Effects of the perinatal environment on the central dopamine system and body weight gain in obesity-prone rats

A dissertation

submitted by

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In partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in

Pharmacology and Experimental Therapeutics

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

Date

May 2011

ADVISOR:
Dr. Emmanuel N. Pothos

List of original papers

This dissertation includes the following articles:

Geiger BM, Behr GG, Frank LE, Caldera-Siu AD, Beinfeld MC, Kokkotou EG, and Pothos EN. Evidence for defective mesolimbic dopamine exocytosis in obesity-prone rats. *The FASEB Journal.* 2008; 22: 2740-2746.

Geiger BM, Haburcak M, Avena NM, Moyer MC, Hoebel BG, and Pothos EN. Deficits of mesolimbic dopamine neurotransmission in rat dietary obesity. *Neuroscience* 2009; 159: 1193-1199.

Geiger BM, Cappellucci LA, and Pothos EN. An obesogenic perinatal environment alters central dopamine signaling and energy balance through a microRNA-related mechanism. *Nature Neuroscience*. In submission.

Geiger BM, Cappellucci L, Umberg E, Frank L, and Pothos EN. Early elevation of central dopamine release in obesity-prone rats aids in preventing hyperphagia. *The FASEB Journal*. In preparation.

Geiger BM, Cappellucci L, Frank L, Karouani M, and Pothos EN. Perinatal influences on central dopamine signaling and the development of obesity of male offspring. In preparation.

Articles written during, but not directly related to, this dissertation:

Geiger BM, Frank LE, Caldera-Siu AD, Pothos EN. Survivable Steretotaxic Surgery in Rodents. JoVE 2008; 19. http://www.jove.com/index/Details.stp?ID=880, doi: 10.3791/880

Geiger BM, Umberg E, Pothos EN. The Reward Deficiency Hypothesis: Implications for Obesity and other Eating Disorders. In Diet, Brain and Behavior: Practical Implications eds. H.R. Lieberman and R. Kanarek. Taylor and Francis. In press

Allen P, Batra P, **Geiger BM**, Wommack T, Gilhooly C, Pothos EN. Is Dietary Obesity an Addictive Disorder? Neurobiology, Food Environment and Policy Effects of Reclassification. Physiology and Behavior. In press

Abstract

Obesity, which develops as the result of increased energy intake and/or decreased energy expenditure, has rapidly become one of the largest public health concerns in the world. The explosive prevalence of the obesity epidemic challenges the notion that a homeostatic system of energy balance is in complete control of body weight regulation. The central reward system, particularly the midbrain dopamine pathways, mediates the hedonic value of palatable food and may override the homeostatic control of energy balance. In this work, we use a multi-level approach that includes *in vivo* microdialysis and behavioral studies, *ex vivo* slice electrophysiology measurements of stimulated dopamine release, and cellular measurements of mRNA and protein expression to study the influence of dopamine on the development of obesity in inbred obesity-prone (OP) rats. In female animals that are dietary obese or obesity prone, we show decreases of approximately 50% in electrically stimulated dopamine release. These significant deficits in dopamine signaling are linked to decreased sensitivity to amphetamine injections. In the obesity-prone animals, we have also established deficits in tyrosine hydroxylase (TH) and vesicular monoamine transporter-2 (VMAT2) mRNA and protein expression. Obesity-resistant (OR) females that have been exposed to an obesogenic prenatal environment through embryonic transplantation on day E1 to obesity-prone dames show similar deficits in central dopamine neurotransmission that are linked to accelerated body weight gain and decreased spontaneous activity during the pre-pubescent period. Dopamine replacement in obesity-prone females during this period results in a decrease of

approximately 10% of both food intake and body weight and more than twice as much stimulated dopamine release in the nucleus accumbens than in obesity-prone control animals. In conclusion depressed dopamine release can be induced by exposure to an obesogenic environment during the prenatal period. Sustained deficits in dopamine during the pre-pubescent period may lead dietary obese and obesity-prone animals to compensate by overeating, particularly palatable food. Selective targeting to increase the synthesis and activity of presynaptic regulators of the mesolimbic dopamine system during this period constitutes a promising approach for both the prevention and treatment of dietary obesity in offspring that are otherwise prone to developing obesity.

Acknowledgments

The work described in this dissertation was conducted in the Program in Pharmacology and Experimental Therapeutics, Tufts University, Boston, MA from 2006-2011. I have had the privilege to complete this work under the supervision of my advisor and mentor, Dr. Emmanuel Pothos. He has offered me a wonderful opportunity to conduct exciting and cutting-edge research in the obesity field in his laboratory. This work has also been influenced by the direction of the other members of my dissertation committee, Drs. Margery Beinfeld, Martin Beinborn and Efi Kokkotou. Finally, the other members of Dr. Pothos laboratory, past and present, have provided a great deal of support and assistance in the completion of the work described in this dissertation.

Funding for this project came from a number of sources including an F31 Ruth L. Kirschstein Pre-doctoral National Research Service Award (DA023760) from the National Institute on Drug Abuse. Other funding sources included the Smith Family Foundation and the National Institute of Diabetes and Digestive and Kidney Diseases. Several Tufts Center for Neuroscience Research Core Facilities, including the Transgenic, Expression Array, and Behavioral Core Facilities also provided considerable support for these experiments.

I would also like to acknowledge the faculty and students in the Program of Pharmacology and Experimental Therapeutics for their understanding and support throughout my time here. In particular, I would like to thank my

classmates Kristina Weisman and Linsey Stiles for being there from the first day of biochemistry through the completion of this work. Their friendship and support have been invaluable during this journey.

The love and support of my family has been crucial to the successful completion of this work. My parents have provided encouragement throughout this process even though they weren't always sure what exactly I was doing. My siblings and their families have often provided much needed comic relief. My boys, Ben and JJ, have helped me to maintain balance in my life, which often has included watching Sesame Street or sneaking in a quick game of hide and seek before heading out the door to catch the train in the morning. Their unconditional love when I come through the door after a long day in the laboratory has turned many frustrating days around.

Most of all, I have to thank my husband, Mike. Whether it was staying behind to watch the boys alone so I could present my work at conferences halfway across the country or helping me with any number of technical issues over the years, he has been a constant shoulder for me to lean on. He has celebrated all of my successes and helped me through those times when I couldn't see the light at the end of the tunnel. Without his unwavering support and love, I would have not completed this work.

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List of Abbreviations

α-MSH- α-melanocyte-stimulating hormone

ACSF- artificial cerebrospinal fluid

AgRP- Agouti-related protein

BMI- body mass index

cAMP- cyclic adenosine monophosphate

CART- cocaine and amphetamine-related transcript

CCK- cholecystokinin

Cdk5- cyclin dependent kinase 5

COMT- catechol-o-methyltransferase

CRH- corticotropin-releasing hormone

D1-dopamine D1-type receptor

D2- dopamine D2-type receptor

D2S- dopamine D2-type receptor, short form

DA- dopamine

DAT- dopamine plasma membrane transporter

DIO- diet-induced obese

DOPAC- dihydroxyphenylacetic acid

DS- dorsal striatum

E1- embryonic day 1

ELISA- enzyme-linked immunosorbent assay

5-FDU-5-fluorodeoxyuridine

Fen-Phen- fenfluramine/phentermine

fMRI- functional magnetic resonance imaging

FTO- fat mass and obesity associated gene

GLP-1- glucagon-like peptide

HF- high fat

HPLC-EC- high-performance liquid chromatography with electrochemical detection

HS- high sucrose

HVA- homovanillic acid

IACUC- Institutional Animal Care and Use Committee

L-dopa- levodopa

MAO-A- monoamine oxidase A

MAO-B- monoamine oxidase B

MC3- melanocortin receptor - 3

MC4- melanocortin receptor - 4

MCH- melanin concentrating hormone

MEF2- myocyte enhancer factor 2

miRNA- microRNA

MSNs- medium spiny neurons

NAc- nucleus accumbens

NAcS- nucleus accumbens shell

NIH- National Institutes of Health

NOMI- nomifensine

NPY- neuropeptide y

Nr4a2- nuclear receptor subfamily 4, group A, member 2 (also known as Nurr1)

OLETF- Otsuka Long Evans Tokushima Fatty

OP- obesity-prone

OP/OP- OP embryo exposed to an OP environment

OP/OR- OP embryo exposed to an OR environment

OR- obesity-resistant

OR/OP- OR embryo exposed to an OP environment

OR/OR- OR embryo exposed to an OR environment

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PET- positron emission tomography

Pitx3- paired-like homeodomain 3

PFC- prefrontal cortex

POMC- proopiomelanocortin

PPARG- peroxisome proliferator-activated receptor gamma

Slc6a2- gene coding for DAT

Slc18a2- gene coding for VMAT2

SN- substantia nigra

SNpc- substantia nigra pars compacta

SOD- superoxide dismutase

T3- 3,3',5-triiodo-L-thyronine

TBP- TATA-binding protein

TH- tyrosine hydroxylase

TRH- TSH-releasing hormone

TSH- thyroid-stimulating hormone

VMAT2- neuronal vesicular monoamine transporter

VTA- ventral tegmental area

WHO- World Health Organization

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1 Introduction and Background

Obesity, which develops as the result of increased energy intake and/or decreased energy expenditure, has rapidly become one of the largest public health concerns in the world. In order to combat the rising cost of medical care due to this condition and improve health in millions of people around the world, a great deal of research is being done to identify why people are gaining excess weight, how the brain controls energy balance and which treatments are most effective at treating or preventing the development of obesity.

To understand obesity a great deal of research has focused on the molecular mechanisms responsible for the homeostatic control of energy balance. However, other systems that override this system, particularly the central reward system that mediates the hedonic value of palatable food, also likely contribute to the extraordinarily large number of people that battle obesity. The common pathway for central reward for both drugs of abuse and natural rewards, like food, is the central dopamine pathway. The experiments described in this dissertation attempt to provide insight into the role of central dopamine signaling in a model of obesity predisposition. Furthermore, we investigate how interactions between an obesogenic environment during gestation and lactation and the development of central dopamine neurotransmission can lead to hyperphagia and obesity in the offspring. Finally, we evaluate the effectiveness of early intervention with this system as a preventative treatment for obesity in children prone to gain excess weight.

1.1 The Obesity Epidemic

1.1.1 Definition, epidemiology and co-morbidities of obesity

Obesity is defined as “abnormal or excessive fat accumulation that presents a risk to health” (WHO, 2000). In order to easily identify those people who meet this definition, the World Health Organization (WHO) established a International Classification System using body mass index (BMI) to define overweight and obesity in adults (WHO, 2000). BMI is a metric that adjusts an individual’s weight based on their height and is reported as kg/m^2. The standards used in the WHO are currently used for both men and women of all ethnic backgrounds. In general, a person is considered normal weight if his/her BMI is between 18.5 and 24.99. Overweight individuals have a BMI between 25 and 29.99 and obese individuals have a BMI greater than or equal to 30 (WHO, 2000).

Based on the above classification system, obesity has grown to epidemic proportions in the United States and around the world. Current estimates are that more than 30% of the US adult population is obese and as many as two-thirds of the population are overweight (Ford et al., 2010; Ogden et al., 2006). The direct costs of obesity are extremely high because obesity is not only associated with cardiovascular disease and diabetes which are expensive to treat by themselves, but is also related to cancers, mental disorders, fertility and orthopedic issues. The annual direct medical cost burden of obesity in the United States is estimated to be close to $150 billion in adults (Hammond and Levine, 2010).

1.1.2 Current treatments for obesity

Current treatment options for obesity include lifestyle changes, bariatric surgery and obesity therapeutics. The most common lifestyle changes that are recommended are changes to an individual's diet and daily exercise routine. Over the years, a number of diet regimens have been recommended, based on the latest trends in dieting. These diets may result in relatively higher or lower amounts of initial weight loss. However, the weight loss is usually not maintained and the best dieting outcomes are seen with simple low calorie diets, which result in around 5-10% weight loss after 6 months. Unfortunately, in many cases, a significant amount of weight that is lost even in the low calorie diets is regained by 2 years (Dansinger et al., 2005; Ebbeling et al., 2007; Wing and Phelan, 2005).

Of all of the current treatment options, bariatric surgery has provided the best weight loss results, especially in patients who have received the appropriate counseling before and after the procedure. Several types of bariatric surgery are currently performed including: gastric banding, gastric sleeves, Roux-en-Y gastric bypass, and biliopancreatic diversions. The average amount of excess weight loss that is maintained for at least a year following surgery is 50% (Buchwald et al., 2009; Pories et al., 1995). Furthermore, numerous comorbid conditions also improve following bariatric surgery including diabetes, hyperlipidemia, high cholesterol, hypertension and sleep apnea (Buchwald et al., 2009). However, surgery is expensive and many people who would qualify for surgery are

unwilling to risk this type of invasive procedure and post-operative lifestyle change in order to lose weight.

Weight loss drugs have been marketed since the early 19th century when amphetamine was marketed as Benzedrine (Harris et al., 1947). However, they have generally been unsuccessful. Currently, orlistat is marketed in the United States for the treatment of obesity. Orlistat prevents the absorption of fat from the gut, resulting in long-term weight loss of only about 5% and uncomfortable side effects, primarily diarrhea (Zhi et al., 1995). Other products that have been sold in the past including the combination drug fenfluramine/phentermine (Fen-Phen) and sibutramine have subsequently been withdrawn from the market due to concerns about side effects. Finally, current investigational drugs including another combination drug of buproprion and naltrexone (Contrave) have failed to get FDA approval due to safety concerns. Because of the limited success of lifestyle interventions and weight loss drugs and the high cost to health care of obesity, research efforts to identify drug targets, including those described in this dissertation, continue to be important in this field.

1.2 Obesity in Children

1.2.1 Definition and epidemiology

Obesity in children is defined slightly differently than obesity in adults. In children the obesity scale is also based on BMI but because the normal range for BMI changes as children grow, a child's BMI is typically reported as a BMI-for-age

that compares them to historical BMI charts that have been generated by the Center for Disease Control or International Obesity Taskforce and is given as a percentile. In this system, in the US, children above the 85th percentile are "at risk for overweight" and children above the 95th percentile are considered "overweight". This nomenclature is used to avoid using the term "obese" when referring to children (Must et al., 2006).

Using the above definitions, the prevalence of overweight children has increased to more than 15% of children over the last several decades with more than 30% of children at risk for overweight (Ogden et al., 2008). This high level of overweight in children contributes $14 billion in direct medical costs for children alone with additional costs likely associated with their disease state as they reach adulthood (Hammond and Levine, 2010).

1.2.2 Genetic influences

A number of risk factors have been identified that lead to obesity in children, including parental obesity, socioeconomic status, and race. The link between parental obesity and increased BMI in offspring has led to a great deal of research looking at genetic influences that might increase susceptibility to obesity (Boney et al., 2005; Fraser et al., 2010; Kitsantas and Gaffney, 2010; Svensson et al., 2010). These studies have shown that the inheritance of obesity likely results from a complex network of gene-gene or gene-environment interactions (Hetherington and Cecil, 2010; Loos, 2009).

Based on data from genome wide association studies, the fat mass and obesity associated gene (FTO), the melanocortin receptor-4 (MC4), the peroxisome proliferator-activated receptor gamma (PPARG) and proopiomelanocortin (POMC), among others, have all been significantly related to obesity (Hetherington and Cecil, 2010; Loos, 2009). Food deprivation upregulated FTO expression in the hypothalamus indicating it is likely orexigenic. Furthermore, the obesity-associated risk allele results in increased FTO expression, mimicking a food deprived state (Olszewski et al., 2009). While this locus is significantly associated with obesity it only contributes slightly to the epidemic. In addition, the importance of melanocortin signaling on dietary obesity is evident due to the link between obesity and mutations in the MC4 receptor gene. Mutations in this gene account for almost 5% of early onset obesity in children, making it by far the most common single gene linked to obesity (Loos, 2009; Vimaleswaran et al., 2010). Similar findings have linked the other genes to obesity, however, so far we have been unable to identify one common gene mutation for obesity, underscoring the importance of a polygenic inheritance pattern for the development of this disorder (Loos, 2009; Vimaleswaran et al., 2010).

1.2.3 Parental and environmental influences

As childhood obesity reaches levels that are not fully explained by known genetic mutations (Freedman et al., 2006; Ogden et al., 2006), a major research focus has become the role of parental influences, including prenatal maternal diet,

early postnatal environment and parental attitudes toward food and body weight on the development of obesity in children. Several animal studies have investigated the role of the prenatal and early postnatal environment on the development of obesity in children in an effort to identify the relative contributions of direct parental influences and early postnatal environmental factors. In rodent studies where the maternal diet consisted of high levels of fat and/or sucrose, which can induce obesity in the mother, the offspring were found to have alterations in key hypothalamic appetite regulatory peptides, like orexin, melanin-concentrating hormone (MCH), and galanin, and enhanced obesity, particularly if they were weaned onto a high-fat diet. (Chang et al., 2008; Chen et al., 2008; Gorski et al., 2006; Morris and Chen, 2009; Tamashiro et al., 2009; Walker et al., 2008). In studies of early postnatal over nutrition, similar effects of sensitivity to high-fat diet and altered leptin and insulin signaling have been demonstrated. (Chen et al., 2008; Glavas et al., 2010; Schroeder et al., 2010) The evidence suggesting that these offspring have an increased sensitivity to palatable diets also indicates that central reward systems may be altered in these animals.

Developmental programming of obesity likely occurs through modifications of the offspring's genome including alterations in DNA methylation, changes in microRNA (miRNA) expression and differences in transcription factor activity. Diet can alter the amount of available methyl donors and change DNA methylation levels in the promoter regions of genes. MicroRNAs are short RNA sequences that have also been implicated in the control of gene expression and

may be modified by environmental changes, like diets deficient in folate or other methyl donors (Davis and Ross, 2008). Finally, transcription factors play an important role in the regulation of gene expression. External influences are critical in the regulation of transcription factor activity as alterations in signals like lipid availability (Choi et al., 2010) and calcium signaling (Smith et al., 2006) have been shown to alter transcription factor activity. Again, based on these findings, diet and excess body weight of the mother could have significant effects on the expression and activity of her offspring's genes that are critical to maintaining a healthy body weight.

1.3 The (Neuro)biology of Obesity

1.3.1 Peripherally secreted peptides and hormones

When considering the mechanisms responsible for the development of obesity, we should first consider the peripheral signals that induce hunger and satiety sensations through interactions with the central nervous system. In the periphery, there is a wide range of signals that are released from a number of different organ systems. In the stomach, ghrelin is released prior to a meal and is involved in the control of food intake, growth hormone secretion and the development of adiposity (Abizaid et al., 2006; Kojima et al., 1999; Tschop et al., 2000; van der Lely et al., 2004). Following a meal, numerous other hormones and peptides are released including insulin and pancreatic polypeptide from the pancreas, cholecystokinin (CCK) and glucagon-like peptide (GLP-1) from the intestine all of which act to decrease food intake (Boguszewski et al., 2010).

Peptides and hormones are also released from adipose tissue, including leptin and adiponectin, to indicate the amount of stored energy that is available.

In addition to the secreted hormones and peptides, the nutrients taken in during the meal also provide indications of the current energy state of the individual. Following a high-fat meal, higher levels of free fatty acids are present in the body. The same is true for sugars, amino acids and other vitamins and minerals that have been ingested in a given meal. Each of these nutrients, in addition to providing energy themselves, also provide information related to the amount of energy available and whether more food should be ingested. This information is passed through the vagus nerve to the brainstem and also directly to the hypothalamus and other higher centers of the brain (Gibson et al., 2010; Lenard and Berthoud, 2008).

1.3.2 Central homeostatic control of energy balance

Homeostatic control of food intake and energy expenditure is mediated in the hypothalamus. The primary neurons that have been identified in this system are the Agouti-related protein (AgRP)/neuropeptide Y (NPY) and POMC/cocaine and amphetamine related peptide (CART) neurons. The AgRP/NPY and POMC/CART neurons both project from the arcuate nucleus to the same second level neurons in the lateral hypothalamus where they exert opposing effect on the same receptors, the melanocortin receptors, MC3 and MC4 (Garfield and Heisler, 2009). POMC is converted to α-melanocyte-stimulating hormone (α-

MSH), which is an agonist of these receptors, while the AgRP inhibits these receptors. This system allows for modulation of the activity of the melanocortin neurons based on the external signals to the primary neurons of either excess energy intake or depleted energy stores. In adults, the connections between the arcuate and the lateral hypothalamus and paraventricular nucleus are well-developed and tightly controlled. In contrast, these systems are not fully developed in younger animals allowing for plasticity and environmental factors in the young may have a more profound effect on this system than they do in adults (Remmers and Delemarre-van de Waal, 2010).

While the POMC, AgRP, melanocortin circuit may provide primary control of energy balance in the hypothalamus, numerous other modulators are also present that integrate signals from and/or propagate signals to other brain regions. These signals are present in order to adjust for experiences or other external factors that might influence food intake or energy expenditure. Some of these signals include the orexigenic peptides in the lateral hypothalamus, orexin and melanin-concentration hormone (MCH) and the anorexigenic peptides in the paraventricular nucleus, corticotropin-releasing hormone (CRH) and TSH-releasing hormone (TRH), to name a few. The complexity of this system indicates that we have developed a very sophisticated system for energy balance, particularly with regards to food intake, which must be overridden for a chronic state of positive energy balance to exist.

1.3.3 Non-homeostatic signals for food reward

The rapid increase in obesity over the past several decades indicates that homeostatic mechanisms of energy balance are not in complete control of food intake and energy expenditure in humans. If the maintenance of ideal body weight is exclusively controlled by homeostasis, one would expect that only a small percentage of the population would become obese due to genetic mutations in the circuit described previously. Instead, almost two-thirds of the population is in a state of positive energy balance indicating other mechanisms like non-homeostatic pathways, that reinforce the value of food, must also be contributing to the neurobiology of food intake. In fact, several mechanisms have been identified where the homeostatic system may be overridden by external factors.

Within the hypothalamus, galanin has been shown to signal for preference for dietary fat and animals will eat beyond their energy needs if given excess galanin. These findings are confirmed in galanin knockout mice, which eat significantly less high-fat diet than the wild-type controls and in a self-administration study that found that galanin increased food intake of a palatable diet when it was freely available (Adams et al., 2008; McNamara and Robinson, 2010). Interestingly, galanin elevation can increase body weight even without increased food intake if the mice are given a normal diet due to suppression of energy expenditure and other peripheral metabolic changes (Poritsanos et al., 2009). These results indicate that in the current food environment of highly-

palatable food, galanin may be altered in obesity leading to a preference for overconsuming high-fat diets. Furthermore, galanin has also been linked to the opioid system. However, these interactions appear complex since the galanin knockout mice exhibit enhanced sensitivity to the rewarding properties of opiates while dietary fat has similar effects on both endogenous opioid and galanin expression in the hypothalamus (Chang et al., 2007; Hawes et al., 2008).

Outside of the hypothalamus, the opioid system also provides a mechanism by which homeostatic signaling may be overridden in order to derive more pleasure from food through interactions of the hypothalamus and striatum (Kelley et al., 2005a). μ-Opioid receptors are widely distributed throughout the brain and their actions in the ventral striatum and amygdala appear important in the processing of the hedonic properties of palatable food, particularly food that is high in fat (Wassum et al., 2009; Will et al., 2004). Increased stimulation of these receptors leads to increased intake of highly palatable foods (Zhang et al., 1998). Repeated exposure to high-fat and high sucrose diets leads to the down-regulation of the endogenous opioid enkephalin and in a diet-induced obesity model release of these endogenous opioids was also altered (Kelley et al., 2003; Smith et al., 2002). It is hypothesized that this system evolved to promote increased intake of highly palatable energy dense food when food sources were scarce, but the current food environment allows for consistent activation of this system and the consequent obesity epidemic (Kelley et al., 2002).

Finally, the midbrain monoamines, in particular, dopamine have been shown to have reinforcing actions, particularly to rewarding stimuli like drugs of abuse or natural rewards (i.e. food and sex). In response to a food challenge, dopamine release and turnover in the rat nucleus accumbens (NAc), dorsal striatum (DS) and medial prefrontal cortex (PFC) is increased (Hernandez and Hoebel, 1988, 1990; Mark et al., 1992; Yoshida et al., 1992). Furthermore, dopamine in these areas is known to increase with exposure to food-associated stimuli and motor activity related to attainment of the food (Bradberry et al., 1991; Mogenson and Wu, 1982; Salamone et al., 1991). Because this dissertation focuses on the role of dopamine in models of obesity-predisposition, more detailed descriptions of dopamine, its functions and measurement of its release kinetics are given in Chapter 2.

1.3.4 Interactions between peripheral signals and central hedonic circuits

A number of the hormones and peptides that are released in the periphery have been shown to interact with midbrain dopamine neurons. Leptin is one hormone that has significant effects on central dopamine signaling. Leptin receptors are present in both the VTA and substantia nigra (SN) and are co-localized with tyrosine hydroxylase, a marker for dopaminergic neurons (Fulton et al., 2006; Hommel et al., 2006; Leinninger et al., 2009; Opland et al., 2010). In acute studies of leptin treatment, leptin increases activity of the dopamine system (Perry et al., 2010). In leptin deficient *ob/ob* mice, evoked dopamine release and tyrosine hydroxylase activity is decreased (Fulton et al., 2006). In leptin deficient

models, treatment with leptin can reverse the dopamine deficits and decrease food intake (Pfaffly et al., 2010).

Insulin readily crosses the blood brain barrier and is known to interact with insulin receptors that are widely expressed throughout the brain (Woods et al., 2000). Furthermore, insulin has been shown to alter the expression and activity of the dopamine reuptake transporter (DAT) on nerve terminals (Figlewicz and Benoit, 2009; Figlewicz et al., 1994; Lute et al., 2008; Williams et al., 2007). While insulin itself reduces dopamine release, when given with cocaine, it actually enhances the cocaine induced dopamine release. These results indicate that insulin functions to maintain the intracellular stores of dopamine in the midbrain (Schoffelmeer et al., 2011). In addition, the presence of insulin increased behavioral impulsivity in rats when cocaine was also given (Schoffelmeer et al., 2011). Together these results indicate that insulin influences the hedonic pathways, and could lead to the loss of control seen in overconsumption and dietary obesity.

Peripheral peptides have also been shown to interact with central dopamine signaling. Dopamine release enhances CCK exposure in the nucleus accumbens and alterations in central dopamine signaling are seen in the Otsuka Long Evans Tokushima Fatty (OLETF) rats that are deficient in CCK-1 receptors (Anderzhanova et al., 2007; Beinfeld et al., 2002; Hajnal et al., 2008; Martin et al., 1986). Ghrelin is also known to interact with central hedonic pathways. In

obese individuals, circulating levels of ghrelin are decreased (Briggs et al., 2010). Acute treatment with ghrelin elevates dopamine neurotransmission in the midbrain, perhaps as part of the anticipatory component of food intake (Abizaid et al., 2006; Malik et al., 2008). Together, these studies indicate that an animal's ability to process peripheral signals appropriately is important in maintaining body weight. Alterations in the processing of these signals can lead to excessive intake and weight gain.

1.3.5 Interactions between homeostatic and hedonic pathways

Within the brain, interactions between the homeostatic pathways of the hypothalamus and hedonic pathways of the midbrain have also been identified. Two primary connections that have been identified to date are through orexin and melanocortin signaling. Neurons containing orexin are located in the lateral hypothalamus and some of these project to the VTA (Fadel and Deutch, 2002). Orexin receptors are co-localized with tyrosine hydroxylase in the VTA and activation of these neurons results in increases in both locomotion and dopamine (Korotkova et al., 2003; Narita et al., 2006). In animals resistant to developing obesity, orexin levels are elevated as is locomotor activity. By giving daily injections of orexin, these animals lose weight, primarily due to changes in locomotor activity (Novak and Levine, 2009; Teske et al., 2006). In animals prone to developing obesity these effects are not as strong (Novak et al., 2006).

The hypothalamic peptide, MCH has also been shown to influence feeding behavior in the hedonic areas of the brain, particularly the nucleus accumbens. The *MCH -/-* mouse is a lean mouse that exhibits elevated dopamine signal and sensitivity to psychostimulant activation of central dopamine pathways (Pissios et al., 2008). Furthermore, MCH has been shown to decrease neuronal firing in the of the medium spiny neurons of the NAc leading to increases in food intake (Sears et al., 2010). Finally, like insulin, MCH potentiates the hyperactivity seen with cocaine treatment, though likely through a different mechanism as the MCH receptors are localized on the neurons that also contain the D1 and D2 dopamine receptors (Chung et al., 2009).

1.4 Animal Models of Obesity

1.4.1 Diet induced obesity models

Obesity research in animals is approached through a number of different types of models. Diet-induced obesity models consist of animals that are fed a highly palatable diet that will induce obesity. There are a number of types of diets that are used including choice diets where animals are presented with a number of different items that are high in sugar, fat or both (Mercer and Archer, 2008). Other diets are more controlled and consist of elevated levels of fat, sucrose or both (Buettner et al., 2007; Mercer and Archer, 2008). These diets may be given either as a purified diet or from a specific food item, like Ensure (Levin and Dunn-Meynell, 2002a; Mercer and Archer, 2008). In developmental studies, these models have been useful in understanding the role of macronutrient changes in

the prenatal diet on the development of obesity. They also provide an opportunity to investigate mechanisms by which feeding behavior is altered in offspring (Remmers and Delemarre-van de Waal, 2010).

1.4.2 Monogenic and spontaneous obesity models

A second type of animal model that is used to study obesity are spontaneous mutation models, often of a single gene, that develop obesity. In rats, OLETF rats are often used as a genetic model of obesity. These animals are deficient in the CCK-1 receptor, are hyperphagic and become diabetic as they age (Anderzhanova et al., 2007). In addition, expression of other genes appears to be altered in these animals, including the orexigenic peptide, NPY. However, CCK and NPY are colocalized in the hypothalamus so changes in NPY mRNA may be due to the absence of the CCK receptor (Moran and Bi, 2006).

In mice, some commonly used genetic models of obesity are the leptin deficient *ob/ob* mouse and the leptin receptor deficient *db/db* mouse. A few of the other mouse models that have been used are the agouti, *MCH -/-* and *FTO -/-.* The agouti mouse model is a model where the Agouti gene produces either a black or yellow coat color depending on the transcription. This model has been useful in understanding epigenetics as well as obesity as the yellow Agouti gene is also associated with adult-onset obesity and diabetes (Dolinoy, 2008). The *MCH -/-* and *FTO -/-* are both lean models, where knocking out the gene results in decreased food intake (Fischer et al., 2009). These models, and many others,

provide insight into the specific functions of single genes that are associated with obesity, but the number of disease states that are associated with obesity and the large number of people who are affected by this indicate that the pathophysiology of obesity is likely due to changes in multiple genes.

1.4.3 Polygenic models of obesity predisposition

A third type of model that is used to study obesity and the inheritance of obesity, is the polygenic model of obesity predisposition. In rats, the selectively bred Sprague-Dawley rat model of obesity predisposition, the obesity-prone (OP) rats gain approximately 20% more weight than their obesity-resistant (OR) counterparts. This rat strain was generated by selecting the top 20% and bottom 20% weight gainers of outbred Sprague-Dawley rats and breeding within each group. The first generation obesity-prone offspring gained more weight than the obesity-resistant offspring when fed a high-fat diet. By the F5 generation, the obesity-prone offspring gained excess weight without being fed a high-fat diet (Levin and Dunn-Meynell, 2002b; Levin et al., 1997; Levin and Keesey, 1998). We take advantage of the body weight difference with no changes in diet of this model in this dissertation to dissect the direct effects of obesity predisposition on the development of central dopamine signaling and obesity.

1.5 Research Objectives and Organization of Dissertation

The explosive prevalence of obesity points to an important role for central mechanisms coding food reward. Prominent among these systems is midbrain dopamine signaling, which has already been linked to dietary obesity in a number

of different studies. However, in most of these models both diet history and body weight gain are different between the control group and the tested group indicating that either parameter could be responsible for the differences reported. Models of genetic predisposition to dietary obesity allow us to investigate the link between genetic predisposition and dietary obesity and central dopamine without differences in diet history and before body weight differences develop. The present study constitutes a systematic effort to investigate the role of the perinatal environment on dietary obesity predisposition, central dopamine neurotransmission and susceptibility to palatable food and psychostimulants like *d*-amphetamine. The central hypothesis for this dissertation is that the central dopamine deficits in obesity-prone rats are partly due to the intrauterine environment of the mother and predispose the offspring to decreased central dopaminergic neurotransmission, hyperphagia and weight gain. Upregulation of the dopamine system through an increase in dopamine synthesis during gestation will at least partly reverse these dopaminergic deficits and the obesity phenotype. To test this hypothesis we use a multi-level approach from the living awake animal to cellular mechanisms including: *in vivo* microdialysis to study extracellular dopamine levels and dopamine turnover, carbon fiber amperometry in acute coronal brain slices and primary dissociated neuronal cultures to study real time neurotransmitter exocytosis and its kinetics, immunocytochemistry to measure protein levels in cell culture, and quantitative real-time PCR to study changes in mRNA and microRNA levels. A more detailed description of dopamine's neurochemistry and carbon fiber amperometry is presented in

Chapter 2: Using Carbon Fiber Amperometry to Measure Dopamine Release Kinetics.

First, we investigated the role of diet-induced obesity on dopamine neurotransmission in animals fed a cafeteria-style diet of high sugar or high fat foods. In this study, we hypothesized that the cafeteria style diet would rapidly induce obesity in Dawley-Dawley rats and that this increase in weight gain would be associated with decreased basal and stimulated dopamine release in the nucleus accumbens. The entire study is described in Chapter 3: Deficits of mesolimbic dopamine neurotransmission in rat dietary obesity. We also investigated the relative contributions of body weight differences and diet composition to the decreased dopaminergic tone in dietary obese animals. To separate the effects of diet and body weight, we took advantage of the obesity-prone rat model. In this model, rats that are prone to develop dietary obesity are approximately 20% heavier by young adulthood than the obesity-resistant counterstrain when they are fed laboratory chow (Levin et al., 1997). In this study, we hypothesized that inbred obesity-prone rats exhibit similar dopaminergic tone and release kinetics as the dietary obese rats, even before the obesity phenotype develops. We further hypothesized that this attenuated dopamine release is at least partly due to differences in TH and VMAT2 levels that are present at all ages. The entire study is described in Chapter 4: Evidence for defective mesolimbic dopamine exocytosis in obesity-prone rats.

The intrauterine environment plays an important role in the development of

obesity in offspring. Both maternal high fat diet and poor maternal nutrition have been shown to increase the likelihood that the offspring will be obese (Chen et al., 2008; Gorski et al., 2006; Jackson et al., 1996; Mitra et al., 2009; Srinivasan et al., 2006; Tamashiro et al., 2009). Also, while many obese animals exhibit dopamine deficits, underweight rats have been shown to have decreased central dopaminergic signaling (Pothos et al., 1995a; Pothos et al., 1995b). We hypothesized that the central dopamine deficits that are present in obesity-prone rats are partly due to the intrauterine environment of the mother and predispose the offspring to decreased central dopaminergic neurotransmission, hyperphagia and weight gain. We further hypothesized that the mechanism by which the altered dopamine signaling developed was through transcriptional regulators of dopamine synthesis and exocytosis genes. To isolate the intrauterine environment, we transplant embryos on embryonic day 1 (E1) from an obesity-prone animal to an obesity-resistant surrogate and vice versa. We then measured central dopamine release, body weight gain, food intake and locomotion of the female offspring. The entire study is described in Chapter 5: An obesogenic perinatal environment alters central dopamine signaling and energy balance through a microRNA-related mechanism.

Dopamine neurons develop in rats as early as E12 so both known negative and positive influences on dopamine signaling during this time frame could influence the development of central dopamine neurotransmission in the offspring. In fact, treatment of pregnant mice with levodopa (L-dopa) has been shown to increase

dopamine release in the nucleus accumbens of the offspring of L-dopa exposed mice (Ren et al., 2011; Smidt and Burbach, 2007). In this study, we hypothesize that dietary alterations for the mother that influence central dopamine signaling during the perinatal period transfer those changes to the offspring. Additionally we hypothesize, that upregulation of the dopamine system through an increased availability of L-dopa will at least partly reverse the dopaminergic deficits and the obesity phenotype seen in obesity-prone rats. To test this hypothesis, we first feed Sprague-Dawley dames high-fat or high-sucrose diets during the perinatal period and measure dopamine release and body weight gain in their offspring. Additionally, we provide pregnant obesity-prone dames with L-dopa in their drinking water during gestation and measure dopamine release, body weight gain, food intake and locomotion in their offspring. Finally, we treat young female obesity-prone rats with L-dopa or a combination of L-dopa and carbidopa in their drinking water for ten weeks and measured their body weight gain, food intake and dopamine release. The results of this study are described in Chapter 6: Elevation of central dopamine through levodopa treatment in young obesity-prone rats reduces food intake and body weight.

A significant amount of evidence is present indicating that estrogen influences central dopamine signaling indicating that differences in dopamine development could occur between male and female offspring. To this point, our results have focused on the female offspring. In our final study, we investigate the development of central dopamine signaling in the male offspring of the perinatal

studies earlier described in this dissertation. We hypothesize that maternal influences on central dopamine signaling and the obesity phenotype will be different in males than in females. To test this hypothesis, we tracked body weight gain, central dopamine release and in some cases, food intake and locomotion of the male offspring of all of our perinatal studies. The entire study is described in Chapter 7: Perinatal influences on central dopamine signaling and the development of obesity of male offspring.

In conclusion, taken together the findings in the dissertation provide a framework by which dopamine influences the obesity phenotype. From these findings, we provide a model by which changes in transcription factors and microRNA expression lead to deficits in dopamine signaling in obesity-prone animals. This low dopamine likely leads to compensatory food intake and the resulting obesity. Proper targeting of this system may be useful in providing therapeutics for or preventing the onset of obesity in children pre-disposed to obesity. A full discussion of the model and therapeutic potential for targeting central dopamine as well as future directions for this work are described in Chapter 8: General discussion, implications and future work.

2 Using Carbon Fiber Amperometry to Measure Dopamine Release Kinetics

Throughout this dissertation we use carbon fiber amperometry to measure real-time release of dopamine from both cellular and *ex vivo* slice preparations. This chapter focuses on dopamine and the use of this electrophysiological tool to measure its release kinetics.

2.1 Dopamine

2.1.1 Dopamine neurochemistry

Dopamine is a neurotransmitter that is synthesized from the amino acid tyrosine. In the first step of the synthesis, tyrosine is converted to levodopa (L-dopa) by the rate-limiting enzyme for dopamine synthesis, tyrosine hydroxylase. L-dopa is then converted to dopamine by aromatic L-amino acid decarboxylase (also known as dopa decarboxylase) (Youdim et al., 2006). In cells that contain dopamine beta hydroxylase and phenylethanolamine *N*-methyltransferase (i.e. chromaffin cells from the adrenal glands), dopamine is further processed to norepinephrine and epinephrine (Goridis and Rohrer, 2002).

As a neurotransmitter, dopamine is packaged into synaptic vesicles in the presynaptic nerve terminal and released into the synaptic cleft following neuronal depolarization. Packaging of dopamine into the synaptic vesicles occurs through the vesicular monoamine transporter (VMAT). Two isoforms of VMAT are present in rodent species. In the periphery, VMAT1 is used to package catecholamines and serotonin while VMAT2 is the primary isoform present in the brain (Erickson et al., 1996; Schutz et al., 1998). VMAT2 is coupled to a proton

pump, which removes two protons from the vesicle for each dopamine molecule that VMAT2 transports in. This allows the transporter to generate extremely high concentrations of dopamine in the synaptic vesicles in preparation for release following depolarization of the cellular membrane (Eiden et al., 2004; Fon et al., 1997; Reimer et al., 1998).

Dopamine is cleared from the nerve terminal through multiple mechanisms. It can bind with dopamine receptors on both the post-synaptic medium spiny neurons (MSNs) and the presynaptic nerve terminal. Five dopamine G-protein coupled receptors have been identified, D1 to D5. Dopamine receptors are further divided into two types, D1-like (D1 and D5) and D2-like (D2, D3, and D4) (Beaulieu and Gainetdinov, 2011; Girault and Greengard, 2004). D1-like receptors are found on the post-synaptic MSNs and are coupled to G_s subunits, which allow them to activate adenylyl cyclase and increase cyclic adenosine monophosphate (cAMP) production (Beaulieu and Gainetdinov, 2011; Girault and Greengard, 2004). Members of the D2-like family of receptors are found both pre-synaptically and post-synaptically. The presynaptic D2 receptor is the short form of the receptor and is an autoreceptor. Activation of this receptor results in phosphorylation of tyrosine hydroxylase on Ser40, which inhibits its activity. Postsynaptically, D2-like receptors are coupled to a G_i subunit, thereby inhibiting adenylyl cyclase and cAMP production (Beaulieu and Gainetdinov, 2011).

Dopamine is transported back into the presynaptic terminal from the synapse by the dopamine reuptake transporter, DAT. This transporter is located on the presynaptic cell membrane and pumps dopamine out of the synapse back into the neuron (Chen et al., 2004; Eriksen et al., 2009). Cocaine and nomifensine are examples of two substances that may be used to selectively inhibit the activity of the transporter (Eriksen et al., 2009). Furthermore, the directionality of the transporter may be reversed by amphetamine, which causes intracellular levels of dopamine to increase by competing for the VMAT2 transporter in the presynaptic terminal (Sulzer et al., 1995).

Dopamine is metabolized both in the presynaptic terminal and in the synapse itself through one of two types of enzymes, either the monoamine oxidases (MAO-A and MAO-B) or catechol-o-methyltransferase (COMT) (Youdim et al., 2006). These metabolic pathways result in the formation of two dopamine metabolites, 3,4 dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). High-performance liquid chromatography with electrochemical detection (HPLC-EC) of these metabolites can show whether turnover of dopamine is altered between groups.

2.1.2 Development of dopaminergic neurons

In the central nervous system, there are two primary populations of dopamine containing neurons. These dopaminergic neurons have cell bodies in either the VTA or the substantia nigra pars compacta (SNpc). These regions are also often

referred to as the A9 and A10 regions. Projections from the VTA to the nucleus accumbens are referred to as the mesolimbic dopaminergic projections. Dopamine neurons also project from the VTA to the prefrontal cortex (mesocortical projections). Finally, dopamine neurons originating in the SNpc project primarily to the dorsal striatum forming the nigrostriatal projections (Smits et al., 2006).

In rats, development of these neurons begins during the prenatal period around gestational day 13 (Golden, 1973; Santana et al., 1992). At birth, the system is still developing as total dopamine levels in the early postnatal rat brain is only a fraction of what it is in adult brains (Loizou, 1972; Santana et al., 1992). Postnatally, dopamine neuronal projections from both the substantia nigra and ventral tegmental area continue to develop through approximately postnatal days 21-35 (Lieb et al., 1996; Park et al., 2000; Santana et al., 1992). Because the dopamine system is still developing at weaning, we measure dopamine release kinetics at both the P21-P35 stage and at P105 when the animals reach young adulthood and the dopamine system is fully developed.

From a molecular perspective, differences between the development of the nigrostriatal and mesolimbic pathways exist. Primarily differences in the activity of the transcription factors, paired-like homeodomain 3 (Pitx3) and nuclear receptor subfamily 4, group A, member 2 (Nr4a2 or Nurr1) in the substantia nigra appear to be related to the preferential degeneration of these neurons in

Parkinson's disease (Kadkhodaei et al., 2009; Smits et al., 2006). However, in study reward and reinforcement we may be able to take advantage of these differences in order to identify molecular mechanisms that might be acting exclusively on the reward centers of the brain.

2.1.3 Dopamine function in the central nervous system

Central dopamine is involved in a variety of processes. The nigrostriatal dopaminergic neurons are involved with the control of movement. In Parkinson's disease, movement issues arise when these neurons are specifically deteriorated (Barzilai and Melamed, 2003; Smits et al., 2006). In the mesocorticolimbic system, the dopamine neurons function to control emotions (specifically those projecting to the prefrontal cortex) and reward processing (specifically those projecting to the nucleus accumbens) (Smits et al., 2006; Tzschentke and Schmidt, 2000). Because these two pathways make up the majority of dopamine neurons it is interesting to note that from an energy balance perspective, dopamine is involved in both energy expenditure (i.e. nigrostriatal control of movement) and food intake (i.e. nucleus accumbens food reward processing). Therefore, disruptions in these neurotransmitter systems could have profound effects on the development of obesity by altering the relative levels of energy expenditure and energy intake.

2.2 Carbon Fiber Amperometry

2.2.1 Sample preparation

In this dissertation, we measure dopamine release kinetics from two types of sample preparations. To measure release from brain tissue of animals, we use an *ex vivo* slice preparation. We can also measure individual vesicular release using neuronal cultures derived from P0 pups.

To prepare slices, rat brains were rapidly placed into ice-cold oxygenated sucrose solution (210 mM sucrose, 3.5 mM KCl, 1.0 mM $CaCl_2$, 4.0 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 10 mM glucose, 26 mM $NaHCO_3$) on a Leica VT1000S vibratome, and cut in 300 µm coronal slices. Once cut, the slices were placed into a separate oxygenated bath containing oxygenated artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 2.0 mM KCl, 1.25 mM KH_2PO_4, 2.0 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 11 mM Glucose, pH=7.3) for one hour.

For quantal size recording from neuronal cells, postnatally derived (P0-P1) primary dissociated cultures of VTA neurons were prepared (Pothos et al., 1998a). These primary neuronal cultures are plated on a layer of glial cells. Therefore, glial cultures are prepared first at least 10 days prior to preparation of the neuronal cultures. To prepare glial cultures, two pups, P0-P5, are euthanized and their brains removed. The cortex is separated and placed in carbogenated papain solution (7.8 mL cysteine water, 20u/mL papain, 2mL H&B concentrate, 10µL 5N HCl, 20 µL 0.5% phenol red, 10 µL 0.5M kynurenate) at 34°C for two hours for digestion of the tissue. Cysteine water consists of 500mM CaCl2 and 20 mM cysteine. H&B concentrate consists of 4 M NaCl, 1 M KCl, 1 M $NaHCO_3$,

1 M NaH_2PO_4, 1 M $MgSO_4$, 134 mM EDTA, and 2.5 M glucose. Following digestion, the cells are rinsed with glial media (per 200 mL media: 180mL Modified Eagle's Medium, 20 mL calf serum, 1.5 mL 45% glucose, 240 μL penicillin/streptomycin, 40 μL 25mg/mL insulin and 0.5mL 200mM glutamine without ascorbic acid) that has been warmed to 34°C. Mitosis is inhibited in these cells after 7-10 days by adding 20 μL of 100X 5-fluorodeoxyuridine (5-FDU) solution (1.65 mg/mL uridine and 6.7 mg/mL 5-FDU) to the outside of each dish. The cells are triturated gently to break up any remaining tissue. 1-2 days prior to neuronal culture preparation, the glial media is removed and neuronal media is added to the glial cultures.

To isolate neurons, brains are dissected from 10-12 P0-P1 pups, rinsed in phosphate buffered saline (PBS) and the VTA is isolated under the microscope. VTA segments are placed into a carbogenated papain solution at 34°C for two hours for digestion of the tissue. Following digestion, the cells are rinsed with neuronal media (per 200mL: 0.5 g 5% bovine serum albumin, 94 mL Modified Eagle's Medium, 80 mL Dulbecco's Modified Eagle's Medium, 20mL F-12 Ham's nutrient mixture, 1.5 mL 45% glucose, 0.5 mL glutamine (200mM), 2.0 mL Diporzio concentrate, 0.1 mL liquid catalase, 200 μL kynurenic acid (0.5M), and 50 μL 5N HCl) that has been warmed to 34°C and conditioned on glia in flasks overnight. Diporzio concentrate contains 200 nM progesterone, 125 nM cortisol, 6.21 mL Hank's buffered saline solution, 25 μg/mL insulin, 30 nM Na_2SeO_3, 30 nM T3 (3,3',5-triiodo-L-thyronine), 5 μg/mL superoxide dismutase (SOD), 15 nM

putrescine, 100 μg/mL transferring (di Porzio et al., 1980). The cells are triturated gently to break up any remaining tissue. Cells were plated at a density of 100,000 cells per well in dishes that had a coating of glial cells on the bottom of the dish. After plating 100 μL of GDNF was added to each dish. Mitotic inhibition is achieved the next day by adding 20 μL of 100X 5-FDU solution to the outside of each dish. Neuronal cultures are used 3 – 8 weeks post-plating for quantal size experiments.

2.2.2 Electrode preparation

To record release we use carbon fiber disc electrodes that are hand-made in our laboratory. These electrodes are made by placing a 7 μm diameter carbon fiber into a glass filament (1.0 mm OD x 0.75 mm ID, 4" long; A-M systems, Inc. Carlsborg, WA, USA). The glass is then heated in the center to 310°F and rapidly pulled apart using a Flaming/Brown Micropipette puller (Sutter Instrument Co., Novato, CA, USA) to form a fine tip. Any carbon fiber that is extending beyond the end of the glass tip is cut and the carbon fiber is glued to the interior of the glass using the Epo-Tek glue kit (Epoxy Technology, Billerica, MA, USA). The glue is dried for at least 4 hours in a 90°C oven. The tip of the electrode is then cut and beveled at a 15° angle (K.T. Brown Type Micro-pipette Beveller; Model BV-10; Sutter Instrument Co., Novato, CA, USA) to remove the glue cap over the tip of the carbon fiber. Electrodes are tested for response by adding 10 μL of 3-5 mg/mL dopamine solution to a dish containing the tip of the electrode submerged in ACSF.

To stimulate release in the slice preparations, we use a bipolar stimulating electrode from Plastics One (Roanoke, VA, USA). The electrodes are stainless steel wire, 20 mm long and 0.125 mm diameter, twisted and insulated with polyimide (MS303/3B/SPC). To stimulate release in neuronal cell cultures, we pull a capillary tube (dimensions) to a fine point using the micropipette puller (Sutter Instruments Co., Novato, CA, USA). The tube is filled with 40 mM potassium and 6 mM calcium solution (102.4 mM NaCl, 40 mM KCl, 1.0 mM NaH_2PO_4, 10 mM Hepes, 6.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose; pH 7.4). The tip of the electrode is cut with a sharp blade so that a small drop escapes from the tube when 10-12 psi of nitrogen is blown into the tube.

2.2.3 Electrode placement and testing parameters

In this dissertation we tested dopamine release from the dorsal striatum (DS), nucleus accumbens shell (NAcS) and prefrontal cortex (PFC). The selected regions exhibit dense TH staining in nerve terminals, showing rich innervation from the dopamine cell bodies of the midbrain (Paxinos and Watson, 2007).

To test dopamine release kinetics from a specific site, the appropriate brain slice is chosen and placed in the slice chamber. The slice chamber is bathed with oxygenated ACSF flowing at a rate of 1 mL/min at 37^{o}C. The slice is held down into the chamber with a wire holder. Electrodes are placed ~50 μm into the slice, with the reference electrode (Ag/AgCl wire) inserted into the ACSF bath and the

voltage set to +700 mV (Axopatch 200B, Axon Instr.). The bipolar, twisted wire, stimulating electrode was placed within 100-200 μm of the carbon fiber electrode. A constant monophasic current stimulus of 2 msec at +500 μA was delivered by an Isoflex stimulus isolator (AMPI Inc.) triggered by a constant-current stimulator (Grass Instr. Co. Model S88). For each site, this stimulus was repeated 5 times at 5-minute intervals. The resulting response peak was digitized and analyzed as described below.

For neuronal cultures, recordings were performed in physiological saline (150 mM NaCl, 2.0 mM KCl, 1.0 mM NaH_2PO_4, 10 mM Hepes, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose; pH 7.4) and maintained at 37ºC. Cells were stimulated using 40 mM potassium and 6 mM calcium iso-osmotically substituted for sodium in the normal medium. Current was acquired at 160 kHz. Quantal events within 20 sec following stimulation were included in the analysis.

2.2.4 Data collection and analysis

For the data reported in Chapters 3 & 4, the response of the amperometric electrode (change in baseline) was monitored and quantified by Superscope software (GW Instruments, Inc.). For the data described in Chapters 5, 6 and 7, data was collected using Axograph software (written by Dr. John Clemens).

Amperometric peaks were identified as events greater than 3.5X the rms noise of the baseline. The event width was the duration between a) the baseline intercept

of the maximal incline from the baseline to the first point that exceeded the cut-off and b) the first data point following the maximal amplitude that registered a value of $\leq$ 0 pA. The maximum amplitude (i_{max}) of the event was the highest value within the event. The width at half-height ($t_{1/2}$) was the duration of the peak from a) the baseline intercept of the maximal incline from the baseline to the first point that exceeded the cut-off and b) the first data point following the maximal amplitude that registered a value of $\leq i_{max}$.

To determine the total number of molecules (N) released, the total charge of the event between the baseline intercepts was determined by calculating the area under the response curve, and the number of molecules estimated by the relation N =Q/nF, where Q is the charge, n the number of electrons donated per molecule, and F is Faraday's constant (96,485 coulombs per equivalent). Estimates were based on an assumption of two electrons donated per oxidized molecule of dopamine (Ciolkowski et al., 1994).

In conclusion, dopamine is a key neurotransmitter for the processing of food reward and carbon fiber amperometry allows us a unique opportunity to detect differences in evoked dopamine release in our animal models. This *ex vivo* approach is advantageous because it isolates the dopaminergic nerve terminals and, consequently, can directly point to presynaptic mechanisms that might be responsible for any observed differences in dopamine release. Throughout this dissertation, we use this technique to evaluate the role of dopamine

neurotransmission in models of dietary obesity and obesity predisposition to aid in the identification of novel drug targets for the prevention and treatment of obesity.

3 Deficits of mesolimbic dopamine neurotransmission in rat dietary obesity

Abstract

Increased caloric intake in dietary obesity could be driven by central mechanisms that regulate reward-seeking behavior. The mesolimbic dopamine system, and the nucleus accumbens in particular, mediates both food and drug reward. We investigated whether rat dietary obesity is linked to changes in dopaminergic neurotransmission in that region. Sprague-Dawley rats were placed on a cafeteria-style diet to induce obesity or a laboratory chow diet to maintain normal weight gain. Extracellular dopamine levels were measured by *in vivo* microdialysis. Electrically evoked dopamine release was measured *ex vivo* in coronal slices of the nucleus accumbens and the dorsal striatum using real-time carbon fiber amperometry. Over 15 weeks, cafeteria-diet fed rats became obese (>20% increase in body weight) and exhibited lower extracellular accumbens dopamine levels than normal weight rats (0.007 vs. 0.023 pmoles/sample). Dopamine release in the nucleus accumbens of obese rats was stimulated by a cafeteria-diet challenge, but it remained unresponsive to a laboratory chow meal. Administration of *d*-amphetamine (1.5 mg/kg i.p.) also revealed an attenuated dopamine response in obese rats. Experiments measuring electrically evoked dopamine signal *ex vivo* in nucleus accumbens slices showed a much weaker response in obese animals (12 vs. 25 x 10^6 dopamine molecules per stimulation). The results demonstrate that deficits in mesolimbic dopamine neurotransmission are linked to dietary obesity. Depressed dopamine release may lead obese animals to compensate by eating palatable “comfort” food, a stimulus that released dopamine when laboratory chow failed.

3.1 Introduction

In this study, we investigated whether chronic exposure (15 weeks) of rats to a high-energy, palatable cafeteria diet causes changes in nucleus accumbens dopamine. This highly palatable diet is successful in inducing dietary obesity in rats and is the most relevant to the development of human obesity (Sclafani and Springer, 1976). Furthermore, the cafeteria diet allowed us to distinguish between high-fat and high-carbohydrate preferences and whether such preferences impacted on mesolimbic dopamine release. We found that Sprague-Dawley rats took the majority of their daily caloric intake from high-carbohydrate sources and developed diet-induced obesity (DIO). Furthermore, they demonstrated depressed basal dopamine release in the nucleus accumbens and an attenuated dopamine response to a standard chow meal or systemic administration of *d*-amphetamine.

3.2 Experimental Procedures

3.2.1 Animals

Female albino Sprague-Dawley rats (Taconic), were matched for a body weight of 300 grams each at the age of 3 months. Female animals were chosen because, in contrast to male rats, the body weight of laboratory-chow fed females is relatively stable over time (Figure 3.2A). Animals were housed individually in the same room under a 12h reverse light/dark cycle (lights on: 6 PM, lights off: 6 AM). Under these conditions we observed no impact of the estrous cycle phase

on mesolimbic dopamine release (Geiger et al., 2008). All experiments have been approved by the Tufts University and Tufts Medical Center Institutional Animal Care and Use Committee (IACUC) Committee.

3.2.2 Cafeteria Diet Composition

Animals were divided into the cafeteria DIO group (also described as the dietary obese group below) and the laboratory chow-fed group (normal weight group). All groups were fed *ad libitum*. The cafeteria diet included high-fat components such as Crisco (33% vegetable shortening, 67% Purina powder), salami, cheddar cheese and peanut butter; and high-carbohydrate components such as sweetened condensed milk (Magnolia brand mixed with water, 1:1), chocolate chip cookies, milk chocolate, bananas, marshmallows and a 32% sucrose solution. This highly palatable diet has been shown to be very effective in inducing dietary obesity in rats and mimic the development of human obesity (Sclafani and Springer, 1976). Each of the components was available at all times and changed four times a week. The cafeteria DIO group, in addition to palatable food, was also given *ad libitum* access to laboratory chow (Purina). To identify diet preferences, the intake of each of the components of the cafeteria diet was measured over two 48-h periods during the eleventh week of the diet. Body weights were recorded once each week.

3.2.3 Stereotaxic Surgery

Stereotaxic surgery was performed during week seven of the study (n=24 cafeteria DIO rats, n=32 lab chow rats). Animals were anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg i.p.) for implantation of bilateral 10 mm, 21 gauge stainless-steel microdialysis guide cannulas aimed at the posterior nucleus accumbens shell region. The stereotaxic coordinates were 10 mm anterior to interaural zero, 1.2 mm lateral to the midsagittal sinus and 4 mm ventral to the level skull surface. The probe dialysis fiber extended another 4 mm ventral to reach the target site (Paxinos and Watson, 2007). Following surgery, all animals were returned to their cages and continued on their dietary regimen.

3.2.4 Microdialysis and HPLC-EC Procedure

Microdialysis was performed during week 14 of the study to allow for adequate recovery from surgery. For each microdialysis session animals were placed individually in microdialysis cages and probes were placed in the microdialysis cannulas 12 to 15 h before the first sample was collected. The site of implantation (left versus right) was counterbalanced. Microdialysis probes were of the concentric type, made locally and have shown a 10% recovery of neurochemicals in *in vitro* tests as described earlier (Hernandez et al., 1986). The probes were perfused with a Ringer's solution (142 mM NaCl, 3.9 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.4 mM Na_2HPO_4, 0.3 mM NaN_2PO_4) at a rate of 1µl/min. The dialysate was collected in 40 µl vials containing 5 µl of

preservative (0.1M HCl and 100 μM EDTA) to slow the oxidation of monoamines. Collection of samples began in the middle of the dark cycle, and all food was removed 3 h prior to sampling for all animals. Samples were collected at 30-min intervals for at least 2 h of baseline, followed by a systemic injection of *d*-amphetamine (1.5 mg/kg i.p.; Sigma). From each sample, 25 μl of dialysate was injected into an amperometric Antec HPLC-EC system (GBC Inc., Boston, MA) with a 10 cm Rainin column and a phosphate mobile phase buffer for the separation of dopamine and the dopamine metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The resulting peaks were then detected by the electrochemical detector at an oxidation potential of 400 mV, recorded and quantified by amplitude. Placement of the microdialysis probe in the target site was verified at the end of the experiment by histological examination of the probe tract following fixation of the brain with paraformaldehyde. For animals presented with a 30-min laboratory chow or cafeteria-diet meal challenge instead of d-amphetamine, all groups were food-deprived for 12 hrs prior to the microdialysis experiment to ensure adequate motivation to eat.

3.2.5 Slice Electrophysiology

Dopamine release kinetics were measured in the nucleus accumbens and dorsal striatum following exposure to the cafeteria diet as described in Chapter 2.

3.2.6 Tissue Micropunches

Cafeteria DIO or laboratory chow-fed rats (n=11/group) were euthanized as in the previous experiment and 1 mm diameter punches of the dorsal striatum and nucleus accumbens were taken from 300 um brain slices. The punches were then exposed to 40 mM KCl solution for three minutes to stimulate dopamine release. Extracellular dopamine levels were then measured using the HPLC method described above.

3.2.7 Data Analysis

Two-way ANOVA (group x time) with repeated measures and Fisher post-hoc analysis as appropriate was used for the analysis of the microdialysis data. One-way ANOVA was used for all other assays. For the slice experiments, results from 5 different stimulations on the same slice were averaged per slice before the ANOVA was run. Results are expressed as mean ± standard error of the mean (SEM).

3.3 Results

3.3.1 Dietary obese rats have a strong preference for highly palatable food

Cafeteria DIO rats showed a strong preference for sweet milk (74.4 ± 6.4 g; 241 ± 21 kcal) and the 32% sucrose solution (31.4 ± 4.1 g; 40 ± 5 kcal) (Fig 3.1a,b, $F(9,127)=116.9854$, $p<0.01$). In addition, these animals ate significantly less of the Purina chow (5.66 ± 1.02 g) compared to the laboratory chow fed animals

(54.7 ± 2.3 g; $F(1,27)=419.681$, $p < 0.01$). After 14 weeks on the cafeteria diet, rats gained 53.7% of their initial body weight to a final weight of 444.9 ± 19.0 g. At the same period, rats on laboratory chow reached a final weight of 344.0 ± 10.8 (Fig 3.2a, $p<0.01$).

3.3.2 Dietary obese rats have low basal dopamine and reduced amphetamine-stimulated dopamine release

At week 14 of the study, cafeteria DIO rats exhibited lower extracellular dopamine levels in the nucleus accumbens, compared to laboratory chow-fed rats (0.007 ± 0.001 pmols/25 µL sample vs. 0.023 ± 0.002 pmols/25 µL sample; respectively, Fig 3.2b, $F(1,19)=11.205$; $p<0.01$), as measured by *in vivo* microdialysis. Baseline levels of the dopamine metabolites, DOPAC and HVA, were also found to be significantly lower in the cafeteria DIO rats. DOPAC levels in cafeteria DIO rats were 3.13 ± 0.42 pmoles vs. 8.53 ± 0.56 pmoles in laboratory chow-fed rats ($F(1,10)=14.727$, $p<0.01$). HVA levels were 1.0 ± 0.28 pmoles vs. 4.28 ± 0.33 pmoles respectively ($F(1,20)=6.931$, $p<0.05$). After the establishment of a steady baseline of dopamine, rats were given a 1.5 mg/kg i.p. injection of amphetamine. The total release of stimulated dopamine levels was less in cafeteria DIO rats compared to laboratory chow-fed animals (Fig 3.2b, $F(9,162)=2.659$, $p < 0.01$).

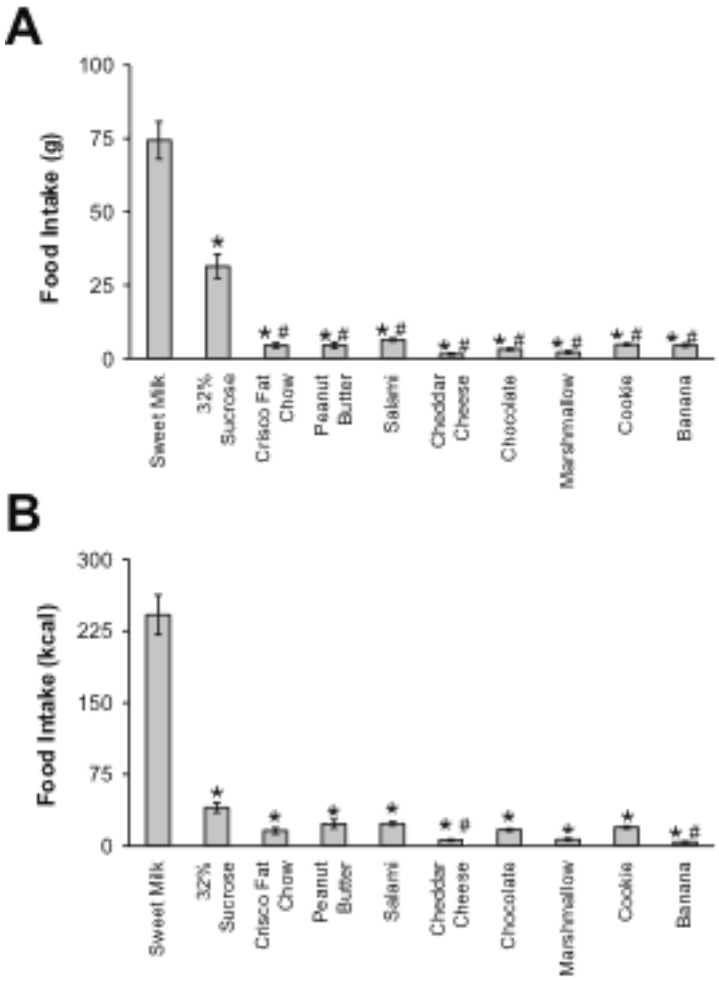

Figure 3.1: Cafeteria diet component preferences in obese rats.

Average consumption of cafeteria diet components in grams (A) and kcal (B) over two 48-h periods during week 11 of dietary regimen show a preference for sweet milk and sucrose solution (mean ± SEM; * $p < 0.01$ relative to sweet milk; # $p < 0.05$ relative to 32% sucrose solution).

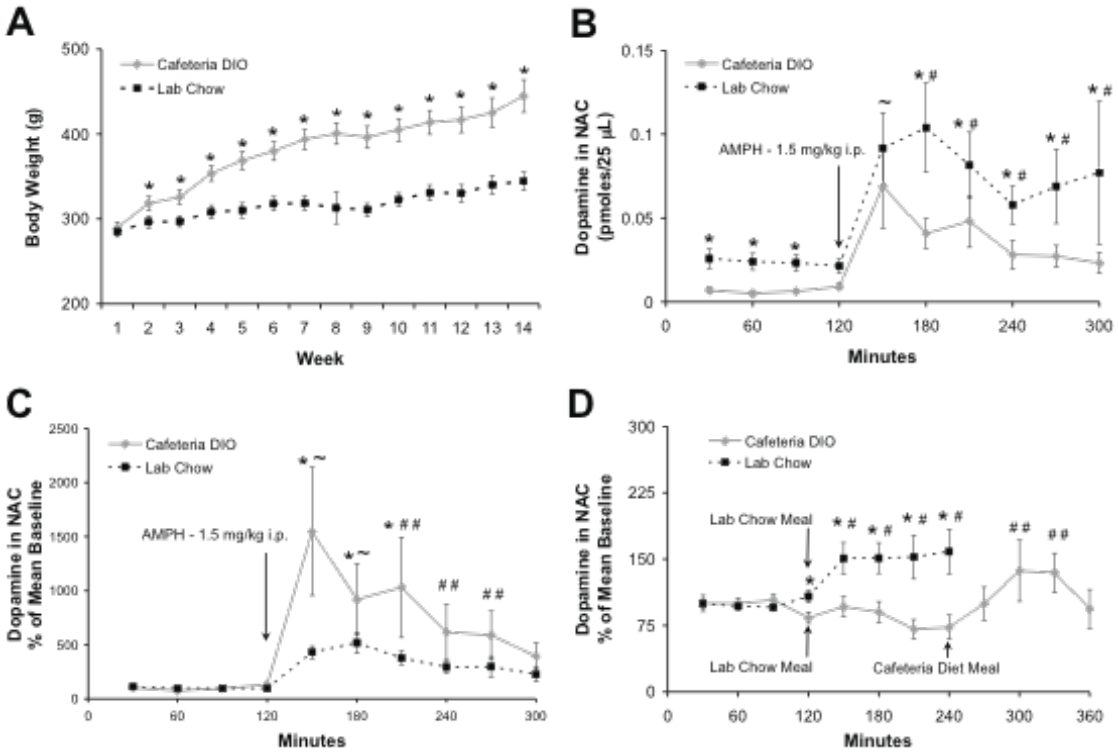

Figure 3.2: Basal and amphetamine-challenged nucleus accumbens dopamine levels are decreased in dietary obese rats.

A) Body weight of cafeteria DIO rats during a 14-week period was significantly more than the lab chow-fed group beginning at week 2 of the dietary regimen (*p < 0.01 by one-way ANOVA). **B)** Basal and amphetamine-challenged extracellular dopamine levels during week 14 in the nucleus accumbens of cafeteria DIO rats (n=9) was significantly lower than in chow-fed rats (n=13). **C)** The percent increase from baseline was higher in the cafeteria DIO rats than in the chow-fed rats (* p < 0.01 between groups, ~ p < 0.05 within both groups, # p < 0.05 within lab chow fed and ## p < 0.05 within obese group relative to the last baseline sample prior to the amphetamine injection). **D)** A plain chow meal was presented to both the cafeteria DIO (n=18) and chow-fed groups (n=22) after 4 baseline samples. Only the chow-fed group showed significant increases in % baseline dopamine after the meal. The cafeteria meal that was presented to a subset (n=8) of the cafeteria DIO group 2.5 h after the regular chow meal resulted in a significant increase in dopamine release. (** p < 0.05 between groups, # p < 0.05 within the lab chow fed group and ## p<0.05 within the cafeteria DIO group relative to the final baseline sample immediately prior to the lab chow or cafeteria meal).

3.3.3 Dietary obese rats release dopamine in the nucleus accumbens when eating highly palatable food, not plain laboratory chow

Figure 3.2d shows that the levels of extracellular dopamine in the cafeteria DIO rats did not increase detectably in response to a meal of laboratory chow. Animals ate on average 1.3 ± 0.4 grams of chow over 30 min. However, when a subset of these animals (n=8) were then fed the cafeteria diet for 30 min, dopamine increased 19.3% from 0.027 ± 0.003 to 0.033 ± 0.004 pmols/25 µL sample ($F(11,187)=8.757$, $p<0.01$). DOPAC levels also increased by 17.13 ± 6.14%. In contrast, dopamine levels in the laboratory chow-fed animals increased by 51.10 ± 17.31% ($F(7,119)=3.902$, $p<0.01$) one hour after the chow meal (animals ate on average 5.7 ± 0.8 grams, significantly more than the DIO animals; $F(1,33)=26.459$, $p<0.01$). However, we do not expect that the lower food intake by the DIO animals is the direct cause for the lack of dopamine release in these animals since food intake as low as 0.6 g has been reported to stimulate dopamine release in the nucleus accumbens of rats (Martel and Fantino, 1996). Furthermore, other studies have shown that differences in the amount of dopamine released are not necessarily directly correlated to the amount of food present but may also be affected by other stimuli such as satiety level of the animal, palatability and novelty effects of the food presented (Hoebel et al., 2007). A cafeteria diet was not given as a challenge to laboratory chow-fed animals because it was expected to induce novelty effects that would confound any comparisons with the cafeteria DIO animals.

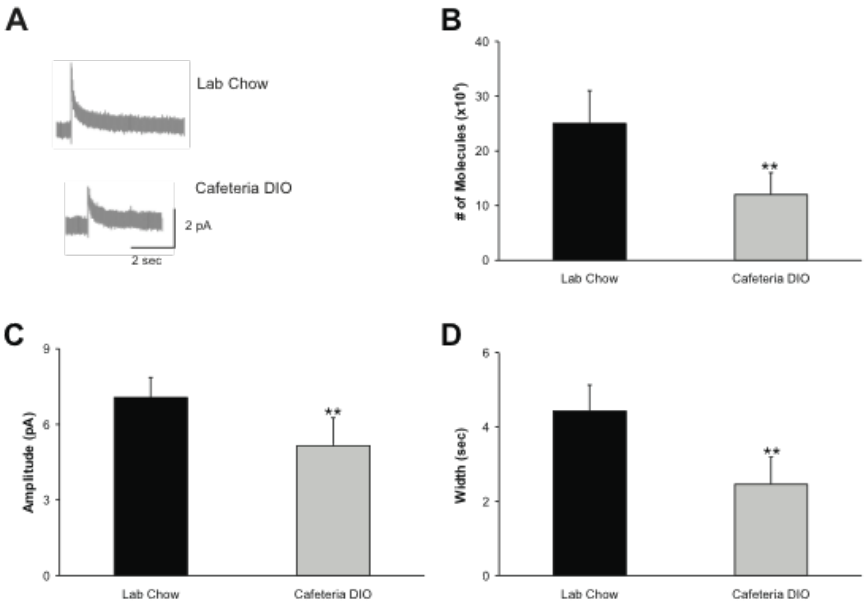

Figure 3.3: Evoked dopamine release from the nucleus accumbens in brain slices

A) Representative traces from acute coronal nucleus accumbens slices of chow-fed animals (top; n=30 stimulations in 7 slices) and cafeteria DIO animals (bottom; n=24 stimulations in 5 slices). The average number of molecules released **(B)**, event amplitude **(C)**, and width **(D)** from the dietary obese animals were significantly lower than in chow-fed animals (** $p < 0.05$).

3.3.4 Electrically stimulated dopamine release is attenuated in acute coronal brain slices from dietary obese rats.

Figure 3.3a shows representative amperometric traces from nucleus accumbens shell slices of normal vs. dietary obese rats (n=30 stimulations in 7 slices vs. 24 stimulations in 5 slices respectively). Cafeteria DIO rats had lower electrically evoked dopamine release than laboratory chow-fed rats ($12 \times 10^6 \pm 4 \times 10^6$ vs. $25 \times 10^6 \pm 6 \times 10^6$ molecules; Fig 3.3b, $F(1,52)=2.1428$). This difference in evoked dopamine release reflects both a decrease in event amplitude (5.16 ± 1.10 pA in cafeteria DIO rats vs. 7.06 ± 0.80 pA in laboratory chow-fed rats; Fig

3.3c, $F(1,52)=2.4472$) and width (2.45 ± 0.73 sec in cafeteria DIO rats vs. 4.43 ± 0.70 sec in laboratory chow-fed rats, Fig 3.3d, $F(1,52)=3.851$, $p<0.05$).

Figure 3.4 shows that the same trends were present in dorsal striatal slices of the dietary obese rats. Representative traces from the laboratory chow-fed (n=31 stimulations in 7 slices) and cafeteria DIO (n=15 stimulations in 4 slices) groups are shown in Figure 3.4a. The electrically evoked dopamine release from the striatum was $0.8 \times 10^6 \pm 0.1 \times 10^6$ in cafeteria DIO rats vs. $44 \times 10^6 \pm 11 \times 10^6$ molecules ($F(1,45)=6.0546$, $p < 0.05$; Fig 4b) in the laboratory chow-fed animals. Again this reflects a decrease in both the event amplitude (2.77 ± 0.42 vs. 9.20 ± 1.88 pA; $F(1,45)=7.8468$, $p < 0.01$) and width (0.22 ± 0.03 vs. 5.90 ± 0.98 sec; $F(1,45)=17.2823$, $p < 0.01$) in the cafeteria DIO group (Figs 3.4c,d).

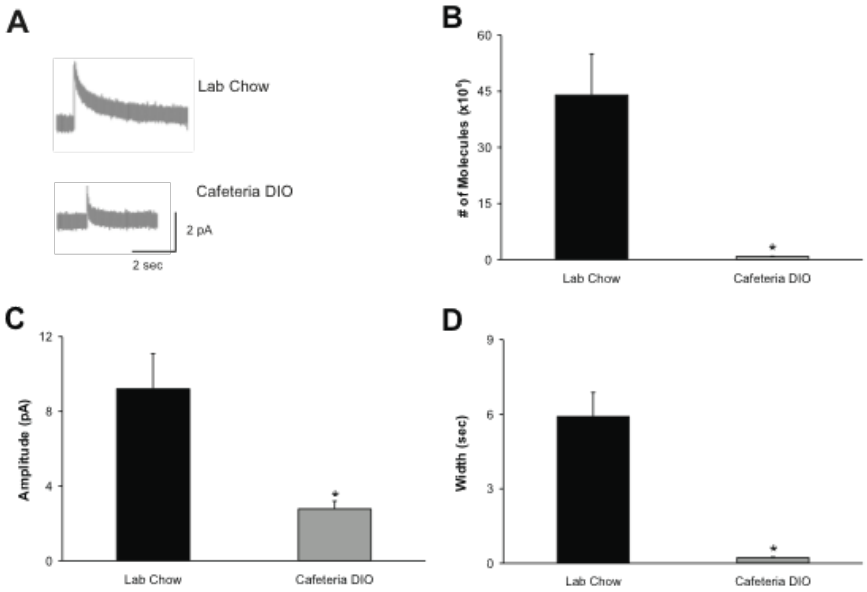

Figure 3.4: Evoked dopamine release from the dorsal striatum in brain slices
A) Representative traces from acute coronal dorsal striatum slices of chow-fed animals (top; n=31 stimulations in 7 slices) and cafeteria DIO animals (bottom; n=15 stimulations in 4 slices). The average number of molecules released **(B)**, event amplitude **(C)**, and width **(D)** from the dietary obese animals were significantly lower than in chow-fed animals (*$p < 0.01$).

3.3.5 Potassium-stimulated dopamine release in tissue micropunches is reduced in the nucleus accumbens and striatum of dietary obese rats

Extracellular dopamine levels following potassium chloride stimulation were measured by HPLC-EC and are shown in Figure 3.5. Extracellular dopamine levels were 0.16 ± 0.08 pmoles/sample in the accumbens micropunches of obese animals (n=10 micropunches) compared to 0.65 ± 0.23 pmoles/sample in the micropunches from the control animals (n=11 micropunches; Fig 3.5a; $F(1,19)=4.1911$, $p < 0.05$). Extracellular dopamine levels were 5.9 ± 1.7

pmoles/sample in the striatal micropunches from obese (n=8 micropunches) rats and 11.3 ± 1.9 pmoles/sample in the same site from control (n=11 micropunches) rats (Fig 3.5b; $F(1,17)=7.5064$, $p < 0.01$).

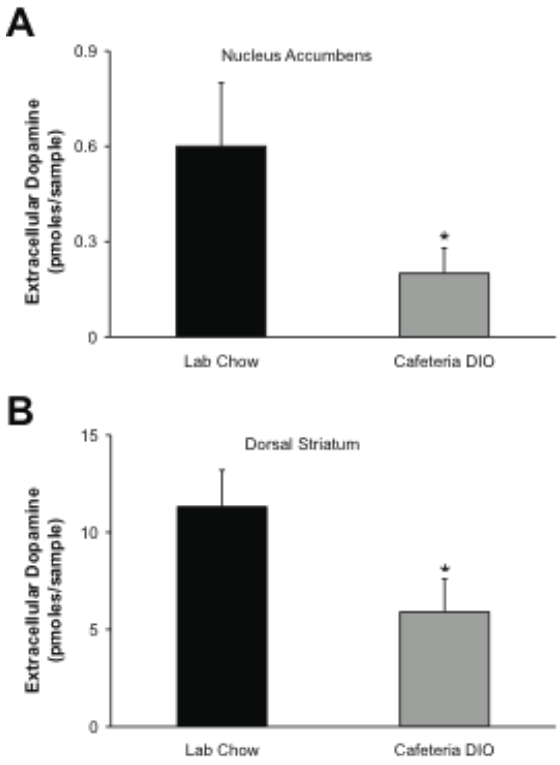

Figure 3.5: Extracellular dopamine levels from potassium-stimulated tissue micropunches
Amount of dopamine released from **(A)** nucleus accumbens (n=11 micropunches from each group) and **(B)** dorsal striatum (n=8 micropunches from obese and n=11 micropunches from controls) was significantly lower in cafeteria DIO animals than in chow-fed animals (* $p < 0.01$).

3.4 Discussion

In this study rats became overweight from eating a cafeteria diet with a preference for high-carbohydrate foods. In their overweight state, they showed lower basal extracellular dopamine as well as chow-stimulated or amphetamine-stimulated dopamine in the nucleus accumbens. In studies using drugs of abuse, animals will work to keep dopamine levels in the nucleus accumbens above a certain level (Ranaldi et al., 1999; Wise et al., 1995a; Wise et al., 1995b). In the

present study, the abused “substance” is palatable food, so the low extracellular dopamine in the accumbens leads to increased consumption of palatable food.

Obese rats also showed attenuated levels of electrically stimulated dopamine in brain slices and potassium-stimulated dopamine in tissue micropunches from the nucleus accumbens and dorsal striatum. A central presynaptic deficit in dopamine exocytosis is, therefore, evident in dietary obesity since depression of evoked dopamine release is present both *in vivo* and in acute striatal and accumbal brain slices and tissue micropunches from dietary obese animals. A potential site of presynaptic alteration is the plasma membrane dopamine reuptake transporter, DAT. The slice electrophysiology studies allow us to distinguish between differences in dopamine release versus reuptake kinetics. The difference in spike width suggests in principle that dietary obese animals may have not only less evoked release but also changes in reuptake due to differences in active DAT transporter sites on the plasma membrane. In Zucker fatty (*fa/fa*) rats, increased mRNA levels of the DAT transporter have been reported in the VTA (Figlewicz et al., 1998). The possibility of increased dopamine clearance is compatible with the decreased evoked dopamine signal in DIO rats in the present study.

We should note that amphetamine’s dopamine releasing ability was not attenuated in the obese animals (in terms of percent change from baseline) and this may “conspire” along with the lower dopamine absolute levels to drive the

motivation of obese animals to obtain dopamine releasing stimuli. Amphetamine is a weak base that displaces dopamine from the vesicles to the cytosol and leads to increase of extracellular dopamine via reverse transport (Sulzer and Rayport, 1990). In cases of severe deficits in dopamine vesicular pools, as for example in the case of the vesicular transporter VMAT2 deficient mice, injection of amphetamine transiently stimulates new dopamine synthesis in the cytosol (Fon et al., 1997). An amphetamine-induced transient increase in cytosolic dopamine may explain the temporary increase in percent change of accumbens dopamine in the obese animals over that observed in normal weight animals and may contribute to the obese animals' susceptibility to dopamine releasing stimuli along with the lower absolute extracellular dopamine levels in the accumbens.

What would be the mechanisms likely to mediate the presynaptic dopamine deficit in obese animals and drive their dietary preferences? The link between food preference and nucleus accumbens dopamine is clearly shown in the blunted response of the dietary obese animals to chow, but not to a palatable diet. Our findings complement recent work showing that a D1 receptor agonist enhanced the preference of rats for highly palatable food (Cooper and Al-Naser, 2006). In addition, nucleus accumbens dopamine is activated in rats trained to binge on sucrose (Avena et al., 2008b), further supporting the involvement of central dopamine in the preference for palatable food rich in carbohydrates. We have demonstrated the central dopamine deficit reported in the present study in additional models of obesity, including the *ob/ob* leptin deficient mouse(Fulton et

al., 2006; Geiger et al., 2008). Thus, one possible signal linking palatable food consumption and accumbens dopamine release might be leptin. In humans with a congenital leptin deficiency, replacement of leptin reduces their hyperphagia and changes the activation of their ventral striatum with respect to visualization of palatable food (Farooqi et al., 2007). In rats it has also been shown that leptin will decrease self-administration of sucrose (Figlewicz et al., 2006; Figlewicz et al., 2007). Other orexigenic inputs such as ghrelin and orexin have also been shown to be involved in the activation of the midbrain dopamine system (Abizaid et al., 2006; Helm et al., 2003; Narita et al., 2006; Rada et al., 1998). It would be interesting to further examine whether switching dietary obese animals to a normal laboratory chow on a chronic basis would maintain their preference for palatable food and the associated accumbens dopamine response to it independent of the expected changes in leptin, ghrelin or orexin and other signals related to appetite regulation.

In conclusion, the findings in this study show that the mesolimbic dopamine system plays a critical role in preference for high-energy diets, hyperphagia and the resulting dietary obesity. The nucleus accumbens and dorsal striatum dopaminergic neurotransmission are depressed in dietary obese rats. The animals can temporarily restore dopamine levels by eating highly palatable, high-energy food. These results suggest that selective targeting of presynaptic regulators of the mesolimbic dopamine system constitute a promising approach for the treatment of dietary obesity.

4 Evidence for defective mesolimbic dopamine exocytosis in obesity-prone rats

Abstract

The association between dietary obesity and mesolimbic systems that regulate hedonic aspects of feeding is currently unresolved. In the present study we examined differences in baseline and stimulated central dopamine levels in obesity-prone (OP) and obesity-resistant (OR) rats. OP rats were hyperphagic and showed a 20% weight gain over obesity-resistant rats at week 15 of age, when fed a standard chow diet. This phenotype was associated with a 50% reduction in basal extracellular dopamine, as measured by a microdialysis probe in the nucleus accumbens, a projection site of the mesolimbic dopamine system that has been implicated in food reward. In electrophysiology studies, electrically evoked dopamine release in slice preparations was significantly attenuated in OP rats, not only in the nucleus accumbens but also in additional terminal sites of dopamine neurons such as the dorsal striatum and medial prefrontal cortex, suggesting that there may be a widespread dysfunction in mechanisms regulating dopamine release in this obesity model. Moreover, dopamine impairment in OP rats was apparent at birth and associated with changes in expression of several factors regulating dopamine synthesis and release: VMAT2, TH, DAT and D2s. Taken together, these results suggest that an attenuated central dopamine system would reduce the hedonic response associated with feeding and induce compensatory hyperphagia leading to obesity.

4.1 Introduction

While a small number of humans become obese because of single gene mutations, greater than 99.9% of human obesity is the result of an interaction between predisposing genes and diet history (Mutch and Clement, 2006). To address potential altered brain neural circuitry associated with obesity predisposition, we studied the mesolimbic dopaminergic system in inbred obesity-prone (OP) rats before and after the onset of obesity. The rat model that we studied has been developed after selective breeding of high and low-weight gainers amongst outbred Sprague-Dawley rats after 2 weeks on a high-energy diet (Considine et al., 1996; Levin and Dunn-Meynell, 2002b; Levin et al., 1997). By the F5 generation, the obesity-prone rats fed a standard chow gain 31% (males) and 22% (females) more weight than the obesity-resistant ones and exhibit metabolic abnormalities similar to obese humans. Our study of the rat mesolimbic system in different ages has shown that obesity-prone rats have lower basal extracellular dopamine levels, lower evoked dopamine release and lower dopamine quantal size most likely due to intrinsic differences in the expression of genes that regulate dopamine exocytosis.

4.2 Experimental Procedures

4.2.1 Animals

Fifteen and 4 week old female (for slice recordings and microdialysis) inbred obesity-resistant and obesity-prone rats (Levin et al., 1997) from Charles River Laboratories, Cambridge, MA were housed in reverse light-dark cycle (lights off

8:00AM, on 8:00PM) and used in the middle of the dark cycle when rodents are normally active. Laboratory chow intake was measured in individually housed animals at the following time points: 1 hr, 2 hr, 3 hr, 6 hr and 12 hr after the onset of the dark cycle; and 12 hr after the onset of the light cycle.

4.2.2 Stereotaxic surgery and microdialysis

Guide cannulas (12 mm; Bioanalytical Systems, Indianapolis, IN) were implanted according to a Tufts IACUC-approved animal protocol. The stereotaxic coordinates used for the nucleus accumbens were: 1.0 mm anterior to bregma, 1.2 mm lateral to midsagittal sinus, and 6.0 mm ventral to the skull surface (Paxinos and Watson, 2007). The dialysis probe extended another 2 mm ventral to the guide cannula. Correct probe placement was verified by histology at the completion of the study. Following 1-week recovery from surgery, microdialysis probes were implanted into the guide cannulas 15 hours prior to the start of the microdialysis session, at which point food was removed. Artificial cerebral spinal fluid (ACSF; 149 mM NaCl, 1 mM NaH_2PO_4, 3 mM KCl, 1 mM $MgCl_2$ and 1.4 mM $CaCl_2$, pH 7.4) was pumped through the probe to allow equilibration. Samples of the interstitial fluid were taken every 30 minutes for 2 hours and analyzed using HPLC-EC as described in Chapter 3.

The estrous cycle of these animals was measured using the EC-40 vaginal impedance instrument (Fine Sciences Tools, Inc). Based on the correlation of impedance to estrous cycle stage, the animals tested were in proestrous or

diestrous and no difference was detected in basal dopamine levels between the estrous cycle stages (data not shown). Furthermore, no cycle-induced changes in evoked dopamine release were detected when stimulated dopamine release was measured in acute striatal slices of normal females by fast-scan cyclic voltammetry (Walker et al., 2000).

4.2.3 Locomotion testing

Locomotion testing was done using Smart-Frame activity boxes (Hamilton-Kinder) and Motor Monitor software (Hamilton-Kinder). 4-week-old obesity-prone and obesity-resistant females were habituated to the activity boxes for 4 days prior to testing. On the testing day, basal locomotion levels were measured for 3 hours prior to an i.p injection of *d*-amphetamine (1.5 mg/kg). Locomotor response to the amphetamine injection was measured for 5 hours following the injection.

4.2.4 Brain slice electrophysiology

Carbon fiber amperometry was performed in the nucleus accumbens, dorsal striatum and prefrontal cortex of 4-week-old and 15-week-old obesity resistant and obesity prone females as described in Chapter 2.

4.2.5 Amperometric recordings from dissociated neuronal cultures

Postnatally derived (P0-P1) primary dissociated cultures of ventral tegmental area (VTA) neurons were prepared from obesity-prone and obesity-resistant

pups as described elsewhere (Pothos et al., 1998a). Cells were plated at a density of 100,000 cells per well. They were used 2 - 7 days post-plating for expression experiments and 3 – 8 weeks post-plating for quantal size experiments. Recordings were performed as described in Chapter 2.

4.2.6 Real-time polymerase chain reaction (PCR)

RNA from primary dissociated cell cultures was isolated using the RNAqueous Mag 96 kit (Ambion). The purified RNA was reverse transcribed using the mMulv reverse transcriptase kit and random primers containing 12 nucleotides (New England Biolabs). Real-time PCR was performed for tyrosine hydroxylase (TH) using previously published PCR primers (Dijk et al., 2004) and the SYBR Green detection system. For all other genes, PCR was performed using PCR primers in combination with FAM-labeled MGB probes (Applied Biosystems) and the Taq-Man detection system. Primer sets and probes are shown in Table 4.1. 45 cycles of real-time PCR were performed using an Applied Biosystems 7700 sequence detector and Platinum QPCR Supermix UDG reagents (Invitrogen) Specific gene expression was calculated relative to levels in the obesity-resistant group and normalized to cyclophilin.

Table 4.1: Primers and probes used for real-time qPCR reactions

Gene	Primers	Probe
Cyclophilin	F: AATGGCACTGGTGGCAAGTC R: GCCAGGACCTGTATGCTTCAG	TCTACGGAGAGAAATT
TH	F: GCCATGAGCTGTTGGGACAT R: CCCCAGAGATGCAAGTCCAAT	Sybr Green
VMAT2	F: CTACCAGCACACAGCACACT R: TCATTCAGAAGGTCTCTGTCTTCACT	CCACTGTCCCTTCGGACTG
$G\alpha_q$	F: ACAACAAGATGTGCTTAGAGTTCGA R: GACCATTCTGAAGATGACACTCTGT	CAGGGATCATTGAGTACCC
D2 - presynaptic	F: GTCAGAAGGGAAGGCAGACA R: GCACATTGCCAAAGACGATGATAAA	CAGCATGGCATAGTAGTTG

4.2.7 Immunohistochemistry

Cultures were fixed using 4% paraformaldehyde solution and then washed with PBS containing 0.1% TritonX100. After blocking with 10% horse serum, cultures were probed with a mouse monoclonal antibody for TH (Chemicon, 1:200) or a rabbit polyclonal antibody against the C terminus of VMAT2 (Chemicon, 1:100), followed by incubation with a secondary biotinylated antibody and an avidin-biotin-HRP complex (Vector Laboratories). The antibody complex was visualized using DAB and hydrogen peroxide.

4.2.8 Statistical analysis

One-way ANOVA (Excel, Microsoft Corporation) was used to compare obesity-prone and obesity-resistant rats in all experiments. Results were expressed as mean ± s.e.m.

4.3 Results

4.3.1 Extracellular dopamine levels in the nucleus accumbens of adult obesity-prone and obesity–resistant rats

At 15 weeks of age, obesity-prone female rats consumed 14% more food on normal chow (16.34 ± 1.24 g vs. 14.25 ± 0.17 g, $p<0.05$, Fig. 4.1A) and were 20% heavier (263 ± 13.5 g vs. 220.5 ± 10.5 g, $p<0.01$, Fig. 4.1B), compared to the obesity-resistant rats. When basal extracellular dopamine levels in the nucleus accumbens were measured during a two-hour period in the middle of the dark cycle and in the absence of food, they were almost 2-fold lower in obesity-prone animals compared to obesity-resistant animals (0.024 ± 0.005 pmol vs. 0.042 ± 0.003 pmol respectively; $p<0.02$; Fig. 4.1C).

4.3.2 Stimulated dopamine release is low in obesity-prone rats

The lower extracellular dopamine levels in obesity-prone rats could be the direct result of a reduction in dopamine release by the accumbens neuronal terminals. To examine this possibility in obesity-prone versus obesity-resistant rats we measured electrically evoked dopamine release *ex vivo* in acute coronal slice preparations of the following brain regions: posterior nucleus accumbens shell, mediodorsal striatum and medial prefrontal cortex.

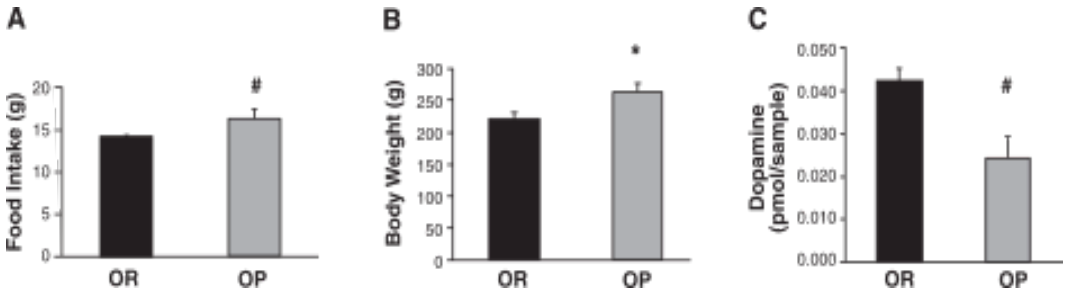

Figure 4.1: Elevated body weight of adult obesity-prone rats is linked to increased chow intake and decreased extracellular dopamine levels in the nucleus accumbens.
(A) Food intake during the dark cycle of obesity-prone animals (n=4 in triplicate) is greater than food intake of obesity resistant animals (n=6 in triplicate). **(B)** Body weight of the young adult obesity-prone rats (n=3) used for microdialysis was significantly higher than weight of obesity resistant (n=4) rats. **(C)** Basal accumbens extracellular dopamine in freely moving obesity-prone rats (n=3) was significantly lower than in obesity resistant rats (n=4). *$p<0.01$, $^{\#}p<0.05$

In the nucleus accumbens shell, the mean amplitude of the evoked dopamine signal was approximately 3-fold lower in obesity-prone rats compared to obesity-resistant rats (9 ± 1 pA vs. 30 ± 6 pA respectively; $p<0.01$, Fig. 4.2A,B). The integration of the dopamine signal yielded an average of $36.8 \times 10^6 \pm 4.8 \times 10^6$ dopamine molecules released per stimulation in obesity-prone animals versus $78.6 \times 10^6 \pm 11.5 \times 10^6$ dopamine molecules in obesity-resistant animals ($p<0.01$, Fig. 4.2C). In the dorsal striatum, the mean amplitude of the evoked dopamine signal was 15 ± 3 pA in obesity-prone rats vs. 22 ± 2 pA in obesity-resistant rats ($p<0.01$, Fig. 4.2B). The integration of the dopamine signal yielded an average of $34.7 \times 10^6 \pm 4.3 \times 10^6$ dopamine molecules released per stimulation in obesity-prone vs. $116.9 \times 10^6 \pm 12.9 \times 10^6$ dopamine molecules in obesity-resistant animals ($p<0.01$, Fig. 4.2C). In the medial prefrontal cortex, the mean amplitude of the evoked dopamine signal was 7 ± 1 pA in obesity-prone vs.

15 ± 2 pA in obesity-resistant rats ($p<0.01$, Fig. 4.2B). The integration of the dopamine signal yielded an average of 21.9 X 10^6 ± 3.0 X 10^6 dopamine molecules released per stimulation in obesity-prone animals vs. 40.4 X 10^6 ± 3.8 X 10^6 dopamine molecules in obesity-resistant animals ($p<0.01$, Fig. 4.2C).

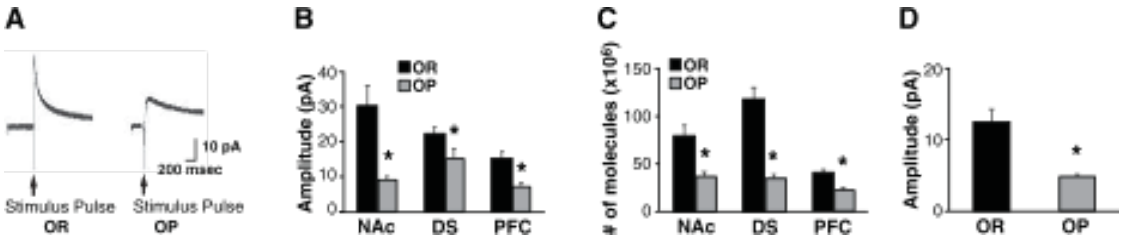

Figure 4.2: Stimulated dopamine release is attenuated in the nucleus accumbens, dorsal striatum and prefrontal cortex of adult obesity-prone rats.
(A) Representative amperometric traces of stimulated dopamine release from acute coronal slices. **(B)** Average peak amplitude (pA) and **(C)** number of molecules released after stimulation of dopamine release was significantly lower in obesity-prone rats than in obesity-resistant rats in the nucleus accumbens (NAc, obesity-prone: n=45 stimulations in 9 slices, obesity-resistant : n=53 stimulations in 11 slices), dorsal striatum (DS, obesity-prone: n=40 stimulations in 8 slices, obesity-resistant: n=40 stimulations in 8 slices) and prefrontal cortex (PFC, obesity-prone: n=32 stimulations in 7 slices, obesity-resistant: n=35 stimulations in 9 slices). **(D)** In 4-5 week old rats, the average peak amplitude (pA) after stimulation of dopamine release was also significantly lower in obesity-prone rats than in obesity-resistant rats (obesity-prone: n=37 stimulations in 10 slices, obesity-resistant: n=56 stimulations in 13 slices). *$p<0.01$

Similar results were obtained in younger (4-week old) rats. At this age, the average body weight of obesity-prone rats was not statistically different from that of obesity-resistant rats (79.9 ± 7.3 grams vs. 66.1 ± 4.4 grams respectively). The mean amplitude of the evoked dopamine signal was 4.7 ± 0.3 pA in obesity-prone rats vs. 12.3 ± 1.9 pA in obesity-resistant rats ($p<0.01$, Fig. 4.2D). Also, in the younger rats, the basal levels of activity and the locomotor response to an

amphetamine injection was significantly lower in the obesity-prone rats compared to the obesity-resistant rats ($p<0.01$, Fig. 4.3)

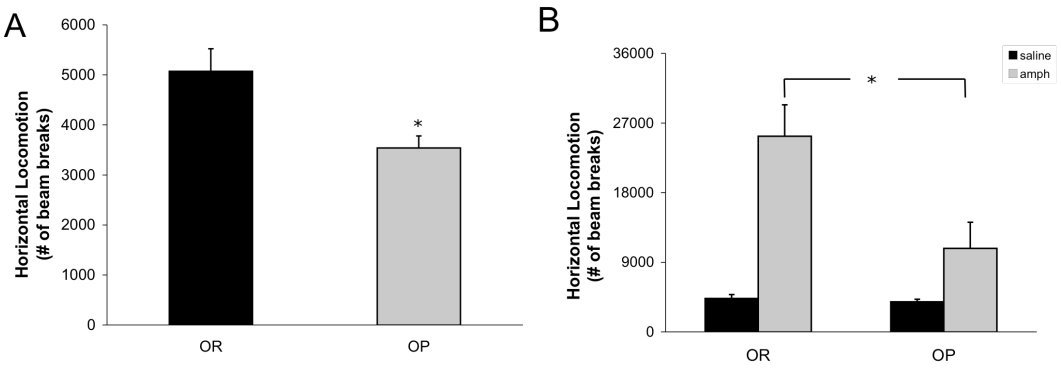

Figure 4.3: Reduced locomotion and response to amphetamine injection in juvenile obesity-prone (OP) rats.
A) Basal levels of horizontal locomotion were decreased in OP rats compared to obesity-resistant (OR) rats. **B)** Response to amphetamine injection was also decreased in the OP rats compared to the OR rats. n=4 animals per treatment group. *p<0.01 by one-way ANOVA.

Taken together, these results indicate that stimulated dopamine release is significantly lower in obesity-prone rats and may account for their lower levels of basal extracellular dopamine in the nucleus accumbens (Fig. 4.1C). In addition, this deficit in dopamine release appears to be global for all central dopamine systems, which suggests an anomaly in presynaptic mechanisms regulating dopamine release.

4.3.3 Dopamine quantal size is reduced in obesity-prone rats

To determine whether differences in dopamine release were related to differences in dopamine exocytosis from individual vesicles, we measured the quantal size of dopamine in cultures of primary dissociated dopamine neurons

from obesity-prone and obesity-resistant neonates (P0-P1). These neurons were isolated from the ventral tegmental area (VTA) where the dopamine cell bodies reside and project to the nucleus accumbens. Representative amperometric traces are shown in Fig. 4.4A and the quantal size distribution is shown in Fig. 4.4B. The average quantal size in cultures derived from obesity-prone rats was 1131 ± 99 molecules, which was significantly lower than the average quantal size in cultures from obesity-resistant rats (2032 ± 152 molecules, $p<0.01$, Fig. 4.4C). The average event amplitude was significantly lower in cultures from obesity-prone neonates (6.59 ± 0.35 pA vs. 10.56 ± 0.66 pA, $p<0.01$, Fig. 4.4D). Not only does this finding provide a potential mechanism for lower dopamine release, but it also indicates that this attenuation in dopamine signal is present at birth.

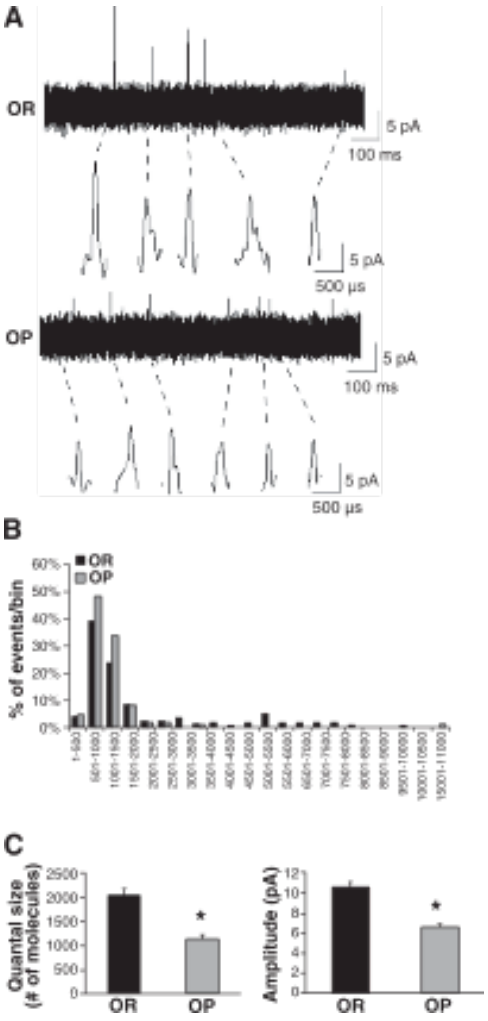

Figure 4.4: Reduced dopamine quantal size in VTA-derived neurons from P0-P1 obesity-prone pups.
(A) Representative amperometric traces from VTA cultures of obesity-resistant (top) and obesity-prone (bottom) neonates. Individual events are shown at higher resolution below. **(B)** Quantal size distribution in cultures from neonatal obesity-prone and obesity-resistant animals. Note the obesity-prone distribution is skewed to the left due to lack of events in the higher quantal size bins. **(C-D)** Quantal size and amplitude of stimulated dopamine release from VTA-derived neuronal cultures from obesity-prone pups (grey bars, n=110 events) is lower than in cultures derived from obesity resistant pups (black bars, n=182 events). *p<0.01

4.3.4 Altered expression of regulators of dopamine synthesis and release in obesity-prone rats

Potential mechanisms for the decreased dopamine signal seen in Fig. 4.1 and 4.2 could be decreased dopamine biosynthesis and decreased vesicular loading. Two key proteins involved in these processes are TH, the rate-limiting enzyme for dopamine synthesis, and VMAT2, the neuronal transporter responsible for packaging dopamine into vesicles. The relative amount of TH mRNA expression in cultures from the obesity-prone animals was 50-fold lower compared to the obesity-resistant group (0.019 ± 0.002 vs. 1.00 ± 0.12, p<0.01, Fig. 4.5A).

Consistent with the mRNA data, TH protein expression was reduced by 2-fold in cultures from the obesity-prone rats (206 ± 19 TH-positive cells in obesity-prone vs. 434 ± 104 TH-positive cells in obesity-resistant, $p<0.01$, Fig. 4.5B), indicating reduced dopamine synthesis. In the same cultures, the relative VMAT2 mRNA expression was significantly lower in obesity-prone rats (0.62 ± 0.03 vs. 1.62 ± 0.15, $p<0.01$, Fig. 4.5C), which would implicate decreased vesicular packaging of dopamine. Moreover, the mRNA expression of $G\alpha_q$, an endogenous negative regulator of VMAT2 activity (Holtje et al., 2003) was significantly higher in cultures from obesity-prone neonates than in cultures from obesity-resistant neonates (1.02 ± 0.01 vs. 0.58 ± 0.1, $p<0.01$, Fig. 4.5C). These changes in VMAT2 mRNA expression correlate to changes in VMAT2 protein expression. The mean count of VMAT2-immunopositive sites in cultures derived from obesity-prone pups was significantly lower than in cultures from obesity-resistant pups (248 ± 29 vs. 658 ± 47 positive cells, $p<0.01$, Fig. 4.5D,E). Additional regulators of dopamine signaling were also found to have significantly lower expression in cultures from obesity-prone neonates than obesity-resistant neonates (Fig. 4.5F). Dopamine plasma membrane transporter (DAT) expression was 0.82 ±0.06 versus 1.5 ± 0.2 ($p<0.01$) and D2S presynaptic autoreceptor expression was 0.49 ± 0.05 versus 1.26 ± 0.21 ($p<0.01$).

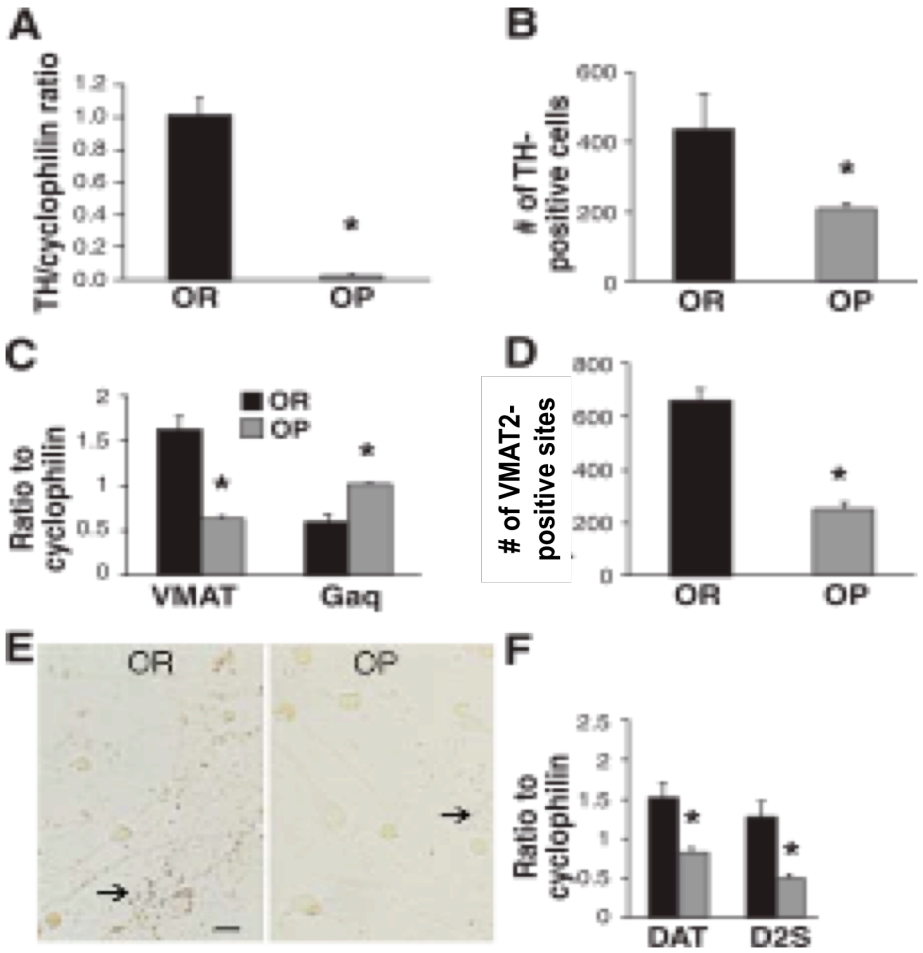

Figure 4.5: Lower mRNA and protein expression regulators of dopamine synthesis and exocytosis VTA cell cultures from obesity-prone neonatal animals.

(A) mRNA expression of TH was significantly lower in dopamine neuronal cultures derived from obesity-prone rats (grey bar) than in those from obesity-resistant rats (black bar, n=5 pooled cultures/group in triplicate). **(B)** TH immunopositive cells in cultures from obesity-prone rats (n=11 cultures) were significantly less than in those from obesity-resistant rats (n=6 cultures). **(C)** mRNA expression of VMAT2 was significantly lower while mRNA expression of endogenous VMAT2 downregulator $G\alpha_q$ was significantly higher in cultures from obesity-prone rats than in those from obesity-resistant rats (n=5 cultures/group). **(D)** VMAT2 immunopositive sites in cultures from obesity-prone rats (n=12 cultures) were significantly less than in those from obesity-resistant rats (n=16 cultures). **(E)** Representative VMAT2 immunostaining in VTA cultures from obesity-resistant and obesity-prone pups, 7 days post plating. Arrows point to VMAT2-positive sites. Scale bar=100µm; 20x magnification. **(F)** Lower mRNA expression of the DAT transporter and the presynaptic dopamine autoreceptor D2S in cultures from obesity-prone (grey bars) than obesity-resistant neonates (black bars, n=5 cultures/group). *p<0.01

4.4 Discussion

This study represents the first demonstration that there are impairments in all midbrain dopamine systems in obesity-prone rats and that these deficiencies are distinctly in place early in postnatal life. The pathophysiological mechanisms responsible for this dopamine phenotype involve a significant reduction in basal and stimulated dopamine release and in dopamine quantal size in obesity-prone animals.

Most of the data presented here focuses on the changes in the dopamine availability in the nucleus accumbens and the underlying regulatory mechanisms in obesity-prone animals. Interestingly, we also show decreased stimulated dopamine in the dorsal striatum and prefrontal cortex indicating a global presynaptic deficit in central dopamine in animals with predisposition to obesity. Such a non-site specific defect apparently implies deficits in universal presynaptic regulators of dopamine neurotransmission. Indeed, TH, VMAT2, the DAT plasma membrane transporter and D2-type receptor message were reduced in obesity-prone rats. However, TH and VMAT2 directly regulate dopamine exocytosis through lower biosynthesis (TH) and vesicular packaging (VMAT2), while DAT and dopamine receptors respond to changes in exocytosis. Therefore, low TH and VMAT2 expression constitutes the most likely underlying mechanism accounting for the central dopamine deficits observed in obesity-prone rats. It has been previously shown that VMAT2 activity is downregulated through vesicle associated G-proteins (i.e. trimeric GTPase Go_2 and $G\alpha_{q,}$ (Holtje

et al., 2000; Holtje et al., 2003). The same molecules (VMAT2, $G\alpha_q$, Go_2) are involved in the mechanism of action of amphetamine and other psychostimulants that are also VMAT2 substituents and displace dopamine from vesicles (Sulzer and Rayport, 1990). Furthermore, we have previously shown VMAT2 to directly regulate dopamine quantal size through vesicular filling (Fon et al., 1997; Pothos et al., 2000). Overexpression of the transporter leads to higher quantal size and quantal content (frequency of release), while mice deficient for VMAT2 have correspondingly deficient synaptic dopamine release. It is thus quite tempting to speculate that genetic polymorphisms in this gene (VMAT2) may negatively correlate with BMI in humans and account for at least some forms of human obesity.

What signal could contribute as a common denominator in the TH, VMAT2 and dopamine deficiencies observed in obesity prone animals? Is this signal extrinsic or intrinsic to the dopamine neuron? One possibility is leptin. Other studies have suggested that systemic inputs from neurons expressing LepRB receptors in the lateral hypothalamus (Leinninger and Myers, 2008) modulate TH expression. In patients with congenital leptin deficiency, leptin administration reverses their blunted ability to discriminate between the rewarding properties of food and, at the neuronal level, alters activation in the ventral striatum. This interaction suggests that leptin could modulate feeding-related mesolimbic sensitivity to the visual presentation of food stimuli (Farooqi et al., 2007). Others point to neuroanatomical links between the nucleus accumbens and the lateral

hypothalamic nuclei via melanin concentrating hormone or orexin signaling (Berthoud, 2005; Harris et al., 2007). However, the deficits reported in the present study have been maintained in preparations (coronal slices, dissociated cell cultures) without systemic input from elsewhere in the brain. Furthermore, the regulation of the VMAT2 transporter appears to be intrinsic to the dopamine neuron through heterotrimeric G protein endogenous downregulators (Holtje et al., 2000; Holtje et al., 2003), one of which (Ga_q) was found elevated in neurons from obesity-prone animals in the present study. Therefore, it is evident that the defects in mesolimbic dopamine exocytosis in obesity-prone animals are due at least partly to aberrant intrinsic signaling in VTA dopamine neurons that is independent from lateral hypothalamic or other systemic inputs. Leptin receptors on VTA neurons (Fulton et al., 2006; Hommel et al., 2006) could trigger this intrinsic deficit.

How do the present findings fit with what is known about the role of mesolimbic dopamine in feeding? The role of mesolimbic dopamine in food and drug reward and addiction has been the subject of much investigation (Di Chiara et al., 2004; Kelley et al., 2005b; Salamone et al., 2005). Interestingly, underweight food-deprived adult rats also have lower levels of basal extracellular accumbens dopamine than normal weight rats (Pothos et al., 1995a; Pothos et al., 1995b; Pothos et al., 1998b). Overall, it appears that low basal accumbens dopamine levels irrespective of nutritional status or body weight are linked to enhanced motivation for food intake. In the case of underweight animals, enhanced

motivation for not only food intake but also other dopamine-releasing agents like drugs of abuse has been established (Carroll, 1985). In addition, sugar binging in rats potentiates accumbens dopamine release similarly to drugs of abuse (Avena et al., 2008b). Those studies that have focused on the link between mesoaccumbens dopamine and normal feeding report that mesolimbic dopamine mediates the appetitive or consummatory phase of feeding. Others and we have observed a dopamine increase in the nucleus accumbens following a meal (Ahn and Phillips, 2002; Bassareo and Di Chiara, 1999; Hernandez et al., 1988; Martel and Fantino, 1996; Pothos et al., 1995a). One interpretation of such findings is that mesoaccumbens dopamine release is a signal that pertains to satiation. When the dopamine signal is attenuated, satiation is not achieved and hyperphagia ensues as a compensatory response to elevate depressed dopamine levels.

Evidence directly linking mesoaccumbens dopamine signaling to obesity is limited. However, we have reported low extracellular dopamine levels in the nucleus accumbens of rats fed a high-energy cafeteria-type diet and low dopamine signal following electrical stimulation of the accumbens in ob/ob mice (Fulton et al., 2006; Pothos et al., 1998b). Furthermore, functional inhibition of the nucleus accumbens shell via the GABAergic inhibitory interneurons induces hyperphagia in *ad libitum* fed animals (Kelley et al., 2005b). These observations are consistent with our findings that hyperphagic obesity-prone rats have reduced dopamine signaling in the nucleus accumbens, although this deficit is

now established to precede the expression of the obesity phenotype. The notion that decreased nucleus accumbens dopamine signaling leads to increased feeding is compatible with the finding that obese humans have reduced central D2 receptor levels (Wang et al., 2001) and that administration of dopamine agonists to *ob/ob* mice normalizes hyperphagia (Bina and Cincotta, 2000; Scislowski et al., 1999).

In conclusion, these experiments describe the first direct evidence that a low midbrain dopamine signal is present in animals with a predisposition to dietary obesity. Deficits in mesolimbic dopamine neurotransmission are an essential component of a neurochemical phenotype of obesity predisposition that induces a compensatory behavioral response, hyperphagia, to elevate central dopamine release. Taken together, these results underscore the importance of motivational and hedonic pathways in addition to hypothalamic homeostatic pathways in the regulation of appetite and feeding behavior; and strengthen the argument that obesity could be approached as an addictive disorder and not only as a metabolic imbalance. Targeting presynaptic components of the mesolimbic dopamine system may provide an approach to both screen for and effectively treat obesity predisposition.

5 An obesogenic perinatal environment alters central dopamine signaling and energy balance through a microRNA-related mechanism

Abstract

The development of obesity in the offspring of obese mothers has been partially linked to the prenatal environment. Using a selectively inbred rat model of obesity predisposition, we have previously shown decreased dopamine tone in obesity-prone rats. Because these deficits are present early in life and development of dopamine neurons begins during gestation, we completely alter the perinatal environment of the offspring via one or two-cell embryo transplantation from an obesity-resistant mother into an obesity-prone dame and vice versa. We found that exposure to an obesogenic perinatal environment decreases dopamine release and spontaneous activity in female offspring through changes in expression of Pitx3 and miR-133b. These results indicate that the association of the perinatal environment to obesity is critical to the development of the central dopamine system and epigenetic mechanisms of transcriptional regulation are a potential target for developing therapeutics to combat dietary obesity.

5.1 Introduction

Most previous studies involving the role of the perinatal environment on the development of obesity in the offspring use a high-fat or high-sucrose maternal diet to evaluate the effects of the resulting maternal obesity on the offspring (Buckley et al., 2005; Chen et al., 2008; Giraudo et al., 2010; Mitra et al., 2009; Srinivasan et al., 2006; Tamashiro et al., 2009; Vucetic et al., 2010). This approach does not allow for separation between the effects of the obese phenotype and dietary macronutrient composition. In this study, we determine the direct contributions of the perinatal obese versus OR maternal phenotype on development of the obese phenotype and central dopamine signaling in the offspring by using the selectively inbred OP Sprague-Dawley rat model, where the OP rats gain approximately 20% more weight than the OR rats when both groups are fed the same chow diet (Geiger et al., 2008; Levin et al., 1997). We completely switch the perinatal environment by transplanting one- or two-cell embryos from an OR mother into an OP mother and vice versa. This approach allows us two distinct advantages over previous maternal obesity studies including alteration of the environment without altering the maternal diet and controlling the litter size by the number of embryos we transplant. Our findings indicate that in female OR offspring exposed to an OP perinatal environment decreased central dopamine signaling is linked to an increase in body weight gain and lower levels of spontaneous activity prior to pubescence. These effects are mediated by a negative feedback circuit between miR-133b and Pitx3 that leads to deficits in the expression of key dopamine-related genes in the VTA.

5.2 Experimental Procedures

5.2.1 Animals

For the oviduct transplant surgery, 8-10 week old male and female OP and OR and vasectomized male Dawley-Dawley rats were purchased from Charles River Laboratories (Cambridge, MA, USA). All animals were kept in a reverse light-dark cycle facility, lights off at 7:00 am and lights on at 7:00 pm and used in the middle of the dark cycle when they are normally active. Body weight was measured weekly on all offspring. All experiments were conducted according to procedures approved by the Tufts University Institutional Animal Care and Use Committee.

5.2.2 Oviduct transplant

On Day 0, the estrous cycle was synchronized in donor and foster females using [des-Gly^{10}, D-Ala^{6}]-LH-RH ethylamide acetate (L4513, Sigma, St. Louis, MO, USA). Beginning on day 3, females were mated with either males of the same strain (donors) or vasectomized males (fosters). Within 24 hours of the appearance of plugs in the cages (usually day 5), donor females were anesthetized. The oviducts were harvested and the embryos were flushed using media containing EmbryoMax FHM HEPES Buffered medium (Millipore, MR-025-D) and 300 mg/mL hyaluronidase (Sigma, H4272). Embryos were then implanted into the oviduct through the infundibulum of an anesthetized foster female. Twenty-five to thirty one-cell or two-cell embryos were implanted into

each female to provide an average litter size of 8.1 ± 0.6 pups (n=22 litters). A schematic of the groups generated through this surgery is shown in Figure 5.1.

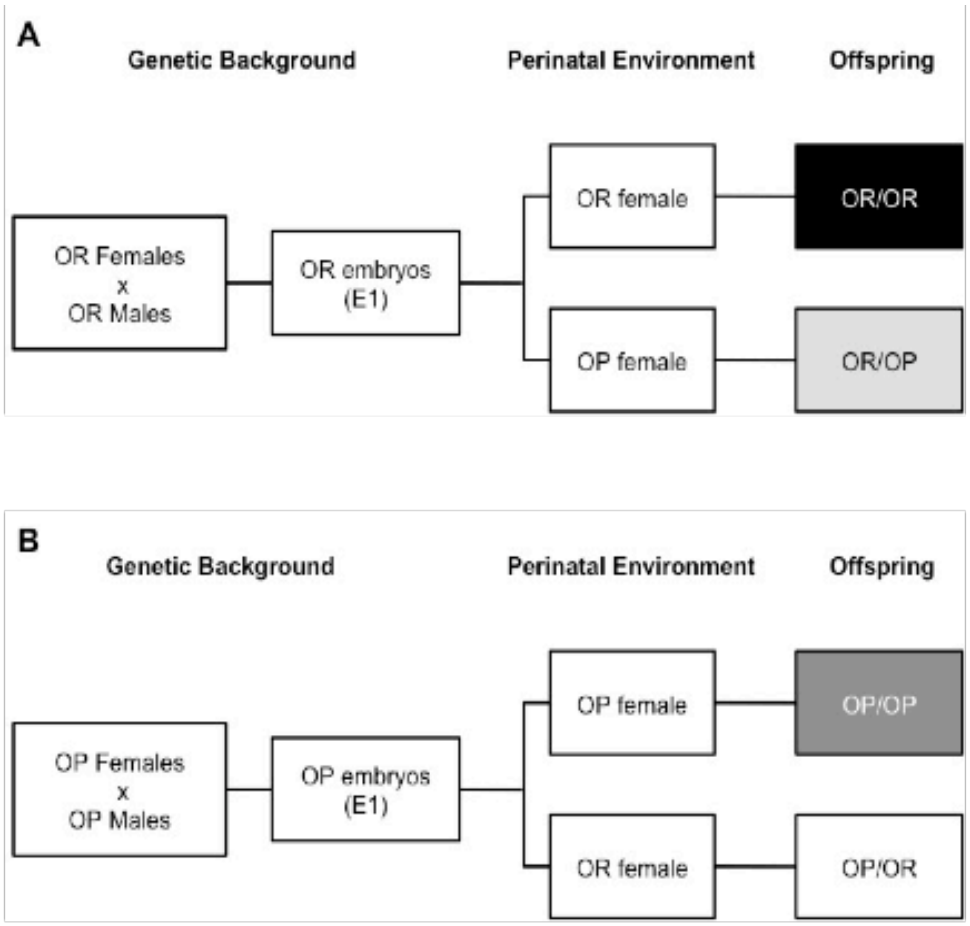

Figure 5.1: Transplanted offspring were generated by harvesting one or two cell OR and OP embryos and transplanting them into a pseudo-pregnant OR or OP dame.
A) Schematic representation of OR embryo transplantation at embryonic day 1. OR/OP offspring are OR animals that were exposed to an OP perinatal environment and OR/OR offspring are OR animals that were transferred to another OR foster mother. **B)** Schematic representation of OP embryo transplantation at embryonic day 1. OP/OR offspring are OP animals that were exposed to an OR perinatal environment and OP/OP offspring are OP animals that were transferred to another OP foster mother.

5.2.3 Brain slice electrophysiology

3 to 5-week old and 14 to 16-week old female offspring were euthanized using ketamine/xylazine and brains were removed. Coronal brain slices (300 μm) were generated and brain slice electrophysiology was completed as described in Chapter 2.

5.2.4 Oral glucose tolerance test, insulin and leptin measurements

Animals were fasted overnight and an oral glucose tolerance test was administered the following morning. 200 mg/kg glucose were given by oral gavage. Blood samples were taken at t=0, 30, 60, 90, and 120 minutes following the gavage. Glucose measurements were taken using a AlphaTRAK blood glucose meter (Abbott Labs, Abbott Park, IL, USA) at each timepoint. Plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA) (EZRMI-13K, Millipore, Billerica, MA**)** according to the manufacturer's directions for each timepoint. Fasting plasma leptin levels were also measured by ELISA (EZRL-83K, Millipore, Billerica, MA) according to the manufacturer's directions. The ELISAs were read using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA).

5.2.5 Food intake and activity measurements

Intake of laboratory chow and activity levels were measured when the animals were one-month and four-months old using PanLab oxylet system metabolic cages. (PanLab S.L., Barcelona, Spain).

5.2.6 Real-time PCR

The ventral tegmental area was dissected and immediately frozen on dry ice. For mRNA expression, total RNA was extracted using TriZOL reagent (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The purified RNA was reverse transcribed using the mMulv reverse transcriptase kit and random primers containing 12 nucleotides (New England Biolabs, Beverly, MA, USA). Real-time PCR using Sybr Green detection was performed for the genes listed in Table 5.1. The primers listed in Table 5.1 were designed using Primer3 software(Rozen and Skaletsky, 2000). Forty-five cycles of real-time quantitative PCR were performed using a Stratagene MX3000P (Qiagen, Valencia, CA, USA). Specific gene expression was calculated relative to levels in the OR/OR or OP/OP group and normalized to TBP.

Table 5.1: Genes and primers used for mRNA expression experiments.

Gene Name	**Gene Symbol**	**Protein**	**Primers**
TATA-box binding protein	*Tbp*	TBP	F: 5'-cggtttgctgcagtcatcat-3' R: 5'-gtgcacaccattttcccaga-3'
Tyrosine hydroxylase	*Th*	TH	F: 5'-gggtacaaaaccctcctcct-3' R: 5'-ccgacacttttcttgggaac-3'
Vesicular monoamine transporter-2	*Slc18a2*	VMAT2	F: 5'-ctaccagcacacagcacact-3' R: 5'-tcattcagaaggtctctgtcttcact-3'
Dopamine reuptake transporter	*Slc6a2*	DAT	F: 5'-tgggtatcgacagtgcaatgg-3' R: 5'-ccgatgtagcagctggaactc-3'
Nuclear receptor subfamily 4, group A, member 2	*Nr4a2*	NURR-1	F: 5'-ggctgttgggatggttaaag-3' R: 5'-atagtcagggtttgcctgga-3'
Paired-like homeodomain 3	*Pitx3*	Pitx3	F: 5'-agatgcaggcactccacac-3' R: 5'-catgtcggggtagcgatt-3'

For microRNA expression, total RNA was extracted using QiaZOL reagent (Qiagen, Valencia, CA, USA) and purified using the miRNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The purified RNA was reverse transcribed using the miScript reverse transcriptase kit (Qiagen, Valencia, CA, USA). miRNA specific primers were purchased from Qiagen (Valencia, CA, USA) for miR-RNU6b, miR-133b, miR-124a, miR-218 and miR-9 and PCR amplification was done using the miScript Sybr Green kit from Qiagen (Valencia, CA, USA) according to the kit instructions. Specific gene expression was calculated relative to levels in the OR/OR group and normalized to miR-RNU6b.

5.2.7 Statistical analysis

Data was analyzed using a one-way ANOVA (Excel; Microsoft, Redmond, WA) between groups in all experiments. Results are expressed as mean +/- SEM.

5.3 Results

5.3.1 Dopamine neurotransmission is decreased in multiple brain sites in female OR offspring that have been exposed to an obesogenic perinatal environment

In our previous studies, we have shown that OP females have decreased real-time dopamine neurotransmission from coronal slice preparations compared to the OR controls (Geiger et al., 2008). To determine if this deficit was a result of the perinatal environment, we compared dopamine release from the NAcS of juvenile OR females that had been exposed to an OP perinatal environment

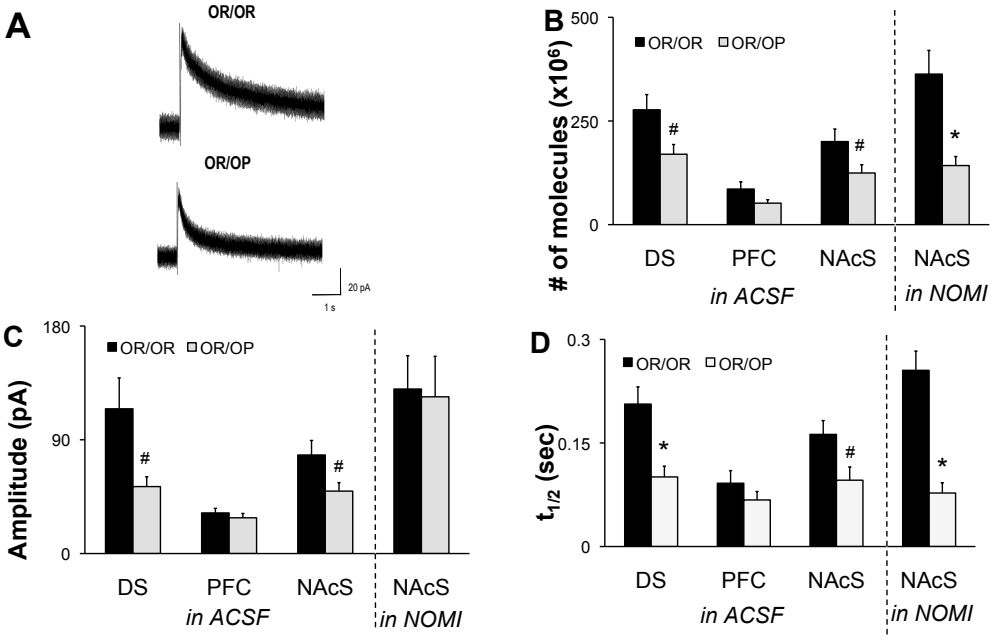

Figure 5.2: Perinatal exposure to an OP environment results in decreased evoked dopamine release of juvenile OR females.
A) Representative amperometric traces of electrically stimulated dopamine release in acute coronal nucleus accumbens shell (NAcS) slices. Mean evoked dopamine molecules (**B)**, peak amplitude **(C)**, and $t_{1/2}$ **(D)** in artificial cerebrospinal fluid (ACSF) from the dorsal striatum (DS), prefrontal cortex (PFC), and NAcS and in the NAcS after a 30-minute wash of 3 mM nomifensine (NOMI) in 3 to 5-week old OR/OR (black bars) and OR/OP (light grey bars) females. n ≥ 30 stimulations from at least 6 slices; N=4-7 animals per group and brain site tested. Data are mean ± sem. * p<0.01; #p<0.05 by one-way ANOVA.

(OR/OP offspring) to that from the control group of OR females that had transplanted into an OR perinatal environment (OR/OR offspring; Fig. 5.1A). Representative amperometric traces of the dopamine release peak following a single stimulus pulse are shown in Figure 5.2A. The number of molecules released is based on the integration of this peak. We found a deficit of approximately 35% in dopamine release in the NAcS of OR/OP females (OR/OP: 124.6 x 10^6 ± 19.9 x 10^6 molecules, OR/OR: 200.3 x 10^6 ± 30.3 x 10^6 molecules; n = 43-53 stimulations from 10-11 slices; N=6-7 pups; p<0.05; Fig. 5.2B). These

effects were mediated by differences in peak amplitude and width at half-height ($t_{1/2}$; Fig. 5.2C,D) and remained following a 30-minute wash in 3 mM nomifensine, an inhibitor of the dopamine reuptake transporter (OR/OP: 142.5 x 10^6 ± 21.9 x 10^6 molecules vs. OR/OR: 363.1 x 10^6 ± 57.2 x 10^6 molecules; n = 30 stimulations from 6 slices; $p<0.01$; N=6 pups). In addition, the difference in $t_{1/2}$ between the OR/OP and OR/OR groups was now even greater, indicating that blocking dopamine reuptake had less of an effect on dopamine release in OR/OP females than in OR/OR females (Fig. 5.2D).

To determine whether the deficits seen in the nucleus accumbens were present in other dopaminergic projections, we measured evoked dopamine release in the DS, which receives dopaminergic input from the SN and the PFC, which receives dopaminergic input from the VTA. In these areas, we found deficits of about 40% in the OR/OP compared to the OR/OR offspring (DS – OR/OP: 169.8 x 10^6 ± 23.5 x 10^6 molecules vs. OR/OR: 277.0 x 10^6 ± 36.8 x 10^6 molecules, n = 49-55 stimulations from 10-11 slices; N= 6 pups; $p<0.05$; PFC – OR/OP: 51.8 x 10^6 ± 8.3 x 10^6 molecules vs. OR/OR: 85.9 x 10^6 ± 17.5 x 10^6 molecules, n = 34-40 stimulations from 7-8 slices; N= 4 pups; $p=0.06$; Fig. 5.2B). These results show that an OP perinatal environment causes a universal deficit in central dopamine signal in young OR females.

5.3.2 Early deficits in dopamine neurotransmission persist into adulthood in female OR offspring that have been exposed to an OP perinatal environment

To determine the duration of the dopamine deficits seen in the juvenile females, we measured central dopamine release in young adult OR/OP females. Similar deficits to those seen in the juvenile OR/OP females were present in the NAcS of the adult females (OR/OP: 112.1 x 10^6 ± 13.0 x 10^6 molecules; OR/OR: 209.6 x 10^6 ± 26.1 x 10^6 molecules; n=38-63 stimulations from 8-13 slices; N=5-8 pups; $p<0.01$; Fig. 5.3A,B). These deficits also correspond to lower dopamine peak amplitudes as opposed to differences in $t_{1/2}$ (Fig. 5.3C,D). After a 30-minute wash in 3 mM nomifensine, these deficits remained (OR/OP: 118.2 x 10^6 ± 16.8 x 10^6 molecules vs. OR/OR: 385.2 x 10^6 ± 53.7 x 10^6 molecules; n = 29-30 stimulations from 6 slices; N=6 pups; $p<0.01$) and $t_{1/2}$ was now significantly lower in the OR/OP animals.

The dopamine deficits seen in the other projections of the juvenile females were also present in the adults. Dopamine release in both the DS and PFC was approximately 35% lower in the OR/OP offspring compared to the OR/OR offspring (DS – OR/OP: 207.5 x 10^6 ± 25.3 x 10^6 molecules vs. OR/OR: 323.2 x 10^6 ± 29.2 x 10^6 molecules; n = 40-58 stimulations from 8-12 slices; N=5-7 pups; $p<0.01$; PFC – OR/OP: 97.0 x 10^6 ± 17.3 x 10^6 molecules vs. OR/OR: 133.7 x 10^6 ± 14.8 x 10^6 molecules; n = 48-50 stimulations from 10 slices; N=5-6 pups; $p<0.01$; Fig. 5.3B). These deficits correspond to lower dopamine peak amplitudes as opposed to differences in $t_{1/2}$ (Fig. 5.3C,D). These results indicate that the OP

environment has detrimental effects on central dopamine release in all of the major dopamine signaling pathways of the OR/OP offspring into young adulthood.

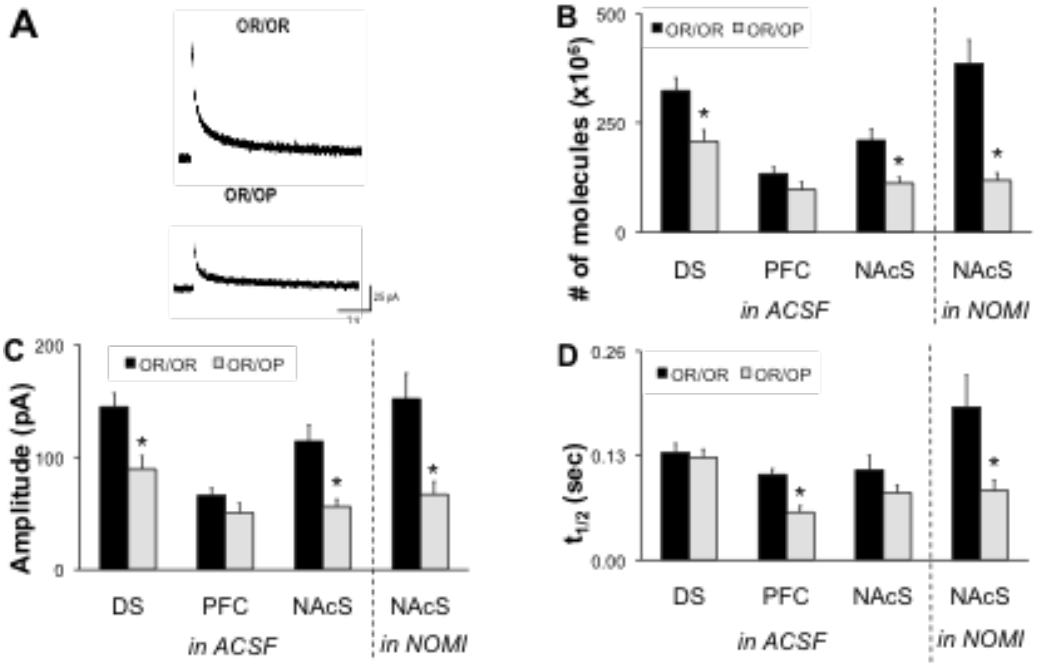

Figure 5.3: Dopamine deficits seen in juvenile OR/OP rats are also present in 15-week-old OR/OP female rats.
A) Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal NAcS slices. Mean evoked dopamine molecules **(B)**, peak amplitude **(C)** and $t_{1/2}$ **(D)** in ACSF from the DS, PFC, and NAcS and in 3 mM NOMI in the NAcS in 15-week-old female OR/OR (black bars) and OR/OP (light grey bars) offspring. n ≥ 29 stimulations from at least 6 slices; N=6-8 animals per group and brain site tested. Data are mean ± sem. * $p<0.01$; #$p<0.05$ by one-way ANOVA.

5.3.3 Decreased dopamine signaling as a result of an OP perinatal environment is related to increased body weight gain and decreased activity during adolescence in OR rats

To evaluate the influence of decreased dopamine signaling on the obesity phenotype, we measured body weight gain, food intake and activity levels of the offspring. Exposure to an OP perinatal environment caused increased body

weight gain in female offspring during the prepubescent period. Immediately following weaning at 3 weeks old through 8 weeks old, the OR/OP offspring gained 416±9% of their body weight at weaning compared to 375±8% % for the OR/OR offspring (n=18 animals per group; $p<0.01$; Fig. 5.4B). By adulthood, there was no difference in body weight between the OR/OP females (202±5 g) and the OR/OR females (213±3 g; Fig. 5.4A). These differences in body weight gain were associated with an increase in insulin response and fasting leptin of 12-week-old OR/OP females compared to the OR/OR controls, though no differences in glucose response were detected (Fig. 5.4C-F).

To determine if energy balance was altered in the OR/OP animals, we measured 24-hour intake of laboratory chow and activity levels in one-month-old female offspring. In this case, we found significantly decreased levels of activity in the OR/OP groups compared to the OR/OR animals (Fig. 5.5B). The OR/OP females had significantly lower spontaneous movement over the course of 24 hours (OR/OR: 7172±533 activity counts vs. OR/OP: 5582±587 activity counts, $p=0.05$, n=5 pups). The decreased activity level in the OR/OP animals drove the increased rate of body weight gain during this period as we found only mild, non-significant differences in food intake during the dark cycle, with the OR/OR females eating slightly more than the OR/OP group (OR/OR: 13.2±0.5 g vs. OR/OP: 11.3±0.8 g; n=7-8; $p=0.06$; Fig. 5.5A). Similar trends were also present in the adult animals, though they did not reach significance (Fig. 5.5C,D). These results indicate that dopamine deficits resulting from the OP perinatal

environment cause increased body weight gain and decreased activity in OR/OP females during adolescent.

5.3.4 Exposure to an OR perinatal environment results in increased dopamine release in OP females

In order to determine if the dopamine deficits previously shown in the OP animals could be avoided by exposure to an OR perinatal environment, we compared dopamine release from the offspring of one or two-cell OP embryos transplanted into pseudo-pregnant OR dames (OP/OR offspring) and their control group, OP embryos transplanted into pseudo-pregnant OP dames (OP/OP offspring; Fig. 5.1B). In the NAcS of the OP/OR juvenile female offspring, we measured a 50% increase of evoked dopamine release in the OP/OR animals both before and after a 30-minute treatment with nomifensine (Before nomifensine – OP/OR: $154.8 \times 10^6 \pm 23.0 \times 10^6$ molecules vs. OP/OP: $61.5 \times 10^6 \pm 9.3 \times 10^6$ molecules; n = 30-40 stimulations from 6-8 slices; N=4 pups and after nomifensine – OP/OR: $200.6 \times 10^6 \pm 56.8 \times 10^6$ molecules vs. OP/OP: $76.5 \times 10^6 \pm 13.7 \times 10^6$ molecules; n = 10-17 stimulations from 2-4 slices; N=2-4 pups; $p<0.05$; Fig. 5.6A,B). This difference was primarily due to higher mean peak amplitude as no significant differences were detected in the $t_{1/2}$ of the dopamine response peak either before or after the nomifensine treatment (Fig. 5.6C,D).

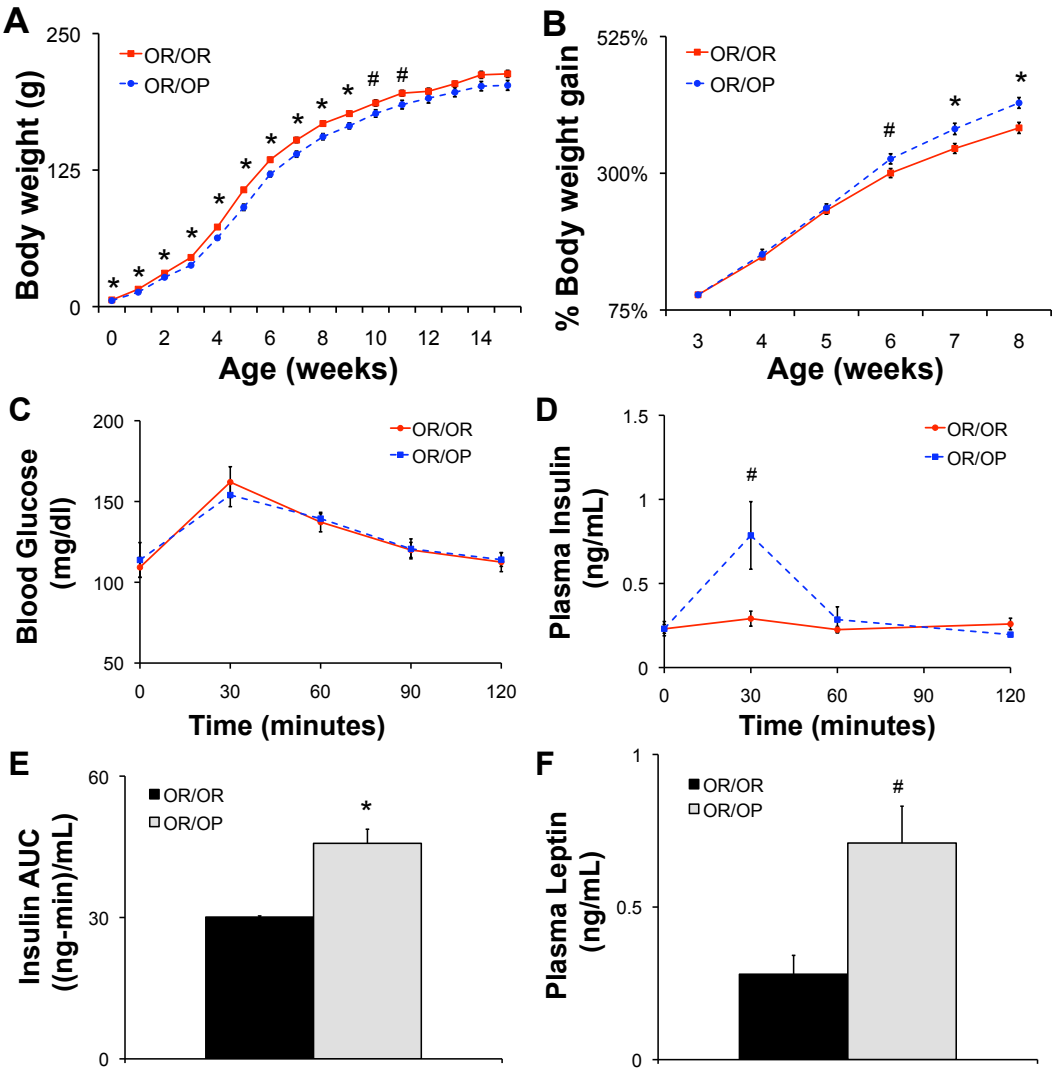

Figure 5.4: Exposure of an OR female rat to an obesogenic environment during the perinatal period results in increased weight gain during adolescence, elevated insulin response and elevated fasting plasma leptin.

(A) Weekly body weights of female OR/OR and OR/OP animals (n=18). **(B)** Body weight gain from weaning at 3 weeks old through 8 weeks old in female OR/OR and OR/OP offspring (n=18). **(C)** Blood glucose levels were not different between groups following a 200 mg/kg oral glucose gavage. **(D)** Plasma insulin levels were significantly higher in OR/OP females 30 minutes after an oral glucose gavage. **(E)** Area under the concentration vs. time curve for insulin was significantly greater in the OR/OP females than in the OR/OR females. **(F)** Fasting plasma leptin levels were higher in the OR/OP females. n = 4-5 12-week-old OR/OR and OR/OP females per group . *p<0.01, #p<0.05 by one-way ANOVA.

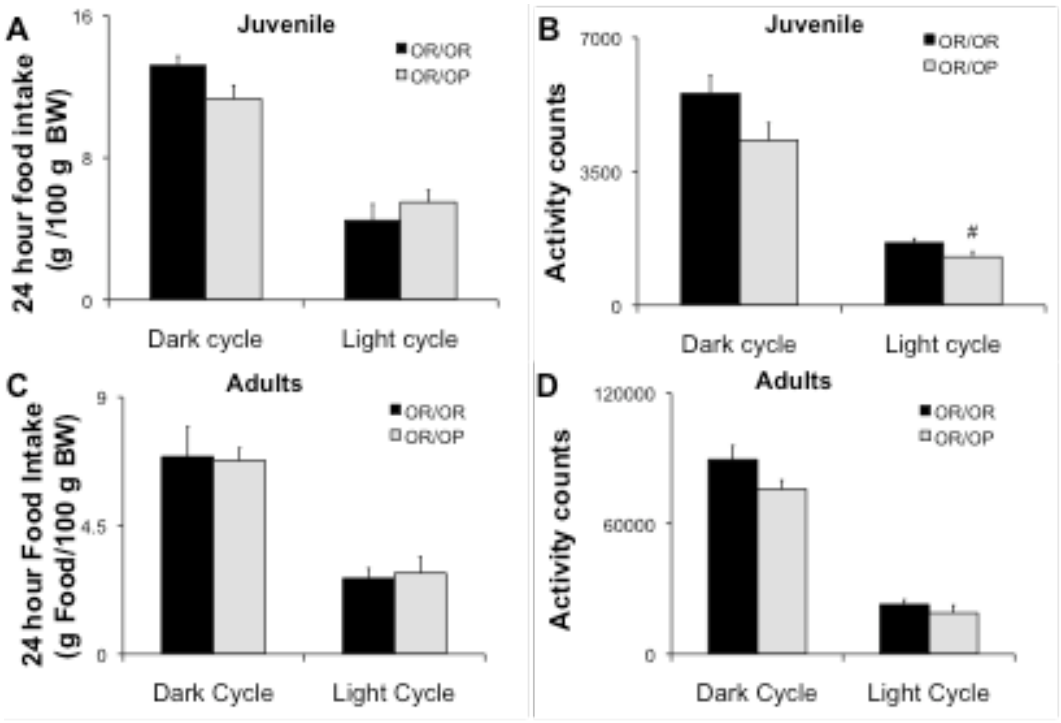

Figure 5.5: Exposure of an OR female rat to an obesogenic environment during the perinatal period results in decreased activity during adolescence.
(A) Average intake of laboratory chow during the dark and light cycles of the OR/OR (black bars) and OR/OP (light grey bars) females (n=7-8). **(B)** Activity levels during the dark and light cycles in the OR/OR and OR/OP group (n=5). **(C)** Food intake is not different in adult OR females that have been exposed to an OP perinatal environment. **(D)** OR/OP females showed a trend toward decreased activity levels during the dark cycle (p=0.06). n=6-8 animals per group. Data are mean ± sem. * p<0.01; # p<0.05 by one-way ANOVA.

In the DS of juvenile OP/OR females, the evoked dopamine release was not significantly different from the evoked dopamine release from the OP/OP group (OP/OR: 114.0 x 10^6 ± 17.4 x 10^6 molecules vs. OP/OP: 103.4 x 10^6 ± 17.4 x 10^6 molecules; n = 30-40 stimulations from 6-8 slices; N=4 pups; p<0.01; Fig. 5.6B,C), even though the peak amplitude was higher in these animals. The lack of difference is likely due to the trend towards a decreased $t_{1/2}$ in these animals (Fig. 5.6D). In the PFC, evoked dopamine release was higher in the OP/OR

female offspring (OP/OR: 177.9 x 10^6 ± 29.5 x 10^6 molecules vs. OP/OP: 31.8 x 10^6 ± 4.4 x 10^6 molecules; n = 25-40 stimulations from 5-9 slices; N=4 pups; p<0.01; Fig. 5.6B-D), indicating that early in life, the OR perinatal environment has a greater influence on the mesolimbic and mesocortical projections than the nigrastriatal projections.

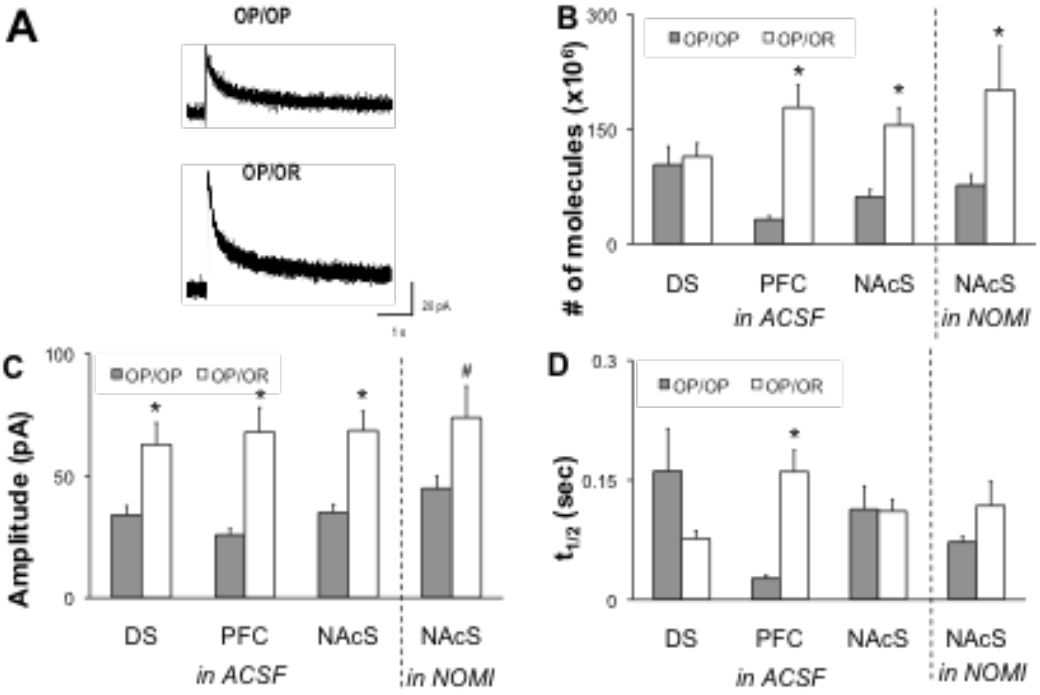

Figure 5.6: Perinatal exposure to an OR environment protects OP offspring from deficits in evoked dopamine release from the nucleus accumbens and dorsal striatum seen in OP/OP controls during adolescence.

(A) Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal NAcS slices. Mean evoked dopamine molecules **(B)**, peak amplitude **(C)**, and $t_{1/2}$ **(D)** in ACSF from the DS, PFC, and NAcS and in 3 mM NOMI from the NAcS of 3 to 5-week-old OP/OP (dark grey bars) and OP/OR (white bars) females. n ≥ 10 stimulations from at least 2 slices; N=2-4 animals per group and brain site tested. Data are mean ± sem. * p<0.01; #p<0.05 by one-way ANOVA.

5.3.5 The protective effects of the OR environment on dopamine neurotransmission develop in the striatum of the OP/OR females by adulthood

In the juvenile female OP/OR offspring, we did not detect a difference in dopamine signaling in the DS. By adulthood, we found that the number of dopamine molecules released from the DS of the female OP/OR offspring was approximately 80% greater than that of the adult female OP/OP offspring (OP/OR: 446.8 x 10^6 ± 35.9 x 10^6 molecules vs. OP/OP: 117.1 x 10^6 ± 18.2 x 10^6 molecules; n = 35-43 stimulations from 7-9 slices; N=4-6 pups; $p<0.01$; Fig. 5.7B). This effect was related to both the amplitude and the $t_{1/2}$ of the evoked dopamine peak (Fig. 5.7C,D).

In addition, a similar elevation of dopamine neurotransmission to that seen in the juvenile females was measured in the NAcS and PFC of the OP/OR females (NAcS - OP/OR: 240.4 x 10^6 ± 26.4 x 10^6 molecules vs. OP/OP: 86.0 x 10^6 ± 14.7 x 10^6 molecules; n = 33-47 stimulations from 7-10 slices; N=4-6 pups; $p<0.01$; PFC – OP/OR: 217.4 x 10^6 ± 28.7 x 10^6 molecules vs. OP/OP: 127.4 x 10^6 ± 25.4 x 10^6 molecules; n = 29-34 stimulations from 6-7 slices; N=3-4 pups; $p<0.01$; Fig. 5.7A,B). The enhanced dopamine release in the OP/OR animals remained following a 30-minute wash with nomifensine (OP/OR: 586.3 x 10^6 ± 56.4 x 10^6 molecules vs. OP/OP: 253.9 x 10^6 ± 62.5 x 10^6 molecules; n = 14-30 stimulations from 3-6 slices; N=3-6 pups; $p<0.01$; Fig. 5.7B). These data point to a protective effect of the OR environment on the development of the central dopamine system through upregulation of the entire system by adulthood.

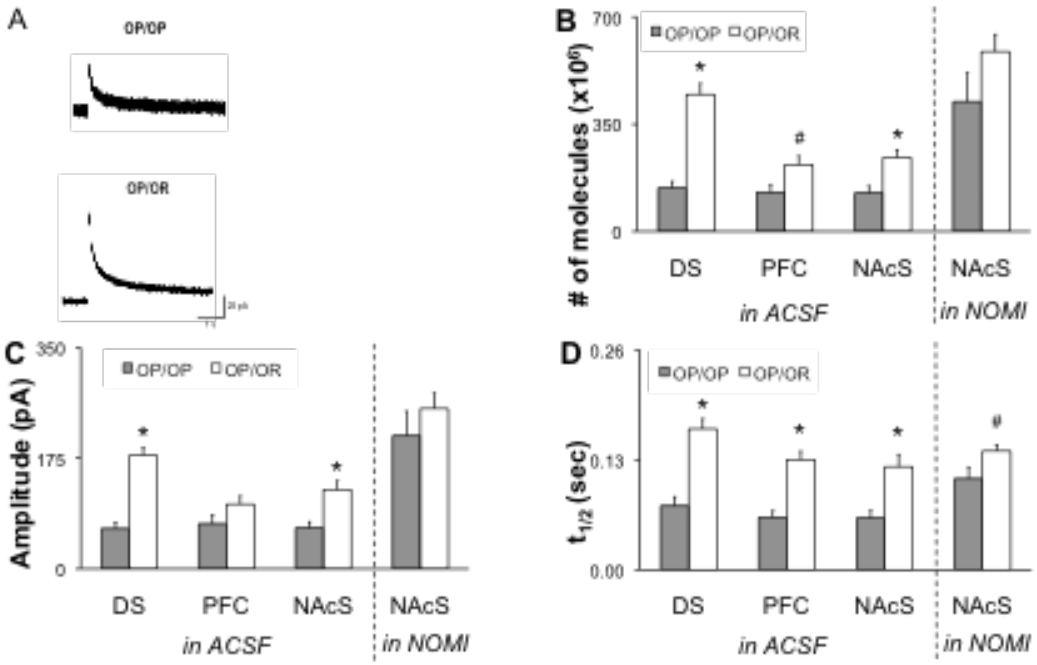

Figure 5.7: Elevations in evoked dopamine release in juvenile females that were exposed to an OP environment early in life are enhanced into adulthood.
(A) Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal NAcS slices. Mean evoked dopamine molecules **(B)**, peak amplitude **(C)**, and $t_{1/2}$ **(D)** in ACSF from the DS, PFC and NAcS and in 3 mM NOMI in the NAcS of 15-week-old OP/OP (dark grey bars) and OP/OR (white bars) females. n ≥ 14 stimulations from at least 3 slices; N=3-6 animals per group and brain site tested. Data are mean ± sem. * $p<0.01$; #$p<0.05$ by one-way ANOVA.

5.3.6 Exposure of an OP animal to an OR perinatal environment results in decreased food intake

As with OR animals exposed to an OP perinatal environment, we did not detect significant differences in body weight at fifteen weeks old, the female OP/OR offspring (283±10 g) compared to the OP/OP offspring (272±10 g; n=11-12 animals; NS; Fig. 5.8A). In addition, even though the OP/OR female offspring gained weight more quickly in the first two weeks following weaning, this

increased rate of body weight gain was much more modest than the increased body weight gain seen in the OR/OP females from the first experiment and the difference in body weight gain disappeared by 8 weeks of age (OP/OP: 497±14% vs. OP/OR: 524±10%, n=11-12 animals per group; Fig. 5.8B). There were no differences in glucose, insulin response or fasting leptin in these animals (Fig. 5.8C-F).

When we measured parameters of energy balance in these animals, we did see differences even though there were no differences in body weight related parameters. Of particular interest is the decreased food intake by the OP/OR during the dark cycle (OP/OP: 12.2±0.4 g; and OP/OR: 10.4±10.5 g n=5-6; $p<0.05$; Fig. 5.9A). There was no difference in activity levels between the groups over 24 hours (OP/OR: 5005±224 activity counts, and OP/OP: 4492±989 activity counts; n=3-6; NS; Fig. 5.9B). Food intake during the dark cycle remained lower in the adult OP/OR offspring. In addition, the activity level of these animals was also decreased as adults (Fig. 5.9C,D). In contrast to the OR/OP animals where decreased dopamine is linked to decreased activity, elevation of dopamine signaling following exposure to the OR perinatal environment results in decreased food intake, indicating differences in the mechanisms by which each environment alters central dopamine signaling.

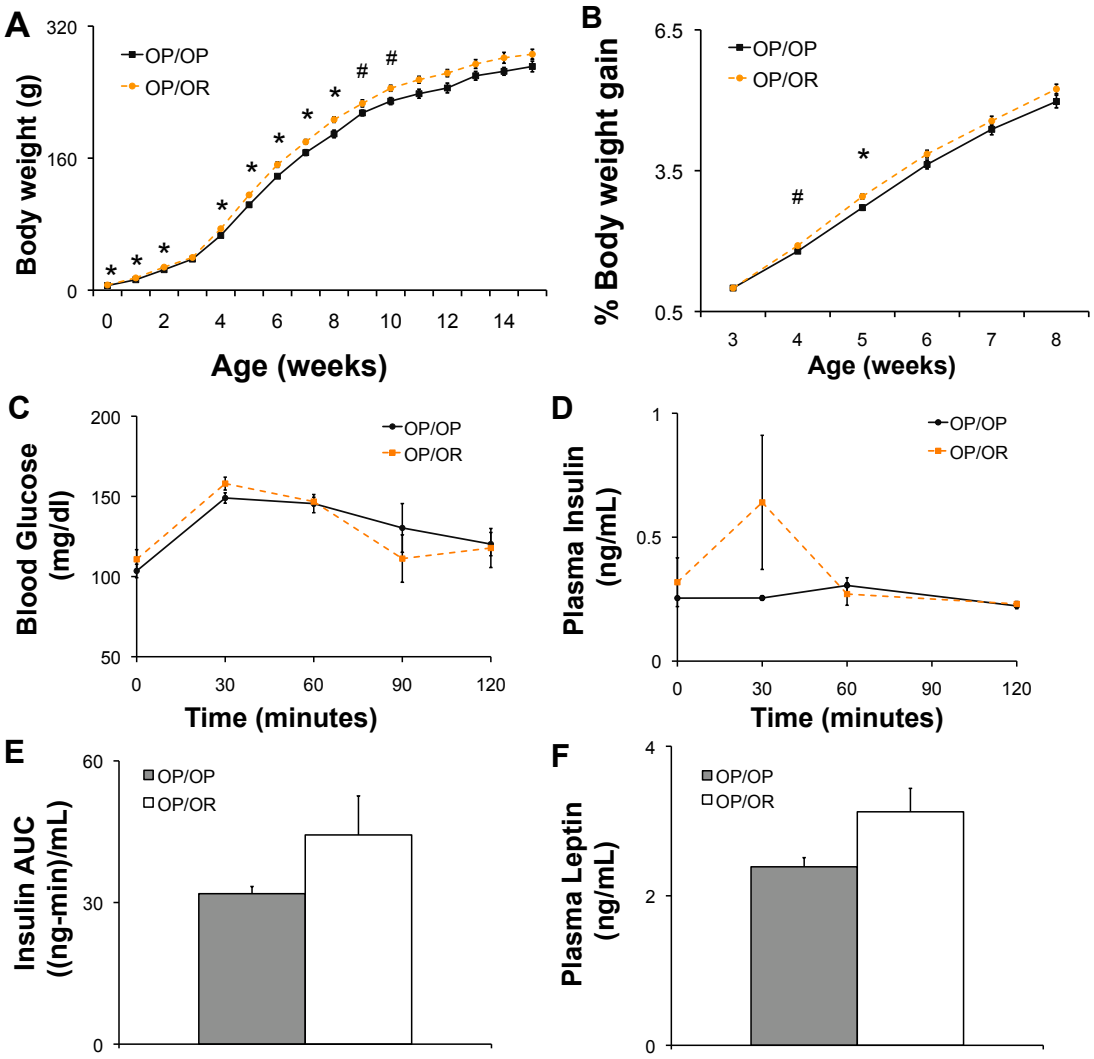

Figure 5.8: Perinatal exposure of an OP female rat to an OR environment results in decreased food intake during adolescence. **(A)** Weekly body weights of female OP/OP and OP/OR offspring (n=11-12). **(B)** Body weight gain from weaning at 3 weeks old through 8 weeks old in OP/OP and OP/OR females (n=11-12). Blood glucose levels **(C)**, plasma insulin levels **(D)** and area under the concentration vs. time curve for insulin **(E)** were not different between groups following a 200 mg/kg oral glucose gavage. **(F)** Fasting plasma leptin levels were not significantly different between groups. n=4-5 12-week-old OP/OP and OP/OR animals per group. Data are mean ± sem. * $p<0.01$; # $p<0.05$ by one-way ANOVA.

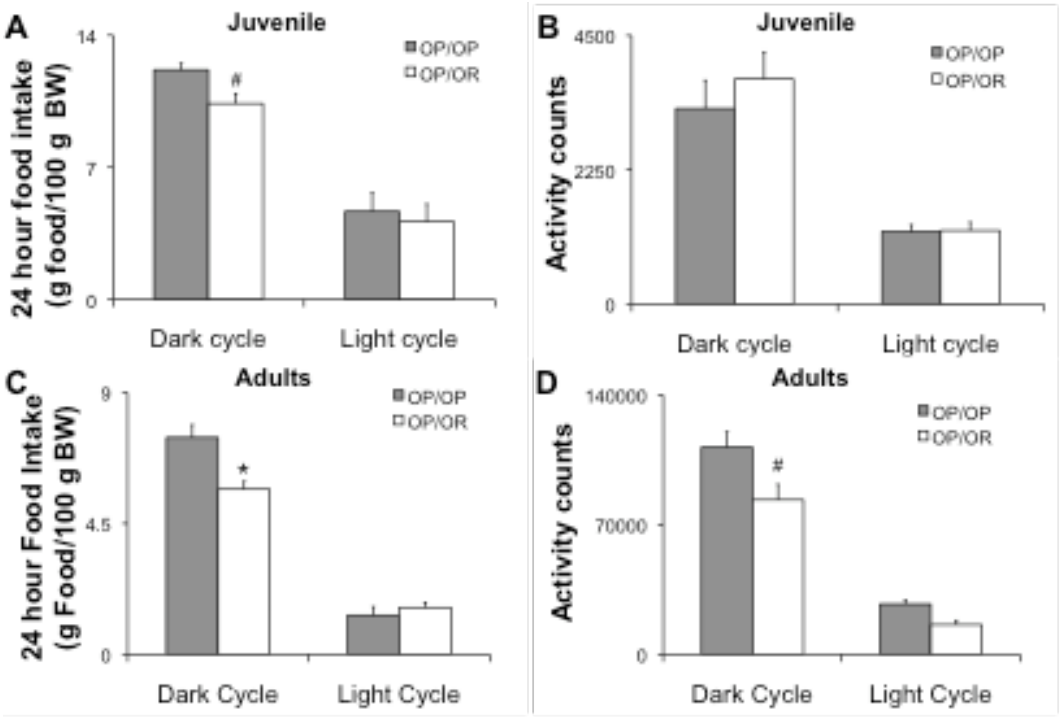

Figure 5.9: Perinatal exposure of an OP female rat to an OR environment results in decreased food intake.
(A) Average intake of laboratory chow during the dark and light cycles in 5 week old OP/OP (dark grey bars) and OP/OR (white bars) females (n=5-6). **(B)** Activity levels during the dark and light cycles in 5 week old OP/OR and OP/OP females (n=3-6). Data are mean ± sem. * $p<0.01$; # $p<0.05$ by one-way ANOVA.**(C)** Food intake was significantly lower in OP females that had been exposed to an OR perinatal environment. **(D)** OP/OR females also had decreased activity levels during the dark cycle compared to the OP/OP females. *$p<0.01$, #$p<0.05$ by one-way ANOVA. n=6-7 animals per group.

5.3.7 Alterations in dopamine signaling that result from the perinatal environment are related to changes in gene expression

To determine the mechanism by which dopamine signaling might be altered in these animals we used quantitative PCR to measure gene expression of pre-synaptic regulators of dopamine synthesis, packaging and metabolism from the VTA of the adult female offspring. Tyrosine hydroxylase (TH) is the rate-limiting enzyme of dopamine synthesis, the dopamine reuptake transporter (DAT) clears

dopamine from the synapse, and the vesicular monoamine transporter (VMAT2) is involved in sequestration of dopamine into synaptic vesicles. We found that the mRNA expression of *Th*, *Slc6a2* (the gene coding for DAT) and *Slc18a2* (the gene coding for VMAT2) are approximately 60%, 40% and 65% lower, respectively, in the OR/OP offspring compared to the OR/OR offspring ($p<0.05$, Fig. 5.10A). In addition, we detected significant increases in mRNA expression of *Slc6a2* and significant decreases in mRNA expression of *Slc18a2* in the OP/OR offspring compared to the OP/OP offspring (Fig. 5.10B). These results indicate that by simply changing the perinatal environment, dopamine signaling is altered through changes in gene expression.

5.3.8 MicroRNAs involved in regulation of transcription factors are altered in animals exposed to an obesogenic perinatal environment

Two common regulatory mechanisms of gene expression are through transcription factors or microRNAs. In this study, we measured mRNA expression in the OR/OR and OR/OP females of the genes coding for two transcription factors known to regulate TH, DAT, and VMAT2, *Nr4a2* (also known as Nurr1) and *Pitx3*, paired-like homeodomain 3. Similar to the reduction in expression of genes coding for TH, DAT, and VMAT2, we detected a decrease in mRNA expression of *Pitx3* but not *Nr4a2* in the OR/OP offspring (Fig. 5.10C). We also measured miRNA expression of several microRNAs that have been shown to either affect *Pitx3* expression or development of dopamine neurons (Huang et al., 2010; Kim et al., 2007). Here we show a 2-fold increase in miR-

133b correlating to the decrease measured in *Pitx3* and a 3-fold increase in miR-124a in the OR/OP females (Fig. 5.10D). Taken together these results indicate that the OP perinatal environment influences the expression of miR-133b, which in turn down regulates expression of *Pitx3*. Since *Pitx3* regulates expression of *Th*, *Slc6a2*, and *Slc18a2*, downregulation of *Pitx3* leads to lower levels of these genes. Decreased levels of these genes results in reduced overall dopaminergic neurotransmission that is correlated with decreased activity and increased body weight gain in the OR/OP offspring.

5.4 Discussion

To our knowledge, this is the first study that has employed embryonic transplantation to determine the effects of an obesogenic perinatal environment on the offspring. Most similar studies have either modified the mother's diet or cross-fostered offspring to an obese mother immediately following birth. In the first approach, the relative effects of diet versus the obese phenotype of the mother are inseparable. In the second approach, effects of obesity during gestation are ignored. Our approach allows us to eliminate differences in diet while simultaneously taking into account prenatal and early postnatal effects of maternal obesity.

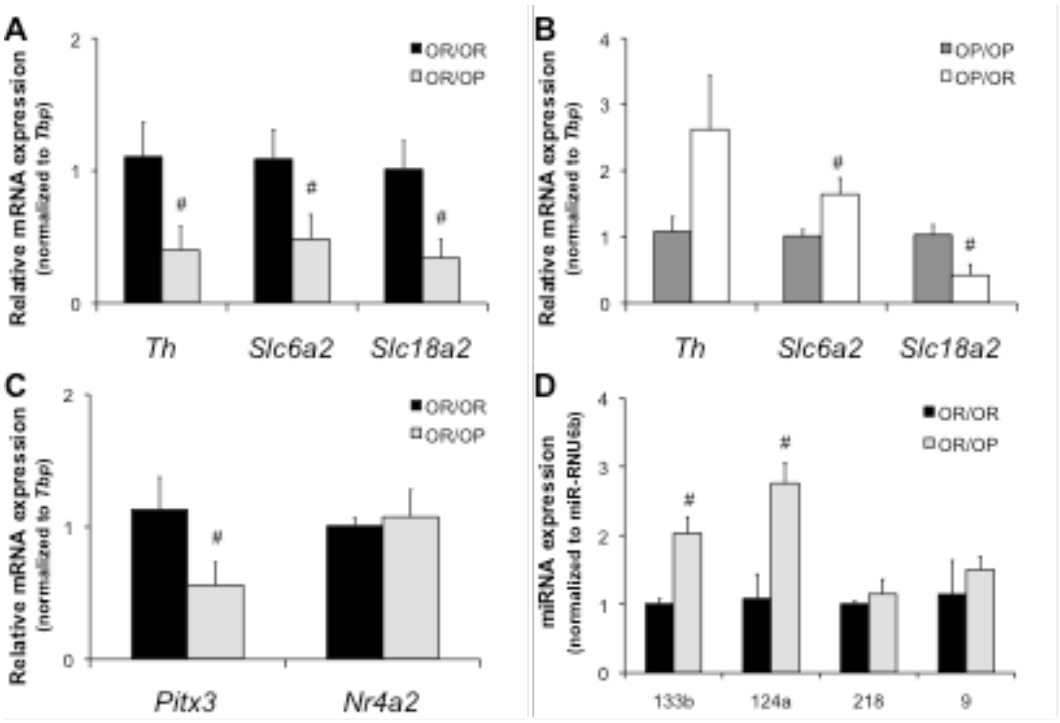

Figure 5.10: Key regulators of dopamine synthesis, sequestration and clearance are down-regulated in the VTA of adult OR/OP females in response to changes in expression of transcription factor and microRNAs.
(A) *Th*, *Slc6a2* (gene coding for DAT), and *Slc18a2* (gene coding for VMAT2) mRNA expression levels from the VTA of 15-week-old OR/OR (black bars) and OR/OP (light grey bars) females. Data are normalized to *Tbp* and calculated relative to the OR/OR group. **(B)** *Slc6a2* (the gene coding for DAT) was significantly greater in OP/OR females compared to OP/OP controls and *Th* also tended to be higher in these animals. *Slc18a2* (the gene coding for VMAT2) was significantly decreased in the OP/OR group compared to the OP/OP group. All genes were normalized to *Tbp*. n=4-5 animals per group. **(C)** Pitx3 and Nr4a2 mRNA expression in the VTA in 15-week-old OR/OR (black bars) and OR/OP (light grey bars) female offspring. **(D)** Expression of microRNAs associated with dopamine signaling and neurogenesis in the VTA of 15-week-old OR/OR (black bars) and OR/OP (light grey bars) female offspring. n=2-6 animals per group. Data are mean ± sem. #p<0.05 by one-way ANOVA.

We find that even without manipulation of the mother's diet the obesogenic perinatal environment has deleterious effects on both central dopamine mechanisms and the activity of the OR/OP offspring which results in excess body

weight gain during the prepubescent period. The decreased levels of activity that we detect in the OR/OP group are expected as we seen decreased striatal dopamine signal. We also found that the OR perinatal environment has beneficial effects on central dopamine exocytosis and food intake, while it does not change activity levels of the female offspring. These results indicate that mechanisms related to changes seen in the offspring are dependent on the type of exposure during the perinatal period. The differences in mRNA expression of the transcription factors support this idea that obesogenic and non-obesogenic perinatal environments have opposite effects on the same central signaling pathway through different mechanisms of transcriptional regulation.

Our results indicate that dopamine plays multiple roles in the development of obesity due to its involvement in both food intake and spontaneous activity. In the animals that had been exposed to the obesogenic environment, we detect differences in their activity level. If we were to provide these animals with a palatable, high-calorie diet during adolescence, we hypothesize that their food intake would increase and because of their already apparent lack of activity, they would gain weight more quickly than the controls. However, when only provided with laboratory chow, our animals seem to have compensatory mechanisms to protect them from altering the adult body weight compared to the control groups. Animals that have been exposed to an obesogenic environment early in life, but are weaned to chow have repeatedly shown this pattern (Gorski et al., 2006; Gorski et al., 2007; Tamashiro et al., 2009; Vucetic et al., 2010). In other studies

in OP rats, these animals have lower dopaminergic tone and are particularly susceptible to elevated food intake and excess weight gain when fed a high-fat diet (Geiger et al., 2008; Levin et al., 1997; Levin and Keesey, 1998). We have also shown that in outbred rats exposure to a highly palatable diet decreases overall dopaminergic tone and results in preferential dopamine response to the palatable diet compared to laboratory chow (Geiger et al., 2009). A recent study of the offspring of mothers fed a high-fat diet also showed deficits in the central dopamine system and a greater preference for both sucrose and fat compared to the control offspring (Vucetic et al., 2010). These results indicate that dopamine also codes for macronutrient preference and given the choice, the animals with low dopaminergic tone would prefer the high-energy, high-palatable food.

MicroRNAs are short non-coding RNAs that regulate expression of numerous genes. In this study, we show for the first time a link between altered microRNA expression in the central nervous system and perinatal exposure to an obesogenic environment. We have found a universal deficit in dopamine that is correlated to decreased expression of genes coding for TH and DAT in the VTA in OR/OP animals, similar to the deficits that we have previously shown in OP animals (Geiger et al., 2008). In a systematic approach looking at regulators of gene expression, we also found that mRNA expression of the transcription factor, *Pitx3*, was decreased and expression of the microRNA, miR-133b was increased in these animals. In support of these finding, a negative feedback loop has been previously described for *Pitx3* and miR-133b (Kim et al., 2007). In this study, we

also show increased expression of miR-124a. The cellular function of miR-124a in dopamine neurons is not clear. It is known that miR-124a is preferentially expressed in the brain and that adults with type-2 diabetes have increased serum levels of miR-124a (Kong et al., 2011; Lim et al., 2005). These results indicate that miR-124a may play a role in regulating insulin signaling in the central nervous system and is likely linked to the changes seen in insulin following the oral glucose challenge. In other studies using OP rats, perinatal levels of insulin and leptin, both of which influence neurogenesis, have been shown to be altered in the OP mothers indicating that these peripheral signals may have central effects on dopamine neurogenesis in our model (Gorski et al., 2006; Gorski et al., 2007).

Besides *Pitx3*, other transcriptional regulators may also provide control over the synaptic plasticity of dopamine neurons that has been associated with obesity. Myocyte enhancer factor-2 (MEF2) activity is altered in the nucleus accumbens following cocaine exposure leading to changes in both synaptic density and reward sensitivity (Pulipparacharuvil et al., 2008). Furthermore, MEF2 activity is inhibited by cyclin dependent kinase-5 (cdk-5), which has been shown to be upregulated following exposure to a high-fat diet (Teegarden et al., 2009). Links between Cdk-5 and dopamine neurotransmission, particularly with regard to dendritic spine density, have also been shown (Meyer et al., 2008; Smith et al., 2006). Cdk-5 activity has also been shown to be upregulated in adipose tissue after high-fat diet feeding and its regulation of PPARg activity is the target for the

anti-diabetic therapeutic, rosiglitazone (Choi et al., 2010). We hypothesize that this could be a universal mechanism of regulation of mRNA expression in multiple organ systems that are altered by obesity.

In conclusion, in this first of its kind study, we use a unique approach of transplanting embryos from an OP or OR female into a surrogate dame from the opposite strain. We show decreased dopamine signal in animals that are exposed to an OP perinatal environment. In contrast, the opposite effect on dopamine was shown in animals exposed to an OR perinatal environment. Interestingly, we also found that these alterations in dopamine signal correlate to changes in activity and food intake in animals that have been exposed to an OP or OR perinatal environment, respectively. Furthermore, in animals that had been exposed to an obesogenic perinatal environment, mRNA expression of key dopamine regulators is decreased through a mechanism involving microRNA regulation of transcription factors. This mechanism provides previously unidentified microRNA targets for the treatment of hyperphagia and inactivity associated with dietary obesity.

6 Elevation of central dopamine through levodopa treatment in young obesity-prone rats reduces food intake and body weight

Abstract

Childhood obesity has become a significant public health concern and with well-established links to maternal obesity. Central dopamine signaling, which codes for food and drug reward, is known to be low in both genetic and diet-induced models of obesity as early as postnatal day 1. Therefore, we hypothesized that maternal environmental influences can alter central dopamine release and elevation of dopamine through the dopamine replacement therapy, levodopa (L-dopa), can lead to decreased food intake and body weight gain. Using carbon fiber amperometry, we found that the adult female offspring of mothers that had been fed either a high-fat or high-sucrose diet during the perinatal period had approximately 50% less evoked dopamine release in the nucleus accumbens shell than control offspring. Female offspring of mothers that had been given L-dopa in their drinking water during gestation had elevated dopamine release as adults, but not immediately following weaning. However, chronic treatment of L-dopa in their drinking water during the linear growth phase immediately following weaning led to a 7% decrease in food intake and a 20g difference in body weight in obesity-prone females. These results indicate that maternal nutrition is critical for the development of central dopamine signaling and direct upregulation of this system may provide a treatment option, especially in children that are prone to developing obesity.

6.1 Introduction

Based on the previous studies, the central dopamine system may provide an ideal target for the treatment and prevention of diet-induced obesity. In Parkinson's disease treatment, the dopamine deficits are often successfully treated using dopamine replacement therapy, particularly the dopamine precursor, L-dopa. Interestingly, a number of studies have shown that plasma levels of L-dopa are inversely related to BMI in patients with Parkinson's disease and may contribute directly to weight loss in these patients (Bachmann et al., 2009; Lorefalt et al., 2009; Palhagen et al., 2005). In obesity, effective pharmacological treatments must also have very low risk associated with them. Most evidence suggests that the common side effects of L-dopa, specifically dyskinesias, do not manifest themselves in humans or animals that do not have significant damage to the nigrostriatal pathways, providing an ideal opportunity to test whether dopamine replacement may be effective as a chronic treatment for obesity or obesity prevention using a well-characterized therapeutic agent (Paille et al., 2004; Perry et al., 1984; Rajput et al., 1997).

In this series of experiments, we hypothesized that maternal high-fat or high-sucrose perinatal diets have detrimental effects on long-term central dopamine release kinetics in the offspring. Elevation of central dopamine in dopamine-deficient obesity-prone animals through increased L-dopa exposure prenatally and postnatally will cause anorexigenic effects resulting in decreased body weight gain.

6.2 Experimental Procedures

6.2.1 Animals

Sprague-Dawley females and males were used for the maternal diet study and obesity-prone females and males were used for the L-dopa treatment studies. All animals were purchased from Charles River Laboratories (Cambridge, MA, USA). The animals were kept on a 12-hour reverse light cycle with lights off at 7:00 am and lights on at 7:00 pm. Testing was completed during the middle of the dark cycle when the animals are normally alert and active. All experiments were conducted using the minimal number of animals necessary to limit animal use and suffering according to published guidelines of the U.S. National Institutes of Health (NIH) and the Institutional Animal Care and Use Committee (IACUC) of Tufts University and Tufts Medical Center.

6.2.2 Perinatal exposure to high-fat or high-sucrose maternal diets

Three-week-old Sprague-Dawley females were weight matched and divided into three groups, control, high-fat and high-sucrose. The control diet consisted of 3.6 kcal/g divided into 18.1% protein, 69.0% carbohydrates and 12.9% fat (Harlan-Teklad TD.08314, Madison, WI, USA). The high-fat diet contained 5.2 kcal/g divided into 18.1% protein, 22.0% carbohydrates and 59.8% fat (Harlan-Teklad TD.08316, Madison, WI, USA) The high-sucrose diet contained 3.8 kcal/g divided into 18.1% protein, 69.4% carbohydrates and 12.3% fat (Harlan-Teklad TD.08315, Madison, WI, USA). The primary difference between the control and

high sucrose diet was the source of the carbohydrates. The control diet contained 455.4 g/kg corn starch, 100 g/kg maltodextrin and 100 g/kg sucrose. The high-sucrose diet contained 570 g/kg sucrose and 75.4 g/kg corn starch. The animals were maintained on their diets until 8 weeks of age at which point they were mated with males that had been fed the control diet. The female offspring from each group were weaned at 3 weeks of age to laboratory chow. Weekly body weights were measured through 15 weeks of age.

6.2.3 Prenatal exposure to L-dopa

Eight- to ten-week-old obesity-prone females were mated with obesity-prone males. On embryonic day 1, the females were separated from the males and given either 2mg/mL L-dopa (Sigma; St. Louis, MO, USA) & 0.025% L-ascorbic acid (Sigma; St. Louis, MO, USA) in their drinking water or 0.025% L-ascorbic acid alone in their drinking water for the duration of the pregnancy. The drinking water for all animals was changed twice weekly. Weekly body weights of the offspring were measured along with 24-hour food intake and basal levels of locomotion at 13 weeks old.

6.2.4 Postnatal treatment with L-dopa

Three-week-old obesity-prone females were weight matched and separated into three groups. The control group was given 0.05% L-ascorbic acid in their drinking water. The L-dopa-treated group was given 5 mg/mL and 0.05% L-ascorbic acid in their drinking water. The L-dopa/carbidopa-treated group was

given 2mg/mL L-dopa, 0.5 mg/mL carbidopa and 0.05% L-ascorbic acid in their drinking water. Treatments continued for 10 weeks with twice weekly changes of the drinking water. Daily food intake and body weight data was collected. Locomotion was measured during the fifth week of treatment.

6.2.5 Locomotion testing

Twenty-four hour locomotion testing was done using Smart-Frame activity boxes (Hamilton-Kinder) and Motor Monitor software (Hamilton-Kinder). Animals were habituated to the activity boxes for 4 days prior to testing. On the testing day, body weight and food intake was measured as well as activity for 24 hours beginning and ending in the middle of the dark cycle.

6.2.6 Brain slice electrophysiology

Carbon fiber amperometry was performed on slices from the nucleus accumbens, dorsal striatum and prefrontal cortex as described in Chapter 2.

6.2.7 Statistical Analysis

Data are shown as mean ± sem and were analyzed using one-way ANOVA.

6.3 Results

6.3.1 Decreased dopamine release in offspring of dames fed a high-fat or high-sucrose perinatal diet.

In our first set of experiments, we studied the effects of a high-fat or high-sucrose maternal diet during the perinatal period on the development of the obesity phenotype and dopamine neurotransmission in the offspring. The female offspring of the high-fat fed mother weighed 70.7 ± 1.7 g (n=16) at weaning compared to 65.6 ± 1.7 g (n=15; $p<0.05$) for the female control offspring. Similarly to other maternal diet studies that wean the offspring onto chow, this difference in body weight disappeared shortly after weaning and no difference in body weight was detected between groups in the adult female offspring from either the high-sucrose or high-fat fed dames (Figure 6.1A&B) (Tamashiro et al., 2009; Vucetic et al., 2010).

We have previously shown that dopamine neurotransmission is decreased in rats that have been fed a cafeteria-style diet that is high in sucrose and fat (Geiger et al., 2009). Therefore, we investigated in this experiment whether a maternal diet that is high in either one of these macronutrients has an influence on dopamine neurotransmission in the offspring. To measure dopamine release in real-time, carbon fiber amperometry was used in acute coronal slices from the dorsal striatum, nucleus accumbens shell and prefrontal cortex in the young adult female offspring (Figure 6.1C&D; Table 6.1). In this experiment we found that similarly to the decreased dopamine release seen in obese models, the adult offspring of the mothers that had been fed a high-fat diet also had 40% to 60% lower dopamine release in all areas of the brain tested. In the offspring of the

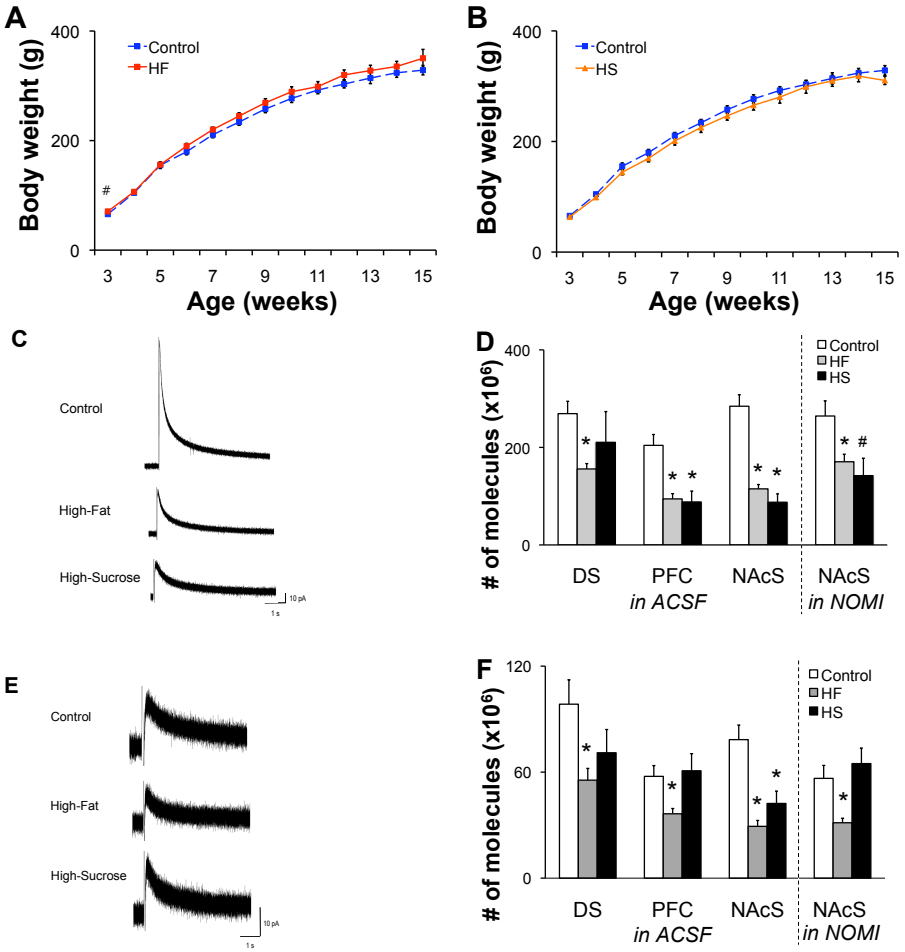

Figure 6.1: Female offspring of mothers fed a perinatal high-fat diet show elevated body weight at weaning and deficits in dopamine signaling that persist into young adulthood.

Body weight of the female offspring of high-fat diet (HF) fed mothers **(A)** but not high-sucrose diet (HS) fed mothers **(B)** was significantly greater at weaning than in the control offspring (n=7-9 offspring per group). **(C)** Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal nucleus accumbens shell slices in 15-week-old female offspring of HF, HS and control fed mothers. **(D)** Reduced dopamine exocytosis in the dorsal striatum (DS), prefrontal cortex (PFC), and nucleus accumbens shell (NAcS) before and after treatment with nomifensine (NOMI) in 15-week-old female offspring of HF diet fed mothers. (n ≥ 19 pulses from 4 slices from 4 animals) **(E)** Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal nucleus accumbens shell slices in 3 to 5-week-old female offspring of HF, HS and control fed mothers. **(F)** Dopamine deficits seen in adult offspring of HF-fed mothers were already present in 3 to 5-week-old female offspring. (n ≥ 22 pulses from 5 slices from 4 animals). Data are mean ± sem. * $p<0.01$; #$p<0.05$ by one-way ANOVA.

Table 6.1: Evoked dopamine release from offspring in maternal diet study

	Control	HF	HS	p- value (HF, HS)
15 week old females				
Dorsal Striatum (ACSF)				
Molecules (x10^6)	269.3 ± 25.4 *(55,11,7)*	155.8 ± 11.1 *(48,11,8)*	210.4 ± 63.2 *(19,4,4)*	<0.01, NS
Amplitude (pA)	117.4 ± 11.2	58.3 ± 4.8	79.3 ± 23.6	<0.01, NS
$t_{1/2}$ (sec)	0.11± 0.02	0.10 ± 0.02	0.17 ± 0.09	NS, NS
Prefrontal Cortex (ACSF)				
Molecules (x10^6)	204.2 ± 22.3 *(54,11,7)*	94.4 ± 10.8 *(50,10,7)*	88.1 ± 22.4 *(29,6,4)*	<0.01, <0.01
Amplitude (pA)	89.5 ± 10.0	30.6 ± 2.9	41.3 ± 9.9	<0.01, <0.01
$t_{1/2}$ (sec)	0.10 ± 0.01	0.10 ± 0.02	0.02 ± 0.01	NS, <0.01
Nucleus Accumbens Shell (ACSF)				
Molecules (x10^6)	284.4 ± 23.6 *(54,11,7)*	115.0 ± 8.9 *(45,9,7)*	87.6 ± 17.2 *(19,4,4)*	<0.01, <0.01
Amplitude (pA)	112.3 ± 10.4	45.0 ± 4.1	44.2 ± 11.6	<0.01, <0.01
$t_{1/2}$ (sec)	0.21 ± 0.09	0.16 ± 0.05	0.07 ± 0.04	NS, NS
Nucleus Accumbens Shell (NOMI)				
Molecules (x10^6)	264.4 ± 31.0 *(35,7,7)*	170.6 ± 15.5 *(35,7,7)*	142.1 ± 35.7 *(19,4,4)*	<0.01, <0.05
Amplitude (pA)	109.7 ± 19.3	61.4 ± 6.1	58.4 ± 15.3	<0.05, NS
$t_{1/2}$ (sec)	0.15 ± 0.02	0.14 ± 0.02	0.08 ± 0.03	NS, NS
3 to 5 week old females				
Dorsal Striatum (ACSF)				
Molecules (x10^6)	98.5 ± 13.8 *(40, 8,6)*	55.5 ± 6.7 *(40,8,5)*	71.0 ± 13.2 *(34,7,4)*	<0.01, NS
Amplitude (pA)	30.2 ± 4.3	22.1 ± 2.1	21.3 ± 3.1	NS, NS
$t_{1/2}$ (sec)	0.12 ± 0.05	0.08 ± 0.02	0.13 ± 0.04	NS, NS
Prefrontal Cortex (ACSF)				
Molecules (x10^6)	57.6 ± 6.1 *(42,9,6)*	36.4 ± 3.0 *(54,11,6)*	60.8 ± 9.7 *(44,9,5)*	<0.01, NS
Amplitude (pA)	15.5 ± 1.6	23.4 ± 3.2	28.2 ± 4.8	<0.01, <0.05
$t_{1/2}$ (sec)	0.04 ± 0.01	0.03 ± 0.01	0.08 ± 0.05	NS, NS
Nucleus Accumbens Shell (ACSF)				
Molecules (x10^6)	78.4 ± 8.3 *(40,8,6)*	29.3 ± 3.3 *(40,9,5)*	42.3 ± 7.0 *(39,8,5)*	<0.01, <0.01
Amplitude (pA)	22.9 ± 2.7	10.7 ± 0.9	19.4 ± 2.8	<0.01, NS
$t_{1/2}$ (sec)	0.02 ± 0.01	0.01 ± 0.003	0.02 ± 0.01	NS, NS
Nucleus Accumbens Shell (NOMI)				
Molecules (x10^6)	56.5 ± 7.3 *(33,7,6)*	31.4 ± 2.5 *(22,5,4)*	64.8 ± 8.8 *(25,5,5)*	<0.01, NS
Amplitude (pA)	19.3 ± 2.6	11.1 ± 0.7	48.1 ± 11.0	<0.05, <0.01
$t_{1/2}$ (sec)	0.05 ± 0.01	0.01 ± 0.001	0.05 ± 0.01	<0.05, NS

Data = mean ± sem, HF=high-fat fed mother, HS=high-sucrose fed mother, n=(# of events, # of slices, # of animals), NS=not significant by one-way ANOVA compared to controls

high-sucrose fed dames, the dopamine release was decreased approximately 70% and 55% in the nucleus accumbens and prefrontal cortex, respectively.

To determine whether differences in the activity of the dopamine reuptake transporter (DAT) are linked to the deficits seen in dopamine signaling in the nucleus accumbens, we blocked reuptake using 3 mM nomifensine and measured dopamine release from the same site in the nucleus accumbens. In this case we found that the differences in dopamine signaling remained in the offspring of both the high-fat and high-sucrose fed mothers. However, the deficit was less than it was prior to treatment with nomifensine in these animals, indicating small differences in transporter activity may be present (Figure 6.1D, Table 6.1).

The timing of the development of the dopamine deficits also differs between the high-sucrose and high-fat offspring. Immediately following weaning, the dopamine deficits were already present in the high-fat female offspring. However, dopamine signaling was only decreased in the nucleus accumbens shell in the female offspring of the high-sucrose fed dames during the same time period (Figure 6.1E&F; Table 6.1). Furthermore, this effect did not remain following treatment with nomifensine.

6.3.2 Treatment of obesity-prone mothers during gestation with L-dopa results in increased evoked dopamine in the adult female offspring

Because we had detected depletions in dopamine signaling following perinatal exposure to diets known to directly cause dopamine depletion, we wanted to examine whether we could get the opposite result if we expose offspring to dopamine enhancers during the perinatal period. To test this, we treated obesity-prone females with L-dopa during gestation and measured evoked dopamine release in adult females using carbon fiber amperometry. Representative traces of the electrical stimulation from the nucleus accumbens shell both before and after treatment with 3 μM nomifensine, a dopamine reuptake blocker, are shown in Figure 6.2A. In the nucleus accumbens shell, the evoked dopamine release was enhanced in the L-dopa treated offspring both before and after treatment with 3 μM nomifensine. The enhanced evoked dopamine release in the nucleus accumbens shell of the L-dopa treated female offspring was related to increased amplitude and width at half-height ($t_{1/2}$) of the evoked dopamine peak both before and after treatment with nomifensine (Figure 6.2B,C&D, Table 6.2).

Similar results were seen in the dorsal striatum and prefrontal cortex of the L-dopa-treated adult female offspring. In the dorsal striatum, we found increased levels of evoked dopamine release in the adult female offspring of the L-dopa treated dames compared to the control offspring due to a significantly higher amplitude and $t_{1/2}$ of the dopamine release peak (Figure 6.2B,C&D, Table 6.2). Enhanced dopamine release was also present in the prefrontal cortex of the L-dopa treated offspring. In this case, this elevation in evoked dopamine release was primarily related to increased $t_{1/2}$ of the evoked dopamine peak in the L-dopa

treated female offspring. The spike amplitude in the prefrontal cortex was slightly, but not significantly, greater in the L-dopa treated female offspring (Figure 6.2B,C&D, Table 6.2)

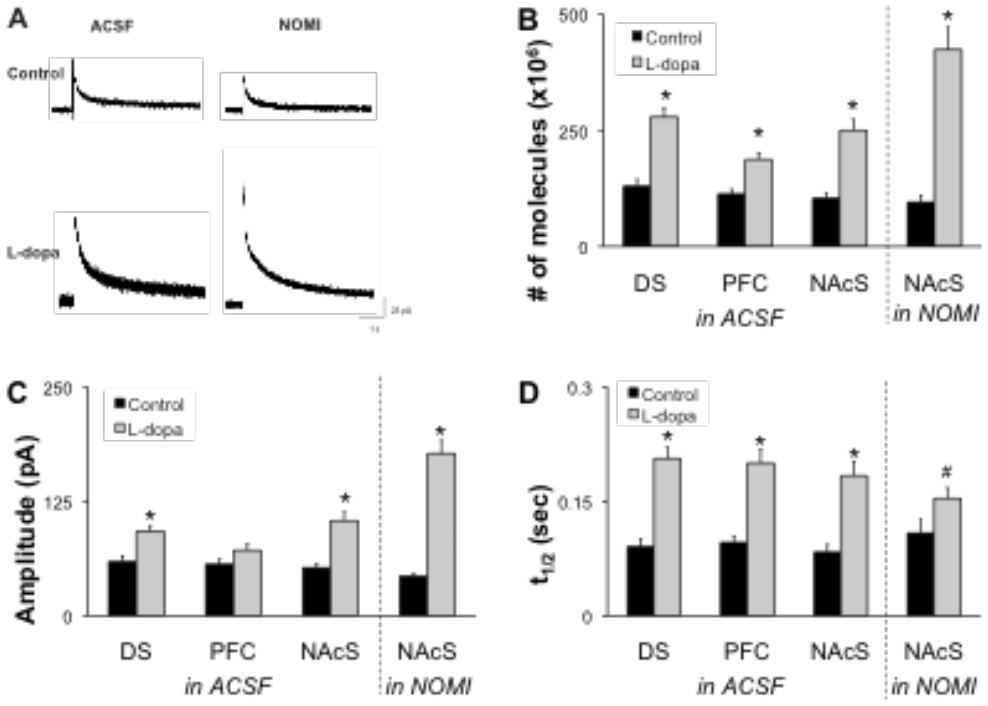

Figure 6.2: L-dopa treatment of the mother (2 mg/mL L-dopa with 0.025% ascorbic acid in their drinking water during gestation) enhanced evoked dopamine release in the midbrain dopamine projections of the 15-week-old female offspring.
Control mothers were given 0.025% ascorbic acid in their drinking water alone. Litters were standardized at 8 pups on P0 if the litter contained more than 8 pups. **(A)** Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal nucleus accumbens shell slices before and after treatment with 3 mM NOMI in offspring of 15-week-old female offspring of L-dopa treated and control treated mothers. The mean evoked dopamine molecules signal **(B)**, spike amplitude **(C)**, and width at half-height ($t_{1/2}$) **(D)** was elevated in the DS, PFC, and NAcS before and after treatment with 3 mM NOMI for 30 minutes in 15-week-old female offspring of the L-dopa treated mothers. n ≥ 33 pulses from 7 slices from 7 animals. Data are mean ± sem. * $p<0.01$; #$p<0.05$ by one-way ANOVA.

Table 6.2: Evoked dopamine release from female offspring of L-dopa treated dames during gestation.

	15 week old females		3 to 5 week old females		p- value (Ad, Juv)
	Control	**L-dopa**	**Control**	**L-dopa**	
Dorsal Striatum (ACSF)					
Molecules (x10^6)	129.4 ± 15.0 *(63,13,7)*	278.8 ± 19.5 *(75, 15, 8)*	159.9 ± 17.4 *(42,9,6)*	162.2 ± 16.8 *(39,8,5)*	<0.01, NS
Amplitude (pA)	59.1 ± 6.3	92.4 ± 6.3	99.1 ± 11.7	74.7 ± 9.9	<0.01, NS
$t_{1/2}$ (sec)	0.09 ± 0.01	0.21 ± 0.02	0.09 ± 0.01	0.16 ± 0.02	<0.01,<0.01
Prefrontal Cortex (ACSF)					
Molecules (x10^6)	129.4 ± 15.0 *(63,13,7)*	186.7 ± 14.9 *(70,14,7)*	180.4 ± 32.5 *(42,9,6)*	105.2 ± 13.2 *(25,5,3)*	<0.01, NS
Amplitude (pA)	56.2 ± 5.8	71.5 ± 7.0	75.1 ± 10.9	62.4 ± 8.3	NS, NS
$t_{1/2}$ (sec)	0.10 ± 0.01	0.20 ± 0.02	0.13 ± 0.02	0.09 ± 0.01	<0.01, NS
Nucleus Accumbens Shell (ACSF)					
Molecules (x10^6)	103.8 ± 10.5 *(60,12,7)*	250.0 ± 23.7 *(75,15,8)*	264.9 ± 25.4 *(54,11,6)*	187.6 ± 31.1 *(39,8,5)*	<0.01, NS
Amplitude (pA)	52.6 ± 4.4	103.8 ± 9.5	131.9 ± 13.4	77.6 ± 10.8	<0.01,<0.05
$t_{1/2}$ (sec)	0.08 ± 0.01	0.18 ± 0.02	0.11 ± 0.01	0.15 ± 0.02	<0.01,<0.05
Nucleus Accumbens Shell (NOMI)					
Molecules (x10^6)	95.4 ± 14.4 *(33,7,7)*	423.6 ± 48.6 *(40,8,7)*	195.1 ± 30.9 *(25,5,5)*	276.3 ± 24.6 *(20,4,4)*	<0.01,<0.05
Amplitude (pA)	43.2 ± 3.5	177.2 ± 14.5	98.9 ± 16.0	111.8 ± 17.9	<0.01, NS
$t_{1/2}$ (sec)	0.11 ± 0.02	0.15 ± 0.01	0.13 ± 0.02	0.18 ± 0.03	<0.05, NS

Data = mean ± sem, n=(# of events, # of slices, # of animals), NS=not significant by one-way ANOVA compared to controls

6.3.3 Enhanced dopamine release in the adult female offspring is associated with small differences in food intake, body weight and locomotor activity

Our original hypothesis was that with increased dopamine, we would see a corresponding decrease in body weight and food intake in these animals. While the body weight of the female offspring of the L-dopa treated mothers was slightly lower than the body weight of the control offspring, this difference was not significantly different between groups (n=11 L-dopa and n=12 control pups, NS; Figure 6.3A) In addition, the female offspring of the L-dopa treated mothers tended to eat less per day than the female offspring of the control treated

mothers (20.4 ± 0.8 g/day vs. 22.9 ± 1.8 g/day; Figure 6.3B). Finally, when we measured activity levels in these animals, we found no significant differences over the course of the day, even though the female offspring of the L-dopa treated mothers tended to move significantly more for a brief period during the middle of the dark cycle (Figure 6.3C&D). These results indicate that while L-dopa treatment could have long-term beneficial effects on offspring of a treated mother, these effects may not be in place early in life when the animals are growing more rapidly.

In fact, when we measured dopamine release in 3- to 5-week-old female offspring, we found that areas that had elevated dopamine release in the adult offspring had either decreased dopamine release or no difference in evoked dopamine signal in the juvenile offspring. The evoked dopamine release from the nucleus accumbens shell of the female offspring of the L-dopa treated dames tended to be lower than the evoked dopamine release from the offspring of the control dames. This is related to a decrease in amplitude in these animals. The peak amplitude in the nucleus accumbens shell was significantly lower from the L-dopa treated female offspring. In addition, there were no significant differences in the mean evoked dopamine signal from the dorsal striatum or prefrontal cortex in L-dopa treated offspring compared to control offspring (Figure 6.3E&F, Table 6.2).

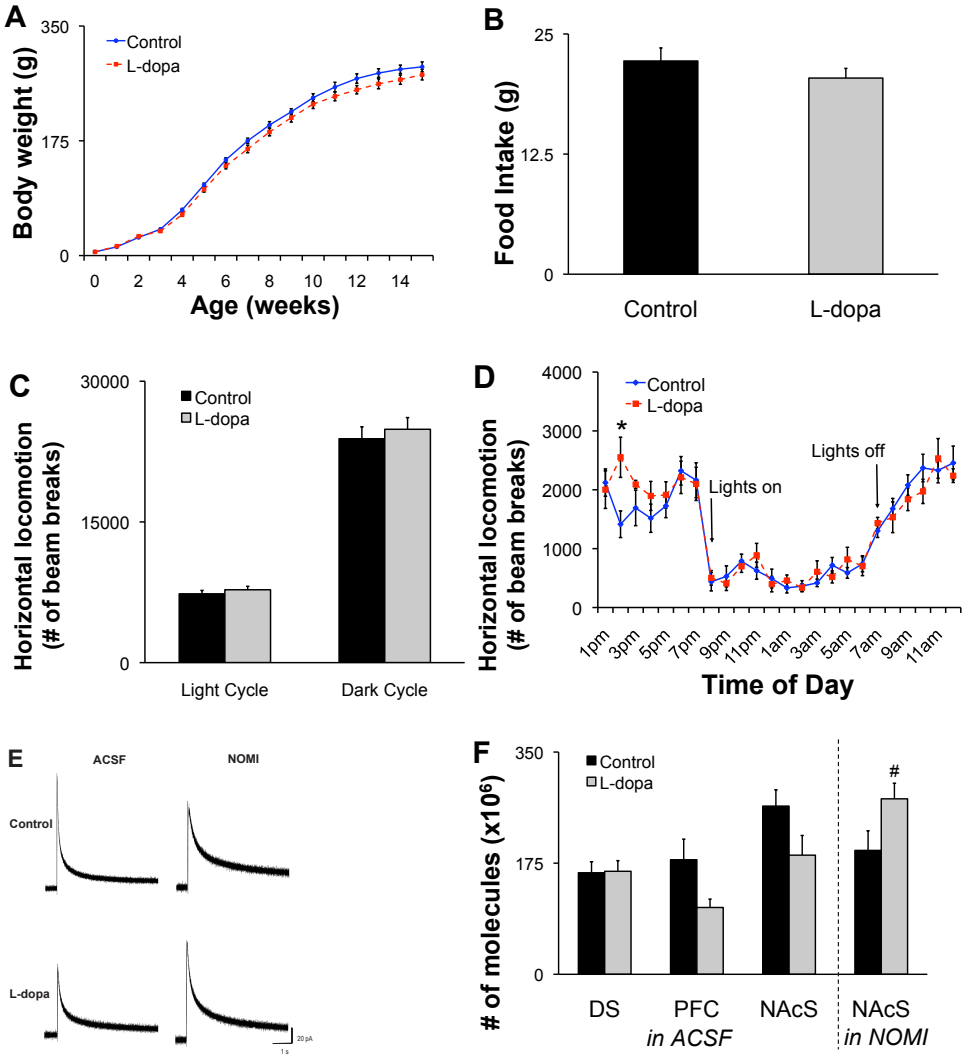

Figure 6.3: The obesity phenotype of female offspring of L-dopa treated mothers during gestation is linked to dopamine exocytosis during the rapid growth period immediately following weaning.
Weekly body weights (n=11 L-dopa and n=12 control pups) **(A)** and 24 hour food intake at 14-weeks-old (n=10 L-dopa and n=11 control pups) **(B)** of the female offspring of the L-dopa treated and control treated mothers were similar. Overall basal activity levels **(C)** in 14-weeks-old female offspring of the L-dopa treated and control mothers were not significantly different, however the L-dopa treated offspring did move more during a brief period **(D)** in the middle of the dark cycle (n=10 L-dopa and n=11 control pups). **(E)** Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal nucleus accumbens shell before and after treatment with 3 μM NOMI from 3 to 5 week old offspring of L-dopa treated and control treated mothers. **(F)** The mean evoked dopamine molecules signal was significantly increased in the NAcS only following a 30-minute NOMI wash of the 3 to 5-week old female offspring of the L-dopa treated mothers. n ≥ 20 pulses from 4 slices from 3 animals. Data are mean ± sem. *p<0.01, #p<0.05 by one-way ANOVA.

However, treatment with nomifensine indicates that differences in the kinetics of dopamine release are present in these animals. Specifically, in the nucleus accumbens, following treatment with nomifensine, the number of evoked dopamine molecules in L-dopa treated female offspring was significantly greater than in the control offspring. In addition, $t_{1/2}$, which is an index of how rapidly dopamine is cleared from the synapse following the stimulus pulse, was higher in both the dorsal striatum and nucleus accumbens of the L-dopa treated offspring (Figure 6.3F, Table 6.2). These results indicate that DAT activity is altered early in life. This change in DAT activity may have the long-term consequence of upregulating dopamine neurotransmission in the adult offspring, but does not lead to the beneficial effects of elevated dopamine during the juvenile period on food intake and body weight.

6.3.4 L-dopa and L-dopa/carbidopa treatment result in decreased body weight and food intake and increased dopamine release in obesity-prone females

Because it appears that prenatal exposure to L-dopa doesn't alter central dopamine signaling dramatically during the rapid growth phase immediately following weaning, we hypothesized that by using L-dopa or a combination of L-dopa and carbidopa, a decarboxylase inhibitor, to increase dopamine during this period, we could alter the obesity phenotype in obesity-prone animals. To determine the role of decreased dopamine on body weight and food intake in these animals, we gave them drinking water treated with either 5 mg/mL L-dopa

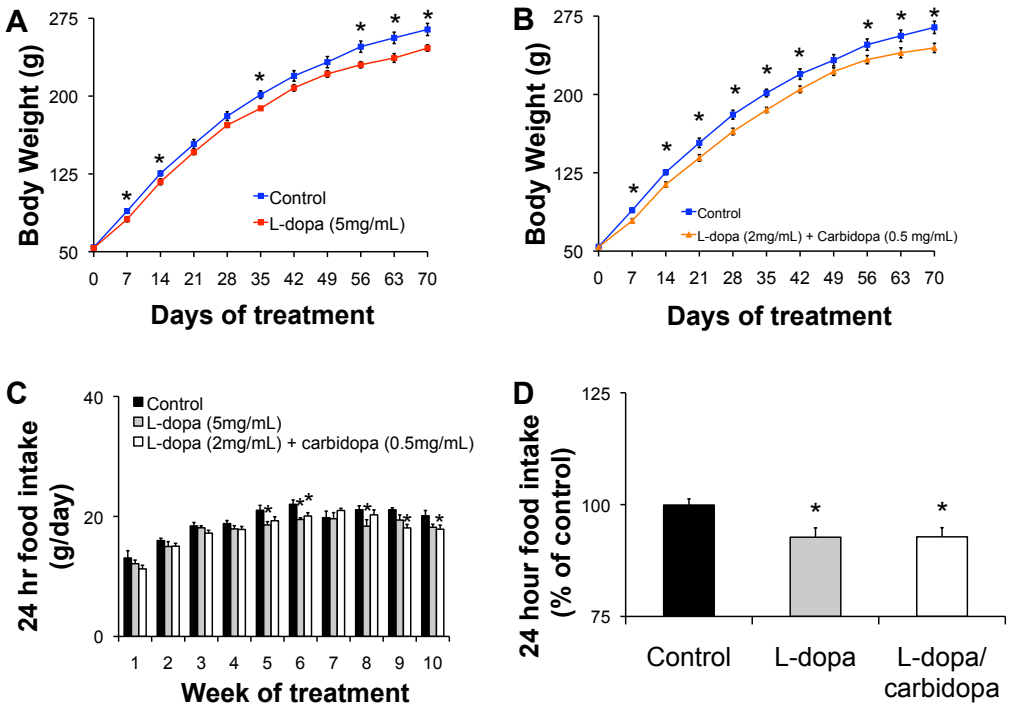

Figure 6.4: Body weight and food intake is decreased in obesity-prone females given 5 mg/mL L-dopa or 2 mg/mL L-dopa/0.5 mg/mL carbidopa with 0.05% ascorbic acid in their drinking water for 10 weeks beginning at 3 weeks old were altered compared to controls.
Control females were given 0.05% ascorbic acid in their drinking water alone. **(A)** Body weight of L-dopa treated (5mg/mL) obesity-prone females was significantly lower following one week of treatment. This effect persisted through the tenth week of treatment. **(B)** Body weight of obesity-prone females treated with a combination of L-dopa (2mg/mL) and carbidopa (0.5 mg/mL) was significantly lower following one week of treatment. This effect persisted through the tenth week of treatment. **(C)** Average daily intake tended to be lower in the treated groups compared to the control groups throughout the study, with differences intake beginning in week 5 reaching significance. **(D)** Overall food intake over the course of the study was significantly lower in the L-dopa and L-dopa/carbidopa treated obesity-prone females compared to controls. n = 6 animals per group. Data are mean ± sem. * $p<0.05$ by one-way ANOVA.

or a combination of 2 mg/mL L-dopa and 0.5 mg/mL carbidopa for 10 weeks beginning at 3 weeks of age. Differences in body weight occurred as early as the first week following treatment with the final body weight of the treated groups being 246.3 ± 3.1 g (n=6) for the L-dopa treated and 244.5 ± 4.5 (n=6) for the L-dopa/carbidopa treated animals compared to 264.2 ± 5.9 g for the controls (Figure 6.4A&B). These differences in body weight were associated with 7% lower level of food intake throughout the duration of the study (Figure 6.4C&D).

Measurement of dopamine release using carbon fiber amperometry in these animals indicated that by the end of the treatment period, they had increase in evoked dopamine release. Representative traces of the evoked dopamine spikes from the nucleus accumbens shell are shown in Figure 6.5A. We found that both treatments significantly increased evoked dopamine release in all areas tested (Figure 6.5B, Table 6.3). The evoked dopamine release from the nucleus accumbens shell was approximately 275% greater for the L-dopa treated females and 150% greater for the L-dopa/carbidopa treated group compared to the control group. These differences remained in the presence nomifensine where evoked dopamine release from the nucleus accumbens of the L-dopa and L-dopa/carbidopa treated females was approximately 275% and 250% greater than the evoked dopamine release from the control females, respectively. These increased levels of dopamine release were correlated with changes in both the amplitude and width at half-height ($t_{1/2}$) of the evoked dopamine spike (Figure 6.5C&D, Table 6.3).

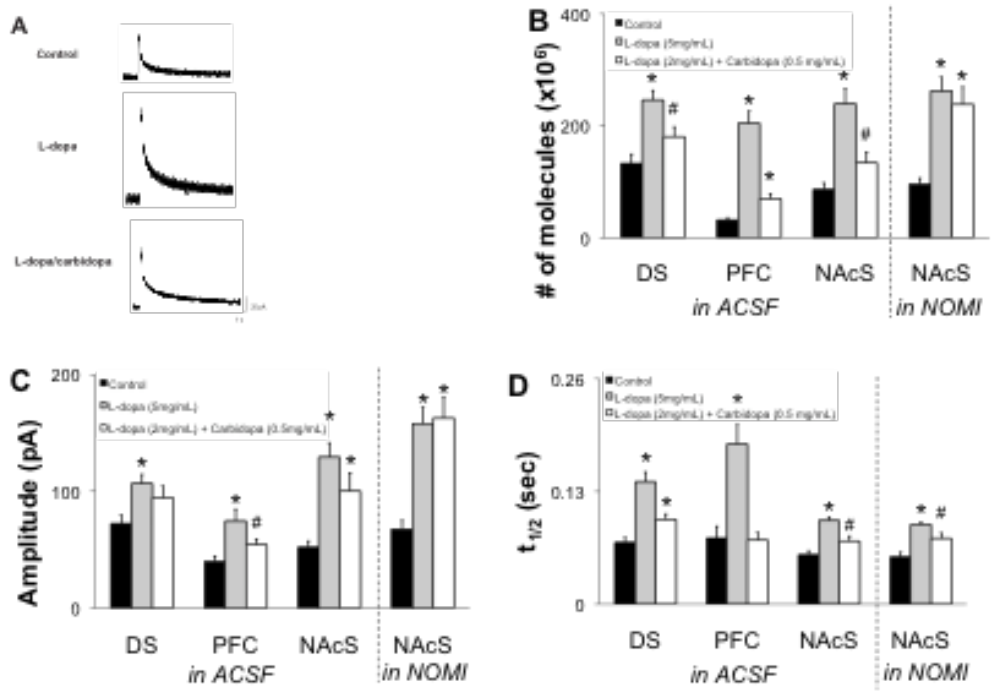

Figure 6.5: L-dopa treatment in obesity-prone females during adolescence results in increased dopamine release kinetics after 10 weeks of treatment. **(A)** Representative amperometric traces of electrical stimulation-evoked dopamine release in acute coronal nucleus accumbens shell. The mean evoked dopamine release **(B)**, spike amplitude **(C)**, and $t_{1/2}$ **(D)** was significantly increased in the DS, PFC and the NAcS before and after 30 minutes exposure to 3mM NOMI in the L-dopa and L-dopa/carbidopa treated females. (n ≥ 25 pulses from 5 slices from 5 animals. Data are mean ± sem. * p<0.01; #p<0.05 by one-way ANOVA.

Like in the nucleus accumbens shell, the evoked dopamine release from the dorsal striatum and prefrontal cortex was greater in the treated animals than the control groups. The evoked dopamine release from the dorsal striatum of the L-dopa and L-dopa/carbidopa treated groups was 185% and 135% greater than in the control group, respectively. (Figure 6.5B,C,&D, Table 6.3). Differences in both peak amplitude and $t_{1/2}$ contributed to the differences seen in the overall dopamine release in the dorsal striatum. The evoked dopamine release from the prefrontal cortex of the L-dopa and L-dopa/carbidopa was also significantly greater compared to the controls. Again, both amplitude and $t_{1/2}$ contributed to the differences seen in overall dopamine release in the L-dopa treated animals.

In the animals given the combination treatment, differences in peak amplitude had the greatest effect on the overall number of molecules released (Figure 6.5B,C,&D, Table 6.3). These results indicate that elevation of central dopamine signaling throughout the rapid growth phase of obesity-prone animals can lead to decreased food intake and body weight.

Table 6.3: Evoked dopamine release from L-dopa and L-dopa/carbidopa-treated females

	Control	L-dopa	Combo	p- value (L-dopa, Combo)
Dorsal Striatum (ACSF)				
Molecules (x10^6)	132.8 ± 16.7 *(54,11,6)*	246.1 ± 17.2 *(50,10,5)*	180.4 ± 17.7 *(54,11,6)*	<0.01, <0.05
Amplitude (pA)	72.4 ± 7.2	106.7 ± 7.8	94.5 ± 10.0	<0.01, NS
$t_{1/2}$ (sec)	0.07 ± 0.01	0.14 ± 0.01	0.10 ± 0.01	<0.01, <0.01
Prefrontal Cortex (ACSF)				
Molecules (x10^6)	32.0 ± 4.0 *(57,12,6)*	204.7 ± 21.5 *(49,10,5)*	70.0 ± 8.7 *(59,12,6)*	<0.01, <0.01
Amplitude (pA)	39.8 ± 4.9	74.7 ± 10.1	54.4 ± 4.6	<0.01, <0.05
$t_{1/2}$ (sec)	0.08 ± 0.01	0.19 ± 0.02	0.07 ± 0.01	<0.01, NS
Nucleus Accumbens Shell (ACSF)				
Molecules (x10^6)	86.4 ± 11.8 *(55,11,6)*	239.5 ± 26.9 *(45,9,5)*	134.1 ± 19.0 *(55,11,6)*	<0.01, <0.05
Amplitude (pA)	52.1 ± 5.5	129.4 ± 12.1	100.3 ± 15.5	<0.01, <0.01
$t_{1/2}$ (sec)	0.06 ± 0.01	0.10 ± 0.01	0.07 ± 0.01	<0.01, <0.05
Nucleus Accumbens Shell (NOMI)				
Molecules (x10^6)	95.6 ± 11.0 *(30,6,6)*	262.4 ± 24.7 *(25,5,5)*	238.5 ± 30.8 *(27,6,6)*	<0.01, <0.01
Amplitude (pA)	67.7 ± 7.4	157.9 ± 14.0	162.7 ± 17.8	<0.01, <0.01
$t_{1/2}$ (sec)	0.05 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	<0.01, <0.05

Data = mean ± sem, n=(# of events, # of slices, # of animals), NS=not significant by one-way ANOVA compared to controls

6.4 Discussion

In this study, we show that the maternal diet profoundly influences central dopamine signaling throughout the lifespan of the offspring. Furthermore, by giving obesity-prone animals, that are known to have low central dopamine signal as early as postnatal day 1, L-dopa during gestation, we found that we could increase central dopamine signaling as these animals reach adulthood. This improvement in central dopamine signaling in adulthood was still developing during the rapid growth phase immediately following weaning and directly increasing dopamine signaling during this time period specifically resulted in decreased food intake and body weight in obesity-prone animals. These results provide us with a potential treatment time period during which elevation of central dopamine signal may be beneficial in individuals prone to obesity. A similar time window for treating obesity proneness has also been suggested in other studies using the obesity-prone rat model (Gorski et al., 2007; Patterson and Levin, 2008).

In the first part of this study, we investigated how specific differences in diet composition during the perinatal period affects the dopaminergic neuronal development. Here we show that elevations in dietary fat have more significant effects on dopamine neurotransmission, particularly immediately following weaning than does exposure to high levels of sucrose during the perinatal period. These results are similar to those shown previously where offspring of mothers fed a high-fat diet during late stage pregnancy and lactation exhibit reduced

sensitivity to an amphetamine injection and reduced levels of dopamine in the nucleus accumbens (Naef et al., 2011; Naef et al., 2008). Elevated levels of sucrose also alter the development of dopamine signaling in these animals by the time they reach adulthood in a pattern that seems specific to the mesocorticolimbic pathway. Previous studies looking at differences between high-sucrose and high-fat diets have also reported behavioral and mechanistic differences in the effects of these diets. A rodent model of sucrose-bingeing shows characteristics similar to those seen with opioid dependence. However, if animals are bingeing on a high-fat diet the behavioral phenotype is markedly different than the animals that have been given sucrose (Avena et al., 2008a; Avena et al., 2008b, 2009; Berner et al., 2008). Together, these studies suggest that high-fat and high-sucrose diets both affect the development of central dopamine signaling pathways, however, through separate mechanisms.

The “reward deficiency hypothesis” was first posited by Blum et al in the mid 1990s (Blum et al., 1995b). This hypothesis has been applied to both drug and food addiction as an explanation for why people with low dopamine signal may overuse these substances in a compensatory effort. Therefore, elevation of central dopamine may allow for the successful treatment of hyperphagia (and the resulting obesity) in children. Evidence of the anorexigenic actions of elevated dopamine dates back to the early 20th century when amphetamine was marketed as Benzedrine for weight loss (Harris et al., 1947). In this study, we investigated the use of L-dopa, which elevates central dopamine through increasing levels of

dopamine precursors in the dopamine synthesis pathway, as a preventative treatment for hyperphagia and excessive weight gain in animals that are prone to developing obesity. We found that timing of the treatment is critical in these animals. Its use in mothers during the perinatal period alters development of central dopamine signaling in such a way that it is elevated by adulthood with little effect on the obesity phenotype of the offspring. However, if L-dopa is used in the rapid growth phase of these animals between weaning and adulthood, we see decreases in both food intake and body weight gain, providing evidence that this treatment strategy may be useful in children that are prone to develop obesity.

As with any therapeutic, side effects of an obesity prevention drug are a significant consideration. In this study, we used the most common treatment for Parkinson's disease, L-dopa, in part because of the large body of literature indicating its safety and efficacy at increasing dopaminergic tone in Parkinson's patients. The primary side effect of L-dopa treatment for Parkinson's is the development of dyskinesias over time, an effect that has been studied in a number of models using healthy cohorts. In most of these studies, no dyskinesias were found following treatment with L-dopa in a variety of species and at several different doses (Paille et al., 2004; Perry et al., 1984; Rajput et al., 1997). One study in macaques did show the development of dyskinesias at very high doses of L-dopa at the period when the peak plasma concentration would have been present (Pearce et al., 2001). In our study, we designed our dosing

strategy to avoid the normal pharmacokinetic curve seen with once daily dosing to avoid both adverse side effects to the drug at high plasma concentrations of L-dopa and compensatory eating when L-dopa plasma levels drop. By giving the L-dopa in the rats' drinking water so that they ingested low levels of the drug throughout the day, maintaining a more steady state level of the drug.

In conclusion, we show in this study that treatment to elevate central dopamine signaling during adolescence can be an effective way to decrease food intake and body weight gain in animals that are prone to obesity. While this study provides strong evidence that targeting dopamine in children may be an effective way to prevent obesity, we test only dopamine replacement. In addition to simply upregulating the dopamine synthesis pathway, we could also target other areas of dopamine neurotransmission, like transporters in the synaptic terminals or pre- and post-synaptic receptors. Finding a potential target for obesity-proneness is critical because we also show in this study that a maternal diet that is high in fat can be enough to predispose the animals to deficiencies in dopamine signaling, which has been linked to hyperphagia and obesity in numerous studies.

7 Perinatal influences on central dopamine signaling and the development of obesity in male offspring

Abstract

While this dissertation has focused its analysis on the effects of perinatal environments on female offspring, obesity affects males as well as females. For this reason, we investigate the role of the perinatal environment on the development of obesity and central dopamine signaling in male offspring. We use a variety of paradigms to compare effects of obesogenic environments due to diet or genetic predisposition as well as treatments targeted at prevention of the development of obesity including exposure to an obesity-resistant environment and increased levels of dopamine precursors during gestation. We find that maternal high-fat diet has the greatest influence on body weight on the male offspring and exposure to this environment during the perinatal period leads to selective deficits in dopamine signaling in adulthood. Prenatal exposure to the dopamine precursor, L-dopa, doesn't alter body weight but does lead to elevated dopamine release and decreased food intake in adult male offspring. Finally, chronic exercise results in elevated dopamine release. Early exposure to exercise has been shown to inhibit the obese phenotype in obesity-prone males. Therefore, elevation of central dopamine signaling during the critical time period of growth immediately following weaning through exercise may prevent the development of obesity in male offspring with a predisposition to obesity.

7.1 Introduction

While obesity affects a large percentage of both men and women, differences in how and when obesity-related disorders develop are present between genders (Carr, 2003; Nedungadi and Clegg, 2009; Shi and Clegg, 2009). In particular, fat pad distribution, leptin signaling and insulin sensitivity all seem to be regulated differently based on the availability of testosterone or estrogen (Clegg et al., 2006; Cowell et al., 1997; Hamdy et al., 2006; Palmert et al., 1998). In this dissertation so far, we have focused our analysis on the role of the intrauterine environment in the female offspring. This chapter describes the effects on male offspring of the same perinatal modifications reported in Chapters 5 and 6. We found that high-fat diet has a profound effect on body weight of male offspring and selectively decreases dopamine release in the nucleus accumbens and prefrontal cortex of these animals. Furthermore, this change in body weight is specific to the maternal diet as obesity-resistant animals that are placed in an obesogenic environment do not gain excess weight and the adult males have similar dopamine signaling as the control obesity-resistant males. Protection against dopamine deficits by either exposure to an obesity-resistant perinatal environment or through upregulation of dopamine precursors during gestation leads to long-term enhancement of central dopamine release with little effect on the obesity phenotype. Early exercise has been shown to be one of the most effective ways improve the obesity phenotype of the obesity-prone males (Patterson et al., 2008; Patterson and Levin, 2008). Here we show that 3-weeks of exercise can dramatically improve dopamine signaling providing a mechanism

by which long term changes in body weight are maintained after exercise is stopped.

7.2 Experimental Procedures

7.2.1 Animals

Sprague-Dawley females and males were used for the maternal diet study and obesity-prone females and males were used for the oviduct transplant surgeries and L-dopa treatment studies. Additionally, vasectomized male Sprague-Dawley rats were used in the oviduct transplant studies. The exercise study used male Wistar rats. All animals were purchased from Charles River Laboratories (Cambridge, MA, USA). All animals were kept in a reverse light-dark cycle facility, lights off at 7:00 am and lights on at 7:00 pm and used in the middle of the dark cycle when the are normally active. All experiments were conducted according to procedures approved by the Tufts University Institutional Animal Care and Use Committee in order to use the minimal number of animals necessary to limit animal use and suffering.

7.2.2 Perinatal exposure to high-fat or high-sucrose maternal diets

Three-week-old Sprague-Dawley females were weight matched and divided into three groups, control, high-fat and high-sucrose. The control diet consisted of 3.6 kcal/g divided into 18.1% protein, 69.0% carbohydrates and 12.9% fat (Harlan-Teklad TD.08314, Madison, WI, USA). The high-fat diet contained 5.2 kcal/g divided into 18.1% protein, 22.0% carbohydrates and 59.8% fat (Harlan-Teklad TD.08316, Madison, WI, USA) The high-sucrose diet contained 3.8 kcal/g

divided into 18.1% protein, 69.4% carbohydrates and 12.3% fat (Harlan-Teklad TD.08315, Madison, WI, USA). The primary difference between the control and high sucrose diet was the source of the carbohydrates. The control diet contained 455.4 g/kg corn starch, 100 g/kg maltodextrin and 100 g/kg sucrose. The high-sucrose diet contained 570 g/kg sucrose and 75.4 g/kg corn starch. The animals were maintained on their diets until 8 weeks of age at which point they were mated with males that had been fed the control diet. The male offspring from each group were weaned at 3 weeks of age to laboratory chow. Weekly body weights were measured through 15 weeks of age.

7.2.3 Oviduct transplant surgery

The estrous cycle was synchronized in donor and foster females using [des-Gly^{10}, D-Ala^{6}]-LH-RH ethylamide acetate (L4513, Sigma, St. Louis, MO, USA). Females were mated with either males of the same strain (donors) or vasectomized males (fosters). Within 24 hours of the appearance of plugs in the cages, donor females were anesthetized. The oviducts were harvested and the embryos were flushed using media containing EmbryoMax FHM HEPES Buffered medium (Millipore, MR-025-D) and 300 mg/mL hyaluronidase (Sigma, H4272). Embryos were then implanted into the oviduct through the infundibulum of an anesthetized foster female. Twenty-five to thirty one-cell or two-cell embryos were implanted into each female to provide an average litter size of 8.1 ± 0.6 pups (n=22 litters). The body weights of the male offspring were measured and recorded weekly. Figure 7.1 is a schematic of the surgeries.

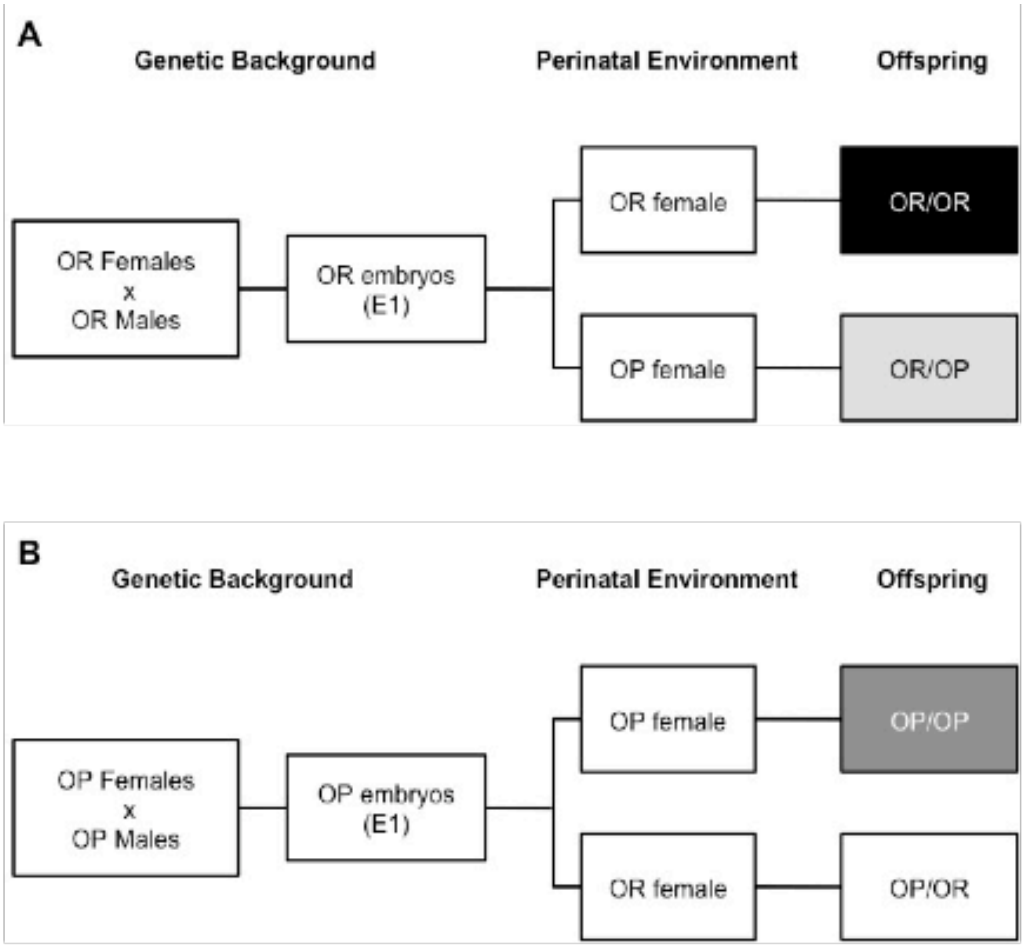

Figure 7.1: Transplanted offspring were generated by harvesting one or two cell OR and OP embryos and transplanting them into a pseudo-pregnant OR or OP dame.

A) Schematic representation of OR embryo transplantation at embryonic day 1. OR/OP offspring are OR animals that were exposed to an OP perinatal environment and OR/OR offspring are OR animals that were transferred to another OR foster mother. **B)** Schematic representation of OP embryo transplantation at embryonic day 1. OP/OR offspring are OP animals that were exposed to an OR perinatal environment and OP/OP offspring are OP animals that were transferred to another OP foster mother.

7.2.4 Prenatal exposure to L-dopa

Eight- to ten-week-old obesity-prone females were mated with obesity-prone males. On embryonic day 1, the females were separated from the males and given either 2mg/mL L-dopa (Sigma; St. Louis, MO, USA) & 0.025% L-ascorbic acid (Sigma; St. Louis, MO, USA) in their drinking water or 0.025% L-ascorbic acid alone in their drinking water for the duration of the pregnancy. The drinking water for all animals was changed twice weekly. Weekly body weights of the male offspring were measured along with 24-hour food intake and basal levels of locomotion at 13 weeks old.

7.2.5 Chronic exercise study

Male Wistar rats were given unrestricted access to a 2m-circumference running wheel in their cage for three weeks prior to testing. On the day of testing, the exercise wheels were removed 2 hours prior to euthanasia and brain removal.

7.2.6 Brain slice electrophysiology

Dopamine release kinetics were measured in all animals as described in Chapter 2.

7.2.7 Locomotion testing

Twenty-four hour locomotion testing was done using Smart-Frame activity boxes (Hamilton-Kinder) and Motor Monitor software (Hamilton-Kinder). Animals were habituated to the activity boxes for 4 days prior to testing. On the testing day,

body weight and food intake was measured as well as activity for 24 hours beginning and ending in the middle of the dark cycle.

7.2.8 Data Analysis

Data are shown as mean ± sem and were analyzed using one-way ANOVA.

7.3 Results

7.3.1 Maternal high fat diet results in increased body weight and selective decreases in central dopamine neurotransmission

In the first study, we investigated the role of maternal high-fat and high-sucrose diets on the body weight and central dopamine neurotransmission of male offspring. We found that the male offspring of mothers fed a high-fat diet (HF) were 12% heavier than the male offspring of mothers fed a control diet by early adulthood. This difference in body weight began by 5 weeks of age and persisted through adolescence. Interestingly, we did not see this difference in body weight in male offspring of mothers fed a high sucrose (HS) diet (Fig. 7.2A&B).

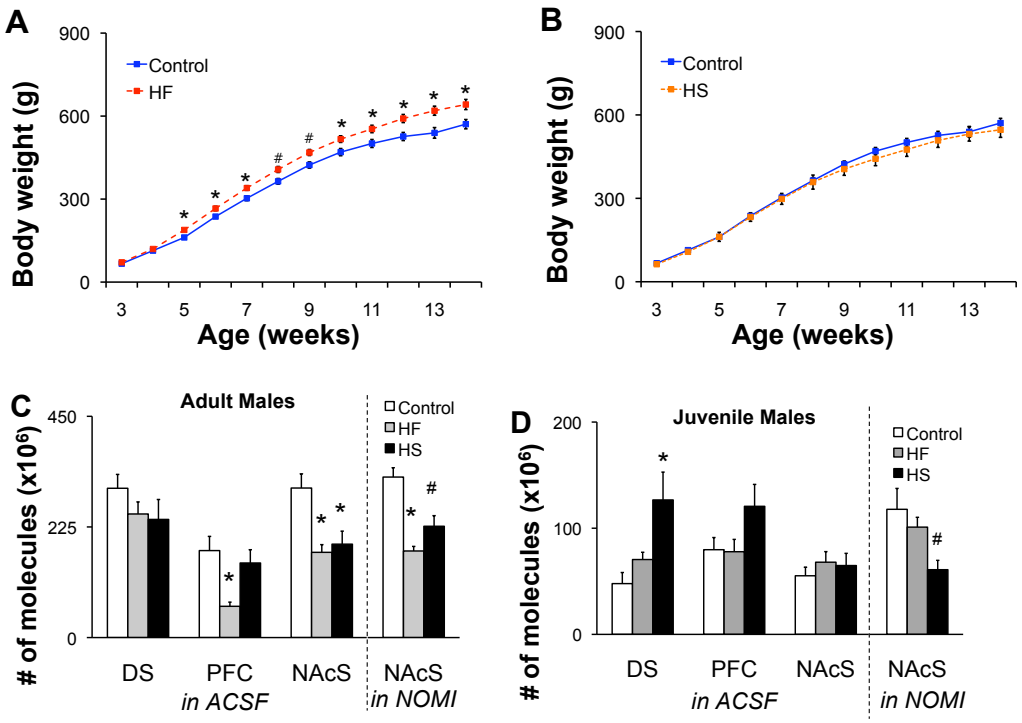

Figure 7.2: Maternal high-fat diet leads to increased body weight and decreased mesocorticolimbic dopamine release in adult male offspring.

A) Body weight of male offspring of mothers fed a high-fat diet (HF) is significantly greater than the control offspring beginning at 5 weeks of age through 14 weeks of age (n=8-9 animals per group). **B)** Body weight of the male offspring of mothers fed a high-sucrose diet (HS) was not different than the body weight of male offspring of mothers fed a high-sucrose diet through 14 weeks of age (n=7-9 animals per group). **C)** Electrically stimulated dopamine release was significantly lower in both the prefrontal cortex and nucleus accumbens shell of the 14 to 16 week old HF males and was only significantly decreased in the nucleus accumbens shell of the 14 to 16 week old HS males. These deficits persisted in the nucleus accumbens following a 30-minute wash with 3μM nomifensine (NOMI; n=20-53 stimulations from 4-11 slices; N=4-7 animals per group per site). **D)** Electrically stimulated dopamine release was only altered in the dorsal striatum and the nomifensine-treated nucleus accumbens of 3 to 5 week old HS males (n=25-48 stimulations from 5-10 slices; N=3-5 animals per group per site). Data are mean ± sem. * $p<0.01$, # $p<0.05$ by one-way ANOVA.

We have previously shown that changes in the obese phenotype is related to changes to central dopamine release, we hypothesized that the male offspring of the high-fat fed mothers would have lower dopamine signaling than the control offspring and the offspring of the high-sucrose fed mothers would have similar dopamine release kinetics as the control offspring. To test this hypothesis, we used carbon fiber amperometry to measure real-time dopamine release in the 15-week-old males following an electrical stimulus pulse. In the nucleus accumbens shell of these animals, the number of dopamine molecules released was approximately 40% lower in both the HF and the HS group compared to the control group (Fig. 7.2C; Table 7.1). This deficit was related to a decrease in the amplitude of the stimulus peak in both groups. We also measured dopamine release from the dorsal striatum and prefrontal cortex to determine if the deficit in dopamine release extended to other midbrain projection sites. At 15 weeks of age, we detected a significant deficit in dopamine release from the prefrontal cortex of the HF offspring but not the HS offspring compared to the control offspring (Fig. 7.2C; Table 7.1). In the dorsal striatum, we found no significant differences in dopamine release between any of the groups. (Fig. 7.2C; Table 7.1).

Table 7.1: Evoked dopamine release from maternal diet study offspring

	Control	HF	HS	p- value (HF, HS)
14 to 16 week old males				
Dorsal Striatum (ACSF)				
Molecules (x10^6)	303.6 ± 28.2 *(46,10,6)*	251.4 ± 24.5 *(43,9,6)*	240.3 ± 40.5 *(25,5,4)*	NS, NS
Amplitude (pA)	105.0 ± 10.6	105.2 ± 12.4	104.1 ± 20.0	NS, NS
$t_{1/2}$ (sec)	0.23± 0.12	0.06 ± 0.02	0.21 ± 0.07	NS, NS
Prefrontal Cortex (ACSF)				
Molecules (x10^6)	176.9 ± 19.0 *(53,11,7)*	63.6 ± 9.3 *(29,6,5)*	151.7 ± 21.6 *(24,5,4)*	<0.01, NS
Amplitude (pA)	60.4 ± 7.3	24.1 ± 2.2	60.9 ± 11.2	<0.01, NS
$t_{1/2}$ (sec)	0.09 ± 0.01	0.14 ± 0.09	0.08 ± 0.02	NS, NS
Nucleus Accumbens Shell (ACSF)				
Molecules (x10^6)	304.1 ± 28.9 *(40,8,6)*	173.3 ± 8.5 *(37,8,6)*	190.0 ± 27.0 *(19,4,4)*	<0.01, <0.01
Amplitude (pA)	114.1 ± 13.1	68.7 ± 4.7	71.5 ± 13.4	<0.01, <0.05
$t_{1/2}$ (sec)	0.12 ± 0.03	0.11 ± 0.04	0.20 ± 0.08	NS, NS
Nucleus Accumbens Shell (NOMI)				
Molecules (x10^6)	326.5 ± 28.7 *(30,6,6)*	176.1 ± 15.7 *(30,6,6)*	226.4 ± 26.5 *(20,4,4)*	<0.01, <0.05
Amplitude (pA)	114.1 ± 14.1	60.5 ± 8.2	80.2 ± 11.9	<0.01, NS
$t_{1/2}$ (sec)	0.26 ± 0.08	0.08 ± 0.03	0.17 ± 0.10	<0.05, NS
3 to 5 week old males				
Dorsal Striatum (ACSF)				
Molecules (x10^6)	47.8 ± 10.5 *(39,8,5)*	70.5 ± 6.9 *(46,10,5)*	126.6 ± 26.2 *(35,7,4)*	NS, <0.01
Amplitude (pA)	65.2 ± 22.2	47.7 ± 6.8	49.9 ± 12.5	NS, NS
$t_{1/2}$ (sec)	0.05 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	NS, NS
Prefrontal Cortex (ACSF)				
Molecules (x10^6)	79.8 ± 11.5 *(42,9,5)*	77.9 ± 11.6 *(35,7,4)*	120.6 ± 20.6 *(29,6,3)*	NS, NS
Amplitude (pA)	31.9 ± 4.9	39.0 ± 4.0	38.9 ± 6.7	NS, NS
$t_{1/2}$ (sec)	0.05 ± 0.01	0.08 ± 0.03	0.20 ± 0.05	NS, <0.01
Nucleus Accumbens Shell (ACSF)				
Molecules (x10^6)	55.2 ± 8.2 *(40,8,5)*	68.0 ± 10.0 *(48,10,5)*	60.9 ± 11.5 *(34,7,4)*	NS, NS
Amplitude (pA)	32.6 ± 6.2	35.1 ± 4.1	24.6 ± 5.3	NS, NS
$t_{1/2}$ (sec)	0.03 ± 0.01	0.07 ± 0.02	0.04 ± 0.01	<0.05, NS
Nucleus Accumbens Shell (NOMI)				
Molecules (x10^6)	117.8 ± 19.7 *(25,5,5)*	101.0 ± 9.3 *(25,5,5)*	64.8 ± 8.8 *(29,6,4)*	NS, <0.05
Amplitude (pA)	65.7 ± 14.6	56.7 ± 4.8	26.6 ± 3.5	NS, <0.01
$t_{1/2}$ (sec)	0.06 ± 0.02	0.14 ± 0.03	0.10 ± 0.05	<0.05, NS

Data = mean ± sem, HF=high-fat fed mother, HS=high-sucrose fed mother, n=(# of events, # of slices, # of animals), NS=not significant by one-way ANOVA compared to controls

We first detected a difference in body weight of the HF group compared to the control group at 5 weeks of age. To determine if the dopamine deficits seen in the adult animals were already in place before the body weight difference developed, we measured central dopamine release in the same brain regions of offspring that were 3 to 5 weeks of age. In this age range, no differences in central dopamine signaling were detected in the HF male offspring (Fig. 7.2D; Table 7.1). In addition, we saw that the HS male offspring had selectively elevated dopamine signal in the dorsal striatum and blocking reuptake in the nucleus accumbens in these animals resulted in decreased dopamine release at this age (Fig. 7.2D; Table 7.1). Together, these results indicate that maternal high-fat diet selectively affects the development of the corticolimbic dopamine projections in the male offspring after the increased body weight has begun to develop and the high-sucrose diet results in specific changes in the nigrostriatal dopamine projections early in life that protect from excess weight gain by adulthood.

7.3.2 An obesogenic perinatal environment results in decreased body weight that is linked to decreased dopamine reuptake in male offspring

To determine if the differences seen in male offspring of mothers fed a high-fat diet was the result of the diet or if similar results would occur in an obesogenic environment without exposure to high-fat diet, transplanted embryos from obesity-resistant to obesity-prone mothers on E1 (OR/OP). The control group used was OR embryos transplanted to another OR mother on E1 (OR/OR). In

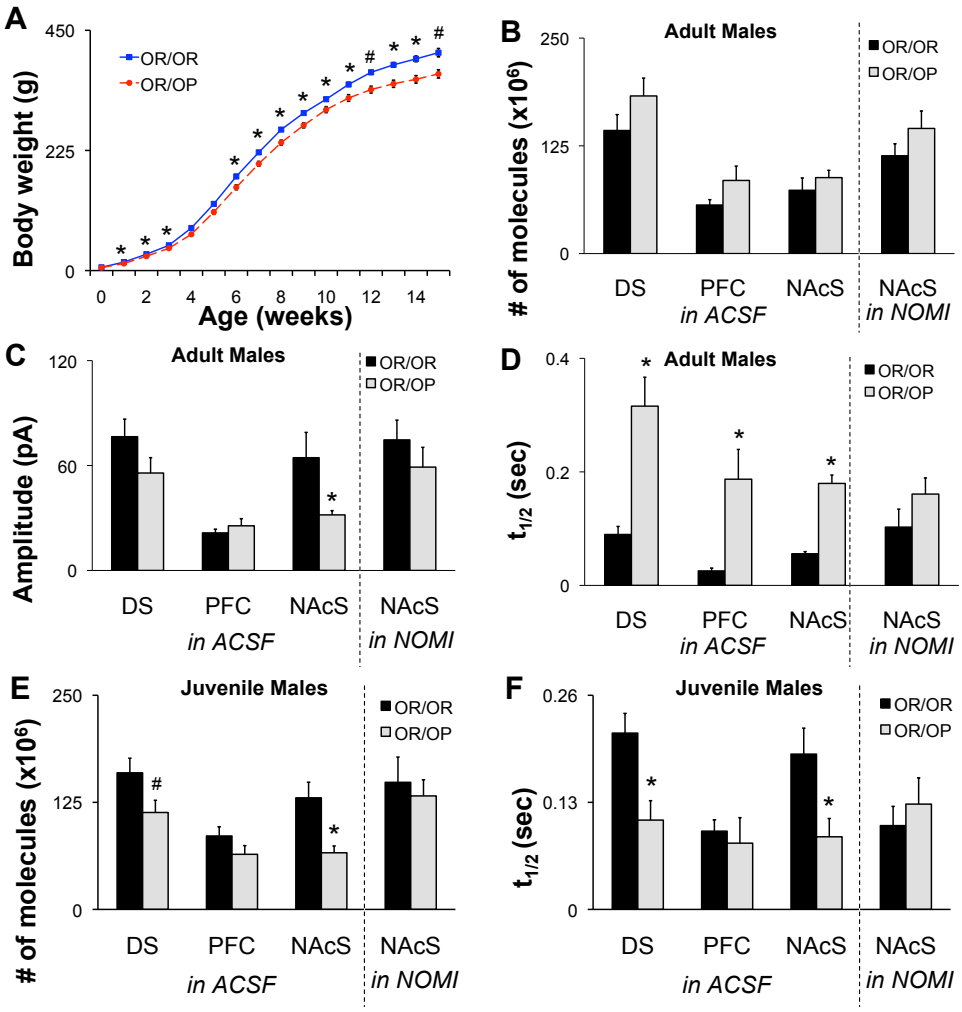

Figure 7.3: Specific differences in reuptake are linked to lower body weight in OR/OP animals.

A) Body weight of young adult OR/OP males was significantly lower than the body weight of OR/OR males (n=9 OR/OR and n=18 OR/OP males). No significant differences in total evoked dopamine release **(B)** was seen in young adult males, because peak amplitude **(C)** tended to be lower while $t_{1/2}$ **(D)** was significantly higher. These differences disappeared following treatment with the dopamine reuptake blocker, nomifensine (NOMI). (n=24-50 stimulations from 5-10 slices; N = 2-7 animals per group per site). Total evoked dopamine release **(E)** was significantly lower in the dorsal striatum and nucleus accumbens shell of the 3 to 5 week old OR/OP males due to a smaller $t_{1/2}$ **(F)** in these sites. These differences were eliminated following treatment with nomifensine. (n=20-58 stimulations from 4-12 slices; N = 3-7 animals per group per site). Data are mean ± sem. *p<0.01 and #p<0.05 by one way ANOVA.

contrast to the male offspring of the high-fat fed mothers, the male OR/OP offspring weighed significantly less than the OR/OR males from immediately following weaning at P21 through young adulthood where a 10% difference in body weight had developed between groups (OR/OR: 408.2 ± 7.9 g vs. OR/OP: 368.4 ± 7.7 g; Fig. 7.3A).

When we measured central dopamine release kinetics in these animals at 15 weeks of age, we detected no differences in the total number of molecules released in any of the projections sites tested (Fig. 7.3B, Table 7.2). However we did notice that the event width at half height ($t_{1/2}$) was significantly greater in all projections tested. However, since the amplitude of the response peak tended to be lower, the total number of molecules detected did not differ even though less dopamine was likely being released (Fig. 7.3C&D, Table 7.2). To determine whether the change in peak width was due to differences in dopamine reuptake we blocked reuptake using nomifensine and measured dopamine release in the nucleus accumbens shell. In this case, we found that the previous differences detected in both event width and amplitude disappeared following treatment with nomifensine.

We also measured dopamine release kinetics from these animals at weaning to determine if elevated dopamine signal was present, leading to lower body weight early in life. Instead, we found that dopamine release was lower in the dorsal striatum and nucleus accumbens shell of the OR/OP males similarly to the

Table 7.2: Evoked dopamine release from male transplanted offspring

	OR/OR	OR/OP	OP/OP	OP/OR	p-value (OR,OP)
14 to 16 week old males					
Dorsal Striatum (ACSF)					
Molecules (x10^6)	142.8 ± 18.5 *(34,7,5)*	182.9 ± 20.7 *(50,10,7)*	137.1 ± 31.3 *(42,9,6)*	188.6 ± 16.2 *(32,7,5)*	NS, NS
Amplitude (pA)	76.5 ± 10.1	55.7 ± 8.8	69.5 ± 10.7	69.5 ± 9.4	NS, NS
$t_{1/2}$ (sec)	0.09± 0.01	0.32 ± 0.05	0.06 ± 0.01	0.12 ± 0.01	<0.01, <0.01
Prefrontal Cortex (ACSF)					
Molecules (x10^6)	56.4 ± 6.2 *(45,9,4)*	84.9 ± 16.7 *(24,5,2)*	46.4 ± 8.0 *(38,8,6)*	80.9 ± 12.0 *(34,7,3)*	NS, <0.05
Amplitude (pA)	21.5 ± 2.2	25.5 ± 4.1	29.2 ± 3.4	27.5 ± 4.4	NS, NS
$t_{1/2}$ (sec)	0.03 ± 0.01	0.19 ± 0.05	0.03 ± 0.01	0.05 ± 0.01	<0.01, NS
Nucleus Accumbens Shell (ACSF)					
Molecules (x10^6)	73.5 ± 14.3 *(30,6,5)*	88.1 ± 8.5 *(45,9,7)*	55.8 ± 8.4 *(37,8,6)*	214.0 ± 22.7 *(30,6,5)*	NS, <0.01
Amplitude (pA)	64.5 ± 14.5	31.8 ± 2.4	42.2 ± 5.5	95.3 ± 15.2	<0.01, <0.01
$t_{1/2}$ (sec)	0.06 ± 0.004	0.18 ± 0.03	0.04 ± 0.01	0.13 ± 0.02	<0.01, <0.01
Nucleus Accumbens Shell (NOMI)					
Molecules (x10^6)	113.5 ± 13.9 *(25,5,5)*	145.2 ± 20.3 *(30,6,6)*	69.2 ± 17.0 *(25,5,5)*	235.1 ± 37.6 *(24,5,5)*	NS, <0.01
Amplitude (pA)	74.7 ± 9.2	59.1 ± 11.3	48.2 ± 11.1	90.0 ± 20.8	NS, NS
$t_{1/2}$ (sec)	0.10 ± 0.03	0.16 ± 0.03	0.03 ± 0.01	0.14 ± 0.02	NS, <0.01
3 to 5 week old males					
Dorsal Striatum (ACSF)					
Molecules (x10^6)	159.5 ± 17.1 *(49,10,6)*	113.2 ± 14.2 *(58,12,7)*	129.0 ± 16.2 *(54,11,6)*	138.8 ± 22.6 *(30,7,4)*	<0.05, NS
Amplitude (pA)	48.1 ± 3.5	50.6 ± 6.4	42.8 ± 3.8	62.4 ± 7.3	NS, <0.05
$t_{1/2}$ (sec)	0.21 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.12 ± 0.02	<0.01, NS
Prefrontal Cortex (ACSF)					
Molecules (x10^6)	85.7 ± 10.7 *(43,9,5)*	64.3 ± 10.2 *(48,10,5)*	54.5 ± 6.6 *(58,12,6)*	124.0 ± 15.7 *(32,7,4)*	NS, <0.01
Amplitude (pA)	44.6 ± 6.7	36.2 ± 4.9	28.8 ± 2.5	58.9 ± 6.0	NS, <0.01
$t_{1/2}$ (sec)	0.10 ± 0.01	0.08 ± 0.03	0.04 ± 0.01	0.11 ± 0.01	NS, <0.01
Nucleus Accumbens Shell (ACSF)					
Molecules (x10^6)	130.3 ± 18.3 *(50,10,5)*	66.1 ± 8.0 *(54,11,6)*	84.9 ± 13.9 *(55,11,6)*	132.8 ± 21.9 *(35,7,4)*	<0.01, <0.05
Amplitude (pA)	41.5 ± 3.9	44.2 ± 5.6	35.2 ± 3.5	57.4 ± 6.9	NS, <0.01
$t_{1/2}$ (sec)	0.19 ± 0.03	0.09 ± 0.02	0.07 ± 0.01	0.12 ± 0.01	<0.01, <0.01
Nucleus Accumbens Shell (NOMI)					
Molecules (x10^6)	148.4 ± 29.5 *(20,4,3)*	132.5 ± 18.9 *(30,6,6)*	83.3 ± 18.0 *(30,6,6)*	110.5 ± 22.4 *(20,4,4)*	NS, NS
Amplitude (pA)	88.1 ± 20.5	80.9 ± 9.6	42.0 ± 5.1	52.3 ± 9.6	NS, NS
$t_{1/2}$ (sec)	0.10 ± 0.02	0.13 ± 0.03	0.05 ± 0.01	0.10 ± 0.01	NS,<0.01

Data = mean ± sem, n=(# of events, # of slices, # of animals), NS=not significant by one-way ANOVA compared to OR/OR or OP/OP control

differences we have previously described in Chapter 5 in the adult female offspring from these groups. In addition, blocking reuptake with nomifensine eliminated the differences in dopamine release like in the 15-week-old males by eliminating differences in the width of the response peak. (Fig. 7.3E&F, Table 7.2)

These results indicate that dopamine reuptake is less active in male offspring that have been exposed to an obesogenic environment beginning very early in life. This deficit in dopamine reuptake prolongs exposure to the dopamine signal in the synaptic cleft following a stimulus like food, leading to lower food intake and/or higher activity levels, tipping the energy balance in favor of lower weight gain in these animals. Furthermore, these results are strikingly similar to those seen in animals that have been food restricted, where a 20% decrease in body weight leads to a decrease in basal nucleus accumbens dopamine(Pothos et al., 1995a; Pothos et al., 1995b). These results indicate that the male OR/OP offspring may be programmed to remain in an underweight state even when they have *ad lib* access to food.

7.3.3 An obesity-resistant perinatal environment enhances central dopamine release

In addition to investigating the role of the obesogenic environment on body weight and central dopamine signaling, we were also interested in whether an obesity-resistant perinatal environment could protect against excess weight gain

in an obesity-prone male and increase central dopamine signaling. To test this, we transplanted obesity-prone embryos into obesity-resistant mothers on E1 (OP/OR). The control group used was OP embryos transplanted to another OP mother on E1 (OP/OP). In this case, the male OP/OR offspring actually weighed more at weaning than the OP/OP males. However, this difference in body weight disappeared by adolescence and by young adulthood the OP/OP and OP/OR males did not differ significantly in body weight.(OP/OP: 502.7 ± 7.0 g vs. OP/OR: 517.8 ± 7.6 g; Fig. 7.4A).

To investigate the role of central dopamine signaling on the pattern of body weight gain in these animals, we measured dopamine release in these animals at weaning when a body weight difference was present and early adulthood when it had disappeared. At early adulthood, we show that total dopamine release was elevated in the mesocorticolimbic projections but not the nigrostriatal projections (Fig. 7.4B, Table 7.2). Treatment with nomifensine didn't eliminate the difference in dopamine release in the nucleus accumbens of the older males, indicating that mechanisms other than simply differences in reuptake are contributing to the changes in dopamine signaling in these animals by the time they reach young adulthood. This observation is also supported by elevations in both the amplitude and the $t_{1/2}$ in the nucleus accumbens shell (Fig. 7.4C&D, Table 7.2).

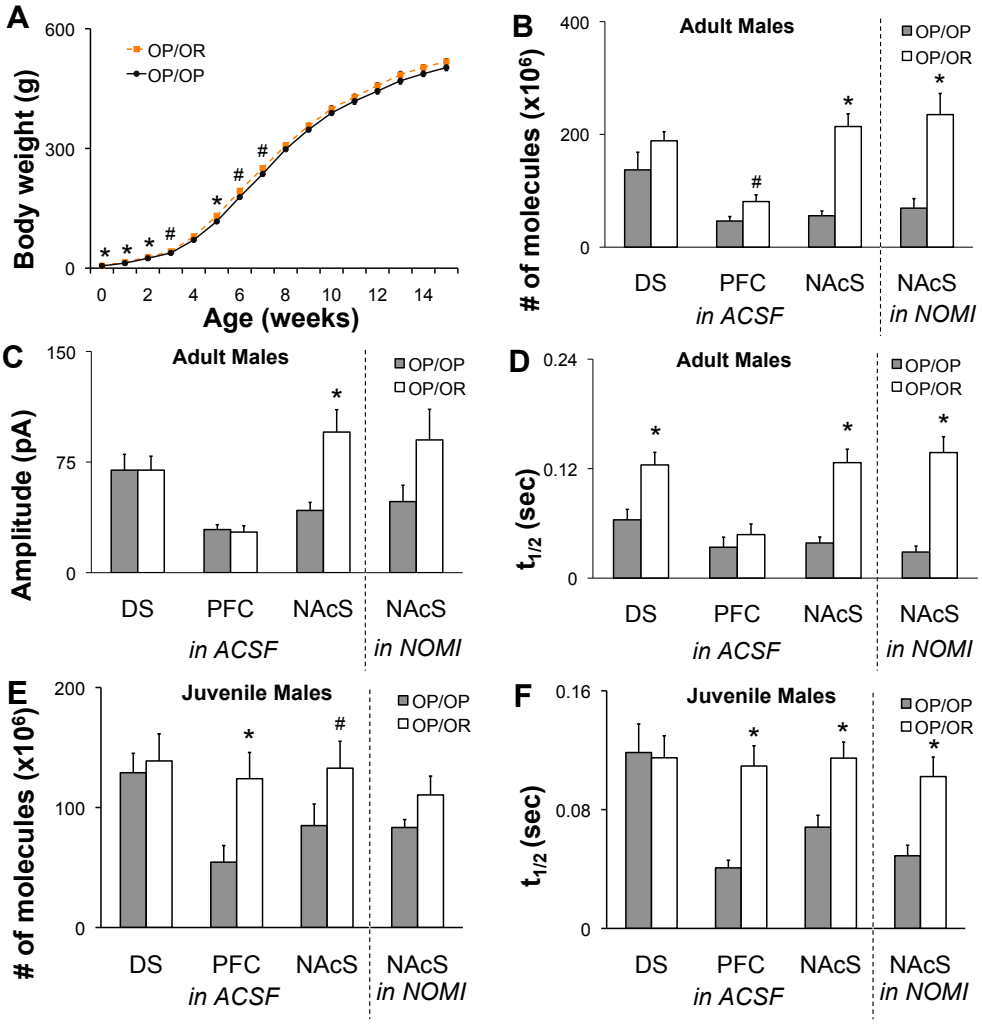

Figure 7.4: OP/OR male offspring have selectively elevated dopamine release in the mesocorticolimbic dopamine pathway.
A) Body weight of juvenile OP/OR males was significantly higher than the body weight of OP/OP males (n=13 OP/OP and n=10 OP/OR males). These differences did not persist into adulthood. Total evoked dopamine release **(B)** was higher in the prefrontal cortex and nucleus accumbens shell but not dorsal striatum. Peak amplitude **(C)** and $t_{1/2}$ **(D)** was significantly higher in the nucleus accumbens shell. This difference persisted following treatment with the dopamine reuptake blocker, nomifensine (NOMI). (n=24-42 stimulations from 5-9 slices; N = 3-6 animals per group per site). Total evoked dopamine release **(E)** was significantly higher in the prefrontal cortex and nucleus accumbens shell of the 3 to 5 week old OR/OP males due to increased $t_{1/2}$ **(F)** in these sites. (n=20-58 stimulations from 4-12 slices; N = 4-6 animals per group per site). Data are mean ± sem. *p<0.01 and #p<0.05 by one way ANOVA.

Because we saw a weight difference in these animals at weaning, we also measured dopamine release in the OP/OR and OP/OP animals at this age. We found that the OP/OR males had selectively increased dopamine release in the nucleus accumbens shell and prefrontal cortex compared to the OP/OP males, like what we had seen in the adult males (Fig. 7.4E&F, Table 7.2). However, when we blocked dopamine reuptake in the nucleus accumbens shell with nomifensine, we found that the differences in dopamine release were no longer present, indicating the reuptake plays a larger role in dopamine release kinetics at this age than it did in the adult animals. We have previously shown in the female OP/OR offspring that elevated central dopamine signaling does not protect against body weight gain in the female offspring. In the males, it appears that the elevated dopamine signaling is related to decreased body weight gain as the males reach adulthood, but not when they are in the rapid growth phase from weaning through adolescence.

7.3.4 Elevation of dopamine precursors during gestation results in long-term changes in central dopamine signaling

Because we see elevated body weight and elevated dopamine signaling in the nucleus accumbens shell and prefrontal cortex of OP/OR animals, we investigated whether the body weight change was related to the change in dopamine signal from the mother. To measure the direct effect of elevated dopamine during gestation, we gave pregnant OP dames 2 mg/mL L-dopa in their drinking water. The male offspring of the L-dopa treated mothers did not

significantly differ in body weight from weaning to adulthood (L-dopa: 510.5 ± 7.9 g vs. control: 497.1 ± 12.1 g; Fig. 7.5A). However, the L-dopa treated male offspring did eat (5.9 ± 0.2 g/100 g BW) and move significantly less during the dark cycle (19,893 ± 554 total beam breaks) than the control male offspring (6.5 ± 0.3 g/100 g BW and 22,009 ± 717 total beam breaks; Fig. 7.5B&C).

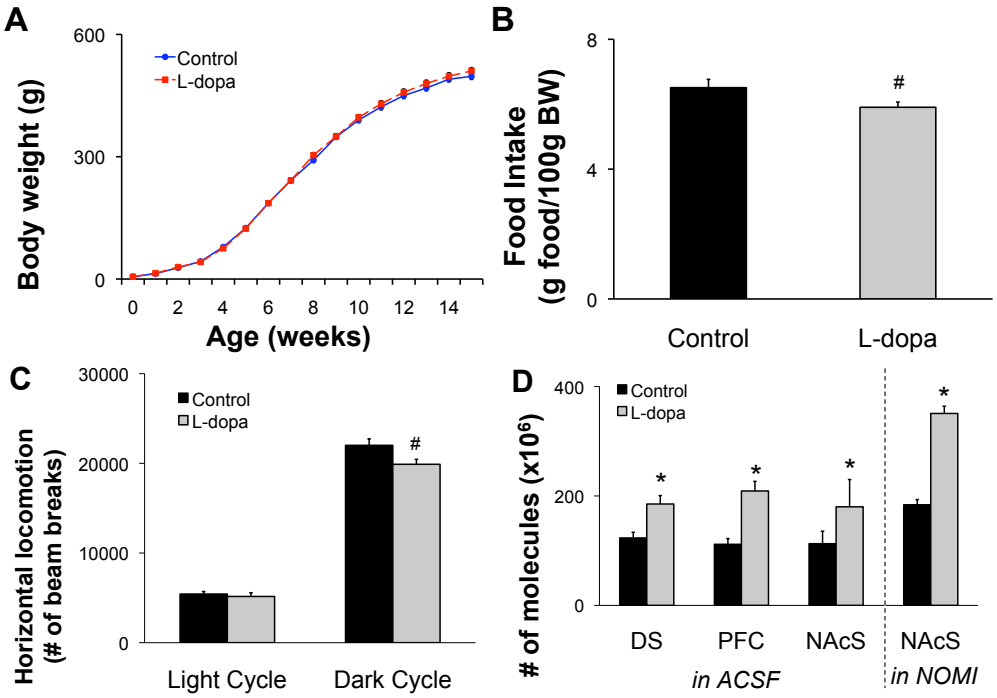

Figure 7.5: Elevated central dopamine signaling is linked to lower food intake and activity in male offspring of L-dopa treated mothers.
A) Body weight did not differ between L-dopa treated and control male offspring from birth to young adulthood. (n=9 control and n=11 L-dopa) **B)** Daily food intake of the male offspring of L-dopa treated mothers was significantly less than daily food intake of control offspring (n=9 per group). **C)** Activity levels as measured by the total number of infrared beam breaks was significantly lower during the dark cycle for the L-dopa treated male offspring compared to the control offspring (n=9 per group). **D)** Total evoked dopamine release was greater in all projections of young adult male offspring compared to control offspring (n=30-75 stimulations from 6-15 slices; N=6-8 animals per group per site). Data are mean ± sem. *p<0.01, #p<0.05 by one-way ANOVA.

Table 7.3: Evoked dopamine release from male offspring of L-dopa treated dames during gestation.

	14 to 16 week old males		3 to 5 week old males		p- value (Ad,Juv)
	Control	L-dopa	Control	L-dopa	
Dorsal Striatum (ACSF)					
Molecules (x10^6)	123.3 ± 10.3 *(74,15,8)*	185.3 ± 15.4 *(60,12,6)*	199.6 ± 27.6 *(44,9,5)*	161.4 ± 27.8 *(35,7,5)*	<0.01, NS
Amplitude (pA)	60.6 ± 4.7	72.6 ± 9.0	75.0 ± 12.3	8.7 ± 13.4	NS, NS
$t_{1/2}$ (sec)	0.14 ± 0.02	0.18 ± 0.02	0.27 ± 0.06	0.11 ± 0.01	NS,<0.05
Prefrontal Cortex (ACSF)					
Molecules (x10^6)	111.7 ± 9.6 *(68,14,8)*	209.1 ± 13.2 *(65,13,7)*	138.7 ± 27.8 *(44,9,5)*	143.9 ± 32.0 *(15,3,2)*	<0.01, NS
Amplitude (pA)	44.6 ± 3.5	62.8 ± 3.4	63.5 ± 11.9	55.0 ± 5.2	<0.01, NS
$t_{1/2}$ (sec)	0.13 ± 0.02	0.24 ± 0.02	0.11 ± 0.02	0.15 ± 0.02	<0.01, NS
Nucleus Accumbens Shell (ACSF)					
Molecules (x10^6)	112.8 ± 10.5 *(75,15,8)*	180.2 ± 17.6 *(58,12,6)*	117.9 ± 21.0 *(47,10,5)*	96.9 ± 13.4 *(39,8,5)*	<0.01, NS
Amplitude (pA)	63.4 ± 5.6	69.0 ± 7.5	53.7 ± 8.0	50.2 ± 5.6	NS, NS
$t_{1/2}$ (sec)	0.10 ± 0.01	0.22 ± 0.05	0.21 ± 0.05	0.13 ± 0.01	<0.01, NS
Nucleus Accumbens Shell (NOMI)					
Molecules (x10^6)	183.9 ± 22.9 *(40,8,8)*	350.8 ± 49.9 *(30,6,6)*	165.2 ± 20.9 *(23,5,4)*	141.3 ± 20.2 *(25,5,5)*	<0.01, NS
Amplitude (pA)	88.4 ± 12.3	131.2 ± 17.8	48.2 ± 4.6	65.9 ± 6.4	<0.05,<0.05
$t_{1/2}$ (sec)	0.18 ± 0.02	0.24 ± 0.05	0.25 ± 0.04	0.15 ± 0.01	NS, <0.05

Data = mean ± sem, n=(# of events, # of slices, # of animals), Ad = comparison of 14 to 16 week old animals, Juv = comparison of 3 to 5 week old animals, NS=not significant by one-way ANOVA compared to controls

We also measured stimulated dopamine release in these animals and found that the young adult male offspring of the L-dopa treated mothers exhibited increased dopamine release in all projections tested (Fig. 7.5D). However, this enhanced release developed over the course of the animal's life as no differences in total dopamine signal were present immediately following weaning in these animals (Table 7.3). These results indicate that the elevated dopamine release in the adult animals has a more significant effect on food intake than locomotion in these animals as we would expect that elevated dopamine release in the dorsal striatum would lead to increased activity. Furthermore, the later development of

the elevated dopamine release didn't protect these animals from gaining the same amount of weight as the control males during the rapid growth phase immediately following weaning.

7.3.5 Chronic exercise increases central dopamine neurotransmission in male rats

It has been shown previously in male OP rats that early exposure to exercise has profound effects on the development of the obesity phenotype in these animals (Patterson et al., 2008; Patterson and Levin, 2008). Additionally, we have shown that elevation of dopamine during the same time period has positive effects on the obesity phenotype in OP females. In this study, we tested whether chronic exercise can increase central dopamine release kinetics in male rats similarly to the effects that L-dopa treatment had on central dopamine release in female rats. We found that dopamine signaling was elevated in both the dorsal striatum and nucleus accumbens shell of these animals (Fig. 7.6A&B, Table 7.4). Furthermore, blockade of the dopamine reuptake transporter had little effect on dopamine release in these animals, indicating that mechanisms other than reuptake are responsible for the enhanced dopamine signal. These results indicate that exercise may provide a viable approach to the prevention of obesity in children prone to the development of obesity by altering the development of their food reward system and protecting them from compensatory eating brought on by low central dopamine signal.

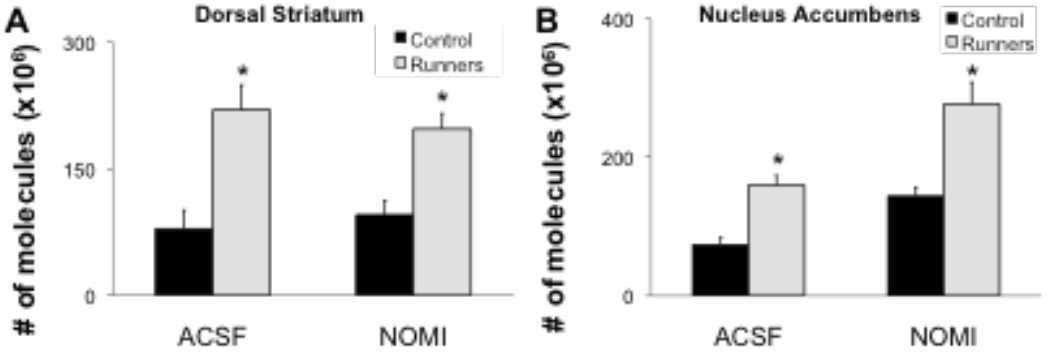

Figure 7.6: Chronic exercise results in enhanced dopamine signaling in male Wistar rats.

Dopamine release is elevated in both the dorsal striatum **(A)** and nucleus accumbens shell **(B)** before and after treatment with 3 μM nomifensine (n=10-15 stimulations from 2-3 slices; N=2-3 animals per group). Data are mean ± sem. *p<0.01 by one-way ANOVA.

Table 7.4: Evoked dopamine release from exercised male rats.

	Control	Runner	p- value
Dorsal Striatum (ACSF)			
Molecules (x10^6)	78.9 ± 22.0 *(15,3,3)*	219.3 ± 29.6 *(15,3,3)*	<0.01
Amplitude (pA)	41.8 ± 5.1	68.2 ± 5.9	<0.01
$t_{1/2}$ (sec)	0.18 ± 0.04	0.42 ± 0.05	<0.01
Dorsal Striatum (NOMI)			
Molecules (x10^6)	95.3 ± 17.2 *(10,2,2)*	197.0 ± 18.1 *(15,3,3)*	<0.01
Amplitude (pA)	43.6 ± 1.7	85.9 ± 14.3	<0.05
$t_{1/2}$ (sec)	0.17 ± 0.01	0.24 ± 0.03	NS
Nucleus Accumbens Shell (ACSF)			
Molecules (x10^6)	73.8 ± 10.6 *(10,2,2)*	159.8 ± 13.9 *(15,3,3)*	<0.01
Amplitude (pA)	51.0 ± 13.2	65.0 ± 3.9	NS
$t_{1/2}$ (sec)	0.10 ± 0.01	0.14 ± 0.02	NS
Nucleus Accumbens Shell (NOMI)			
Molecules (x10^6)	143.7 ± 13.3 *(10,2,2)*	276.6 ± 31.7 *(15,3,3)*	<0.01
Amplitude (pA)	66.6 ± 5.4	101.5 ± 11.5	NS
$t_{1/2}$ (sec)	0.21 ± 0.03	0.25 ± 0.04	<0.05

Data = mean ± sem, n=(# of events, # of slices, # of animals), NS=not significant by one-way ANOVA compared to controls

7.4 Discussion

This study investigates the effects of the perinatal environment on central dopamine signaling and the development of the obesity phenotype in the male offspring. In the case of maternal high-fat diet, we show that the males have increased body weight and selectively decreased dopamine signaling in the nucleus accumbens and prefrontal cortex as adults. These differences in both body weight and dopamine signaling are not present in these animals at weaning. This finding is in contrast to what we have shown in the female offspring where no differences in adult body weight are present and deficits in central dopamine signaling are present in not only the nucleus accumbens and prefrontal cortex but also in the dorsal striatum. These results indicate that the male offspring are particularly sensitive to the high fat diet of the mother, which lead to specific long-term alterations in central dopamine signaling in these animals only after a body weight difference has developed.

We also show that the effect on body weight is specific to the perinatal high-fat diet exposure as an obesity-resistant male pup that is exposed to an obesogenic environment actually weigh less than the control animal in adulthood. In comparison, the OR/OP female offspring had more rapid body weight gain during the pre-pubescent period compared to the OR/OR females. Furthermore, both the males and the females had decreased dopamine release in the dorsal striatum and nucleus accumbens at weaning. However, in males these differences disappeared by adulthood, while they persisted in females.

One potential mechanism by which these differences in body weight and dopamine signaling between males and females could occur is through the opposing effects of testosterone and estrogen on dopamine neurotransmission. Testosterone appears to suppress dopamine signaling and studies in vasectomized males have shown that number TH-IR positive cells in the SN and VTA increase in the month following the vasectomy, but these differences are reversed if testosterone replacement is given (Johnson et al., 2010a). The opposite effects on TH immunoreactivity appear to be true following an ovariectomy (Johnson et al., 2010b). Colocalization of both androgen and estrogen receptors with TH-positive cells have been shown in these areas. Androgen receptors are particularly prevalent neurons projecting to the nucleus accumbens and prefrontal cortex while estrogen receptors seem to be more highly expressed in the striatal regions that control movement (Kritzer, 1997). Estrogen has also been shown to be neuroprotective in models of Parkinson's disease, especially if the timing of a low-dose of estrogen preceded neuronal damage (Chen et al., 2006; Liu and Dluzen, 2007). In addition, obesity-related disorders are higher in women who have reached menopause (Carr, 2003; Shi and Clegg, 2009). We hypothesize that exposure to altered levels of estrogen in an obesogenic environment during the perinatal period could lead to early deficits in estrogen signaling and therefore deficits in central dopamine signaling in the OR/OP females as they reach adulthood. These deficits may not persist in the males past puberty, since the male dopamine system is likely less affected by the neuroprotective effects of estrogen by young adulthood. More studies need to be

done to confirm the links between testosterone, estrogen, dopamine signaling and obesity.

Elevation of dopamine precursors during gestation had no effect on the body weight of the male offspring even though by adulthood the males had increased dopamine release. Patterson et al. have shown that 3-weeks, but not 2 weeks of early exercise in obesity-prone males results in decreased body weight that is sustained following elimination of exercise in these animals (Patterson et al., 2008). It has also been shown in male rats that 6 weeks of exercise, but not 2 weeks, has significant effects on the upregulation of central dopamine markers and can eliminate conditioned place preference to cocaine (Greenwood et al., 2011; Thanos et al., 2010). Here we show that 3 weeks of chronic exercise results in elevated dopamine release, similarly to the elevated dopamine release seen in females that had been chronically treated with L-dopa. Taken together these results indicate that the food reward system is still developing during the rapid growth phase of childhood into adolescence and targeting interventions that raise central dopamine signaling specifically during this time frame may provide the necessary protection against the development of obesity in children with a predisposition for obesity. Furthermore, the duration of these interventions is critical as long-term up-regulation of this system requires extended exposure to the increased levels of dopamine.

In conclusion, this study shows that the maternal environment has significant effects on both the obesity phenotype and central dopamine release kinetics in male offspring. From these studies we find that both the type of environmental factor and timing of exposure are important in the development of obesity or protection from excess weight gain in male offspring. Furthermore, like with the female offspring, the obesogenic environment had greater detrimental effects on the phenotype male offspring than the obesity-resistant environments had positive benefits, especially if the elevated dopamine signal is not in place at weaning. In fact, the only beneficial treatment for protection against excess weight gain in male obesity-prone rats has been early exercise intervention (Patterson et al., 2008; Patterson and Levin, 2008). Here we show that chronic exercise upregulates central dopamine release. We have also identified the rapid growth phase immediately following weaning as a critical period in dopaminergic development, therefore targeting interventions that can raise dopamine exocytosis during this period could lead to viable strategies for the prevention of obesity in obesity-prone children.

8 General Discussion, Implications and Future Work

8.1 Summary of key findings

This dissertation has focused on understanding the interactions between the perinatal environment, dietary obesity predisposition and central dopamine neurotransmission. In the first set of experiments described in Chapter 3, we demonstrate a link between the preference for a high-energy diet and the mesolimbic dopamine system that leads hyperphagia and dietary obesity in Sprague-Dawley rats. Specifically, we find decreased dopaminergic neurotransmission in the nucleus accumbens and dorsal striatum of the dietary obese rats that can be temporarily restored by eating highly palatable, high-energy food. The second set of experiments, which is described in Chapter 4, expands the finding of low midbrain dopamine to animals with a predisposition to dietary obesity. In these animals, the low dopamine signal is widespread and present very early in life, prior to the development to the development of the obese phenotype, and induces a compensatory behavioral response, hyperphagia, to elevate central dopamine release. Furthermore, this deficit is linked to changes in expression of key presynaptic regulators of dopamine synthesis and exocytosis.

In the experiments investigating the role of the obesogenic perinatal environment (described in Chapters 5 and 6), we found that simply exposing obesity-resistant embryos to an obesogenic perinatal environment was sufficient to alter central dopamine signaling in the female offspring and have detrimental effects on the obesity phenotype. Like in the previous set of experiments, these deficits were

linked to changes in presynaptic regulation of dopamine synthesis and exocytosis. They were also linked to changes in both transcription factor levels and microRNA expression. Interestingly, the obesity-resistant environment also altered central dopamine signaling, but failed to have the protective effects on the obese phenotype that were expected. Similarly, treatment of mothers during gestation with L-dopa to increase dopaminergic tone had little effect on the obesity phenotype of the obesity-prone offspring while resulting in long-term upregulation of central dopamine signaling. However, if L-dopa was given during the prepubescent period, food intake and body weight were decreased as dopamine levels were increased, providing insight into how and when targeting central dopamine signaling may be an effective treatment for obesity.

In the final analysis described in Chapter 7, we investigated how male offspring responded to the maternal environments described in Chapters 5 and 6. We found that the maternal environment has significant effects on both the obesity phenotype and central dopamine release kinetics in male offspring. From these studies we find that both the type of environmental factor and timing of exposure are important in the development of obesity or protection from excess weight gain in male offspring. Furthermore, like with the female offspring, the obesogenic environments had greater detrimental effects on the phenotype male offspring than the obesity-resistant environments had positive benefits, especially if the elevated dopamine signal is not in place at weaning. Here we show that chronic exercise upregulates central dopamine release, a treatment that has

been previously shown to have beneficial effects on the obesity phenotype of obesity-prone males (Patterson et al., 2008).

8.2 The Reward Deficiency Hypothesis

8.2.1 Reward deficiency in addiction

"Reward deficiency syndrome" describes a phenomenon where people with low central dopamine levels, particularly in the nucleus accumbens, tend to overconsume food or abusable drugs to compensate for the low reward signal. (Blum et al., 1995b). This hypothesis is based on the association of the Taq1 A1 allele of the DRD2 gene to an increased susceptibility to multiple types of addictions including: alcoholism, drug use, and excessive gambling or sex (Blum et al., 2000; Blum et al., 1990; Blum et al., 1995a). Not only does this allele result in decreased D2 activity and hyporesponsiveness of the central dopamine system (Blum et al., 2000), but imaging studies in substance dependent humans also show decreased D2 receptor activity (Volkow et al., 2004a; Volkow et al., 2004b). Together, these results indicate that a decreased dopamine signal is a significant contributor to the development of substance dependence in an individual.

8.2.2 Reward deficiency in obesity

Reward deficiency has also been implicated in obesity. A number of imaging studies in obese humans have investigated alterations in dopamine signaling. Functional magnetic resonance imaging (fMRI) studies have shown negative

correlations between BMI and activation of the caudate nucleus both following consumption of a palatable milkshake and in anticipation of food (Stice et al., 2008a; Stice et al., 2008b). Positron emmision tomography (PET) studies measuring the activity of the D2 receptor show decreased D2 activity in obese subjects, like that seen in drug addicts. (Wang et al., 2001; Wang et al., 2004). To further support these findings, the Taq1 A1 allele of the DRD2 gene is associated with decreased striatal activity in obese individuals (Blum et al., 1996; Stice et al., 2008a). Behaviorally, the D2 receptor knockout mouse exhibits phenotypes associated with dopamine deficiency including hyperphagia and reduced amphetamine induced hyperactivity, even though dopamine levels appear normal (Garcia-Tornadu et al., 2010b; Holmes et al., 2004). This apparent dichotomy is though to be the result of a compensatory increase in expression of DAT, which may replace dopamine, but not the signal transduction induced by the D2 receptor (Holmes et al., 2004; Parish et al., 2002). Disruption of the D2 receptor has also been shown to negatively influence insulin secretion and glucose tolerance in rodents, further linking central dopamine and metabolism (de Leeuw van Weenen et al., 2011; Garcia-Tornadu et al., 2010a).

Recent studies of the influence of bariatric surgery on perceived rewarding properties of food have helped begin to understand the functional implications of low dopamine signal on the development of obesity. Following surgery, patients have indicated that they sense sweet taste differently and rodent studies have shown that following surgery, these animals find higher concentrations of sugar

to be unpleasant (Burge et al., 1995; Hajnal et al., 2010; Miras and le Roux, 2010; Shin et al., 2010). Recent studies support the alterations in central reward associated with gastric bypass surgery by showing that D2 receptor availability is increased in patients following Roux-en-Y gastric bypass surgery (Steele et al., 2010).

While the surgery data is providing valuable insight into the role of dopamine in obesity, the functional implications of dopamine deficits are still extremely difficult to decipher in humans, mechanisms and timing of the development of dopamine deficits have been studied in a number of animal models. In Chapter 3, we show that dietary obese rats exhibit reduced central dopamine neurotransmission. Also in these animals, we show decreased sensitivity of the dopamine response to both laboratory chow and amphetamine (Geiger et al., 2009). These findings are supported by a number of other studies in dietary obese or high-fat fed animals. In high-fat fed rats, dopamine signaling was reduced in the nucleus accumbens, even before the animals became obese (Davis et al., 2008; York et al., 2010). Also, cafeteria diet fed animals showed reduced response to reward that was linked to deficits in striatal D2 receptors as their obesity developed (Johnson and Kenny, 2010). Taken together all of these studies indicate that either the diet or the obesity itself leads to deficits in central dopamine signaling leading the obese animals to consume more food as a compensatory response. However, they do not address the relative contributions of the genetic background, the diet and the excess body weight of these animals.

To eliminate some of the confounding factors of diet and body weight differences in the models where obesity is induced by changes in the rodents' diets, reward deficiency has been studied in genetic models of obesity and obesity-predisposition. In Chapter 4, we show in selectively bred obesity-prone animals that compared to obesity-resistant animals, dopamine signal is lower even before a weight difference develops (Geiger et al., 2008). These deficits are linked to hypoactivity and an attenuated response to amphetamine injection that results from decreased expression of both tyrosine hydroxylase and VMAT2 (Geiger et al., 2008). In outbred rats selected for obesity predisposition after five days on a high-fat diet, similar deficits in dopamine neurotransmission were also present before the animals became obese (Rada et al., 2010). Dopaminergic neurotransmission has also been studied in the leptin deficient *ob/ob* mouse model. This mouse is extremely obese due to extreme hyperphagia and hypoactivity that has been linked to decreased dopamine signaling (Fulton et al., 2006; Hommel et al., 2006). Furthermore, opposite effects were seen in a lean model, the *MCH -/-* mouse, where leanness was linked to increased dopaminergic neurotransmission (Pissios et al., 2008). This evidence supports that hypothesis that decreased dopamine signaling results in excessive weight gain through increased food intake and decreased locomotion and that these deficits may be present in individuals predisposed to obesity before the obesity develops.

8.2.3 Competing hypotheses to the "reward deficiency hypothesis"

The "reward sensitization" hypothesis posits that people who are susceptible to overconsumption of rewarding substances have enhanced sensitivity to the rewarding properties of these substances. Therefore, obese individuals are hyperphagic because highly palatable food is more rewarding to them (Davis and Fox, 2008). In support of this hypothesis, the CLOCK mutant mouse, which does not have a functional CLOCK gene is obese, exhibits increased response to the cocaine reward, which is linked to elevated tyrosine hydroxylase levels. (Manev and Uz, 2006; McClung et al., 2005; Turek et al., 2005). However, these differences may be reconciled based on a recent study by Davis and Fox in overweight and obese humans. They found and inverted U-shaped relationship between BMI and sensitivity to reward, where BMIs in the overweight range were correlated with increased reward sensitivity and BMIs in the obese range were correlated with decreased reward sensitivity (Davis and Fox, 2008).

Also disputing the idea that the decreased D2 signaling is associated with obesity is the observation that treatment with D2 receptor antagonists, like olanzapine and clozapine, cause significant weight gain. Inhibiting the D2 autoreceptor results in increased dopamine synthesis. The development of the obese phenotype indicating in this case that elevated dopamine results in overconsumption and obesity. However, rodent studies indicate that these drugs are not selective for D2, but also alter serotonin signaling and both effects are required for weight gain to occur (Kirk et al., 2009; Reynolds and Kirk, 2010).

Together, these studies indicate that although other mechanisms might also contribute to the development of obesity, reward deficiency plays an important role in the altered energy balance of obese individuals.

8.2.4 Transgenerational obesity and reward deficiency

Evidence of transmission of the obese phenotype from mother to child through the maternal diet and in mothers that are prone to obesity indicates that epigenetic modifications of genes associated with central dopamine signaling including altered DNA methylation, changes in expression of transcription factors and altered miRNA levels may be a contributing factor to the development of decreased dopamine release in obesity (Boney et al., 2005; Buckley et al., 2005; Chen et al., 2008; Geiger et al., 2008; Gorski et al., 2006; Levin et al., 1997). Offspring of high-fat diet fed mice have deficits in dopamine signal that are associated with altered DNA methylation of the DAT gene. This altered gene expression would lead to decreased synthesis of dopamine and increased food intake (Vucetic et al., 2010). In our studies described in Chapter 5, we show altered microRNA and transcription factor levels in obesity-resistant animals that have been exposed to an obesogenic environment. In particular, we show that exposure during the perinatal period to an obesogenic environment results in downregulation of the transcription factor Pitx3 and upregulation of the microRNA miR-133b. This interaction has been shown to be a negative feedback loop and is involved in regulation of TH and DAT gene expression, where decreased Pitx3 levels are associated with decreased dopaminergic tone (Kim et al., 2007).

Paternal influences may also contribute to transgenerational obesity. Paternal low-protein diet has been linked to increased expression of metabolic genes in the offspring (Carone et al., 2010). Paternal alcoholism has also been linked to obesity in their children and paternal links to diet-induced obesity and excessive food intake have been shown (Fortuna, 2010; Munafo et al., 2007; Yazbek et al., 2010). We did not evaluate paternal influences directly in the studies described here. Further evaluation is needed to elucidate the contribution of the fathers beyond genetics on the obese phenotype and central dopamine signaling in the obesity-prone rats.

8.2.5 Mechanistic model for dopamine depletion

Dopamine depletion is seen throughout several models of both obesity and addiction. However, prior to this work, the mechanism by which this universal depletion occurs has not been well established. Changes in expression of key dopamine synthesis and exocytosis regulators are present in many of these models, indicating that at least one, if not multiple, higher level regulators are responsible for the overall depletion of this system (Fulton et al., 2006; Geiger et al., 2008; Geiger et al., 2009; Johnson and Kenny, 2010). Here we propose a mechanism in which microRNAs and transcription factors together play a role in the long-term down-regulation of central dopamine signaling. Specifically, if female obesity-resistant offspring are exposed to an obesogenic maternal environment during the perinatal period, they exhibit increased expression of

miR-133b and decreased Pitx3 expression. Pitx3 controls the expression of dopamine synthesis and exocytosis related genes including tyrosine hydroxylase and DAT (Kim et al., 2007). Downregulation of these genes by Pitx3 leads to decreased presynaptic dopamine and decreased stimulated dopamine release. In these animals we see decreased spontaneous activity and increased body weight gain, especially during the rapid growth phase immediately following weaning. Figure 8.1 summarizes the mechanisms we have shown as well as hypothesized interactions that may be driving dopamine depletion and the resulting hyperphagia in obesity. Along with the molecules that we have identified, other transcription factors, calcium-regulated kinases and microRNAs may also be involved in this process. Further studies are needed to determine whether higher levels of regulation are involved than those identified in this work.

8.2.6 Potential for dopamine replacement as a therapeutic

To combat this syndrome, elevation of central dopamine should result in reduced food and drug intake. In fact, amphetamine, a well known agonist of central dopamine signaling, was marketed as Benzedrine in the 1930s and became one of the first weight loss drugs (Harris et al., 1947). Since then numerous other agonists of the dopamine system have also been shown to reduce food intake and/or contribute to weight loss (Cooper et al., 2006; Cooper et al., 1990; HA and Cooper, 1994; van der Hoek and Cooper, 1994).

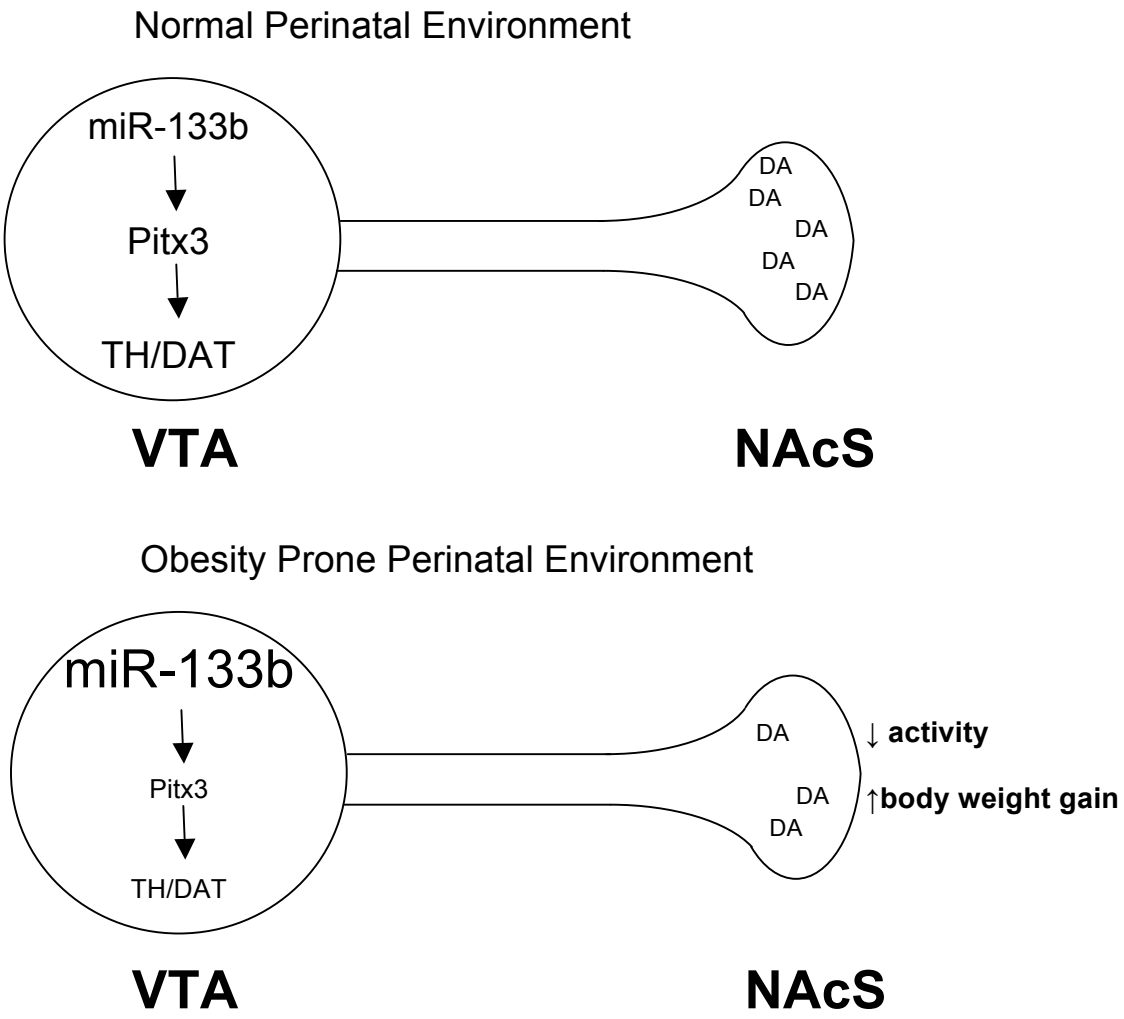

Figure 8.1: Proposed mechanism for decreased dopamine signaling in OR females exposed to an obesogenic environment.
Exposure to an obesogenic environment upregulates miR-133b, which leads to, decreased expression of the transcription factor, Pitx3. Because Pitx3 regulates expression of TH and DAT, downregulation of Pitx3 leads to decreased expression of these genes and an overall decrease in dopamine. Decreased pre-synaptic dopamine leads to decreased spontaneous activity and increased body weight gain during adolescence in the female offspring.

In addition to providing a potential mechanism by which dopamine depletion may be occurring in obesity, this work also identifies a time frame where intervening with this system may help to prevent the onset of obesity in children that are predisposed to obesity, especially females. We found by giving female obesity-prone rats dopamine replacement therapy in their drinking water during the rapid growth phase immediately following weaning significantly decreased food intake and body weight gain in these animals. Furthermore, the only treatment that has successfully resulted in decreased body weight gain in obesity-prone males is 3 weeks of exercise during this same phase of their life (Patterson et al., 2008). Together these studies indicate that targeting dopamine replacement, potentially through increased exercise, during childhood in individuals prone to developing obesity could help prevent the development of obesity in some people.

8.3 Future directions

This work has provided insight into the relationships between the development of obesity and central dopamine signaling. However, more studies are needed to understand the molecular mechanisms driving the changes in dopamine signaling. More detailed studies into transcription factor and miRNA activity and function, DNA modifications that result from changes in diet, and the relevant signaling pathways that are involved are needed to isolate specific targets for the development of treatments and preventative interventions for obesity. We also note several differences between how the females and males respond to the intrauterine environments they are exposed to. The mechanisms behind these

differences are also not well understood and more work is needed to elucidate the specific roles of testosterone and estrogen in the development of the central dopamine signaling pathways and obesity phenotype due to excess food intake.

When assessing the safety and efficacy of dopamine replacement therapy as a treatment for obesity more studies are needed. First, elevation of dopamine signaling has been used previously in weight loss drugs that were later shown to cause individuals to become dependent on the substances. If we plan to target this system, any therapeutic must be shown to not cause dependence in the patients receiving the treatment and studies are needed to identify substances that would meet these criteria. Furthermore, the studies described here treat only with L-dopa, which specifically targets dopamine synthesis. However, as with all neurotransmitters, the dopamine system contains multiple facets including vesicular packaging, clearance by both membrane transporters and metabolizing enzymes and signal transduction through a variety of receptors. Dopamine replacement treatments that target more than one part of the dopamine system may provide synergistic effects on decreasing food intake and body weight. For example, we could complete a study similar to the one described here where we upregulate dopamine synthesis using L-dopa, enhance downstream signaling by treating with a D1 agonist and inhibit reuptake by blocking DAT to determine if we can achieve synergistic effects in this system. This synergy could be beneficial as, aside from surgical options, the best treatments for obesity currently only provide an 8-10% decrease in body weight.

Finally, identification of children prone to develop obesity is critical in the effort to prevent obesity. In order to do this, easily accessible biomarkers are needed. To date, no plasma markers related to the central dopamine deficits have been identified. More studies are needed to determine whether peripheral markers are present and if we can use these markers to provide targeted interventions for individuals who are most likely to develop obesity.

8.4 Overall Conclusion

In conclusion, the studies described in this dissertation underscore the importance of motivational and hedonic pathways in addition to hypothalamic homeostatic pathways in the regulation of appetite and feeding behavior; and strengthen the argument that obesity should be approached not only as a metabolic imbalance but as an addictive disorder that contributes to the imbalance. We have identified the rapid growth phase immediately following weaning as a critical period in dopaminergic development. Selective targeting of presynaptic regulators of the mesolimbic dopamine system during this period, including transcription factors associated with synaptic plasticity and specific microRNAs, constitutes a promising approach for both the prevention and treatment of dietary obesity in obesity-prone children.

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