DIET-INDUCED OBESITY: DOPAMINERGIC AND BEHAVIORAL MECHANISMS AS OUTCOMES AND PREDICTORS

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DIET-INDUCED OBESITY:
DOPAMINERGIC AND BEHAVIORAL MECHANISMS
AS OUTCOMES AND PREDICTORS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By

Vidya Narayanaswami

Lexington, Kentucky

Director: Dr. Linda P. Dwoskin, Professor of Pharmaceutical Sciences
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2012

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ABSTRACT OF DISSERTATION

DIET-INDUCED OBESITY:
DOPAMINERGIC AND BEHAVIORAL MECHANISMS
AS OUTCOMES AND PREDICTORS

Obesity and drug abuse share common neural circuitries including the mesocorticolimbic and striatal dopamine reward system. In the current study, a rat model of diet-induced obesity (DIO) was used to determine striatal dopamine function, impulsivity and motivation as neurobehavioral outcomes and predictors of obesity. For the outcome study, rats were randomly assigned a high-fat (HF) or a low-fat (LF) diet for 8 wk. Following the 8-wk HF-diet exposure, rats were segregated into obesity-prone and obesity-resistant groups based on maximum and minimum body weight gain, respectively, and neurobehavioral outcomes were evaluated. For the predictor study, neurobehavioral antecedents were evaluated prior to an 8-wk high-fat diet exposure and were correlated with subsequent body weight gain. Striatal D2 receptor density was determined by in vitro kinetic analysis of [3H]raclopride binding. DAT function was determined using in vitro kinetic analysis of [3H]dopamine uptake, methamphetamine-evoked [3H]dopamine overflow and no net flux in vivo microdialysis. DAT cell-surface expression was determined using biotinylation and Western blotting. Impulsivity and food-motivated behavior were determined using a delay discounting task and progressive ratio schedule for food-reinforcers, respectively. Relative to obesity-resistant, obesity-prone rats exhibited 18% greater body weight, 42% lower striatal D2 receptor density, 30% lower total DAT expression, 40% lower in vitro and in vivo DAT function, 45% greater extracellular dopamine concentration, and 2-fold greater methamphetamine-evoked [3H]dopamine overflow. Obesity-prone rats exhibited higher motivation for food, but were less impulsive relative to obesity-resistant rats. Neurobehavioral antecedents of DIO included greater motivation for high-fat reinforcers in rats subsequently shown to be obesity-prone relative to obesity-resistant. Impulsivity, DAT function and extracellular dopamine concentration did not predict the DIO-phenotype. Thus, motivation for food is linked to both initiation and maintenance of obesity. Importantly, obesity results in decreased striatal DAT function, which may underlie the maintenance of compulsive food intake in obesity.
KEYWORDS: Diet-induced obesity, dopamine transporter, impulsivity, motivation, striatum

Vidya Narayanaswami

December 14, 2012
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DOPAMINERGIC AND BEHAVIORAL MECHANISMS 
AS OUTCOMES AND PREDICTORS

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December 14, 2012
Dedicated to My Parents R. Narayanaswami and Pushkala,
Sister Swati
&
Late Grand Parents, Raman and Lakshmi
ACKNOWLEDGEMENTS

With immense pleasure, I take this opportunity to thank all those individuals whose support and guidance have been instrumental in the successful completion of my dissertation work.

First and foremost, I offer my sincere gratitude to my mentor, Dr Linda Dwoskin, for her guidance and constant strive for perfection that have been major driving forces in my professional and personal development. I would like to thank her for providing me with transdisciplinary research training that has strengthened my research capabilities and scientific writing skills over the years, and has also instilled in me important qualities that include; independent and critical thinking, team spirit, competitive spirit and the ability to adapt in a collaborative work environment.

I would also like to thank my committee members; Dr. Lisa Cassis, Dr. Kim Nixon and Dr. Ming Gong for their guidance and important insights over the years. I sincerely thank Dr. Vicki King for agreeing to serve as an outside examiner for my dissertation defense. I am grateful to Dr. Michael Bardo and Dr. Alexis Thompson for their expert guidance, training and insightful discussions that have allowed me to address important questions in my dissertation work. I deeply appreciate the support offered by Catina Rossoll and Charolette Garland through all these years.
Special thanks to all current and past Dwoskin laboratory members; Gabi Deaciuc, Kiran Babu Siripurapu, Justin Nickell, Mahesh Darna, Andrew Smith, David Horton, Yolanda Williams, Zac Cao and Sucharita Somkuwar. Thank you all for your timely support and friendship that has made this journey a joyful and memorable learning experience.

I would also like to express my heartiest gratitude to all my friends for their unconditional love, tolerance and support through all these years and for being my family away from home.

My final and heartfelt acknowledgement goes out to my wonderful parents, Narayanaswami and Pushkala; my late grandparents, Raman and Lakshmi; my elder sister Swati, and my brother-in-law Jayesh, for having faith in me and for being there through thick and thin. Thank you all for being extremely tolerant and for encouraging me to persist and achieve my goals. The upbringing, love and support that you all have bestowed upon me have enabled me to reach this point in life.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti-related protein</td>
</tr>
<tr>
<td>ARH</td>
<td>arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>ATP-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>adenosine 5'-triphosphate magnesium salt</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BOLD</td>
<td>blood-oxygen level dependent</td>
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<tr>
<td>BPD-DS</td>
<td>biliopancreatic diversion with a duodenal switch</td>
</tr>
<tr>
<td>BRFSS</td>
<td>Behavioral Risk Factor Surveillance System</td>
</tr>
<tr>
<td>CaMKIIα</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;/calmodulin-dependent protein kinase α</td>
</tr>
<tr>
<td>CART</td>
<td>cocaine and amphetamine related transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin; CNS, central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyl transferase</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
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<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DIO</td>
<td>diet-induced obesity</td>
</tr>
<tr>
<td>DLPFC</td>
<td>dorsolateral prefrontal cortex</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial hypothalamus</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>DOPAC</td>
<td>dihydroxy phenylacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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ERK1/2, extracellular signal-regulated kinases 1 and 2
fMRI; functional magnetic resonance imaging
FR, fixed ratio
GABA, gamma amino butyric acid
GLP-1, glucagon-like peptide
HEPES, $N$-[2-hydroxyethyl] piperazine-$N'$-[2-ethanesulfonic acid]
HF, high-fat
HFCS, high-fructose corn syrup
5HT, serotonin
IASO, International Association for the Study of Obesity
IOTF, International Obesity Task Force
LAGB, laparoscopic adjustable gastric banding
LHA, lateral hypothalamus
LF, low-fat
MAO, monoamine oxidase
MAPK, mitogen-activated protein kinase
α-MSH, α- melanocyte stimulating hormone
mPFC, medial prefrontal cortex
NAc, nucleus accumbens
NCI; National Cancer Institute
NHANES, National Health and Examination Survey
NPY, neuropeptide Y
NST/AP, nucleus of the solitary tract/area postrema
OFC, orbitofrontal cortex
PBS, phosphate buffered saline
PEI, polyethyleneimine
PI3K, phosphoinositide 3 kinase
PKA, protein kinase A
PKC, protein kinase C
PVN, paraventricular nucleus
PICKI, PDZ domain containing protein interacting with C-kinase 1
POMC, pro-opiomelanocortin
PR, progressive ratio
PYY, peptide YY
PP2A, protein phosphotase 2A
RYGB, roux-en-Y gastric bypass
SDS, sodium dodecyl sulfate
SG, sleeve gastrectomy
sulfo-NHS-biotin, sulfosuccinimidobiotin
Tris-HCl, Tris [hydroxyl methyl] aminomethane hydrochloride
TMDs, transmembrane domains
TRH, thyrotropin-releasing hormone
US, United States
VMH, ventral medial hypothalamus
VMAT2, vesicular monoamine transporter 2
VTA, ventral tegmental area
WHO, World health organization
ZDF, Zucker Diabetic Fatty.
Chapter One

Overall Introduction

I. Obesity: A global epidemic

Obesity is a complex medical condition that is characterized by excessive, abnormal fat accumulation as a result of increased intake of energy-dense foods and decreased physical activity (World Health Organization (WHO) factsheet N° 311). Body mass index (BMI), a proportion of body weight to height, is commonly employed to classify overweight and obese individuals and has been considered as a reliable indicator of body fat (Mei et al., 2002; Flegal et al., 2009). Individuals with BMI greater than or equal to 25 are classified as overweight and those with BMI greater than 30 are classified as obese.

Over the past two decades, the prevalence of obesity has increased in the United States (US) and worldwide and has contributed to serious health and economic consequences. The dramatic rise in obesity has been attributed to genetic susceptibility and environmental factors that include availability of high-energy dense foods and sedentary life style that lacks adequate physical activity. The prevalence of obesity in the US during 2009-2010 was 35.5% among adult men, 35.8% among adult women, and approximately 17% among children and adolescents, as evidenced by reports from the National Health and Examination Survey (NHANES). Furthermore, reports from the Center for Disease Control and
Prevention’s Behavioral Risk Factor Surveillance System (BRFSS) indicate that in 2012, all states in the US had a prevalence of obesity greater than 20%, and 12 states (including Kentucky) had a prevalence of more than 30%. The current trends in obesity in the US have led to the estimation that total healthcare costs attributable to obesity could reach $861 to $957 billion by 2030, which would account for 16-18% of total US health expenditures. Obesity was previously recognized as a health problem only in developed countries. However, the prevalence of overweight and obesity is evident in developing countries as well. Importantly, WHO reports indicate that in 2010, around 43 million children were overweight, out of which approximately 35 million overweight children lived in developing countries. Based on the current trends, WHO predicts that by 2015, about 2.3 billion adults will be overweight and over 700 million people will be classified as obese.

Metabolic syndrome is a constellation of metabolic abnormalities that increases the risk for cardiovascular diseases and other severe health problems including type-2 diabetes and stroke (Eckel et al., 2005). Obesity significantly contributes to the metabolic syndrome. Specifically, abdominal adiposity is known to result in metabolic abnormalities that include; hyperglycemia, dyslipidemia and hypertension (Mokdad et al., 2001; Bloomgarden, 2002; Montani et al., 2002; Grundy et al., 2005). Compared to non-obese women, obese women exhibit a greater probability (~ 60-fold) of being diagnosed with type-2 diabetes (Colditz et al., 1990). Further, overweight and obese men were
shown to exhibit increased risk for ischemic and hemorrhagic stroke (Kurth et al., 2002).

In addition to the metabolic syndrome, obesity is also shown to increase the risk for certain types of cancer. The National Cancer Institute (NCI) Surveillance, Epidemiology, and End Results data in 2007 estimated that in the US, about 4% of cancer cases in men and 7% in women were due to obesity. Importantly, the contribution of obesity to cancer was as high as 40% for cancers like, endometrial and esophageal adenocarcinoma (NCI, 2012). The current trend in obesity is estimated to lead to an additional 500,000 cases of cancer in the US by 2030. Thus, there is compelling evidence that demonstrates metabolic, cardiovascular and cancer-related risks associated with obesity. The escalating rates of obesity and associated co-morbidities necessitate effective long term pharmacotherapy to treat this complex medical condition.

II. Homoeostatic regulation of energy balance

Obesity results from a long-term positive energy balance such that there is increased food intake and decreased energy expenditure (Spiegelman and Flier, 2001). Homeostasis involves the maintenance of equilibrium by adjusting physiological processes. Importantly, homeostatic energy balance involves mechanisms that adjust food intake and energy expenditure to maintain a body weight set point. Regulation of appetite and body weight involves a complex
interaction between multiple neural systems, peripheral energy stores and external cues that influence food availability and palatability. A fundamental overview of both homeostatic and higher brain reward centers that regulate food intake and metabolism is provided in sections II, III and IV of this chapter.

II a. Peripheral signals: Satiety, hunger and adiposity

Maintenance of energy balance involves extensive coordination between brain and peripheral mechanisms. In order to establish energy balance, homeostatic neuronal circuits in the central nervous system (CNS) integrates two major peripheral factors that are triggered upon food ingestion. These include: a) satiety and hunger; b) adiposity. These peripheral factors can be further classified into episodic and tonic factors (Halford and Blundell, 2000). Episodic factors are triggered upon recent food consumption and constitute both satiety and hunger factors. Tonic factors include adiposity factors that arise from tissue stores to indicate the depletion or repletion of energy stores and thereby regulate food intake based on the state of body’s energy stores.

Satiety. Satiety is a state of being full after eating such that hunger and eating behaviors are consequently inhibited (Blundell, 1991). Satiety factors that inhibit appetite include pre-absorptive stomach distension and post absorptive fuel metabolism (Harrold et al., 2012). Chemicals released by the gastrointestinal tract during the process of food digestion also contribute towards satiety signals. Peripheral hormones that regulate satiety include; cholecystokinin (CCK),
glucagon-like peptide-1 (GLP-1), peptide YY (PYY) and amylin. CCK is a peptide hormone released from the I-cells of the proximal intestinal tract following detection of fat and protein in the gut (Liddle et al., 1985). CCK is derived from the pro-CCK gene through post-translational modification which creates a number of biologically active peptides that differ in the length of amino acids (4 to 83 amino acids), the predominant molecular form being CCK-58 (Rehfeld et al., 2001). CCK-induced satiety signaling is mediated via CCK receptors expressed in the intestinal tract, brain stem and the hypothalamus (Ballinger et al., 1995; Blevins et al., 2000; Degen et al., 2001). GLP-1 is a peptide hormone that is synthesized by the L-cells of distal small intestine and is released in response to carbohydrate and fat digestion (Lavin et al., 1998; Thomsen et al., 1999). Two forms of GLP-1 that are found in the circulatory system include; GLP-1(7–36) amide and GLP-1(7–37) amide, the predominant variant being GLP-1(7–36) (Marks et al., 1991). GLP-1 is known to mediate appetite control via insulin release, glucagon inhibition, absorption and metabolism of macronutrients (Naslund et al., 1999; Flint et al., 2001). GLP-1 receptors are widely expressed in the CNS and peripheral tissues (Bullock et al., 1995; Gutzwiller et al., 2004). PYY is a 36-amino acid peptide hormone released from the L-cells of distal small intestine in response to the detection of fatty acids, fibers and bile in the gut (Onaga et al., 2002). PYY receptors are widely expressed in the CNS. In addition to appetite control, PYY is also shown to increase energy expenditure (Sloth et al., 2007). Amylin is a 37-amino acid peptide hormone that is released along with insulin by the pancreatic β-cells in response to high-blood glucose levels and is
shown to reduce both food intake and body weight *via* actions at the level of the brain stem (Rushing et al., 2001).

**Hunger.** Hunger is a physiological response to the orosensory effects of food stimuli, as well as to the decline in blood glucose levels that involves the motivation to seek and consume food (Melanson et al., 1999; Harrold et al., 2012). Importantly, glucose availability is known to influence the expression of orexigenic and anorexigenic peptides, thereby regulating food intake (Levin, 2006). Ghrelin is a 28-amino acid appetite-stimulating peptide hormone that is found in the highest concentrations in the gut (Korbonits and Grossman, 2004). Additionally, hypothalamic neurons are shown to secrete ghrelin. Ghrelin crosses the blood brain barrier and initiates feeding *via* ghrelin receptors expressed in the hypothalamus (Cummings, 2006; Harrold et al., 2008). Ghrelin levels are dependent on nutritional status such that peak ghrelin concentrations are found prior to meal initiation (Cummings et al., 2001). Ghrelin levels decrease upon food consumption and the postprandial ghrelin suppression is shown to be proportional to ingested caloric load (Callahan et al., 2004). Compared to lean control, obese individuals are more sensitive to the appetite-stimulating effects of ghrelin (Druce et al., 2005).

**Adiposity.** Three major adiposity factors that serve as feedback signals and are modulated by food intake and adipose tissue mass include; leptin, insulin and adiponectin. Leptin, a product of leptin gene (*Lep*(ob)), is a protein of
molecular weight 18,000, that contains a signal sequence which is cleaved to produce the mature leptin hormone of molecular weight 16,000 (Zhang et al., 1994). Leptin is secreted by adipocytes in proportion to fat mass and is known to decrease food intake and increase energy expenditure by acting via neuronal leptin receptors in multiple brain regions (Maffei et al., 1995; Porte et al., 2002). Leptin-induced decreases in food intake and body weight are predominantly mediated by hypothalamic leptin receptors (Fei et al., 1997; Elmquist et al., 1998; Myers et al., 2008). Insulin, a metabolic hormone synthesized in the pancreas by the β-cells of the islets of Langerhans, also increases proportionally with adiposity (Woods et al., 1974). Insulin is a polypeptide hormone that is composed of two peptide chains of 21 (A) and 30 (B) amino acids, which are linked by two disulfide bonds (Brange and Langkjoer, 1993). Similar to leptin, insulin is also shown to decrease food intake and body weight via insulin receptors in the hypothalamus (Menendez and Atrens 1991). Adiponectin, a 244-amino acid long polypeptide, is another adipocyte-derived protein that is shown to decrease body weight and lipid levels and facilitate insulin action (increases insulin sensitization) (Berg et al., 2001; Qi et al., 2004; Kadowaki and Yamauchi, 2005).

Collectively, energy homeostasis involves the central integration of peripheral hormonal signals that include; short-term, meal-related afferent satiety and hunger signals from the gut (CCK, GLP-1, PYY, amylin and ghrelin) and longer-term afferent signals from the adipocytes (leptin and adiponectin) and the pancreatic β-cells (insulin).
Il b. Brain circuits involved in homoeostatic regulation

Energy homeostasis is known to be regulated by neuronal circuits in the hypothalamus and the brain stem. Importantly, these brain circuits integrate signals from circulating hormones, sensory factors that include smell, taste and sight of food; as well as peripheral factors that are governed by ingestion of food, absorption and metabolism.

**Hypothalamus.** Multiple neuronal circuits within the hypothalamus are implicated in the primary regulation of energy homeostasis (Wynne et al., 2005; Morrison and Berthoud, 2007). Specifically, the arcuate nucleus of the hypothalamus (ARH) is known to stimulate and inhibit food intake via two distinct neuronal populations. A sub population of neurons within the ARH expresses orexigenic peptides, neuropeptide Y (NPY) and agouti-related protein (AgRP). Activation of the NPY/AgrP neurons increases food intake and decreases energy expenditure, as evidenced by brain infusion studies conducted in rats (Broberger et al., 1998, Hahn et al., 1998). NPY infusion into the hypothalamus was shown to increase feeding and delay the onset of satiety (Stanley and Libowitz, 1985). Central infusion of AgRP increased food intake and body weight and also reversed leptin-induced inhibition of food intake and body weight (Ebihara et al., 1999).
In contrast, pro-opiomelanocortin (POMC) neurons in the ARH produce anorexigenic peptides, α-melanocyte stimulating hormone (α-MSH) and cocaine and amphetamine related transcript (CART). α-MSH, a 13-amino acid peptide, is generated from the precursor hormone, POMC (Eipper and Mains, 1980). CART is a neuropeptide that also serves as a neurotransmitter (Lambert et al., 1997). In particular, CART peptides (55–102 amino acids) have been implicated in the regulation of energy homeostasis (Murphy, 2005). Activation of POMC neurons inhibits food intake and increases energy expenditure, as demonstrated by brain infusion and knockout studies in rodents (Elias et al., 1998a; Kristensen et al., 1998). The anorexigenic effects of α-MSH are mediated via MC3R and MC4R receptors expressed in the lateral ARH (Watson and Akil, 1979). MC4R null mice exhibit hyperphagia and are obese (Huszar et al., 1997). MC3R null mice are shown to develop obesity regardless of increases in food intake, suggesting the role of MC3R in fat metabolism (Chen et al., 2000). Interestingly, AgRP is known to selectively antagonize MC3R and MC4R (Ollmann et al., 1997). Mutations of MC3R/MC4R are shown to be associated with morbid obesity in humans (Krude et al., 1998; Yeo et al., 1998). In rats, central administration of CART and anti-CART antibodies is shown to decrease and increase food intake, respectively (Lambert et al., 1997). Also, CART knockout mice exhibit increased body weight when fed a high-fat (HF) diet (Asnicar et al., 2001).

Extensive reciprocal connections exist between the ARH and other hypothalamic regions including the paraventricular nucleus (PVN), dorsomedial
hypothalamus (DMH), lateral hypothalamus (LHA), perifornical area and ventral medial hypothalamus (VMH) (Elias et al., 1998b; Kalra et al., 1999). These brain regions receive projections from NPY/AgRP neurons and POMC neurons in the ARH and also comprise of second-order neurons that express neuropeptides involved in energy homeostasis. Orexigenic peptides, termed as orexins or hypocretins (orexin-A (33 amino acid residues) and orexin-B (28 amino acid residues)), are excitatory neuropeptides that are synthesized in the LHA/perifornical area and are implicated in feeding behavior (De Lecea et al., 1998; Sakurai et al., 1998). Acute central administration of orexins is shown to promote hyperphagia and delay the onset of satiety in rats (Rodgers et al., 2002). Further, orexin knockout mice exhibit narcolepsy, implicating the role of orexins in arousal (Chemelli et al., 1999). Cell bodies within the LHA consist of another potent orexigenic peptide, melanin-concentrating hormone (MCH). Chronic central infusion of MCH was shown to increase food intake and body weight in rats (Qu et al., 1996). Neurons of the PVN express anorexigenic peptides that include; thyrotropin-releasing hormone (TRH), corticotrophin-releasing hormone (CRH) and brain derived neurotrophic factor (BDNF). TRH is a tripeptidal hormone whereas CRH is a 41-amino acid peptide hormone and neurotransmitter (Fekete et al, 2000; Sarkar and Lechan, 2003). The role of brain BDNF in energy homeostasis comes from genetic mutation studies in which BDNF conditional mutant mice exhibited increased body weight and increased food intake compared to control (Rios et al., 2001). Further, BDNF infusion into the VMH of mice with deficient MC4R signaling suppressed hyperphagia and
excessive weight gain in response to a HF-diet (Xu et al., 2003). This result lead to the interpretation that BDNF may regulate energy balance downstream of MC4R.

**Brain stem.** The nucleus of the solitary tract/area postrema (NST/AP) in the medulla of the brain stem is also involved in integrating afferent signals and relaying them further to other regulatory centers in the CNS including the ARH (Harrold et al., 2012). Peripheral gustatory signals that include gastric distension as well as signals (CCK) involved in meal termination are integrated in the NST via receptors on the vagal nerve (Berthoud et al., 2002). Further, destruction of NST is shown to result in elevated consumption of palatable foods (Hyde and Miselis, 1983). Receptors for leptin, ghrelin, amylin and NPY and for components of the melanocortin system have also been identified in the brainstem (Fodor et al., 1994; Mountjoy et al., 1994; Dumont et al., 1998; Berthoud, 2002; Grill and Kaplan, 2002).

Monoamine neurotransmitters that regulate food intake and that are synthesized in brain stem areas include; norepinephrine and serotonin (5HT) (Schwartz et al., 2000). Norepinephrine is synthesized predominantly in the dorsal vagal complex and the locus coeruleus. Norepinephrine synthesizing neurons project to the spinal cord, hypothalamus, thalamus and cortex. Importantly, increased norepinephrine in the PVN and LHA is shown to promote hyperphagia and body weight gain in rats (Oltmans, 1983; Leibowitz et al., 1984). The 5HT system consists of cell bodies in the caudal brainstem that project
widely throughout the brain. 5HT-receptor signaling is shown to suppress food intake (Leibowitz et al., 1988). There are seven families of 5HT receptors (5-HT₁₋₇), comprising at least 14 distinct receptor subtypes out of which 5HT₁₅R, 5HT₂CR and 5HT₆R subtypes are shown to be the principal mediators of anorectic effects in rodents (Garfield and Heisler, 2009). Overall, brain stem mechanisms appear to be important in the regulation of energy balance.

Taken together, both the hypothalamus and the brain stem consist of distinct neuronal circuits, signaling molecules and reciprocal connections that regulate energy homeostasis. Additionally, peripheral satiety, hunger and adiposity signals are also integrated within the hypothalamic and brainstem circuits to regulate feeding and metabolism.

III. Non-homeostatic regulation of energy balance: The brain reward system

The brain reward system consists of neural circuits that regulate a) reward/saliency, b) motivation, c) learning/conditioning and, d) inhibitory control and executive function (Wise, 2006; Volkow et al., 2008, Kenny, 2011). Reward/saliency is mediated through nucleus accumbens (NAc), ventral pallidum, medial orbitofrontal cortex (OFC) and hypothalamus; motivation is mediated through dorsal striatum; inhibitory control and executive function is mediated through cingulated gyrus, dorsolateral prefrontal cortex (DLPFC) and
lateral OFC and, learning/conditioning is mediated through hippocampus, amygdala and dorsal striatum (Volkow et al., 2008).

Increased ease of access to palatable foods and overconsumption is responsible for the increasing prevalence of obesity (Volkow and Wise, 2005; Swinburn et al., 2009). Importantly, the rewarding and pleasurable properties of palatable foods trigger excessive consumption, despite having met the energy requirements. The metabolic consequences of food are regulated by homeostatic functions; however, the reinforcing and hedonic consequences of food are regulated by the reward system (Lutter and Nestler, 2009; Berthoud et al., 2011). In obesity, the brain reward system appears to override the homeostatic control of appetite, such that feeding behavior is regulated by hedonic rather than physiological needs (Flier, 2004; Palmiter, 2007; Shomaker et al., 2010).

Orosensory and post-ingestive consequences of palatable foods engender greater preference compared to less palatable foods, and promote excessive food consumption (Warwick and Weingarten, 1995; Sclafani, 2001; Macht and Mueller, 2007). Consumption of palatable foods is also associated with sensory reward processing of food and food-related cues that regulate preference and hedonic value of certain diets (Rolls, 2011). The role of the brain reward system in hedonic food consumption is evident from human imaging studies that employed the blood-oxygen level dependent (BOLD) functional magnetic resonance imaging fMRI technique. In these studies, compared to lean
individuals, obese humans exhibited enhanced baseline resting activity in the somatosensory cortex as well as greater activation of insula and midbrain areas upon tasting palatable foods (Wang et al., 2002a). These results suggest that obesity is associated with increased sensitivity to the rewarding properties of food. Human imaging studies have also demonstrated activation of brain reward relevant regions in response to palatable food-related visual and olfactory cues. Compared with non-food objects, viewing photographs of fattening foods resulted in significantly greater activation in the brainstem, hypothalamus, amygdala, DLPFC, OFC, insular cortex, dorsal striatum and NAc of healthy, non-obese women (Schur et al., 2009). In another study, obese women exhibited greater activation of the hippocampus in response to palatable food related odors, compared to lean control (Bragulat et al., 2010). Palatable food related odors elicited greater BOLD responses in the ventral tegmental area (VTA), NAc and medial prefrontal cortex (mPFC), compared to nonappetitive odors, when responses were collapsed across obese and control groups (Bragulat et al., 2010). Immediate early gene-expression (c-fos) studies conducted in rats following exposure to palatable foods demonstrated increased activation of reward-related brain regions that include; striatum, NAc, amygdala and limbic structures, consistent with human findings (Angeles-Castellanos et al., 2007). Recent animal studies also indicate the role of habenula in hedonic food intake. Deep brain stimulation of the later habenula was shown to decrease sucrose self-administration in rats (Friedman et al., 2011). Furthermore, lesioning of the lateral habenula resulted in delayed extinction response despite substitution of
sucrose with water, indicating increased sucrose-seeking behavior. Collectively, nonhomeostatic feeding behavior appears to be regulated by brain regions implicated in learning, reward processing and motivation.

Dopamine, one of the major neurotransmitters of the reward system, is known to facilitate conditioning to food stimuli as well as to regulate appetitive and food-motivated behaviors (Mark et al., 1994; Martel and Fantino, 1996; Wang et al., 2002b). The reinforcing properties of food are predominantly mediated by dopaminergic activation of the reward circuits (Epstein and Leddy, 2006). Ingestion of palatable foods results in dopamine release in the NAc and dorsal striatum, brain regions that mediate primary reward and motivation, respectively (Martel and Fantino, 1996; Small et al., 2003; Kelley, 2004).

In addition to dopamine, NAc-mediated signaling of opioids, gamma amino butyric acid (GABA), and glutamate have also been implicated in food reward. Opioid signaling in the NAc shell is shown to modulate food palatability and nonhomeostatic feeding (Peciña and Berridge, 2005). Intra-accumbens infusion of μ-opioid receptor agonist, D-Ala², N, Me-Phe⁴, Gly-ol⁵-enkephalin, in satiated rats resulted in increased consumption of palatable foods and also greater preference for HF- compared to high-carbohydrate diets (Zhang et al., 1998). Opioid-mediated stimulatory effects on palatable food ingestion was blocked by systemic administration of a non-selective opioid receptor antagonist, naltrexone. Stimulation of inhibitory GABA system and blockade of excitatory glutamate
system in the NAc shell is shown to produce increases in food intake (Kelley et al., 2005). Intra-accumbens shell injections of muscimol and baclofen, selective GABA-A and GABA-B agonists, respectively, increased feeding in satiated rats (Stratford and Kelley, 1997). Glutamatergic signaling in the NAc is shown to negatively regulate feeding behavior, as evidenced by stimulation of food intake upon blockade of AMPA/kainate glutamate receptors in the NAc shell (Maldonado-Irizarry et al., 1995; Reynolds and Berridge, 2003). The amygdala is another important component of the brain reward system that is known to regulate reward-mediated food intake. Importantly, reciprocal connections between the central nucleus of the amygdala and the NAc as well as the basolateral amygdala and forebrain regions have been suggested in the involvement of opioid-mediated eating and assessment of food palatability (Kim et al., 2004).

IV. Interaction between homeostatic and reward mechanisms

Emerging evidence demonstrates functional interaction between homeostatic and reward circuits that regulate food intake. Reciprocal connections have been reported between the NAc and the LHA (Stratford and Kelley, 1999). Importantly, peripheral hormones that include leptin, insulin and ghrelin are shown to module feeding behavior independent of their actions in the hypothalamus (Palmiter, 2007). Ghrelin levels rise upon fasting, and leptin and insulin levels increase with corresponding increases in fat and glucose levels,
resulting in activation of respective hypothalamic receptors to regulate food intake and increase energy expenditure. In addition to widespread distribution in the hypothalamus, leptin, insulin and ghrelin receptors are also shown to be co-localized with tyrosine hydroxylase, a marker of dopamine neurons and rate limiting enzyme of dopamine synthesis of (Figlewicz et al., 2003; Abizaid et al., 2006). Leptin inhibits dopamine neuron firing rate and decreases extracellular dopamine in the NAc (Krugel et al., 2003; Hommel et al., 2006). On the other hand, ghrelin activates dopamine neuron firing; however, the stimulatory action is shown to be dependent on glutamate signaling (Abizaid et al., 2006). Insulin is shown to increase dopamine transporter (DAT) mRNA, suggesting a reduction in dopamine signaling (Figlewicz et al., 1994; 2003).

Further, adiposity hormones are shown to influence food reward (Tzschentke, 2007). Rewarding effects of food can be measured using self-administration (operant procedures) and conditioned place preference (CPP). During operant procedures, animals are food deprived and trained to lever-press for food pellets in operant conditioning chambers. CPP is a behavioral conditioning paradigm using compartmentalised test chambers in which the animal is presented with a positive reinforcer (e.g., food) that is paired with distinct environmental cues (Bardo and Bevins, 2000). Preference for the compartment previously associated with the positive reinforcer provides an indicator of preference for that reinforcer. Decreased sucrose self-administration, decreased CPP for sucrose pellets and reversal of CPP for HF food was
demonstrated following intracerebroventricular infusions of insulin and leptin (Figlewicz et al., 2003, 2004, 2006). Leptin and insulin exert catabolic actions by decreasing food intake and increasing energy expenditure (Woods et al., 1996; Barzilai et al., 1997). While obesity is associated with increased leptin and insulin levels, decreased central leptin and insulin signaling in obesity results in reduced sensitivity to central catabolic effects (Levin and Dunn-Meynell, 2002; Levin et al., 2004; Clegg et al., 2005). Following leptin injections, obesity-prone rats exhibited decreased leptin-induced immunoreactive phosphorylated signal transducer and activator of transcription 3 (pSTAT3) expression in the hypothalamic arcuate, ventromedial, and dorsomedial nuclei, respectively (Levin et al., 2004). Intracerebroventricular injections of insulin were shown to decrease food intake in obesity-resistant rats; however, no such effect was found in the obesity-prone rats (Clegg et al., 2005). Thus, defective hypothalamic signaling of adiposity hormones leads to disruption in energy homeostasis. Also, defective leptin- and insulin-signaling in the dopamine reward pathway may result in disinhibition of dopamine neurons, thereby leading to increased dopamine signaling and food-motivated behavior.

Leptin regulation of the dopamine reward pathway is evident from studies conducted in leptin deficient \( \text{ob/ob} \) mice. Compared to wild type, \( \text{ob/ob} \) mice exhibited decreased levels of tyrosine hydroxylase in midbrain dopamine neurons, reduced evoked dopamine release in NAc, decreased somatodendritic vesicular stores of dopamine in the VTA and substantia nigra, and decreased
striatal D2 receptors (Fulton et al., 2006; Roseberry et al., 2007; Pfaffly et al., 2010). Importantly, peripheral leptin treatment ameliorated the prior existing dopaminergic deficiencies in \textit{ob/ob} mice (Fulton et al., 2006; Pfaffly et al., 2010). Furthermore, leptin receptor-expressing neurons in the LHA are shown to innervate the VTA (Leinninger et al., 2009). Also, leptin action on hypothalamic neurons is shown to restore tyrosine hydroxylase and mesolimbic dopamine levels in leptin-deficient mice (Leinninger et al., 2009). Diet-induced obese (DIO) rats exhibit leptin resistance in the VTA as evidenced by impaired leptin-stimulated phosphorylation of signal transducers and activators of transcription 3 (STAT3) (Matheny et al., 2011). In addition to the inhibitory effect of leptin signaling on striatal dopaminergic transmission, an effect of D2 receptors (D2R) on leptin signaling is suggested by decreases in circulating leptin levels following administration of D2R agonist, bromocriptine (Doknic et al., 2002; Kok et al., 2006). Thus, leptin and D2R signaling appear to act in a reciprocal manner to regulate homeostatic and hedonic aspects of feeding behavior. Taken together, energy balance appears to involve a complex crosstalk and functional interaction between homeostatic and reward mechanisms.

V. Role of macronutrients in food addiction

The increasing prevalence of obesity has been attributed to the ease of availability and excessive consumption of energy-dense foods as well as a sedentary lifestyle that lacks adequate physical activity (Drewnowski and Specter, 2004; Rolls, 2009). Compared to non-obese individuals, obese
individuals consume diets higher in energy density, as evidenced by a cross-sectional survey of food intake (Ledikwe et al., 2006). Importantly, energy density of fat is twice as high as that of protein and carbohydrates (Bray et al., 2004). In addition to increased energy density, sensory preferences for fat as well as levels of body fat are shown to positively correlate with amount of dietary fat (Dreon et al., 1988; Mela and Sacchetti, 1991). HF-diets are shown to produce greater weight gain than high-carbohydrate diets, as evidenced by both human and animal studies (Warwick and Schiffman, 1992). Further, HF-diets are shown to induce increases in body weight gain even if caloric intake does not exceed that of high-carbohydrate diet-fed controls (Warwick et al., 2002). Overall, dietary fat appears to be an important factor that regulates the development of obesity.

V a. Dietary preference and effects

Energy intake is influenced by internal episodic factors that include; hunger and satiety signals, as well as external factors such as availability and food properties. Measures of food preference serve as sensitive predictors of food consumption (Blundell and Hill, 1988). Preference for specific food types are modulated by orosensory properties and macronutrient composition (Drewnowksi et al., 1992; Sclafani, 2001). Importantly, proteins, fats and carbohydrates differ in energy density and generate distinct physiological responses that influence meal size and post-ingestive inhibition (Blundell and King, 1996).
Controversy exists regarding macronutrient-preference in obesity. While some human studies report increased preference and consumption of carbohydrates in obese compared to non-obese individuals, other studies suggest a major role of dietary fat in human obesity (Wurtman and Wurtman, 1984; Heraief et al., 1985; Lieberman et al., 1986). Obese women preferred sensory stimuli that are relatively low in sugar, but rich in fat, as evidenced by taste preference studies using sugar/fat mixtures (Drewnowski, 1985). In another study, obese women were shown to consume more fat calories than lean women (Romieu et al., 1988). Preferences for typical American foods that are major sources of carbohydrate, protein and fat calories were determined in obese men and women (Drewnowski et al., 1992). Obese men preferred protein/fat mixtures while obese women preferred carbohydrate/fat mixtures. Also, carbohydrates were listed less frequently, and only if they were also major sources of fat, or were sweet. Consistent with human findings, animal studies also demonstrate increased fat preference in obesity, as evidenced by a macronutrient choice procedure (Okada et al., 1992). Relative to inbred obesity-resistant (S5B\PI) rats, inbred obesity-prone (Osborne–Mendel) rats exhibited increased choice for fat compared to carbohydrate and protein diets. Generally, these studies suggest that obesity is associated with increased preference for foods that are a major source of fat.

Orosensory properties and post-ingestive effects of HF-diets engender greater preference and overeating (Kern et al., 1993; Warwick and Weingarten,
The significance of flavor and the influence of post-ingestive nutrient effects on food choice and intake have been investigated using self-regulated intragastric feeding techniques. Enhancing the flavor of a solution paired with intra-gastric carbohydrate infusions significantly increased carbohydrate selection and total energy intake (Ackroff and Sclafani, 2006). Further, when the flavors were equalized, infusions of a HF-diet stimulated greater overeating compared to infusions of an isocaloric high-carbohydrate diet (Warwick and Weingarten, 1995; Lucas et al., 1998). Thus, alterations in nutrient composition (fat-carbohydrate ratio) of the intra-gastric infusions appear to influence energy intake. Importantly, flavor and post-ingestive nutrient effects are not independent since nutrient feedback is known to influence flavor preferences and food consumption (Sclafani, 2001).

Recent studies have revealed a link between increased consumption of high-fructose corn syrup (HFCS) and the prevalence of obesity (Bray, 2008). Despite lack of differences in the number of total calories consumed, rats with access to HFCS for 12 h daily for 8 wk gained significantly greater body weight than rats with access to 10% sucrose for an equivalent duration of time (Bocarsly et al., 2010). Further, following long-term exposure to HFCS for 6-7 months, rats exhibited increased weight gain and fat deposition and also increased circulating triglyceride levels. HFCS contains a greater proportion of fructose compared to sucrose. Importantly, fructose molecules are transformed into glycerol and fatty acids, which are eventually taken up by adipose tissue, leading to increased
adiposity (Hallfrisch, 1990). Insulin released by dietary sucrose increases leptin release and thereby inhibits food intake (Saad et al., 1998). On the contrary, HFCS decreases circulating insulin and leptin levels (Teff et al., 2004). Thus, unlike sucrose, intake of fructose delays satiety thereby contributing to increased food intake and body weight. Taken together, long term consumption of HFCS appears to induce obesity-related parameters in animals; however, comparisons between HFCS and HF-diets are yet to be investigated.

Excessive intake of palatable foods that contain high-levels of fat and sugar has been attributed to the physiological effects of these diets on hunger and satiety peptides (Charlotte Erlanson-Albertsson, 2005). Rats fed a diet rich in unsaturated fat demonstrate elevated levels of the satiety hormone POMC, whereas no effects of saturated fat on POMC levels were found (Dziedzic et al., 2007). When infused into the ileum of healthy humans, triacylglycerols with unsaturated fatty acids increased CCK levels whereas triacylglycerols with saturated fatty acids did not (Maljaars et al., 2009). On the other hand, sucrose is shown to increase levels of hunger peptide NPY and satiety peptide α-MSH. Thus, differential expression of appetite regulating peptides may govern intake of specific food types.

The reward system has been implicated also in the hedonic consumption of high-energy dense foods (Blundell and King, 1996). Involvement of the opioid system in palatable food consumption is supported by increases in hypothalamic
expression of opioid peptides and opioid receptors upon ingestion of palatable foods (Kim et al., 1996; Welch et al., 1996). Further, consumption of palatable foods is shown also to activate the opioid system in the NAc (Zhang et al., 2003). Intracerebroventricular injection of opioids is shown to stimulate intake of sucrose and fat (Ookuma et al., 1997; Zhang and Kelley, 2002). Compared to less-palatable foods, ingestion of high-palatable foods is shown to induce greater dopamine release in NAc and striatum (Martel and Fantino, 1996; Small et al., 2003). These results suggest that sensory properties influence the rewarding effects of food. Nonetheless, the effect of specific macronutrients (HF, high-carbohydrate) on dopamine release is yet to be determined.

In contrast to dopamine, 5HT produces satiety that is suggested to be mediated by hypothalamic inhibition of NPY expression (Lawton et al., 1995; Halford and Blundell, 2000). Importantly, brain 5HT levels are shown to be affected by circulating levels of tryptophan and certain macronutrients (Halford and Blundell, 2000). Depressed patients exhibit deficiency in 5HT neurotransmission and therefore tend to compensate by overeating high-carbohydrate diets that increase brain serotonin turnover (Wurtman and Wurtman, 1995). Fenfluramine, an anorexic agent, which is believed to release 5HT as well as inhibit 5HT reuptake has been shown to specifically reduce fat intake (Lawton et al. 1995; Bray, 2001). Enterostatin, an anorexigenic peptide, produced by proteolytic cleavage of pancreatic procolipase in the gut has been shown to inhibit fat intake in rats (Erlanson-Albertsson, 1981; Erlanson-
Albertsson and York, 1997). Importantly, this inhibitory response is known to be mediated by both 5HT and opioidergic systems. Overall, the reinforcing effects of palatable foods appear to be regulated by vital neurotransmitters of the reward system that include dopamine, opioids and 5HT.

VI. Striatal dopaminergic system in obesity

Dopamine is a critical monoamine neurotransmitter that is known to regulate food intake by modulating appetitive motivational processes via the reward pathway (Wise, 2006). Ingestion of food results in dopamine release in the NAc, the primary reward center of the brain (Koob and Bloom, 1988; Bassareo and Di Chiara, 1999; Kelley, 2004). Importantly, dopaminergic activation of the reward pathway is dependent on the sensory and rewarding properties of food such that high-palatable foods induce greater dopamine release in the NAc compared to less-palatable foods (Martel and Fantino, 1996). Repeated stimulation of the dopaminergic system upon excessive consumption of palatable foods is believed to trigger neurobiological adaptations that may result in compulsive hedonic food intake (Volkow et al., 2008).

Dopaminergic activation in the NAc is critical for primary reward processes associated with food palatability (Szczypta et al., 2001; Wise, 2006). Nonetheless, the involvement of dopamine in the dorsal striatum appears to be critical for motivational process involved in general food consumption for survival.
Furthermore, striatal dopamine involvement in obesity is suggested by reductions in preference of palatable foods as well as general food consumption following tyrosine hydroxylase gene inactivation. While gene rescue in the NAc and striatum restored preference, only striatal rescue restored general consumption (Szczypka et al., 2001). Striatal dopamine involvement in motivation for food is supported also by high progressive ratio (PR) breakpoints in rats over-expressing striatal delta Fos B, a transcription factor that is believed to be expressed in response to sustained dopamine signaling (Olausson et al., 2006).

Dysfunction of the dopamine reward system has been implicated in obesity. Obese humans exhibit decreased striatal D2-receptor density and decreased striatal activity (BOLD signals), compared to non-obese individuals (Wang et al., 2001; Stice et al., 2008). TaqI A restriction fragment length polymorphism is downstream from the D2R gene and individuals carrying the A1 allele of the polymorphism exhibit 30–40% decreased striatal D2 receptor levels compared to those lacking the A1 allele (Jönsson et al., 1999; Ritchie and Noble, 2003). Similar to drug addiction, the TaqI A1 allele of the DRD2 gene also is suggested to influence susceptibility to obesity (Comings and Blum, 2000). Importantly, individuals with the A1 allele exhibit a strong inverse relationship between striatal response to food receipt and BMI (Stice et al., 2008). Also, A1 allele carriers’ exhibit decreased glucose metabolism in striatal and cortical brain areas that regulate hedonic responses to food (Jönsson et al., 1999).
Collectively, striatal D2 receptor function appears to regulate response to palatable foods, and diminished D2 receptor signaling may increase vulnerability to obesity.

Consistent with obese humans, decreased striatal D2 receptor levels were found in obese rats (Johnson and Kenny, 2010). Furthermore, lentivirus-mediated knockdown of striatal D2 receptors resulted in increased thresholds for electrical brain stimulation reward in rats with extended access to a palatable cafeteria diet, compared to lenti-control rats (Johnson and Kenny, 2010). Furthermore, striatal D2-knockdown was shown to induce compulsive food-seeking behavior in rats with extended access to a cafeteria diet, as evidenced by increased resistance to aversive stimuli-induced disruption of food consumption (Johnson and Kenny, 2010). Taken together, overconsumption of palatable foods and the development of obesity appear to trigger addiction-like neuroadaptive responses in the striatal dopamine system that may contribute to the development of compulsive eating.

VII. Dopamine neurochemistry

Dopaminergic pathways arise from projections originating from different brain regions that synthesize dopamine. The major dopamine pathways include: the mesolimbic, mesocortical, nigrostriatal and tuberofundibular pathways (Fig. 1). The mesolimbic pathways orginates from the midbrain VTA and innervates the NAc (ventral striatum), amygdala and olfactory tubercle and parts of the
limbic system and is known to mediate reward and motivation (Koob and Bloom, 1988; Koob, 1992; Kelley, 2004). The mesocortical pathway consists of dopaminergic projections from the VTA to different regions of the frontal cortex that influence learning, memory and inhibitory control (Moal and Simon, 1991; Koob and Volkow, 2010). The tuberoinfundibular pathways arises from dopamine synthesizing cells in the PVN and ARH and innervates the median eminence of the hypothalamus and the anterior pituitary gland where dopamine inhibits the release of prolactin hormone (Feldman et al., 1997; Vallone et al., 2000).

The nigrostriatal pathway consists of dopaminergic projections from the substantia nigra compacta which innervates the dorsal striatum (caudate-putamen). While dopamine function in the dorsal striatum is primarily involved in motor control, emerging evidence demonstrates the role of striatal in motivation and habit formation (Ito et al., 2002; Yin et al., 2005; Everitt and Robbins, 2005; Olausson et al., 2006). The involvement of striatum in motivation for food is supported by findings that rats over-expressing striatal delta Fos B (transcription factor expressed in response to sustained dopaminergic activation) exhibit increased progressive ratio (PR) responding for food. During PR responding, rats are trained to respond on a lever to obtain food reinforcers and the number of responses required to obtain a single food reinforcer is increased progressively within a behavioral session. The response ratio at which the rat ceases to respond is called the PR breakpoint, indicative of motivation (Richardson and Roberts, 1996). Habitual behavior is indicated by operant performance for reward.
that is insensitive to post-training changes in the value of the outcome (Yin et al., 2005). The development of a stimulus-response habit involving the dorsolateral striatum was demonstrated using the outcome devaluation procedure in rats with lesions in the dorsolateral striatum and sham control rats (Yin et al., 2005). Rats were trained to press a lever for sucrose reward delivered on an interval schedule. The sucrose was then devalued by pairing its consumption with illness induced by an injection of lithium chloride before the effect of this treatment was assessed in an extinction test (lever-press did not result in sucrose reinforcement). In contrast to sham operated controls, rats with dorsolateral striatum lesions reduced their lever performance upon devaluation of the outcome. These results indicate that operant performance in the rats with dorsolateral lesions was controlled by goal expectancy, such that devaluation of the goal decreased subsequent performance (Yin et al., 2005). Furthermore, microdialysis studies demonstrate that prolonged cocaine-seeking increases dopamine release in the dorsal striatum but not ventral striatum, suggesting the role of striatal dopamine in habitual behaviors (Ito et al., 2002). Additionally, autoradiographic studies demonstrate reductions in dopamine D2 receptors in the dorsal striatum following chronic, but not acute cocaine self-administration in monkeys, emphasizing the role of striatum in habit formation (Nader et al., 2002).

Dopamine is a catecholamine neurotransmitter synthesized in dopaminergic neurons that constitutes about 80% of the catecholamine content
in the brain (Feldman et al., 1997; Vallone et al., 2000). The synthesis, release and uptake mechanisms that regulate dopamine function are illustrated in Fig. 2. Dopamine is synthesized from the precursor aromatic amino acid L-tyrosine (Cooper et al., 2003). L-tyrosine undergoes hydroxylation into L-3, 4-dihydroxyphenylalanine (L-DOPA) in the presence of a rate-limiting catalytic enzyme, tyrosine hydroxylase and its cofactors, including Fe^{2+}, O_2 and tetrahydropteridine (Cooper et al., 2003). Further, L-DOPA undergoes decarboxylation to form dopamine. Decarboxylation occurs in the presence of the catalytic enzyme, aromatic L-amino acid decarboxylase (AADC) and cofactor, pyridoxal phosphate (vitamin B6). The synthesized dopamine is stored in synaptic vesicles of presynaptic terminals until release into the extracellular space.

Under the influence of an action potential at the nerve terminal, the synaptic membrane undergoes depolarization resulting in influx of calcium into the nerve terminal (Blaustein et al., 1978; Bean and Roth, 1991). Subsequently, calcium-dependent dopamine release into the extracellular space occurs as a result of fusion of the synaptic vesicles with the membrane. Extracellular dopamine binds to dopamine D1-like and D2-like receptors. Importantly, dopamine release is also modulated by presynaptic D2 autoreceptors such that D2 agonists inhibit and D2 antagonists enhance evoked release of dopamine (Roth, 1984; Dwoskin and Zahniser, 1986; Boyar and Altar 1987; Westerink and De Vries, 1989). Autoreceptors are shown to be localized at the soma, dendrites
and nerve terminals of dopaminergic neurons (Sesack et al., 1994). Stimulation of D2 autoreceptors in the somatodendritic region slows the firing rate of dopaminergic neurons, whereas stimulation of autoreceptors located on dopamine nerve terminals results in inhibition of dopamine synthesis and release (Wolf and Roth, 1990). In order to terminate dopamine signaling, dopamine is transported from the extracellular space back into the nerve terminal by the membrane dopamine transporter (DAT). Once in the cytosol, dopamine is packaged back into synaptic vesicles by the vesicular monoamine transporter-2 (VMAT2) for storage and subsequent release into the extracellular space. Cytosolic dopamine is metabolized into dihydroxyphenylacetic acid (DOPAC), by undergoing oxidative deamination in the presence of monoamine oxidase (MAO), a mitochondrial outer membrane-bound flavoprotein (Edmondson et al., 2004). Dopamine is also metabolized extraneuronally by catechol-O-methyl transferase (COMT) into 3-methoxy-4-hydroxyphenylacetaldehyde which is further metabolized into homovanillic acid by MAO (Grossman et al., 1992).

**VII a. Dopamine receptors**

Dopaminergic effects are mediated by specific dopamine membrane receptors. Dopamine receptors belong to the family of 7-transmembrane domain, G-protein coupled receptors (Gingrich and Caron, 1993). Following receptor synthesis in the endoplasmic reticulum (ER) and oligosaccharide processing in the Golgi apparatus, dopamine receptors are targeted to the plasma membrane (Missale et al., 1998; Drake et al., 2006). Activation of dopamine receptors
results in G-protein coupling that governs subsequent receptor-mediated effects. The agonist-occupied receptor is then internalized following phosphorylation by G-protein receptor kinases and β-arrestin binding. Following internalization, receptors either undergo recycling, trafficking back to the plasma membrane or get sorted for lysosomal degradation. These events may be dependent on the magnitude and duration of dopamine signaling. Desensitization (loss of activity) of dopamine receptors may be a result of sustained agonist-stimulation (Hausdorff et al., 1989).

On the basis of biochemical and pharmacological properties, dopamine receptors are further classified into D1-like and D2-like receptors (Missale et al., 1998). The D1-like receptor family includes D1 and D5 receptors, and the D2-like receptor family includes D2, D3 and D4 receptors. D1-like receptor subtypes couple to the G-protein Gs and activate adenylyl cyclase (Monsma et al., 1990). The D2-like receptor subtypes couple to the G-protein Gi, inhibit adenylyl cyclase and Ca^{2+} channels and activate K^{+} channels and arachidonic acid synthesis (Piomelli, 1993; Jackson and Westlind-Danielsson, 1994; Missale et al., 1998). While the D1-like receptors are exclusively postsynaptic, D2-like receptors are also expressed presynaptically, in addition to their postsynaptic expression. Specifically, the gene for the D2 receptor gives rise to two receptor isoforms, D2L and D2S receptors, that differ in the presence and absence of a 29 amino-acid segment in the third cytoplasmic loop, respectively (Missale et al., 1998). D2L receptors are primarily postsynaptic. On the other hand, D2S receptors are
located presynaptically and serve as autoreceptors. Importantly, autoreceptor-stimulation exerts a negative feedback on dopamine neurotransmission by inhibition of dopamine synthesis and release and increases in DAT function (Dwoskin and Zahniser, 1986; Westerink and De Vries, 1989; Wolf and Roth, 1990; Meiergerd et al., 1993; Cass and Gerhardt, 1994).

D1 receptors are mainly expressed in the dorsal striatum, NAc, olfactory tubercle, cerebral cortex, amygdala, island of Calleja and in the subthalamic nucleus (Jackson and Westlind-Danielsson, 1994). In contrast to the widespread expression of D1 receptors, D5 receptor expression is restricted to the hippocampus, lateral mammilary nucleus and the parafascicular nucleus of the thalamus. D2 receptors are predominantly expressed in the dorsal striatum, NAc core, olfactory tubercle, prefrontal, cingulate, temporal and enthorinal cortices, amygdala, hippocampus, hypothalamus, substantia nigra pars compacta and the VTA. Importantly, D2 receptors are also located peripherally in the retina, kidney, vascular system and pituitary gland (Jackson and Westlind-Danielsson, 1994).

D1-like receptors are shown to mediate gustatory learning and palatable food preference (Fenu et al., 2001; Cooper and Al-Naser, 2006). A conditioned taste aversion paradigm was employed to investigate the role of dopamine receptors in gustatory learning. This paradigm is shown to demonstrate Pavlovian learning induced by the association of the gustatory conditioned stimulus (saccharin or sucrose) with the malaise-inducing unconditioned stimulus
An unconditioned stimulus automatically triggers a response. A conditioned stimulus is the stimulus that over repeated exposure becomes associated with the unconditioned stimulus. D1 antagonist (SCH 23390) (12.5–50 μg/kg, s.c.), administered 5 min after the conditioned stimulus (saccharin solution), impaired the acquisition of conditioned taste aversion in a paradigm consisting of saccharin–lithium association. On the contrary, administration of the specific D2/D3 antagonist raclopride (100 and 300 μg/kg, s.c.) failed to impair the acquisition of conditioned taste aversion, indicating the specific role of D1 receptors in gustatory learning. Due to lack of a selective ligand for D5 receptors, the independent role of D5 receptors in feeding behavior is not clear. The role of D2 receptors in obesity is evidenced by increased thresholds for electrical brain stimulation reward in rats with lentivirus-mediated knockdown of striatal D2 receptors and extended access to a palatable cafeteria diet, as compared to lenti-control rats (Johnson and Kenny, 2010). Compulsivity is a behavior that persists despite negative health consequences (Koob and Volkow, 2010). Increased resistance to aversive stimuli-induced disruption of food consumption in striatal D2-knockdown rats indicates compulsive food-seeking behavior in rats with extended access to a cafeteria diet (Johnson and Kenny, 2010). Administration of SB-277011-A, a selective D3 receptor antagonist, is shown to attenuate sucrose-seeking behavior induced by sucrose-associated cue reintroduction in rodents, suggesting the role of D3 receptors in relapse (Cervo et al., 2007). In depth study of the specific role of the various dopamine receptor types in obesity is yet to be conducted.
VII b. Monoamine transporters: DAT and VMAT2

Dopaminergic neurotransmission mediates a wide array of functions including locomotor activity, cognition, reward mechanisms and motivated behaviors (Iversen and Iversen, 2007; Palmiter, 2008). The intensity and duration of dopamine signaling is dependent on the re-uptake of dopamine from the extracellular space back into the nerve terminals by plasma membrane DAT (Torres, 2006). Furthermore, cytosolic dopamine is transported back into the vesicles for storage and subsequent release by VMAT2 (Erickson et al., 1992; Schuldiner, 1994). Thus, both DAT and VMAT2 regulate extracellular dopamine concentrations, and thereby, represent critical mechanisms in the termination of dopaminergic effects at presynaptic and postsynaptic dopamine receptors.

i) DAT

DAT belongs to the family of Na⁺ and Cl⁻ dependent transporters, which includes transporters for other related monomines such as norepinephrine and 5HT as well as inhibitory neurotransmitters like GABA and glycine (Masson et., 1999). DAT also is considered as a marker of dopaminergic neurons due to its exclusive expression in dopamine-synthesizing neurons (Torres, 2006). Translocation of dopamine into the nerve terminal by DAT is accompanied by co-transport of two Na⁺ ions and one Cl⁻ ion (Reith et al., 1997; Rudnick, 1997). DAT consists of 12 transmembrane domains (TMDs), a large glycosylated loop between TMDs 3 and 4, and intracellular carboxy and amino terminal domains
(Shimada et al., 1991). Synthesis and assembly of DAT occurs within the ER and Golgi complex of neurons. Oligomerization of DAT has been suggested as an important mechanism for the efficient exit of DAT from the ER (Sorkin et al., 2003; Torres et al., 2003). Following proper folding and assembly within the ER, DAT oligomers interact with components of the coat protein complex II, and this interaction is known to regulate DAT transport from the ER to the Golgi complex for glycosylation (Sucic et al., 2011). Although DATs are targeted to the plasma membrane, they shuttle between the cell-surface and endosomal compartments. An immunofluorescent study conducted in rat dopamine neuronal cultures demonstrated predominant expression of DAT in the plasma membrane and recycling endosomes of presynaptic structures (Rao et al., 2011). On the contrary, in the somatodendritic compartments, DAT was detected in early, late and recycling endosomal compartments suggesting differential pathways and/or protein interactions regulating DAT trafficking mechanisms between cell-surface and endosomal compartments (Rao et al., 2011; Sager and Torres, 2011). Proteins that are known to regulate DAT degradation include clarithrin coat accessory proteins and ubiquitin ligases (Sorkin et al., 2006). Nonetheless, detailed molecular mechanisms associated with DAT degradation are not completely understood.

**Regulation of DAT function**
DAT-mediated clearance of extracellular dopamine requires effective targeting of DAT to the cell-surface of dopaminergic neurons (Zahnisier and Sorkin, 2004). Importantly, DAT activity is regulated by a wide array of cellular and exogenous factors that include; activation of intracellular second-messenger systems (Gulley and Zahnisier, 2003; Torres et al., 2003; Vaughan, 2004; Ramamoorthy et al., 2011), presynaptic G-protein coupled receptors (Meigerd et al., 1993; Cass and Gerhardt, 1994, Bolan et al., 2007; Zapata et al., 2007), protein-protein interactions between DAT and other transmembrane or cytoskeletal scaffolding proteins (Sager and Torres, 2011), and interaction with DAT substrates and inhibitors (Saunders et al., 2000; Loder and Melikian, 2003).

**Autoreceptor-mediated regulation of DAT.** Activation of D2 and D3 autoreceptors modulate DAT function. *In vitro* kinetic analysis conducted in striatal synaptosomes demonstrated increased $V_{\text{max}}$ of dopamine transport in the presence of D2/D3 agonist, quinpirole (Meigerd et al., 1993). This finding was further supported by *in vivo* electrochemical assays that reported inhibition of DAT function upon localized intrastriatal application of raclopride, a selective D2 receptor antagonist (Cass and Gerhardt, 1994). Addition of D2/D3 receptor agonist quinpirole to human embryonic kidney (HEK) cells co-expressing human DAT (hDAT) and D2S revealed increased DAT function and cell-surface expression (Bolan et al., 2007). Furthermore, D2S-mediated increase in DAT function was shown to be dependent on extracellular signal-regulated kinases 1 and 2 (ERK1/2) and independent of phosphoinositide 3 kinase (PI3K).
In vitro kinetic analysis and in vivo electrochemical studies revealed increased NAc DAT function upon treatment with PD128907, a selective D3 agonist (Zapata and Shippenberg, 2002). Furthermore, D3 receptor-mediated increases in DAT activity was also observed in HEK cells and mouse neuroblastoma cells co-expressing hDAT and D3 receptors (Zapata et al., 2007). In the same study, acute activation of D3 receptors was reported to enhance DAT function. Also, the D3-mediate increase in DAT function was shown to be PI3K and mitogen-activated protein kinase (MAPK) dependent, and was also associated with increased DAT cell-surface expression. On the contrary, sustained D3 receptor activation decreased DAT function and DAT cell-surface expression (Zapata et al., 2007). Thus, D2- and D3-mediated regulation of DAT function appears to occur via similar signaling mechanisms.

**Second-messenger systems.** Cellular signaling cascades that involve phosphorylation are a critical regulator of DAT activity. Importantly, DAT phosphorylation may result in alteration in intrinsic activity of the transporter, alterations in affinity for ligands, as well as alterations in membrane distribution of DAT (Mortensen and Amara, 2003). Protein kinases and phosphotases that affect DAT function include protein kinase C (PKC), protein kinase A (PKA), PI3K, protein tyrosine kinase, Ca^{2+}/calmodulin kinase, protein phosphotase 1 and MAPK families (Melikian, 2004; Vaughan, 2004; Foster et al., 2006; Hoover et al., 2007). The role of phosphotases in the regulation of DAT has been studied using a pharmacological inhibitor of PP1 and PP2A, okdaic acid (Vaughan et al.,
1997). Dose-dependent increases in DAT phosphorylation and decreases in dopamine uptake were found in striatal synaptosomes following okdaic acid treatment (Vaughan et al., 1997). Insulin signaling via tyrosin kinase receptors is critical for DAT function and expression (Doolen and Zahniser, 2001). Blockade of insulin receptor-mediated signaling with tyrosine kinase inhibitors revealed decreased dopamine clearance and decreased DAT expression (Doolen and Zahniser, 2001). Furthermore, inhibition of downstream components of the insulin signaling pathway that include PI3K and protein kinase B (Akt), have been shown to result in decreased DAT function and surface expression (Carvelli et al., 2002; Garcia et al., 2005).

PKC is the most extensively investigated kinase with respect to regulation of DAT function. While PKC, PI3K and MAPK signaling pathways influence phosphorylation of DAT serine and threonine residues, the regulatory effects of PI3K and MAPK on DAT is opposite to that of PKC (Schmitt and Reith, 2010). In contrast to PKC, activation of PI3K and MAPK results in upregulation of DAT activity (Carvelli et al., 2002; Lin et al., 2003). As described previously, these signaling cascades can be activated by GPCRs (D2 and D3 receptors). Further, PI3K and MAPK pathways also are essential for tyrosine kinase-mediated upregulation of DAT function and cell-surface expression (Hoover et al., 2007). On the contrary, PKC activation results in decreased $[^3H]$dopamine uptake and $[^3H]$mazindol (DAT ligand) binding to human DATs expressed in Xenopus oocytes, thereby suggesting a role of phosphorylation in promoting internalization
of plasmalemmal DAT (Zhu et al., 1997). Also, PKC-mediated DAT internalization was postulated as a result of increased clarithrin-dependent endocytosis and decreased recycling from endosomal compartments (Daniels and Amara, 1999; Loder and Melikian, 2003; Sorkina et al., 2005). Unfortunately, the relationship between PKC-mediated DAT phosphorylation at serine and threonine residues and DAT membrane distribution remains unclear. Nonetheless, it has been hypothesized that activation of PKC may result in activation of DAT-associated scaffolding or skeletal proteins that in turn regulate DAT trafficking (Vaughan, 2004; Foster et al., 2006). Taken together, distinct intracellular signaling cascades differentially regulate DAT function.

**Protein-protein interactions.** DAT interacting proteins can be classified as signaling molecules, GPCRs, presynaptic proteins and other miscellaneous proteins. Signaling molecules include protein phosphotase 2A (PP2A), PKCβ, cGMP-dependent protein kinase Iα and neuronal nitric oxide synthase. Interaction of PP2A and PKCβ with DAT has been demonstrated by co-immunoprecipitation (Bauman et al., 2000; Johnson et al., 2005). Importantly, PKCβ knockout mice exhibit decreased dopamine uptake by DAT and decreased cell-surface DAT expression, thereby suggesting that PKCβ facilitates DAT function (Chen et al., 2009). Other signaling molecules have been shown to co-immunoprecipitate with DAT; however, their precise role is yet to be established.
Functional regulation of DAT by GPCRs, namely D2 receptors, is evidenced by experiments in which D2 receptor stimulation resulted in increased DAT function and expression (Cass and Gerhardt, 1994; Bolan et al., 2007). Direct interaction between D2 and DAT resulting in a functional protein complex was revealed (Lee et al., 2007). The first 23 residues of the amino terminus of DAT co-immunoprecipitated with the D2 receptors from striatal lysates and conversely, residues 242-344 from the third intracellular loop of D2 receptors co-immunoprecipitated with DAT (Lee et al., 2007). Further, disruption of the D2-DAT complex by a cell membrane permeable interfering peptide decreased DAT function. An orphan GPCR, GPR37, has also been reported to co-immunoprecipitate with DAT. GPR37 null mice exhibit increased DAT function and plasma membrane DAT levels in striatal synaptosomes (Marazitti et al., 2004).

Presynaptic protein-α-synuclein has been shown to form a complex with presynaptic hDAT in cotransfected HEK-293 cells through direct binding of the non-A β amyloid component of α-synuclein to the carboxyl-terminal tail of hDAT (Lee et al., 2001). Further, the α-synuclein-hDAT complex formation has been shown to facilitate DAT trafficking to the membrane, thereby facilitating dopamine uptake. On the otherhand, overexpression of syntaxin1A decreased DAT function and cell-surface DAT expression in a non-neuronal LLCPK1 cell line (Cervinski et al., 2010). Also, overexpression of other miscellaneous proteins like Hic-5, PDZ domain containing protein interacting with C-kinase 1 (PICK1) and SCAMP2 in
HEK-293 cells transfected with hDAT revealed decreased DAT function and decreased cell-surface DAT expression (Torres et al., 200; Carneiro et al., 2002; Muller et al., 2006). Furthermore, functional interaction between DAT and synaptic vesicle protein, synaptogyrin-3 was revealed by studies in which overexpression of synaptogyrin-3 in PC12 and MN9D cells demonstrated increased DAT function without alterations in cell-surface DAT levels (Egana et al., 2009). Overall, protein-protein interactions regulate DAT trafficking and function.

**Regulation of DAT reverse transport**

In addition to dopamine uptake, reverse transport of dopamine via DAT occurs in the presence of pharmacological agents including amphetamines (Sulzer et al., 2005). DAT reverse transport of dopamine involves distinct second messenger systems and protein interactions. In both rat striatum and PC12 cells, direct PKC activation by phorbol esters has been shown to enhance amphetamine-induced dopamine efflux, whereas PKC inhibitors block this effect (Gianbalvo, 1992; Kantor and Gnegy, 1998; Kantor et al., 2001). Furthermore, PKC activation results in phosphorylation of N-terminal serines in DAT (Foster et al., 2002). Importantly, N-terminal phosphorylation of DAT has been shown to be essential for amphetamine-mediated dopamine efflux (Khoshbouei et al., 2004). Studies conducted using the patch-clamp technique in the whole cell configuration demonstrated that amphetamine-induced DAT reverse transport
requires intracellular Ca\textsuperscript{2+} that possibly activates PKC leading to DAT phosphorylation and DAT-mediated dopamine efflux (Gnegy et al., 2004).

In addition to PKC, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase \textalpha (CaMKII\textalpha) is also a critical regulatory component of amphetamine-induced dopamine efflux via DAT. Chronoamperometry measurements revealed decreased amphetamine-induced dopamine efflux in response to a CaMKII\textalpha inhibitor, K93 (Fog et al., 2006). Importantly, in the same study, a physical association between CaMKII\textalpha and DAT C-terminus was established and subsequent disruption of this association resulted in a decrease in the amount of DAT-mediated dopamine efflux evoked by amphetamine. CaMKII\textalpha activation inhibits Akt, a central component of the insulin signaling pathway (Wei et al., 2007). Therefore, amphetamine-induced decreases in dopamine uptake and expression may involve defective insulin signaling.

DAT association with syntaxin 1A also has been implicated in DAT reverse transport. Syntaxin1A is a SNARE component (soluble N-ethylmaleimide sensitive factor attachment protein receptor) that is critical for synaptic vesicular release. Syntaxin 1A interaction with DAT increases amphetamine-induced dopamine efflux (Binda et al., 2008). Furthermore, pharmacological inhibition of CaMKII revealed that the syntaxin 1A-DAT association in response to amphetamine requires CaMKII activity (Dipace et al., 2007). Based on these findings, a model for amphetamine-induced DAT reverse transport has been
formulated (Robertson et al., 2009). In the presence of amphetamine, CaMKIIα binds to the C-terminus of DAT that result in subsequent N-terminal phosphorylation facilitating syntaxin1A -DAT association. This series of events is postulated to promote the shift of DAT from an outward to inward facing conformation that facilitates dopamine efflux from the terminal into the extracellular space.

In addition to promoting dopamine efflux, amphetamines also decrease dopamine uptake function and cell-surface expression (Saunder et al., 2000). PKC activation leads to decreased DAT cell-surface expression; however, DAT residues required for PKC-induced DAT internalization are not required for amphetamine-induced DAT trafficking from the cell membrane (Daniels and Amara, 1999; Loder and Melikian, 2003; Boudanova et al., 2008). While PKC plays a prominent role in amphetamine-induced dopamine efflux, it may not be involved in amphetamine-induced DAT trafficking. Interestingly, in another study, PKC-induced regulation of DAT was attributed to the membrane localization of DAT (Foster et al., 2008). Briefly, within the membrane, DATs exist in two subpopulations, namely the lipid-raft DATs and non-raft DATs. PKC-induced phosphorylation of DAT was shown to occur to a greater extent in the lipid-raft DATs compared to the non-raft DATs. These results suggest that the non-raft population of DAT mediate amphetamine-induced DAT internalization, whereas the lipid-raft population is responsible for amphetamine-mediated dopamine efflux (Foster et al., 2008; Robertson and Galli, 2009).
**ii) VMAT2**

VMATs translocate monoamines from the cytosol into synaptic vesicles via a proton electrochemical gradient that is generated by the vacuolar type H+-adenosine triphosphotase (Yelin and Schuldiner, 2002). VMAT1 and VMAT2 are the two pharmacologically distinct VMAT isoforms and both contain 12 TMDs with intracellular amino and carboxy termini (Erickson et al., 1992; Erickson and Eiden, 1993). VMAT1 is expressed in chromaffin cells of the adrenal medulla and enterochromaffin cells of the intestinal tract and VMAT2 is expressed in monoaminergic neurons and sympathetic postganglionic neurons (Weihe et al., 1994; Peter et al., 1995; Erickson et al., 1996).

VMAT2 is solely responsible for dopamine transport from the cytosol into synaptic vesicles for storage and subsequent release and is also reported to have higher affinity for monamine substrates (Peter et al., 1994; Schuldiner, 1994). The proteins and pathways involved in VMAT2 synthesis, assembly and trafficking still remains obscure (Sager and Torres, 2011). However, VMAT2 is differentially localized to the synaptic vesicles in the nerve terminals and to the tubulovesicular structures in the somatodendritic compartments (Nirenberg et al., 1996, 1997). Hsc70, a chaperone protein was shown to colocalize with VMAT2 in synaptic vesicles (Requena et al., 2009). Physical and functional coupling of VMAT2 with tyrosine hydroxylase and AADC in the synaptic vesicles has been reported (Cartier et al., 2010). Taken together, a model was proposed in which
the chaperone protein Hsc70 was suggested to promote functional interaction between VMAT2, tyrosine hydroxylase and AADC that would facilitate dopamine synthesis in the synaptic vesicle membrane followed by loading into the vesicles by VMAT2 (Sager and Torres, 2011). The proposed mechanism also suggests minimization of intracellular dopamine diffusion, oxidation and toxicity. Thus, VMAT2 plays an important role in dopamine homeostasis.

VIII. Treatment options for obesity

Recent reports from the International Association for the Study of Obesity (IASO) and International Obesity Task Force (IOTF) indicate that about 475 million adults and up to 50 million children in the world are obese (IASO, 2012). Importantly, obesity leads to many serious diseases including dyslipidaemia, hypertension, stroke, myocardial infarction, type-2 diabetes and certain cancers (IASO, 2012). The increasing prevalence of obesity has resulted in several health and financial problems. Thus, prevention and treatment of obesity has become a major public health concern.

VIII a. Dietary intervention and exercise

Obesity is known to result from excessive consumption of high-energy density foods and decreased physical activity (Rolls, 2009). A negative energy balance induces weight loss and this can be achieved by decreasing intake of
energy-dense foods and increasing energy expenditure by exercise. The influence of energy density on weight loss was evident from a 1-year clinical trial conducted in obese men and women in which individuals were food restricted and different groups were provided foods with different energy density (Rolls et al., 2005). The magnitude of weight loss was greater with low-energy foods compared to that of high-energy foods. Decreased energy intake when accompanied with exercise is proven to accelerate weight loss. Exercise minimizes the loss of lean body mass and increases the metabolic rate thereby facilitating weight loss (Despres and Lamarche, 1993). Importantly, exercise also ameliorates the metabolic complications and co-morbidities of obesity that include heart diseases and cancer (Ross et al., 2000, 2004; Sigal et al., 2006).

**VIII b. Pharmacotherapeutic intervention**

A healthy diet and lifestyle by itself have failed to ameliorate the obesity epidemic, thereby necessitating the implementation of effective pharmacotherapeutic strategies along with dietary and lifestyle modification. The US FDA guidelines for registration of an anti-obesity therapy stipulate a weight loss of 5% more than placebo after 1 year of treatment, and the guidelines in Europe require a 10% weight loss from baseline, including effects of diet and exercise (Cawthrone, 2007). Anti-obesity agents induce weight loss by one of the following mechanisms that include increase in metabolism, increase in satiety, decrease in appetite and decrease in fat absorption. Despite compliance with
weight loss guidelines, the majority of the drugs launched for the treatment of obesity over the last two decades have been withdrawn due to noncompliance, safety profile and increased risk of cardiovascular and psychiatric complications.

The majority of past and current treatment options for obesity fall into two categories that include appetite suppressants and gastrointestinal fat blockers (Kordik and Reitz, 1999). Appetite suppressants that have been withdrawn include; 1) Amphetamines that reverse monoamine transporters, thereby increasing extracellular monoamine concentrations; 2) Fen-phen: A combination of fenfluramine and dexfenfluramine that elevates extracellular 5HT levels by stimulating 5HT release and inhibiting its reuptake; 3) Rimonabant, a cannabinoid receptor (CB1) antagonist; 4) Sibutramine, a 5HT and norepinephrine uptake inhibitor (Ioannides-Demos, 2011).

**Currently approved medication for obesity**

**Orlistat.** Orlistat ((S)-((S)-1-((2S, 3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl) 2-formamido-4-methylpentanoate) is the first lipase inhibitor for obesity that was approved by the FDA in 1999 and currently has been granted an “over the counter license” in the US and Europe (Heal et al., 2012). Importantly, orlistat is the only available for long-term treatment for obesity such that it induces weight loss and allows weight maintenance when used in conjunction with a reduced-calorie diet (Kushner, 2012). Orlistat is a gastrointestinal lipase inhibitor that
blocks fat absorption from the gut (Mcneeley and Benfield, 1998; Lucas and Kaplan-Machlis, 2001; Padwal and Majumdar, 2007).

The long-term efficacy of orlistat for weight loss has been demonstrated in several randomized controlled trials that range from 2-4 years of therapy (Hauptman et al., 2000; Heymsfield et al., 2000; Torgerson et al., 2004). Moreover, orlistat is shown to produce improvements in blood pressure, insulin resistance and serum lipid levels (Torgerson et al., 2004; Siebenhofer et al., 2009). The most commonly experienced side-effects of orlistat are gastrointestinal and include diarrhoea, flatulence, bloating, abdominal pain and dyspepsia (Siebenhofer et al., 2009). The FDA received 32 reports of serious liver injury in patients using orlistat between 1999 and October 2008, including 6 cases of liver failure (FDA drug safety communication, 2010). In May 2010 the label for orlistat was revised and a warning of severe liver injury was included.

Cetilistat (2-(Hexadecyloxy)-6-methyl-4H-3, 1-benzoxazin-4-one) is another gastric- and pancreatic-lipase inhibitor that is currently under development Alizyme and Takeda Pharmaceuticals (Charmot, 2012). Importantly, this drug is reported to exhibit fewer adverse effects compared to orlistat (Kopelman et al., 2007, 2010).

Lorcaserin (Belviq®). The ability of fenfluramines to suppress appetite by activation of 5HT receptors prompted the investigation of lorcaserin ((1R)-8-Chloro-1-methyl-2, 3, 4, 5-tetrahydro-1H-3-benzazepine), a selective 5HT2C
agonist, as a potential anti-obesity agent (Chakrabarti, 2009). Phase 3 clinical trials of lorcaserin (10 mg twice a day) have demonstrated effective weight loss compared to placebo, along with a good safety profile (Anderson et al., 2010; Fidler et al., 2010; Smith et al., 2010). The most frequent adverse events reported were headache, dizziness and nausea, but these were not significantly different between treatment and placebo groups. Unlike fenfluramine, there was no increase in the rate of cardiac valvulopathy following 2-year treatment with lorcaserin (Smith et al., 2010). The FDA recently approved Belviq (lorcaserin hydrochloride), as an addition to a reduced-calorie diet and exercise, for chronic weight management (FDA News Release, 2012a)

**Topiramate and phentermine (Qsymia).** Topiramate (2, 3: 4, 5-Bis-O-(1-methylethylidene)-beta-D-fructopyranose sulfamate) is a GABA agonist and an approved antiepileptic drug that has been evaluated in combination with phentermine (2-Amino-2-methyl-1-phenylpropane) for the treatment of obesity. Obese patients administered an extended release formulation of the combination Qsymia (topiramate-7.5 mg and phentermine-46 mg) demonstrated greater weight loss compared to those treated with either of the drug alone or placebo (Kaplan, 2005). Since this combination is shown to increase heart rate, regular monitoring of heart rate is recommended for all patients taking Qsymia (Pollack, 2010). The FDA recently approved Qsymia for obesity treatment (FDA News Release, 2012b).
Potential anti-obesity drugs under investigation: Monotherapy and combination therapy candidates

Withdrawal of several centrally-acting appetite suppressants over the last two decades has led to the development of novel pharmacotherapeutic options that provide promising long-term results in the amelioration of obesity and associated co-morbidities. In addition to the development of novel drugs, drugs that are approved for other indications also are being evaluated currently for the treatment of obesity (Gadde and Allison, 2009; Valentino et al., 2010). There are also several combination drug therapies in phase 3 clinical trials that include pramlintide and metreleptin, bupropion and naltrexone, bupropion and zonisamide, and phentermine and topiramate.

Monotherapy

Tesofensine. Tesofensine (1R, 2R, 3S, 5S)-3-(3, 4-dichlorophenyl)-2-(ethoxymethyl)-8-methyl-8-azabicyclo[3.2.1]octane) is a novel potent, non-selective uptake inhibitor of dopamine, norepinephrine and 5HT. Tesofensine was initially developed for the treatment of Alzheimer's and Parkinson's disease. Efficacy was not found for the treatment of these neurological conditions (Astrup et al., 2008a). Interestingly, meta-analysis of the results revealed that tesofensine (0.125-1.0 mg once daily) produced dose-dependent weight loss and that approximately 32% of obese patients achieved ≥5% weight reduction following
14 wk of treatment (Astrup et al., 2008b). While no effects on blood pressure were found, an increase in the dose of tesofensine produced increases in heart rate. Also, psychiatric complications were evident at the highest dose of tesofensine where 6.1% of obese subjects reported depressed mood compared with 0% on placebo (Astrup et al., 2008b). Moreover, these adverse events occurred in a patient group that had been pre-selected to exclude those with known psychiatric disorders. While adverse effects were reported in human obese patients, tesofensine revealed encouraging results in DIO rats. In addition to tesofensine-induced weight loss, the obese rats also exhibited reductions in abdominal and subcutaneous fat mass, reductions in plasma lipids and increased insulin sensitivity (Hansen et al., 2010). The ability to decrease body weight and improve various cardiometabolic risk factors in the DIO rat model supports its clinical development as an anti-obesity drug. Overall, tesofensine appears to be an impressive drug candidate for weight reduction; however, further development is required to eliminate the psychiatric side-effects.

**Pramlintide.** Pramlintide was originally used for the treatment of type-2 diabetes and is currently under investigation for the treatment of obesity (Gadde and Allison, 2009; Aronne et al., 2010). Pramlintide is a synthetic analog of the pancreatic peptide hormone Amylin. In a 4 month study, subcutaneous injections of pramlintide (120 µg three times a day and 360 µg twice a day) prior to meals, in conjunction with lifestyle intervention, demonstrated significant weight loss, reduced meal size and decreased food cravings in obese humans compared to
placebo group (Smith et al., 2007). The proposed anti-obesity mechanism is via decreases in appetite and increases in satiety through delayed gastrointestinal motility. However, nausea was reported as a common side-effect in patients treated with pramlintide. In another 16-wk phase 2 clinical trial employing obese subjects with or without type-2 diabetes, pramlintide (240 µg, three time a day) produced ≥5% weight loss compared to the placebo group (Aronne et al., 2007). Importantly, the extent of weight loss was not different in pramlintide-treated patients, either with or without nausea, indicating that the weight loss was not mediated by nausea. Pramlintide is also being explored for potential combination therapy as an anti-obesity agent.

**Liraglutide and exenatide.** Liraglutide and exenatide are GLP1 analogs that were developed originally and approved for the treatment of type-2 diabetes (Vilsbull et al., 2007). These analogs are known to exert their anti-obesity effect via stimulation of leptin secretion and by delay of gastric emptying. While liraglutide has entered phase 3 clinical trials, exenatide is currently being evaluated in phase 2 clinical trials for the treatment of obesity (Meade, 2009). Overweight patients treated with exenatide exhibited significant weight loss as well as an improvement in glycemic control, compared to the placebo group (Blonde, 2006). In addition to weight reduction, long-term use of liraglutide and exenatide has been shown to decrease systolic blood pressure and levels of glycosylated hemoglobin (Madsbad, 2009; Nauck et al., 2009).
Combination therapy

**Pramlintide combination therapy.** A combination of pramlintide and recombinant methyl human leptin, metreleptin, is being evaluated for the treatment of obesity (Ravussin et al., 2009). In a 24-wk phase 2 clinical trial conducted in obese patients, greater weight loss was demonstrated in patients treated with the combination of pramlintide (360 µg twice a day) and metreleptin (5 mg twice a day), compared to those treated with either one of the two drugs alone. Currently, pramlintide is also being evaluated in combination (in pairs) with sibutramine, phentermine and exenatide (Meade, 2009; Aronne et al., 2010). Common side-effects reported with these combination treatments are nausea, and increased heart rate and blood pressure (Aronne et al., 2010).

**Bupropion and naltrexone (Contrave).** Bupropion ((±)-2-(tert-Butylamino)-1-(3-chlorophenyl)propan-1-one), an inhibitor of dopamine and norepinephrine transporters, is used to treat depression and nicotine addiction (Dwoskin et al., 2006). Naltrexone (17-(cyclopropylmethyl)-4, 5α-epoxy-3, 14-dihydroxymorphinan-6-one) is a selective opioid receptor antagonist used to treat opiate and alcohol dependence (Heal et al., 2012). A combination of bupropion and naltrexone was explored as a potential treatment option for obesity since both these drugs are shown to activate POMC neurons and increase the release of the α-MSH, an anorexigenic neuropeptide (Cone, 2005; Greenway et al., 2009). An open-label 24 wk study using a sustained release formulation of naltrexone (32 mg) and bupropion (360 mg) combination was conducted in
overweight and obese women with depression (Mcelroy et al., 2010). In addition to weight loss and decreased food intake, significant improvements in depressive symptoms and glycemic control were achieved in the treatment group compared to placebo. The proposed mechanism of action of the bupropion-naltrexone combination is to inhibit the reinforcing and motivational aspects of food addiction (Kushner, 2008). Nausea was the most commonly reported adverse event.

**Bupropion and zonisamide.** Weight-loss is a common side-effect of the anti-convulsant drug, zonisamide, and this side-effect prompted the evaluation of this drug as a potential treatment option for obesity (Gadde et al., 2003). Zonisamide (1, 2-benzoxazol-3-ylmethanesulfonamide) is a potent inhibitor of carbonic anhydrase isoenzymes and this pharmacological mechanism has been proposed to mediate its weight-loss effect (De Simone et al., 2008). A 24 wk phase 2 clinical trial of the bupropion (360 mg)-zonisamide (360 mg) combination (sustained release formulation) demonstrated greater weight loss (9.2%) than either drugs alone or placebo (Greenway et al., 2006, 2009). The most common adverse events reported were headache, nausea and insomnia.

**VIII c. Surgical treatment for obesity: Bariatric surgery**

In 1991, the National Institutes of Health Consensus Development Panel indicated that patients eligible for bariatric surgery include those with a BMI>40 kg/m² and those who were well informed about the risks associated with surgical
procedures (Greenway, 1996). The most recent guidelines of the American Diabetes Association redefined the criteria for metabolic or bariatric surgery by indicating that individuals with a BMI $>$ 35 kg/m$^2$ and those with obesity-related co-morbidities are eligible candidates for bariatric surgery (Kirkman et al., 2009). Importantly, only obese individuals who are unable to achieve weight loss through diet, exercise and pharmacotherapy are recommended to opt for surgical procedures. Bariatric procedures are broadly classified into two categories that include restrictive procedures and diversionary procedures (Murr et al., 2010). Restrictive procedures include laparoscopic adjustable gastric banding (LAGB) and sleeve gastrectomy (SG), in which weight loss is predominantly achieved due to the restriction of nutrient intake as a result of reduction in stomach size (O’Brien et al., 2001; Karmali et al., 2010). Diversionary procedures include roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion with a duodenal switch (BPD-DS), which restricts food intake and also diverts nutrients from the stomach and duodenum (Murr et al., 2010). In RYGB, a small stomach pouch is created with a stapler device, and connected to the distal small intestine (Wickremesekara et al., 2005). BPD-DS is a more complex bariatric surgery that includes removal of a large part of the stomach, disconnection of the stomach from the duodenum, and connection to the distal part of the small intestine (Buchwald et al., 2009).

Bariatric surgery is shown to successfully induce sustained weight loss and reduce obesity-related complications including reduction in serum
triglyceride and cholesterol levels, and improvement in hypertension (Foley et al., 1992; Buchwald et al., 2005; Nugent et al., 2008). Side-effects of bariatric procedures include bleeding, infection, leaks from the site where the intestines are sewn together, diarrhea, blood clots as well as poor absorption of vitamins and minerals (Murr et al., 2010). Bariatric surgery is contraindicated in patients with depression, psychosis, binge eating disorders, current drug and alcohol abuse, cardiac diseases, severe coagulopathy and those with major anesthesia-related risks (Nandagopal et al., 2010). Success rates of bariatric surgery in children less than 18 years of age remains controversial.

In summary, despite the prevalence of obesity, the current treatment options are limited to bariatric surgery and a single approved anti-obesity medication, orlistat. The scarcity of pharmacotherapeutic interventions and withdrawal of several anti-obesity medications due to tolerance and safety issues reveals the complexity of the regulation of appetite and body weight. Recent anti-obesity approaches that include combination therapies targeted towards more than one pharmacological target are postulated to exert greater and long-term weight loss compared to monotherapies (Kushner, 2012). Overall, the most efficacious treatment for obesity appears to be a combination of dietary restriction, exercise and pharmacotherapy.

IX. Rationale for employing a DIO model in the current study
Obesity involves a complex interaction between central and peripheral hormonal factors, in both homeostatic and reward systems. In addition to increased high-caloric intake and decreased physical activity, the development of obesity is also strongly influenced by genetics (Mutch and Clement, 2006). Considering the complex etiology of obesity, the development and selection of appropriate animal models that reflect human obesity is crucial to elucidate the underlying physiological and molecular mechanisms and also for the evaluation of potential pharmacotherapies.

An appropriate animal model ideally provides the majority of pathophysiological characteristics associated with the disease under study, thereby attributing optimum face and predictive validity (Heidbreder, 2011). Research over the past two decades suggests rodents as the predominant model of human obesity (Vickers et al., 2011). Importantly, several neuroanatomical and functional similarities exist between rodents and humans, with respect to central and peripheral regulation of energy homeostasis. Also, rodents have a well developed orosensory and digestive system, enabling them to develop taste preferences and consume a wide variety of foods. Rodent models of obesity can be classified into two major categories, i.e, genetically-manipulated and environmentally-induced obesity models (Kanasaki and Koya, 2011). Additionally, obesity models have been generated by employing chemical and surgical techniques whereby, localized lesions were produced in different regions.
of the hypothalamus including PVN, ARH and VMH (Nemeroff et al., 1978; Bray et al., 1989; Tokunaga et al., 1989).

**Genetic models of obesity.** Single gene defects are shown to disrupt function of the leptin-melanocortin pathway, thereby resulting in human obesity (Farooqi and Rahilly, 2006). Extensive animal research has explored the genetic basis of obesity enabling the generation of obesity rat models from the propagation of specific rodent strains that exhibit spontaneous mutations of genes, receptors or proteins (Tschop and Heiman, 2001). Abnormal functioning of cellular enzymes may contribute to these spontaneous mutations resulting in homeostatic imbalance. In contrast to animal models generated from spontaneous mutants, genetically-engineered mutant models include transgenics and knockouts that are generated by disruption or overexpression of a specific gene, protein or receptor. Rodent models resulting from spontaneous or engineered mutations may result in one or more of the following conditions; hyperphagia, hypothermia, hyperglycemia, hyperinsulinemia, hormonal deficiency, early or late onset of obesity. Obesity strains identified and propagated based on spontaneous mutations are illustrated in Table 1. Mutant obesity strains derived by transgenic gene overexpression and disruption are illustrated in Table 2.

While genetically-engineered models have been extensively employed to study the role of endogenous factors, physiological pathways and the mechanism
of action of therapeutic compounds, interpretation of the results obtained from these studies is complex (Tschop and Heiman, 2001). Deletion or alteration of genes may trigger activation of compensatory mechanisms leading to misinterpretation of the normal contribution of the gene *per se* (Nelson, 1997). Importantly, gene products play active roles in different processes and pathways such that genetic alteration may cause modifications in the associated processes and pathways.

*Environmentally-induced obesity: The DIO model.* Genetically-engineered animal models of obesity have been developed based on single gene mutations identified in obese humans. However, owing to the polygenetic nature of obesity, one must exercise caution while employing genetic animal models to address questions regarding mechanisms involved in obesity (Korner et al., 2008). In addition to internal genetic factors, the prevalence of obesity is attributed to the increased ease of access to high-caloric palatable foods and decreased physical activity (Rahilly and Farooqi, 2008). Thus, obesity involves a complex polygenetic interaction with an obesogenic environment. Furthermore, inter-individual differences exists with respect to susceptibility to obesity such that some humans become obese and others resist the development of obesity despite exposure to an obesogenic environment (Sims et al., 1968). Interestingly, a similar differentiation into two distinct obesity-prone (OP) and obesity-resistant (OR) phenotypes was found in rodents that were exposed to a HF-diet. Rat
strains were shown to vary in their propensity to develop DIO (Schemmel et al., 1970).

The rodent model of DIO has been extensively used to study factors that underlie the development and maintenance of obesity, upon exposure to a high-caloric diet (Levin et al., 1997). Upon 90 days of exposure to a HF-diet, outbred Sprague Dawley rats exhibited a bimodal weight gain pattern in which approximately 50% of the rats employed developed obesity and the remaining rats were obesity-resistant (Levin et al., 1987). Compared to OR rats, the DIO OP rats exhibited hyperphagia, increased body weight and visceral adiposity, increased levels of cholesterol and triglycerides, hyperinsulinemia, hyperglycemia, defective leptin and insulin signaling as well as increased concentrations of angiotensin peptides suggesting obesity-related metabolic and cardiovascular complications (Levin et al., 1987, 1997, Dobrian et al., 2000; Levin and Dunn-Meynell, 2002; Boustanly et al., 2004; Clegg et al., 2005). Thus, the DIO model mimics human obesity with respect to several neurohormonal factors and cardio-metabolic conditions and represents polygenetic interactions with an obesogenic environment (Madsen et al., 2010; Vickers et al., 2011). Importantly, long-term metabolic and pharmacological characterization demonstrates that the polygenetic DIO model efficiently simulates human obesity conditions (Madsen et al., 2010; Vickers et al., 2011).
The predictive validity of genetic models is limited with respect to screening of anti-obesity agents (Vickers et al., 2011). This drawback is evidenced by failure of leptin-replacement therapy in normal obese patients. While leptin-replacement therapy produced dramatic weight loss in leptin-deficient monogenetic ob/ob mice and humans with congenital leptin deficiency, similar weight loss results were not found in clinical trials conducted in normal obese patients using recombinant leptin (Heymsfield et al., 1999; Farooqi and O’Rahilly, 2009). This failure was attributed to the polygenic nature of obesity which is associated with increased leptin-resistance in addition to increased leptin levels (Correia and Haynes, 2007). In contrast to the genetic models, the DIO model has been employed successfully to predict clinical efficiency of weight loss drugs including, d-fenfluramine, sibutramine, orlistat and rimonabant (Hauptman et al., 2000; Madsen et al., 2010).

Taken together, genetic models with spontaneous or engineered single gene mutations are crucial in understanding the physiological role of specific genes, receptors or hormones in the regulation of energy homeostasis (Tschop and Heiman, 2001; Vickers et al., 2011). Moreover, these genetic techniques provide an insight of novel-targets that can be explored for molecular intervention. However, since obesity is polygenic by nature, the environmentally-induced obesity model appears to mimic human obesity to a greater extent in comparison with genetic models. Thus, the DIO model appears to be an appropriate animal model for the investigation of mechanisms.
underlying the development and maintenance of obesity in response to an obesogenic environment.

X. In vivo methodologies employed to assess dopamine function and behavior in obesity

X a. No net flux microdialysis

Microdialysis is a widely used in vivo technique that enables the monitoring and quantification of neurotransmitters, peptides and hormones in the extracellular space (Smith et al., 1992; Chefer et al., 2006). Over the past few decades, the microdialysis technique has advanced significantly facilitating the analysis of monoamines, aminoacids, glucose and opioid peptides (Ungerstedt et al., 1982; Imperato and Di Chiara, 1984; Zetterstrom et al., 1988; De Boer et al., 1992; Bourdelais and Kalivas, 1992). Furthermore, microdialysis can be conducted in various organ/tissue systems (eg., adipose tissue, brain, heart, kidney) across species (eg., rats, dogs, rhesus monkey and rabbits) (Hallstrom et al., 1989, Kuzmin et al., 1992; Linderoth et al., 1992; Saunders et al., 1993; Wang and Sawchuck, 1995). The main principle underlying microdialysis is the law of diffusion, such that substances diffuse along their concentration gradient, from a region of higher concentration to a region of lower concentration, across the microdialysis membrane (Friedman, 1986). The impact of osmotic and
hydrostatic pressure differences across the membrane are considered to be negligible during microdialysis (Benveniste, 1989; Bungay et al., 1990).

The microdialysis technique has been extensively employed to monitor and quantify extracellular concentrations of neurotransmitters such as dopamine (Smith et al., 1992; Justice, 1993; Acri et al., 2001). The principal device employed in the microdialysis procedure is the probe that facilitates exchange of substances between the extracellular space in the tissue of interest and the perfusate. The microdialysis probe membrane is semi-permeable and typically allows diffusion of substances with molecular mass < 20,000 Da. While microdialysis is an invasive technique, experimental modifications over the years have limited successfully the extent of tissue damage. As opposed to the surgical implantation of the microdialysis probe itself, a sterile guide cannula is stereotaxically implanted in the animal’s brain (Chefer et al., 2009). Following recovery from surgery, a removable microdialysis probe is inserted into the animal’s brain and artificial cerebrospinal fluid (aCSF) is infused into the probe at a perfusion rate ranging from 0.3-3 µl/min. During continuous perfusion of aCSF along the length of the microdialysis probe, diffusion of analytes occurs across the probe membrane along the concentration gradient and the dialysate samples are collected for subsequent quantification. The sensitivity of the analytical technique employed for subsequent quantification governs the sample collection volume and perfusion time. Typically, dialysate collection times range from 1 to
20 min. A diagrammatic representation of microdialysis experimental set up and microdialysis probe is provided in Fig. 3.

Brain microdialysis experiments can be categorized into two types that include conventional microdialysis and the no net flux microdialysis technique. During conventional microdialysis, aCSF devoid of the neurotransmitter is perfused through the probe and the concentration of the neurotransmitter diffused into the probe is determined. Extracellular neurotransmitter concentration is governed by release, metabolism and uptake processes. Thus, interpretation of the data derived from conventional microdialysis experiments is limited such that the specific mechanism contributing to extracellular neurotransmitter concentration is not known. In contrast, in no net flux microdialysis, aCSF containing a range of concentrations of the neurotransmitter is perfused through the probe and the concentration of the neurotransmitter gained or lost by the probe is measured (Justice, 1993). The concentration range of the neurotransmitter is based on the anticipated concentration of that neurotransmitter in the extracellular space. The neurotransmitter concentration lost or gained by the probe is plotted as a function of the concentration perfused into the probe. Linear regression analysis of the no net flux plot yields the slope which is called extraction fraction or in vivo probe recovery ($E_d$) and the X-intercept, which is the point of no net flux, represents the extracellular neurotransmitter concentration.
Alterations in $E_d$ are regulated by changes in diffusional resistance or changes in flow rate (Chefer et al., 2009). Since the flow rate is held constant during the experiment, $E_d$ values are majorly governed by diffusional resistance. In comparison to the probe membrane and perfusate, the tissue offers maximum diffusional resistance to the analyte (Bungay et al., 1990). Resistance mechanisms in the tissue include tortuosity (diffusional path) and mechanisms that regulate analyte clearance (Bungay et al., 1990, 2003; Chefer et al., 2009).

Importantly, alterations in $E_d$ are shown to be dependent on changes in monoamine uptake and independent of release and metabolism processes (Smith and Justice, 1994; Bungay et al., 2003). Most importantly, studies have demonstrated a relationship between $E_d$ and processes that control neurotransmitter clearance. Pharmacological manipulations that inhibit synthesis, release or metabolism of dopamine were shown to produce alterations in extracellular NAc dopamine concentrations. However, only pharmacological inhibition of dopamine uptake produced decreases in $E_d$ (Smith and Justice, 1994). Furthermore, electrochemical and radioligand uptake techniques have demonstrated that alterations in $E_d$ provide a sensitive index of changes in dopamine uptake (Chefer et al., 2000, 2005; Thompson et al., 2000). Another study demonstrated reductions in $E_d$ for norepinephrine and serotonin upon administration of the norepinephrine uptake inhibitor, desipramine, and serotonin uptake inhibitor, paroxetine, respectively (Cosford et al., 1996).
Thus, no net flux microdialysis appears to be a useful in vivo technique that facilitates determination of extracellular neurotransmitter concentration as well as neurotransmitter uptake mechanisms. Importantly, this technique was employed in the current study to evaluate in vivo striatal DAT function and extracellular dopamine levels.

X b. Operant behavioral procedures to assess impulsivity and motivation

Obesity has been speculated to be a neurobehavioral disorder (Blum et al., 2006; Volkow and O'Brien, 2007). The American Psychiatric Association’s Diagnostic and Statistical Manual of Mental Disorders (DSM), list the diagnostic criteria for behavioral disorders that include depression, substance abuse and other personality disorders. Interestingly, the DSM-IV criteria for substance of abuse dependence consist of symptoms that also correspond to behaviors in obesity, few of which include persistent desire for food, maintenance of overeating despite knowledge of adverse physical and psychological consequences caused by excessive food consumption (Volkow and O'Brien, 2007). This striking similarity in behaviors suggests that obesity may be considered as “food addiction” and should be included as a mental disorder in DSM-V (Volkow and O'Brien, 2007).
The emerging neurobehavioral commonalities between obesity and drug addiction have facilitated the implementation of operant behavioral paradigms in obesity research. Operant conditioning procedures have been employed extensively to evaluate the rewarding and motivational aspects of drugs of abuse as well as food (Richardson and Roberts, 1996; Fleur et al., 2007; Hajnal et al., 2007; Baladi and France, 2009). During operant conditioning, rats are trained to perform an operant response to obtain reward (Lawrence and Illius, 1989). Behaviors are measured in operant conditioning chambers that consist of retractable levers for responding, hopper containing food pellet reinforcers, and a food tray (Fig. 4; top). Food is a powerful positive reinforcer whereby the rewarding effect of food increases the probability of a subsequent response (Epstein and Leddy, 2006). During operant procedures, animals are food restricted in order to increase hunger and decrease satiety, thereby facilitating operant responding for food.

Motivation for food reinforcement and lack of inhibitory control has been implicated in hedonic and nonhomeostatic intake of palatable foods (Volkow et al., 2008; Appelhans et al., 2011). In the current study, impulsivity and motivation were evaluated as behavioral mechanisms that may underlie DIO. Impulsivity was determined using the delay discounting task and motivation for food was evaluated using the PR schedule of reinforcement. Detailed information of these behavioral assays is provided in chapter three. However, a brief introduction to
the delay discounting task and PR schedule of reinforcement is provided in this section.

**Delay discounting task.** Impulsivity is a multidimensional behavioral trait that involves urgent actions, lack of premeditation and perseverance and increased sensation seeking behaviors (Evenden, 1999; Whiteside and Lynam, 2001). Different behavioral tasks have been employed to address different aspects of impulsivity that are dissociable at a neuroanatomical level (Winstanley et al., 2004; Roesch and Bryden, 2011). Behavioral procedures that are employed to evaluate impulsivity include go/no go task, stop-signal reaction time task and five-choice serial reaction task, that measure impulsive action, and the delay discounting procedure that measures impulsive choice (Winstanley et al., 2006). Delay discounting is defined by the choice of a smaller, immediate reward over a larger delayed reward and this choice of immediate reward is considered to model impulsive behavior (Ainslie, 1975).

During the delay discounting task, rats undergo operant training sessions that consist of forced trials and free trials (Evenden and Ryan, 1996). During the forced trials, rats are provided access to one of the two levers for a specific period of time during which the cue light above the extended lever remains illuminated. Responses on the extended lever results in delivery of either one or three food reinforcers. During the free trials, both levers are extended and both stimulus lights are illuminated. While one lever delivers one food reinforcer
immediately following a response, response on the other lever delivers three reinforcers following a delay. The delay to obtain the larger reward is either increased or decreased dependent on the choice of the lever that deliver three or one food reinforcer, respectively. Impulsivity is defined as a behavior that lacks inhibitory control (Whiteside and Lynam, 2001). With that perspective, greater choice for the immediate, smaller reward compared to the larger delayed reward is defined as an impulsive choice. Thus, the delay to discount the larger reward is inversely proportional to impulsivity (Fig. 4; bottom-left).

**PR schedule of reinforcement.** Considerable evidence indicates that alterations in the ratio of responses to rewards strongly influence operant behavior (Collier, 1980). Motivation for reinforcement is evaluated using operant procedures conducted using the fixed ratio (FR) and PR schedule of reinforcement. In the FR schedule of reinforcement, rats are required to emit a fixed number of responses to obtain a reinforcer (Lawrence et al., 1988). For example, during an FR-1 schedule of reinforcement, 1 response yields one reinforcer, during an FR-2 schedule, 2 responses yield one reinforcer and so on. Thus, during the FR schedule, the number of responses required to obtain a single reinforcer remains constant within an experimental session. During the PR schedule of reinforcement, the number of responses required to obtain a single reinforcer is increased progressively within an experimental session, whereby the animal works harder for every successive reinforcer (Hudos, 1961; Lawrence and Illius, 1989; Richardson and Roberts, 1996). Compared to FR, the PR schedule
for reinforcement provides a sensitive measure of motivation (Kennedy and Baldwin, 1972; Dantzer, 1976). The objective of conducting the PR schedule is to escalate the response requirement to obtain the successive reinforcer until the animal ceases to respond. The point at which the animal stops responding is known as the PR breakpoint (Richardson and Roberts, 1996). The breakpoint is also defined as the largest response ratio completed within an experimental session (Shippenberg and Koob, 2002). The breakpoint represents maximum effort expended to obtain reinforcement and hence is indicative of motivation for reward (Richardson and Roberts, 1996; Fig 4; bottom-right).

XI. Overall hypotheses and specific aims

Obesity is regulated by multiple neural networks that include the homeostatic and reward system. In addition to hypothalamic mechanisms, the elucidation of higher brain reward mechanisms in obesity is imperative for identification of potential targets for pharmacological intervention. The overall objective of the current dissertation research is to delineate neurobehavioral mechanisms that underlie the development of DIO. A rat model of DIO was used to investigate striatal dopamine function, impulsivity and motivation as neurobehavioral outcomes and predictors of obesity. The current dissertation research can be broadly classified into the outcome study and the predictor study.
**Outcome study.** Striatal D2 receptor density, VMAT2 function, DAT function and expression, extracellular dopamine, impulsivity and food-motivated behavior were evaluated as neurobehavioral outcomes of DIO.

**Hypothesis 1 (chapter two):** DIO is associated with decreased striatal D2 receptors, increased VMAT2 function, decreased DAT function and expression, and increased extracellular dopamine concentration. The specific aims formulated to test the hypothesis were.

**Specific aim 1.** As an outcome of DIO, striatal D2 receptor density was evaluated *in vitro* using $[^3]H$raclopride saturation analysis in striatal membrane preparations.

**Specific aim 2.** As an outcome of DIO, striatal VMAT2 and DAT function were evaluated *in vitro* using saturation analysis of $[^3]H$dopamine uptake into striatal vesicular and synaptosomal preparations, respectively.

**Specific aim 3.** As an outcome of DIO, methamphetamine-induced reverse transport of DAT was evaluated *in vitro* using superfused striatal slices.

**Specific aim 4.** As an outcome of DIO, striatal DAT cellular expression was evaluated using biotinylation and Western blot assays.

**Specific aim 5.** As outcomes of DIO, striatal dopamine uptake and extracellular dopamine concentration were evaluated *in vivo* using no net flux microdialysis.
**Hypothesis 2 (chapter three):** DIO is associated with increased impulsivity and increased food-motivated behavior. The specific aims that were formulated to test the proposed hypothesis follow.

**Specific aim 1.** As an outcome of DIO, impulsivity was evaluated using the delay discounting task.

**Specific aim 2.** As outcomes of DIO, food-motivated behavior and persistence of food-seeking behavior were evaluated using the PR schedule of reinforcement and extinction procedures, respectively.

**Predictor study.** Impulsivity, motivation for HF-reinforcers, striatal DAT function and extracellular dopamine concentration were evaluated as neurobehavioral antecedents of DIO to determine whether these factors serve as predictors of DIO.

**Hypothesis 3 (chapter four):** Pre-existing individual differences in the levels of impulsivity, motivation, striatal DAT function and extracellular dopamine concentration will predict the development of DIO in the rat model. The specific aims formulated to test the proposed hypothesis follow.

**Specific aim 1.** As predictors of DIO, impulsivity and food-motivated behavior were evaluated using the delay discounting task and PR schedule of reinforcement, respectively.

**Specific aim 2.** As predictors of DIO, striatal DAT function and extracellular dopamine concentration were evaluated *in vivo* using no net flux microdialysis.
Table 1

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<thead>
<tr>
<th>Spontaneous mutant rodent strains</th>
<th>References</th>
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<tr>
<td><strong>Except the dw/dw and ZDF rat strains that develop obesity in response to a HF-diet, remaining spontaneous mutant strains develop obesity in response to a standard chow diet</strong></td>
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<tr>
<td><strong>ob/ob mice</strong></td>
<td>Zhang et al., 1994; Campfield et al., 1995</td>
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<td>Leptin gene mutation results in leptin hormone deficiency</td>
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<td><strong>db/db mice</strong></td>
<td>Chen et al., 1996</td>
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<td>Leptin receptor mutation results in leptin resistance</td>
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<td><strong>fa</strong> mutation in Zucker, Koletsky and <strong>Zucker Diabetic Fatty (ZDF) rats</strong></td>
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<td>Leptin receptor mutation results in decreased surface receptor expression</td>
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<td>Disruption in posttranslational processing and secretion of pro-insulin and POMC</td>
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<td>**<strong>Growth hormone-deficient dwarf (dw/dw) rat</strong></td>
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<td>Ectopic expression of the agouti gene</td>
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Table 2

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<th>Genetically engineered mutant rodent strains</th>
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<td><strong>Except for the MCH over expressing mice that develop obesity in response to a HF-diet, remaining mutant strains develop obesity in response to a standard chow diet.</strong></td>
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<td>CRF overexpression in mice</td>
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<td>Adipose tissue-specific glucose transporter (GLUT4) overexpression in mice</td>
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<td>**MCH overexpression in mice</td>
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<td>Melanocortin receptor knockout mice (MC3 and MC4 )</td>
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<td>5-HT_{2C} receptor knockout mice</td>
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<td>Neuropeptide Y receptor knockout; NPYR-Y_1, NPYR-Y_2</td>
<td>Kushi et al., 1998</td>
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<td>Peroxisome proliferator-activated receptor alpha (PPAR_{α}) knockout mice</td>
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<td>Bombesin receptor 3 (BRS-3) knockout mice</td>
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<td>Brain specific, insulin receptor knockout mice</td>
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<td>Brown adipose tissue deficient mice</td>
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Figure 1: Saggital rat brain section displaying major dopamine reward pathways.
Figure 2

- Tyrosine
- L-DOPA
- Dopamine (DA)
- Tyrosine hydroxylase
- DOPA decarboxylase
- VMAT2
- MAO
- DOPAC
- DA
- DAT
- D1-like receptors
- D2-like receptors
- D2-autoreceptors
Figure 2: Schematic representation of dopamine synthesis, release, uptake and metabolism.
Figure 3: Diagrammatic representation of *in vivo* brain microdialysis.

Microdialysis set up (top); microdialysis probe (bottom).
Figure 4
**Figure 4:** Operant chamber and behaviors evaluated. Impulsivity and food-motivated behavior in rats were measured using behavioral procedures conducted in operant chambers (top). Impulsivity was measured using a delay discounting task in which rats were given a choice between an immediate small food reward and a delayed larger food reward. Response on one lever delivered one sucrose pellet immediately, whereas response on the other lever delivered three pellets after an adjusted delay. The delay to obtain the larger reinforcer served as the measure of impulsivity. The mean adjusted delay is inversely proportional to impulsivity such that rats with low mean adjusted delay are categorized as high-impulsive and vice-versa (bottom-left). Motivation for food reinforcers was evaluated using a PR schedule in which the number of responses required to obtain a single food reinforcer was increased progressively within a session. As the number of responses required to obtain a single food reinforcer is increased progressively, the response rate gradually declines. The response ratio at the end of the session or the point at which the rat ceases to respond is known as the PR breakpoint, indicative of motivation to obtain food reward (bottom-right).
Chapter Two

Evaluation of striatal D2 receptor density, VMAT2 function, DAT function and expression, and extracellular dopamine levels as outcomes of DIO

I. Introduction

Obesity is associated with greater preference for and non-homeostatic overeating of palatable, HF-foods (Drewnowski et al., 1992; Lucas et al., 1998; Sclafani, 2001; Gaillard et al., 2008). Both orosensory properties and nutritional content confer rewarding properties to food (Warwick and Weingarten, 1995; Martel and Fantino, 1996). A considerable body of evidence suggests that the ability of palatable foods to establish and maintain response habits and preference is mediated by dopamine function within the reward neurocircuitry (Wang et al., 2001; Small et al., 2003; Wise, 2006). Importantly, ingestion of palatable foods activates brain reward centers leading to dopamine release in the NAc and striatum, associated with primary reward and habit formation, respectively (Martel and Fantino, 1996; Small et al., 2003; Kelley, 2004). The resulting dopaminergic activation is known to mediate the primary reinforcing effects and incentive motivation for food, which further influence hedonic and non-homeostatic food intake in obesity (Berthoud, 2002; Wise, 2006).

NAc dopamine mediates primary reward; however, a shift in control from dopamine pathways in NAc to striatum is believed to occur coincident with the
development of habitual behaviors (Di Chiara et al., 2004; Koob and Volkow, 2010). Inhibition of dopamine synthesis by inactivation of the tyrosine hydroxylase gene results in reduced preference for palatable foods (Szczyapka et al., 2001). Gene rescue in NAc and/or striatum restores preference, whereas only striatal rescue restores food consumption. Repeated dopaminergic activation is known to cause expression of delta Fos B, a transcription factor that is hypothesized to regulate behaviors and neuroadaptions associated with dopaminergic signaling (Nestler et al., 2001). The involvement of striatum in motivation for food is supported by findings that rats over-expressing striatal delta Fos B exhibit high PR breakpoints (Olausson et al., 2006). Following extended access to HF-food, obese rats exhibit increased thresholds for electrical brain stimulation reward, increased resistance to aversive stimuli-induced disruption of food consumption and decreased striatal D2 receptors (Johnson and Kenny, 2010). In obese humans, both D2-receptor density and neuronal activity in striatum are decreased compared to non-obese individuals (Wang et al., 2001; Stice et al., 2008). Thus, dysregulated striatal function may underlie excessive food intake in obesity.

Extracellular dopamine concentrations are regulated by DAT and VMAT2, which translocate dopamine across the plasmalemma from the extracellular space into the cytosol, and from the cytosol into synaptic vesicles, respectively (Sulzer et al., 2005). Inhibition of transporter function and/or reversal of these striatal transporters by drugs of abuse increases extracellular dopamine
concentrations, and the resulting dopaminergic stimulation is known to mediate
drug associated reward (Fleckenstein and Hanson, 2003; Kahlig and Galli, 2003;
Riddle et al., 2005). The role of VMAT2 in food intake and obesity has not been
studied; however, knockout studies and genetic analysis implicate the role for
DAT in food intake. DAT-deficient mice exhibit increased extracellular dopamine
concentration and greater food intake, compared to wildtype-mice (Pecina et al.,
2003). Genetic-linkage analysis reveals human DAT gene polymorphisms with a
greater frequency of short alleles (7 or 9 repeats) to be associated with reduced
DAT expression and binge eating (Heinz et al., 2000; Fuke et al., 2001;
Shinohara et al., 2004). Thus, DAT appears to play a prominent role in regulating
binge eating. Importantly, elevated extracellular dopamine concentration as a
result of dysregulated VMAT2 and DAT transporter function may contribute to
excessive food intake and the development of obesity.

The current study investigated striatal dopaminergic function following the
development of DIO. Striatal D2 receptor density, VMAT2 function, DAT function
and expression, and extracellular dopamine concentration were evaluated as
outcomes of DIO.

**Hypothesis:** DIO is associated with decreased striatal D2 receptor density,
increased VMAT2 function, decreased DAT function and expression, and
increased extracellular dopamine concentration. The specific aims formulated to
test the hypothesis follow.
Specific aim 1. Determine striatal D2 receptor density as an outcome of DIO using saturation analysis of [3H]raclopride binding to striatal membrane preparations.

Specific aim 2. Determine striatal in vitro VMAT2 and DAT function as outcomes of DIO using saturation analysis of [3H]dopamine uptake into striatal vesicular and synaptosomal preparations, respectively.

Specific aim 3. Determine in vitro methamphetamine-induced reverse transport of DAT as an outcome of DIO using superfused striatal slices.

Specific aim 4. Determine in vitro striatal DAT cellular expression as an outcome of DIO using biotinylation and Western blot assays.

Specific aim 5. Determine in vivo striatal dopamine uptake and extracellular dopamine concentration using no net flux microdialysis.

II. Methods

II a. Materials. Adenosine 5'-triphosphate magnesium salt (ATP-Mg\(^{2+}\)), ascorbate oxidase, chromasolv\(^{\circledR}\), D-glucose, D-methamphetamine, dopamine hydrochloride, ethylenediaminetetraacetic acid (EDTA), N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), nomifensine maleate, pargyline hydrochloride, polyethyleneimine (PEI), (S)-sulpiride and Tris [hydroxyl methyl] aminomethane hydrochloride (Tris-HCl) were purchased from Sigma-Aldrich (St. Louis, MO). Perchloric acid (70%) was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Diazepam and ketamine hydrochloride were purchased from
N.L.S. Animal Health (Pittsburgh, PA). [³H]Dopamine (3, 4-ethyl-2-[N-
³H]dihydroxyphenyl-ethylamine; specific activity, 33.7 Ci/mmol), [³H]raclopride
(methoxy-³H; specific activity, 82.8 Ci/mmol) and tissue solubilizer (TS-2) were
purchased from PerkinElmer Life Sciences (Boston, MA). Phosphate buffered
saline (PBS), polyacrylamide, sodium dodecyl sulfate (SDS), Triton-X 100 buffer
and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA).
Immunopure immobilized monomeric avidin gel and sulfosuccinimidobiotin (sulfo-
NHS-biotin) were purchased from Pierce Chemical (Rockford, IL). Immobilon-P
transfer membranes (0.45 µm pore size) were purchased from Millipore Co
(Bedford, MA). Primary antibodies recognizing rat DAT (C-20; goat polyclonal
antibody), protein phosphatase 2A (PP2A; sc-13601; mouse monoclonal
antibody) and secondary antibodies; donkey anti-goat (sc-2020) and chicken
anti-mouse (sc-2954), were purchased from Santa Cruz Biotechnology, Inc.
(Santa Cruz, CA). β-Actin (A 5441, mouse monoclonal antibody was purchased
from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from
Fisher Scientific Co. (Pittsburgh, PA). (2R, 3S, 11bS)-2-Ethyl-3-isobutyl-9,10-
dimethoxy-2,2,4,6,7,11b-hexahydro-1H-pyrido[2,1-a]isoquinolin-2-ol (Ro4-1284)
was a gift from Hoffman-La Roche Ltd. (Basel, Switzerland). All other chemicals
were purchased from Fisher Scientific Co. (Pittsburgh, PA).

**II b. Animals.** Outbred adult male Sprague Dawley rats (350-400 g;
Charles River Laboratories Inc., Wilmington, MA) were housed individually in
solid-bottom cages with bedding and received standard rat chow (Teklad
mouse/rat diet (2018), 18% of calories from fat and total density of 3.1 kcal/g; Harlan Laboratories Inc., Indianapolis, IN) and water ad libitum. Prior to the initiation of experimental procedures, rats were fed standard rat chow for one week during acclimatization to the animal facility. All experimental procedures were approved by the Institutional Animal Care and Use Committee at University of Kentucky.

**II c. DIO model.** A well characterized, naturalistic DIO model employing outbred rats was used to mimic human obesity (Levin et al., 1997, Boustany et al., 2004). The model was previously characterized as exhibiting hyperphagia, increased visceral, epididymal and retroperitoneal adiposity, hypertension, increased plasma levels of angiotensin peptides, cholesterol and triglycerides, and defective leptin- and insulin-signaling (Dobrian et al., 2000; Levin and Dunn-Meynell, 2002; Boustany et al., 2004; Clegg et al., 2005). Rats were fed for 8 wk either a moderately HF-diet (HF; n = 24; D12266B, 31.8% kcal from fat and total density of 4.41 kcal/g) or a low-fat diet (LF; n = 6-8; D12489B, 10.6% kcal from fat and total density of 3.9 kcal/g; Research Diets Inc. New Brunswick, NJ). The rationale for including the LF group was to parse out the effects of obesity per se and diet per se. Importantly, the LF group serves as a more appropriate control than the standard chow group, since unlike standard chow, the protein content and source of macronutrients is not different between the LF- and HF-diets.
Food intake was determined daily and body weight determined 3-times weekly throughout the 8-wk dietary treatment period. During food intake measurements, the cages were scanned for spillage of any uneaten food particles in the bedding to determine the exact amount of food consumed. Following the 8-wk period, rats fed the HF-diet were segregated into OP and OR groups based on body weight gain (top and bottom third, respectively; n = 6-8 rats/group; Fig. 5) (Levin et al., 1997; Boustany et al., 2004). The rats with body weight gain intermediate to those with maximum and minimum body weight gain (OP and OR rats, respectively) were not employed for the neurobehavioral assays (Fig. 6: top). Energy intake was calculated by multiplying daily food intake (g) by total kcal/g of the respective diet consumed.

**II d. Experimental design and procedures.** To evaluate the dopaminergic outcomes of DIO, striatal D2 receptor density, VMAT2 function, DAT function and expression, and extracellular dopamine concentration were determined in OP, OR and LF groups. The details of each study follow.

**Study 1:** As an outcome of DIO, striatal D2 receptor density ($B_{max}$) and affinity ($K_d$) were evaluated *in vitro* using [$^3$H]raclopride binding saturation analysis. Between-subject differences in the kinetic parameters were determined in OP, OR and LF groups (n = 6 rats/group). Thus, treatment group was a between-subject factor and [$^3$H]raclopride concentration a within-subject factor. For these analyses, brains were obtained from OP, OR and LF rats previously employed to
determine other outcomes that included the evaluation of *in vivo* striatal DAT function (chapter 2, study 6) and extracellular dopamine concentration (chapter 2, study 6), followed by the evaluation of impulsivity (chapter 3, study 1).

**[^3H]Raclopride binding.** Striatal D2 receptor density was determined using saturation kinetic analysis of[^3H]raclopride binding according to previous methods (Sun et al., 2003). Rats employed in this study include those animals that previously underwent microdialysis (chapter 2, study 6). The alternate striatum (n = 6 rats/group), that was not implanted with a microdialysis probe during no net flux microdialysis experiments (chapter 2 study 6) were stored at -80°C for the binding assay. Striata were thawed and homogenized using a motor-driven glass Teflon homogenizer with 10 up-and-down strokes (500 rpm) at 0°C in 5 ml of 50 mM Tris-HCl buffer containing 120 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂ and 1 mM EDTA, pH 7.4. Homogenates were incubated with[^3H]raclopride (0.2-20 nM) in buffer (total volume, 250 μl) for 120 min at 22°C. Nonspecific binding was determined in the presence of (S)-sulpiride (10 μM), a selective D2 receptor antagonist, that displaces[^3H]raclopride bound to D2 receptors (Sun et al., 2003). Thus,[^3H]raclopride binding determined in the presence of (S)-sulpiride (10 μM) enables the detection of[^3H]raclopride that is bound to the entire membrane preparation, except the D2 receptors (nonspecific binding). Reactions were terminated by filtration onto buffer-soaked Unifilter-96 GF/B filter plates using a Packard Filter Mate Harvester (PerkinElmer Inc., Downers Grove, IL). Filter plates were washed 3 times with ice-cold buffer, dried
for 60 min at 45°C, bottom-sealed and each well filled with 40 μl of scintillation cocktail (MicroScint 20; Perkin Elmer Inc.). Radioactivity on the filters was determined by liquid scintillation spectrometry (TopCount NXT; PerkinElmer Inc.). Protein concentrations were determined with bovine serum albumin as the standard (Bradford, 1976).

Study 2. As an outcome of DIO, maximal velocity ($V_{\text{max}}$) and affinity ($K_{\text{m}}$) of [³H]dopamine uptake by striatal VMAT2 into synaptic vesicle preparations were evaluated in vitro using saturation analysis. Between-subject differences in kinetic parameters were determined in OP, OR and LF groups (n = 6 rats/group). Thus, treatment group was a between-subject factor and [³H]dopamine concentration was a within-subject factor. Rats in this study had no prior experimental manipulations.

**In vitro kinetic analysis of [³H]dopamine uptake into VMAT2.** VMAT2 function was assessed in striatal synaptic vesicle preparations from separate groups of OP, OR and LF rats (n = 6 rats/group) using saturation kinetic analysis of [³H]dopamine uptake, based on previous methods (Nickell et al., 2010). Vesicles were prepared by homogenizing striata with 10 passes of a Teflon pestle homogenizer (clearance ~ 0.009") in 14 ml of ice-cold sucrose solution (0.32 M sucrose and 5 mM sodium bicarbonate, pH 7.4). Homogenates were centrifuged at 2,000 $g$ for 10 min at 4°C, and resulting supernatants were centrifuged at 10,000 $g$ for 30 min at 4°C. Pellets were suspended in 2 ml of 0.32
M sucrose solution and exposed to osmotic shock via addition of 7 ml of ice cold water followed by immediate restoration of osmolarity by addition of 900 µl of 0.25 M HEPES and 900 µl of 1 M potassium tartrate solution. Samples were centrifuged at 20,000 g for 20 min at 4°C. Resulting supernatants were centrifuged at 55,000 g for 1 h at 4°C, followed by addition of 100 µl of 1.0 M MgSO₄, 100 µl of 0.25 M HEPES and 100 µl of 1.0 M potassium tartrate to the supernatants. Samples were centrifuged at 100,000 g for 45 min at 4°C. Final pellets were resuspended in 2.4 ml of assay buffer (25 mM HEPES, 100 mM potassium tartrate, 50 µM EGTA, 100 µM EDTA, 1.7 mM ascorbic acid, 2 mM ATP-Mg⁺², pH 7.4). Nonspecific [³H]dopamine uptake was determined in the presence of Ro-4-1284 (10 µM), a selective VMAT2 inhibitor (Nickell et al., 2010). Thus, vesicular [³H]dopamine uptake determined in the presence of Ro-4-1284 (10 µM) indicates [³H]DA uptake through mechanisms other than VMAT2 (nonspecific uptake). Reactions were initiated by addition of 100 µl of vesicular preparation to 350 µl assay buffer containing [³H]dopamine (0.1 nM-5 µM) in the absence or presence of 10 µM Ro4-1284; final assay volume was 500 µl. Assay tubes were incubated at 34°C for 8 min. [³H]Dopamine uptake was terminated by placing the samples on ice followed by rapid filtration through Whatman GF/B glass fiber filters (presoaked with 0.5% PEI in MilliQ water for 3 h). Filters were washed 3 times with ice-cold assay buffer containing 2 mM MgSO₄ (without 2 mM ATP-Mg⁺²) using a Brandel cell harvester (Model MP-43RS, Biochemical Research and Development Laboratories Inc., Gaithersburg, MD). Radioactivity
was determined by liquid scintillation spectrometry (Model B1600TR, Perkin Elmer Inc.). Protein concentrations were determined as previously described.

**Study 3.** As an outcome of DIO, maximal velocity ($V_{\text{max}}$) and affinity ($K_m$) of $[^3\text{H}]$dopamine uptake by striatal DAT into synaptosomal preparations were evaluated *in vitro* using saturation analysis. Between-subject differences in kinetic parameters were determined in OP, OR and LF groups (n = 6-8 rats/group). Thus, treatment group was a between-subject factor and $[^3\text{H}]$dopamine concentration was a within-subject factor. Rats in this study had no prior experimental manipulations.

*In vitro kinetic analysis of $[^3\text{H}]$dopamine uptake at DAT.* DAT function was determined using saturation kinetic analysis of $[^3\text{H}]$dopamine uptake into striatal synaptosomal preparations from separate groups of OP, OR and LF rats (n = 12-14 rats/group from two separate series of DAT uptake assays), based on previous methods (Zhu et al., 2003). Striata were homogenized in 20 ml of ice-cold sucrose solution (0.32 M sucrose and 5 mM sodium bicarbonate, pH 7.4) with 16 passes of a Teflon pestle homogenizer. Homogenates were centrifuged at 2,000 $g$ for 10 min at 4°C, and the resulting supernatants were centrifuged at 20,000 $g$ for 15 min at 4°C. Resulting pellets were resuspended in 2.4 ml of ice-cold assay buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO$_4$, 1.25 mM CaCl$_2$, 1.5 mM KH$_2$PO$_4$, 10 mM D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline and 0.1 mM L-ascorbic acid, saturated with 95% O$_2$/5% CO$_2$, pH 7.4).
Nonspecific $[^3$H]$dopamine uptake was determined in the presence of nomifensine (10 μM), a selective DAT inhibitor (Maragos et al., 2002). Thus, synaptosomal $[^3$H]$dopamine uptake determined in the presence of nomifensine (10 μM) indicates $[^3$H]$DA uptake through mechanisms other than DAT (nonspecific uptake). Reactions were initiated by the addition of 50 μl of synaptosomes to 350 μl assay buffer in the absence or presence of nomifensine, and incubated at 34°C for 5 min followed by addition of $[^3$H]$dopamine (0.1 nM-5 μM) in a final volume of 500 μl. Incubation continued for 10 min at 34°C. Reactions were terminated by the addition of 3 ml of ice-cold assay buffer, followed by filtration through Whatman GF/B glass fiber filters (presoaked with 1 mM pyrocatechol for 3 h to minimize nonspecific binding of $[^3$H]$DA). Filters were processed, radioactivity and protein concentrations determined as previously described.

**Study 4.** As an outcome of DIO, methamphetamine-induced reverse transport of DAT in superfused striatal slices was determined *in vitro* in OP, OR and LF groups (n = 6-8 rats/group). Thus, treatment group was a between-subject factor, and methamphetamine concentration and time were within-subject factors. Rats in this study had no prior experimental manipulations.

**Methamphetamine-evoked $[^3$H]$dopamine overflow assay.** Under normal physiological conditions, DAT translocates dopamine from the extracellular space into the presynaptic terminal; whereas methamphetamine
inhibits dopamine uptake and promotes DAT reverse transport, thereby increasing extracellular dopamine concentrations (Liang and Rutledge, 1982; Parker and Cubeddu, 1986; Dwoskin et al., 1988; Sulzer et al., 2005). DAT reverse transport was assessed in striatal slice preparations from separate groups of OP, OR and LF rats (n = 6-8 rats/group), based on previous methods (Miller et al., 2001). Coronal slices (500 µm, 6-8 mg) were incubated for 30 min in Krebs' buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1 mM NaH₂PO₄, 1.3 mM CaCl₂, 11.1 mM D-glucose, 25 mM NaHCO₃, 0.11 mM L-ascorbic acid, and 0.004 mM EDTA, pH 7.4, saturated with 95% O₂/5% CO₂ at 34°C), and then incubated for an additional 30 min in buffer containing 0.1 µM [³H]dopamine. Each slice was transferred to a superfusion chamber and superfused (1 ml/min) with Krebs' buffer containing pargyline (10 µM), an MAO inhibitor. After 60 min of superfusion, three samples (5 ml) were collected to determine basal [³H]dopamine outflow. Superfusion continued in the absence or presence of methamphetamine (1-30 µM) for 60 min to determine methamphetamine-evoked [³H]dopamine overflow as a result of DAT reverse transport. Using a repeated-measures design, each chamber containing a single slice was exposed to one methamphetamine concentration which remained in the buffer until the end of the experiment. At the end of the experiment, each slice was solubilized in TS-2 tissue solubilizer. Radioactivity in the superfusate samples and striatal slices were determined by liquid scintillation spectrometry as previously described.
Study 5. As an outcome of DIO, striatal DAT protein in total, intracellular and cell-surface fractions was determined in vitro using biotinylation and Western blot assay in OP, OR and LF groups (n = 8 rats/group). Treatment group was a between-subject factor, and individual fractions were within-subject factors. Rats in this study had no prior experimental manipulations.

**Biotinylation and Western blot assays.** DAT cellular expression was determined in separate groups of OP, OR and LF groups (n = 8 rats/group) using biotinylation and Western blot assays, as previously described (Zhu et al., 2005). β-Actin was used to monitor protein loading within a fraction (total, non-biotinylated (intracellular) and biotinylated (cell-surface) fraction). To monitor the integrity of the samples, PP2A was used because this protein is located predominantly in the intracellular compartment, and should not be biotinylated since biotin is cell-impermeable. Only cell-surface proteins are expected to be biotinylated.

Striatal synaptosomes (500 μg protein/sample) were incubated for 1 h at 4°C with continual shaking in 500 μl of 1.5 mg/ml sulfo-NHS-biotin in PBS/Ca/Mg buffer (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 9.6 mM Na₂HPO₄, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.3). After incubation, samples were centrifuged (8,000 g, 4 min, 4°C). In order to remove the free sulfo-NHS-biotin, the resulting pellets were resuspended in 1 ml of ice-cold 100 mM glycine in PBS/Ca/Mg buffer and centrifuged (8,000 g, 4 min, 4°C) and resuspended three times. Final
pellets were resuspended in 1 ml of ice-cold 100 mM glycine in PBS/Ca/Mg buffer and incubated with continual shaking for 30 min at 4°C. Samples were then centrifuged (8,000 g, 4 min, 4°C). Resulting pellets were resuspended in 1 ml of ice-cold PBS/Ca/Mg buffer and centrifuged (8,000 g, 4 min, 4°C) and resuspended and centrifuged twice more. Final pellets were lysed by sonication for 2-4 s in 300 μl of Triton X-100 buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, 250 μM phenylmethysulfonyl fluoride, pH 7.4), followed by incubation and continual shaking for 20 min at 4°C. Lysates (300 μl) were centrifuged (21,000 g, 20 min, 4°C).

An aliquot of the supernatant fraction (100 μl) was assayed for total DAT immunoreactivity. The remaining supernatant volume was incubated with continuous shaking in the presence of monomeric avidin beads in Triton X-100 buffer (100 μl/sample) for 1 h at room temperature followed by centrifugation (17,000 g, 4 min, 4°C). The resulting supernatant (300 μl; fraction) represented the non-biotinylated fraction, and was assayed for intracellular immunoreactive DAT. Laemmli buffer was added to the total synaptosomal and non-biotinylated fractions before samples were stored at -20°C (1:1 ratio of Laemmli buffer and respective striatal fraction). Pellets from the previous centrifugations contained the avidin-adsorbed biotinylated proteins, which were then resuspended in 1 ml of 1% Triton X-100 buffer and centrifuged (17,000 g, 4 min, 4°C), and this was repeated twice. Final pellets were incubated with 100 μl of Laemmli buffer (62.5
mM Tris-HCl, 20% glycerol, 2% SDS, 0.05% β-mercaptoethanol, and 0.05% bromphenol blue, pH 6.8) for 20 min at room temperature to elute the biotinylated cell-surface proteins. Following incubation, samples were centrifuged (17,000 g, 4 min, 4°C) and 100 μl of the resulting supernatant (biotinylated fraction) were stored at -20°C until assay for cell-surface, immunoreactive DAT.

Immunoreactive DAT protein in total, non-biotinylated (intracellular) and biotinylated (cell-surface) fractions were determined using gel electrophoresis and Western blot assay. Samples were thawed; proteins were separated by 8% SDS-polyacrylamide gel electrophoresis for 90 min at 150 V and transferred to Immobilon-P transfer membranes (0.45-μm pore size; Millipore Corporation, Billerica, MA) in transfer buffer (50 mM Tris, 250 mM glycine, 3.5 mM SDS) using a Trans-Blot Electrophoretic semi-dry transfer cell (Bio-Rad) for 60 min at 25 V. Transfer membranes were incubated with blocking buffer (5% dry milk powder in PBS containing 0.5% Tween 20) for 1 h at room temperature. DAT and control protein immunoreactivity were assessed in parallel. Transfer membranes containing DAT protein (75 kDa mol wt) and control proteins (β-actin: 42 kDa, PP2A: 34 kDa) were incubated overnight at 4°C with goat polyclonal DAT antibody (1:500 dilution in blocking buffer) and with mouse monoclonal β-actin and PP2A antibodies (1:10,000 and 1:500 dilution in blocking buffer, respectively). Transfer membranes were washed four times with blocking buffer at room temperature. Transfer membranes containing DAT were incubated for 1 h at 22°C with donkey anti-goat DAT secondary antibody (1:1000 dilution in
blocking buffer). Transfer membranes containing β-actin and PP2A were incubated for 1 h at 22°C with chicken anti-mouse secondary antibody (1:5000 dilution in blocking buffer). Chemiluminescence was used to detect the blots by spraying the membranes with Hy GLO™ chemiluminiscent (CL) horseradish peroxidase antibody detection reagent (Denville Scientific Inc., Metuchen, NJ) followed by development using hyblot CL autoradiography film (Denville Scientific Inc.). Multiple autoradiographs were obtained using different exposure times, and immunoreactive bands were quantified by densitometric scanning using Image J software (NIH, Bethesda, MD).

**Study 6.** As outcomes of DIO, striatal dopamine uptake (extraction fraction) and extracellular dopamine concentration were evaluated *in vivo* using no net flux microdialysis in OP, OR and LF groups (n = 8 rats/group). Treatment group was a between-subject factor and dopamine concentration was a within-subject factor. Rats employed in this study were also used for the behavioral assessment of impulsivity (chapter 3, study 1) followed by the evaluation of the kinetic parameters of D2 receptor binding (chapter 2, study 1).

**No net flux microdialysis.** *In vivo* striatal DAT function and basal extracellular dopamine concentration were evaluated using the no net flux microdialysis method as previously described (Acri et al., 2001). Rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and diazepam (5 mg/kg, i.p.) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL).
An intracerebral guide cannula (MD 2251, Bioanalytical System Inc., West Lafayette, IN) was implanted. Stereotaxic coordinates were: AP = 1.2 mm, anterior to bregma: ML = 2.8 mm, lateral to midline and DV = -4 mm ventral to dura (Paxinos and Watson, 1986). The cannula was fixed to the skull using stainless steel screws and dental cement. Following surgery, rats were administered carpofen (5 mg/kg, s.c.) and allowed to recover for 3 days in the home-cage. Then, a microdialysis probe (MD 2200, Bioanalytical System Inc.) was inserted into the guide cannula, such that it extended 2 mm beyond the end of the cannula. The probe tubing was suspended through a swivel spring assembly attached to the wall of the Plexiglas chamber (25 × 44 × 38 cm), and connected to a microinfusion pump (KDS250, Holliston, MA). The microdialysis probe was perfused at 1.2 μl/min for 120 min, and after that at 2.2 μl/min for 90 min with aCSF (145 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1.2 mM CaCl2, 2 mM sodium phosphate, 0.25 mM ascorbic acid, 5.4 mM D-glucose, pH 7.4, filtered through a 0.2 μm sterile filter). Probes were perfused (2.2 μl/min) with aCSF containing 0, 5, 10 and 20 nM dopamine (dopaminein) in a randomized order of presentation for 60 min each (20 min for equilibration and 40 min for sample collection; dopamineout). Dialysates were collected in tubes containing 5 μl of 0.1 N perchloric acid, frozen on dry ice and stored at −80°C until HPLC analysis. Dopamine standards were prepared and frozen coincident with dialysate.

Dopamineout was quantified using HPLC with electrochemical (EC) detection as described previously (Nickell et al., 2010). Dialysates and dopamine
standards were thawed, ascorbate oxidase (30 μl; 81 U/ml) added to each sample (30 μl) and the resulting solution analyzed by HPLC-EC. The HPLC-EC consisted of a model 126 pump and model 508 autosampler (Beckman Coulter Inc., Fullerton, CA), an ODS Ultrasphere C18 reverse-phase 3 μm column (80 mm × 4.6 mm), a Coulometric-II detector with model 5020 guard cell (+0.60 V) and a model 5011 analytical cell maintained at 50 mV and 350 mV (ESA Inc., Chelmsford, MA). HPLC mobile phase was 0.07 M citrate/0.1 M acetate buffer (175 mg/L octylsulfonic acid-sodium salt, 650 mg/L of NaCl and 7% methanol, pH 4; flow rate, 1.5 ml/min). Separations were performed at room temperature, and 5 to 6 min was required to process each sample. Retention time of dopamine standards was used to identify dopamine peaks. Peak heights were measured to quantify the amount of dopamine based on standard curves generated before and after sample analysis using 32 karat software (Beckman Coulter). Detection limit for dopamine was 1 pg/100 μl.

Following the behavioral assessment of impulsivity (chapter 3, study 1), whole brains were obtained and fixed in Chromasolv® for sectioning (Leica 1850 M, Nussloch, Germany) for determination of probe placement.

**Il e. Data analysis.** Data are presented as mean values ± S.E.M., and ‘n’ represents the number of animals tested for each group. Data were analyzed using GraphPad Prism 4.0 program (GraphPad Software Inc., San Diego, CA) and SPSS version 9.0 (SPSS Science, Chicago, IL).
Time dependent differences in body weight and energy intake between groups were determined by two-way ANOVAs, with OP, OR and LF as a between-subject factor and time as a within-subject factor. One-way ANOVAs were used to analyze between-group differences in body weight, energy intake and % body weight gain after 8 wk of dietary treatment.

Specific [\(^3\)H]raclopride binding to D2 receptors and specific [\(^3\)H]dopamine uptake at VMAT2 and DAT were determined by subtracting nonspecific binding or uptake from total binding or uptake (Fig. 7). One-way ANOVAs determined between-group differences in D2 receptor density (B\(_{\text{max}}\)) and affinity (log K\(_d\)) for [\(^3\)H]raclopride binding, and maximal velocity (V\(_{\text{max}}\)) and affinity (log K\(_m\)) for [\(^3\)H]dopamine uptake.

For methamphetamine-evoked [\(^3\)H]dopamine overflow, each superfusate-[\(^3\)H] was divided by tissue-[\(^3\)H] at the time of sample collection and expressed as percentage of tissue-[\(^3\)H]. Basal [\(^3\)H]dopamine outflow was determined from the 3 samples collected prior to methamphetamine exposure. To determine between-group differences in methamphetamine-evoked [\(^3\)H]dopamine outflow in striatal superfusates, a three-way ANOVA was conducted on the time-course data with treatment group as a between-subject factor and methamphetamine concentration and time as within-subject factors. Methamphetamine-evoked [\(^3\)H]dopamine overflow was calculated by summing increases in superfusate-[\(^3\)H]

For Western blot analysis, DAT densitometry values were normalized to its respective $\beta$-actin band to account for variations in loaded protein and corrected by multiplying by the sum of the volume in each fraction (100 µl total + 300 µl non-biotinylated fraction + 100 µl biotinylated fractions) divided by the specific fraction volume applied to SDS–polyacrylamide gel (20 µl, total; 40 µl, non-biotinylated; 10 µl, biotinylated). This value was divided by the ratio of the fraction volume to the synaptosomal volume (100:300 for total; 200:300 for non-biotinylated and biotinylated fractions). One-way ANOVAs determined between-group DAT density differences within each fraction.

For no net flux microdialysis, the difference between perfusate and dialysate dopamine concentration [$dopamine_{in} - dopamine_{out}$] for each rat was plotted as a function of perfusion concentration [$dopamine_{in}$]. Slope of the linear regression represents extraction fraction ($E_d$: index of DAT function) and the regression line x-axis intercept represents the no net flux point (extracellular dopamine concentration) (Justice, 1993). One-way ANOVAs analyzed between-group differences in extraction fraction and extracellular dopamine concentration.
*Post hoc* analyses were conducted using Tukey’s or Newman Keul’s test, as appropriate.

III. Results

**III a. Time course of body weight gain and energy intake during the 8-wk exposure to the HF- and LF-diets**

Body weight and energy intake for OP, OR and LF groups during the 8-wk exposure to the HF- and LF-diets are illustrated in Fig. 6. Analysis of the body weight data revealed a significant group x time interaction ($F_{14, 1000} = 12.72$, $p < 0.0001$). Prior to diet exposure, body weight did not differ between groups (Fig. 6; top). At 3 wk of diet exposure, body weight for the OP group was greater than that for OR and LF groups. At the end of the diet exposure (wk 8), the OP group exhibited 15% and 12% greater body weight than the OR and LF groups, respectively. The mean daily energy intake across the 8-wk period was 14% greater in the OP group compared to the OR group (119 ± 2 and 102 ± 2 kcal per day, respectively) ($F_{7, 602} = 7.15$, $p < 0.0001$; Fig. 6; bottom).

**III b. In vitro striatal D2 receptor density**

Striatal D2 receptor density was evaluated using saturation analysis of $[^3]H$raclopride binding to striatal membrane preparations (Fig. 8). OP rats exhibited 42% lower $B_{\text{max}}$ compared to OR rats ($F_{2, 13} = 6.08$, $p < 0.01$; Fig. 8). No between-group differences in $K_d$ were found.
III c. In vitro striatal VMAT2 function

Striatal VMAT2 function was assessed using kinetic analysis of \([^3H]dopamine\) uptake in isolated striatal synaptic vesicles (Table 3). No between-group differences in \(V_{\text{max}}\) and \(K_m\) were found.

III d. In vitro striatal DAT function

\(V_{\text{max}}\) and \(K_m\) of specific \([^3H]dopamine\) uptake into striatal synaptosomes was evaluated using kinetic analysis (Fig. 9). \(V_{\text{max}}\) for \([^3H]dopamine\) uptake at DAT was 40% lower in OP compared to OR rats (\(F_{2, 35} = 6.285, p < 0.01\)), and not different from LF rats. No between-group differences in \(K_m\) were found.

III e. Methamphetamine-induced reverse transport of DAT in striatum, in vitro

Time course of methamphetamine-evoked \([^3H]dopamine\) overflow was assessed using superfused rat striatal slices (Fig. 10). ANOVA of the time course data revealed a group x concentration x time interaction (\(F_{112, 910} = 1.312, p < 0.05\)). No differences in basal \([^3H]dopamine\) outflow prior to methamphetamine addition to the buffer were observed among groups. Across the time-course of the experiment, \([^3H]dopamine\) overflow increased following addition of methamphetamine to the buffer, peaked 10-15 min after methamphetamine addition, and then over time declined towards basal levels despite the continued presence of methamphetamine. Although methamphetamine produced a
concentration-dependent increase in [³H]dopamine, the pattern of the concentration response was different among OP, OR and LF groups. The concentration-dependent increase in [³H]dopamine for the OR group reached plateau at a lower concentration of methamphetamine (3 µM) compared to the OP and LF groups. The significant three-way interaction from the ANOVA was evaluated further by a two-way ANOVA at the time point of peak effect for each group, and a group x concentration interaction was found (F_{6, 45} = 3.52; p < 0.05). *Post hoc* analysis revealed that only at the highest concentration (30 µM), the effect of methamphetamine to evoke [³H]dopamine at the peak response was greater in the OP group compared to OR group. Also, the effect of methamphetamine (30 µM) to evoke [³H]dopamine at the peak response was greater in the LF group compared to the OP group.

Collapsed across the time-course, two-way ANOVA on the methamphetamine-evoked total [³H]dopamine overflow data revealed a group x concentration interaction (F_{8,75} = 6.45, p < 0.0001; Fig. 11). *Post hoc* analysis revealed that total [³H]dopamine overflow was greater in the OP group compared to the OR group at 10 and 30 µM methamphetamine. Also, at these concentrations, total [³H]dopamine overflow was lower in the OP group compared to LF.

*III f. Striatal DAT cellular expression*
Striatal DAT cellular expression was evaluated using biotinylation and Western blot analysis. DAT and β-actin immunoreactive bands located at ~75 and 42 kDa, respectively, were compared among groups for each fraction (Fig. 12). β-Actin levels were not different among groups within a fraction. PP2A immunoreactive bands located at ~34 kDa were not detected in the biotinylated fraction, indicating that biotinylation was performed efficiently. Total striatal DAT levels were 30% and 48% higher in the OR group compared to OP and LF groups, respectively (F_{2, 17} = 6.143, p < 0.05). No between-group differences in the biotinylated (F_{2, 17} = 3.035, p > 0.05) and non-biotinylated fractions (F_{2, 17} = 0.586, p > 0.05) were found.

III g. In vivo striatal DAT function and extracellular dopamine concentration

In vivo striatal DAT function and basal extracellular dopamine concentration were determined using the no net flux microdialysis method. Representative chromatograms of dopamine standards and microdialysates are illustrated in Fig. 13. The regression line for each group is illustrated in Fig. 14: top. Striatal extraction fraction (index of DAT function) for the OP group was about 40% lower than for OR and LF groups (slope = 0.54 ± 0.1, 0.92 ± 0.1, 0.96 ± 0.1, respectively; F_{2, 16} = 5.313, p < 0.05; Fig. 14: bottom-left). The basal extracellular dopamine concentration in OP striatum was 45% greater than in OR striatum (F_{2, 16} = 4.767, p < 0.05), but not different from LF striatum (Fig. 14:
bottom). Schematic representation of microdialysis probe placements in the dorsal striatum for all groups of rats is provided in Fig. 15.

IV. Discussion

The current study delineates alterations in striatal dopaminergic function following the development of DIO. Striatal D2 receptor density was decreased in the outbred DIO rat model, similar to human obesity (Wang et al., 2001). Striatal VMAT2 function was not altered following the development of DIO; however, striatal DAT function and expression were decreased and extracellular dopamine concentration increased following the development of DIO.

Primary reward associated with food intake has been shown to be mediated by NAc dopamine (Kelley, 2004). Following ingestion of a HF-diet for 12 wk, NAc dopamine turnover in rats with free-access (obese) or restricted-access (non-obese) to a HF-diet was decreased compared to rats with free-access to standard chow (Davis et al., 2008), suggesting alterations in either dopamine release, uptake and/or metabolism. In contrast, extracellular NAc dopamine was decreased in inbred adult obese rats compared to rats inbred for resistance to obesity and fed standard chow (Geiger et al., 2008). A caveat of both these latter studies is that NAc was not dissected into shell and core, which are functionally distinct regions and subserve primary reward and habitual behavior, respectively (Bassareo and Di Chiara, 1999; Kelley, 1999; Kelley,
Dopamine release from NAc shell, striatum and mPFC slices was shown to be decreased in inbred obese rats compared to inbred obesity-resistant rats (Geiger et al., 2008). Further, a decrease in expression of mRNA for VMAT2, tyrosine hydroxylase, DAT and D2 was found in cultured VTA neurons from inbred obesity-prone compared to inbred obesity-resistant rats (Geiger et al., 2008). Collectively, deficient dopamine function in NAc appears to contribute to decreased primary reward and to the development of the obesity phenotype. While decreased mesolimbic dopamine function is suggested to promote compensatory hyperphagia leading to obesity, the current study focused on striatal dopaminergic mechanisms.

A shift in underlying dopaminergic control from NAc to dorsal striatum coincides with the development of habitual behaviors (Ito et al, 2002; Kelley, 2004; Everitt and Robbins, 2005; Koob and Volkow, 2010). A decrease in dorsal striatal postsynaptic D2 receptors was found in the current study using an outbred-DIO model, consistent with results from obese humans (Wang et al., 2001). Decreased D2 receptor density may have preceded the development of obesity or resulted from pre-existing decreases in DAT function and/or increased extracellular dopamine levels associated with repeated food reward. Further, alterations in VMAT2 and DAT function following the development of DIO was investigated since these transporters regulate extracellular dopamine concentrations. Increased VMAT2 function in DIO may contribute towards increased extracellular dopamine levels such that VMAT2-mediated uptake of
cytosolic dopamine into synaptic vesicles will facilitate dopamine storage for subsequent release. In contrast to this proposed hypothesis, no between-group differences in striatal VMAT2 function were found suggesting that striatal VMAT2 function is not altered following the development of DIO.

Both *in vitro* and *in vivo* studies demonstrated decreased striatal DAT function and increased extracellular dopamine concentration in the OP compared to the OR group. Importantly, obesity (not the diet *per se*) decreased DAT function since OP and OR rats received the same HF-diet. Furthermore, the ability of the OR phenotype to resist the development of obesity, despite receiving *ad libitum* access to the HF-diet, may be due to compensatory increases in DAT function and decreases in extracellular dopamine concentration. Overall, decreases in striatal DAT function as a result of obesity may be important for the maintenance of obesity.

Clearance of extracellular dopamine is primarily regulated by DAT localized at the cell-surface (Zahniser and Sorkin, 2004). A possible explanation for the decrease in DAT function in DIO is a decrease in cell-surface DAT expression. Thus, in DIO, the effect of methamphetamine was anticipated to be decreased as a result of diminished cell-surface DAT expression. Surprisingly, methamphetamine-induced DAT reverse transport was increased in OP relative to OR. In contrast, dopamine uptake was decreased in OP compared to OR. Differential effect of obesity on DAT uptake and reverse transport may be
explained by differential regulation of dopamine uptake via DAT and dopamine release via DAT reverse transport. Uptake is regulated by D2 autoreceptors and DAT interacting proteins like, PICK1 and synaptogyrin-3 (Cass and Gerhardt, 1994; Torres et al., 2001, Egana et al., 2009). Reverse transport is dependent on PKC and CaMKII, whereas uptake is not (Kantor and Gnegy, 1998; Fog et al., 2006). Thus, obesity may differentially alter the bidirectional function of DAT, i.e., dopamine uptake and dopamine reverse transport, by modulating distinct signaling mechanisms.

DAT cellular localization was evaluated as a potential mechanism underlying altered DAT function in OP and OR. Previous studies showed reduced DAT density in striatum from rats fed a HF-diet for 20 days compared with chow-fed controls (South and Huang, 2008). The current study extends previous work showing a decrease in total DAT protein in OP compared to OR striatum, also consistent with decreased striatal dopamine uptake in OP compared to OR. However, no differences in cell-surface or intracellular DAT expression were found, suggesting that regulation of DAT function in DIO is trafficking-independent. Alternative mechanisms underlying decreased DAT function and expression in OP striatum may include differential regulation of DAT synthesis, degradation and recycling.

Overall, the current study demonstrates decreased striatal DAT function and increased extracellular dopamine concentration following the development of
DIO. Further, these striatal alterations may be responsible for the compensatory decrease in post-synaptic D2 receptors. Importantly, the reported striatal dopaminergic mechanisms in DIO may lead to an increase in reward threshold that maintains excessive and habitual food intake in obesity.
Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>$V_{\text{max}}$ (fmol/min/mg protein)</th>
<th>$K_{m}$ (μM)</th>
</tr>
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<tbody>
<tr>
<td>LF</td>
<td>$318 \pm 26.3^a$</td>
<td>$0.15 \pm 0.05^a$</td>
</tr>
<tr>
<td>OP</td>
<td>$300 \pm 28.3$</td>
<td>$0.16 \pm 0.06$</td>
</tr>
<tr>
<td>OR</td>
<td>$285 \pm 24.6$</td>
<td>$0.15 \pm 0.05$</td>
</tr>
</tbody>
</table>
Table 3: Kinetic analysis of [3H]dopamine uptake at striatal VMAT2. aData are expressed as mean ± S.E.M; n = 6 rats/group.
HF-diet (31.8% Kcal fat) or LF-diet (10.6% Kcal fat)

Segregation into OP and OR

Neurochemical assays

Week 1 Week 2-7 Week 8 EVALUATION OF OUTCOMES

Monitor body weight and food intake

Obesity Resistant group (OR): minimum body weight gain

Obesity Prone group (OP): maximum body weight gain

Figure 5
Figure 5: Development of the DIO model for the evaluation of dopaminergic outcomes. Adult male Sprague Dawley rats received the HF- or LF- diet for 8 wk. Following the 8-wk diet exposure, rats fed the HF-diet were segregated into OP and OR groups based on body weight gain (top third and bottom third, respectively). Neurochemical assays were conducted in OP, OR and LF groups (Picture courtesy of Victoria English, Dr. Cassis’ Laboratory).
Figure 6

- **Body weight gain, gm**
- **Week**
- **Body weight (gm)**
- **Energy intake (kcal)**

- **OP**
- **OR**
- **LF**

* and # symbols indicate statistical significance.
Figure 6: Time course of body weight gain and energy intake during the 8-wk exposure to the HF- and LF-diets. Top: Representative scatter plot that illustrates the body weight gain in rats (n = 22) following the 8-wk HF-diet exposure. The dots in red and blue represent the body weight gain in rats that were segregated as OP and OR phenotypes, respectively. The dots in black represent the body weight gain in rats that were intermediate to that of OP and OR. These rats were not employed for the neurobehavioral assays. Bottom: DIO is associated with increased body weight and energy intake. Since mean body weight and mean energy intake for OP, OR and LF groups were not different between assays, data for each group were collapsed across the different assays conducted. The number of rats per group employed for the different assays was: in vitro VMAT2 function, n = 6/group; in vitro DAT function, n = 12-14/group (two separate series of DAT uptake assays); methamphetamine (METH)-induced DAT reverse transport, n = 6-8/group; DAT cellular localization, n = 8/group; in vivo DAT function and extracellular dopamine concentration, n = 8/group. The group of rats used to determine in vivo striatal DAT function was employed also to determine impulsivity and striatal D2 receptor density. Top: OP rats exhibited greater body weight compared to OR and LF rats from wk 3-8 of diet exposure. Data are pooled from groups that were employed in studies 1-6 (methods section) and expressed as the mean (SEM are smaller than the size of the symbol); n = 40-44 rats/group; *p < 0.001, indicates OP different from OR and LF. OP rats had greater energy intake compared to OR rats across the 8-wk period of diet exposure. Data are pooled from groups that were employed in studies 1-6
(methods section) and expressed as the mean (SEM are smaller than the size of the symbol); n = 40-44 rats/group; *p < 0.001, indicates OP different from OR.
Figure 7

[Diagram showing the relationship between \(^{3}\text{H}\)Raclopride binding and \(^{3}\text{H}\)DA uptake, with data points and error bars for total, nonspecific, and specific binding and uptake.]

\[^{3}\text{H}\]Raclopride binding, pmol/g protein/µm

\[^{3}\text{H}\]DA uptake, pmol/min/mg

Legend:
- Total
- Nonspecific
- Specific

B\(_{max}\)/V\(_{max}\)

K\(_d\)/K\(_m\)
**Figure 7:** Typical saturation kinetic analysis curves for binding ([³H]raclopride) and uptake ([³H]dopamine uptake) assays. Striatal D2 receptor density was assessed using saturation analysis of [³H]raclopride binding to striatal membranes. Striatal VMAT2 function and DAT function were assessed using saturation kinetic analysis of [³H]dopamine (DA) uptake into striatal vesicular and synaptosomal preparations, respectively. Specific [³H]raclopride binding to D2 receptors and specific [³H]DA uptake at VMAT2 and DAT were determined by subtracting nonspecific binding or uptake from total binding or uptake. B$_{\text{max}}$ represents D2 receptor density and K$_{\text{d}}$ represents affinity: concentration of [³H]raclopride that binds to 50% of the maximal number of DA D2 receptors. V$_{\text{max}}$ represents the maximal velocity of [³H]DA uptake at VMAT2/ DAT and K$_{\text{m}}$ represents the affinity of [³H]DA for VMAT2/ DAT (concentration of [³H]DA transported at $\frac{1}{2}$ V$_{\text{max}}$).
Figure 8

[Graph showing specific binding in pmol/g protein vs. [3H]Raclopride (nM) for OP, OR, and LF.]

[Bar graphs showing B_max and K_d values for OP, OR, and LF.]
**Figure 8:** Saturation analysis of $[^3\text{H}]$raclopride binding to striatal membranes from OP, OR and LF groups. DIO is associated with decreased striatal D2 receptor density. Striatal D2 receptor density was assessed using saturation analysis of $[^3\text{H}]$raclopride binding to striatal membranes. Nonspecific binding was determined in the presence of (S)-sulpiride (10 μM). Specific $[^3\text{H}]$raclopride binding to striatal D2 receptors in OP, OR and LF groups (top). $B_{\text{max}}$ of the OP group was lower compared to the OR group (bottom-left). No between-group differences in $K_d$ were found (bottom-right). Data are expressed as pmol/g protein (mean ± SEM) for specific $[^3\text{H}]$raclopride binding and $B_{\text{max}}$ and as nM (mean ± SEM) for $K_d$; n = 4-5 rats/group; *p < 0.05, indicates OP different from OR.
Figure 9
Figure 9: Kinetic analysis of $[^{3}\text{H}]$DA uptake at striatal DAT from OP, OR and LF groups. DIO is associated with decreased *in vitro* striatal DAT function. Kinetic analysis of $[^{3}\text{H}]$DA uptake was conducted in the presence of nomifensine (10 µM). Specific $[^{3}\text{H}]$DA uptake at striatal DAT of OP, OR and LF groups (top). $V_{\text{max}}$ of striatal $[^{3}\text{H}]$DA uptake at DAT was lower in the OP group compared to the OR group (bottom-left). No between-group differences in $K_{m}$ were found (bottom-right). Data are expressed as pmol/min/mg protein (mean ± SEM) for specific $[^{3}\text{H}]$DA uptake at striatal DAT and $V_{\text{max}}$ and as µM (mean ± SEM) for $K_{m}$; n = 10-13 rats/group; *p < 0.05, indicates OR different from OP and LF.
Figure 10
Figure 10: Time course of methamphetamine-evoked $[^3]$H]DA in striatal superfusates from OP (top), OR (middle) and LF (bottom) groups. DIO is associated with increased methamphetamine-evoked $[^3]$H]DA (% tissue) in striatal superfusates. Time course of methamphetamine-evoked $[^3]$H]DA overflow was assessed using superfused rat striatal slices. Superfusion buffer contained 10 µM pargyline. Striatal slices were superfused in the absence (control) or presence of methamphetamine (1-30 µM) for 60 min. Arrow indicates time point at which methamphetamine (METH: 1-30 µM) was added to the buffer. Also, methamphetamine remained in the buffer until the end of the experiment. Data are expressed as mean ± S.E.M; n = 5-7 rats/group; *p < 0.01, indicates OP different from OR and LF.
**Figure 11:** Methamphetamine-evoked striatal \[^{3}H\]DA overflow from OP, OR and LF groups. DIO is associated with increased methamphetamine-evoked striatal \[^{3}H\]DA overflow. In response to methamphetamine (10 or 30 µM), striatal \[^{3}H\]DA overflow was higher in the OP group compared to the OR group, but lower compared to the LF group. Also methamphetamine-induced striatal \[^{3}H\]DA overflow was lower in the OR group compared to the LF group. Data are expressed as mean ± S.E.M; n = 5-7 rats/group; *p < 0.01, indicates OP different from OR and LF.
Figure 12

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Non-biot</th>
<th>Biot</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LF</td>
<td>OP</td>
<td>OR</td>
</tr>
<tr>
<td>75 kDa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 kDa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34 kDa</td>
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DAT

β-actin

PP2A

DAT Immunoreactivity (arbitrary units)

Total

Non-biotinylated

Biotinylated
Figure 12: Striatal DAT cellular expression in OP, OR and LF groups. DIO is associated with decreased total striatal DAT expression. Biotinylation was conducted in striatal synaptosomes obtained from OP, OR and LF groups. Total striatal DAT levels were higher in the OR group compared to that of OP and LF groups. No between-group differences in striatal DAT levels in the non-biotinylated and biotinylated fractions were found. Representative immunoblot (top). Mean DAT immunoreactivity in total, non-biotinylated and biotinylated fractions from LF, OP and OR groups (bottom). Data are mean ± SEM; n = 6 rats/group; *p < 0.05, indicates OR different from OP and LF.
Figure 13
**Figure 13:** Representative HPLC chromatograms. Top: Representative chromatogram of dopamine standards; 5, 10, 20 and 40 nM. Bottom: Representative chromatogram of a microdialysis sample. Retention time of dopamine: 4-5 min.
Figure 14
**Figure 14:** *In vivo* striatal DAT function and basal extracellular DA concentration in OP, OR and LF groups. DIO is associated with decreased *in vivo* striatal DAT function and increased basal extracellular DA concentration. No net flux *in vivo* microdialysis was conducted in the dorsal striatum of OP, OR and LF groups. The slope of the regression line represents the extraction fraction (E_d) and the intersection of the regression line with x-axis represents the no net flux point i.e. extracellular DA concentration (top). Striatal extraction fraction for the OP group was lower than for OR and LF groups (p < 0.05; bottom left). The basal extracellular DA concentration in OP striatum was greater than in OR striatum (p < 0.05; bottom right). Data are expressed as nM (mean ± SEM) for DA exchange and basal extracellular DA and as mean ± SEM for E_d; n = 5-6 rats/group; *p < 0.05, indicates OP different from OR and LF; #p < 0.05, indicates OP different from OR.
**Figure 15:** Schematic representation of probe placement within the dorsal striatum. Lines indicate the 2mm microdialysis membrane (n = 5-6/ group). Numbers to the right indicate distance from bregma. (Adapted from Paxinos and Watson, 1986)
Chapter Three
Evaluation of impulsivity and food-motivated behavior as outcomes of DIO

I. Introduction

Obesity is a neurobehavioral disorder which involves excessive intake of palatable foods that persists despite the threat of fatal health consequences (Volkow and Wise, 2005). Increased preference for palatable, HF-foods is attributed to favorable orosensory properties and post-ingestive effects of HF-diets (Warcik and Weingarten, 1995; Lucas et al., 1998; Sclafani et al., 2001; Gaillard et al., 2008). Importantly, the reinforcing effects of palatable foods are shown to be mediated by activation of striatal dopaminergic systems (Martel and Fantino, 1996; Small et al., 2003). Furthermore, decreased striatal D2 receptor availability in obese humans compared to control supports a role for striatal dopaminergic system involvement in obesity (Wang et al., 2001). Striatal D2-deficiency in obesity is hypothesized to trigger motivational processes that increase food consumption, and thereby, compensate for an understimulated dopaminergic system (Wang et al., 2009).

In addition to the involvement of striatal dopamine pathways that regulate reward and motivation, brain circuits that regulate inhibitory control (impulsivity), salience attribution and decision making have also been implicated in obesity. In obesity, enhanced sensitivity to palatable foods is evident from higher than
normal baseline metabolism of $[^{18}\text{F}]$fluoro-deoxyglucose (marker of brain function) in the somatosensory cortex of obese subjects compared to control (Wang et al., 2002a). Decreased striatal D2 receptor availability in obese humans was shown to be associated with decreased metabolic activity in DLPFC, medial OFC and anterior cingulate gyrus (Volkow et al., 2008). The reported association between striatal D2 receptor availability and prefrontal metabolism suggests dopaminergic modulation of prefrontal cortical function that regulate inhibition, salience attribution and decision making processes (Volkow et al., 2008).

Obese individuals exhibit greater preference for diets high in fat and carbohydrates, compared to lean individuals (Drewnowski et al., 1992). Reinforcing effects of palatable foods are shown to be greater in obese compared to non-obese humans, as demonstrated in studies conducted using the PR schedule of reinforcement (Epstein et al., 2007). While food reinforcement is an important determinant of motivation to eat, it may also interact with other psychological variables that influence food intake. Importantly, sensitivity to palatable food reward was shown to drive overeating only when accompanied by insufficient inhibitory control (Appelhans et al., 2011). In overweight and obese women, sensitivity to palatable food reward and inhibitory control was assessed using the power of food scale and delay discounting task for monetary rewards, respectively (Appelhans et al., 2011). Higher food reward sensitivity predicted greater palatable food intake in overweight and obese women, only at low levels of inhibitory control. Further, women with a binge-
eating disorder were shown to exhibit increased impulsivity compared to control, as measured using an impulsivity and novelty seeking scale (Claes et al., 2002; Vervaet et al., 2003). Higher disinhibition scores was found in obese compared to non-obese individuals, based on a three factor eating questionnaire, that assess hunger, restraint and inhibition (Boschi et al., 2001). Another study demonstrated a positive correlation between impulsivity, assessed using the delay discounting procedure and dietary disinhibition (Yeomans et al., 2008). Thus, coincident with increased motivation for palatable foods, increased impulsivity, a multifaceted behavioral construct, also plays a role in obesity (Whiteside and Lynam, 2001; Mobbs et al., 2010).

Overall, nonhomeostatic food intake in obesity appears to be regulated by lack of inhibitory control and increased motivation. The current study investigated impulsivity and food-motivated behavior as behavioral outcomes of DIO.

**Hypothesis:** DIO will be associated with increased impulsivity and increased food-motivated behavior. The specific aims that were formulated to test the proposed hypothesis follow.

**Specific aim 1.** As an outcome of DIO, impulsivity will be evaluated using the delay discounting task.

**Specific aim 2.** As outcomes of DIO, food-motivated behavior and persistence of food-seeking behavior will be evaluated using the PR schedule of reinforcement and extinction procedures, respectively.
II. Methods

**II a. Animals.** Outbred adult male Sprague Dawley rats (350-400 g; Charles River Laboratories Inc., Wilmington, MA) were housed individually in solid-bottom cages with bedding and received standard rat chow and water *ad libitum*. Prior to the initiation of experimental procedures, rats were fed standard chow for one week during acclimatization to the animal facility. Experimental procedures were approved by the Institutional Animal Care and Use Committee at University of Kentucky.

Following acclimatization, rats were randomly assigned to groups fed for 8 wk either a HF-diet (n = 24) or a LF-diet (n = 8). Food intake was determined daily and body weight determined 3 times weekly. Energy intake was derived by multiplying the daily food intake (g) by the total kcal/g of the respective diet consumed. Following an 8-wk HF-diet exposure, rats were segregated into OP and OR groups as previously described in chapter two (n = 8/group) (Fig. 16).

**II b. Behavioral apparatus.** Behavioral experiments were conducted in operant conditioning chambers (28 cm × 24 cm × 25 cm; ENV-001; MED Associates, St. Albans, VT) enclosed within sound-attenuating chambers (ENV-018M; MED Associates). Operant chambers had a metal rod floor, Plexiglas front and back walls and aluminum side walls. A house light (28 V) was centered on the left side wall 2.5 cm from the ceiling and illuminated the chamber. Food
pellets were dispensed from a food hopper (ENV-203M-45; MED Associates) into a recessed food tray (5 cm × 5 cm × 3 cm), which was centered on the right side wall 2 cm above the floor. Retractable levers (4.5 cm) were located on the right side wall 6 cm above the floor on either side of the recessed food tray. White stimulus lights (28 V; 3 cm in diameter) were located 3 cm above each lever. Chambers were connected to a personal computer interface and controlled using MED-PC software (SG-502). Behavioral procedures were carried out during the light cycle.

**II c. Experimental design and procedures.** Impulsivity, motivation for HF- and LF- food reinforcement, and extinction to food-reinforced responding were evaluated as behavioral outcomes of DIO. The details of each study follow.

**Study 1.** As an outcome of DIO, impulsivity was evaluated using the delay discounting task in OP, OR and LF groups (n = 6-8/group). Treatment group was a between-subject factor and session was a within-subject factor. Rats employed in this study were previously used to evaluate striatal DAT function and extracellular dopamine concentration *in vivo* (chapter 2, study 6).

*Delay discounting task.* Impulsivity was evaluated as an outcome of DIO, using the delay discounting task (Marusich and Bardo, 2009). To facilitate lever responding, rats received all of their daily food allowance during a 3-h period after each operant session; OP and OR groups received the HF-diet and
the LF group received the LF-diet. Each delay discounting session consisted of 15 blocks, and each block consisted of 4 trials, such that 60 trials were administered across a 2-h session. Each trial lasted for 60 s including an adjusted inter-trial interval (ITI). During ITIs, both cue lights and house light were off and lever presses had no programmed consequences. The first 2 trials of each block were forced trials; only one of the two levers was extended into the chamber and the stimulus light above the extended lever was illuminated. Presses on the extended lever during the forced trials resulted in immediate retraction of the lever, followed by delivery of either one or three sucrose-based 45 mg pellets (F0021 dustless precision pellets, Bio-Serv Inc., Frenchtown, NJ). In subsequent free-choice trials, both levers were extended and both stimulus lights were illuminated. One lever delivered one sucrose pellet immediately following a response. A response on the other lever delivered three sucrose pellets following a delay. The position of the lever delivering one reinforcer vs. three reinforcers alternated daily. The initial delay for the delivery of the three pellets was 0 s. The delay was adjusted depending on which lever was chosen subsequently. If the lever delivering three pellets was chosen, then a 1-s increase in the delay resulted upon reinforcement of the next response on this lever. Alternatively, if the lever delivering one pellet was chosen, then a 1-s decrease in the delay resulted following a response on the lever delivering three pellets. A minimum delay of 0 s and a maximum delay of 45 s was imposed between the response and the delivery of the three-pellet reinforcer. During the delay, the stimulus lights were off and the house light remained illuminated. The
delay imposed during the last free choice trial for each session was employed as the initial delay on the next session.

**Study 2.** As outcomes of DIO, motivation for food reinforcement and persistence of food-seeking behavior were evaluated in OP, OR and LF rats (n = 8 rats/group). To evaluate motivation for food reinforcement, rats were initially trained on a FR schedule followed by a PR schedule of reinforcement. Rats in this study experienced no prior experimental manipulations. Persistence of food-seeking behavior was evaluated using extinction procedures that were conducted in the same groups of rats employed for the FR and PR schedules of reinforcement. For FR, PR schedules of reinforcement and during extinction procedures, treatment group was a between-subject factor and session was a within-subject factor.

*Food-reinforced operant behavior during FR and PR schedules of reinforcement.* Food-reinforced operant behavior was assessed in separate groups of naïve OP, OR and LF rats using a FR and PR schedule of reinforcement. To facilitate lever responding, rats received all of their daily food allowance during a 3-h period after each operant session; OP and OR groups received the HF-diet and the LF group received the LF-diet. For the operant conditioning studies, rats received custom made HF and LF dustless 45 mg pellet reinforcers (F06378 HF precision pellets with 31.2% Kcal from fat; F06379 LF precision pellets with 10.6% Kcal from fat; Bio-Serv Inc.). Operant procedures
were employed as described previously (Green et al., 2002), with minor modifications. Initially, rats were introduced to the chambers with the levers retracted, and non-contingent food pellets became available in the food tray at 45 s intervals for 12 min. Subsequently, two daily 1-h autoshaping sessions occurred during which both levers were extended into the chamber for 100 s. Cue light above the active lever was illuminated only during the period when the levers were extended into the chamber. Following 100 s, both levers retracted immediately followed by delivery of a food pellet reinforcer. If a response occurred on the active lever prior to the elapse of 100-s lever extensions, the levers were retracted and a food pellet was delivered. Inactive lever responses were recorded, but had no programmed consequence. The position of the active lever was counterbalanced across sessions. Following autoshaping, rats were reinforced on a FR-1 schedule for. The duration of each FR schedule was one hour session per day for three days, during which one lever press resulted in the delivery of one food pellet with no illumination of the cue light. Lever response requirements were gradually increased from FR-2 to FR-3 to FR-5 to FR-10, the terminal schedule. During this phase of training, OP and OR groups received the HF pellet reinforcers, and the LF group received the LF pellet reinforcers. For each incremental FR schedule, requirements for stable responding was defined as less than 20% variability in the number of responses across 3 consecutive sessions and a minimum of a 2:1 ratio of active to inactive lever responses. Stable responding was attained for each schedule within the three FR 1-h sessions that were conducted across there days.
Subsequently, the groups were switched to a PR schedule of reinforcement and the session duration was increased to 3 h. During the PR sessions, both levers were extended; however, responses on only active lever resulted in reinforcer delivery. Responding on the other lever (inactive lever) had no programmed consequence. Following delivery of each reinforcer, the response requirement for the next reinforcer delivery was increased based on the following equation: $[5e^{\text{response number} \times 0.2}]-5$ (1, 2, 4, 6, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603 …; number of active lever responses required to obtain a single reinforcer) (Richardson and Roberts, 1996). For 3 PR sessions, OP and OR groups received the HF pellet reinforcers, and the LF group received the LF pellet reinforcers. For the next 3 PR sessions, OP and OR groups received the LF pellet reinforcers, and the LF group received the HF pellet reinforcers. The last ratio completed at the end of the 3-h session was defined as the PR breakpoint, and served as an index of motivation.

**Extinction of food-reinforced operant behavior.** Following the PR schedule of reinforcement, the same groups of rats underwent extinction for daily 1-h sessions for ten consecutive days to determine the effect of DIO on persistence of food-seeking behavior. During extinction, responses on either lever did not result in the delivery of food pellets. After each extinction session, OP and OR groups were given 3 h access to the HF-diet in the home-cage and the LF group given 3-h access to the LF-diet in the home-cage.
Il d. Data analysis. Data are presented as mean values ± S.E.M., and ‘n’ represents the number of animals tested for each group. Data were analyzed using GraphPad Prism 4.0 program (GraphPad Software Inc., San Diego, CA) and SPSS version 9.0 (SPSS Science, Chicago, IL).

Time dependent differences in body weight and energy intake between groups were determined by two-way ANOVA, with OP, OR and LF as a between-subject factor and time as a within-subject factor. One-way ANOVAs analyzed between-group differences in body weight, energy intake and % body weight gain after 8 wk of dietary treatment. The level of caloric restriction imposed by the 3-h access to daily food allowance was derived based on the food intake during the 3-h access, expressed as a percentage of daily food intake during the 8-wk ad libitum access.

Mean adjusted delay was obtained from the delay discounting task as a measure of impulsivity. Rats with mean adjusted delay (averaged for the last 5 sessions of free choice trials) below the median split were categorized as high-impulsive, and those with mean adjusted delay above the median were categorized as low-impulsive. Two-way ANOVAs with OP, OR and LF as a between-subject factor and FR schedule and extinction session as within-subject factors analyzed between groups differences in number of reinforcers earned and lever responses. One-way ANOVAs on mean adjusted delay and PR
breakpoint determined between-group differences. *Post hoc* analyses were conducted by Tukey’s test to compare between two groups.

**III. Results**

**III a. Time course of body weight gain and energy intake during the 8-wk exposure to the HF- and LF-diet**

Body weight and energy intake for OP, OR and LF groups during the 8-wk exposure to the HF- or LF-diets are illustrated in Fig.17. Analysis of the body weight data revealed a significant group x time interaction \((F_{14, 147} = 20.19, p < 0.0001)\). Prior to diet exposure, body weight did not differ between groups (Fig. 17; top). At 3 wk of diet exposure, body weight for the OP group was greater than that for OR and LF groups. At the end of the diet exposure (wk 8), the OP group exhibited 18% and 11% greater body weight than the OR and LF groups, respectively. Determination of energy intake for OP and OR groups revealed that OP rats had greater energy intake (14%) than OR rats across the 8-wk period \((F_{7, 98} = 6.5, p < 0.0001;\) Fig. 17; bottom). Interestingly, despite increases in body weight rats across the 8-wk period, energy intake remained relatively constant across this period.

**III b. Impulsivity**

The level of caloric restriction imposed by the 3-h access to daily food allowance during the behavioral assays was not different between OP and OR
groups (58% and 54%, respectively). Rats from the OP, OR and LF groups were evaluated in the delay discounting task and the mean adjusted delay was averaged across the last five sessions (Fig. 18). The mean adjusted delay for each group varied by less than 5 s across the last 5 sessions of testing. The median for all of the animals tested was 10.5 s. Rats with a mean adjusted delay below and above the median were categorized as high-impulsive and low-impulsive, respectively. OP rats exhibited 30% greater mean adjusted delay compared to OR rats ($F_{2, 14} = 4.219, p < 0.05$), indicating that OP rats were less impulsive than OR rats.

**III c. Food-reinforced operant behavior**

Food-reinforced operant behavior in OP, OR and LF rats was determined using FR and PR schedules of reinforcement. During the FR schedule, number of reinforcers earned and number of active and inactive lever presses for OP, OR and LF groups are illustrated in Fig. 19. The number of reinforcers earned was relatively constant across the incremental increases in the FR schedule requirement (Fig. 19; top), and the number of response on the active lever (Fig 19; bottom left), but not the inactive lever (Fig 19; bottom right), increased correspondingly. ANOVA on the number of reinforcers earned revealed a significant interaction of group x FR schedule ($F_{8, 84} = 2.751, p < 0.05$). *Post hoc* analysis showed that at FR-10, the number of reinforcers earned by OP and OR groups was lower than that for the LF group ($p < 0.05$). Parallel results were obtained for the number of active lever responses, in that a significant interaction
of group x FR schedule ($F_{8, 84} = 4.068, p < 0.001$) was revealed. The number of active lever responses during the FR 10 schedule was lower in OP and OR groups compared to the LF group ($p < 0.001$). Inactive lever responses were not different between groups across FR schedules ($F_{8, 84} = 0.91, p > 0.05$).

Following completion of the FR schedule of responding, the PR schedule was initiated to determine the breakpoint (motivation) for food reinforcement for each group (Fig. 20). During PR sessions 1-3, OP and OR groups earned HF-reinforcers and the LF group earned LF-reinforcers. Main effects of group ($F_{2, 21} = 4.14, p < 0.01$) and session ($F_{2, 42} = 8.09, p < 0.01$) were found; however, the group x session interaction was not significant ($F_{2, 42} = 0.41, p > 0.05$). During PR sessions 4-6, OP and OR groups earned LF-reinforcers, and the LF group earned HF-reinforcers. A main effect of group ($F_{2, 20} = 5.35, p < 0.01$) was found; there was no main effect of session ($F_{2, 40} = 0.1, p > 0.05$) and no group x session interaction ($F_{4, 40} = 2.32, p > 0.05$). The mean breakpoint for OP rats was greater than that for OR rats, when responding was for either HF ($F_{2, 22} = 3.655, p < 0.05$) or LF reinforcers ($F_{2, 18} = 4.639, p < 0.05$).

**III d. Extinction of food-reinforced operant responding**

The persistence of operant responding during extinction, a time period in which responding did not result in delivery of reinforcers, was determined for OP, OR and LF groups (Fig. 21). A main effect of extinction session was found ($F_{9, 189}$...
= 69.8, p < 0.0001); however, there was no main effect of group ($F_{2, 21} = 0.27, p > 0.05$) and no group x session interaction ($F_{18, 189} = 1.35, p > 0.05$).

IV. Discussion

The current study demonstrates decreased impulsivity and increased motivation for food reinforcers following the development of DIO, in the outbred rat model. Decreased impulsivity in obesity reported in the current study is not consistent with previous findings that obese humans are impulsive (Weller et al., 2008). Nonetheless, increased motivation for HF-reinforcers following the development of DIO is consistent with the observation that the orosensory properties and post-ingestive effects of HF-dengender overeating of HF-foods (Kern et al., 1993; Warwick and Weingarten, 1995).

Obesity and drug addiction are hypothesized to share common underlying neurobehavioral mechanisms (Robinson and Berridge, 2003; Volkow et al., 2008; Kenny, 2011). Personality traits including high sensation seeking and high impulsivity (lack of inhibition, premeditation and perseverance) predispose and are affected by drug addiction (Ersche et al., 2010). Furthermore, drug addiction is shown to be associated with increased impulsive choice, assessed using the delay discounting procedure in human addicts and rats following repeated drug exposure (Coffey et al., 2003; Gipson and Bardo, 2009; Setlow et al., 2009). In the current study, impulsivity also measured using delay discounting was
decreased following development of DIO, which contrasts with a report showing increased impulsivity in obese women as measured by delay discounting (Weller et al., 2008). Different outcomes between the animal model and the human studies regarding impulsivity may be due to differences in species, sex or reinforcers employed. Importantly, both studies employed delay discounting which measures only one facet of impulsivity. Impulsivity is a multidimensional behavioral trait that involves urgent actions, lack of premeditation, lack of perseverance, and an increase in sensation seeking behaviors (Whiteside and Lynam, 2001). Therefore, interpretation of current and previous studies is limited somewhat by delay discounting, which measures only one facet of the construct of impulsivity, i.e impulsive choice. Impulsivity consists of both reward seeking and lack of inhibition personality factors. Since OP rats exhibited increased responding for the larger delayed reward as opposed to the smaller immediate reward, it is likely that reward-based motivational processes may have overcome impulsive choice in the animal model, but not in the human study.

OP and OR rats did not differ in acquisition of FR responding for HF-reinforcers suggesting that between group-differences in learning were not found. However, PR breakpoint was greater in OP than OR rats. Further, PR breakpoint was higher in OP rats regardless of whether the reinforcer was HF or LF, indicating that caloric density was not a factor in food motivation. Also, OP rats exhibited a greater number of active lever responses during extinction sessions, compared to OR and LF rats. However, one caveat of the extinction results is
that extinction training was conducted following the PR sessions. Evaluation of PR breakpoint prior to the extinction procedure may have diminished the food seeking behavior at higher response ratios during the PR schedule. Compulsivity is typically characterized by persistence of responding when the reinforcer is no longer available (extinction), increased PR breakpoints (motivation), and increased resistance to punishment (Belin et al., 2011). The observation that the OP group exhibits a greater level of motivation for both HF- and LF-reinforcers compared to the OR group suggests the development of compulsive behavior in DIO (Belin et al., 2011).

Controversy exists regarding the effect of obesity on motivation for food across studies using different animal models of obesity. In contrast to the current results, PR breakpoint for HF-reinforcers did not differ between DIO-prone Osborne-Mendel and the DIO-resistant S5B\PI inbred rats fed standard chow in the home-cage (Thanos et al., 2011). Similarly, no differences in PR breakpoint for sucrose pellets were found between out-bred Sprague Dawley rats with 12-wk free-access (obese) or restricted-access (non-obese) to a HF-diet in the home-cage (Davis et al., 2008). However, both groups receiving the HF-diet in the home-cage had decreased PR breakpoint for sucrose pellets compared to another group with free-access to standard-chow in the home-cage, suggesting that the reward value of sucrose was decreased following long-term access to HF-diet. Interestingly, a decreased PR breakpoint for sucrose reinforcement was found in Sprague Dawley rats selectively inbred for obesity compared to those
inbred for resistance to obesity and fed standard chow in the home-cage (Davis et al., 2008). Discrepancies between these previous results and those of the current study showing an increase in PR breakpoint and motivation for HF-food in the OP group may be due to differences in the animal model of obesity, the reinforcer type (sucrose vs. HF), and/or the home-cage diet. Importantly, sucrose and HF-diets exhibit differences in orosensory properties and satiety mechanisms (Warwick and Weingarten, 1995; Gaillard et al., 2008; Avena et al., 2009). However, it is unlikely that the HF home-cage diet is responsible for the increased PR breakpoint in OP rats in the current study, since PR breakpoint for HF- or LF-reinforcers was not different between OR and LF rats, even though they received different home-cage diets.

Consistent with the current results, CCK-1 receptor-deficient OLETF rats and leptin-receptor deficient Zucker rats fed standard chow in the home-cage exhibited increased PR breakpoints for sucrose reinforcers compared to lean controls (Glass et al., 1999; Hajnal et al., 2007). Similarly, outbred Wistar rats fed a HF/high-sugar home-cage diet demonstrated increased PR breakpoint for sucrose reinforcers compared to chow-fed rats (Fleur et al., 2007). The current study is consistent with and extends these findings by showing increased motivation for HF- and LF-reinforcers in the OP group compared to the OR group, both of which received ad libitum access to the HF-diet in the home cage.
Interestingly, the LF group was not differentially motivated to obtain the LF vs. HF reinforcers during the PR schedule. One explanation for the lack of an increase in responding (breakpoint) is that the LF rats did not find the novel HF reinforcers more rewarding than the LF reinforcers, such that both types of reinforcers were similarly rewarding. Alternatively, it is possible that an extended training would have revealed group differences based on the palatability of the pellet reinforcer. However, during acquisition of operant responding under the FR schedule, the LF rats responded more for LF reinforcers than OP and OR rats for HF reinforcers, but only under the FR-10 schedule of responding. This may be explained by the lower caloric density and lower satiation associated with LF reinforcers compared to HF reinforcers.

In summary, the current results reveal decreased impulsivity and increased motivation for food reinforcement following the development of DIO. Decreased impulsivity in DIO was an unanticipated outcome. Nonetheless, increased food-motivated behavior may underlie the maintenance of habitual and excessive food intake in obesity.
Segregation into OP and OR

Monitor body weight and food intake

EVALUATION OF OUTCOMES

Week 1
Week 2-7
Week 8

HF-diet (31.8% Kcal fat) or LF-diet (10.6% Kcal fat)

Obesity Resistant group (OR): minimum body weight gain

Obesity Prone group (OP): maximum body weight gain

Behavioral assays

Figure 16
**Figure 16:** Development of the DIO model for the evaluation of behavioral outcomes. Adult male Sprague Dawley rats received the HF- or LF- diet for 8 wk. Following the 8-wk diet exposure, rats fed the HF-diet were segregated into OP and OR groups based on body weight gain (top third and bottom third, respectively). Behavioral assays were conducted in OP, OR and LF groups (Picture courtesy of Victoria English, Dr. Cassis’ Laboratory).
**Figure 17:** Time course of body weight gain and energy intake during the 8-wk exposure to the HF- and LF-diets. DIO is associated with increased body weight and energy intake. *Top:* OP rats exhibited greater body weight compared to OR and LF rats from wk 3-8 of diet exposure. Data are pooled from groups that were employed in studies 1 and 2 (methods section) and expressed as the mean (SEM are smaller than the size of the symbol); n = 16 rats/group; *p < 0.001, indicates OP different from OR and LF. *Bottom:* OP rats had greater energy intake compared to OR rats across the 8-wk period of diet exposure. Data are pooled from groups that were employed in studies 1 and 2 (methods section) and expressed as the mean (SEM are smaller than the size of the symbol); n = 16 rats/group; #p < 0.001, indicates OP different from OR.
Figure 18

Mean adjusted delay, s

*OP  OR  LF
Figure 18: Mean adjusted delay of OP, OR and LF groups. OP rats exhibited a greater mean adjusted delay compared to OR rats. Rats were trained on a delay discounting task for 28 consecutive sessions. One lever delivered one sucrose pellet immediately following a response. Response on the other lever delivered three sucrose pellets following an adjusted delay. Mean adjusted delay to obtain the larger delayed food reinforcer was calculated at the end of each session by averaging the delays on free choice trials. Data are expressed as mean ± SEM of adjusted delay across the last 5 sessions; n = 5 rats/group; *p < 0.05, indicates OP different from OR.
Figure 19
Figure 19: Reinforcers earned and lever responses during FR schedule of reinforcement in OP, OR and LF groups. During the FR-10 schedule of reinforcement, the number of reinforcers earned and the number of active lever responses emitted was lower in OP and OR groups compared to the LF group. No between-group differences in inactive lever responses were found. OP, OR and LF groups were reinforced on FR-1 schedule for three 1-h sessions, during which one active lever press resulted in delivery of one food pellet reinforcer. Lever response requirements were gradually increased from FR-2 to FR-3 to FR-5, until FR-10. For each FR response requirement, OP and OR groups pressed the active lever to obtain HF-reinforcers; LF groups pressed the active lever to obtain LF-reinforcers. Data are expressed as number of food reinforcers earned (top) and number of active and inactive lever presses (bottom) and are presented as mean ± SEM; n = 8 rats/group; *p < 0.001, indicates OP and OR different from LF.
Figure 20
**Figure 20:** PR breakpoint of OP, OR and LF groups. The OP group exhibited a higher PR breakpoint for both HF- and LF-reinforcers compared to the OR group. During sessions 1-3 of the PR schedule, OP and OR groups earned HF-reinforcers and LF group earned LF-reinforcers. During sessions 4-6, OP and OR groups earned LF-reinforcers; LF received HF-reinforcers. PR breakpoint of OP, OR and LF groups for each PR session (top). Mean PR breakpoint for the HF- and LF-reinforcers (bottom). Data are expressed as mean ± SEM; n = 5-8 rats/group; *p < 0.05, indicates OP different from OR.
Figure 21

Active lever responses vs. Extinction session for different conditions: OP, OR, and LF.
**Figure 21:** Extinction of operant responding in OP, OR and LF groups. No between-group differences in active lever responses were found during extinction. Data are expressed as mean ± SEM; n = 7-8 rats/group.
Chapter Four

Evaluation of behavioral and dopaminergic predictors of the development of DIO

I. Introduction

Obesity is shown to be associated with increased motivation for palatable foods and increased impulsivity, a multifaceted behavioral construct (Whiteside and Lynam, 2001; Weller et al., 2008; Appelhans et al., 2011). Increased sensitivity to palatable food reward in obesity has been shown to drive overeating only when accompanied by insufficient inhibitory control (Appelhans et al., 2011). Personality traits that include high sensation seeking and high impulsivity (lack of inhibition, premeditation and perseverance) are not only affected by drug addiction, but also serve as behavioral predictors of drug addiction (Ersche et al., 2010). To the extent that obesity and drug addiction share common underlying neurobehavioral mechanisms (Kenny, 2011), an individual’s level of motivation and impulsivity may also serve as behavioral predictors of obesity.

Ingestion of palatable foods activates striatal dopaminergic systems and the resulting dopaminergic activation is suggested to influence hedonic and nonhomeostatic food intake in obesity (Berthoud, 2011; Wise, 2006). Decreased striatal D2 receptors are reported in obese humans compared to control (Wang
et al., 2001). However, it is not clear whether decreases in striatal D2 levels preceded the development of obesity.

Activation of D2 autoreceptors is shown to increase DAT function (Cass and Gerhardt, 1994). Given that decreases in striatal D2 levels may precede the development of obesity, it is likely that corresponding alterations in DAT function may exist prior to the development of obesity. The role of DAT is implicated in obesity based on previous reports that DAT-deficient mice exhibit increased extracellular dopamine concentration and greater food intake, compared to wildtype-mice (Pecina et al., 2003). Furthermore, the current results described in chapter 2 demonstrate decreases in striatal DAT function and increases in extracellular dopamine levels following the development of DIO. Taken together, these striatal dopaminergic mechanisms may be consequences of obesity or may have preceded the development of obesity.

Identification of predictors of obesity will facilitate better pharmacological and behavioral intervention. Evaluation of predictors of obesity is difficult in humans, but can be investigated in controlled studies using animal models, such as the outbred DIO rat model. In the current study, impulsivity, food-motivated behavior and striatal dopamine function were evaluated as neurobehavioral predictors of DIO.
Hypothesis: Pre-existing individual differences in the levels of impulsivity, motivation, striatal DAT function and extracellular dopamine concentration will predict the development of DIO in a rat model. The specific aims formulated to test the proposed hypothesis follow.

Specific aim 1. As predictors of DIO, impulsivity and motivation for HF food reinforcers will be evaluated using the delay discounting task and PR schedule of reinforcement, respectively.

Specific aim 2. As predictors of DIO, striatal DAT function and extracellular dopamine concentration will be evaluated using in vivo no net flux microdialysis.

II. Methods

II a. Materials. Ascorbate oxidase, Chromasolv®, D-glucose and dopamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Perchloric acid (70%) was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Diazepam and ketamine hydrochloride were purchased from N.L.S. Animal Health (Pittsburgh, PA). All other chemicals were purchased from Fisher Scientific Co. (Pittsburgh, PA).

II b. Animals. Outbred adult male Sprague Dawley rats (350-400 g; Charles River Laboratories Inc., Wilmington, MA) were housed individually and received standard rat chow and water ad libitum. Rats were maintained on
standard chow during acclimatization to the environment and assessment of neurobehavioral predictors. Behavioral procedures were carried out in operant chambers during the light cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

II c. Experimental design and procedures. Impulsivity, motivation for HF-reinforcers, striatal DAT function and extracellular dopamine concentration were evaluated as neurobehavioral predictors of DIO. Following the evaluation of predictors using behavioral and neurochemical assays, rats were fed the HF-diet for 8 wk. Following the 8-wk HF-diet exposure, rats were segregated into OP and OR groups as previously described in chapter two (methods section) (Fig. 22). The LF group was not employed in the evaluation of the predictors of obesity, because the LF-diet does not result in obesity. The details of each experiment follow.

Study 1. As a predictor of DIO, impulsivity was determined using the delay discounting task that was conducted exactly as described previously in the methods section of chapter three. An experimentally naïve group of rats (n = 22 rats) was used for this study. Session was a within-subject factor. To facilitate lever responding, rats were provided with 15 g of standard chow following each operant session. Following the delay discounting task, this group was used to evaluate motivation for food reinforcement (Study 2).
Study 2. As a predictor of DIO, motivation for HF food reinforcement was determined using the PR schedule of reinforcement that was conducted for daily 3-h sessions for 6 consecutive days. The PR schedule was conducted exactly as described previously in the methods section of chapter three. Session was a within-subject factor. To increase hunger and thereby facilitate lever responding, rats were provided with 15 g of standard chow following each operant session. Subsequently, this group of rats was used to evaluate in vivo striatal DAT function and extracellular dopamine concentration (Study 3).

Study 3. As predictors of DIO, striatal DAT function and extracellular dopamine concentration in vivo were determined using the no net flux microdialysis assay that was conducted exactly as described in the methods section of chapter two. Dopamine concentration was a within-subject factor. Rats employed in this study were previously used to evaluate impulsivity and motivation for food reinforcement (Studies 1 and 2).

Following microdialysis, rats were fed the HF-diet for 8 wk. Body weight and food intake were monitored across the 8-wk period, as described previously. Following the 8-wk HF-diet exposure, rats were segregated into OP and OR groups. Whole brains were obtained and fixed in Chromasolv® for sectioning (Leica 1850 M, Nussloch, Germany) for determination of probe placement.
**II e. Data analysis.** Data are presented as mean values ± S.E.M., and ‘n’ represents the number of animals tested for each group. Data were analyzed using GraphPad Prism 4.0 program (GraphPad Software Inc., San Diego, CA).

Time dependent differences in body weight and energy intake between groups were determined by two-way ANOVA, with OP and OR as a between-subject factor and time as a within-subject factor. Student’s unpaired t-test was used to determine between-group differences in body weight, energy intake and % body weight gain after 8 wk of dietary treatment. Mean adjusted delay was obtained from the delay discounting task as a measure of impulsivity. Rats with mean adjusted delay (averaged for the last 5 sessions of free choice trials) below the median were categorized as high-impulsive and rats with mean adjusted delay above the median were categorized as low-impulsive. For no net flux microdialysis, the difference between perfusate and dialysate dopamine concentration \( [\text{dopamine}_{\text{in}} - \text{dopamine}_{\text{out}}] \) for each rat was plotted as a function of perfusion concentration \( [\text{dopamine}_{\text{in}}] \). Slope of the linear regression represents extraction fraction \( (E_d; \text{index of DAT function}) \) and the regression line x-axis intersect represents the no net flux point (extracellular dopamine concentration) (Justice, 1993). Pearson’s correlation coefficients determined relationships between % body weight gain and each behavioral and neurochemical measure that include mean adjusted delay, PR breakpoint, extraction fraction and extracellular dopamine concentration.
III. Results

**III a. Development of DIO**

Body weight and energy intake for OP and OR groups during the 8-wk exposure to the HF-diet are illustrated in Fig. 23. Analysis of the body weight data revealed a group x time interaction ($F_{7, 77} = 17.2$, $p < 0.0001$). Prior to diet exposure, body weight did not differ between groups (Fig. 23: top). At 3 wk of diet exposure, body weight for the OP group was greater than that for the OR group. At the end of the 8-wk HF-diet exposure, the OP group exhibited 20% greater body weight than the OR group. Also, the OP group exhibited 17% greater energy intake than the OR group, across 8 wk ($F_{7, 77} = 2.21$, $p < 0.05$; Fig. 23: bottom).

**III b. Impulsivity does not predict the development of DIO**

To determine if impulsivity predicts the development of DIO, the delay to obtain the larger sucrose reinforcer was determined prior to an 8-wk exposure to the HF-diet. As illustrated in the scatter plot (Fig. 24), both OP and OR groups included rats that exhibited low mean adjusted delay. The mean adjusted delay varied by less than 5 s across the last 5 days of testing all of the animals. The median of the group was 7.5 s. Rats with mean adjusted delay below and above 7.5 s were categorized as high- and low-impulsive groups, respectively. Linear regression analysis of the data showed no correlation between mean adjusted delay and % body weight gain (Pearson $r = 0.227$, $p = 0.35$).
III c. Motivation to obtain HF-reinforcers predicts the development of DIO

To determine if motivation to obtain HF-food predicts the development of DIO, PR breakpoint for HF-reinforcers was determined prior to an 8-wk HF-diet exposure. Linear regression analysis of the data showed a positive correlation between PR breakpoint for HF-reinforcers and % body weight gain (Pearson r = 0.51, p < 0.05, Fig. 25; top). Mean PR breakpoint for HF-reinforcers across the 6 sessions was greater in OP compared to OR rats (p < 0.01; Fig. 25; bottom).

III d. In vivo striatal DAT function and basal extracellular dopamine concentration do not predict the development of DIO

To determine if striatal DAT function and basal extracellular dopamine concentration predicts the development of DIO, in vivo microdialysis, using the no net flux microdialysis method, was conducted prior to an 8-wk HF-diet exposure. Linear regression analysis of the data showed no correlation between extraction fraction (index of DAT function) and % body weight gain (Pearson r = 0.275, p = 0.3, Fig. 26; top). Also, no correlation was found between striatal basal extracellular dopamine concentration and % body weight gain (Pearson r = -0.291, p = 0.275, Fig. 26; bottom).

IV. Discussion
The current study provides novel findings that motivation to obtain HF-food predicts the development of obesity in an outbred animal model of DIO, whereas surprisingly, impulsivity does not predict DIO. A pre-existing dopamine reward deficiency has been suggested to contribute to compensatory overeating of carbohydrates leading to human obesity (Blum et al., 2006). The current results indicate that striatal DAT function and extracellular dopamine do not predict DIO. Thus, deficiency in striatal DAT function does not underlie obesity resulting from consumption of a HF-diet.

Personality traits including reward-seeking and high impulsivity (inability to resist cravings, lack of premeditation and perseverance) are known to regulate drug addiction in humans (Whiteside and Lynam, 2001). Given that common neurobehavioral mechanisms underlie obesity and drug addiction (Volkow et al., 2008; Kenny, 2011), hallmarks of drug addiction including impulsivity and motivation were evaluated in the DIO model. Impulsivity evaluated using delay discounting is shown to predict psychostimulant intake in animal models (Perry and Caroll, 2008; Marusich and Bardo, 2009). In the current study, impulsivity did not predict DIO, suggesting that distinct mechanisms underlie the predisposition for drug addiction and obesity. Nonetheless, interpretation of the current study is limited somewhat by the delay discounting task, which measures only one facet of the construct of impulsivity. Therefore, evaluation of other facets of impulsivity might provide a broader perspective in highlighting the role of impulsivity in the development of obesity.
Lack of correlation between mean adjusted delay and body weight gain suggests that impulsivity does not predict the development of DIO. Unlike impulsivity, motivation for HF-reinforcers predicted the development of DIO, as indicated by a positive correlation between PR breakpoint for HF-reinforcers and body weight gain. The current results are consistent with previous studies in which PR responding for sucrose pellets were determined in Wistar rats that were subsequently exposed to a high-fat high-sucrose (HFHS) diet or a chow-diet for 4 wk (Fleur et al., 2007). In contrast to the chow-fed control, a positive correlation was found between PR responding for sucrose pellets and abdominal fat stores in rats fed the HFHS-diet. However, unlike the current study, rats fed the HFHS-diet were not segregated into OP and OR phenotypes. Although both current and previous studies report pre-existing increases in food-motivated behavior, the two studies differ with respect to reinforcers, home-cage diet, duration of diet exposure and obesity model employed. In the previous study, pre-existing increases in motivation for sucrose reinforcers did not translate into increased energy intake upon 4-wk exposure to the HFHS diet. Conversely, in the current study, a pre-existing increase in PR breakpoint for HF-reinforcers was consistent with increased energy intake in the OP phenotype throughout the 8-wk exposure to the HF-diet. This discrepancy may be attributed to distinct postingestive mechanisms associated with HF- and sucrose-reinforcers/diets (Warwick and Weingarten, 1995; Avena et al., 2009; Gaillard et al., 2008). While preference for high-fat was not determined in the current study, previous studies have demonstrated increased choice for fat compared to carbohydrate and
protein diets in inbred obesity-prone (Osborne-Mendel) compared to obesity-resistant (S5B\PI) rats during a macronutrient choice procedure (Okada et al., 1992). Similar increases in fat preference may underlie increased motivation for HF-reinforcers in the current outbred DIO model.

Reward associated with food intake is known to be mediated by NAc dopamine; however, the development of habitual behavior appears to involve a shift in underlying dopaminergic control from NAc to dorsal striatum (Ito et al., 2002; Kelley, 2004; Everitt and Robbins, 2005; Koob and Volkow, 2010). In the current study, striatal DAT function and extracellular dopamine concentration did not predict obesity that developed following an 8-wk HF-diet exposure, leading to large body-weight differences of 150 g. Decreased extracellular dopamine in NAc-shell has been reported to predict obesity, and excessive food intake was interpreted as a compensation for low basal dopamine in NAc-shell (Rada et al., 2010). However, these results were obtained only after 5 days of HF-diet, the between-group body-weight differences were only 20 g, and the lower dopamine levels may have resulted from transient exposure to the HF-diet per se. In contrast, in the current study, striatal dopamine did not predict obesity when evaluated prior to the HF-diet exposure. That is, individual differences in striatal dopamine prior to the HF-diet exposure did not correlate with long-term body-weight gain.
The lack of pre-exisiting individual differences in striatal dopamine function between OP and OR phenotypes is supported by previous reports that suggest dorsal striatal mechanisms to be recruited only upon development of habitual and compulsive behaviors (Ito et al., 2002; Everitt et al., 2008; Wise, 2009). Therefore, prior to repeated exposure to the HF-diet, alternate neural mechanisms that mediate reward sensitivity and associative learning may regulate the susceptibility to DIO. Further studies evaluating NAc dopamine as a predictor of obesity are needed.

In summary, motivation for HF-food, but not impulsive behavior, predicts the development of DIO. Both striatal DAT function and basal extracellular dopamine concentration did not predict the development of obesity. Nonetheless, motivation for HF-food likely promotes excessive food intake associated with the development of obesity.
Figure 22

Behavioral and neurochemical assays

HF diet (31.8% Kcal fat)

Segregation into OP and OR

EVALUATION OF PREDICTORS

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Week 2-7</th>
<th>Week 8</th>
</tr>
</thead>
</table>

Monitor body weight and food intake

Obesity Resistant group (OR): minimum body weight gain

Obesity Prone group (OP): maximum body weight gain
Figure 22: Evaluation of neurobehavioral predictors of DIO. Adult male Sprague Dawley rats were fed standard chow during the evaluation of neurobehavioral predictors and were subsequently exposed to the HF-diet for 8 wk. Food intake was determined daily and body weight determined 3 times weekly. Following the 8-wk exposure to the HF-diet, rats were segregated into OP and OR groups based on body weight gain (top third and bottom third, respectively). (Picture courtesy of Victoria English).
Figure 23: Development of DIO in rats employed to evaluate the neurobehavioral predictors. Rats were maintained on standard chow during assessment of neurobehavioral predictors of DIO and were subsequently exposed to a HF-diet for 8 wk. Rats were segregated into OP and OR groups based on body weight gain. Top: OP rats exhibited greater body weight compared to OR rats from wk 3-8 of the dietary treatment. Bottom: OP rats exhibited greater energy intake compared to OR rats from wk 1-6. Data are expressed as the mean (SEM are smaller than the size of the symbol); n = 6 rats/group; *p < 0.05, indicates OP different from OR.
Figure 24

Pearson $r = 0.227$
$p = 0.35; n = 19$
**Figure 24:** Lack of correlation between mean adjusted delay and % body weight gain. Mean adjusted delay was determined prior to an 8-wk exposure to the HF-diet. No correlation between mean adjusted delay and % body weight gain was found. Data points represent individual rats (n = 19; Pearson r = 0.227, p = 0.35).
Figure 25

**Graph 1:**

- **Y-axis:** % Body weight gain
- **X-axis:** PR breakpoint
- **Pearson r = 0.51**
- **p < 0.05, n = 19**

**Graph 2:**

- **Y-axis:** PR breakpoint
- **X-axis:** OP and OR
- **Statistical Significance:** *
Figure 25: Positive correlation between PR breakpoint for HF-reinforcers and % body weight gain. Mean PR breakpoint for HF-reinforcers across the 6 sessions was determined prior to an 8-wk exposure to the HF-diet. Top: A positive correlation between PR breakpoint for HF-reinforcers and % body weight gain was found. Data points represent individual rats (n = 19; Pearson r = 0.51, p < 0.05). Bottom: In the same group of rats, the OP group exhibited a greater PR breakpoint for HF-reinforcers compared to the OR group. Data are expressed as mean ± SEM; n = 6 rats/group; *p < 0.01, indicates OP different from OR.
Figure 26

Pearson $r = 0.275$
$p = 0.30$, $n = 16$

Extracellular DA, nM

Pearson $r = -0.291$
$p = 0.275$, $n = 16$
Figure 26: Lack of correlation between in vivo striatal dopamine function and % body weight gain. No net flux *in vivo* microdialysis was conducted prior to an 8-wk exposure to the HF-diet. Data points represent individual rats. Linear regression of the data shows no correlation between striatal extraction fraction and % body weight gain (top). Also, no correlation between striatal basal extracellular DA concentration and % body weight gain was found (bottom).
Chapter Five

Overall Discussion

I. Review

Obesity has emerged as a major worldwide health concern. Latest reports from the IOTF indicate that around 475 million adults and 40-50 million children in the world are obese (IASO, 2012). The dramatic rise in obesity rates has been attributed to multiple factors that include; genetic susceptibility, increased ease of access to diets rich in fat, sugar and salt, and a sedentary lifestyle lacking adequate physical activity (Deedwania, 2004; Farooqi and O’Rahilly, 2006; Valentino et al., 2010). Obesity is also associated with several metabolic disorders, cardiovascular complications and certain types of cancer (IASO, 2012). Evidently, escalating obesity rates have serious health and economic consequences. Thus, there is a critical need for the development of safe and effective strategies to treat this medical condition, and prevent further escalation of the obesity epidemic.

Obesity results from a long-term positive energy balance such that energy intake exceeds energy expenditure. Obesity research over the past few decades has unveiled potential central and peripheral anti-obesity molecular targets (Valentino et al., 2010). In particular, comprehensive understanding of hypothalamic mechanisms that regulate energy homeostasis has increased over
the past few decades. The homeostatic system comprises of hunger, satiety and adiposity hormones which act on hypothalamic and brainstem circuits to regulate food intake and energy expenditure (Hommel et al., 2006; Morton et al., 2006). Importantly, dysfunction in one or more components of the homeostatic system has been implicated in the development of obesity (Druce et al., 2005; Meyers et al., 2008). While homeostatic systems regulate energy homeostasis under normal physiological conditions, persistent indulgence in palatable HF foods is believed to involve the higher brain reward systems (Palmiter, 2007). Furthermore, hormonal regulators of energy homeostasis that include leptin, insulin and ghrelin are shown to act on the brain reward system, specifically the mesoaccumbens dopamine system (Kenny, 2011).

Obesity is associated with increased preference and excessive consumption of palatable HF foods (Sclafani et al., 2001; Gaillard et al., 2008). Importantly, activation of the mesolimbic and the striatal dopamine reward systems are implicated in the reinforcing and motivational effects of palatable foods (Martel and Fantino, 1996; Small et al., 2003; Olausson et al., 2006). Further, a shift in control from dopamine pathways in the NAc to striatum is believed to occur coincident with the development of habitual behaviors (Wise, 2009; Koob and Volkow, 2010). The involvement of striatum in motivation for food is supported by findings that rats over-expressing striatal delta Fos B exhibit high PR breakpoints for food reinforcement (Olausson et al., 2006). Human brain imaging studies demonstrate an inverse relationship between BMI and striatal D2 receptors.
receptor density, suggesting striatal dopaminergic dysregulation in obesity (Wang et al., 2001; Volkow et al., 2008). Furthermore, obese rats are shown to exhibit increased thresholds for electrical brain stimulation reward, increased resistance to aversive stimuli-induced disruption of food consumption, and decreased striatal D2 receptors (Johnson and Kenny, 2010). Thus, dysregulated striatal dopaminergic function may underlie excessive food intake in obesity.

Extracellular dopamine levels are primarily regulated by DAT, which translocates dopamine back into the neuron, thereby terminating activation of presynaptic and postsynaptic dopamine receptors (Sulzer et al., 2005). Implication of DAT in food reward is suggested by results from DAT knockout studies in which DAT deficient mice exhibited increased extracellular dopamine and greater food intake, compared to wild-type mice (Pecina et al., 2003). Furthermore, genetic linkage analysis revealed that DAT polymorphism is associated with binge-eating (Shinohara et al., 2004). Thus, elevated dopamine as a result of dysregulated DAT function may contribute to alterations in food intake and the development of obesity. Furthermore, motivation for palatable foods and impulsivity also play an important role in obesity (Weller et al., 2008; Mobbs et al., 2010).

Current pharmacotherapies for obesity remain limited with respect to weight loss, amelioration of metabolic abnormalities, safety and tolerance (Cawthrone, 2007; Kushner, 2012). Further investigation of the role of higher
brain reward systems and behavioral mechanisms in obesity may facilitate pharmacological and behavioral interventions for the treatment and prevention of obesity. Thus, the purpose of this dissertation was to investigate the role of the striatal dopamine reward system, impulsivity and motivation in obesity. Importantly, striatal dopaminergic and behavioral mechanisms were evaluated as both outcomes and predictors of obesity using a rat model of DIO.

**Neurobehavioral outcomes of DIO.** Evaluation of the neurochemical and behavioral outcomes of DIO are detailed in chapters 2 and 3, respectively. As neurochemical outcomes of DIO, striatal D2 receptor density, VMAT2 function, DAT function and expression, methamphetamine-induced DAT reverse transport, and extracellular dopamine concentration were evaluated in the outbred DIO rat model. Striatal D2 receptor density was decreased in OP relative to OR rats, consistent with human obesity (Wang et al., 2001). Decreases in striatal D2 receptor density may be compensatory to increased VMAT2 function, decreased striatal DAT function and increased extracellular dopamine concentration. No between-group differences in striatal VMAT2 function were found suggesting that VMAT2 function is not altered following the development of DIO. However, OP rats exhibited decreased striatal DAT function (*in vitro* and *in vivo*) and increased extracellular dopamine concentration compared to OR rats. In contrast with the decrease in DAT function, an increased effect of methamphetamine to induce DAT reverse transport was found in OP relative to OR striatum. DAT cellular localization was evaluated as a potential mechanism
underlying altered DAT function in DIO. Total DAT protein was decreased in OP relative to OR striatum; however, no differences in cell-surface or intracellular DAT expression were found. These results suggest that regulation of DAT function in DIO is trafficking-independent.

Impulsivity and food motivated behavior were evaluated as behavioral outcomes of DIO. The OP rats exhibited decreased impulsivity in the delay discounting task, compared to the OR rats. These results are in contrast to a study showing increased impulsive choice exhibited by obese women in a delay discounting task (Weller et al., 2008). Importantly, motivation for both HF and LF reinforcement was greater in OP compared to OR rats.

**Neurobehavioral predictors of DIO.** Human studies are limited by their ability to determine if impulsivity, motivation, DAT function and extracellular dopamine concentration serve as predictors of DIO. In the current study, these neurobehavioral factors were evaluated in an outbred rat model, prior to an 8-wk HF-diet exposure. Results demonstrated that motivation to obtain HF-food predicts the development of obesity, whereas impulsivity does not predict DIO. Also, striatal DAT function and extracellular dopamine concentration did not predict DIO.

Collectively, results from the outcome and predictor studies demonstrate that as a predictor, motivation for HF-reinforcers was greater in rats which subsequently became obese upon the 8-wk exposure to the HF-diet, compared
to those that did not become obese when exposed to the same diet for the same period of time. The greater level of motivation continued to be observed in the OP rats, compared with OR rats, once DIO was established, indicating that this factor is involved in both the initiation and maintenance of obesity. In contrast, impulsivity, DAT function and extracellular striatal dopamine levels did not predict the development of obesity upon exposure to the HF-diet. A pre-existing dopamine reward deficiency has been suggested to contribute to compensatory overeating of carbohydrates leading to human obesity (Blum et al., 2006). The current results indicate that a deficiency in striatal DAT function is an outcome of DIO, but does not underlie obesity resulting from consumption of a HF-diet. Once DIO was established, the level of impulsivity decreased, DAT function decreased and extracellular striatal dopamine concentration increased in OP rats relative to OR rats, indicating that obesity has an impact on activation of the striatal dopamine system and on impulsive behavior. Although the impact on the dopaminergic system was expected, the decrease in impulsivity was an unanticipated outcome. A working model of dopaminergic outcomes in OP and OR phenotypes are illustrated in Fig. 27.

II. Mechanisms underlying decreased DAT function and increased methamphetamine-induced DAT reverse transport in DIO

Extracellular levels of dopamine are primarily regulated by DAT function (Sulzer et al., 2005). Importantly, DAT-deficient mice exhibit increased
extracellular dopamine and greater food intake compared to wildtype-mice
(Pecina et al., 2003). Based on these prior observations, the hypothesis of the
current study was that striatal DAT function will be decreased and extracellular
dopamine levels will be increased in obesity. Consistent with this hypothesis,
both our current *in vitro* and *in vivo* results demonstrate decreased striatal DAT
function and increased extracellular dopamine levels following the development
of DIO. Since clearance of extracellular dopamine is primarily regulated by DAT
localized at the cell-surface of dopaminergic neurons (Zahniser and Sorkin,
2004), one explanation for the decrease in DAT function is a decrease in cell-
surface DAT expression. Thus, the expectation was that obesity will be
associated with diminished impact of methamphetamine as a result of diminished
cell-surface DAT expression available for methamphetamine-induced reverse
transport.

Refuting the proposed hypothesis, the current results revealed increased
methamphetamine-induced DAT reverse transport in OP relative to OR striatum.
Obesity may differentially alter DAT functions, i.e., dopamine uptake and
dopamine reverse transport, by modulating the specific signaling mechanisms
that regulate these functions. In the current study, striatal dopamine uptake was
decreased in OP compared to OR, which could be explained by a decrease in D2
autoreceptor function in OP, considering previous observations that D2
autoreceptors regulate DAT function (Cass and Gerhardt, 1994).
The decreased DAT function in OP relative to OR could be alternatively explained by activation of PKC, which has been previously shown to decrease dopamine uptake at hDAT expressed in *Xenopus* oocytes (Zhu et al., 1997). With respect to DAT reverse transport, PKC activation is shown to increase amphetamine-induced dopamine reverse transport in both striatal slices and HEK 293 cells transfected with DAT (Kantor and Gnegy, 1998; Khoshbouei et al., 2004; Robertson and Galli, 2009). Thus, PKC activation may underlie the unanticipated increase in methamphetamine-induced reverse transport of DAT in the current study.

Another explanation for the decreased dopamine uptake and increased methamphetamine-induced DAT reverse transport is differential partitioning of membrane DAT into lipid-raft and non-raft micro-domains. Previous studies suggest that cell-surface DAT can be fractionated into at least two different micro-domains (i.e., raft and non-raft membrane compartments; Foster et al., 2008). Rafts are composed of a greater percentage of lipids than the non-raft compartments. DAT distribution was reported to be 35% in raft and 65% in non-raft striatal membranes from standard housed rats. Importantly, phosphorylation of DAT by PKC is shown to occur to a greater extent in the lipid raft compartment of the membrane, compared with the non-raft compartment (Foster et al., 2008). Thus, DAT partitioning between lipid-raft and non-raft membrane compartments may underlie the decrease in dopamine uptake and increase in methamphetamine-induced reverse transport in obesity.
III. Pattern of neurobehavioral responses between OP, OR and LF groups

The design of the current studies included the LF group only for the evaluation of the outcomes of obesity, not for the evaluation of the predictors of obesity. Since the LF-diet does not result in obesity; including the LF group in the predictor studies simply would not have provided information about predictors of obesity.

Generally, the pattern of response between OP and OR was consistent, i.e., there was a difference between these groups and the direction depended on the outcome measured. However, comparisons between OP and LF vs. OR and LF were not always consistent in the studies that evaluated the neurobehavioral outcomes of DIO. For several neurochemical outcome measures, including dopamine uptake, DAT expression and extracellular dopamine concentration, no differences were found between OP and LF groups. Also, no differences were found between OR and LF groups on behavioral outcome measures of impulsivity, nor for motivation for food. Thus, OP and OR rats were fed the HF-diet, and LF rats were fed the LF-diet, yet neither the OP nor the OR groups were different from the LF group with respect to these neurobehavioral outcomes. An interpretation of this result is that these outcome measures are not altered by the diet per se, but instead, altered by the neurobiological mechanisms underlying obesity.
A different pattern emerged when comparisons were made between the OP and LF groups for methamphetamine-induced reverse transport of DAT. The effect of methamphetamine was lower in the OP group than in the LF group; further, the effect of methamphetamine was lower in the OR group compared with the OP group. Thus, an outcome of prolonged exposure to the HF-diet *per se* may be a downregulation of the signaling mechanisms that regulate the interaction of methamphetamine with DAT, resulting in decreased dopamine release into the extracellular space in both OP and OR groups relative to the LF group. Importantly, since the OP group exhibited a greater effect of methamphetamine compared to the OR group, it appears that obesity mitigated the effect of the diet *per se* on this specific outcome measure. Thus, both the development of obesity and effects of the diet *per se* contribute to the observed differential between-group effects of methamphetamine.

A somewhat different pattern emerged in the no net flux microdialysis studies evaluating *in vivo* DAT function. In contrast to the pattern of effect for methamphetamine, the OP group exhibited lower DAT function *in vivo* compared with the LF group; however, the OR group was not different from the LF group, indicating that the diet *per se* does not affect this outcome measure. Importantly, the OP group exhibited a lower DAT function *in vivo* compared to the OR group and this effect is not dependent on the diet since both OP and OR groups were fed the HF-diet. Thus, obesity alone appears to contribute to differential *in vivo* DAT function between the OP and OR groups.
Overall, the LF group is comprised of an unknown distribution of both OP and OR phenotypes. Based on sampling distribution, the pattern of response might vary across experiments. Thus, cross-experiment direct statistical comparisons to LF are inappropriate and were not conducted.

IV. Alternate explanations for decreased impulsivity and increased food-motivated behavior following the development of DIO

Increased sensitivity to palatable-food reward in obese humans is shown to drive overeating only when accompanied by insufficient inhibitory control (Appelhans et al., 2011). Based on prior reports, the hypothesis of the current study was that high impulsivity may underlie the inability to resist excessive eating in obesity. In contrast, OP rats demonstrated decreased impulsivity i.e. increased choice for the larger delayed reward in the delay discounting task, compared to OR rats. Consistent with the current results, decreased impulsivity in a delay discounting task was reported in rats with excitotoxic lesions of the OFC (Winstanley et al., 2004). Despite increases in the delay to the larger reward, OFC-lesioned rats did not shift preference for the smaller immediate reward, suggesting that the delay failed to devalue the large reward. Thus, dysregulated OFC function may underlie decreased impulsivity in OP compared to OR rats.
In humans, self-reported impulsivity is shown to negatively correlate with activation in different cortical areas that mediate inhibitory control that includes; superior medial frontal gyrus, bilateral ventral PFC, dorsal amygdala, and right dorsolateral PFC (Horn et al., 2003; Asahi et al., 2004; Brown et al., 2006). Interestingly, higher BMI is shown to be associated with lower baseline metabolism in PFC and cingulate gyrus, along with associated impairments in inhibitory control processes (Volkow et al., 2009). Furthermore, a structural MRI study reported lower gray matter density in the medial frontal gyrus of the PFC in obese versus lean individuals, suggesting dysregulated inhibitory control in obesity (Pannacciulli et al., 2006). Thus, impairments in neural systems that mediate inhibitory control and executive functions may increase the vulnerability to indulge in excessive eating behaviors (Wang et al., 2009). Mesocorticolimbic function was not investigated in the current DIO model. Insight into these alternate mechanisms may provide additional explanation for the observed decrease in impulsivity following the development of DIO.

In the current study, sucrose reinforcers were employed during the delay discounting task. Previous studies have investigated the influence of the quality and quantity of the delayed reinforcer on the rate of discounting food rewards (Calvert et al., 2010). The amount and quality of the delayed reinforcer employed i.e., precision food pellets vs. cellulose; saccharin vs. quinine were varied across experimental conditions (Calvert et al., 2010). Rate of discounting was derived as a function of the amount and type of the reinforcer employed as well as the delay
until the receipt of the food reinforcer (Myerson and Green, 1995). Despite increased preference for precision food pellets over cellulose and for saccharin over quinine, no differences in the rate of discounting for the different types of reinforcer were found. These results suggest that the quantity and quality of the reinforcer type do not influence discounting of the delayed reward. Thus, despite increased preference for fat over carbohydrate diets (Okada et al., 1992), employment of HF-reinforcers over sucrose pellets in the current study may not have yielded different results with respect to impulsivity measured using the delay discounting task.

In contrast to decreased impulsivity following the development of DIO, increased food-motivated behavior was found in OP relative to OR rats. In the current study, motivation for HF-reinforcers was determined as opposed to sucrose reinforcers, since obesity is known to result from excessive consumption of HF-diets. Decreased DAT function and increased extracellular striatal dopamine levels may underlie increased food-motivated behavior in OP rats. Dopamine is implicated in incentive motivation for food reinforcement (Wise, 2004). DAT-deficient mice that exhibit elevated extracellular striatal dopamine levels also demonstrate enhanced PR responding for palatable foods, compared to wildtype control (Caignard et al., 2006). Delta FosB is a transcription factor that accumulates in dynorphin-expressing medium spiny neurons of both the NAc and dorsal striatum upon repeated exposure to natural rewards or drugs of abuse (Nestler, 2008; Perroti et al., 2008). DAT-deficient mice also exhibited increased
delta FosB expression in the NAc-core and dorsal striatum, consistent with previous reports that suggest upregulation of delta FosB expression upon chronic dopaminergic stimulation (Nestler, 2008). Importantly, over expression of striatal delta FosB is shown to increase PR responding for palatable foods indicating that delta FosB expression increases food-motivated behavior (Olausson et al., 2006). Furthermore, increased striatal delta FosB expression was detected in adult mice that had access to palatable HF or sucrose diets, and this effect was associated with enhanced motivation for palatable foods (Wallace et al., 2008). In addition to striatal delta FosB expression, additional intracellular signaling cascades are implicated in modulating incentive motivation for food (Kenny, 2011). Dopamine receptor signaling in the striatum is shown to increase motivational properties of food via inhibition of cyclin-dependent kinase 5 and also via dephosphorylation of protein phosphatase 1 regulatory subunit 1B (Benavides et al., 2007; Stipanovich et al., 2008). Taken together, activation of transcriptional mechanisms as a result of decreased striatal DAT function, increased extracellular dopamine levels and increased dopaminergic signaling may underlie increased motivation for food reinforcement in DIO. On the other hand, increased DAT function in OR relative to OP rats may enable the OR phenotype to resist transcriptional modulation of food-motivated behavior, despite exposure to the HF-diet.

V. Neurobehavioral predictors of DIO
Motivation for HF-reinforcers predicted the development of DIO; however, impulsivity was not a predictive factor. Thus, neural systems that regulate impulsivity may not regulate predisposition to DIO. Also, striatal DAT function and extracellular dopamine concentration did not serve as predictors of DIO. Given the role of striatal dopamine in food motivated behavior, motivation for HF-reinforcers was not associated with corresponding increases in extracellular striatal dopamine levels for the predictor study. A possible explanation for this observation is that the dorsal striatum does not play a major role in primary reward but appears to be recruited only during the development of compulsive and habitual behaviors (Everitt et al., 2008). Importantly, striatal dopaminergic mechanisms were recruited only after the development of DIO in the current study, suggesting that these mechanisms facilitate the maintenance of habitual and excessive food intake in obesity. Prior to an 8-wk exposure to the HF-diet, alternate reward systems may modulate incentive motivational properties of HF-reinforcers. NAc dopamine mediates primary reward and incentive motivation for food reinforcers (Bassareo and Di Chiara, 1999). Thus, dopamine function in alternate reward systems, that includes the mesocorticolimbic system, may serve as predictors of DIO.

VI. **Neuroendocrine regulation of the striatal dopamine reward system**

Given the interaction between homeostatic and reward systems, sustained stimulation of dopamine reward circuits by adiposity hormones may underlie
increased food-motivated behavior following DIO. Previous studies have demonstrated defective central leptin- and insulin-signaling relative in OP relative OR rats (Levin and Dunn-Meynell, 2002; Clegg et al., 2005). Metabolic hormones that regulate energy homeostasis also have been shown to modulate the activity of the dopamine reward system (Palmiter, 2007). Double-label studies have demonstrated the co-expression of leptin and insulin receptors with tyrosine hydroxylase, a marker of dopamine neurons (Figlewicz et al., 2003). Leptin inhibits dopamine neuron firing rate and decreases extracellular NAc dopamine levels (Krugel et al., 2003; Hommel et al., 2006). Intraventricular insulin administration has been shown to increase DAT mRNA in rat VTA/substantia nigra, suggesting insulin-mediated decreases in extracellular dopamine concentration (Figlewicz et al., 1994). Also, local application of insulin to VTA slice preparations revealed inhibition of evoked somatodendritic dopamine via PI3K and mTOR activation (Mebel et al., 2012). Furthermore, this effect was abolished in the presence of a selective DAT inhibitor, GBR 12909, as well as in VTA slices of DAT knockout mice, suggesting that insulin regulates DAT function (Mebel et al., 2012). Intracerebroventricular leptin and insulin infusion has been shown to decrease sucrose self-administration and reverse CPP for HF-food (Figlewicz et al., 2004, 2006). Knockdown of midbrain leptin receptors in rats has been shown to increase PR responding for sucrose reinforcers (Davis et al., 2011). Taken together, development of DIO and increased food intake may lead to increased adiposity hormones stimulation of the reward circuitry, including decreased DAT function and increased extracellular dopamine.
VII. Implications

The results from the current dissertation research imply that alterations in striatal DAT function and extracellular dopamine concentration occur as a result of DIO. Motivation for HF-food reinforcers predicted the development of DIO and the greater level of motivation persisted following the development of DIO. Decreased DAT function, increased extracellular dopamine concentration and decreased striatal D2 receptors following the development of DIO may contribute towards an increase in reward threshold in the OP phenotype. Overall, these striatal dopaminergic alterations may underlie the maintenance of non-homeostatic food intake in obesity.

VIII. Limitations

In the current study, decreased striatal D2 receptor density was found following the development of DIO. Striatal D2 receptor availability is known to modulate reinforcing properties of food and govern vulnerability to addictive behaviors (Volkow et al., 2003). Reduced striatal D2 receptors is believed to produce a reward deficit such that this deficiency predisposes an individual to a high risk of rewarding, compulsive addictive behaviors, like excessive indulgence in palatable foods, in order to compensate for the understimulated dopaminergic system (Blum et al., 2006; Volkow et al., 2008). Thus, decreased striatal D2 levels may have preceded the development of DIO and hence may serve as a
predictor of DIO, which was not investigated in the current study. As a predictor of DIO, striatal D2 receptor density can be evaluated prior to the 8-wk HF-diet exposure, using positron emission tomography scanning. Also, the role of D1 receptors in obesity is not clear. Thus, in addition to D2 receptors, further studies that investigate the expression and function of striatal D1 receptors in obesity are needed.

The effect of methamphetamine on DAT reverse transport was evaluated in the current DIO model. In addition to DAT reverse transport, methamphetamine also decreases vesicular dopamine uptake and promotes dopamine release from the vesicles (Sulzer et al., 2005). Striatal VMAT2 function was not altered following the development of DIO. However, the effect of methamphetamine on VMAT2 function following the development of DIO was not investigated. Thus the current study provides limited understanding of the role of striatal VMAT2 in DIO.

Extracellular striatal dopamine levels were evaluated as a function of dopamine uptake mechanisms. Synthesis, metabolism and release mechanisms also contribute towards extracellular dopamine levels; however these mechanisms were not investigated in the current study. Importantly, D2 autoreceptor-stimulation exerts a negative feedback on dopamine neurotransmission by inhibition of dopamine synthesis and release and increases in DAT function (Dwoskin and Zahniser, 1986; Meiergerd et al., 1993; Cass and
Gerhadt, 1994). Further studies investigating D2 autoreceptor function in the DIO model are needed to provide better understanding of mechanisms that contribute towards decreased DAT function and increased extracellular DA levels in obesity.

Given that total DAT protein levels were decreased in OP relative to OR rats, this effect was not localized to either the cell-surface or the intracellular locus using the biotinylation assay. The biotinylation assay enables the isolation of a relatively purified cell-surface fraction as opposed to the intracellular fraction. As such, lack of corresponding alterations in intracellular DAT levels may have been overlooked during the biotinylation assay due to technical limitations.

Alterations in $E_d$ values obtained in the no net flux microdialysis assay reflect changes in tissue resistance mechanisms that include tortuosity and uptake mechanisms (Bungay et al., 1990; Chefer et al, 2009). Considerable evidence demonstrates the influence of uptake mechanisms on $E_d$ (Bungay et al., 2003). However, it is also likely that tissue lipid content may have contributed towards alteration in tissue tortuosity thereby resulting in corresponding alterations in $E_d$ values in the current study (e.g., decrease in tortuosity will increase $E_d$ values and vice-versa). The influence of tissue tortuosity on alterations in $E_d$ is unclear. Also, theoretically, as $E_d$ approaches 1 the source of resistance to diffusion may switch to the membrane (Bungay et al., 1990). Thus, alterations in $E_d$ may not reflect resistance mechanisms in the tissue alone.
However, it is not clear when this switch occurs. Therefore, it is likely that $E_d$ values closer to or greater than 1 may represent a ceiling effect under the experimental conditions employed.

In the current study, extinction procedures were conducted following the PR schedule of reinforcement which complicates the interpretation of the extinction results. Importantly, OP and OR rats exhibited different levels of responding during the PR schedule such that extinction training started at different levels for these groups. Alternatively, rats can be trained on a FR schedule (eg. FR5) that would first equalize responding between the two groups, following which extinction procedures can be conducted.

**IX. Future directions**

Future studies may include experiments that elucidate alternate mechanisms that underlie altered DAT function following the development of DIO. Cell-surface DATs are distributed into lipid-raft and non-raft microdomains (Foster et al., 2008). Greater phosphorylation of DAT by PKC occurs in the lipid-raft compared to the non-raft compartment. Importantly, PKC activation decreases DA uptake and increases amphetamine-induced DA reverse transport (Zhu et al., 1997; Khoshbouei et al., 2004). Taken together, an alternate interpretation of decreased DA uptake and increased methamphetamine-induced DA reverse transport in DIO is that cell-surface DAT in the OP striatum may be
localized to the lipid raft, relative to the non-raft microdomain. DAT localization in lipid raft and non-raft microdomains could be investigated in the DIO model, using discontinuous sucrose density centrifugation of striatal synaptosomal preparations (Foster et al., 2008). Different gradient fractions obtained could be assayed for DAT protein by Western blot analysis. Lipid composition of intact striatal tissue and isolated membrane rafts could be determined using mass spectrometry to further elucidate the effects of HF- and LF-diets on DAT function and localization.

To the extent that the dopamine reward system is malleable, interventions that increase striatal DAT function and thereby normalize dopaminergic transmission may be efficacious in the treatment of obesity. Drugs that increase DAT function, for example, D2 agonists (Meiergerd et al. 1993; Cass and Gerhardt, 1994), may serve as potential treatment options for obesity. Importantly, acute administration of bromocriptine, a D2 receptor agonist, is shown to normalize obesity-associated metabolic profile abnormalities through mechanisms independent of body weight and food intake (Kok et al., 2006). Based on current results, D2-agonists can be evaluated for their ability to normalize DAT function, extracellular dopamine concentration and food-motivated behavior in DIO. Presynaptic D2 receptors are high affinity receptors compared to postsynaptic receptors. In order to achieve selective stimulation of D2 autoreceptors lower doses of D2 agonists can be evaluated.
In addition to decreases in food intake, increases in energy expenditure is also critical for obesity intervention. There is considerable evidence that suggests environmental enrichment as a potential behavioral intervention for the prevention and treatment of obesity. Novel stimuli provided in an enriched environment are shown to increase physical activity and dopamine release in reward-relevant pathways (Rebec et al., 1997). Furthermore, environmental enrichment is shown to decrease body weight and produce alterations in DAT function in rats fed standard chow. Also, rats exposed to environmental enrichment during development are shown to be protected against the rewarding effects of stimulant drugs of abuse (Bardo et al., 2001; Zhu et al., 2004, 2005). Enrichment-induced protection may result directly from increases in physical activity and/or alterations in the dopamine reward system. Considering the common dopamine reward circuitries underlying drug addiction and obesity, environmental enrichment can be evaluated as a potential preventive and treatment strategy for obesity.

X. Final comments

The current dissertation research delineates striatal dopaminergic and behavioral mechanisms as outcomes and predictors of obesity. Results demonstrate that motivation for high-fat food, but not impulsive behavior, predicts DIO and that a higher level of motivation for food persists after the development of obesity. Interestingly, obesity results in decreased striatal DAT function, which may underlie the maintenance of habitual, compulsive food intake associated
with obesity. Pharmacological and behavioral interventions counteracting the alterations in striatal dopaminergic function may prove efficacious in the treatment of obesity.
Figure 27
**Figure 27:** Schematic of DIO effects on striatal DA. Striatal D2 receptor density and DAT function were decreased and extracellular DA concentration increased in OP compared to OR. Cell-surface DAT expression and VMAT2 function were not different between OP and OR. Closed dots represent DA.
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Professional publications

- **Narayanaswami V**, Thompson AC, Cassis LA, Bardo MT and Dwoskin LP. Diet-induced obesity: Dopamine transporter function, impulsivity and motivation. (*International Journal of Obesity*)

- **Narayanaswami V**, Somkuwar SS, Horton DB, Cassis LA and Dwoskin LP. Angiotensin AT1 and AT2 receptors modulate nicotine-evoked \[^{3}\text{H}\text{DA}\] and \[^{3}\text{H}\text{norepinephrine}\] release. (*In progress, Biochemical Pharmacology*)

- **Narayanaswami V**, Cassis LA, Bardo MT and Dwoskin LP. Obesity: Current and emerging pharmacotherapeutic targets. (*In progress, Pharmacology and Therapeutics*)

Abstracts


- **Narayanaswami V**, Deaciuc AG, Denehy E, Cassis LA, Bardo MT and Dwoskin LP. Diet-induced obesity increases motivation for high-fat and low-fat food and decreases striatal dopamine transporter function. Neural
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