receptor affinity and internalization. Another general experimental limitation is that there is no examination of food seeking behavior or direct quantification of adiposity. In particular, ingestive behavior is characterized by various components (i.e. motivation to eat, seeking food, consuming food, sensory aspects of feeding, etc.) and we cannot detail the exact involvement of leptin-DA interactions in such components using this specific approach. Future studies will focus on bridging the imaging data with a more thorough behavioral approach that will also extend to cocaine seeking. Finally, our findings may apply to other peripheral messengers involved in metabolism (i.e. insulin) and therefore future studies should be aimed at examining associations between such peripheral messengers and central neurotransmitters involved in ingestive behavior. Nevertheless, given the above findings, leptin-DA interactions relevant to feeding and drug addiction should be investigated further as well as in other contexts where DA has been traditionally believed to play a role in such as depression, attention-deficit hyperactivity disorder, Parkinson's disease and others.

Chapter 5

Summary and Conclusions

5.1 Summary

This thesis presents evidence that inherent striatal DA-D2R signaling deficits correlate with increased susceptibility to weight gain and cocaine preference. The deficits observed in the dorsal striatum strongly correlated to weight gain, while those observed in the ventral striatum strongly correlated with cocaine preference. D2R were also found to decrease in response to energy intake (high-fat diet) and cocaine intake similarly in both the dorsal and ventral striatum. Approaches that increase striatal D2R signaling were found to decrease feeding, adiposity and cocaine intake. Changes in D2R occur in response to changes in leptin signaling and were found to be context-dependent, in particular being affected by food availability, body weight and leptin resistance. Leptin was found to exert a biphasic effect on striatal DA-D2R signaling. In particular, lower doses increased DA-D2R signaling, while higher doses produced variable effects on DA-D2R signaling. Finally, the variability in DA-D2R signaling attributed to leptin administration significantly correlated with weight gain but not cocaine preference.

5.2 Conclusions

The experiments described in Chapter 2 show that ventral striatal D2R negatively correlate with future cocaine preference, while dorsal striatal D2R do not. This suggests that D2R deficits previously observed in cocaine abusers may precede cocaine use and potentially originate in the ventral striatum. Furthermore, these results suggest that measuring D2R binding non-invasively through the use of PET imaging may be used to predict future cocaine abuse. The experiments described in this chapter also show that cocaine intake leads to decreases in dorsal and ventral striatal D2R, while genetic upregulation of D2R in the ventral striatum decreases cocaine intake. These findings suggest that striatal D2R play a significant role in cocaine abuse and in particular seem to be involved in both the initiation as well as maintenance of cocaine abuse. Finally, they are one of several likely factors involved in cocaine abuse since genetic upregulation of

D2R attenuated, but did not abolish cocaine intake. Future studies assessing the efficacy of more advanced gene therapy approaches should shed further light on the exact role of D2R on cocaine intake.

Chapter 3 experiments focused on examining the involvement of striatal D2R in obesity and weight gain. These experiments showed that both dorsal and ventral striatal D2R negatively correlate with future weight gain, albeit this effect is stronger in the dorsal striatum. Like in relation to cocaine abuse, this result suggests that D2R deficits previously observed in obese individuals may precede the obesity. Furthermore, since the correlations effect was more pronounced in the dorsal striatum it seems that this region may play a significant role in mediating ingestive behavior and/or metabolism and weight gain, while ventral striatal D2R may be more involved in mediating the rewarding effects of cocaine. Nevertheless, our results suggest that D2R binding using PET can be used as a measure to predict future weight gain susceptibility. Results from this chapter also show that genetic and high-fat diet induced obesity both lead to similar declines in D2R. In these models of obesity, low leptin signaling was especially deemed important for the observed D2R declines. Finally, in another recent study we showed that D2R stimulation improved leptin signaling, decreased feeding and adiposity (Davis et al., 2009).

Finally, Chapter 4 experiments assessed whether peripheral leptin and central D2R interact and whether this interaction is relevant in obesity and/or cocaine abuse. These experiments showed that peripheral administration of leptin at doses which inhibit feeding lead to increases in D2R stimulation by DA ([^{11}C]raclopride D2R binding declined). This finding is consistent with findings from the literature that support low tonic DA levels as leading to increased sensitivity or predisposition to consume food. On the other hand, higher leptin doses, similar to circulating leptin levels found in obesity, lead to variable effects on DA-D2R stimulation, with rats that gained the lowest future body weight showing increases in DA-D2R stimulation while those that gained the most showed decreases in DA-D2R stimulation. These effects were similar for both the dorsal and ventral striatum. Finally, this variability in DA-D2R stimulation by high leptin levels did not correlate with cocaine preference.

In sum, striatal D2R plays an important role in modulating responses to weight gain and cocaine preference. The above findings imply a novel role for D2R in regulating homeostasis via effects on peripheral homeostatic messengers like the adipose hormone leptin. Finally, the experiments show that D2R may serve as both a promising target for drugs aimed at obesity and addiction as well as a kind of biomarker to predict future susceptibility to obesity and substance abuse.

5.3 Implications

This thesis argues that leptin, a peptide/hormone that conveys the status of long-term energy (fat) stores in the body to the brain differentially modulates brain mechanisms that have been previously implicated in mediating the rewarding and motivational properties involved in seeking and consuming food and drugs of abuse. These differential effects were found to depend on individual differences in susceptibility and resistance to weight gain but not cocaine preference. Under normal

physiological conditions, low leptin levels signal to the brain that energy stores are inadequate while high leptin levels indicate that energy requirements are met. The main outcome that underlies the experimental findings described in this thesis is that striatal D2R mediate weight gain as well as cocaine preference and that this effect depends in part on peripheral leptin concentrations. Consequently, leptin-responsive striatal DA neurons may receive signals and initiate the goal-directed behavior required for seeking food and increasing or decreasing adiposity but leptin's effects on cocaine abuse may not be as strong. The direct interaction between an adiposity signal such as leptin and striatal D2R has not previously been investigated. Future experiments studying this relationship will lead to a better understanding of DA and D2R-related mechanisms in energy regulation and how these may be related to compulsive behavior such as overeating and cocaine abuse. In turn, increased understanding of such mechanisms may lead to drug treatments for obesity and drug addiction.

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