BARNES, CHERIE N. M.S. The Effect of Dietary Fat on Dopamine Neurotransmission. (2019)
Directed by Dr. Steven C. Fordahl. 84 pp.

Emerging evidence has revealed that obesity and diets high in saturated fat are linked with pathophysiological changes in the dopaminergic reward system that disrupt satiety signals governing homeostatic food intake. Diets high in saturated fat are also implicated in the development of a metabolic syndrome-like phenotype characterized by obesogenic weight gain, insulin resistance, and chronic inflammation. While there is evidence that anti-inflammatory unsaturated fats promote healthier metabolic profiles and brain health, little is known about the effects of diets high in unsaturated fat on dopamine neurotransmission which plays a role in feeding and satiety circuits. We sought to determine whether a diet high in unsaturated fat, in contrast to saturated fat, would prevent the development of a metabolic disorders and preserve normal dopamine function. To examine this, male C57BL/6 mice were fed, *ad libitum*, a low-fat (LF) control diet or a nutrient-matched diet high in either saturated fat (SFD) or unsaturated flaxseed oil (FSO) for six weeks. We measured food intake and body weight throughout the dietary intervention and after six weeks we assessed metabolic dysfunction with glucose tolerance tests and locomotor behaviors in an open field test. We subsequently measured sub-second dopamine release and uptake from dopamine neurons using *ex-vivo* Fast Scan Cyclic Voltammetry in the nucleus accumbens (NAc). Dopamine kinetics in response to the dopamine D2/D3 receptor agonist quinpirole was also measured to assess dopamine receptor

function. In order to assess the relationship between dietary fat, inflammation, and dopamine neurotransmission, the pro-inflammatory cytokine, interleukin-6 (IL-6) was also measured in the NAc.

Mice fed a SFD consumed significantly more food and gained significantly more weight compared to their LF-fed counterparts. In addition, unlike the LF group, the SFD group displayed anxiogenic locomotor behaviors in open field tests. Interestingly, the FSO group consumed the same amount of food as the SFD group; however, the FSO diet attenuated weight gain and preserved normal blood glucose regulation and locomotor behaviors. Significantly, the SFD group also exhibited dampened phasic dopamine release, impaired dopamine uptake and increased sensitivity to quinpirole, all of which was prevented with the FSO diet. There was also a negative association between dopamine uptake and IL-6 in the SFD group suggesting IL-6 selectively corresponded with reduced dopamine uptake in mice fed saturated versus unsaturated fat. Collectively, we demonstrate that different types of dietary fat have substantially different effects on metabolic phenotype and dopamine terminal regulation. In contrast to a diet high in saturated fat, a diet high in unsaturated fat preserved both normal metabolic and behavioral parameters as well as dopamine signaling in the NAc.

THE EFFECT OF DIETARY FAT ON DOPAMINE NEUROTRANSMISSION

by

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A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2019

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ACKNOWLEDGEMENTS

I want to thank my parents for all of their support. I am also very appreciative to members of my committee, Drs. Erickson and Armah, for their time, advice, and expertise. Most of all, I want to thank Dr. Fordahl for being a generous sponsor and an inspiring mentor. It has been an incredible privilege to work with you.

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CHAPTER I

REVIEW OF LITERATURE

Introduction

Research related to diet-induced obesity has been traditionally viewed through the lens of a homeostatic model of food intake. This model is primarily focused on the ways that metabolic calorie requirements and the peripheral signaling molecules of hunger and satiety communicate with the central nervous system. The lateral hypothalamus, which is the “feeding center” of the brain, is central to energy homeostasis since it is the primary hub that receives peripheral, autonomic orexigenic and anorexigenic signals (Cassidy and Tong, 2017; Coccurello and Maccarrone, 2018). However, food-seeking and food intake behaviors take place within the larger context of all motivated behaviors and are therefore influenced by a rich interplay among multiple brain nuclei as well as peripheral signaling pathways. Within this larger context of motivated behaviors, the mesolimbic dopamine system plays a central role. It receives information related to all salient stimuli from numerous areas of the brain and peripheral endocrine system and subsequently integrates these signals to influence and modulate many functions including motor control, learning, cognitive function, motivation, mood, reward, addiction, and stress (Cassidy and Tong, 2017;

Coccurello and Maccarrone, 2018; Ferrario et al., 2016; Haber, 2011; Volkow et al., 2013, 2011).

The mesolimbic dopamine signaling pathway is comprised of dopaminergic neurons originating in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc). This signaling pathway encodes and incentivizes salient rewards, thereby initiating motivated behaviors by actuating goal-directed movements (Salgado and Kaplitt, 2015). The NAc is of key importance since it is the limbic-motor interface as well as the primary reward center of the brain (Ferrario et al., 2016; Goto and Grace, 2008; Salgado and Kaplitt, 2015; Volkow et al., 2011). As such, the NAc is believed to underlie conditioning that links the memory of salient rewards that include both homeostatic and hedonic experiences with the context in which they occur and subsequently influence the desire and future pursuit of rewarding, salient reinforcers (Goto and Grace, 2008; Salgado and Kaplitt, 2015). Given the central role that the NAc plays in the motivational pursuit of reward and conditioned responses, it is implicated in the addictive effects of many illicit drugs of abuse (Volkow et al., 2013, 2003). More recently, it has been proposed that overconsumption of potent natural rewards, such as highly palatable foods that are high in sugar and fat, might also adversely affect VTA-NAc dopamine signaling. In extreme cases, it is possible that highly palatable foods cause synaptic alterations similar to drugs of abuse (Di Chiara and Bassareo, 2007; Volkow et al., 2013, 2011). This theory would explain pathological behavioral

patterns such as compulsive food seeking and consumption that exceed metabolic needs and therefore lead to obesity (Matikainen-Ankney and Kravitz, 2018).

Role of the Mesolimbic Dopamine System in Food-seeking Behaviors and Energy Homeostasis

The mesolimbic dopamine system communicates with many nuclei in the CNS to modulate behaviors, motivators, and choices. Within the context of food seeking and food consumption behaviors, the VTA-NAc dopamine circuit assigns salience to external stimuli and directs motivated behavior based on both orexigenic and anorexigenic cues from the lateral hypothalamus and arcuate nucleus (Cassidy and Tong, 2017; Ferrario et al., 2016; Ferré, 2017). It also integrates these signals with excitatory inputs from the paraventricular thalamic nucleus related to arousal/motivation as well as executive function/decision making input from the prefrontal cortex (Arias-Carrión et al., 2010; Ferrario et al., 2016; Ferré, 2017). Much like the lateral hypothalamus, the VTA and NAc also receive peripheral orexigenic and anorexigenic signals. For example, neurons of the VTA and NAc have receptors for classic autonomic energy homeostasis hormones/peptides such as GLP-1, leptin, ghrelin, orexin, insulin, and melanocortin (Ferrario et al., 2016). As both peripheral signals and central inputs are received, they are filtered and/or amplified based on their salience, leading to orchestrated, complex, goal-directed behaviors. Specific to feeding behaviors, the VTA-NAc is involved in food-related memory processing with the

hippocampus, emotional reactivity with the amygdala, food-cues for arousal with the thalamus, as well as higher-level executive decision-making related to food intake with the prefrontal and insular cortex (Ferrario et al., 2016; Goto and Grace, 2008). When these signals are in balance, salience associated with food is initiated by hunger cues and subsequently discontinued when adequate calories are consumed to support metabolic energy requirements. In the case of highly-palatable foods, hedonic hunger associated with mesolimbic dopamine can override homeostatic satiety cues, leading to overconsumption of food (Licholai et al., 2018). This phenomenon is significantly exacerbated when there are alterations in dopamine neurotransmission. For example, decreases in dopamine receptor activity can cause reductions in the elicited hedonic pleasure response associated with food and a resultant compensatory overconsumption of food (Ferrario et al., 2016; Kroemer and Small, 2016). In addition, since one of the downstream mesolimbic dopamine targets is the hypothalamic feeding center, reduced dopamine signaling also leads to a resultant decrease in inhibitory satiety cues that serve to discontinue food consumption (Ferrario et al., 2016). In order to understand the underlying pathophysiology of alterations in dopamine neurotransmission, the following sections will discuss the mechanisms involved in dopamine neurotransmission as well as the effects of obesity and HFD on normal dopamine neurotransmission.

Regulation of Dopamine Neurotransmission

Dopamine is a neurotransmitter that affects both the peripheral and central nervous system. Within the central nervous system, dopamine exerts potent effects due to the nature of dopamine neuron morphology, the unique dopamine transmission and receptor signaling mechanisms, as well as the specialized firing patterns of dopamine neurons (Cachope and Cheer, 2014; Goto and Grace, 2008; Grace, 2000; Grace et al., 2007).

The majority of dopamine neurons in the CNS reside in midbrain nuclei of the ventral tegmental area (VTA) and substantia nigra brain (Burke et al., 2017). These neurons have a unique morphology that features dense, diffuse axonal arborization (Cachope and Cheer, 2014; Goto and Grace, 2008; Grace, 2000; Grace et al., 2007). Dopamine neurons have been likened to oak trees where the branches of one neuron fills a large portion of their target regions (Arias-Carrión et al., 2010; Richard Palmiter, 2010). For example, a single dopamine neuron fills about 10% of the striatum, contacting tens of thousands of medium spiny neurons in the NAc, which are one of the predominant neuronal targets of dopamine signaling. The diffuse spread of these dopamine axons leads to a subsequent diffuse mode of dopamine transmission, or “volume transmission” (Cachope and Cheer, 2014; Richard Palmiter, 2010). Unlike synaptic, or “wired” neurotransmission that is ideal for high-speed and precise signaling, the temporally slower and diffuse nature of volume transmission is optimized for the modulation and fine-tuning functions of dopamine (Taber and Hurley, 2014). This

allows dopamine to act as a regulator, amplifier and reinforcer in response to salient stimuli (Arias-Carrión et al., 2010).

Dopamine cell firing causes vesicular release of dopamine into the synaptic cleft via fusion with the pre-synaptic membrane. After dopamine is released, it subsequently binds to a family of G-coupled protein receptors located on target neurons. These dopamine receptors, which include classes D1-D5, are generally categorized as either low-binding-affinity D1-like receptors (D1, D5) or high-binding-affinity D2-like receptors (D2, D3, D4) (Beaulieu and Gainetdinov, 2011). Medium spiny neurons (MSNs), which make up 95% of the neuron population of the NAc, can express both D1 and D2 receptors; however most express either D1-like receptors or D2-like receptors (Burke et al., 2017; Ford, 2014; Scofield et al., 2016). In addition, there is a special population of D2-like autoreceptors (D2 and D3) that are expressed on dopamine neurons in the VTA. These autoreceptors provide inhibitory feedback control over pre-synaptic neuron activity by regulating tonic dopamine levels, DAT activity, dopamine synthesis, as well as the frequency and amplitude of phasic dopamine release (Cachope and Cheer, 2014; Ford, 2014; Siciliano et al., 2015a; Sulzer et al., 2016).

Since there is a pattern of preferential expression of either D1-like receptors or D2-like receptors of MSN's within the NAc, it is interesting to note that there are functional distinctions between D1 and D2 receptors that emerge when one examines their different roles in response to the two different types of firing activity exhibited by dopamine neurons. Dopamine neurons fire regularly at

tonic, slow, pacemaker-like frequencies (2−10 Hz). In addition, they also fire in phasic bursts (~20 Hz). Tonic firing results in the release of small amounts of dopamine that primarily bind high-affinity, inhibitory, D2-like receptors (Arias-Carrión et al., 2010; Ford, 2014). Therefore, it is believed that tonic release largely serves a feedback role. In contrast, phasic firing is exemplified by rapid, discreet, high-frequency spike activity of 2-6 action potentials that are ≥ 20 Hz (Goto and Grace, 2008; Siciliano et al., 2015a; Sulzer et al., 2016; Wenzel et al., 2015). Phasic burst-firing, which occurs in response to biologically salient stimuli, such as unexpected rewards, reward cues, or anticipated receipt of a known reward (e.g. palatable food), leads to the release of large concentrations of dopamine that are sufficient to saturate high-affinity D2 receptors and also activate low-affinity D1 receptors. This high concentration is also sufficient to elicit the dopamine transporter (DAT) activity of dopamine neurons. The DAT serves several critical functions: 1) it quickly removes dopamine from the synaptic space to shape and ultimately terminate phasic signaling events 2) it provides a mechanism for recycling of dopamine for use in subsequent release 3) it also plays a role in regulating tonic extracellular dopamine levels (Cachope and Cheer, 2014; Rice and Patel, 2015; Siciliano et al., 2015a; Sulzer et al., 2016).

Both tonic and phasic firing are involved in the execution of motivated behaviors, but they have different roles in this respect. Interestingly, increased tonic dopamine is associated with enhanced reward seeking since it primes the

dopamine signaling system and therefore elicits increased phasic signaling responses to future stimuli (Beeler and Mourra, 2018; Siciliano et al., 2015a). However, since tonic dopamine primarily activates D2 receptors while phasic release activates both D1 and D2 receptors, it is interesting to compare the effects of activating low-affinity D1 versus high-affinity D2 receptors. The distinct qualities of D1 and D2 receptors related to binding affinity and target receptor effects suggests that they play complementary roles in the downstream effects of dopamine. MSN's in the NAc that predominantly have D2 receptors indirectly project to the ventral mesencephalon via the ventral pallidum and cause reduced motivation, motor output and goal-directed behavior (Arias-Carrión et al., 2010). In contrast, D1 receptor activity is associated with reinforcing reward and promotion of reward-conditioned place preference (Calipari et al., 2016; Lobo et al., 2010; Scofield et al., 2016).

Since mesolimbic dopamine neurotransmission governs reward and goal-seeking behaviors, alterations in dopamine neurotransmission can lead to behavioral changes in food seeking and food intake. For example, the opposing roles of D1 and D2 receptors specifically related to food consumption have been demonstrated in a rodent model by Ball and colleagues. When rats were given a D1-like antagonist it completely blocked discrete cue-induced reinstatement of food seeking. In contrast, administration of D2-like antagonist significantly increased reinstatement responding that elicited cue-induced relapse to food seeking (Ball et al., 2011). A reduction in D2 autoreceptors, which serve an

important feedback role for dopamine release, also affect food seeking and intake behaviors. This was elegantly demonstrated in a novel cre/lox mouse model by Bello and colleagues. They reported that mice lacking D2 autoreceptors (autoDrd2 KO) had elevated dopamine synthesis/release, hyperlocomotion, elevated sensitivity to the psychomotor effects of cocaine, as well as enhanced motivation for food reward (Bello et al., 2011). Changes in DAT also have a profound effect on eating behaviors. The consequences of reduced DAT activity are exemplified by a study using DAT-knockdown mice. Compared to wild-type control, DAT-deficient mice have increased levels of extracellular dopamine and exhibit significantly greater food intake compared to control wild-type mice (Peciña et al., 2003). In humans, similar evidence showed that polymorphisms in the DAT gene associated with decreased DAT activity and DAT expression increased susceptibility to binge eating behaviors (Fuke et al., 2001; Heinz et al., 2000; Shinohara et al., 2004).

The above evidence demonstrates that altered dopamine neurotransmission can lead to behavioral changes in eating behaviors. Conversely, dietary factors and diet-induced obesity can lead to changes in dopamine neurotransmission.

Impact of Diet and Obesity on Dopamine Dysregulation

It is well-established that highly-palatable foods, which are high in fat and sugar, exert strong effects on the dopaminergic reward system (Ferrario et al., 2016; Matikainen-Ankney and Kravitz, 2018). Addiction research classically

employs food reward models since drugs of abuse also exert their effects on the dopaminergic reward system. (Di Chiara and Bassareo, 2007; Volkow et al., 2013, 2011). Drug addiction models are associated with changes in the expression and activity of the regulatory feedback for dopamine neurotransmission which cause pathological behavioral selection and anhedonia resulting in enhanced seeking/consumption of rewards (Coccurello and Maccarrone, 2018; Di Chiara and Bassareo, 2007; Scofield et al., 2016; Volkow et al., 2013, 2011). There is currently a debate about whether to classify chronic overconsumption of food related to obesity as a “food addiction”. However, addiction research has elucidated that pathological changes in dopamine neurotransmission observed in addiction models related to reduced numbers of functional DAT and dopamine receptors are also associated with obesity and high fat diets (Baik, 2013a; Cone et al., 2013; Fordahl et al., 2016; Narayanaswami et al., 2013).

One specific similarity between drug addiction models and obesity/HFD is related to D2 receptors. There is a large body of research demonstrating that many drugs of abuse have addictive effects due to resultant decreases in D2 receptor expression and activity. There is also evidence that obesity and HFD also decrease D2 receptor activity. Imaging studies have shown that D2 receptor expression is significantly decreased in obese subjects in inverse relationship to their body mass index (Volkow et al., 2003; Wang et al., 2001). Similar results were demonstrated in two studies that reported a negative association between

striatal D2 and D3 receptor function with increased BMI (de Weijer et al., 2011; Kessler et al., 2014; Matikainen-Ankney and Kravitz, 2018). In addition, multiple studies of HFD-induced obesity in rodents have demonstrated that overweight and obese rodents on HFD had significantly reduced expression of D2R as well as decreased D2R binding compared to control (Hajnal et al., 2008; Huang et al., 2006; Matikainen-Ankney and Kravitz, 2018). Decreased post-synaptic D2 receptors results in a dampened response to salient stimuli and potentially increase vulnerability to substance abuse since D2 receptors in the NAc provide inhibitory feedback to the VTA (Dobbs et al., 2017). Studies performed by the France lab using dopamine receptor agonists also support the evidence above. Rats who were fed a HFD were more sensitive to the D2/3 receptor agonist, quinpirole, compared to control. The HFD rats had increased sensitivity to quinpirole-induced discriminative stimulus effects and exhibited increased yawning (a behavioral effect of quinpirole) (Baladi et al., 2011; Baladi and France, 2010; Serafine and France, 2013).

Another key commonality observed between drug addiction and HFD-induced obesity models is decreased dopamine re-uptake by the DAT (Baik, 2013a; Cone et al., 2013; Fordahl et al., 2016; Narayanaswami et al., 2013). This is important since DAT uptake of dopamine is the primary mechanism for terminating phasic signaling in response to salient stimuli. The effect of reduced DAT function is delayed termination of phasic burst firing, enhanced tonic signaling, and an overall increase in synaptic dopamine (Sulzer et al., 2016).

Over time, elevated synaptic dopamine leads to a compensatory reduction in post-synaptic dopamine receptors and increased consumption of salient rewards (Blum et al., 2014; Koob, 2013; Narayanaswami et al., 2013). Additionally, decreased DAT function and the resultant high levels of extra-synaptic dopamine results in feedback mechanisms via pre-synaptic D2 autoreceptors. This is significant since the binding of dopamine to pre-synaptic D2 autoreceptors subsequently reduce excitability of dopamine neurons and also reduce synthesis and release of dopamine (Ford, 2014).

Decreased DAT activity observed with HFD-induced obesity is primarily due to decreased trafficking of the DAT to the pre-synaptic membrane. This is supported by a study in HFD-obese rodents where DAT mRNA and DAT protein are not globally affected however, the DAT was localized in cytosolic endosomes rather than the plasma membrane where they are functionally active (Cone et al., 2013). The functional effects of decreased levels of membrane DAT have been reported in studies of HFD-induced obesity where dopamine uptake, measured using voltammetry, was significantly reduced in rodents fed a diet high in fat compared to control (Cone et al., 2013; Fordahl et al., 2016).

The underlying explanation for the role of obesity/HFD in dopamine neuroplasticity that contributes to dysregulated food seeking and food intake behaviors are likely multifactorial. In addition to dopaminergic adaptations, plasticity changes in glutamatergic neurons that act on dopamine neurons in the NAc may also occur with a HFD, resulting in increased dopamine release

(Ferrario et al., 2016). Over time the increased release of dopamine leads to an adaptive decline in dopamine receptor availability via compensatory down-regulation mechanisms. While the above evidence of the effect of HFD/obesity on dopamine release and re-uptake demonstrates that it is implicated in dysregulated dopamine neurotransmission the underlying causes of these adaptive declines are not fully understood. However, insulin putatively plays a central role since it is involved in the regulation of mesolimbic dopamine release by acting in the VTA as well as dopamine clearance in the NAc (Ferrario et al., 2016). In addition, insulin's influence on the mesolimbic dopamine system are underscored by the fact that the mesolimbic dopamine system has a dense population of insulin receptors (Schulingkamp et al., 2000; Sulzer et al., 2016). In addition, diets high in saturated fat and obesity are associated with insulin resistance. Given insulin's role in dopamine neurotransmission, recent research findings related to the roles of insulin and the potential effects of insulin resistance merit a detailed discussion.

Insulin Regulation of Mesolimbic Dopamine Neurotransmission

Insulin is classically regarded as an anabolic hormone that is primarily involved with energy homeostasis and blood glucose regulation. However, it is now understood that insulin can cross the blood brain barrier and work in concert with the mesolimbic dopamine system to modulate goal-directed behaviors associated with food-seeking and food intake (Banks, 2004; Daws et al., 2011; Stouffer et al., 2015).

Compelling evidence of insulin's effects on dopamine signaling have been demonstrated in several human studies that used intranasal insulin (INI) administration. Kullmann and colleagues reported that INI led to decreased food consumption and attention to food-related cues (Kullmann et al., 2015, 2013). Similarly, Tiedemann and colleagues studied the effect of INI versus placebo on fasted subjects who were presented with images of highly-palatable food. Pharmacological fMRI revealed that INI reduced food palatability ratings as well as reduced "value" signaling in mesolimbic regions in subjects with normal insulin sensitivity. In addition, insulin inhibition mechanisms affected forward projections from the VTA to the NAc. Importantly, these effects were not observed in subjects receiving INI who were insulin resistant. (Tiedemann et al., 2017).

The effect of insulin on dopamine neurons have also been demonstrated in rodent models. The specific behavioral effects of insulin reported in rodent models include reduced food anticipatory behavior, decreased place-preference related to highly palatable food, and reduced hedonic feeding (Ferrario et al., 2016; Figlewicz et al., 2006, 2004; Labouèbe et al., 2013; S. Liu et al., 2013; Mebel et al., 2012; Patel et al., 2018). Closer examination of the mechanistic effects of insulin demonstrate that it has pleiotropic effects on dopamine neurons. The Borgland lab reported that insulin acts on dopamine neurons in the VTA by suppressing excitatory synaptic transmission (Labouèbe et al., 2013; Liu et al., 2016; Mebel et al., 2012).

Many of the effects of insulin are initiated when it binds to insulin receptors on dopamine neurons that in turn activate the PI3K/Akt signaling cascade. Once phosphoinositide 3-kinases (PI3K) are activated they initiate a classic signaling cascade by phosphorylating the D-3 position of phosphoinositides to generate PI(3,4,5) P3(PIP3), which then phosphorylates/activates protein kinase B (Akt) by acting at the plasma membrane as a second messenger (Daws et al., 2011). The PI3K/Akt signaling cascade is significant because it enhances surface expression of DAT. The Akt/DAT link has been demonstrated by several research groups, where inhibition of PI3K and Akt resulted in reduced dopamine clearance and surface expression of the DAT (Carvelli et al., 2002; Daws et al., 2011; Garcia et al., 2005). Doolen and colleagues used a different model to investigate insulin's effect on DAT trafficking. They reported reduced synaptic dopamine clearance due to decreased DAT protein levels at the plasma membrane in cells treated with tyrosine kinase inhibitors which block insulin-activated receptors (Daws et al., 2011; Doolen and Zahniser, 2001). The effects of insulin on DAT activity is further supported by studies using fast scan cyclic voltammetry (FSCV). In these studies, direct application of insulin on *ex-vivo* brain slices containing the NAc significantly increased dopamine uptake (Fordahl et al., 2016; Patel et al., 2018; Stouffer et al., 2015). The evidence of these effects are further supported when the underlying mechanisms were investigated using DAT-expressing embryonic cells which exhibited significantly increased dopamine uptake when insulin was applied to the tissue cultures (Carvelli et al., 2002).

Given the effects of insulin on dopamine neurotransmission, it has been proposed that insulin resistant states could contribute to the adaptive decline in dopamine neurotransmission observed with the development of obesity and metabolic syndrome.

Obesity, Saturated Fat and the Etiology of Insulin Resistance with Dopamine Neurotransmission

Changes in dopamine neurotransmission related to obesity/HFD compared with the effects of insulin on dopamine neurotransmission are striking since obesity/HFD and insulin have several significant opposing effects related to regulation of DAT and dopamine receptors. This suggests that that obesity and HFD may affect dopamine neurotransmission by causing insulin resistance and thereby impairing the effectiveness of insulin to act on dopamine neurons. The link between HFD and insulin-related dysregulation of dopamine neurotransmission is supported by two key lines of evidence: 1) There is a growing body of research that demonstrates an association between insulin resistance/diet-induced obesity with dysregulated dopamine neurotransmission 2) It is well-established that obesity and diets high in saturated fat are associated with peripheral insulin resistance.

Repeated exposure to a HFD has been mechanistically linked to impairment of insulin signaling (i.e., insulin-activated signaling kinase), decrease of DAT cell expression and activity, dopamine clearance and amphetamine-mediated effects (Speed et al., 2011). In addition, the association between

obesity and HFD on insulin's effect on dopamine neurotransmission have been demonstrated in hypoinsulinemic and streptozotocin-induced (STZ) diabetic rodent models. Hypoinsulinemic rats had a significantly decreased maximal velocity (Vmax) for dopamine uptake compared to control (Daws et al., 2011; Patterson et al., 1998). Similarly, STZ rats had significantly decreased trafficking and activity of DAT evidenced by decreased in dopamine uptake (Daws et al., 2011; Owens et al., 2012, 2005; Williams et al., 2007). The France lab corroborated the above results using dopamine receptor agonists. They reported that, compared to a chow control, rats on a HFD had increased sensitivity to quinpirole and a parallel decreased insulin sensitivity (Baladi et al., 2011). Evidence of the link between insulin resistance and altered dopamine neurotransmission have also been observed in human imaging research by Caravaggio and colleagues. Their study used PET scans in combination with radiotracers specific to D2/3 receptors and alpha-methyl-para-tyrosine-induced dopamine depletion. They reported that there was a positive association between insulin sensitivity and ventral striatal levels of D2/3R (Caravaggio et al., 2015).

The mechanistic explanation related to obesity/HFD and insulin's regulation of dopamine neurotransmission is not well understood. However, it has been proposed that inflammation plays a role given the well-established effects of obesity and HFD in promoting systemic inflammation and subsequent insulin resistance.

Regarding the effect of fat storage in peripheral tissues, it is well established that obesity induces insulin resistance due to its role in low-grade systemic inflammation (Chen et al., 2015; Rehman and Akash, 2016). Obesity-related inflammation mechanisms are a result of adipokine production and immune cell stimulation. Fat accumulation patterns observed with obesity are primarily due to excess accumulation of biologically active white adipose tissue (WAT). WAT releases pro-inflammatory adipokines including TNF-α, interleukin (IL)-6, and IL-1β. In addition, regions of WAT secrete chemo-attractants such as MCP-1 and MIF that recruit local immune cell populations that express pro-inflammatory M1 macrophages that are present in excess compared to their M2, anti-inflammatory macrophage counterparts. The inflammation due to pro-inflammatory adipokines and activity of the pro-inflammatory M1 macrophages induce insulin resistance by activating serine kinases such as IκB kinase (IKK) and JNK. IKK and JNK kinases directly induce insulin resistance by phosphorylating/inactivating insulin receptor substrate-1 (IRS-1). This directly counters insulin's ability to induce tyrosine phosphorylation of IRS-1 (McArdle et al., 2013).

In addition to inflammatory adipose tissue, diets that are specifically high in saturated fatty acids (SFA) also promote inflammation-induced insulin resistance since SFAs are precursor components of pro-inflammatory signaling pathways. These include the following: 1) SFA is a precursor of diacylglycerols (DAG) that affect PKC activation (Kumashiro et al., 2011); 2) SFAs are also

precursors to ceramides. Ceramides are an inflammatory class of sphingolipids that induce insulin resistance by inhibiting Akt/PKB signaling through two independent pathways. Ceramides catalyze the dephosphorylation of Akt/PKB by activating protein phosphatase 2A and block Akt/ PKB translocation to the plasma membrane (Glass and Olefsky, 2012; Holland et al., 2007). 3) SFAs also stimulate NADPH oxidase production of reactive oxygen species (ROS) that in turn act on ‘‘lipid rafts’’ domains in macrophages and lead to stimulation of inflammation via JNK1 (Rogero and Calder, 2018; Verdile et al., 2015). 4) Finally, one of the best studied roles of SFAs is their ability to induce the release of pro-inflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-1β by causing the dimerization of the toll-like receptors, Tlr2 and Tlr4 (Rogero and Calder, 2018).

Based on the large body of evidence related to the effect of saturated fat on inflammation in peripheral tissues, there has been increased interest in understanding the effect of SFA in inflammation in the CNS. Preliminary studies suggest that SFAs exert pro-inflammatory effects on the CNS by activating immune response. Dietary SFA has been associated with increased production of pro-inflammatory cytokines related to microgliosis and insulin resistance (Erta et al., 2012; Jais and Brüning, 2017; Shih et al., 2015). While this preliminary evidence suggests that SFA is implicated in neuroinflammation, more investigation is needed to understand the full impact of dietary fat on

inflammation in the CNS and its resultant effects on neurotransmission and brain health.

Polyunsaturated Fatty Acids

In contrast to the role that SFAs play in inflammation and insulin resistance, omega-3 polyunsaturated fatty acids (PUFA) exert opposing effects that reduce inflammation and promote insulin sensitivity. Several large human studies have demonstrated an association between increased dietary PUFA and improved insulin sensitivity. Several key observational studies have also reported an inverse association between circulating PUFA levels and the index of insulin resistance (Lalia and Lanza, 2016). PUFAs exert their influence on inflammation and insulin sensitivity via numerous anti-inflammatory/regulatory mechanisms. These include anti-inflammatory effects that are not currently linked to SFA-induced insulin resistance that are outside the scope of this review.

The ability of PUFA to antagonize the actions of SFA-induced insulin resistance has been well documented in numerous animal studies where SFA-induced insulin resistance in both mice and rat studies was attenuated with PUFA supplementation. (Kalupahana et al., 2010; Lalia and Lanza, 2016; Lionetti et al., 2014; X. Liu et al., 2013). The ability of PUFAs to attenuate insulin resistance are mechanistically explained by studies that examined the association between PUFA and insulin signaling pathways in peripheral tissues. In these studies, SFA-induced insulin resistance models demonstrated that PUFA increased mRNA expression of insulin receptor (IR) and insulin receptor

substrate-1 (IRS1), both of which promote insulin signaling through increased activation of IR/IRS1 phosphorylation and subsequent PI3K activity (Lalia and Lanza, 2016).

Pre-clinical cell and animal models provide evidence to explain the underlying mechanistic effects of PUFA on insulin signaling and inflammation. The effects of PUFA on insulin signaling pathways have been attributed in part to PUFA's well-established antagonism of classic pro-inflammatory TLR-associated cytokines, thereby reducing production of TNF-α, IL-6, and IL-1B (Calder, 2015). Some of the mechanistic effects of PUFAs on these cytokines include: 1) Activation of the transcription factor, peroxisome proliferator activated receptor gamma (PPAR-γ), which physically inhibits the translocation of NFκB to the cell nucleus (Rogero and Calder, 2018), 2) Inhibition of the cell-membrane lipid raft signaling between TLR4 and myeloid differentiation primary response gene 88 (MyD88) (Calder, 2015), 3) Activation of the G-protein-coupled receptor 120 (GPR 120) which inhibits both TLR and subsequent downstream TNF-α inflammatory signaling pathways by reducing activation of the NFκB signaling pathway (Calder, 2015; Lalia and Lanza, 2016; Oh et al., 2010).

Conclusion

It is important to understand how diets high in SFA cause dysregulated dopamine signaling in the NAc since it would elucidate the underlying causes of diet-induced obesity associated with excessive/compulsive food seeking and consumption that exceed metabolic needs. The above sections have presented

evidence that 1) Diets high in SFA can cause inflammation, insulin resistance and dysregulated dopamine neurotransmission, 2) Insulin resistance has adverse effects within the mesolimbic dopamine pathway since insulin fine tunes dopamine neurotransmission to reduce food seeking and support meal termination, and 3) Unsaturated fat exerts anti-inflammatory effects that oppose the effects of SFA and improve insulin sensitivity. Despite the overlapping associations described above, there is a paucity of research establishing definitive links related to the inflammatory effects of different dietary fats on the insulin sensitivity of neurons in the VTA-NAc dopamine mesolimbic pathway even though it has one of the densest population of insulin receptors in the CNS (Schulingkamp et al., 2000; Sulzer et al., 2016). For these reasons, addressing research gaps related to effects of dietary fat on inflammation associated with dopamine neurotransmission are warranted. Since diets high in unsaturated fats promote brain health and improve insulin signaling putatively though attenuated inflammation, the anti-inflammatory properties of unsaturated fats, these "healthier" fats may preserve dopamine signaling and prevent adaptations at dopamine terminals in the NAc that contribute to hedonic food intake.

CHAPTER II

THE EFFECTS OF A HIGH SATURATED FAT DIET ON FOOD INTAKE, METABOLIC STATUS, AND LOCOMOTOR BEHAVIORS IN MICE ARE PREVENTED BY UNSATURATED FAT

Introduction

There is a growing body of evidence that implicates diets high in saturated fat with increased risk for the development of metabolic syndrome in humans (Chen et al., 2015; Nettleton et al., 2014; Rehman and Akash, 2016). Similarly, rodent studies investigating the effects of dietary fat report the development of metabolic syndrome-like phenotypes characterized by obesogenic weight gain, insulin resistance, and chronic inflammation after consuming high amounts of saturated fat (Décarie-Spain et al., 2018; Jais and Brüning, 2017; Rogero and Calder, 2018). In addition, rodent studies suggest that normal, homeostatic food intake and locomotor behaviors are also disrupted by high saturated fat feeding (Décarie-Spain et al., 2018; Fordahl et al., 2016; Hryhorczuk et al., 2016; Speed et al., 2011). While a diet high in saturated fat is directly linked to metabolic pathophysiology, weight gain, and behavioral changes, it is not known whether unsaturated fats can attenuate or prevent the metabolic and behavioral effects observed with a high saturated fat diet.

The most significant effects of a high fat (HF) diet leading to excessive food intake are observed when rodents have free access to a HF diet (Hryhorczuk et al., 2016; Jones et al., 2017; Licholai et al., 2018; Yang et al., 2014). Compared to intermittent access to HF food, mice specifically provided an *ad libitum* diet high in saturated fat consumed food well beyond their metabolic calorie needs. This is interesting since it suggests that hedonic hunger may be the primary contributor to overconsumption of a HF diet (Licholai et al., 2018). Since hedonic food intake is regulated by the nucleus accumbens (NAc), it is possible that a HF diet specifically causes plasticity changes in the NAc that lead to hedonic reward that overrides homeostatic satiety cues (Arias-Carrión et al., 2010; Berridge, 2009). This is supported by a study examining the effects of cocaine, which directly affects dopamine signaling in the NAc of HF-fed rats (Serafine et al., 2016). Rats fed a HF diet gained significantly more weight than LF controls, developed metabolic dysfunction, and exhibited increased sensitivity to cocaine-induced locomotion. Another study that compared HF to LF reported anxiodepressive behaviors and increased motivation to consume sucrose in HF-fed mice with metabolic dysfunction. Interestingly, viral inhibition of the inflammatory IKKb/NFkB pathway in the NAc prevented diet-induced anxiodepressive behavior and neuroinflammation caused by the HF diet (Décarie-Spain et al., 2018). In addition to the anxiodepressive phenotype, HF diets are implicated in numerous neural adaptations that lead to escalating intake

of food, compulsive behaviors, and anhedonia (Pandit et al., 2012; Sharma and Fulton, 2013).

It is important to emphasize that the well-established metabolic syndrome-like phenotype described above is produced by diets that are specifically high in saturated fat. However, it is not known whether different types of fat have the same effect. This is an essential question to address since saturated fat is specifically associated with pro-inflammatory states while mono- and polyunsaturated fats have anti-inflammatory effects that are associated with healthy metabolic profiles (Calder, 2015). Indeed, emerging evidence suggests that there are important differences. It has been consistently demonstrated that rodents provided diets high in mono- and polyunsaturated fats do not develop the obesity, insulin resistance, or systemic inflammation observed in rodents fed diets high in saturated fat. In recent studies, these metabolic differences were also accompanied by changes in behaviors such as reduced sensitivity to cocaine and amphetamine as well as reduced anxiodepressive and compulsive sucrose-seeking behaviors (Décarie-Spain et al., 2018; Fordahl et al., 2016; Serafine et al., 2016). Interestingly, the diet-induced inflammation and neuroplastic changes in the NAc observed with a diet high in saturated fat were ameliorated when unsaturated fat was directly infused into neurons that project to the NAc (Hryhorczuk et al., 2018). In addition, unsaturated fat decreased food intake, increased locomotor activity, and attenuated the rewarding-seeking behaviors to attain highly-palatable food.

Since high saturated fat diets and a resultant metabolic syndrome-like phenotype impact locomotor activity and behavioral responses to drugs that act on dopamine signaling while unsaturated fats attenuate responses to dopamine agonists and dopamine neuron activity, we examined whether a diet high in unsaturated fats had similar effects on overall locomotor behaviors. We also examined whether differences in metabolic phenotype between high saturated vs. high unsaturated fats contributed to these effects. In order to verify that our model replicates the metabolic syndrome-like phenotype reported in previous studies, we provided mice with a high saturated fat diet and measured: 1) food intake; 2) weight gain; 3) glucose clearance as a proxy for metabolic dysfunction; and 4) locomotor behaviors in an open field test. Matching groups of mice were fed HF diets containing unsaturated flaxseed oil to test whether unsaturated fat prevents the development of metabolic and behavioral disorders commonly associated with obesity and diets high in saturated fat. We hypothesized that unsaturated fat would attenuate or prevent weight gain, improve blood glucose regulation, and attenuate changes in locomotive behaviors observed with a diet high in saturated fat.

Methods

Animals and Diet

Six-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed three per cage, maintained on a 12 h light/dark cycle with access to water and fed an *ad libidum* low-fat control

diet (LF; 10% kcals from fat; D12450J, Research Diets, n=6) or one of three nutrient matched experimental diets containing 60% kcals from either saturated fat (SFD) (D12492, Research Diets, n=9), saturated fat combined with flaxseed oil (Blend) (1:1 ratio of saturated fats to n3 polyunsaturated fats) (Prelim Form 2, Research Diets, n=6) or flaxseed oil alone (FSO) (Prelim Form 1, Research Diets, n=6). Custom flax diets were similarly nutrient matched to both high and low-fat diets, and calorically matched to the high saturated fat diet (Appendix; Table I). Food intake was measured on M/W/F by weighing the amount of diet consumed and calculating caloric intake based on food disappearance, and averaging intake by the number of mice in each cage. Body weights were measured upon arrival and weekly thereafter. Mice remained on respective diets for 6 weeks prior to experimental tests. All experiments were in compliance with the University of North Carolina at Greensboro Animal Care and Use Committee.

Behavioral Testing

Locomotor activity was assessed using an open field test. Behavioral experiments were initiated during the first four hours of the light cycle and were conducted using plexiglass chambers (30 cm × 30 cm × 60 cm). The chambers were sanitized prior to each experiment and the walls of the plexiglass chambers were wrapped in white coating with white disposable white floor linings to reduce external stimuli variability. Mice were removed from their home cages, immediately placed in the center of the arena and allowed to explore freely for 10 minutes. The total distance traveled (mm) and number of bouts entering the

center were measured using TopScanLite (Version 2.0, CleverSys Inc) for the first 10 minutes after being placed in the arena. The defined center area (45% of perimeter area) and length of time used for behavioral analysis were derived from standard reported practices for open field behavioral testing (Seibenhener and Wooten, 2015).

Intraperitoneal Glucose Tolerance Test

At the end of the 6-week dietary protocol, all mice underwent an intraperitoneal glucose tolerance test (IPGTT) to measure glucose clearance as proxy for metabolic dysfunction. Briefly, mice were placed in clean cages two hours into the dark cycle with access to water, but no food, for a 9 h fast. Blood glucose levels were then measured from the tail vein using a TRUEtrack glucometer and blood glucose test strips (Rite Aid Pharmacy, Camp Hill, PA) to establish fasting blood glucose levels. An i.p. bolus of glucose (2 g/kg in 20% w/v saline) was delivered and repeat blood glucose measurements were subsequently performed at 15, 30, 60, and 120 min thereafter.

Statistical Analysis

Graph Pad Prism v.5 (La Jolla, CA) was used for all statistical analyses. Two-way repeated measures analysis of variance (ANOVA) was used to identify significant differences within and between treatment groups for body weight and IPGTT using Tukey's posthoc analysis to identify significant variations between groups at individual data points, when applicable. One-way ANOVA tests were used to analyze between group differences for food intake, fasting blood glucose,

area under the curve data for IPGTT, and locomotor activity measurements. Pearson's correlational analysis was used to identify relationships between distance travelled x body weight and center entries x body weight. Group data are presented as means ± SEM; statistical significance was set at $p \leq 0.05$.

Results

Food Intake Patterns and Body Weight

Mice in the SFD, Blend and FSO groups consumed significantly more calories (kcals) per day than the LF group (13.9 ± 0.18 g/d, 14.1 ± 0.39 g/d, 13.5 ± 0.31 g/d, and 10.0 ± 0.18 g/d respectively; $p < 0.0001$). There was no significant difference of caloric intake between the SFD, Blend and FSO groups (Figure 2.1A). At the end of the 6-week diet intervention the final body weight of the FSO group (32.6 ± 1.1 g) was significantly greater than the LF group (25.8 ± 0.9 g) ($p < 0.0001$), but significantly less than the SFD group (37.7 ± 0.9 g) ($p < 0.001$). The final body weights in the SFD and Blend (36.9 ± 1.3 g) groups were similar (Figure 2.1B), and the Blend group was significantly greater than the LF and FSO groups ($p < 0.0001$ and $p = 0.0042$ respectively). Therefore, even though there was no statistically significant difference in caloric intake between the SFD, Blend and FSO groups, the FSO group did not gain as much weight on average as either the SFD group or Blend groups ($p < 0.001$). However, the FSO group weighed significantly more than the LF group ($p < 0.0001$). A two-way ANOVA identified a significant effect

of dietary treatment on body weight ($F_{(3, 23)}$ = 20.7; p < 0.0001) (Figure 2.1B), indicating that dietary fat amount, and importantly fat type, both impacted body weight.

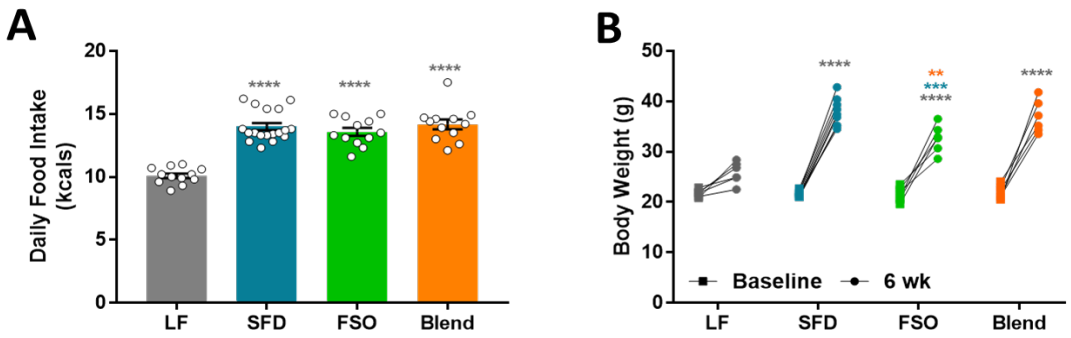

Figure 2.1 Food Intake and Body Weight

(A) Mice fed SFD (n=9), FSO (n=6), and Blend (n=6) diets consumed a similar amount of kilocalories (kcals), which was significantly greater than the LF group (n=6). **(B)** While all of the high-fat diet groups consumed significantly more kcals and gained significantly more weight compared to the LF group, the FSO group gained significantly less than the SFD and Blend groups.
(Group Mean ± SEM. One-way analysis of variance (A); Two-way analysis of variance, Tukey's post hoc (B))
(**p < 0.01, ***p < 0.001, ****p < 0.0001)

Blood Glucose Regulation

To examine the metabolic impact of SFD and unsaturated FSO, we measured fasting blood glucose and blood glucose clearance with IPGTT. Fasting blood glucose was not significantly different between the LF (118.3 ± 6.7 mg/dl) and FSO (109.7 ± 10.8 mg/dl) groups (p = 0.9637) (Figure 2.2A). In contrast, fasting blood glucose was significantly elevated in the SFD group (184.9 ± 13.1 mg/dl) compared to the LF (118.3 ± 6.7 mg/dl) and FSO (109.7 ± 10.8 mg/dl) groups (p = 0.0029 and p = 0.0008, respectively). The fasting blood

glucose in the Blend group (186.7 ± 13.7 mg/dl), which was not significantly different from the HF group, was also significantly higher compared to the LF and FSO groups ($p = 0.0054$ and $p = 0.0017$, respectively) (Figure 2.2A). Measuring the effectiveness of blood glucose clearance, IPGTT analysis revealed impaired blood glucose clearance in the SFD and Blend groups compared to LF and FSO groups. A two-way ANOVA identified a significant effect of time ($F_{(4,128)} = 6.637$; $p < 0.0001$) and dietary treatment ($F_{(3,32)} = 21.95$; $p < 0.0001$), as well as a time x treatment interaction ($F_{(12,128)} = 6.637$; $p < 0.0001$) in blood glucose levels measured during the IPGTT (Figure 2.2B). Tukey's post hoc analysis indicated significant differences in blood glucose between the HF and LF groups at BL ($p < 0.05$), 15min ($p < 0.05$), 30min ($p < 0.001$), 60min ($p < 0.0001$), and 120min ($p < 0.05$) timepoints. The Blend group had significantly higher blood glucose levels compared to the LF group (minutes 30 & 60, $p < 0.001$; minute 120, $p < 0.0001$). There were no significant differences in blood glucose levels between the LF and FSO groups for all timepoints. In contrast to the SFD and Blend groups, the FSO group was similar to the LF group and was significantly lower than the SFD group at the 30 and 60-minute timepoints ($p < 0.01$ and $p < 0.0001$, respectively) as well as the Blend group at the 30, 60, and 120-minute timepoints ($p < 0.001$, $p < 0.0001$, and $p < 0.001$, respectively).

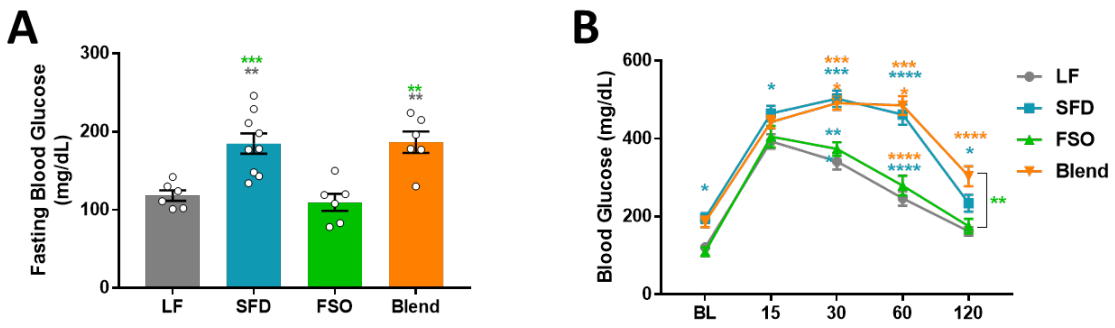

Figure 2.2 Blood Glucose Regulation

(A) Fasting blood glucose was significantly elevated in groups consuming diets containing saturated fat (SFD and Blend), but high amounts of fat as flax seed (FSO) did not elevate fasting blood glucose compared to controls fed LF. **(B)** Blood glucose clearance measured using IPGTT showed significantly slower clearance of blood glucose in SFD and Blend groups compared to LF and FSO groups indicating greater metabolic dysfunction in mice consuming saturated fat. (Group Mean ± SEM, One-way analysis of variance, Tukey's post hoc (A); Two-way analysis of variance, Tukey's post hoc (B))
(*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

Locomotor Activity

There were no differences in the mean total distance traveled (m) between the LF, SFD, FSO and Blend groups during open-field behavioral testing (25.95 ± 3.186 m, 21.45 ± 4.04 m, 25.04 ± 4.561 m, 24.2 ± 3.148 m, respectively) (Figure 2.3A). In addition, when distance travelled was plotted as a function of body weight, there were no significant associations observed between distance travelled and body weight (Figure 2.3B).

In contrast, a one-way ANOVA revealed differences in the number of center bouts, defined as the number of times the center of the subject entered the center of the arena. The mean number of center entries in the LF group were significantly greater compared to the SFD group (p = 0.037) (Figure 2.3D). While

the number of center bouts for the FSO and Blend did not differ from any other group, there was a noticeable trend such that the number of center entries increased with decreased levels of dietary saturated fat (Mean ± SEM of center entries: LF>FSO>Blend>SFD). In addition, analyzing the number of center entries as a function of body weight revealed a significant interaction in the LF group ($F_{(3,18)} = 4.398$; $p < 0.05$). In the LF group, the increased number of center entries was correlated with lower body weight $r = -0.945$; $p = 0.004$) (Figure 2.3E).

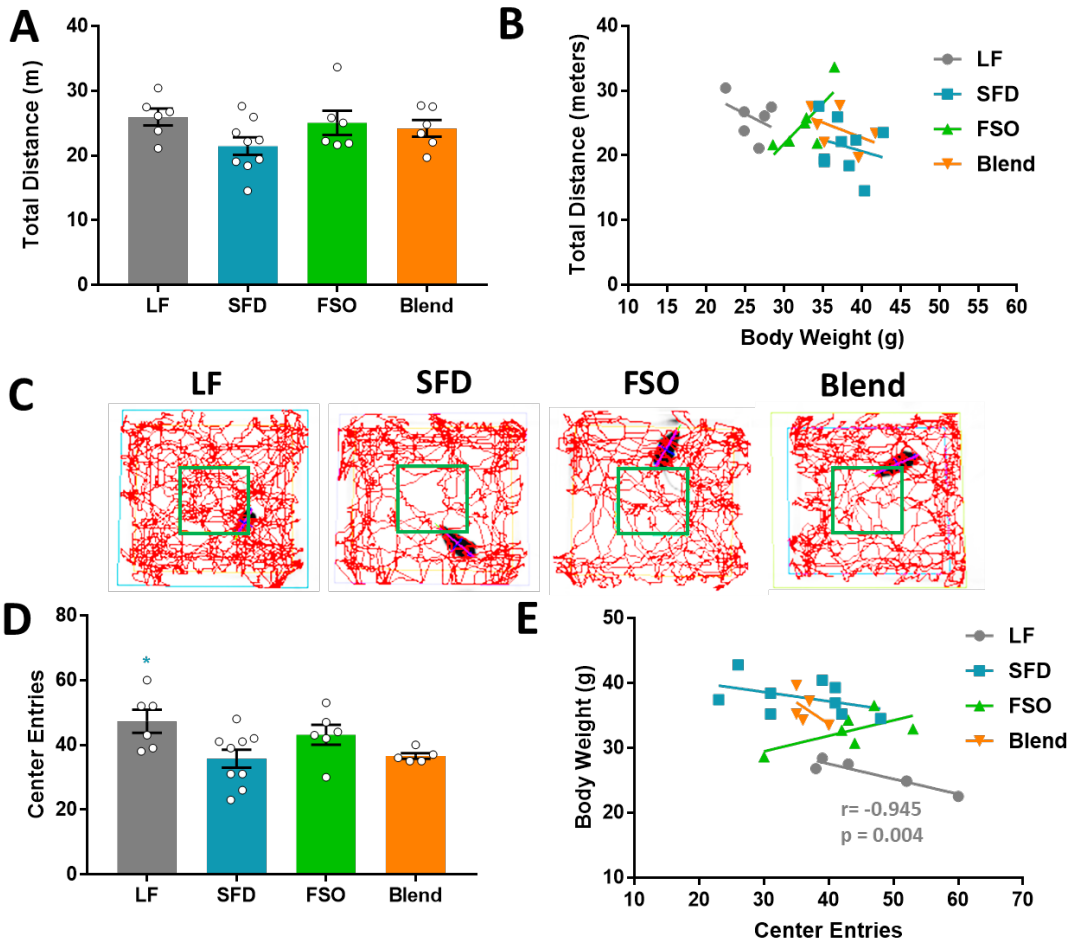

Figure 2.3 Open-field Locomotor Activity

(A) There were no statistically significant differences in mean total distance traveled between groups. **(B)** A linear regression revealed no interaction between distance travelled and body weight or significant differences between groups. Pooled analysis (not graphically represented) showed no significant variability in distanced travelled. **(C)** A visual tracing of locomotor activity in open-field behavioral testing with green box delineating the center of the arena. **(D)** In contrast to distance travelled, the SFD group exhibited increased thigmotaxic tendencies as evidenced by a significantly reduced number of entries into the center of the arena compared to the LF group ($p < 0.05$). **(E)** Moreover, a linear regression revealed an increased number of center entries was associated with lower body weight in the LF group ($r = - 0.945$).
Group Mean ± SEM. One-way analysis of variance (A); One-way analysis of variance, Tukey's post hoc (D) (*$p < 0.05$)

Discussion

This study sought to compare metabolic and consummatory phenotypes in our model of high saturated or unsaturated fat feeding against previous studies that observed obesogenic weight gain, increased food intake, and locomotor changes in mice. We observed that metabolic and behavioral changes associated with classic obesogenic diets are specifically dependent on the type of dietary fats consumed. Similar to other studies, mice with free access to a diet high in saturated fat (SFD) consumed significantly more food and also gained significantly more weight compared to the LF group. However, we also observed that free access to a high-fat diet that contained unsaturated fat from flaxseed oil attenuated weight gain compared to a diet high in saturated fat. Moreover, in contrast to our SFD, the unsaturated FSO diet preserved normal blood glucose clearance and behavior in open-field testing.

High-fat Diet Promotes Increased Food Intake Leading to Obesogenic Weight Gain

We observed similar food intake in the SFD, FSO and Blend groups indicating palatability of all high fat diets were comparable. Interestingly, even though all high-fat diet groups gained significantly more weight than the LF group, it is important to note that weight gain in the FSO group was significantly lower compared to the SFD group. This is consistent with similar observations from the Fulton lab and suggests that a diet low in saturated fat but high in unsaturated fat attenuates weight gain (Décarie-Spain et al., 2018). However, it

is important to note that the beneficial effect of the flaxseed oil substituted for saturated fat in the Blend group did not attenuate the excessive weight gain observed in the SFD group. This difference may provide insight into the other metabolic differences observed between groups described below. But it is also important to note that the Blend diet was higher in both saturated fat as well as n-6 fatty acids compared to the FSO diet. It is possible that the overall amount of unsaturated fat was not adequate to counter the effects of the saturated fat. Additionally the higher n-6:n-3 fatty acid ratio in the Blend diet may have been a confounding factor since there is evidence to suggest that a high n-6:n-3 ratio increases risk of obesity (Simopoulos, 2016).

High-fat Diet Potentiates Metabolic Impairments

We also sought to confirm that, compared to a low-fat diet, free access to a diet high in saturated fat would result in metabolic impairments observed in other studies. We observed that the SFD group had significantly elevated fasting blood glucose as well as reduced blood glucose clearance compared to the LF group. Impaired blood glucose clearance measured by IPGTTs, which is an indirect measure of insulin receptor function, is a validated proxy for assessing insulin resistance (Ayala et al., 2010). Saturated fat has been shown to promote insulin resistance by increasing production of pro-inflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-1β (Rogero and Calder, 2018). In addition, saturated fat is also a precursor for several classes of molecules, such as ceramides and diacylglycerol, which inhibit insulin signaling pathways (Glass and

Olefsky, 2012; Holland et al., 2007). Therefore, the impaired blood glucose clearance observed in the SFD group in this study are possibly due in part to the pro-inflammatory effects of saturated fat.

In contrast to saturated fats, unsaturated fats exert a number of anti-inflammatory effects that are associated with healthy blood glucose regulation (Calder, 2015; Lalia and Lanza, 2016). We hypothesized that substituting flaxseed oil, which is high in unsaturated fat, would prevent or attenuate the effects observed in the SFD group. Interestingly, even though the FSO group gain more weight than the LF group, they exhibited a metabolically distinct phenotype from the other high fat groups, characterized by reduced weight gain and normal fasting blood glucose and IPGTT blood glucose levels. These findings support the argument that the FSO group was metabolically similar to the LF group, indicating that unsaturated fat has less of an overall metabolic impact than saturated fat.

It is also important to note that mice consuming the Blend diet were metabolically similar to those consuming SFD. This suggests that replacing only a modest proportion of dietary saturated fat with unsaturated flax seed oil while still consuming high amounts of overall total dietary fat is not as effective at normalizing metabolic impairments as replacing a majority of dietary saturated fat with unsaturated fat. The lack of treatment effect of unsaturated fat in the Blend group could be due to several factors. First, the Blend group gained more weight than the LF and FSO groups. Since obesity itself promotes a pro-inflammatory

state that induces insulin resistance, the excess weight gained by the Blend group may have contributed to metabolic dysfunction (McArdle et al., 2013). It is also possible that the amount of unsaturated fat in the Blend diet, which was lower than the FSO group, was insufficient to overcome the deleterious effects of saturated fat. In addition, the Blend diet had a higher n-6:n-3 ratio compared to the FSO diet. Increased obesity risk and associated insulin resistance with a high fat diet with a high n-6:n-3 ratio versus a diet higher in n:3 has been reported by Liu and colleagues (X. Liu et al., 2013). They also observed that a diet high in n-6 fat was associated with higher inflammatory cytokines compared to the high n-3 diet. This association between a high n-6:n-3 ratio and inflammation could be related to evidence that a high level of n-6 unsaturated fats, which are precursors to several classes of pro-inflammatory eicosanoids, may counter the anti-inflammatory effects of n-3 unsaturated fats (Calder, 2015).

Anxiogenic Behavior Exhibited with Saturated Fat Diet

To establish whether the SFD diet in our study produced a behaviorally distinct phenotype compared to LF diet observed in previous studies, we assessed anxiogenic behaviors using an open field test. An open field test was used to evaluate behavior since it provides a rapid assessment of well-validated behavioral anxiety in rodents (Seibenhener and Wooten, 2015; Simon et al., 1994). Previous studies have shown that rodents consuming an obesogenic diet high in saturated fats displayed anxiogenic and depressive behaviors evidenced by decreased entries into the center of a novel, open-field environment (Cai et

al., 2018). These behaviors are associated with anxiety associated with exposure to novel environments typified by aversion to large, open areas and a resultant display of thigmotaxis (Seibenhener and Wooten, 2015; Simon et al., 1994). In our study, the SFD group exhibited increased thigmotaxic tendencies as evidenced by a significantly reduced number of entries into the center of the arena compared to the LF group. It is important to note that there was no difference in the total distance travelled between the LF and SFD groups. This suggests that the difference in center bouts observed in the SFD group is not due to overall distance travelled but a preference to limit exploration of a novel environment to walls in the perimeter. This is consistent with a model of anxiogenic behavior reported by the Fulton lab and others who reported a significantly decreased number of center entries in an open field test that was associated with other markers of anxiety and depression (Cai et al., 2018; Décarie-Spain et al., 2018; Sharma and Fulton, 2013). We hypothesized that there would be an observed attenuation in anxiogenic behaviors in the unsaturated fat diet groups compared to SFD. Interestingly, the FSO group had a similar number of center entries compared to the LF group. The FSO group also had a greater number of center entries compared to the SFD group. This difference was not statistically significant; however, the lack of treatment effect for unsaturated fat could be due to a small sample size or the design of the open field testing. Similar studies that report significant differences between treatment

groups used larger arenas and brightly-lit centers that more clearly elicit anxiogenic behaviors in rodents (Seibenhener and Wooten, 2015).

Conclusion

Overall, we show that the type of dietary fat, independent of the quantity of food consumed impacts body weight. Moreover, the type of dietary fat impacts metabolic and behavioral parameters. Mice fed a diet high in saturated fat consumed significantly more food and gained significantly more weight compared to their LF-fed counterparts. In addition, unlike the LF group, the SFD group displayed anxiogenic behaviors in open field tests. These findings verify that our SFD model is consistent with the established metabolic syndrome-like phenotype observed in previous preclinical models of diet-induced obesity. We also observed that an anti-inflammatory diet containing unsaturated fats in the form of FSO, in contrast to the SFD diet, attenuated weight gain and preserved blood glucose regulation and locomotor behaviors normally disrupted by saturated fat.

CHAPTER III

IMPAIRED DOPAMINE NEUROTRANSMISSION IN THE NUCLEUS ACCUMBENS OF C57BL/6 MICE IS ASSOCIATED WITH SATURATED BUT NOT UNSATURATED FAT INTAKE

Introduction

Research related to diet-induced obesity has been traditionally viewed through the lens of a homeostatic model of food intake modulated by the hypothalamus (Cassidy and Tong, 2017; Coccurello and Maccarrone, 2018). However, food intake is also governed by the mesolimbic dopamine signaling pathway. Central to this pathway is the nucleus accumbens (NAc), which is the limbic-motor interface and primary reward center of the brain (Goto and Grace, 2008; Salgado and Kaplitt, 2015). The NAc is believed to underlie conditioning that links the memory of salient rewards that include both homeostatic and hedonic experiences with the context in which they occur and subsequently influence the desire and future pursuit of rewarding, salient reinforcers. This is significant since the NAc specifically encodes salience of palatable foods and is also involved in promoting satiety (Arias-Carrión et al., 2010; Ferrario et al., 2016; Ferré, 2017; O'Connor et al., 2015).

There is currently a debate about whether to classify chronic overconsumption of food related to obesity as a "food addiction". However, it is

well established that highly-palatable foods exert strong effects on the dopaminergic reward system. Specifically, there is growing evidence that high fat (HF) diets and obesity lead to pathophysiological changes in the dopaminergic reward system similar to drug addiction (Di Chiara and Bassareo, 2007; Volkow et al., 2013, 2011). These changes are characterized by a hypodopaminergic phenotype with dampened dopamine response to rewards. This hypodopaminergic phenotype is thought to be initially due in part to reduced dopamine transporter (DAT) function that specifically results in delayed termination of phasic burst firing, enhanced tonic signaling, and an overall increase in synaptic dopamine (Sulzer et al., 2016). Over time, elevated synaptic dopamine concentrations lead to a compensatory reduction in post-synaptic dopamine D2 receptors and increased consumption of salient rewards (Blum et al., 2014; Koob, 2013; Narayanaswami et al., 2013). In addition to changes in post-synaptic D2 receptor function, changes in D2 autoreceptors on dopamine neurons are also observed with both addiction models and HF diet-induced obesity. The effects of HF diets on dopamine autoreceptors is significant since these inhibitory dopamine autoreceptors serve a key feedback role (Ford, 2014; Robinson et al., 2017). D2 autoreceptors modulate dopamine transmission by decreasing dopamine neuron excitability. In addition, dopamine autoreceptors regulate dopamine synthesis and decrease the level of dopamine released (Beaulieu and Gainetdinov, 2011; Ford, 2014).

The underlying mechanisms of HF diet-induced obesity that contribute to impaired DAT activity and the adaptive changes in D2 receptor function are not well understood. However, insulin may play a central role since it is involved in fine-tuning dopamine neurotransmission (Ferrario et al., 2016). Insulin exerts its effects by acting on dopamine neurons in the NAc to increase DAT recruitment to the plasma membrane (Daws et al., 2011; Doolen and Zahniser, 2001). Insulin also functions to fine-tune phasic dopamine bursts which in turn regulate postsynaptic dopamine receptor activation and dampen dopamine neuron firing in the VTA to reduce food seeking and support meal termination (Daws et al., 2011; Mebel et al., 2012; Stouffer et al., 2015). Given the important role of insulin related to dopamine signaling, there is a strong possibility that an association exists between impaired insulin signaling and impaired dopamine neurotransmission related to saturated fat and HF diet-induced obesity. This is supported by the large body of evidence demonstrating that saturated fat and obesity lead to systemic insulin resistance (Chen et al., 2015; Nettleton et al., 2014; Rehman and Akash, 2016). This is also specifically supported by evidence that an obesogenic, high saturated fat diet associated with impaired dopamine uptake and insulin resistance was attenuated when insulin signaling at dopamine terminals was restored (Fordahl and Jones, 2017).

While it has been demonstrated that obesity and diets high in saturated fat promote peripheral insulin resistance by inducing chronic inflammation, unknown is whether inflammation associated with diets high in saturated fat are implicated

in impaired dopamine neurotransmission (Chen et al., 2015; Rehman and Akash, 2016). Additionally, very little is known about the effect of different types of dietary fat on dopamine neuron function. However, it is important to address this gap since most research to date has only studied dopamine neurotransmission using diets that are primarily high in pro-inflammatory saturated fat. In contrast to the pro-inflammatory role that saturated fat has in insulin resistance, omega-3 polyunsaturated fatty acids (PUFA) have an opposing effect that reduce inflammation and promotes insulin sensitivity. This is well documented in numerous mouse and rat studies where saturated fat-induced insulin resistance was attenuated with PUFA supplementation (Kalupahana et al., 2010; Lalia and Lanza, 2016; Lionetti et al., 2014; X. Liu et al., 2013). In contrast to saturated fat, diets high in unsaturated fats have been reported to promote brain health, preserve behavioral responses to dopamine agonists, and improve insulin signaling in other regions of the brain, putatively through attenuated inflammation (Bazinet and Layé, 2014; Coccurello and Maccarrone, 2018; Trépanier et al., 2016). These effects have been attributed in part to PUFA's well-established role in reducing pro-inflammatory cytokines. This is supported by a multitude of cell and animal studies that demonstrate PUFAs reduce the production of cytokines such as TNF-α, IL-6, and IL-1B (Calder, 2015). Collectively, this suggests that the type of dietary fat may differentially impact dopamine signaling by mediating inflammatory status. While it is not currently known whether or how a diet high in unsaturated fat will affect dopamine signaling within the NAc, the

anti-inflammatory properties of unsaturated fats suggest that these “healthier” fats may preserve normal dopamine signaling.

Given the divergent roles that saturated and unsaturated fatty acids have on inflammation and insulin resistance, as well as the impact that reduced insulin signaling has on dopamine neurotransmission, we hypothesized that a diet high in saturated fat would induce the production of pro-inflammatory cytokines which in turn impair dopamine signaling, but anti-inflammatory unsaturated fats would not initiate cytokine production and would therefore preserve dopamine signaling. This study specifically examines whether preserving inflammatory balance with a diet rich in PUFAs would maintain proper control of the dopamine release and uptake mechanisms in the NAc. To test these hypotheses, we measured sub-second dopamine release and uptake at dopamine terminals in the NAc using *ex-vivo* fast scan cyclic voltammetry (FSCV) in mice fed diets high in saturated or PUFA rich flaxseed oil (FSO). We also measured the dopamine terminal response to quinpirole, a dopamine D2 receptor agonist, as well as levels of the inflammatory cytokine interleukin-6 (IL-6) in the NAc.

Methods

Animals and Diet

Six-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed three per cage, maintained on a 12 h light/dark cycle with free access to water and either a low-fat control diet (LF; 10% kcals from fat; D12450J, Research Diets, n=6) or one of three nutrient

matched experimental diets containing 60% kcals from either saturated fat (SFD; D12492, Research Diets, n=9), saturated fat combined with flaxseed oil (Blend; 1:1 ratio of saturated fats to n3 polyunsaturated fats) (n=6) or flaxseed oil alone (FSO; n=6). Custom flax diets were similarly nutrient matched to both high and low-fat diets, and calorically matched to the high saturated fat diet. Food intake was measured on M/W/F by weighing the amount of diet consumed and calculating caloric intake based on food disappearance. Total food consumed was divided by the number of mice in each cage. Body weights were collected upon arrival and weekly thereafter. Mice remained on respective diets for 6 weeks prior to experimental tests. All experiments followed the University of North Carolina at Greensboro Animal Care and Use Committee guidelines.

Fast Scan Cyclic Voltammetry

FSCV was used to characterize baseline dopamine release and uptake within the NAc core. FSCV is a useful tool to study dopamine neurotransmission in the NAc due to its excellent temporal and spatial resolution as well as its specificity and sensitivity for dopamine. Importantly, FSCV can measure the kinetics of dopamine uptake via the DAT, which is a key commonality observed between drug addiction and HF diet-induced obesity models. Since our experimental design also included use of the dopamine receptor agonist, quinpirole, we were able to characterize dopamine receptor functionality in the same locations where dopamine release and uptake are measured to more fully characterize overall dopamine neurotransmission in the NAc.

All voltammetry experiments were conducted after the 6-week dietary protocol, within two weeks after IPGTT using a Latin square design, and began ~3 h into the light cycle. Experimental procedures were executed and modeled as previously described (Fordahl and Jones, 2017). Briefly, mice were rendered unconscious using 5% isoflurane, decapitated, and the brain swiftly removed. The brain was hemisected with one hemisphere submerged in ice cold artificial cerebral spinal fluid (aCSF) (NaCl 126 mM, NaHCO3 25 mM, D glucose 11 mM, KCl 2.5 mM, CaCl2 2.4 mM, MgCl2 1.2 mM, 452 NaH2PO4 1.2 mM, L-ascorbic acid 0.4 mM, pH adjusted to 7.4) for slicing on a compresstome (Precisionary Instruments; Greenville, NC), and the other was used to excise NAc tissue for IL-6 quantification. Next, 300 μm brain slices containing the NAc (from +1.45 to +0.74 from bregma) were transferred to the voltammetry chamber and allowed to equilibrate for 60 min at 31.5 °C while bathed in oxygenated (95% O2/5% CO2) aCSF at 100 mL/min. Dopamine was recorded using a triangular waveform applied to a glass capillary-pulled carbon-fiber working microelectrode (70-100 μm length, 7 μm diameter). Briefly, the working electrode penetrated brain slices within the NAc core ~50 μM in depth and a bipolar stimulating electrode (Plastics One, Roanoke, VA, 8IMS3033SPCE) was placed on the surface of the slice approximately 100-200 μm away from the working electrode. The working electrode was maintained at a potential of -0.4 V versus a Ag/AgCl reference electrode and subsequently ramped up to +1.2 V and back to -0.4 V at a scan rate of 400 V/s every 100ms. Dopamine release was evoked with a single

electrical pulse (20Hz, 4 ms pulse width, 350 μA stimulation amplitude) from the stimulating electrode every 3 min. Next, dopamine release was evoked using 5-pulse stimulations (20Hz, 4 ms, 350 μA) to characterize phasic dopamine release. All recording electrode placements were grouped in the NAc core. Dopamine signals were acquired and modeled using Demon Voltammetry Software, based on Michaelis−Menten kinetics. Baseline recordings were obtained from one to two slices from each animal. To measure dopamine D2 auto-receptor function at dopamine terminals, quinpirole (Tocris; Minneapolis, MN; Cat. No. 1061) was subsequently washed over the slices with cumulative half-log applications to obtain a dose response curve (1-100 nM) at the same location as baseline collections. Dopamine current was converted to concentration by electrode calibration after each experiment using a flow cell, adding 3 μM of dopamine in combination with the Demon Analysis Software.

IL-6 Protein Quantification

The ventral striatum containing the NAc was rapidly dissected from the contralateral brain hemisphere used for voltammetry, and tissue was stored at −80 °C until use. An enzyme linked immunosorbent assay (ELISA) kit (Cayman Chemical, 583371) was used to quantify IL-6 protein content in NAc tissue per the manufacturer's instructions. Briefly, NAc tissue was homogenized in phosphate buffered saline (0.02 mol/L pH7.2) containing HALT™ protease inhibitor cocktail (ThermoFisher, 1860932) and tissue was further lysed by ultrasonication. Homogenates were centrifuged at 5000g for 5 min, and the

supernatant was used for BCA protein assay (ThermoScientific, Rockford, IL, 23225) and the IL-6 ELISA kits. IL-6 content was normalized to tissue protein and is reported as pg IL-6/mg protein.

Statistical Analysis

Graph Pad Prism v7.04 (La Jolla, CA) was used for all statistical analyses. One-way analysis of variance (ANOVA) tests were used to analyze between group differences for dopamine release, Vmax, and IL-6 in NAc. Group data are presented as means ± SEM and statistical significance was set at $P \leq 0.05$. A two-way repeated measures ANOVA was used to identify significant differences within and between treatment groups for voltammetry dose response curves using Tukey's posthoc analysis to identify significant variations between groups at individual data points, when applicable. Pearson's correlational analysis was used to identify relationships between dopamine release and body weight, Vmax and body weight, dopamine release and NAc IL-6, and Vmax and NAc IL-6.

Results

Effect of Dietary Fat on Dopamine Release and Uptake

Slice voltammetry was used to characterize differences in dopamine release and uptake in the NAc after 6-week exposure to the respective diets. No differences in tonic dopamine release were detected between groups (1.64 ± 0.72 μM, LF (n = 16); 1.38 ± 0.56 μM, SFD (n = 18); 1.17 ± 0.53 μM, FSO (n = 12); 1.36 ± 0.64 μM, Blend (n = 9) (Figure 3.1A). However, a significant effect of diet on dopamine uptake was observed ($F_{(3,46)} = 4.325$; $p = 0.001$).

Tukey's posthoc analysis revealed the maximal rate of dopamine uptake (Vmax) was significantly reduced in the SFD group (1.77 ± 0.34 μM, n = 18) compared to the LF (2.21 ± 0.31 μM, n = 10) and FSO (2.18 ± 0.28 μM, n = 12) groups ($p < 0.05$), indicating a reduction in DAT function (Figure 3.1B). No difference was observed between the LF and FSO groups suggesting that high amounts of dietary unsaturated fat prevented or attenuated impaired DAT function observed with a diet high in saturated fat. The Blend group (2.00 ± 0.55 μM, n = 10) was not significantly different from the LF, SFD or FSO groups (Figure 3.1B). Overall, there was no association between body weight and dopamine release (Figure 3.1C); however, Pearson's correlational analysis revealed a significant association between weight gain and impaired dopamine clearance in the SFD group ($r = -0.407$, $p < 0.05$). There was no association between body weight and dopamine uptake in the LF, FSO or Blend groups (Figure 3.1D).

The SFD group (172.9 ± 15.19, n = 9) also had significantly lower phasic neuron firing compared to the LF (195.6 ± 19.56, n = 7) and FSO (195.6 ± 25.21, n = 5) groups ($p < 0.05$). There were no other differences between the LF and FSO groups. The Blend group (182.8 ± 13.38, n = 4) was not significantly different compared to the LF, SFD, or FSO groups (Figure 3.1E).

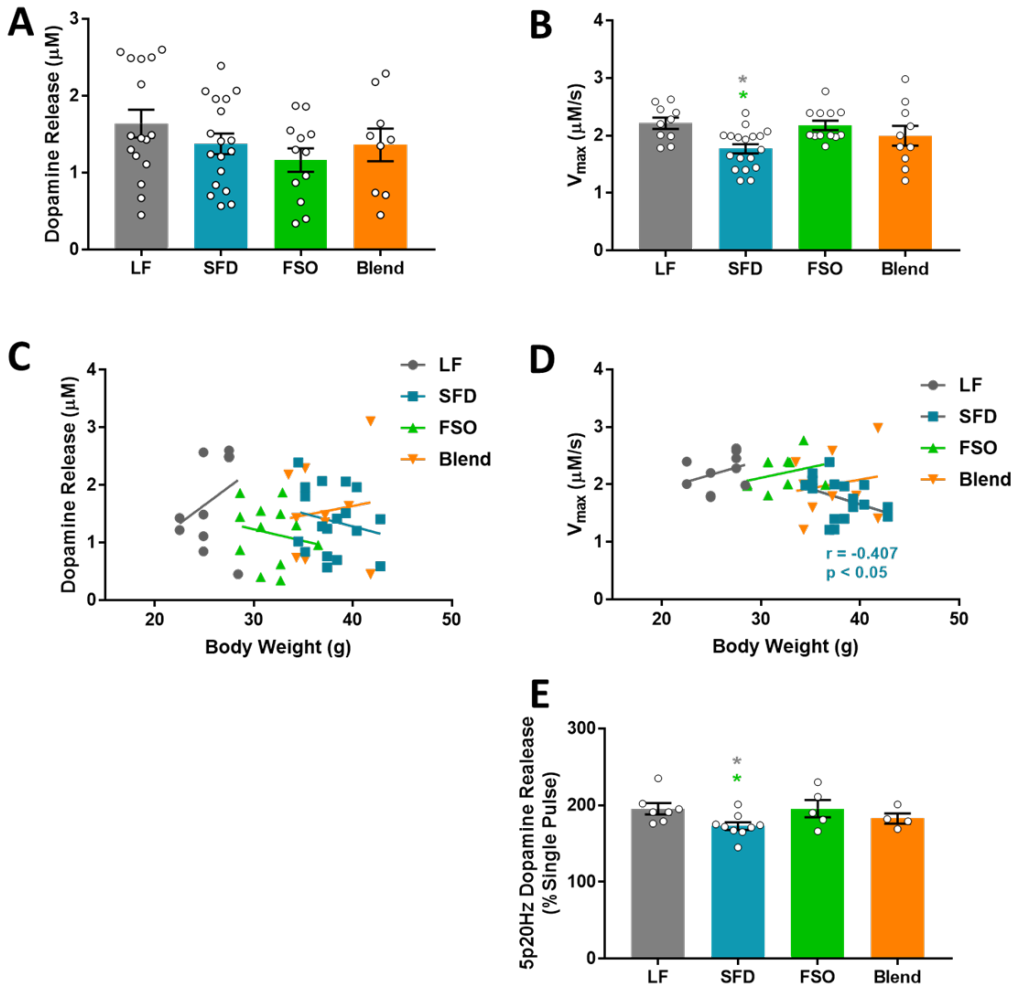

Figure 3.1 Dopamine Release and Uptake.

Ex-vivo fast scan cyclic voltammetry in slices containing the nucleus accumbens was performed to characterize differences in dopamine release and uptake. **(A)** No differences in dopamine release, evoked by a single pulse stimulation, were detected between groups. LF (n = 16); SFD (n = 18); FSO (n = 12); Blend (n = 9). **(B)** Vmax, the maximal rate of dopamine uptake from the synapse was significantly reduced in the SFD group (n = 18) compared to the LF (n = 10) and FSO (n = 12) groups ($p < 0.05$). **(C)** There was no association between body weight and dopamine release evoked by single pulse stimulations **(D)** There was a significant negative association between Vmax and body weight was observed in the HF group ($r = -0.407$, $p < 0.05$) **(E)** Multi-pulse (5p20Hz) stimulation was applied to slices to elicit dopamine release observed with phasic neurotransmission. The SFD group (n = 9) also had significantly lower dopamine release compared to the LF (n = 7) and FSO (n = 5) groups ($p < 0.05$).

(Group Mean ± SEM. One-way analysis of variance (A); One-way analysis of variance, Tukey's post hoc (B); Pearson correlation (C, D); One-way analysis of variance, Tukey's post hoc (E))
(*$p < 0.05$)

Effect of Dietary Fat on D2 Autoreceptor Function in the NAc

After collecting baseline parameters, dose response curves for quinpirole (a D2/D3 receptor agonist) were collected to characterize differences in D2 autoreceptor activity. A two-way ANOVA was used to identify differences in quinpirole sensitivity, detected by measuring reduced dopamine release with increasing quinpirole concentrations. There was a significant main effect of quinpirole dose on reduced dopamine release observed within each group ($F_{(4, 52)} = 177.8$; $p < 0.0001$), but no between group effects were observed when Tukey's multiple comparisons test was applied (Figure 3.2A). However, there was a leftward shift in the dose effect of quinpirole in the SFD group compared to the LF group indicating a slight increase in sensitivity to quinpirole in the SFD group (Figure 3.2B), though not statistically significant ($p = 0.1$).

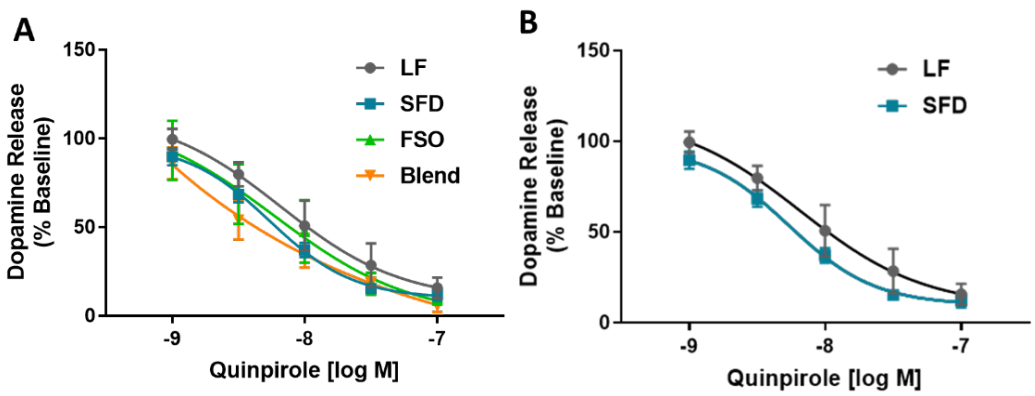

Figure 3.2 Effect of Diet on D2 Autoreceptors

(**A**) Dose response curves for the D2/D3 agonist quinpirole were used to identify changes in dopamine autoreceptor function in NAc slices. There were no significant differences between groups. **(B)** However, the SFD group was more sensitive to quinpirole compared to the LF group as evidenced by the leftward shift in the dose effect of quinpirole indicating a reduced magnitude of evoked dopamine release. (Group Mean ± SEM. Two-way analysis of variance, Tukey's post hoc (A); Two-way analysis of variance, Tukey's post hoc (B))

IL-6 Levels and Dopamine Neurotransmission

Protein levels of IL-6 were measured in tissue in the ventral striatum containing the NAc to assess neuroinflammation. IL-6 in the CNS is upregulated during neuroinflammatory events such as CNS infection, injury, and disease. Previous studies report significantly higher levels of mRNA expression of cytokines including TNF-α, IL-1β, and IL-6 in the CNS with a high-fat diet compared to low-fat controls (Wang et al., 2012a). Overexpression of IL-6 has also been associated with astrogliosis and microglial activation (Erta et al., 2012). There were no significant differences in the IL-6 protein levels between the LF (0.0209 ± 0.0034 pg/mg; n = 6), SFD (0.0193 ± 0.0060 pg/mg; n = 6), and FSO (0.0212 ± 0.0047 pg/mg; n = 6) groups. In addition, the Blend group (0.0262 ±

0.0017 pg/mg; n = 6) was not different from the FSO group; however, the IL-6 levels in the Blend group were significantly higher compared to the LF and SFD groups ($p < 0.05$) (Figure 3A). When dopamine release was examined as a function of IL-6 levels, there were no associations detected (Figure 3B). Similarly, no correlations between dopamine uptake and tissue IL-6 levels were observed; however, a near significant negative correlation was observed between Vmax and IL-6 in the SFD group ($r = -0.719$, $p = 0.053$) (Figure 3C), indicating that higher levels of IL-6 was associated with reduced dopamine clearance in this group.

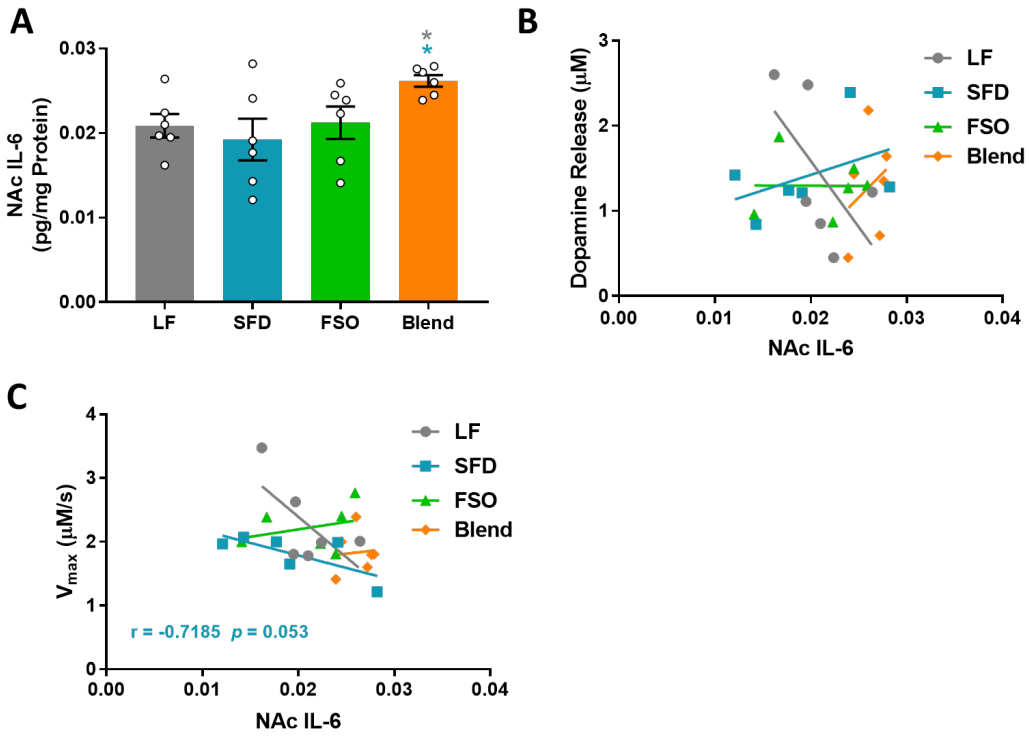

Figure 3.3 IL-6 Levels Related to Dopamine Neurotransmission

(A) Dietary effects on IL-6 protein levels in the nucleus accumbens (NAc) revealed significantly elevated IL-6 in the Blend group compared to LF and SFD groups. **(B)** No relationship was observed between dopamine release and tissue IL-6; **(C)** however, a near significant negative association between dopamine clearance (Vmax) and IL-6 was observed in the SFD group. These data suggest that saturated fat (SFD group) does not elevate basal IL-6, but IL-6 selectively impacted dopamine clearance in mice fed saturated vs. unsaturated fat.
(Group Mean ± SEM. One-way analysis of variance (A); Pearson's correlation (B, C))

Discussion

Effect of Dietary Fat on Dopamine Release and Uptake

It is well-established that diets high in saturated fat produce a metabolic syndrome-like (MetS) phenotype characterized by obesogenic weight gain and impaired blood glucose regulation related to insulin-resistance. Consistent with previous studies, we induced a MetS phenotype in our mice with a diet high in

saturated fat and observed that a diet high in mono- and polyunsaturated fat in the form of flaxseed oil prevented metabolic impairments.

Previous studies show that saturated fat-induced MetS phenotype is also associated with impaired dopamine neurotransmission (Baladi et al., 2011; Fordahl et al., 2016; Fordahl and Jones, 2017; Narayanaswami et al., 2013; Owens et al., 2012; Serafine and France, 2013; South and Huang, 2008; Speed et al., 2011; Williams et al., 2007). We similarly demonstrated that a diet high in saturated fat decreased phasic dopamine release and dopamine uptake, producing dopamine terminals that were dramatically different than a LF diet; however, we report a novel observation that a diet high in polyunsaturated fat specifically preserved normal phasic dopamine release and uptake from dopamine terminals in the NAc. Our findings build on previous studies which reported unsaturated fat, in contrast to saturated fat, prevented dopamine-related behavioral changes in locomotion, sensitization to cocaine, as well as anxiodepressive behaviors (Décarie-Spain et al., 2018; Serafine et al., 2016). Our observation that dampened phasic dopamine release only occurred in the SFD group is pertinent since phasic, burst-release of dopamine specifically occurs in response to salient stimuli such as high-calorie, highly-palatable food (Baik, 2013b, 2013a). This suggests that diets high in saturated fat could promote dysregulated feeding by dampening phasic dopamine release and subsequently reducing the perceived enjoyment of food thereby leading to compensatory intake to achieve the same reward magnitude (Blum et al., 2014;

Matikainen-Ankney and Kravitz, 2018). In addition, D1 receptors preferentially respond to phasic burst firing due to their low affinity for dopamine (Beaulieu and Gainetdinov, 2011; Burke et al., 2017; Ferrario et al., 2016; Ford, 2014; Scofield et al., 2016). Since D1 receptors project to the hypothalamus to promote satiety and terminate feeding, reduced phasic burst firing could further promote dysregulated feeding (O'Connor et al., 2015). This is supported by findings from the Fulton Lab, who reported that a saturated fat diet impaired the D1 receptor/PKA signaling pathway which is involved with the sensitizing and conditioning of response to reward (Hryhorczuk et al., 2016). In contrast, a diet high in monounsaturated fat in the form of olive oil preserved normal enzyme pathway signaling associated with D1 receptors.

In addition to the differences in phasic dopamine release, we observed a deficit in synaptic dopamine clearance in the SFD group compared to the LF and FSO groups. This is consistent with previous reports of in rodents that consume a diet high in saturated fat (Fordahl et al., 2016; Fordahl and Jones, 2017). Here we report the novel observation that type of dietary fat differentially impacts dopamine uptake. Specifically, unsaturated fat in the form of FSO preserved normal dopamine uptake and metabolic parameters, despite causing similar weight gain. It should also be noted that even though the SFD and FSO groups weighed more than the LF group, a negative association between dopamine uptake and body weight was only observed in the SFD group. The fact that the changes in DAT activity were affected by increased adiposity in the SFD group

alone is important to underscore in the context of findings from previous studies where similar changes in DAT activity were observed with high-fat, diet-induced obesity without consideration of the type of fat (Cone et al., 2013; Fordahl et al., 2016; Fordahl and Jones, 2017; Narayanaswami et al., 2013). Our results suggest that the type of dietary fat is an independent driver of impaired dopamine uptake and DAT activity apart from diet-induced obesity.

One possible reason for the observed differences in DAT activity between the SFD group and FSO groups could be associated protein kinase C (PKC) activation. PKC, a primary regulator of DAT surface expression, co-localizes with DAT and leads to the internalization of DAT (Gabriel et al., 2013; O'Malley et al., 2010; Vaughan and Foster, 2013). One of the molecules that activates PKC is diacylglycerol (DAG) (Melis and Pistis, 2012). Recent research has elucidated that different PKC isoforms have varying sensitivity to DAG depending whether DAG contains shorter and saturated fatty acids or longer and polyunsaturated fatty acids (Kamiya et al., 2016). Moreover, there is evidence that saturated fatty acids are preferentially converted to DAG compared to PUFA (Timmers et al., 2011). Therefore, enhanced PKC activation by DAG driven by saturated fat in our SFD group could lead to decreased DAT activity.

However, in addition the putative direct role of PKC and DAG on DAT internalization, extracellular signal regulated kinase (ERK) also regulates DAT activity (Vaughan and Foster, 2013). This is highly relevant to our study where we observe an insulin-resistant phenotype, as insulin acts on the ERK signaling

pathway via PI3K/ Akt kinase signaling to regulate DAT activity (Nash, 2017). Indeed, Fordahl and Jones demonstrated that impaired insulin receptor function on dopamine neurons associated with an insulin-resistant phenotype causes deficits in dopamine uptake. Moreover, saturated fat specifically impairs insulin-activated regulation of dopamine uptake in the NAc in a PI3K/Akt-dependent manner by decreasing dopamine transporter expression on the cell surface and reducing dopamine uptake (Patel et al., 2018; Speed et al., 2011).

In the current study we also observed that the SFD group exhibited an impaired metabolic phenotype that is an indicator of insulin resistance within the NAc (Fordahl and Jones, 2017). However, we uniquely demonstrated that FSO not only preserved blood glucose regulation but also preserved normal dopamine uptake in the NAc. The dramatic difference between the SFD and FSO groups may be explained by the well-established evidence that saturated and unsaturated fats have opposing effects on normal insulin signaling (Kalupahana et al., 2010; Lalia and Lanza, 2016; Lionetti et al., 2014; X. Liu et al., 2013). Therefore, while SFD produced an insulin-resistant state that impaired DAT activity, the preservation of blood glucose regulation with the FSO diet suggests that it preserved overall normal insulin signaling, thereby maintaining normal DAT activity.

Effect of Dietary Fat on D2 Autoreceptor Function in the NAc

To further characterize dopamine terminal responses to saturated and unsaturated fats quinpirole was used to identify changes in dopamine

autoreceptor function. Quinpirole is a D2/D3 receptor agonist that reduces the magnitude of dopamine release with increasing concentrations (Robinson et al., 2017). Accordingly, we observed a reduction in dopamine release as quinpirole dose increased but did not identify a differential effect of diet on autoreceptor activity between dietary treatments. However, the SFD group was more sensitive to quinpirole compared to the LF group as evidenced by the leftward shift in the dose effect of quinpirole in the SFD group compared to the LF. It is possible that increased sensitivity to quinpirole in the SFD group is due to increased dopamine autoreceptor activity as a compensatory response to prolonged dopamine exposure due to the reduced DAT activity observed in the SFD group. The increased D2 autoreceptor sensitivity is potentially an important finding since increased sensitivity to D2 autoreceptor agonists has been associated with the development of a hypodopaminergic state and subsequent dampened dopaminergic responses to salient stimuli (Siciliano et al., 2016, 2015b). Additional studies are needed to investigate these effects this since D2 autoreceptor sensitivity associated with high-fat diet are not well understood. However, the results of this study are consistent with previous reports and are also similar to findings reported by the France Lab (Fordahl and Jones, 2017). The France Lab showed that compared to rats eating standard chow, rats eating high fat chow had increased sensitivity to quinpirole-induced discriminative stimulus effects and exhibited increased yawning (a behavioral effect of

quinpirole) (Baladi et al., 2011; Baladi and France, 2010; Serafine and France, 2013).

IL-6 Levels and Dopamine Neurotransmission

Previous studies observed significantly higher levels of mRNA expression of cytokines including TNF-α, IL-1β, and IL-6 in the CNS (hypothalamus) with a high-fat diet compared to low-fat controls (Wang et al., 2012b). The Fulton lab also reported increased levels of plasma and NAc mRNA levels of TNF-α and IL-1β with a SFD versus a LF diet or a diet high in monounsaturated olive oil (Décarie-Spain et al., 2018). Building on these studies, we specifically assessed IL-6 protein expression within the NAc to assess neuroinflammation. While measuring cytokine protein expression specifically within the NAc was a strength of this study, the NAc affords a small amount of tissue per mouse. This limited our analysis of cytokine expression; therefore, future work could include measurement of additional cytokines such as TNF-α and IL-1β to more fully characterize inflammation.

There were significantly elevated IL-6 levels in the Blend group compared to the LF and SFD groups. This finding was not expected; however, the Blend group had higher levels of n-6 PUFA, which have been associated with elevated IL-6 levels (Calder, 2015; Patterson et al., 2012). Interestingly, we also observed a negative association between dopamine uptake and IL-6 in the SFD group suggesting that even though saturated fat did not elevate basal IL-6, it promoted an IL-6 sensitivity. The increase in sensitivity to IL-6 associated with changes in

dopamine neurochemistry may be a result of microglial activation since SFD has been shown to induce inflammation by activating microglia and is specifically associated with abnormal and prolonged activation of the IL-6 target, STAT3 (Erta et al., 2012; Lee et al., 2003; Vigneswara et al., 2012; Wang et al., 2013). This is significant since cytokines such as IL-6 that are released by microglia are known to cause neuronal insulin resistance (Ferreira et al., 2014). When insulin is unable to exert effects on dopamine neurons we observe a resultant decrease in dopamine uptake in the NAc.

While we did not observe a significant effect of dietary fat on IL-6 protein level expression, neurons, astrocytes, microglia and endothelial cells in the CNS only synthesize modest basal levels of IL-6 (Erta et al., 2012). Reactive IL-6 release from these cell types may only occur when challenged by damaging stimuli such as stroke, neurodegeneration, or agents associated with infection such as bacterial lipopolysaccharide (LPS) (Erta et al., 2012). Studies that induced a mounted inflammatory response with oxidative stress or application of LPS reported elevated levels of cytokines such as IL-6, TNF-α and IL-1β that were associated with changes in dopamine neuron function (Vumma et al., 2017; Wang et al., 2018). It is possible that the low levels of chronic inflammation, as observed with high amounts of saturated fat, may prime these immune response cells for an expedited or enhanced response to such stimuli. Enhanced reactivity of these immune cells may contribute to reports of poor prognosis in obese individuals that suffer from neurological injuries (Bas and Ozdemir, 2017; Cai,

2013). Since IL-6 in the CNS is upregulated during neuroinflammatory events such as LPS-induced injury, future studies could use LPS to initiate a neuroinflammatory response to better characterize diet-related differences.

Conclusion

Overall, this study demonstrated that different types of dietary fat have substantially different effects on dopamine terminal regulation. A diet high in saturated fat resulted in dampened phasic dopamine release and impaired dopamine uptake, all of which were prevented by a diet high in unsaturated fat. These differences may be associated with differential effects of fatty acids on signaling pathways such as DAG-PKC activation. However, given the known effects of insulin on dopamine neurotransmission, it is striking that the FSO diet also preserved blood glucose regulation, which was drastically impaired by high saturated fat. Evidence from previous studies demonstrating the effects of insulin on DAT activity in conjunction with the known effects of dietary fats on insulin sensitivity strongly suggest that the observed difference in DAT activity between the SFD and FSO groups is associated with the insulin sensitivity of dopamine neurons (Fordahl et al., 2016; Jones et al., 2017; Patel et al., 2018). The observed decrease in phasic dopamine release in the SFD group might also be attributed to impaired insulin sensitivity since the overall effect of post-prandial insulin release leads to reduced dopamine signaling (Liu et al., 2016; Mebel et al., 2012). An insulin-resistant state may cause altered dopamine signaling in the long term when chronically prolonged dopamine signaling in the NAc leads to

counter-regulatory neuroplastic changes including a hypodopaminergic, dampened phasic dopamine response. Our findings indicate that type of fat differentially impacts dopamine terminal regulation, which we show is related to the metabolic status of the mice. The impact of dietary fat and altered metabolism on neuroinflammation may contribute to this process, but further inquiry is necessary to characterize this relationship. The impact of dietary fat on dopamine neurotransmission is particularly relevant to neurological control of feeding, since dopamine signaling in the NAc helps initiate satiety (O'Connor et al., 2015). In conclusion, our findings suggest that a diet high in unsaturated fat, in the form of flaxseed oil, may preserve dopamine signaling in the NAc, and presumably improve satiety mechanisms compared with saturated fat.

CHAPTER IV

EPILOGUE

There is a growing body of evidence that implicates diets high in saturated fat with increased risk for the development of metabolic syndrome in humans (Chen et al., 2015; Nettleton et al., 2014; Rehman and Akash, 2016). Similarly, rodent studies investigating the effects of dietary fat report the development of metabolic syndrome-like phenotypes characterized by obesogenic weight gain, insulin resistance, and chronic inflammation after consuming high amounts of saturated fat (Décarie-Spain et al., 2018; Jais and Brüning, 2017; Rogero and Calder, 2018).

Emerging evidence has revealed that obesity and diets high in saturated fat are linked with pathophysiological changes in the dopaminergic reward system that disrupt satiety signals governing homeostatic food intake. Diets high in saturated fat are also implicated in the development of a metabolic syndrome-like phenotype characterized by obesogenic weight gain, insulin resistance, and chronic inflammation (Chen et al., 2015; Décarie-Spain et al., 2018; Jais and Brüning, 2017; Nettleton et al., 2014; Rehman and Akash, 2016; Rogero and Calder, 2018). While there is evidence that anti-inflammatory unsaturated fats promote healthier metabolic profiles and brain health, little is known about the effects of diets high in unsaturated fat on dopamine neurotransmission which

plays a role in feeding and satiety circuits (Bazinet and Layé, 2014; Calder, 2015; Coccurello and Maccarrone, 2018; Trépanier et al., 2016).

The purpose of this project was to determine whether a diet high in unsaturated fat, in contrast to saturated fat, would prevent the development of metabolic disorders and preserve normal dopamine function. Mice fed a diet high in saturated fat (SFD) consumed significantly more food and gained significantly more weight compared to their low fat (LF)-fed counterparts. In addition, unlike the LF group, the SFD group displayed anxiogenic locomotor behaviors in open field tests. Interestingly, the group fed a flaxseed oil (FSO) diet consumed the same amount of food as the SFD group; however, the FSO diet attenuated weight gain and preserved normal blood glucose regulation and locomotor behaviors. Significantly, the SFD group also exhibited dampened phasic dopamine release, impaired dopamine uptake and increased sensitivity to quinpirole, all of which was prevented with the FSO diet. There was also a negative association between dopamine uptake and IL-6 in the SFD group suggesting IL-6 selectively corresponded with reduced dopamine uptake in mice fed saturated versus unsaturated fat. Collectively, we demonstrated that different types of dietary fat have substantially different effects on metabolic phenotype and dopamine terminal regulation. In contrast to a diet high in saturated fat, a diet high in unsaturated fat preserved both normal metabolic and behavioral parameters as well as dopamine signaling in the NAc.

We demonstrated that different types of dietary fats have dramatically different effects on dopamine release and uptake. Previous studies have also shown that saturated fat promotes inflammation while diets high in unsaturated fats promote brain health, preserve behavioral responses to dopamine agonists, and improve insulin signaling putatively though attenuated inflammation. However, additional research is required to understand the role of dietary fats on inflammation associated with dopamine neuron function. IL-6 levels collected in this study were not elevated; however, collection of additional key inflammatory cytokines associated with insulin resistance, such as TNF-α, and IL-1β, would more fully characterize inflammation in the NAc. Moreover, use of lipopolysaccharide to initiate a heightened neuroinflammatory response could better characterize diet-related differences in production of cytokines. Direct measurement of microglia activation within the NAc could also demonstrate the role of microglia and inflammation on dopamine release and uptake.

It is also important to underscore that in contrast to unsaturated fat, the inflammation induced by diets high in saturated fat also causes insulin resistance. Indeed, we demonstrated that the FSO diet preserved glucose regulation while SFD impaired glucose regulation. We did not observe an association between peripheral blood glucose regulation and dopamine regulation, however we did not directly measure the effect of insulin in the NAc. It is therefore warranted to more fully investigate the effects of FSO versus SFD through methods such as the direct application of insulin in the NAc since insulin

fine tunes dopamine neurotransmission in the NAc to trigger satiety circuits and also dampens dopamine neuron firing to reduce food seeking and support meal termination (Ferrario et al., 2016). In addition, it would be important to study the effects of insulin in the NAc in an inflammatory state through experimental methods such as the application of lipopolysaccharide or direct exposure to exogenous cytokines (Bas and Ozdemir, 2017; Cai et al., 2018; Erta et al., 2012; Vumma et al., 2017; Wang et al., 2018). This would discern whether inflammation alone or inflammation-induced insulin resistance in the NAc is implicated in decreased dopamine release and uptake.

It is critical to understand how diets high in saturated fat cause dysregulated dopamine signaling in the NAc since it would provide evidence to link pathological behaviors of diet-induced obesity such as excessive/compulsive food seeking and consumption that exceed metabolic needs. Additionally, identifying whether inflammation causes similar deficits in NAc dopamine neurotransmission as SFD-induced insulin resistance will help us develop anti-inflammatory therapeutic strategies, diet or otherwise, that restore dopamine signaling, promote satiety, and potentially treat diet-induced obesity.

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APPENDIX A

ANIMAL DIETS

Formulated by:
Sara Sturgess
Research Diets, Inc.
May 2017

D12450J, D12492 and Preliminary Formulas

10 kcal% Fat or 60 kcal% Fat Rodent Diet, and Same with 22 gm% Flax Seed Oil or with SFA:n3 Ratio of 1:1

Product #	D12450J		D12492		Prelim Form 1		Prelim Form 2	
%	gm	*kcal*	gm	*kcal*	gm	*kcal*	gm	*kcal*
Protein	19	***20***	26	***20***	26	***20***	26	***20***
Carbohydrate	67	***70***	26	***20***	26	***20***	26	***20***
Fat	4	***10***	35	***60***	35	***60***	35	***60***
Total		*100*		*100*		*100*		*100*
kcal/gm	3.8		5.2		5.2		5.2	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	506.2	*2025*	0	*0*	0	*0*	0	*0*
Maltodextrin 10	125	*500*	125	*500*	125	*500*	125	*500*
Sucrose	68.8	*275*	68.8	*275*	68.8	*275*	68.8	*275*
Cellulose	50	*0*	50	*0*	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*	25	*225*	25	*225*
Flaxseed Oil	0	*0*	0	*0*	170	*1530*	99.3	*894*
Olive Oil, NF	0	*0*	0	*0*	0	*0*	0	*0*
Lard	20	*180*	245	*2205*	75	*675*	145.7	*1311*
Mineral Mix S10026	10	*0*	10	*0*	10	*0*	10	*0*
DiCalcium Phosphate	13	*0*	13	*0*	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*
FD&C Yellow Dye #5	0.05	*0*	0	*0*	0.025	*0*	0.025	*0*
FD&C Red Dye #40	0	*0*	0	*0*	0	*0*	0.025	*0*
FD&C Blue Dye #1	0	*0*	0.05	*0*	0.025	*0*	0	*0*
Total	**1055.05**	***4057***	**773.85**	***4057***	**773.85**	***4057***	**773.85**	***4057***
Flax Seed Oil, gm%	0.00		0.00		21.97		12.83	
Flax Seed Oil, kcal%	0.00		0.00		37.71		22.03	
SFA, gm %	1.01		11.27		5.42		7.85	
SFA, kcal%	2.37		19.34		9.31		13.48	
n3 FA, gm %	0.21		0.69		12.95		7.85	
n3 FA, kcal%	0.48		1.18		22.22		13.47	
n6 FA, gm %	1.75		9.94		7.88		8.74	
n6 FA, kcal%	4.09		17.07		13.52		15.00	
SFA, gm	10.68		87.18		41.96		60.76	
n3 FA, gm	2.18		5.33		100.19		60.74	
Ratio SFA:n3 FA	4.90		16.36		0.42		1.00	