EMMONS, HEATHER A. PH.D. ATTENUATING SATURATED-FAT INDUCED CHANGES IN DOPAMINE NEUROTRANSMISSION. (2024)
Directed by Dr. Steven Fordahl. 118 pp.

Diet-induced obesity (DIO) is associated with chronic, low-grade inflammation and correlates with mood and psychological disorders (i.e., depression and anxiety). Recent studies suggest inflammation plays a role in neurodegenerative diseases such as Parkinson's and Alzheimer's disease. Dopamine acts as a key link between obesity and these other neurological conditions. Research models, timing, and the composition of the obesity-inducing diet contribute to disrupted dopamine signaling. The exact mechanism behind this interruption remains unclear but one theory is the release of pro-inflammatory cytokines from adipose tissue promotes low-grade inflammation. Saturated fats, a major component of DIO, exacerbate the inflammatory response by activating the same innate immune system receptor (toll-like receptor 4, TLR4) on macrophages and microglia. This project investigated three different approaches to reduce inflammation (diet, exercise, and pharmacology) to observe how they affect dopamine signaling (release and reuptake rate) under pro- or anti- inflammatory conditions. Our findings show that a diet high in fat (60% kcals) decreases phasic dopamine release (5p20Hz) and V_{max}. Prophylacytic NSAID treatment with Ketofen counteracts these effects. However, neither dietary changes or exercise has a significant impact. Interestingly, females seem to have some protection from further inflammation when they exercise while consuming a high-fat diet. Overall, this data offers promising avenues for exploring alternative treatments for DIO.

ATTENUATING SATURATED FAT INDUCED CHANGES IN DOPAMINE NEUROTRANSMISSION

by

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A Dissertation
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
Doctor of Philosophy

Greensboro

2024

Approved by

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APPROVAL PAGE

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Date of Acceptance by Committee

June 4, 2024

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ACKNOWLEDGEMENTS

I would like to thank my advisor and committee chair, Dr. Fordahl, in addition to all of my committee members, Dr. Erikson, Dr. Allred, and Dr. Parry. Thank you to all of Dr. Fordahl's lab members, past and present. I would also like to thank all of the faculty and students of the Nutrition department at the University of North Carolina - Greensboro.

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Introduction

Despite ongoing public health efforts, obesity prevalence continues to rise, straining healthcare systems while reducing quality and length of life. Alongside increased adiposity, obesity is associated with metabolic syndrome (MeS), and elevated risk of other chronic diseases like Type II Diabetes[5], cardiovascular disease[51], and certain cancers[41]. MeS is characterized by dyslipidemia, dysregulated blood glucose, and raised blood pressure[292]. These symptoms often persist alongside chronic, low-grade inflammation[286,348]. Highly palatable foods contribute to excess calorie intake and evidence suggests saturated fat specifically promotes systemic inflammation similar to what is observed with MeS[30,52,120]. This chronic inflammation may also affect brain health, potentially influencing mood and motivation[229]. This review examines how dietary saturated fat and obesity-related inflammation modulate dopamine signaling and explores potential modes for attenuation.

Diet-Induced Obesity

Obesity (BMI $\geq$ 30kg/m^2) develops from chronic overconsumption of calories, often from highly palatable foods (high in sugar and/or fat). These foods contribute to the excess caloric intake and disrupted natural satiety signals in the brain[8,162], promoting increased adiposity and fostering the development of Metabolic Syndrome (MeS) – a cluster of symptoms including hypertension, dysregulated blood glucose, and dyslipidemia. Individuals with MeS have increased risk for developing chronic diseases such as diabetes[158,288] and cancer[95]. However, obesity alone is a modifiable risk for developing these ailments in addition to mental health conditions like depression and anxiety[28,131]. MeS and obesity are also associated with low-grade chronic

inflammation, which disrupts several body processes[97,262], and has been theorized to disrupt satiety signals by influencing neurotransmitters like dopamine.

Dopamine is a critical neuromodulator that impacts various physiological processes, including circadian rhythm[178], reward-based learning[79,276,340], and motivation to seek food or reproductive mates[17,18,367]. However, disrupted dopamine in this finely tuned system can lead to a cascade of effects beyond physical changes like weight gain; it can also significantly impact mood and behavior. For instance, dopamine dysregulation has been linked to an increased tendency towards addictive behaviors[247,272]. Dopamine dysregulation in addiction is often characterized by development of anhedonia, leading to compulsive reward seeking behavior exacerbating the problem. Additionally, mood disorders such as depression[309,314] and anxiety[282] are also associated with dopamine signaling abnormalities. This strong correlation between various disorders and dopamine dysfunction suggests a potential underlying mechanism connecting them.

Obesity is a complex condition with significant mental health comorbidities, including depression and anxiety.[124,246] Research suggests a bidirectional relationship between these conditions. One meta-analysis from Luppino et al. (2010) demonstrated that individuals with obesity (BMI$\geq$30kg/m^2) have an increased risk of developing depression, and vice versa[53,211]. This correlation is also observed in adolescents, suggesting a potential link established early in life[53]. Similarly, Gariepy et al. 2010 found an association between obesity and various anxiety disorders[124]. These findings point towards a potential disruption in dopamine signaling and neuromodulation in the context of obesity and related mental health conditions. Given the known role of dopamine in mood and motivation, it is important to explore how neuronal signaling might be affected in the context of obesity.

Neural and Hormonal Regulation of Food Intake

The hypothalamus integrates opposing hormonal signals from insulin, ghrelin, and leptin[14,91,225,274,275,322] to maintain homeostatic food intake and body weight in response to changing metabolic demands[83,203,277]. The delicate hormonal interplay is disrupted by diet-induced obesity (DIO), leading to caloric overconsumption despite adequate energy stores[20,91,311,332]. Anorexigenic cues like POMC and leptin trigger satiety and generally halt feeding[77,121,215], while orexigenic signals like AgRP and ghrelin promote hunger and increase feeding[14,36,66,94,149,215]. However, the interplay between these signals is more complex, influenced by both metabolic state (lean vs obese) and energy status (sated vs fasted). Choi et al (2012) demonstrated that the orexigenic hormone orexin, released in the paraventricular thalamic nucleus, elevated dopamine cell body excitability in the ventral tegmental area (VTA) resulting in increased dopamine release downstream in the nucleus accumbens (NAc), modulating hedonic eating behaviors[59]. DIO associated inflammation directly disrupts the hypothalamus, but also indirectly influences it through changes in dopaminergic reward circuitry that normally integrate within the hypothalamus[50,169,180].

Multiple hormones influence food intake by regulating dopamine signaling in the brain. Excitatory inputs are associated with increased burst firing in the VTA[133,159]. Ghrelin, an orexigenic hormone, that increases in a fasted state elevates dopamine levels, potentially altering the salience of food cues[1,12,166]. Insulin, elevated in a fed state, suppresses dopamine release in the VTA via modulating the dopamine transporter (DAT)[183,205,207,224]; suggesting insulin reduces cravings for palatable foods. Similar to insulin, GLP-1 (a gut hormone), acts as an anorexigenic signal, reducing dopamine release in the VTA and downstream synapses in the NAc, ultimately decreasing food intake[6,43,74,226,227,343]. The NAc is responsible for integrating signals involved in

reward associated learning and food seeking behaviors[356], and also tied to Pavlovian learning[38,49,79,81]. Insulin[115] and GLP-1[11] may exert effects via modulating DAT function in the NAc, leading to altered reward and motivation, explaining the ability to curb salience of palatable foods and addictive drugs[85,108–112,115,207]. Together, these opposing signals regulate food intake and feedback of food-related cues, with increased salience in a fasted state[32,54].

Dopamine

Dopamine is one of the major monoamine neurotransmitters in the brain. In recent years, dopamine is largely thought of as a neuromodulator rather than just a neurotransmitter. Dopaminergic cell bodies originate either in the substantia nigra (SN) or the VTA, with the latter projecting to nuclei within the ventral striatum, known as the NAc. Glutamate is a crucial regulator of dopamine release from medium-spiny neurons of the mesocorticolimbic circuit[182,330]. Remodeling of dopaminergic pathways can alter how glutamate and GABA inputs propagate satiety signals, playing a key role in the ongoing dysregulation of brain circuits involved in eating.

Dopamine influences various body function, including precise motor control[303] and the motivation to seek food[18]. The specific brain region activated determines the outcome, with the NAc playing a key role in encoding reward and learning-based behaviors[176,329]. Brain scans of chronic cocaine users show activation in dopamine-rich regions, highlighting a parallel to Pavlovian conditioned cues[79,81,199,220,337]. Dopamine release is dynamic, adjusting as behaviors and rewards transition from unexpected stimuli to learned responses[84,197]. In obesity research NAc dopamine dysregulation is of particular interest as the direct pathway is linked to reward learning and its dysfunction could contribute to disordered eating patterns triggered by highly palatable, sugary, or fatty foods.[69,80]. Evidence suggests dopamine dysregulation is a critical

factor in DIO[160,326,334,337], potentially leading to excessive calorie consumption beyond metabolic needs. This may be partly explained by disrupted dopamine signaling in the NAc, which can impair the normal feedback to the hypothalamus, a region critical for integrating food intake cues and signaling satiety[242,299].

Studies in humans and animals show that short-term, high-fat intake increases dopamine levels, while chronic consumption leads to a reduction. However, as Carlin et al. (2016) demonstrate the potential for reversing dopamine dysregulation. Their study utilized a low-fat diet for 4 weeks following 12 weeks of a high-fat diet restored dopamine levels in mice[48]. This suggests potential for remodeling dopaminergic neuron synapses in the brain region responsible for food intake motivation and learning[206,270,329,352,353]. Understanding how these synapses are influenced is crucial for identifying potential targets in future clinical treatments for overeating and weight gain. Animal studies support this, demonstrating reduced dopamine receptor availability/density with high-fat diets[16,48,128]. Additionally, Naneix et al. 2017 observed increased dopamine response in animals sensitized to a high-fat diet, both at baseline and after amphetamine injections, suggesting synaptic remodeling[236]. Tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, is downregulated in diet-induced obesity but upregulated with calorie restriction or low-fat diets[73,165,201]. Similar findings were observed in rats fed a high-fat diet for 25 weeks, with a significant decrease in TH associated with increased oxidative stress and microglial activation[33]. Collectively, these data suggest fat may influence TH function, potentially through increased inflammation.

Inflammation and Dopamine: Hypothesized Link in DIO

A growing body of evidence suggests a link between chronic inflammation and dopamine dysregulation in DIO. However, the precise mechanisms and relative role of the NAc remains

unclear. Inflammation, a necessary response to acute injury, can contribute to pathology when chronic[55]. Macrophages and microglia, resident immune cells, dynamically switch between pro-inflammatory (M1) and anti-inflammatory (M2) states upon stimulation.[130]. Research suggests a shift towards the M1 phenotype, characterized by pro-inflammatory cytokine production (TNFα and IL-6)[366], is linked to neurodegeneration[4,350] and obesity[342]. Zhang et al (2007) reviewed the mechanism by which activated microglia release these cytokines[366]. Chronic microglial activation and resulting neuroinflammation are hypothesized to connect neurodegenerative diseases like Alzheimer's[145], Parkinson's[147,296,357], and multiple sclerosis (MS)[34] to dopamine dysregulation similar to what is observed in pre-clinical DIO models[20,116,335]. This hypothesis is supported by evidence that pro-inflammatory cytokines from microglia can indirectly restructure synapses through chronic inflammation[34,144,177]. High-fat diets activate various inflammatory pathways (TLR4, NF-kB, JAK-STAT)[55,89,102,261,287] and induce local inflammation in dopamine-rich brain regions[82,172]. Additionally, saturated fat and metabolic endotoxins can damage the blood-brain-barrier (BBB)[89,107,155], increasing permeability to pro-inflammatory molecules. Davidson et al (2012) demonstrated this by observing increased BBB permeability in DIO rats that consumed a high-fat diet for 4 weeks[74].

Studies show that the hypothalamus experiences the earliest onset of diet-induced insulin resistance[250,291]. Supporting this notion, a study in rats fed a high fat diet (60% kcals fat) demonstrated neuronal changes within the hypothalamus as early as 3 days[308]. The timing of insulin resistance development in the NAc remains unclear. However, a rodent study showed a correlation between dopamine release and reduced blood glucose clearance[116]. Additionally, blocking tyrosine phosphatase 1β attenuated the observed dysregulations in dopamine signaling[116]. Evidence suggests the hypothalamus is the most susceptible brain region to initial inflammation in

the context of DIO[308]. Inflammation can originate locally or systemically, with a damaged BBB allowing peripheral molecules to cross into the brain[231]. This inflammation caused by stressed, hypertrophic adipose tissue alters synaptic and connections, leading to changes in circuitry that become evident as depression, anxiety, or other mood-related disorders. While synaptic remodeling is a normal and necessary process, especially during development, unchecked chronic inflammation can contribute to further complications[86]. The effects of inflammation on NAc function in DIO and binge-eating disorder require further investigation.

Chronic low-grade inflammation, observed in various brain dysfunction diseases[218,324], is a proposed mechanism for synaptic remodeling. This inflammation may originate from systemic pro-inflammatory cytokines breaching a compromised BBB[319], from local release by activated microglia[62,70], or a combination of both. Lipopolysaccharide (LPS), a component of gram-negative bacteria, triggers an inflammatory response in mammals (including rodents and humans)[138,369]. LPS binds the pattern recognition toll-like receptor 4 (TLR4), activating the NF-**κ**B pathway and inducing the production of proinflammatory cytokines, like IL-6 and TNFα[167,208]. Notably, LPS-induced inflammation has also been implicated in decreased dopamine concentration in brain tissue[279]. Interestingly, human studies suggest that systemic inflammation can influence dopamine release in DIO[91,196]. One study observed an increased dopamine response to methylphenidate[244] suggesting a priming of dopamine terminals to be more sensitive upon further insult. Additionally, there is evidence for modulation of dopamine release with consumption of highly palatable foods, potentially through an interaction with the NLRP3 inflammasome[358]. This inflammasome complex is selectively activated by saturated fat intake, as demonstrated in a human study[198].

Research by Wong et al (2009) demonstrated that both LPS and saturated fat can activate MyD88 -dependent and -independent pathways, leading to increased pro-inflammatory cytokine

release[354]. The independent pathway induces delayed activation of the NF-kB pathway, explaining why inflammatory responses differ at various timepoints after dietary change[354]. Fritsche et al (2015) proposed that saturated fats activate this pro-inflammatory cascade via binding the TLR4 receptor[119]. This is further supported by research that showed similar inflammatory effects with saturated, but not unsaturated fats[188,191].

Potential Treatment/Prevention Strategies

Current clinical weight loss strategies often rely on schedule II-controlled stimulants, or analogs of gut peptides. While these medications can be initially effective, stimulants carry a high risk for abuse[98,122,194], and the gut peptide glucagon-like peptide 1 (GLP-1) analogs perform poorly in long-term weight regain. Medications like selective serotonin reuptake inhibitors (SSRIs) have not demonstrated lasting effects on compulsive eating behaviors (i.e. binge-eating disorder) beyond a few weeks[98,122,194]. While some success has been achieved with psychotherapy treatments (i.e., cognitive behavioral therapy), long-term effectiveness remains a challenge[157,164]. In contrast, exercise consistently demonstrates its ability to promote and maintain weight loss over extended durations (2+ years)[58,304]. This is evident in the National Weight Control Registry, which tracks individuals who have lost at least 30 lbs. and kept it off for a year or more. Regular exercise is a common characteristic among these participants[351]. Importantly, exercise not only reduces body weight, but also lowers levels of circulating pro-inflammatory cytokines[2,114,125,163,240]. This makes exercise a promising treatment option, without the potential for inflammation-induced synaptic remodeling, a concern with long-term SSRI use[137,222]. This is especially crucial since individuals struggling with overeating are often predisposed to other addictive behaviors, potentially increasing their risk of abusing medications with addictive qualities.

A significant gap exists in the current literature regarding the investigation of

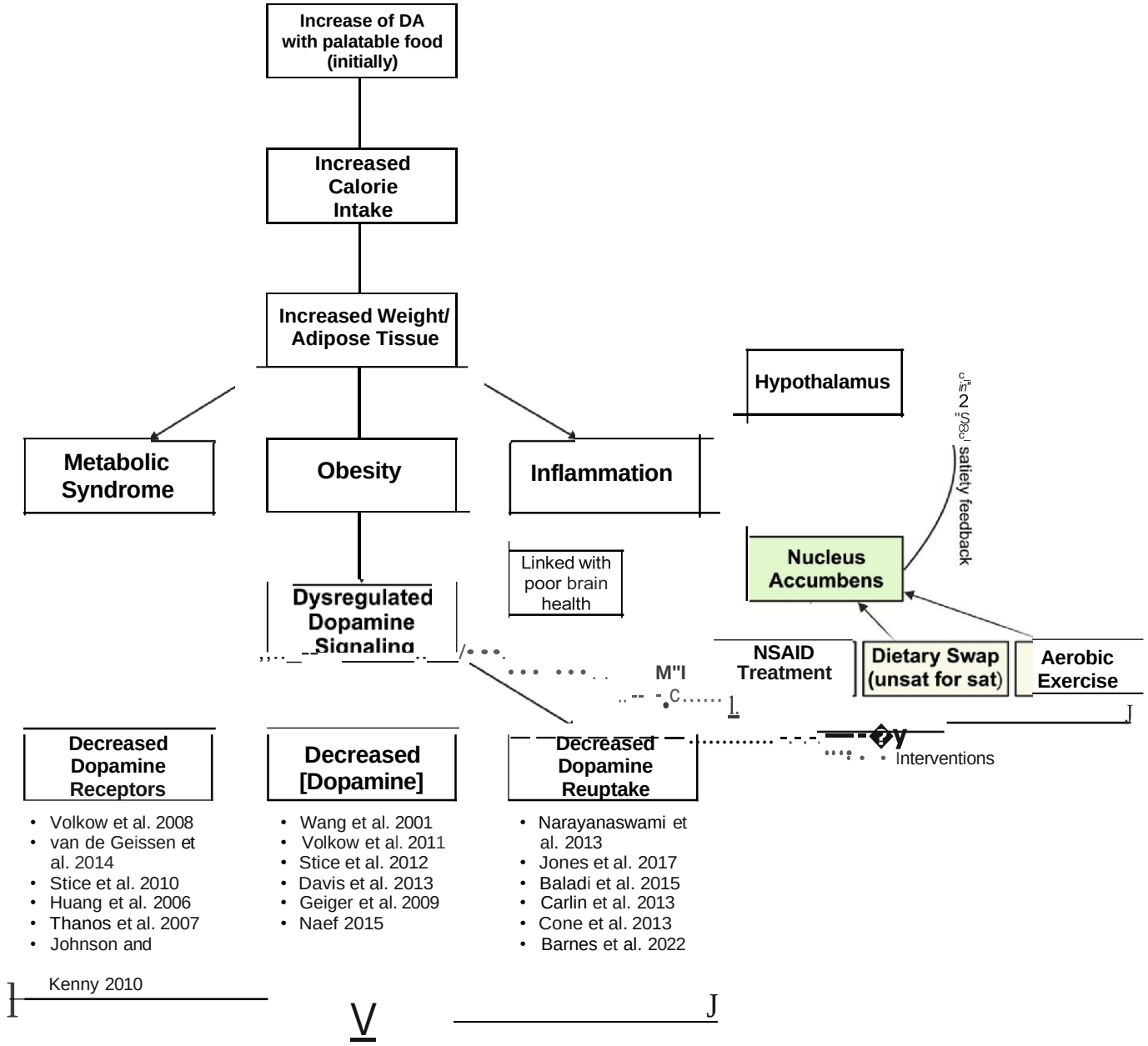
inflammation-reducing therapies and their ability to restore or prevent disruptions in homeostatic dopamine signaling (measured through release and reuptake rate). Furthermore, real-time measurements of dopamine levels at presynaptic terminals would be invaluable in understanding how these dopaminergic neurons function under different dietary conditions. Previous research on dopamine signaling with exercise is limited by reliance on high-performance liquid chromatography (HPLC), which only provides a snapshot of overall dopamine concentration at the time of tissue harvest[280]. Our proposed method (fast-scan cyclic voltammetry) offers a clear advantage by allowing real-time sub-second visualization of dopamine release and reuptake in this DIO research model.

This research addresses these gaps by employing three different approaches to reduce inflammation (pharmacology, diet, and exercise) and analyzing their impact on dopamine signaling. This real-time method has the potential to identify new avenues for weight loss treatment that do not involve medications with a high abuse potential.

Conclusions

Pre-clinical models of DIO demonstrate that excess calorie intake, particularly from high-fat diets, increases adipose tissue and leads to disrupted cellular function. This can lead to ER stress, free radical damage, and the upregulation of pro-inflammatory cytokines like IL-6 and TNFα by hypertrophic fat cells. This systemic inflammation, a hallmark of DIO, further disrupts cellular homeostasis and damages the BBB similar to what is observed in various physical and psychiatric conditions. Therefore, targeting mechanisms to reduce chronic inflammation in the context of DIO holds significant promise for developing non-addictive therapeutic approaches for treating obesity, potentially leading to a deeper understanding of the disease.

Figure 1.1-Conceptual Model



CHAPTER II: DIETARY APPROACH TO RESTORE DOPAMINE SIGNALING

Introduction

Obesity (BMI ≥30kg/m^2) is a metabolic condition historically associated with low-grade inflammation[44,263] and increased risk for chronic diseases like Type 2 Diabetes Mellitus[15,321] and cardiovascular disease.[51,140] Emerging evidence has linked obesity to psychiatric disorders like depression, and anxiety.[9,124,211,228] A unifying contributor to diet-induced obesity, depression and anxiety is disrupted mesolimbic reward processing implicating the neuromodulator dopamine as an underlying mechanism.[231,300] Studies utilizing microdialysis[148,219] and brain imaging techniques[118,290] have demonstrated the release of dopamine is initially elevated by highly palatable foods, however, long-term consumption is correlated with dampened dopamine signaling[126] and neurochemical changes similar to chronic drug abusers.[328,338,339] It has been shown that this state of dysregulation also persists with diet-induced obesity(DIO)[78,127] and neuroinflammation.[100,104] Neuroinflammation is reported with DIO and is an emerging etiology for depression and other psychiatric disorders. However, it is unclear whether reversing weight gain or attenuating diet-induced inflammation can improve disrupted dopamine signaling. Exploring nutritional strategies to restore disrupted dopamine signaling that occurs with diet-induced obesity is a logical step to identify if impairments caused by altered metabolism can be reversed with a clinically relevant therapeutic approach.

A critical component of the mesolimbic reward system is the nucleus accumbens (NAc), which plays a key role in mediating hedonic responses to various stimuli, including food and drugs.[195,213,267,329] Dopamine signaling within the NAc, specifically the dynamics of its release and reuptake, is intricately linked to reward-related behaviors and susceptible to modulation by

environmental factors, including nutrients.[202] Previous research has demonstrated that consuming a diet high in saturated fat dampens dopamine signaling (release and reuptake rate) compared to a low saturated fat diet.[20,332] This decrease in mesolimbic dopamine signaling seen in a state of DIO may impair rewarding aspects of food intake[76,128,294] resulting in overconsumption, further dampening dopamine, and yielding a pattern that can be difficult to halt. How DIO caused by saturated fat intake degrades dopamine related reward processing is unclear but plausibly rooted in inflammation caused by DIO or saturated fat-induced activation of the immune cells.

Chronic, low-grade inflammation is a hallmark of diet-induced obesity, with saturated fat specifically implicated in triggering an inflammatory response comparable to what is observed in the presence of lipopolysaccharide (LPS).[185,251] In addition, diets high in saturated fat have been shown to disrupt gut permeability[186,187] and allow passage of bacterial components, like LPS (part of the outer wall of gram negative bacteria), into the bloodstream.[265] LPS binds Toll-like receptor 4 (TLR4), consequently activating inflammation-associated downstream pathways NF-kB and MAPK[229,318,345] This ultimately results in the release of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). Given that saturated fat can bind to TLR4 and induce inflammation similar to lipopolysaccharide (LPS), it is proposed this could be a shared mechanism disrupting dopamine signaling either directly or indirectly at pre-synaptic terminals in the NAc,[298,310] potentially influencing natural satiety signals.[3,150] This may lead to continued overeating and further production of inflammation leading to a devolving cycle. Therefore, it is hypothesized that the same fat percentage derived from unsaturated sources would not elicit the same downstream effects by attenuating disruptions in neural satiety signals. Although unsaturated fats act as precursors to inflammatory-associated molecules (eicosanoids),[135,146] evidence points to overall anti-inflammatory effects for omega-3 (ω-3)

unsaturated dietary fats.[67] The unsaturated fat in this study is derived from flaxseed oil, which is comprised higher ω-3 to ω-6 unsaturated fatty acid ratio.[7] It has been reported that a lower ω-6:ω-3 fatty acid ratio is associated with an anti-inflammatory state, while a higher ratio propagates inflammation.[67]

Given the link between saturated fats and inflammation,[171,370] along with the observed effects that obesity induced by saturated fat has on pre-synaptic dopamine signaling, we aimed to investigate whether unsaturated fats with anti-inflammatory properties[61,254] would reverse these effects. Therefore, the aim of the present study is to restore dopamine signaling changes reported with high saturated fat feeding[20,91,336] by reducing the amount of saturated fat or replace saturated fat with anti-inflammatory ω-3 fat. We hypothesize that after mice feed on a pro-inflammatory high-fat diet, changing their diet to either an anti-inflammatory ω-3-rich diet or a low-fat diet will improve dopamine release and reuptake rate to resemble dopamine parameters of mice with continuous intake of a low-fat diet. Furthermore, understanding the impact of dietary interventions on dopamine signaling is an important avenue to exploring potential therapeutic strategies for mitigating obesity-related alterations in brain function and behavior.

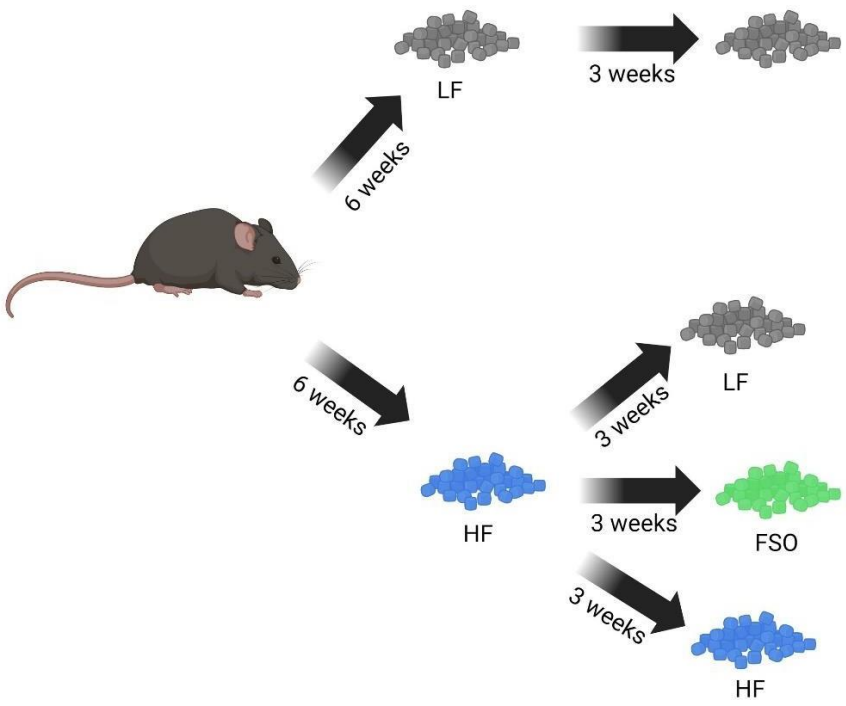
Experimental Procedures

Animals, Diet, and Experimental Design

Six-week-old mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed on a reversed 12-hour light/dark cycle (lights off 0600, lights on 1800). Mice were given free access to food and water and randomly assigned to one of two dietary groups: low-fat (LF) (n=12) (10% total kcals from fat; Research Diets, D12450J) or high-fat (HF) (n=50) (60% of kcals from fat; Research Diets, D12492). They remained on these respective diets for 6 weeks at which point the low-fat group continued on with the LF diet, while the HF group was randomly

separated to one of three groups: 1) continue with HF diet (n=20), 2) switched to the 10% LF diet (HFxLF) (n=16), 3) switched to an isocaloric diet with saturated fat replaced with unsaturated fat from flaxseed oil (HFxFSO (n=14) (60% kcals from fat; Research Diets, Preliminary Formula). These diets were provided for an additional 3 weeks before animals proceeded to voltammetry experiments. All experiments were conducted following approval by the UNC Greensboro Animal Care and Use Committee in compliance with the *Guide for the Care and Use of Laboratory Animals (NIH).*

Figure 2.0-Experimental Design



Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance tests (IPGTT) were performed to examine blood glucose clearance. Mice were fasted 12 hours prior to IPGTT which began three hours into the dark cycle (0900). Fasting body weights were recorded to calculate the volume required for 20% glucose administration. A topical anesthetic (lidocaine 2.5% and prilocaine 2.5% (item #977934 CVS, NC)) was applied to the tail tip, which was then nicked with a surgical blade to produce a droplet of blood for blood glucose measurement with a glucometer (item #323932 CVS, NC). Baseline glucose was measured, then mice received an intraperitoneal injection of 20% glucose (2g/kg bodyweight; Sigma, St. Louis, MO) in sterile saline (0.9% sodium chloride, Hospira; Lake Forest, IL). Blood glucose was measured at 15-, 30-, 60-, and 120-minute intervals. These values were plotted and reported as area under the curve (AUC).

Fast Scan Cyclic Voltammetry (FSCV)

Mice from each treatment group proceeded to FSCV in a Latin-square design. Following brain removal, brains were placed in oxygenated artificial cerebrospinal fluid (aCSF) (126mM NaCl, 25mM $NaHCO_3$, 11mM D-glucose, 2.5mM KCl, 2.4mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM NaH_2PO_4, 0.4mM L-ascorbic acid) (Sigma product codes: NaCl-1002579927, $NaHCO_3$-S5761, D-glucose-1002593418, KCl-1002323941, $CaCl_2$-1002393239, $MgCl_2$-1002368596, NaH_2PO_4-1002355618, L-ascorbic acid-1002334976) and sliced coronally into 300µm thickness with a compresstome (Precisionary Instruments; Greenville, NC) as previously described.[91] Slices were equilibrated for 60 minutes in oxygenated aCSF flowing at a rate of 100mL/hr before beginning experiments. A glass capillary-pulled carbon fiber electrode (C005722, batch 4, carbon metal fiber, 10m length; Goodfellow) (A-M Systems, catalog #60200; Sequim, WA) was placed approximately 50µM into the slice within the NAc next to a bipolar stimulating electrode

(Plastics One; Roanoke, VA) which evoked terminal release of dopamine with a single monophasic electrical pulse (350µA, 4ms) or a five-pulse train at 20Hz (5p20Hz). Dopamine release and uptake was recorded for 15 seconds with a three-minute recovery window between stimulations. Baseline dopamine recordings were considered stable when the current detected for dopamine peak height was within 10% between three or more recordings. An Ag/AgCl reference (Precision Instruments; Sarasota, FL) was used to scan a triangular waveform between -0.4V and 1.2V at a rate of 400V/s every 100ms. Dopamine current (nA) was converted to concentration (µM) using a calculation of electrode sensitivity through DEMON voltammetry and analysis software[362] Michaelis-Menten kinetics were used to determine the concentration of maximal dopamine release [DA] and reuptake rate (V_{max}) with K_m held at 160nM, corresponding with the affinity of dopamine for the dopamine transporter.[355] K_m was only adjusted after the application of amphetamine (AMPH), to measure any diet- or exercise-induced differences in the affinity of dopamine for the DAT.[362] After baseline recordings were established, a multiple-pulse simulation was recorded at 5p20Hz. We then re-established a stable single-pulse baseline prior to addition of AMPH or cytokines. Either dopamine transporter competitive inhibitor, AMPH, at concentrations of 300nM and 3µM, or pro-inflammatory cytokines IL-6 (10nM) or TNFα (300nM) was applied to the aCSF for 30-minutes each. [IL-6 (catalogue no. SRP3330), TNFα (catalogue no. T7539); Sigma-Aldrich, St. Lewis, MO]. IL-6 was reconstituted with 0.1% bovine serum albumin (catalogue no. A7030; Sigma-Aldrich) to facilitate tissue delivery. After a 30-minute perfusion with IL-6 or TNFα, the flow was switched back to aCSF and collections continued for a 30-minute washout period. All recordings were obtained from the NAc core, oriented ventral to ventral lateral of the anterior commissure.

Statistical Analyses

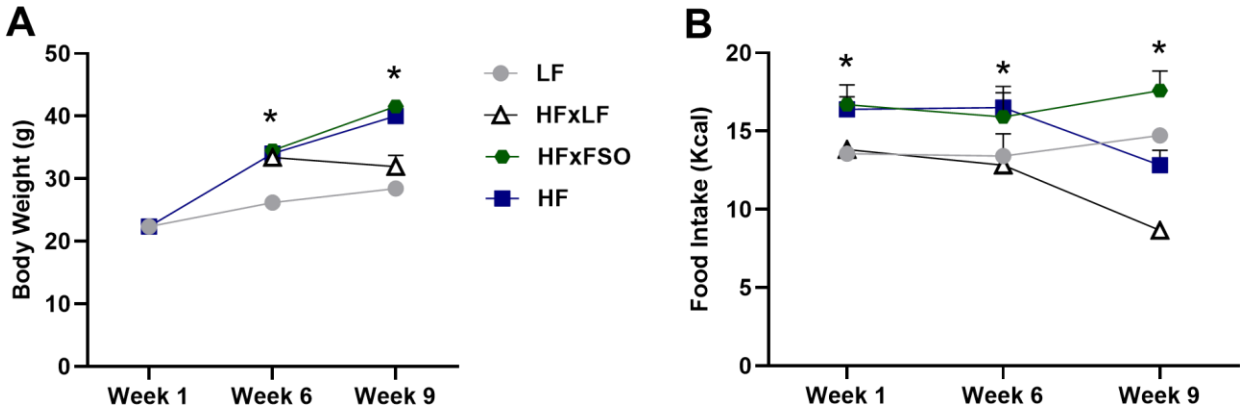
All analyses were performed in GraphPad Prism (v.9.1.1). One-way analysis of variance (ANOVA) was used to identify diet effects in daily food intake, body weight and dopamine related outcomes (release, reuptake rate). Two-way ANOVA was used to identify effects of diet over a span of cytokine exposure and washout. All group differences were assessed using Šidák's or Tukey's post-hoc tests. Results are expressed as mean ± standard error of the mean (SEM).

Results

Body weight gain from a high saturated fat diet was attenuated with switching to a low-fat diet, but not a diet high in unsaturated fat.

Body weight gain and food intake were tracked weekly for the duration of the study (Figure 1). All groups began the experiment at similar body weights (Fig. 1A), while food intake (Kcals) was higher for all HF groups (Fig. 1B) at weeks 1 and 6. A significant increase in body weight gain was seen at week 6 between all HF groups compared to LF fed.

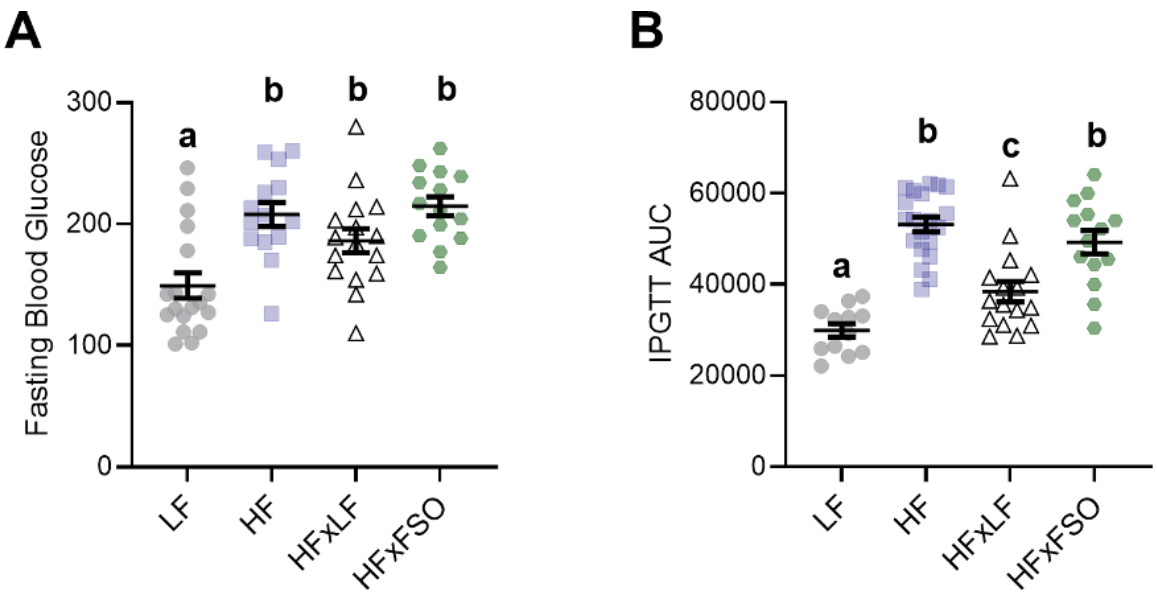
Figure 2.1. Body Weight and Food Intake
Body weight and food intake for dietary crossover groups. Body weight (A) was measured in grams (g) and food intake (B) is expressed in kilocalories (Kcal). Measurements were taken weekly and representative timepoints for weeks 1, 6, and 9 are shown. *p<0.05



Switching to a diet high in unsaturated fat did not improve blood glucose regulation disrupted by a high saturated fat diet.

Blood glucose parameters were measured at the end of week 9 (Figure 2). Fasting blood glucose was elevated for all of the HF groups (HF-p=0.0004; HFxLF-p=0.0336; HFxFSO-p<0.0001)(Fig. 2A) compared to the continual LF group. Intraperitoneal glucose tolerance tests (IPGTT) area under the curve (AUC) was calculated and compared across groups (Fig. 2B). Both HF (p<0.0001) and HFxFSO (p<0.0001) groups diminished the ability for blood glucose clearance compared to LF-fed, while the HFxLF showed more efficient blood glucose clearance when presented with a challenge bolus of glucose when compared to the HF (p<0.0001) and HFxFSO (p=0.0024) groups.

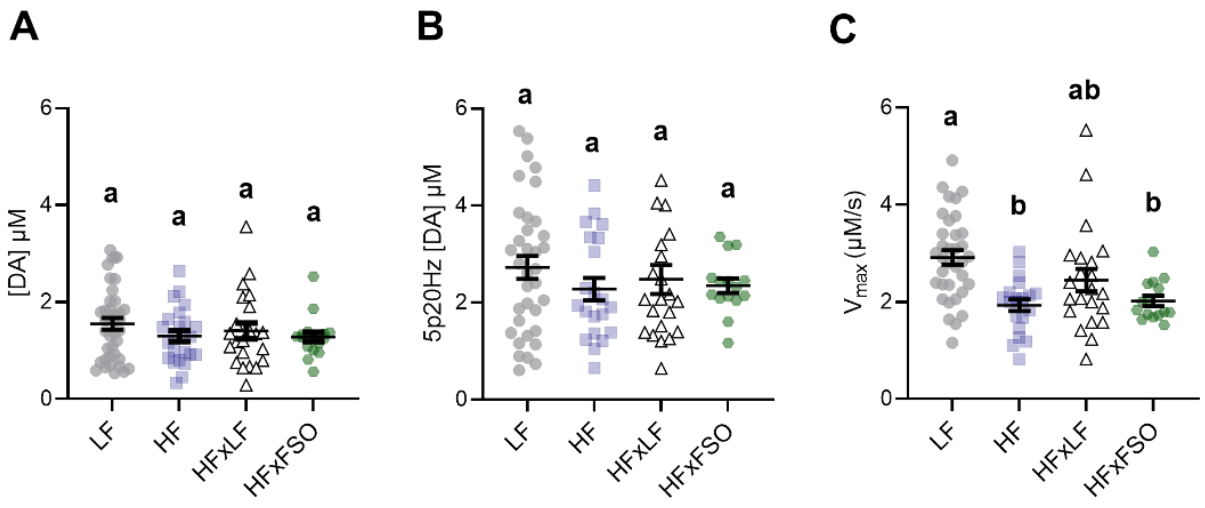
Figure 2.2. Blood Glucose Data
Metabolic glucose data for dietary crossover groups. Distinct letters represent statistically significant differences.



A switch from HF to a LF diet increased V_{max}, while continual HF intake or swapping to a HF (unsaturated) diet had no effect.

Fast-scan cyclic voltammetry was used to measure levels of evoked dopamine release and rate of reuptake (V_{max}) from dopamine terminal fields in the NAc. (Figure 3) Single- and multi-pulse dopamine release yielded no differences between dietary groups. (Fig. 3A-B) However, V_{max} was significantly lower for HF (p=0.0002) and HFxFSO (p=0.0035) groups compared to LF-fed counterparts. (Fig. 3C) No differences were observed in V_{max} for HFxLF group compared to LF, HF, and HFxFSO groups.

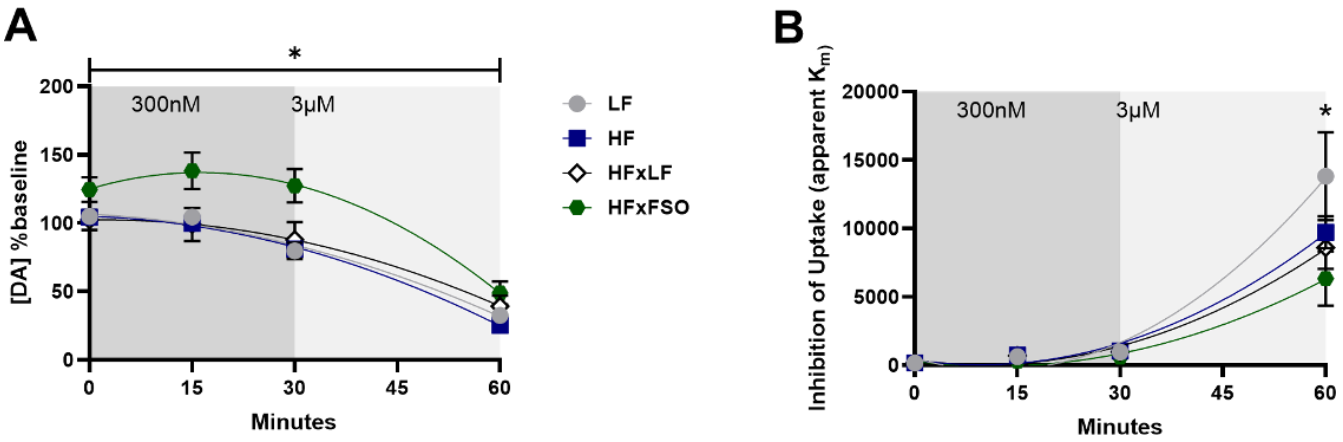
Figure 2.3. FSCV Dopamine Release and Reuptake
Voltammetry data for dietary crossover groups. Single (A) and multi-pulse (B) evoked dopamine release concentration measured in µM. V_{max} (C) is expressed as µM/s. Distinct letters represent statistically significant differences.



Switching from a HF (saturated) to a HF (unsaturated) diet increased early amphetamine-induced evoked dopamine release in the NAc.

The addition of 300nM amphetamine (AMPH) to the aCSF significantly increased ($p=0.0004$) evoked dopamine release in HFxFSO group compared to LF-fed. Dopamine was depleted to 50% at 60 minutes with 3µM AMPH for all dietary groups. (Fig. 4A) Similarly, K_m was significantly lower ($p<0.0001$) at the 60-minute timepoint (3µM) for HFxFSO group compared to LF-fed. (Fig. 4B)

Figure 2.4. FSCV with Amphetamine
Evoked dopamine release (A) and K_m (B) with application of 300nM and 3µM amphetamine (AMPH). Dopamine release is expressed as a % of baseline dopamine release. *$p<0.05$



Exogenous application of TNFα increased dopamine release in the HF and HF crossover groups, while application of IL-6 decreased dopamine release.

After baseline dopamine signals were established, pro-inflammatory cytokines, TNF-α and IL-6, were added to the aCSF bath (Figures 5 and 6, respectively). Two-way ANOVA identified differences between groups ($p<0.0001$). Multiple comparisons revealed that HF-crossover groups (HFxLF, HFxFSO) had increased evoked dopamine levels in the presence of

300nM TNF-α, compared to LF-fed group. (p=0.002) These groups were significantly elevated for the duration of the cytokine exposure and span of washout in aCSF. (Fig. 5) Similarly, the group that was fed HF diet for the entire 9 weeks yielded elevated dopamine release for the same duration that approached significance (p=0.0575). (Fig. 5)

Similarly, a two-way ANOVA revealed statistically significant dopamine release between diet groups (p<0.0001) when IL-6 was added to the aCSF. However, 10nM IL-6 exposure significantly decreased dopamine release for HF (p=0.0004), HFxLF (p=0.0080), and HFxFSO (p=0.0017) groups compared to LF-fed counterparts. (Fig. 6) Neither pro-inflammatory cytokine (TNFα, IL-6) had an effect on V_{max} (data not shown).

Figure 2.5. FSCV with 300nM TNFα
Fast-scan cyclic voltammetry with application of 300nM TNFα followed by washout. Shaded region delineates timeframe when 300nM TNFα was added to aCSF perfusion. [DA] release is expressed as a % of measured baseline. **p<0.01

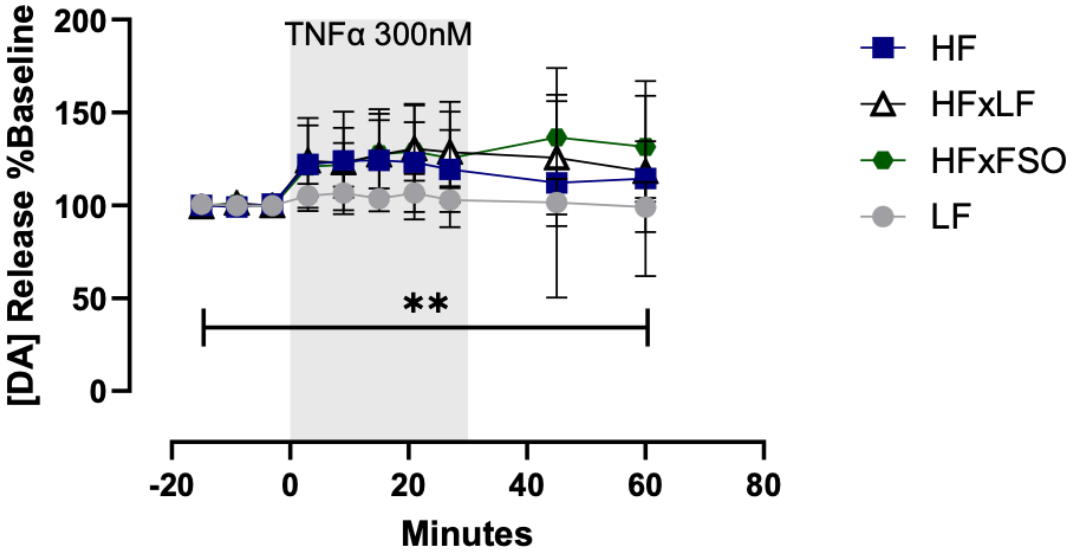
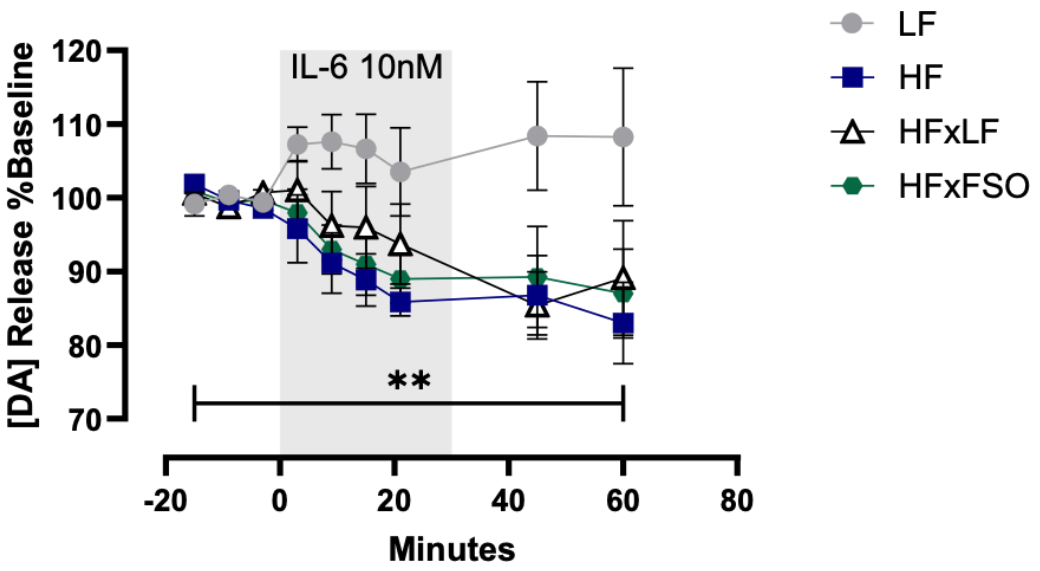


Figure 2.6. FSCV with 10nM IL-6
Fast-scan cyclic voltammetry with application of 10nM IL-6 followed by washout. Shaded region delineates timeframe when 10nM IL-6 was added to aCSF perfusion. [DA] release is expressed as a % of measured baseline dopamine release. **p<0.01



Discussion

This study examined if deficits in dopamine terminal function caused by a diet high in saturated fat (HF) could be reversed if the HF was isocalorically replaced with unsaturated fat (FSO) or reduced to a low-fat profile (LF). Our findings show that a three-week substitution with FSO did not attenuate body weight gain or improve blood glucose regulation. However, replacing the HF diet with a LF diet improved blood glucose clearance and restored dopamine uptake in the NAc, similar to the continual LF-fed group. Slower dopamine reuptake rate (V_{max}) in the continual HF and flaxseed crossover (HFxFSO) groups suggests reduced dopamine transporter function, confirmed by lower inhibition of uptake (apparent K_m) in these groups after AMPH was added to the aCSF. Cytokine application did not impact V_{max}, supporting the conclusion that inflammation does not contribute to the slowed dopamine reuptake rate under our experimental parameters. Although there is still a question whether a long-term accumulation of pro-inflammatory events, observed with chronic inflammation caused by DIO, would affect reuptake rate. Our findings show that acute pro-inflammatory insult with IL-6 and TNFα has a

modest effect on dopamine terminal function but highlights the importance of evaluating how chronic low-grade inflammation from excess adipose tissue may degrade dopamine kinetics over time.

This study demonstrated that switching from a HF to a LF diet (HFxLF) partially restored dopamine reuptake rate, which was decreased in the HF group. However, substituting unsaturated for saturated fat (HFxFSO) did not significantly affect dopamine reuptake (V_{max}). It is important to note that the diet-induced obesity phenotype was established over 6 weeks, while the crossover diet with FSO only lasted 3 weeks before kinetic measurements. A longer crossover dietary intervention to LF or FSO from HF is a necessary next step to determine if the observed improvement in V_{max} reflects a gradual restoration of dopamine transporter function that was initiated by the LF diet. This is supported by studies demonstrating delayed effects of brain structure and function changed by diet. One study showed no changes in neuronal density after a 4 week exposure to HF diet, with reductions only appearing after 8 weeks.[235] Raji et al. employed tensor-based morphometry to analyze brain structure changes over time and discovered detrimental changes in neuronal morphology are exacerbated over time with obesity.[257] Similarly, studies that ameliorated inflammation by treating obesity (through either lifestyle or surgical interventions)[273] typically observed a decrease in inflammatory markers no earlier than 3 months after onset of intervention,[323] with a common reduction timeframe of 6 months.[255] Accelerated reductions in inflammation have only been observed with extreme caloric restriction (very low calorie diets, intermittent fasting),[13,142] which was not employed in our study. Reducing body weight or attenuating inflammation through incorporation of cell membranes and adipose tissue into an unsaturated fatty acid profile may lead to improvements on a similar timescale, as suggested by these data.

Interestingly, AMPH application initially increased evoked dopamine release in the HFxFSO group before depletion of dopamine release typical with AMPH exposure[40,161]. The HFxFSO group did not show improvements in V_{max} like the HFxLF group, nor was the dopamine transporter function enhanced to the extent of the LF group, as indicated by enhanced apparent K_m after AMPH application. Insufficient timeframe may have prevented turnover of membrane phospholipid composition to increase fluidity[71,359]. Our findings suggest the persistence of chronic low-grade inflammation after the dietary crossover point. Acute bouts of inflammation have been observed following a lipid rich meal and endotoxin absorption in the gut could contribute to this inflammation[120]. Saturated fats are capable of binding TLR4 and activating a proinflammatory signaling cascade like what is seen with LPS activation[120]. Lee et al demonstrated this same activation of TLR4 is not induced with unsaturated fatty acids[191]. It has been observed that unsaturated fatty acids are precursors to some anti-inflammatory eicosanoids (resolvins, protectins, etc.)[88]. However, there may still be high levels of saturated fats being released from adipose cell metabolism in the HFxFSO group after the crossover point, increasing the availability of pro-inflammatory eicosanoid substrate. Competing imbalances in inflammatory molecules may explain differential effects of TNFα and IL-6 on dopamine release, with implications for the modulation of dopaminergic function in obesity.

Proinflammatory molecules TNFα and IL-6, exerted different effects in our FSCV dopamine release experiments. TNFα's effects are observed within minutes of exposure to inflammatory insult[271], while IL-6 has a slower timescale release, ranging from hours to days[306]. All HF groups (HF, HFxLF, HFxFSO) had similar responses to TNFα and IL-6, however, changing dietary fat source from HF to FSO increased the sensitivity to inflammatory insult. This is supported by the HFxFSO group's increased sensitivity to AMPH, which exerts

neurotoxic and neuroinflammatory effects[173]. AMPH and its derivatives influence the structure and function of pre-synaptic terminals[39,134]. Additionally, the initial increase in evoked dopamine release in the presence of AMPH suggests an increased sensitivity from changes in cellular membrane fluidity[93,260,359]. The change to FSO supports a role for the cell membrane composition in conjunction with localized AMPH interference in dopamine vesicle packaging/transport[173,325] acutely rescuing impacted neurotransmission observed with increased dopamine release prior to depletion. The altered eicosanoid profile favoring anti-inflammatory molecules like resolvins and protectins may confer neuroprotection[152,283,284] under this acute AMPH-induced inflammatory insult.

Switching to a LF diet effectively decreased body weight and normalized blood glucose metabolism, achieved through the HFxLF group severely restricting their caloric intake following the dietary crossover point; potentially inducing a state of ketosis. While the HF diet is comprised of 20% carbohydrate, exceeding the threshold used to induce ketosis in rodents[268], it could have contributed to ketosis after the dietary crossover point when mice extremely self-restricted their caloric intake (likely due to reduced palatability). Using a pair-feeding paradigm in a future study could investigate the neurological benefits. This would allow for parsing out effects independent of carbohydrate restriction.

Our study provides insights on the complex interaction between fat composition and dopamine signaling in the context of diet-induced obesity. While replacing a HF diet with a LF diet improved dopamine reuptake and blood glucose regulation, substituting saturated fat with flaxseed oil (HFxFSO) did not yield similar benefits. This suggests that early-stage dietary interventions to reduce overall dietary fat intake is superior to replacing fat type for restoring dopamine function in the NAc. This could be due to a decrease in adiposity resulting in an

improved balance of metabolic related hormones (insulin and leptin). However, the long-term consequences of dietary fat composition on dopamine signaling remain to be elucidated. The lack of significant changes in dopamine reuptake following crossover to FSO might be due to this 3-week timeframe being insufficient. Additionally, our study is limited by the difficulty of isolating low-grade inflammation without an acute event like an LPS challenge. Our mouse model may be exhibiting subthreshold levels of inflammation accumulating over time and contribute to the gradual decline of dopaminergic pathway components. Studies employing progressively increasing duration of dietary interventions and an acute inflammatory challenge under this state of DIO are warranted to fully understand these complex interactions.

CHAPTER III: NSAID TREATMENT TO ATTENUATE INFLAMMATION AND RESTORE DOPAMINE SIGNALING*

Introduction

Obesity and intake of high-fat diets are associated with systemic inflammation[190,193]; characterized by the activation of macrophages, resident immune cells that release pro-inflammatory cytokines, interleukin-6 (IL-6) and tumor-necrosis factor alpha (TNFα)[189]. These cytokines repair local tissue damage but contribute to insulin resistance and the development of metabolic syndrome[189]. The development of obesity is associated with elevated IL-6 and TNFα concentrations in the blood[249]. This chronic 'low-grade' inflammation has pleiotropic effects on systemic organs and tissues that contribute to insulin resistance. Pro-inflammatory cytokines can inhibit phosphorylation of serine residues on insulin receptor substrates[243] which promotes characteristic traits of metabolic syndrome like elevated blood glucose and increased cardiovascular disease risk[123]. Additionally, chronic acute inflammation can also cross the blood–brain barrier (BBB), which gates nutrient access to the brain[72], to act centrally on neurons and glial cells[19]. Previous studies have shown that cytokines can cross the BBB resulting in a cascade of potentially detrimental effects throughout the central nervous system, particularly neuronal function[361]. Notably, inflammation has been linked with comorbidities of obesity, like depression and anxiety, by impacting dopamine neurotransmission[100].

Dopamine plays diverse roles in learning, memory, and motivation. Previous research in this field identified increased levels of dopamine in the nucleus accumbens (NAc) following chronic high-fat ingestion[252], showing muted effects in obesity-prone animals[253]. Inflammation may impact dopamine neurotransmission in the (NAc), a brain region that influences motivation

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and food seeking[79]. One mechanism of this inflammatory response includes the activation of toll-like receptors (TLR)-4 and -2 on macrophages and microglia by lipo- polysaccharide (LPS), a molecule in bacterial cell walls, which induces CD14 and MD-2 binding and receptor internalization[60]. CD14 and MD-2 are glycoproteins which act at the cell membrane assisting in the activation of TLR4 signaling a cascade of inflammatory responses[90,364]. Saturated fatty acids promote inflammation directly by binding to MD-2 to induce TLR4 complex formation[170,345], increasing the production and release of pro-inflammatory cytokines[360]. Additionally, nuclear factor-κB and mitogen-activated protein kinase signaling upregulate the release of pro- inflammatory cytokines TNFα and IL-6[170,344,345].

The effect of cytokines on dopamine synthesis has been characterized[239]; however, there is a lack of exploration into how these cytokines directly impact dopamine release and reuptake kinetics at the terminal level. Metabolic syndrome characteristics, specifically inflammation induced by increased adiposity, are associated with elevated systemic production of cytokines which can cross the BBB[336]; while saturated fat directly binds to TLR4 causing microglial cells to release pro-inflammatory cytokines in the brain[345], likely influencing dopamine terminals in the NAc. Coupled with evidence that long-term intake of saturated fat slows dopamine reuptake rate and attenuated the capacity for phasic dopamine release in the NAc core[20,336], this study sought to explore whether changes in dopamine neurochemistry that accompany a high-fat diet are due to pro-inflammatory cytokines altering synaptic regulation of dopamine. To test this, we used *ex vivo* fast-scan cyclic voltammetry (FSCV) to appraise dopamine release and reuptake rate (V_{max}) in the NAc, then we bath applied IL-6 5nM, 10nM and TNFα 30nM, 300nM to observe any changes in synaptic dopamine regulation. These cytokine concentrations were chosen to test

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physiological limits with lower doses analogous to what would be expected in a state of chronic inflammation[16,189,237,248,249]; and higher doses approaching astrocyte toxicity levels[56,75,113,136,307]. We hypothesized that pro-inflammatory cytokines would attenuate dopamine release and reuptake rate in low-fat fed mice, replicating dopamine terminal adaptations previously reported in high-fat fed mice[20,116,336]. We also hypothesized that increasing the amount of dietary fat would enhance the effect of IL-6 and TNFα on dopamine terminals.

Experimental Procedures

Animals, Diet, and Experimental Design

Six-week-old male (n =34) and female (n=34) C57BL/ 6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed three per-cage on a reversed 12-hour light/dark cycle (lights off 0600, lights on 1800). Mice had free access to water and a purified nutrient-matched diet containing either 10% (LF), 30% (HF30), or 60% (HF60) kcals from fat with energy densities of 3.8, 4.3, and 5.2 kcals/g, respectively for six weeks (DIO series D12450J, D12053105, and D12492; Research Diets Inc). The LF diet had a fat composition of 5% lard and 5% soybean oil. HF30 diet contained a higher proportion of lard (saturated fat source) to soybean oil with 25% and 5% of total kcals, respectively. HF60 diet included the highest ratio of lard to soybean oil with 55% and 5% of total kcals, respectively. Diets were all matched for sucrose and protein content with percentage of kcals from fat being varied across experimental groups. Body weight data were collected once a week with food intake monitored and refreshed twice per week. One male fed the HF60 diet died shortly after arrival from Jackson Labs with necropsy revealing no conclusive cause of death, yielding the final group numbers: LF (n=14 male and n=14 female), HF30 (n =6 male and n=6 female), and HF60 (n=13 male and

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n=14 female). Data were collected from three unique cohorts of mice that underwent an identical diet and FSCV experimental timelines. This helped facilitate experimental throughput and ensured reproducibility. The first two cohorts contained n = 4 males and females in the LF and HF60 groups. The HF30 group was added to the third cohort which contained n=6 males and females in all three diet groups. Body weights were measured weekly. At the end of the sixth week, mice were fasted for 12 h before blood glucose was measured with an intraperitoneal glucose tolerance test (IPGTT). Fasting blood glucose was collected from the tail vein and measured using a glucometer (CVS Pharmacy, Woonsocket, RI). Next, an intraperitoneal (IP) bolus of glucose (2 g/kg in 20% w/v saline) was administered, followed by repeat blood glucose measurements at 15, 30, 60, and 120 min following the injection.

After investigating the effects of manipulating dietary fat, we applied LPS and an NSAID treatment to explore positive and negative controls for inflammation and better characterize the influence of DIO on dopamine signaling. Mice were given free access to their respective diet and were separated into LF and HF60 fed groups. After 2 weeks, a subset of the LF-fed group was implanted with micro-osmotic minipumps (ALZET model #1004) (Durect Corporation; Cupertino, CA) for continuous delivery of either physiologic saline (0.9% Sodium Chloride; Hospira, Lake Forest, IL) or 300µg/kg/day lipopolysaccharide (LPS) (Sigma Aldrich; product #L3024) for positive inflammatory control measurements. This LPS dose increases IL-6 and TNFα after 4-week subcutaneous dosing in mice[45]. These mice then underwent IPGTT and FSCV 4 weeks after minipump implantation. Another subset of mice consuming LF and HF60 experimental diets were separated into NSAID treatment groups. They received either physiologic saline or 3mg/kg bodyweight ketofen (Zoetis Inc.; Kalamazoo, MI) via IP injection

for 4 days prior to undergoing FSCV, with the fourth ketofen dose administered 4 hours prior to euthanasia.

Fast Scan Cyclic Voltammetry

Fast-scan cyclic voltammetry (FSCV) was used to measure dopamine release and reuptake rate in the NAc core. FSCV was conducted at least 3-days following IPGTT tests and began 3 h into the dark cycle. Mice were anesthetized using 5% isoflurane prior to brain removal. Brains were sectioned into 300 μm thick coronal brain slices (from +1.54 to +1.10 mm from bregma) using a compresstome (Precisionary Instruments; Greenville, NC) in oxygenated (95% O2/5% CO2) artificial cerebrospinal fluid (aCSF) (in mM: 126 NaCl, 25 NaHCO3, 11 D-glucose, 2.5 KCl, 2.4 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 0.4 L-ascorbic acid, pH adjusted to 7.4). All slices equilibrated for 60 min at 37°C with oxygenated aCSF flowing at 2 mL/min. Voltammetric recordings used a triangular waveform that scanned from −0.4 to +1.2 and back to −0.4 V vs. Ag/AgCl at 400 V/s every 100 ms. The scanning voltage was applied to a carbon fiber electrode pulled through a glass capillary with the carbon fiber (100-150 μM length, 7 μM diameter (Goodfellow, C005722; Huntingdon, UK)) placed at a depth of ∼75 μM into the NAc core. Dopamine release was evoked with a single 4 ms pulse stimulation (1p) or a train of five pulse stimulations at 20hz (5p20hz) (monophasic, 350 μA) from a bipolar stimulating electrode (Plastics One, Roanoke, VA, 8IMS3033SPCE) every 3 min. Data reported are stable baseline recordings using the criteria of three consecutive 1p recordings with <5% variation in nA peak height. Recordings typically stabilized around 60 min after collections began. Once a stable 1p baseline was established, a 5p20hz stimulation was collected from the same location to mimic physiological burst firing[46]. Next, we re-established a stable 1p baseline, then added 5 or 10nM

IL-6 or 30 or 300nM TNFα to the aCSF perfusion [IL-6 (catalogue no. SRP3330), TNFα (catalogue no. T7539); Sigma Aldrich, St. Lewis, MO]. IL-6 was reconstituted with 0.1% bovine serum albumin (catalogue no. A7030; Sigma-Aldrich) to facilitate tissue delivery. After a 30minute perfusion with IL-6 or TNFα, the perfusion was switched back to normal aCSF and collections continued for a 30-minute washout period. All recordings were obtained from the NAc core, oriented ventral to ventral lateral of the anterior commissure. The number of brain slices used to collect FSCV recordings is reported in the figure legends for each experiment. Dopamine signals were acquired and kinetically modeled using Demon Voltammetry Software, based on Michaelis–Menten kinetics, as previously described[362] holding the Km at 160 nM[355] with the assumptions that Km in mice is similar to the well-documented affinity of dopamine for the DAT in rats[355], and that sex or diet does not alter Km. Electrodes were calibrated after each experiment in a flow cell by adding 3 μM dopamine to the remaining experimental aCSF.

Statistical Analyses

Statistical analysis was conducted via GraphPad Prism (v. 9.1.1). Two-way analysis of variance (ANOVA) using repeated measures was used to identify changes in body weight. One-way ANOVA was used to analyze fasting blood glucose and IPGTTs. Two-way ANOVAs were used to compare sex differences for all dopamine parameters measured, followed by one-way ANOVAs to compare effects of diet on baseline dopamine parameters with sexes combined, due to no effect of sex detected for any baseline parameter. Two-way ANOVAs were used to detect sex effects of cytokine exposure on dopamine release. This was followed by two-way repeated measures analysis of time and diet on cytokine-induced changes in dopamine release and

reuptake rate. All post-hoc analyses used Tukey's or Šidák's multiple comparisons tests, when appropriate. Results are expressed as mean± standard error of the mean.

Results

High and moderate saturated fat intake slows blood glucose clearance, but moderate saturated fat does not affect body weight or fasting blood glucose.

In males, there were main effects of time ($F(1, 30)= 117.0$; $p<0.0001$), diet ($F(2, 30)= 8.342$; $p = 0.001$), and subject ($F(30, 30) = 3.235$; $p = 0.001$) with a time x diet interaction ($F(2, 30)= 21.64$; $p < 0.0001$) on body weight gain (Figure 1(A)). Šídák's post-hoc analyses revealed significant weight gain within diet group from week 1–6, LF ($p= 0.002$), HF30 ($p=0.002$), and HF60 ($p<0.0001$), and significantly elevated body weight between the HF60 group compared to LF ($p<0.0001$) and HF30 ($p= 0.002$) during week 6. In females, there were main effects of time ($F(1, 31)=104.3$; $p<0.0001$), diet ($F(2, 31) = 16.82$; $p < 0.0001$), and subject ($F(31, 31)= 2.679$; $p =0.004$), with a time x diet interaction ($F(2, 31)= 29.66$; $p<0.0001$) (Figure 1(A)). Šídák's post-hoc analyses revealed significant weight gain within diet group from week 1–6, LF ($p=0.003$) and HF60 ($p<0.0001$), and significantly elevated body weight between the HF60 group compared to LF ($p<0.0001$) and HF30 ($p <0.0001$) during week 6. We observed the main effects of sex ($F(1, 61)= 11.5$; $p =0.002$) and diet ($F(2, 61) = 52.65$; $p <0.0001$) on percent weight gain, with the less pronounced weight gain in females fed the HF60 diet (Figure 1(B)). Males in HF60 group had significantly higher fasting blood glucose than LF ($p<0.0001$) or HF30 ($p< 0.0001$) counterparts (Figure 1(C)). In females, the same was observed with HF60 subjects yielding significantly higher fasting blood glucose results than LF ($p<0.0001$) or HF30 ($p=0.0002$) groups (Figure 1(C)). IPGTT AUC values were significantly higher in HF60 males as compared to LF

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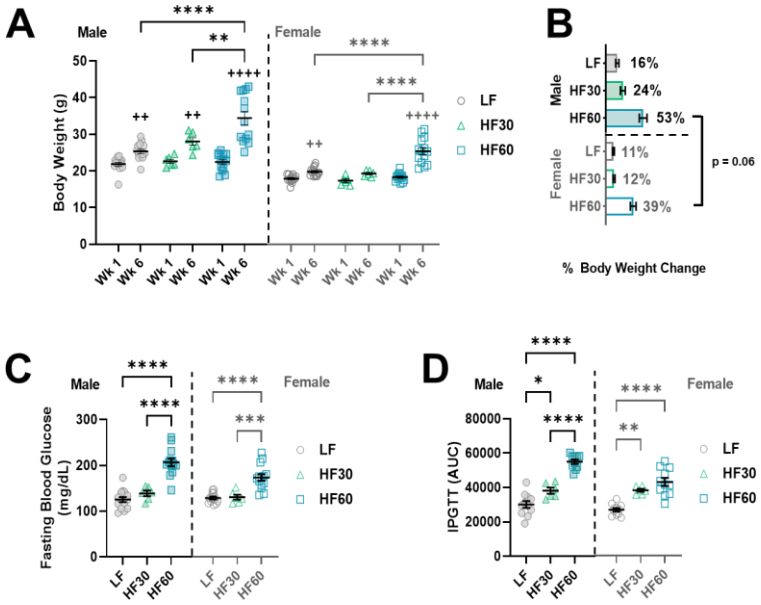
(p<0.0001) and HF30 (p<0.0001) (Figure 1(D)). Additionally, the HF30 group also had significantly higher AUC values than LF group (p=0.017). In females, HF60 subjects yielded significantly higher AUC than LF (p<0.0001) group. HF30 females also showed significantly higher IPGTT AUC than LF (p =0.0012) (Figure 1(D)).

Figure 3.1. Body Weight Changes and Blood Glucose

Body weight changes and blood glucose comparisons. Anthropometric and metabolic changes in a model of diet-induced obesity. (A) Body weight gain (g) of males and females in each experimental diet group (LF, HF30, HF60) over six weeks. (B) Percent body weight change compared body weight gain between diet group and sex. (C) Fasting blood glucose (mg/dL) for males and females in LF, HF30, and HF60 groups. (D) Area under the curve (AUC) for intraperitoneal glucose tolerance test (IPGTT) showing HF30 and HF60 groups (male and female) had greater AUC than the LF control counterpart. Data are presented as group mean± SEM. (* denotes differences between diet groups; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) (+ denotes differences within diet group; +p < 0.05, ++p < 0.01, ++++p < 0.0001)



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No effect of biological sex was observed on baseline dopamine parameters, and the HF30 and HF60 diets reduced dopamine release and reuptake rate compared to LF diet.

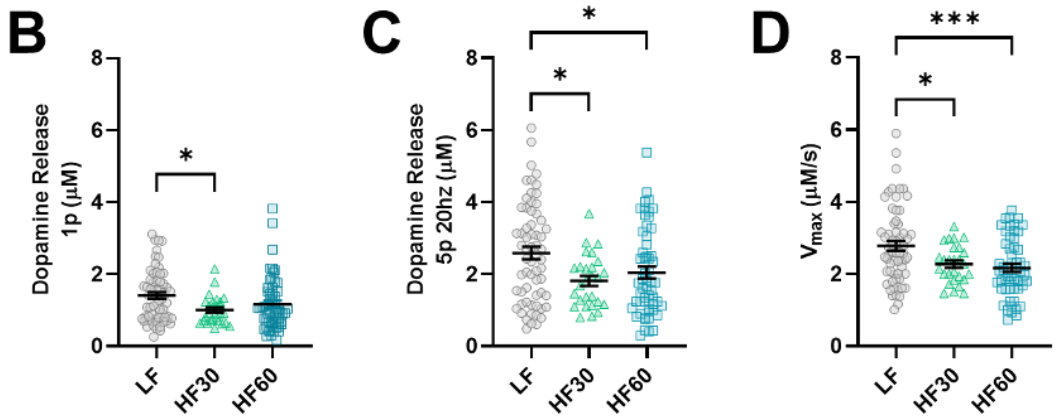
No effect of sex was detected for any dopamine parameter measured (Figure 2(A)), therefore males and females were combined in each diet group for subsequent analyses. One-way ANOVA on grouped sexes indicated a difference in single-pulse dopamine release between diet groups ($F(2, 143)= 3.911$; $p = 0.022$) with Tukey's post-hoc analysis revealing a significant decrease for HF30 mice in 1p dopamine release compared to LF group ($p=0.028$) (Figure 2(B)). A one-way ANOVA of 5p20hz dopamine release identified a main effect of diet ($F(2, 143)=4.985$; $p=0.0081$) with post-hoc analysis revealing significantly decreased 5p20hz release in the HF30 ($p=0.0173$) and HF60 ($p =0.0415$) groups, compared to the LF controls (Figure 2(C)). A main effect of diet was also observed on Vmax ($F(2, 143)= 7.787$; $p = 0.0006$), with significantly slower Vmax in the HF30 ($p=0.0354$) and HF60 ($p=0.0007$) groups compared to LF counterparts (Figure 2(D)).

Figure 3.2. Effects of Sex and Diet on Dopamine Release and Reuptake
Effects of sex and diet on dopamine release and reuptake rate. (A) Two-way ANOVA table showing diet x sex effects for single-pulse (1p) and multi-pulse (5p at 20hz) electrical stimulation of dopamine release (µM) and reuptake rate (Vmax) in the nucleus accumbens (NAc) core. Because we only observed effects of diet on these parameters, male and female data were combined for baseline analyses. Dopamine release after (B) single- (1p) and (C) multi- (5p) pulse stimulations for LF, HF30, and HF60 groups. (D) Vmax (µM/s) measuring maximal dopamine reuptake rate and comparing across experimental groups. Data represent 1–2 recordings collected from up to three slices per mouse used for ex-vivo FSCV. In the LF group n = 49 total recordings were collected in n = 23 slices from n=14 males, and n=26 slices from n=14 females. The HF30 group n=22 total recordings were collected in n=10 slices from n =6 males, and n=12 slices from n=6 females. In the HF60 group n=56 recordings were collected in n=26 slices from n=13 males, and n=20 slices from n=14 females. (B–D) data points with solid fill represent males; symbols with white fill represent females. Data are presented as individual means ± SEM, with *p < 0.05, ***p < 0.001.

A

2way ANOVA	1p Release		5p 20hz Release		Vmax	
Diet & Sex	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
Interaction	F (2, 140) = 1.988	P=0.1409	F (2, 140) = 2.375	P=0.0968	F (2, 140) = 1.007	P=0.3681
Sex	F (1, 140) = 1.358	P=0.2458	F (1, 140) = 2.158	P=0.1441	F (1, 140) = 0.3516	P=0.5541
Diet	F (2, 140) = 4.279	P=0.0157 *	F (2, 140) = 6.290	P=0.0024 **	F (2, 140) = 8.046	P=0.0005 ***

Males & Females Combined For Graphs B, C, & D



IL-6 reduced dopamine release but had no effect on dopamine reuptake rate.

After baseline dopamine parameters were established, cytokines were added to the aCSF perfusion to investigate effects on dopamine release and reuptake rate. In separate experiments, IL-6 5nM or 10nM, added to the aCSF, was bathed over the slices for 30 min followed by a 30-minute washout with standard aCSF. IL-6 5nM had no effect on dopamine release throughout IL-6 exposure (Figure 3(A)) or after the washout period (Figure 3(B)). For all groups, there was no impact of IL-6 5nM on the Vmax of the final washout collection (Figure 3(C)). Representative line traces show peak dopamine release at baseline, after adding IL-6, and following the aCSF washout period, all normalized to the three baseline collections prior to adding IL-6 (Figure 3(D)). Application of 10nM IL-6 resulted in a main effect of the subject

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(F(23, 207)= 7.386; p < 0.0001) and a time x diet interaction (F(18, 207)=3.381; p< 0.0001) on dopamine release (Figure 3(E)). Tukey's post-hoc test revealed a significant reduction in dopamine release at the 15-minute point of the washout between the HF60 and LF groups (p=0.042). The final recording during washout showed dopamine release was significantly lower in the HF60 group compared to the LF (p=0.014) (Figure 3(F)), but no effect was observed on Vmax (Figure 3(G)). Representative line traces show a reduction in dopamine release, measured as peak height, in the HF60 group, compared to control (Figure 3(H)). We observed one sex-specific effect in the 10nM IL-6 group, where dopamine release was reduced in the HF30 males compared to females (Supplemental Figure S1). Dopamine release in HF30 males returned to the same level as HF30 females during washout. All cytokine sex statistics are reported in Supplementary Figure (S1) and figure comparisons are reported in Supplementary Figure (S2).

Figure 3.3. FSCV with 5nM and 10nM IL-6

Effects of IL-6 on dopamine release and reuptake rate. Two concentrations of IL-6 were used to examine dopamine kinetics; 5nML-6 (A–D) and 10nM IL-6 (E–H). Dopamine release is reported as percent of the average µM dopamine release in the last three points with solid fill that represent males while symbols with white fill represent females. baseline collections prior to adding IL-6 for (A) 5nM IL-6 and (E) 10nM IL-6. IL-6 was washed over the slices for 30 min followed by a 30-minute washout with standard aCSF. (B) and (F) Comparison of dopamine release during the final washout collections of 5nM or 10nMIL-6, respectively. (C) and (G) Comparison of the maximal rate of dopamine reuptake (Vmax) during the last aCSF washout collection, expressed as % of baseline, for 5nM or 10nM IL-6, respectively. One FSCV recording was collected per slice for each IL-6 dose. A representative line trace from each dietary group is shown for both 5nM (D) and 10nM (H) IL-6 concentrations. This collection occurred after baseline dopamine parameters were assessed. For 5nM IL-6; LF: n = 6 males and n=8 females, HF30: n=4 males and n=3 females, HF60: n=6 males and n=8 females. For 10nM IL-6; LF: n=4 males and n=4 females, HF30: n=3 males and n =4 females, HF60: n=6 males and n=5 females. Data represent group means±SEM with *p<0.05. (B–C) and (F –G) show data points with solid fill that represent males while symbols with white fill represent females.

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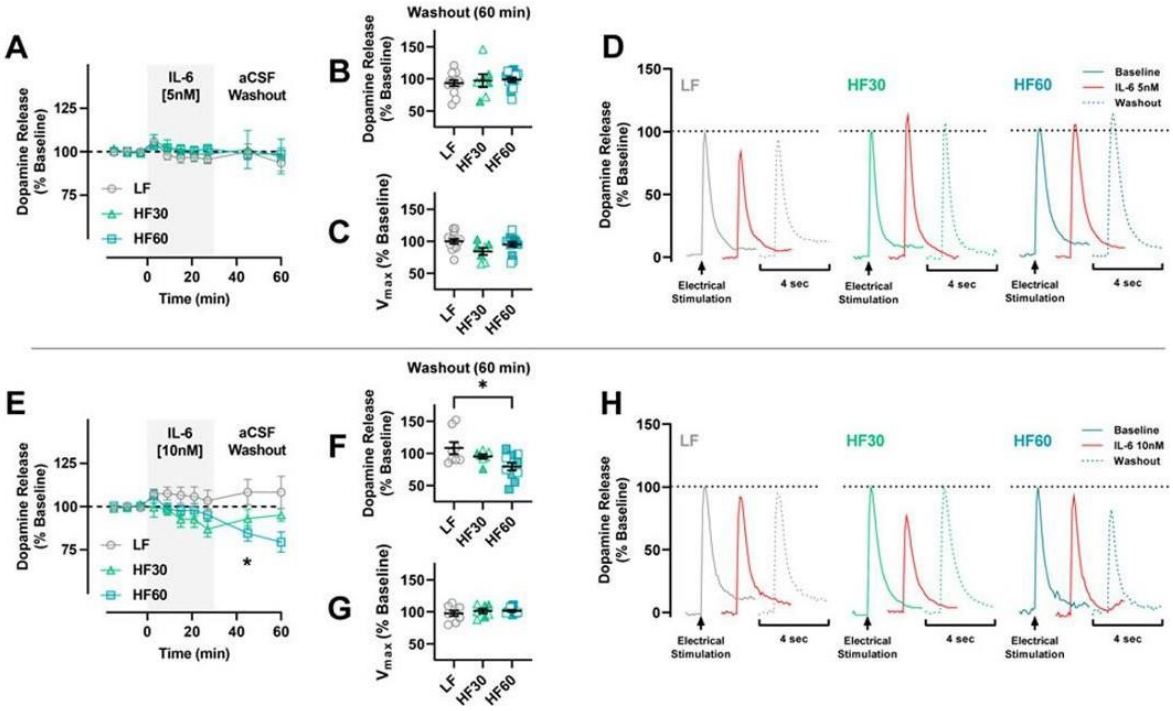

TNFα reduced dopamine release but had no effect on dopamine reuptake rate.

Separate brain slices were bathed in TNFα [30 or 300nM] to investigate effects on dopamine release and reuptake rate. A main effect of time ($F(3.157,87.70)= 2.676$; $p=0.049$) and a time x diet interaction ($F(18,250) = 1.956$; $p = 0.0127$) was observed on dopamine release with 30nM TNFα (Figure 4(A)), but repeated measures test showed no significant differences between diet groups after 30nM TNFα(Figure4(A)). There were no differences detected in overall dopamine release (Figure 4(B)) or Vmax (Figure 4(C)) after TNFα washout. Representative line traces showing dopamine release indicate no reduction in peak height caused by 30nM TNFα in any diet group. Increasing the concentration of TNFα to 300nM yielded main effects of time ($F(2.155,53.88) = 4.999$; $p = 0.009$), diet ($F(2,25)= 3.765$; $p =0.037$), and subject

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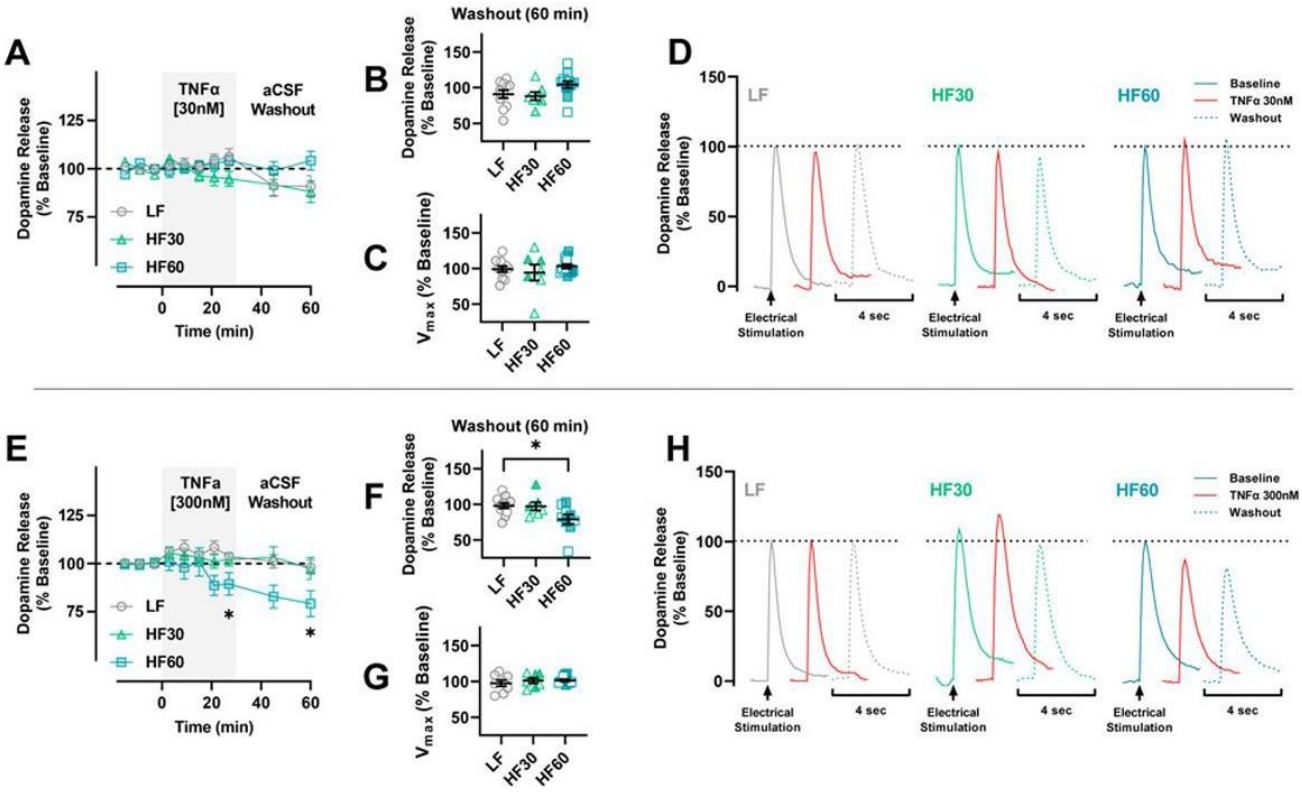
(F(25, 225)=7.989; p<0.001); along with a time x diet interaction (F(18, 225)= 2.762; p =0.0003) on dopamine release (Figure 4(E)). Tukey's post-hoc analysis indicated dopamine release was significantly reduced in the HF60 group compared to LF during the last TNFα collection (p =0.017) and at the end of the washout (p=0.05). Like the higher dose of IL-6, 300nM TNFα significantly reduced dopamine release (p = 0.033) (Figure 4(F)), but not Vmax in the HF60 group compared to the LF group (Figure 4(G)). Representative line traces show a reduction in dopamine release during the TNFα bath and further during washout (Figure 4(H)).

Figure 3.4. FSCV with 30nM and 300nM TNFα

Effects of TNFα on dopamine release and reuptake rate. Two concentrations of TNFα were used to examine dopamine kinetics; 30nM TNFα (A–D) and 300nM TNFα (E–H). Dopamine release is reported as percent of the average µM dopamine release in the last three baseline collections prior to adding TNFα for (A) 30nM TNFα and (E) 300nM TNFα. TNFα was washed over the slices for 30 min followed by a 30-minute washout with standard aCSF. (B) and (F): comparisons of dopamine release during the final washout collections of 30nM or 300nM TNFα, respectively. (C) and (G): comparisons for maximal rate of dopamine reuptake (Vmax) during the last aCSF washout collection, expressed as % of baseline, for 30 or 300nM TNFα, respectively. One FSCV recording was collected per slice for each TNFα dose. A representative line trace from each dietary group is shown for both 30nM (D) and 300nM (H) TNFα, respectively. This collection occurred after baseline dopamine parameters were assessed. For 30nM TNFα; LF: n = 5 males and n = 6 females, HF30: n=4 males and n=4 females, HF60: n=6 males and n=6 females. For 300nM TNFα; LF: n=6 males and n=6 females, HF30: n = 3 males and n=4 females, HF60: n=4 males and n=5 females. Data represent group means±SEM. *p<0.05 (B–C) and (F–G) show data points with solid fill that represent males while symbols with white fill represent females.



NSAID treatment blocked the reduction in dopamine release caused by HF60 diet after exogenous application of 10nM IL-6.

Next, we examined the effects ketofen and LPS imparted on dopamine terminal function. We investigated if the anti-inflammatory effects of ketofen could block the reduction in dopamine observed in the HF60 group following exogenous application of IL-6 and TNFα. We then sought to identify if inducing inflammation with LPS in the LF group would recapitulate the effects observed in the HF60 group. Evoked dopamine release (μM) reflecting phasic firing (5p20Hz) was significantly decreased in the HF60 group compared to LF counterparts (p=0.024) following dietary manipulation as shown in Figure 2C. The HF60 group data (summarized from

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Figure 2) suggest this amount of dietary fat impairs phasic dopamine release. I.p. ketofen injection treatment normalized dopamine release and V_{max} in the HF60 group. (Figure 5A, C)

Next, pro-inflammatory cytokines IL-6 and TNFα were added to the aCSF and dopamine release was measured during cytokine exposure and following in a washout with normal aCSF. Mixed effects analysis yielded a significantly lower dopamine release for HF60 group compared to LF at the end of the 10nM IL-6 washout time ($p=0.015$). Similarly, dopamine release in the HF60 saline group was significantly lower at the end of the 10nM IL-6 washout compared to LF saline group ($p=0.002$). No significant differences were observed during 300nM TNFα exposure or subsequent aCSF washout.

Figure 3.5. FSCV with LPS and NSAID Groups

FSCV measurements of μM dopamine release (A) V_{max} (C) for diet and NSAID treatment groups. (A) and comparison of the maximal rate of dopamine reuptake (Vmax) (C) for each group. (A) and (C) LF(Saline-minipump): n = 10 males and 4 females, LF (LPS-minipump): n = 10 males and 4 females, LF (Saline-i.p.): n = 4 males and 4 females, LF (Ketofen-i.p.): n = 4 males and 4 females, HF60 (Saline-i.p.): n = 4 males and 4 females, HF60 (Ketofen-i.p.): n = 4 males and 4 females. 10nM IL-6 (B) and 300nM TNFα (D) were used to examine dopamine kinetics. Dopamine release is expressed as a percent of the average μM release for last three baseline collections prior to adding cytokine. After baseline was established, IL-6 (B) or TNFα (D) was bathed over brain slices for 30 minutes followed by a 30 minute washout with standard aCSF. Comparison of phasic dopamine release (5p20Hz). Group number for groups in (B)/(D) LF: n = 5 males and 3 females/ 5 males and 3 females, HF60: n = 5 males and 3 females/ 5 males and 3 females, LF (Saline-minipump): n = 6 males and 5 females/ 6 males and 4 females, LF (LPS-minipump): n = 3 males and 3 females/ 3 males and 3 females, LF (Saline-i.p.): n = 3 males and 2 females/ 3 males and 2 females, LF (Ketofen-i.p.): n = 4 males and 4 females/ 4 males and 4 females, HF60 (Saline-i.p.): 4 males and 4 females/ 4 males and 3 females, HF60 (Ketofen-i.p.): 4 males and 3 females/ 4 males and 3 females. Data represent group means±SEM with *p<0.05 and ***p<0.001. (B) and (D) show data points with solid fill that represent males while symbols with white fill represent females.

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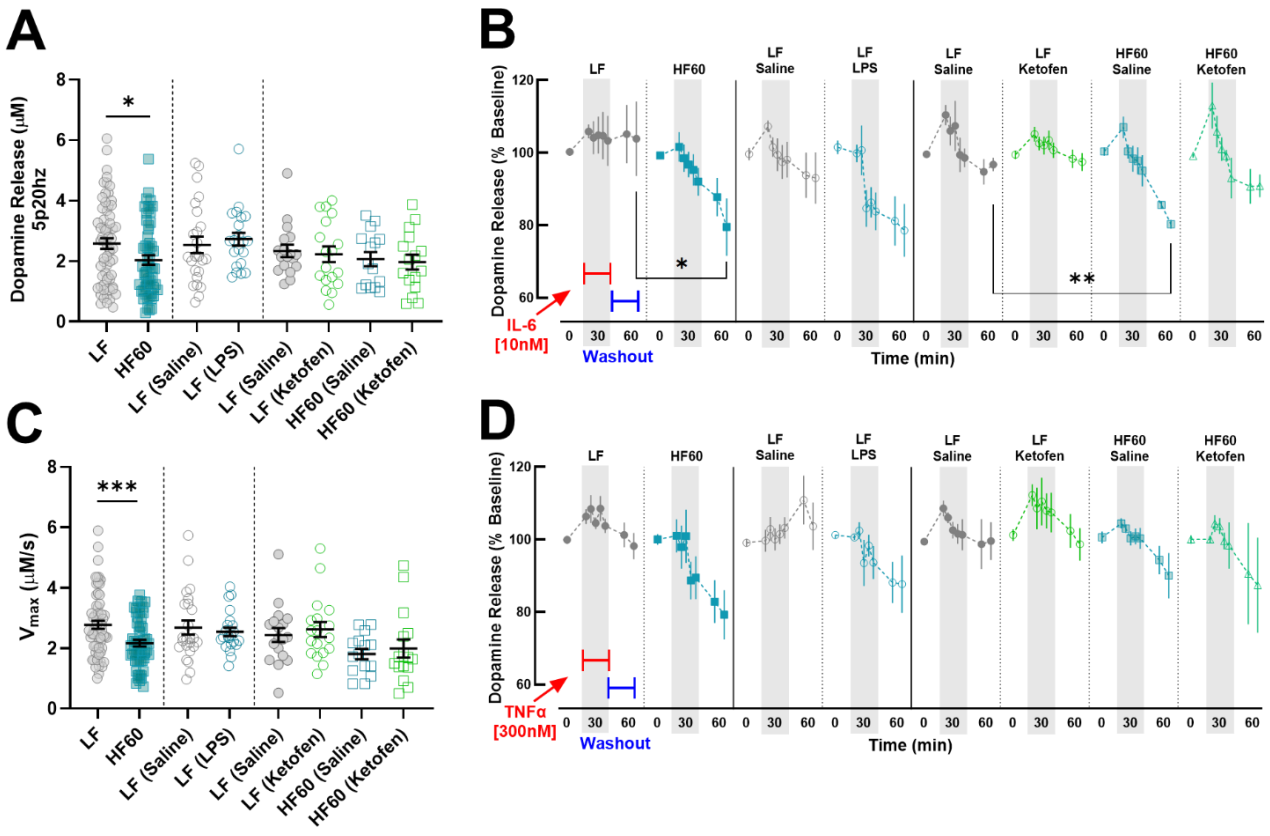

Discussion

This study sought to characterize the effects of proinflammatory cytokines on presynaptic dopamine release and reuptake rate (Vmax) at dopamine terminals in the NAc core. Our data show that IL-6 and TNFα selectively reduce dopamine release in mice fed a high-fat diet (HF60) with minimal to no effect in the HF30 or LF groups during our time-window of cytokine exposure. We did not observe any effects of IL-6 or TNFα on Vmax in any diet group; however, baseline Vmax for dopamine was significantly slowed by the moderate and high-fat diets. Although these noted effects following cytokine exposure were observed during the washout

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timeframe, this could indicate a delayed effect of the drug, attenuating the capacity for evoked dopamine release in HF60 mice. We also explored positive and negative controls for inflammation to compare with dietary results. We found that Ketofen prevented the decline in dopamine release seen in the HF60 group during IL-6 washout. Additionally, LPS reduced dopamine release in the LF group during TNFα and IL-6 washout, although these were not significant. These findings support our hypothesis that pro-inflammatory cytokines would have a greater impact on dopamine parameters with increasing levels of saturated fat. In contrast, our hypothesis that pro-inflammatory cytokines would significantly reduce dopamine release and slow Vmax in LF-fed mice was not supported. This suggests that deficits in baseline dopamine release and reuptake rate in the HF60 group (reported herein and previously[20,116,336]) are likely due to mechanisms other than this paradigm of pro-inflammatory cytokine exposure. We also provide novel evidence that a diet with moderate saturated fat (HF30) has a similar impact on dopamine release and Vmax as a high-fat (HF60) diet, despite the little impact on body weight gain and fasting blood glucose levels. Together, these data show a dose-dependent effect of saturated fat on dopamine release and reuptake kinetics in the NAc and indicate high levels of saturated fat enhance pro-inflammatory effects on dopamine terminals in this region. An excellent model by Felger and Treadway[105] outlined the impacts of inflammation on dopamine, including upregulation of quinolinic acid[256] and nitric oxide[175], resulting in decreased tetrahydrobiopterin availability[175,200,239] required for dopamine synthesis[239]. Further, cytokine administration increased phenylalanine to tyrosine ratio[101], also indicative of reduced dopamine synthesis. Inflammation has not been shown to alter dopamine transporter (DAT) expression[104]. However, chronic pro-inflammatory cytokine administration altered intracellular mitogen-

activated protein kinase signaling[371] and reduced expression of vesicular monoamine transporter 2[168] required for dopamine D2 receptor (D2R)-mediated DAT shuttling[238] and vesicular dopamine packaging, respectively. Together, the impact of inflammation on cellular signaling cascades and proteins involved with dopamine packaging and release could alter synaptic regulation of dopamine neurotransmission. High-fat diet intake was previously shown to inhibit expression[154] or membrane shuttling[65] of the DAT, as well as increase the sensitivity of dopamine D2 autoreceptors[117,259] that inhibit dopamine release and promote reuptake[238]. Reduced baseline V_{max} reportedly stemmed from diet-induced disruption of DAT expression and D2R-mediated shuttling as well as inflammation-mediated reduction of D2R expression and intracellular signaling molecules[371] that are required for DAT function. Results reported herein showed TNFα, and to a lesser extent IL-6, reduced dopamine release more strongly in animals fed an HFD. IL-6 is known to reduce neuronal tetrahydrobiopterin (BH4) concentrations[200] involved in dopamine synthesis, so it is possible that IL-6 may increasingly attenuate dopamine release with prolonged exposure. BH4 is also a cofactor for alkylglycerol monooxygenase (AGMO), an enzyme that plays a role in fatty acid cleavage from phospholipids[349]. It is possible that the increased lipid metabolism in the HF60 group could dilute tetrahydrobiopterin availability leading to less dopamine synthesis and a quicker reduction in dopamine release when compounded with IL-6 exposure. IL-6 has also been associated with reward signaling, as functional MRI scans in healthy participants revealed stress-induced plasma IL-6 predicted lower NAc activation during reward learning[313] and lower functional connectivity between the cingulate cortex and NAc[143]. Conversely, blocking inflammation improves the obese phenotype, exhibited by reduction of fat and sugar preference, food intake, and weight gain after exposure to

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an HFD with added sugar, in TLR4 knockout mice[42]. These reports suggest a functional link between IL-6 and dopamine-mediated behaviors integrated in the NAc. Moreover, psychiatric comorbidities of obesity like depression and Alzheimer's disease are increasingly linked with inflammation impacting dopamine neurotransmission[99,241]. While our model does not examine behavioral outcomes caused by IL-6-induced reductions in dopamine, we do provide one mechanistic avenue as to how dietary fat and obesogenic weight gain could enhance IL-6 effects on dopamine neurotransmission to potentiate these disorders. TNFα acts upstream of many cytokines with more widespread effects than IL-6. Our data revealed an interesting interaction with TNFα wherein the lower 30nM dose slightly reduced dopamine release in the LF and HF30 groups, whereas the higher 300nM dose significantly reduced dopamine release in HF60 mice. It was previously reported that TNFα reduced VMAT2 expression in neuroendocrine cells[168], which is important for vesicular dopamine packaging and would support reduced dopamine release by TNFα. Conversely, TNFα promoted striatal dopamine reuptake and vesicular packaging[234], promoted dopamine neuron cell death[223], and shared activation of similar intracellular signaling cascades as D2R autoreceptors[31]. Therefore, reduced dopamine release after the higher dose of TNFα observed in HF-fed versus LF-fed mice could be due to promotion of DAT shuttling and dopamine packaging in the LF group, whereas HF-fed mice with greater neuroinflammation could be more sensitive to potentiation of cell death induced by TNFα. In support of increased diet-induced neuroinflammation in the NAc, intake of a free-choice cafeteria diet reduced spine density of medium spiny neurons and activated microglia in the NAc core along with increased TNFα and IL-1β but not IL-6 expression, whereas microglial inhibition blocked these effects and reduced food intake and body weight[139]. Saturated fat is

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likely responsible for these effects, as intake of a high saturated fat diet but not monounsaturated fat increased anxiety-like and depressive behaviors in conjunction with plasma cytokine concentrations and transcriptional activity of cytokines and NF-κB in the NAc[82]. Combined with these reports showing increased neuroinflammation in dopaminergic regions, the present findings support that diet-induced obesity promotes NAc sensitivity to proinflammatory cytokines to reduce dopamine neurotransmission.

Two additional mechanistic theories that could explain the inflammatory reduction of dopamine neurotransmission involve HFD-induced central insulin resistance and dietary saturated fat priming glial cells to expedite IL-6 and TNFα effects on dopamine terminals. Prior FSCV investigations showed long-term intake of a high saturated fat diet inhibited insulin-mediated increases in dopamine release and reuptake rate seen in the NAc of LF-fed controls[116] or food restricted animals[297]. However, the role of diet-induced inflammation in NAc insulin resistance is uncharacterized. Saturated fatty acids inhibit insulin receptor substrates, PI3K, Akt, and PPARγ but promote endoplasmic reticulum stress and accumulation of inflammatory diacylglycerol and ceramide that together account for systemic and hypothalamic insulin resistance[35,170]. Additionally, HFD intake upregulates pro-inflammatory cytokines such as TNFα and IL-6[139,143], which dampen insulin signal transduction by altering phosphorylation and expression of adipocyte insulin receptor substrate[153,266]. However, a loss-of-function mutation in the TNFα, TNFα receptor, and TLR4 genes prevented HFD-induced obesity and insulin resistance[315,317]. Given that insulin receptors are present on cholinergic interneurons and presynaptic dopamine terminals in the NAc[297] that function to promote dopamine release[297] and reuptake[238], respectively, it is possible that diet-mediated inflammation promoted insulin

resistance in the NAc and could account for reduced dopamine reuptake in HF-fed mice. Mice used for the IL-6 and TNFα investigations were fed ad libitum, so saturated fat-induced striatal insulin resistance could have been exacerbated by the addition of pro-inflammatory cytokines to reduce dopamine release. Similarly, saturated fats consumed ad libitum could directly activate TLR4 on microglia increasing endogenous levels of IL-6 and TNFα in the HF60 group[60,170,344,345]. This could initiate glial cells response prior to our exogenous application of cytokines, thus enhancing or expediting their effects on dopamine terminals.

NSAID treatment investigated whether observed deficits in dopamine release and reuptake caused by the HF60 diet could be reversed by attenuating diet-induced inflammation. Ketofen attenuated the reduced dopamine release during the washout phase following application of IL-6. Conversely, TNFα did not yield the same dopamine release impairment. This highlights the differences in timescale release of these pro-inflammatory cytokines. TNFα acts rapidly, appearing within minutes of inflammatory insult[249], while IL-6 has longer lasting release, peaking hours to days after the inflammatory cascade is triggered[249,306].

Long-term consumption of a high-fat diet impairs dopamine release capacity. The effects of systemic inflammation and ketofen treatment appear more complex, depending on the specific inflammatory cytokine and stage of the inflammatory response, particularly in animals consuming a HF60 diet. Results from the FSCV experiments performed following ketofen treatment support the hypothesis that IL-6 reduction leads to an increase BH4 availability. Pro-inflammatory IL-6 decreases BH4 concentrations[200], which serves as a cofactor for tyrosine hydroxylase (key in dopamine synthesis)[151,233] leading to decreased dopamine release with long term exposure. Additionally, BH4 aids in the cleavage of fatty acids from phospholipids by

functioning as a cofactor for alkylglycerol monooxygenase (AGMO)[349]. Therefore, increased dietary fats could increase lipid metabolism in the HF60 group and further dilute BH4 availability to facilitate dopamine synthesis and subsequent release. By blocking production of IL-6, ketofen may remove the suppression of BH4 and lead to attenuation of the decline in dopamine synthesis.

Contrary to our initial hypothesis, systemic inflammation induced by LPS minipump administration did not decrease dopamine release compared to saline controls. This suggests that an adaptive mechanism could have been triggered to resolve the continual release of LPS. Conversely, the release of LPS from interscapular minipump implantation might not have mimicked traditional diet-induced metabolic endotoxemia conditions. However, these data suggest that ketofen treatment has the potential to reduce the effects of proinflammatory cytokines on the decay of dopamine release. While long-term use of NSAIDs has known deleterious effects[184,216,278], these findings point to the development of potential molecular targets involved in IL-6 and BH4 regulation that does not also inhibit widely employed COX enzymes.

Conclusions

This investigation offered novel insights into how diet-induced obesity could alter inflammatory responses that influence dopamine kinetics. We expand on previous literature demonstrating that deficits in dopamine neurotransmission occur under moderate saturated fat intake similar to high levels of saturated fat, despite large changes in body weight. This indicates dietary fat influences dopamine kinetics independent of obesogenic weight gain. The moderate level of saturated fat, however, yielded a muted effect of cytokines to reduce dopamine release compared to the high saturated fat group. Additional investigations into glial cell reactivity due

to dietary fat are warranted to further characterize a potential role in high levels of saturated fat priming an immunological response in the brain. Our study had several strengths, including use of males and females over several independent cohorts, showing reproducibility. One limitation included a relatively short dosing window of cytokines, though sufficient to observe rapid onset effects due to diet exposure. Overall, this study supported that high levels of saturated fat intake reduced the capacity for dopamine neurotransmission through an increased sensitivity of the NAc dopamine system to inflammation. These data provide a potential mechanism for how neuroinflammation caused by diet dampens dopamine neurotransmission. Additionally, we found that non-selective NSAID treatment blocked the effects of IL-6 on dopamine release, suggesting a potential target for future therapies.

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CHAPTER IV: MODERATE-INTENSITY AEROBIC EXERCISE ENHANCED DOPAMINE SIGNALING IN DIET-INDUCED OBESE FEMALE MICE WITHOUT PREVENTING BODY WEIGHT GAIN

Introduction

Diet-induced obesity (DIO) is associated with the development psychiatric disorders including depression and anxiety.[10,212] The public health burden of obesity and brain related disorders is caused by certain diet patterns that are linked to a state of chronic low-grade inflammation, shown to dysregulate the dopamine system.[24] Therapeutics to attenuate body weight gain and reduce inflammation may improve brain health. A low-cost alternative to pharmaceutical treatments is moderate-level exercise which is shown to reduce body weight and decrease chronic inflammation potentially improving brain health by attenuating the DIO effects on dopamine signaling.

DIO is caused by excess calories, often in the form of ultra-processed foods and other foods high in saturated fat. In neurons, saturated fat is known to bind the same TLR4 receptor as LPS, mediating a cascade resulting in increased proinflammatory cytokine release.[156,192,346] Additionally, systemic inflammation caused by adipose tissue expansion has been shown to disrupt dopamine mechanisms in reward-related neural pathways.[106] Specifically, systemic inflammation from hypertrophic adipose cells may cause a decreased release of phasic dopamine[96] and changes to post-synaptic receptor density.[103] Adipose tissue releases an array of cytokines (mix of anti- and pro- inflammatory) that are increased with adiposity.[68] Pro-inflammatory cytokines interleukin-6 (IL-6), tumor-necrosis factor alpha (TNF-α), and interleukin-1beta (IL-1β) are released from adipocytes[214], macrophages when an innate immune response is activated[87], and microglia as the resident immune cell type of brain tissue.[210,341] TNF-

α and IL-1β are some of the first cytokines released in response to an inflammatory insult with slower responding cytokines (IL-6) being released on a longer timescale.[305,347] Evidence suggests these cytokines influence dopamine signaling, but questions remain as to the time-course and magnitude of interaction.

While exercise initially elevates markers of inflammation,[37,302] prolonged training yields adaptations and overall protection from activators of the immune system.[217,281] In Parkinson's patients, aerobic exercise was shown to increase dopamine signaling during anticipation of a reward, an effect not seen in controls.[269] Similarly, improvements were also seen in children with ADHD who underwent a moderate-intensity exercise regimen.[245] These data suggest an underlying mechanism that is common to various neurological disorders, hypothesized to involve dopaminergic pathways. However, the potential interaction between pro-inflammatory molecules modulating dopamine neurotransmission is unclear. Exercise has potential to mitigate inflammation caused by adiposity through the release of brain-derived neurotrophic factor (BDNF). BDNF levels increase following acute aerobic exercise protocols,[289,320] and have been shown to enhance neurogenesis[204] and neuronal plasticity.[63] However, the expression of BDNF after a chronic exercise regimen yielded mixed results,[181,289] and further confounded hinging on what type of biological sample type used for analysis (brain tissue vs plasma).[181,289] While plasma BDNF levels were actually shown to decrease after long-term exercise in a population of mid-aged men, they showed improved memory.[181] suggesting that there might be an interaction between BDNF and dopaminergic pathways due to long-term potentiation in the striatum being dopamine-dependent.[22,23,27,264] However, BDNF is shown to increase directly following an exercise bout and might have interactions with dopaminergic pathways, as shown by Bastioli et al., 2022.[25]

Therefore, the aim of this study was to investigate if moderate-intensity exercise could reduce body weight gain and associated implicated in decreased levels of dopamine signaling observed with high-fat diet-induced adiposity. To test this, fast-scan cyclic voltammetry was used to measure pre-synaptic dopamine kinetics in brain slices containing the nucleus accumbens (NAc), a dopamine-rich brain region in the ventral striatum, involved in motivational aspects of decision making and reward processing. Inflammation state was also assessed in mice fed a low-fat (LF) or high-fat (HF) diet with or without a treadmill exercise intervention. We hypothesized that exercise would attenuate body weight gain and diet-induced inflammation in (HF)-fed mice corresponding with dopamine signaling comparable to sedentary, (LF)-fed counterparts.

Experimental Procedures

Animals, Diet, and Experimental Design

Six-week-old mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed on a reversed 12-hour light/dark cycle (lights off 0600, lights on 1800). Mice were given free access to food and water and randomly assigned to one of four groups: 1) LF sedentary (LF), 2) LF exercised (LFex), 3) HF sedentary (HF), or 4) HF exercised (HFex). LF-fed groups were provided a diet with 10% total kcals from fat (Research Diets, D12450J) while HF-fed groups were provided with a diet 60% total kcals from fat (Research Diets, D12492). The mice had access to their respective diets only for six weeks. All experiments were conducted following approval by the UNC Greensboro Animal Care and Use Committee in compliance with the *Guide for the Care and Use of Laboratory Animals (NIH).*

Treadmill Running

The aerobic exercise protocol consisted of forced treadmill running on a Collins treadmill (Braintree, USA). Exercised groups were acclimated to the treadmill and the onset of the exercise program coincided with the onset of experimental diet consumption. All running

activity started with a warm-up period at a rate of 10m/min for 5 minutes. Speed was then gradually increased to 15m/min for the remaining run duration. Mice ran for 30 min/day, 5days/week starting week 1. Duration was progressively increased by 10 minutes/day for each consecutive week to reach a maximum of 60min/day.

Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance tests (IPGTT) were performed to examine blood glucose clearance. Mice were fasted 12 hours prior to IPGTT which began three hours into the dark cycle (0900). Fasting body weights were recorded to calculate volume required for 20% glucose administration. A topical anesthetic (lidocaine 2.5% and prilocaine 2.5% (item #977934 CVS, NC)) was applied to the tail tip, which was then nicked with a surgical blade to produce a droplet of blood for blood glucose measurement with a glucometer (item #323932 CVS, NC). Baseline glucose was measured, then mice received an intraperitoneal injection of 20% glucose (2g/kg bodyweight; Sigma, St. Louis, MO) in sterile saline (0.9% sodium chloride, Hospira; Lake Forest, IL). Blood glucose was measured at 15-, 30-, 60-, and 120-minute intervals. These values were plotted and reported as area under the curve (AUC).

Tissue Collection

After six-weeks of the diet and exercise protocol, mice were rendered unconscious with 5% isoflurane and euthanized, by rapid decapitation. The brain was removed and hemisected with left brain hemisphere delegated for voltammetry preparation and remaining right hemisphere used to dissect out the NAc, which was flash frozen in liquid nitrogen and stored at -80°C for gene expression analysis.

Real-Time PCR

Frozen tissue samples stored at -80°C were lysed via sonification in RNA lysis buffer (catalog #: R1060-1-100, Zymo Research; CA, USA) and then centrifuged at 13,000rpm for 30 seconds. Supernatant was then removed, and RNA was further purified through a series of spin centrifuge reactions following manufacturer's instructions (Quick-RNA^{TM} MiniPrep, catalog #: R1055, Zymo Research; CA, USA). cDNA was created using a reagent kit from (ThermoFisher Scientific, USA; catalog #: 4368813) according to protocol previously described.[312] RT-PCR was run using TaqMan Advanced Master Mix (catalog #: 4444556, Applied Biosystems; USA) and probes for genes: 18s (Mm03928990_g1), Tyrosine Hydroxylase (Mm00447557_m1), Itgam (Mm00434455_m1), Aif1 (Mm00479862_g1), IL-6 (Mm00446190_m1), IL-1B (Mm00434228_m1), TNFα (Mm00443258_m1), and Slc6a3 (Mm00438388_m1). Gene expression was quantified using the $\Delta\Delta^{ct}$ method[209] with 18s used as the endogenous control.

C-Reactive Protein

Trunk blood was collected in (1.5mL Flex-Tubes, catalog #: 022363531; Eppendorf, USA) then centrifuged for 10 min at 3,000 rpm at 4°C and plasma was decanted and stored at -80°C until analysis. ELISA kits were used to analyze CRP protein levels and performed according to manufacturer's instructions. (Catalog #: MCRP00, R&D Systems, Minneapolis, MN)

Fast Scan Cyclic Voltammetry (FSCV)

Mice from each treatment group proceeded to FSCV in a Latin-square design. Following brain removal, brains were placed in oxygenated artificial cerebrospinal fluid (aCSF) (126mM NaCl, 25mM $NaHCO_3$, 11mM D-glucose, 2.5mM KCl, 2.4mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM NaH_2PO_4, 0.4mM L-ascorbic acid) (Sigma product codes: NaCl-1002579927, $NaHCO_3$-S5761,

D-glucose-1002593418, KCl-1002323941, $CaCl_2$-1002393239, $MgCl_2$-1002368596, NaH_2PO_4-1002355618, L-ascorbic acid-1002334976) and sliced coronally into 300µm thickness with a compresstome (Precisionary Instruments; Greenville, NC) as previously described.[92] Slices were equilibrated for 60 minutes in oxygenated aCSF flowing at a rate of 100mL/hr before beginning experiments. A glass capillary-pulled carbon fiber electrode (C005722, batch 4, carbon metal fiber, 10m length; Goodfellow) (A-M Systems, catalog #60200; Sequim, WA) was placed approximately 50µM into the slice within the region of interest (NAc) next to a bipolar stimulating electrode (Plastics One; Roanoke, VA) that evoked terminal release of dopamine with a single monophasic electrical pulse (350µA, 4ms) or a five-pulse train at 20Hz (5p20Hz). Dopamine release and uptake was recorded for 15 seconds with a three-minute recovery window between stimulations. Baseline dopamine recordings were considered stable when the current detected for dopamine peak height was within 10% between three or more recordings. An Ag/AgCl reference (Precision Instruments; Sarasota, FL) was used to scan a triangular waveform between -0.4V and 1.2V at a rate of 400V/s every 100ms. Dopamine current (nA) was converted to concentration (µM) using a calculation of electrode sensitivity through DEMON voltammetry and analysis software[363] Michaelis-Menten kinetics were used to determine the concentration of maximal dopamine release [DA] and reuptake rate (V_{max}) with K_m held at 160nM, corresponding with the affinity of dopamine for the dopamine transporter.[221] K_m was only adjusted after the application of amphetamine (AMPH), to measure any diet- or exercise-induced differences in the affinity of dopamine for the DAT.[363] After baseline recordings were established we collected multiple-pulse recordings at 5p20Hz. We then re-established a stable single-pulse baseline prior to the application of the competitive inhibitor AMPH at concentrations of 300nM and 3µM. Dopamine release [DA] and reuptake rate (V_{max}) were analyzed for differences across groups.

Statistical Analyses

All analyses were performed in GraphPad Prism (v.9.1.1). Three-way ANOVA was used to analyze sex-differences between males and females and kept separate for further analyses. Two-way analysis of variance (ANOVA) was used to identify diet or exercise effects in daily food intake, body weight and dopamine related outcomes (release, reuptake rate). All group differences were assessed using Šidák's or Tukey's post-hoc tests. Results are expressed as mean ± standard error of the mean (SEM). Outliers were identified and removed using the ROUT method, which is based on false discovery rate, and maximum threshold specified was 1%.

Results

Moderate-intensity exercise did not significantly reduce body weight gain or normalize blood glucose during six-weeks of HF-feeding.

Body weight gain was tracked weekly for the duration of the study and expressed as a percent of initial body weight (Figure 1). All groups gained significant weight from weeks 1-6 ($p<0.0001$) (Fig. 1-A males, 1C females), consistent with growth and development from adolescence into early adulthood. A main effect of diet was detected for body weight gain in males ($p<0.0001$), where sedentary HF-fed gained significantly more body weight (13.78g ± 1.45g) than the LF diet (4.64g ± 0.14g) ($p<0.0001$). Moderate-intensity exercise did not significantly influence body weight gain for either diet group (HF- 10.68g ± 1.10g and LF- 3.84g ± 0.6g (Fig. 1A). A similar main effect of diet was observed in females, ($p=0.0003$), where sedentary HF-fed females gained significantly more weight (8.99g ±0.84g) than the LF-fed group (1.93g ±0.21g) ($p<0.0001$). Exercise did not attenuate body weight gain in females (HF- 6.67g ± 1.05g and LF- 1.03g ±0.36g) (Fig. 1C). Body weight gain was also expressed as a percent of weight gain in addition to initial and final body weights with similar results for males (Fig. 1B) and females (Fig. 1D).

Figure 4.1. Body Weight
Measures of body weight for each experimental group**.** Initial body weight from week 1 (Wk1) and final body weight from week 6 (Wk6) are reported for males (A) and females (C) for each of the experimental groups-low fat sedentary (LF), low fat exercised (LFex), high fat sedentary (HF), and high fat exercised (HFex). The change in body weight are reported as % body weight gain for males (B) and females (D). ***p<0.001; ****p<0.0001

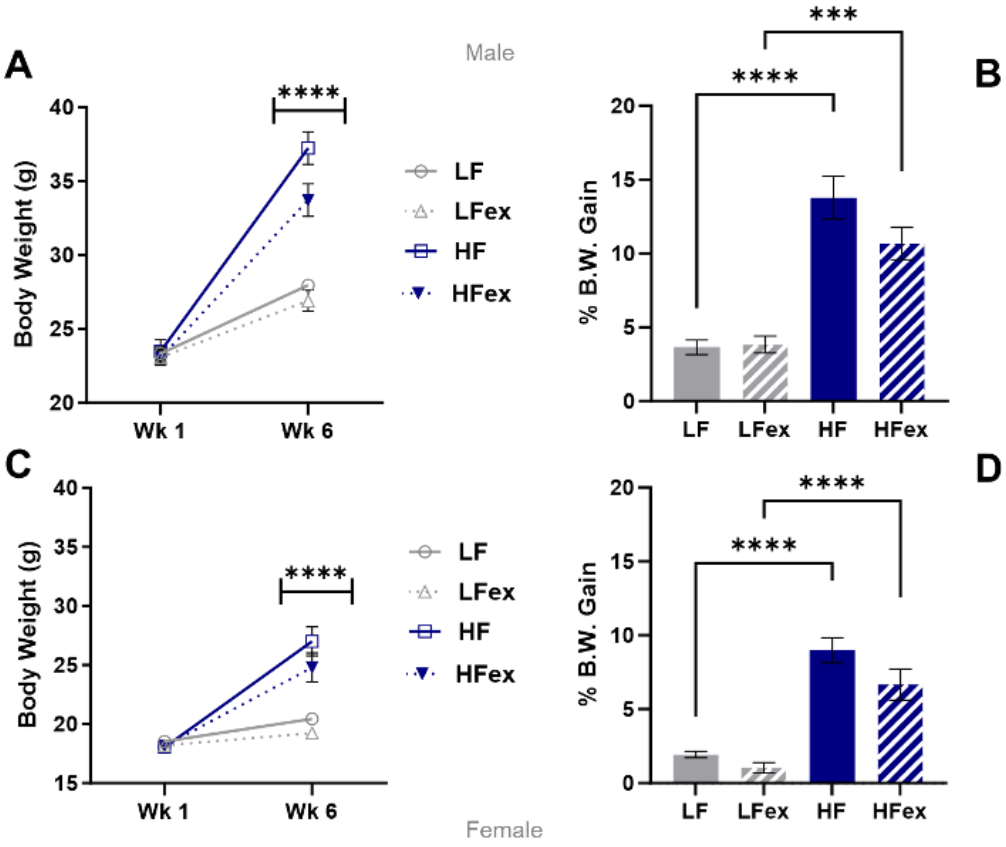
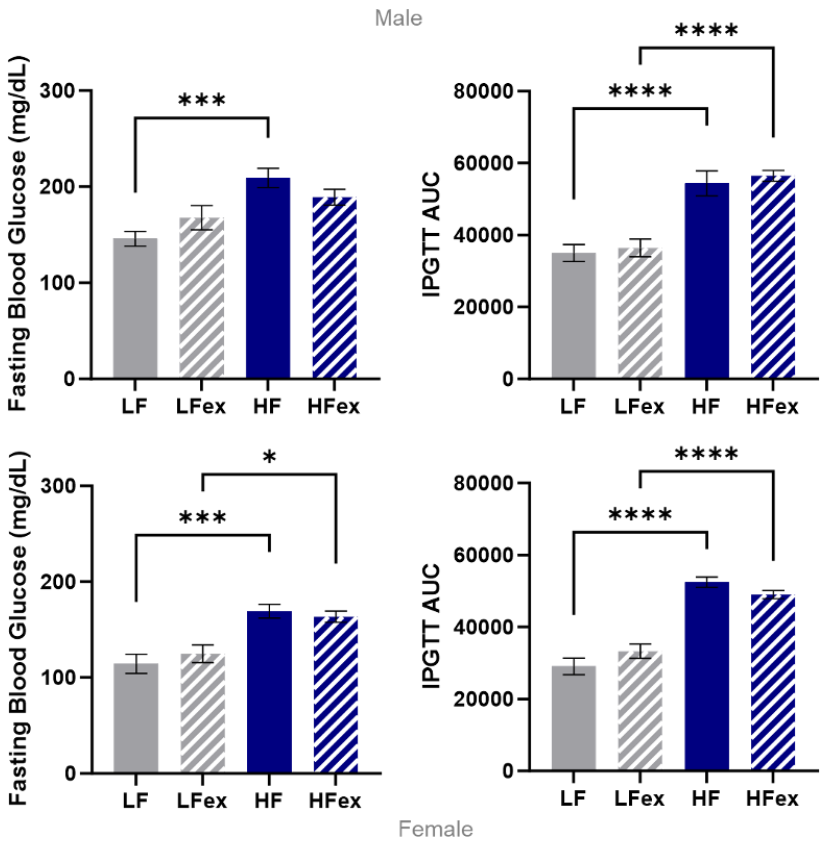


Figure 4.2. Glucose Metabolism
Measurements of Glucose Metabolism. Fasting blood glucose for males (A) and females (C) are shown in addition to area under the curve (AUC) for intraperitoneal glucose tolerance tests (IPGTT) in both males (B) and females (D). *p<0.05; ***p<0.001; ****p<0.0001

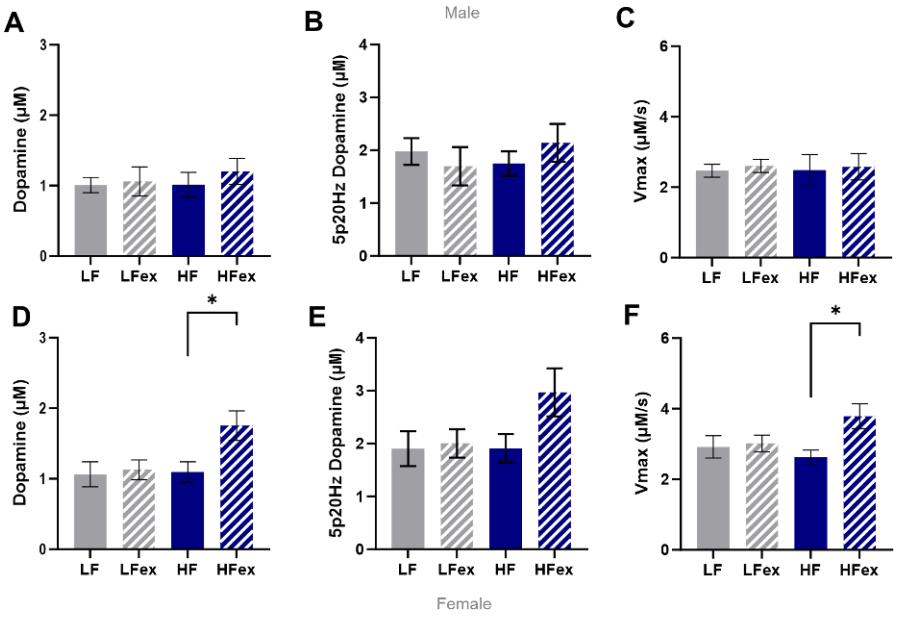


Metabolic phenotyping was assessed using fasting bloody glucose measurements and blood glucose clearance challenge via IPGTT (Figure 2). Fasting blood glucose was significantly elevated in sedentary HF-fed males (Fig. 2A) and females (Fig. 2C) ($p<0.001$). Additionally, HF-exercised females had significantly elevated fasting blood glucose compared to LF-exercised counterparts ($p<0.05$) (Fig. 2C). Area under the curve (AUC) was calculated from (IPGTT) to evaluate blood glucose clearance (Fig. 2B males, 2D females). HF-fed groups had significantly larger AUC in comparison to LF-fed counterparts in both males (Fig. 2B) and females (Fig. 2D) ($p<0.0001$, respectively). Exercise did not influence blood glucose clearance with no differences detected between LF-sedentary and LF-exercised males or females. Similarly, no differences between HF-sedentary and HF-exercised males or females (Fig. 2B males, 2D females).

Moderate-intensity exercise increased single-pulse dopamine release and reuptake rate in HF-fed female mice.

FSCV was used to measure pre-synaptic dopamine release and reuptake rate (Figure 3). No differences in dopamine release from single pulse (Fig. 3A) or five-pulse (5p20Hz) stimulations (Fig. 3B) were observed. Additionally, no differences were observed for males in reuptake rate (V_{max}) (Fig. 3C). In HF-fed females, exercise significantly increased single pulse dopamine release (Fig. 3D) and increased V_{max} (Fig. 3F) ($p<0.05$). However, no differences in dopamine release were seen under evoked multi-pulse conditions (Fig. 3E).

Figure 4.3. FSCV Dopamine Release and Reuptake
Dopamine Release and Reuptake Rate. Evoked single pulse µM dopamine [DA] release for males (A) and females (D) for each experimental group. Dopamine release for evoked multi-pulse is expressed as a % of baseline release for both males (B) and females (E). Enzyme kinetics for reuptake of dopamine is shown as V_{max} (µM/s) for males (C) and females (F). *p<0.05



HF-fed mice displayed faster AMPH-induced depletion of pre-synaptic terminal dopamine and greater sensitivity to inhibition of dopamine transporter reuptake.

AMPH was bathed over brain slices after baseline recordings at 2 different concentrations (300nM and 3µM) for 30 minutes each (Fig. 4). In males, AMPH depleted dopamine terminals in the sedentary HF-fed group compared to the sedentary LF-fed ($p<0.05$) (Fig. 4A). Similarly, exercised HF-males had greater depletion of dopamine release with AMPH compared to exercised LF-males ($p<0.05$) (Fig. 4B). However, exercise had no effect on dopamine depletion within diet groups (Fig. 4C, 4D). In females, no differences in dopamine release were observed following AMPH application for any group comparisons (Fig. 4E-H).

K_m was calculated to quantify level of inhibition for dopamine reuptake into pre-synaptic terminals during AMPH exposure. In males, the only difference observed was between sedentary LF and HF groups. The HF-fed group had significantly lower K_m than the LF-fed group ($p=0.015$) at the 60-minute time point with 3µM AMPH (Fig. 5A). Conversely, exercised males showed no differences regardless of diet ($p=0.807$) (Fig. 5B), suggesting that exercise normalized this effect comparable to sedentary and exercised-LF groups. Likewise, there were no differences within diet group between exercise comparisons for males (sedentary LF vs exercised LF (Fig. 5C); and sedentary HF vs exercised HF (Fig. 5D)).

In females, there were no differences when comparing diet in sedentary groups (Fig. 5E). However, LF-exercised females yielded a significant decrease in K_m at the 60-minute timepoint with 3µM AMPH compared to HF-exercised females ($p=0.018$) (Fig. 5F). Further comparing differences in K_m within diet groups discerned a significantly higher K_m in exercised HF-females than the sedentary HF-females ($p=0.006$) (Fig. 5H). However, no difference between LF-fed females was detected when comparing sedentary and exercised groups (Fig. 5G).

Figure 4.4. FSCV Dopamine Release with Amphetamine
Dopamine release in presence of AMPH. Single-pulse evoked dopamine release [DA] expressed as a % of baseline for males (A-D) and females (E-H) with application of increasing amphetamine (AMPH) concentrations (300nM, 3µM) over 60-mins. 300nM AMPH was applied over first 30 minutes (light grey panels) and 3µM AMPH during second timeframe (darker grey panels). Comparisons are displayed between LF/HF groups (A, E); LFex/HFex groups (B, F); LF/LFex groups (C, G); and HF/HFex groups (D, H). *p<0.05

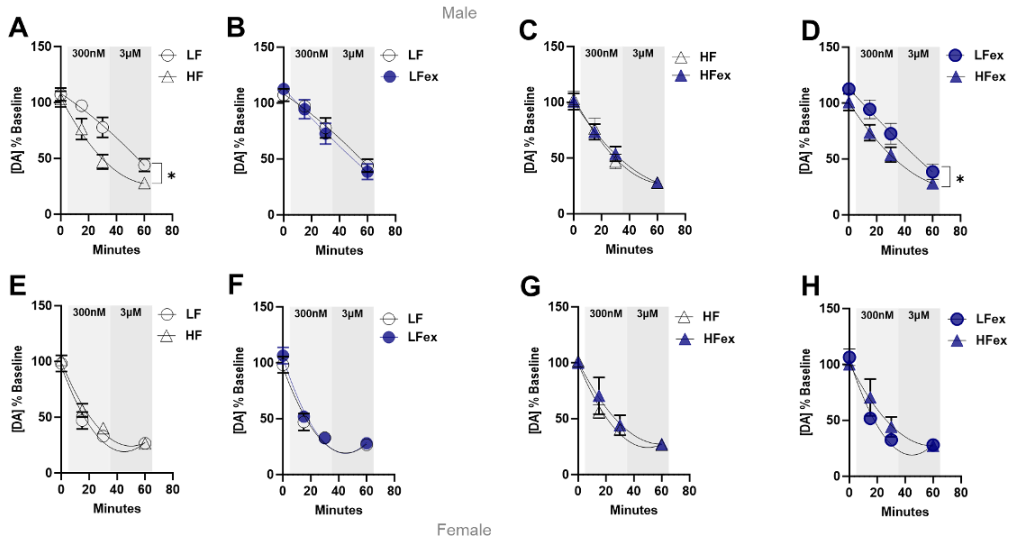
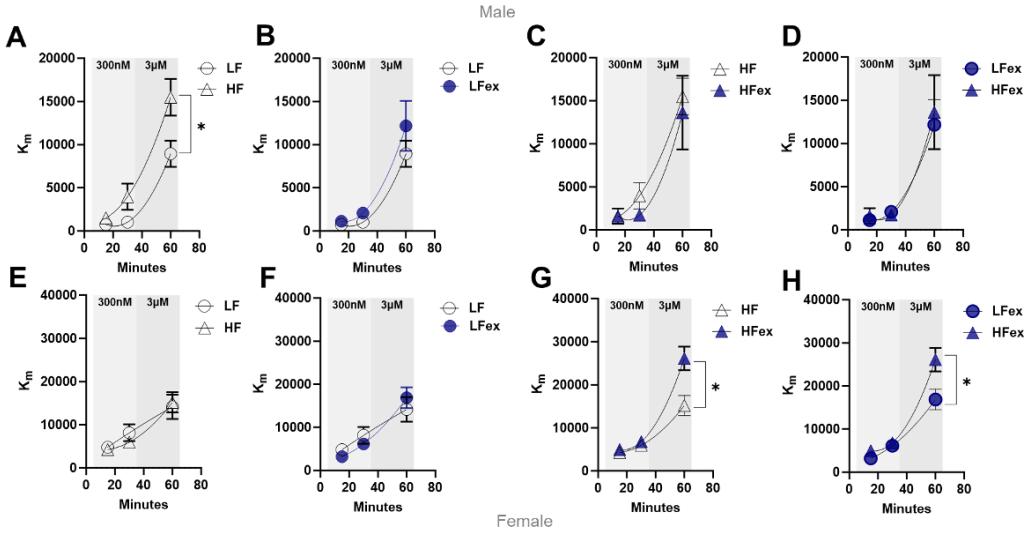


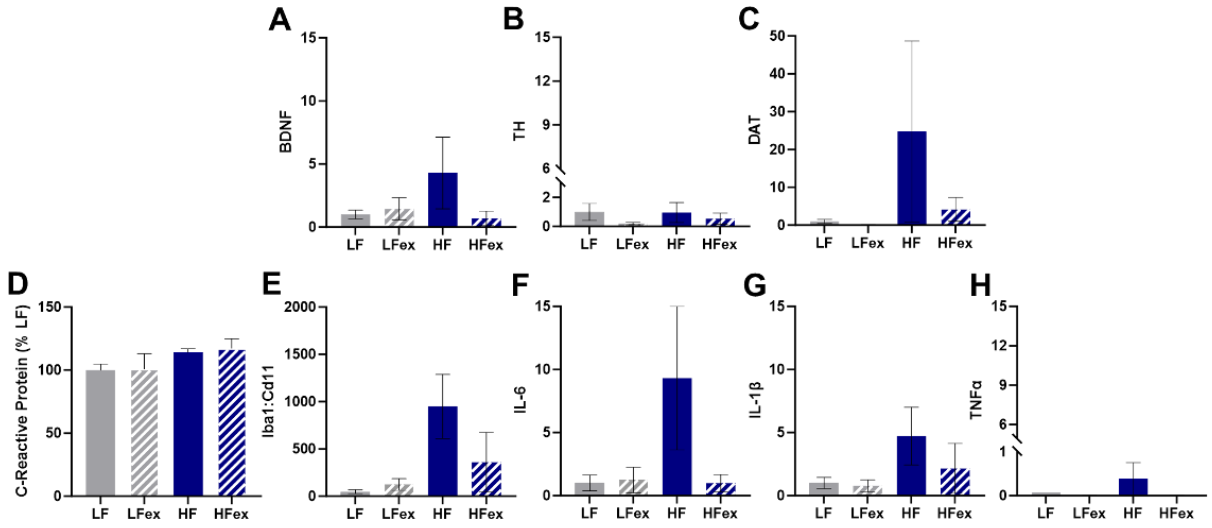
Figure 4.5. FSCV K_m with Amphetamine
Measurement of K_m in presence of AMPH. Changes in K_m for males (A-D) and females (E-H) with increasing amphetamine (AMPH) doses (300nM, 3µM) over a 60-minute timeframe. 300nM AMPH was applied over first 30 minutes (light grey panels) and 3µM AMPH during second timeframe (darker grey panels). Comparisons are shown between groups: LF/HF (A, E); LFex/HFex (B, F); LF/LFex (C, G); HF/HFex (D, H). *p<0.05



Diet type and exercise did not influence gene expression or protein levels of pro-inflammatory biomarkers in male mice.

Gene expression in the NAc was quantified for BDNF (Fig. 5A), TH (5B), DAT (5C), Iba1:Cd11 (5E), IL-6 (5F), IL-1β (5G), and TNFα (5H) all normalized to a housekeeping gene, 18s. Plasma levels of C-reactive protein were measured and normalized to the sedentary LF group (Fig. 5D). No significant differences were detected in male mice for CRP or any gene expression measurements (Figure 5). However, sedentary HF-fed males had a globally enhanced pro-inflammatory gene expression profile compared to sedentary LF males, although no statistically significant comparisons were observed. A noteworthy increase in the ratio of Iba1 to Cd11 was observed for sedentary HF-fed males (949.2 ± 338.9) compared to sedentary LF males (46.84 ± 20.11), mirrored trends in pro-inflammatory gene expression, though not significant (p=0.085) (Fig. 5E).

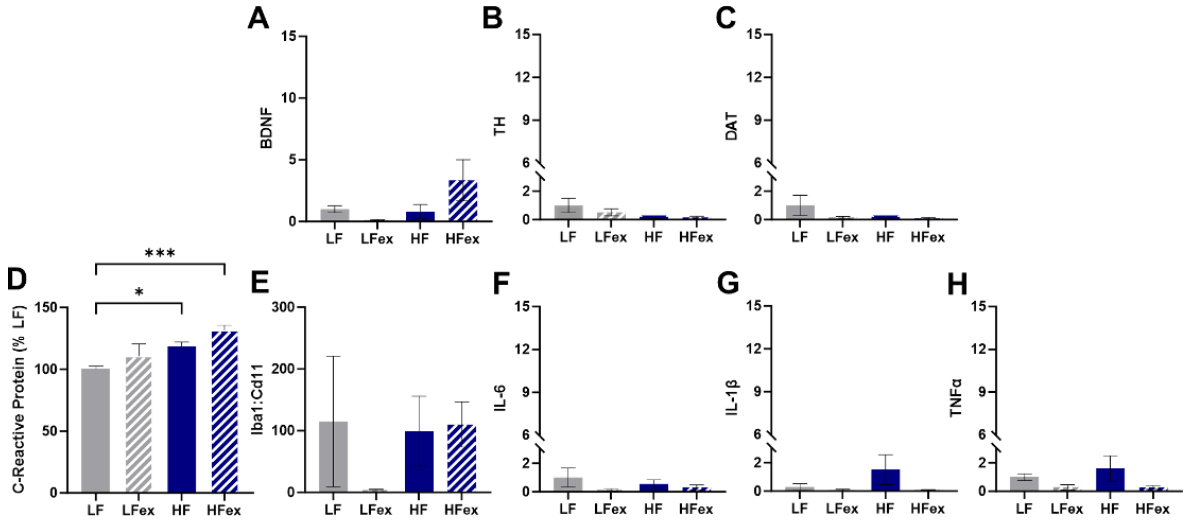
Figure 4.6. Plasma CRP and NAc Gene Expression for Males
Data are normalized to sedentary low-fat (LF) group values. 18s was used as the housekeeping gene to evaluate gene expression for BDNF (A), Tyrosine Hydroxylase-TH (B), Dopamine transporter-DAT (C), ratio of Iba1:Cd11 (E), IL-6 (F), IL-1β (G), and TNFα (H). C-reactive protein levels are expressed as % of LF group.



HF-fed female mice exhibited increased protein levels of pro-inflammatory biomarker CRP with or without exercise.

Gene expression in the NAc and plasma CRP protein levels were quantified in females (Figure 7) in the same way represented in males (Figure 6). No differences in gene expression were detected between female groups (Fig. 7A-C; E-H). However, exercised HF-fed females trended towards increased BDNF levels (3.340 ± 1.66) compared to the sedentary HF-fed group (0.793 ± 0.59); although this was not significant (p=0.109) (Fig. 7A). Conversely, the level of C-reactive protein (CRP) was significantly elevated in both sedentary and exercised HF-females in comparison to the sedentary LF female group (p=0.017 and p=0.0008, respectively). However, exercise did not influence this biomarker within diet groups (sedentary vs exercised LF- or HF-diet groups) (Fig. 4D).

Figure 4.7. Plasma CRP and NAc Gene Expression for Females
Data are normalized to sedentary low-fat (LF) group values. 18s was used as the housekeeping gene to evaluate gene expression for BDNF (A), Tyrosine Hydroxylase-TH (B), Dopamine transporter-DAT (C), ratio of Iba1:Cd11 (E), IL-6 (F), IL-1β (G), and TNFα (H). C-reactive protein levels are expressed as % of LF group.



Discussion

We examined whether moderate-intensity exercise influenced dopamine terminals by attenuating diet-induced inflammation. Exercise caused a modest reduction in weight gain for HF-fed mice with minimal impact on blood glucose regulation. There was a trend toward increased pro-inflammatory gene expression (IL-6, IL-1β and TNFα) in HF-sedentary males and females that was not observed in the HFex groups. Overall, our findings did not support our initial hypotheses largely because we did not observe deficits in dopamine terminal function previously reported after HF feeding.[21,333] or significantly elevated pro-inflammatory gene expression. However, we identified sex-specific effects of exercise on dopamine kinetics and dopamine terminal response to AMPH. Specifically, exercise significantly increased the rate of dopamine reuptake in female mice fed a HF diet. This corresponded with increased potency of AMPH as an uptake inhibitor (Km) in exercised HF-fed female mice. Elevated V_{max} and increased AMPH potency demonstrate enhanced DAT function in exercised females, while male HF-sedentary mice were more susceptible to AMPH-induced dopamine depletion. We also observed sex-specific effects on inflammatory markers. The HF diet engendered a greater inflammatory gene expression profile in the NAc of male mice, but systemic inflammation measured by CRP was significantly elevated in HF-fed females. The dissimilarity between male and female inflammatory profile suggests that low-grade inflammation caused by the HF diet (males) and increased systemic inflammation caused by exercise (females) are likely uncoupled from changes in baseline dopamine terminal kinetics caused by diet and exercise.

The exercise protocol employed was adapted from Chen et al (2017)[57] and intended to attenuate body weight gain in the HF-fed groups. Given a modest non-significant attenuation of body weight in the HF groups our exercise prescription may not have been long enough in duration or at an intensity high enough to overcome the excess number of calories that were

being consumed by the HF-fed groups. Despite significant body weight reductions, exercise did increase dopamine release and reuptake rate in female mice fed a HF-diet. Other pre-clinical studies reported attenuated weight gain with higher intensity exercise[64] (Wang et al 2017) or longer duration protocol.[141] Our next step will be to explore stepwise increases in exercise intensity and/or duration to better match weight control with the literature. More intense exercise may also translate into a larger effect on glucose regulation. This underscores the importance of intensity and length of protocol for improving metabolic parameters that develop alongside metabolic syndrome. Had our study extended the exercise protocol until marked improvements in blood glucose were consistently observed, we may have observed a larger effect on body weight and dopamine signaling endpoints.

Another notable finding in exercised females was elevated BDNF gene expression levels, a well-categorized factor involved in neurogenesis[29,365,372] and neurotransmitter modulation.[232,316,320] BDNF could facilitate terminal changes that supported enhanced dopamine release and V_{max} reported in exercised HF-fed females. Kim et al observed no differences in BDNF gene expression between sedentary and exercised mice.[174] However, this study investigated the coding region of the BDNF probe, like the one used in the present study, while utilizing a probe containing nucleotides for the promoter region of BDNF gene yielded differences between sedentary and high-intensity exercise group only. This was confirmed with measured BDNF protein levels with significantly higher amounts in the high-intensity exercise group.[174] There were no differences in moderate intensity exercise groups akin to the protocol used in this study. Interestingly, BDNF was also elevated in male HF sedentary mice. Similar to enhanced release and V_{max} in the HF female exercise group, the absence of slowed V_{max} and attenuated phasic dopamine release in the HF males, as reported previously,[21,333] could be

attributed to elevated BDNF levels. A previous report in Swiss mice fed a HF diet reported an early increase in BDNF followed by a reduction that coincided with an increased inflammatory response.[258] It is possible the male HF-fed mice benefited from the protective effects at the point we measured dopamine, preceding a greater inflammatory decline. Sedentary females fed a HF diet also exhibited a muted rate of dopamine reuptake (V_{max}) in comparison to LF counterparts. This attenuated reuptake rate was rescued with moderate-intensity aerobic exercise. The consumption of our HF diet (high in palatable saturated fat) is well-documented to influence dopamine neurotransmission through decreasing dopamine receptors[129], and attenuating dopamine release over long-term conditions.[21,293,295,327] Exercise improved the diminished reuptake rate effect in female mice consuming high amounts of saturated fat. Although male mice did trend toward a decrease in evoked phasic release there was not a significant effect for either diet or exercise observed. One explanation could be an issue of timing and differing methylation patterns on the DAT encoding gene. Different methylation patterns were observed in mice fed a high fat diet that was reversed when HFD was removed.[47]

To test pre-synaptic dopamine terminal function with differing levels of exercise, AMPH was bathed over slices during voltammetry at a concentration of 300nM and 3µM for a duration of 30 minutes each. Male HF-fed mice displayed greater depletion of dopamine compared to LF counterparts. This could be due to a priming of the pre-synaptic terminals to exacerbate the effects of a dopamine disrupting compound like AMPH. This finding is similar to a previous report where proinflammatory cytokines were bathed over brain slices during FSCV[92]. The previous report showed that dopamine release in HF-fed mice was significantly reduced after the application of endogenous cytokines, but LF-fed mice showed a small impact of cytokines on dopamine release that was rescued after washout of the drug.[92] Together, these findings suggest

that HF diet may prime dopamine terminals in the male sedentary group for an exaggerated response to exogenous insults like recreational drugs or endogenous insults caused by inflammation. The potency of AMPH to inhibit DAT, as measured by apparent K_m, was increased in HF-sedentary males. Conversely, exercised increased the potency of AMPH in the HF-exercised females compared to their HF-sedentary counterparts. This suggests that there might be a sex and diet interaction affecting AMPH's actions, aligning with previous microdialysis studies showing differences in AMPH-stimulated dopamine release based on estrous cycle.[26] Additionally, dopamine release is altered dependent upon estrous cycle in rodent, due to estradiol having differing effects on VTA neuron excitability. Specifically, estradiol increases excitability during metestrus stage, while decreasing excitability in proestrus and estrus stages.[285] This further points to the need to investigate these effects under different stages of the estrus cycle in female mice in a model of diet-induced obesity.

In males fed a HF-diet there was the greatest impairment of fasting blood glucose, and blood glucose clearance ability, pointing to the greatest metabolic impairment. Exercise was not able to attenuate these outcomes, but exercise did attenuate inflammatory gene expression between HF-sedentary and HF-exercised males. Since exercised females had increased plasma CRP protein levels, elevated NAc BDNF gene expression, and enhanced V_{max} but sedentary males displayed a greater inflammatory gene expression with no effect on CRP, BDNF, or V_{max} there is likely little influence of diet-induced inflammation on baseline dopamine terminal kinetics caused by diet and exercise. It is possible that the chronic low-grade inflammation caused by HF-feeding may alter how dopamine terminals respond to exogenous compounds like dopamine agonists or acute inflammatory insults.

This study was limited in that we only examined gene expression to assess central inflammation. Exposure to a pro-inflammatory stimulus like lipopolysaccharide (LPS) would provide insight into how the HF diet impacts a known inflammatory response. The temporal nature of cytokine release from diet-induced inflammation is difficult to capture, but a timed pro-inflammatory challenge could further illuminate any priming chronic low-grade inflammation has on the central immune system response. A canonical marker of neuroinflammation is activated microglia, measured by a ratio of ionized calcium binding adaptor molecule-1 (Iba1) to cluster of differentiation receptor (Cd11b).[179,301] Utilizing a ratio of the mRNA for these proteins, rather than one in isolation, can provide a more reliable picture of a proinflammatory state. Cd11b is expressed on microglia cells throughout the brain, while Iba1 is indicative of microglia in a reactive state.[132] In the present study, this ratio was markedly increased in sedentary HF-fed males, while in females the only observed difference was a drastic reduction in exercised LF-fed females. In contrast, exercise had no effects on attenuating gene expression of inflammation-related markers. However, this might be due to temporal differences that were not captured. The initial increase in inflammation within the hypothalamus has been shown to occur after only 1 day on an HFD,[331] while gene expression is initially elevated, then diminished over time[230,368]. There may be an adaptive mechanism regulating this transient release of cytokines to prevent from large accumulations over time. Studies have shown increased Iba1+ with chronic long-term feeding, but these were experiments that fed mice for 5 months.

Conclusions

A 6-week moderate-intensity aerobic exercise protocol did not attenuate body weight gain when consuming a diet high in saturated fat. Dopamine reuptake rate was enhanced in exercised females, suggesting a potential sex-linked effect. This was seen without overall metabolic improvements and the reuptake rate might be increased for males with either a longer

protocol or higher intensity. Specifically, since pro-inflammatory profile, as measured in this study, played no role in changing dopamine neurochemistry. However, regardless of no changes within inflammatory gene expression, treadmill exercise independently enhanced the rate of dopamine reuptake in female mice. Future exercise implementation might start at this intensity and duration to initiate neurochemical changes, gradually working up to a high-intensity protocol with longer exercise bouts. Additionally, there is a need to explore intensity and duration combinations to optimize the most sustainable improvements, potentially informing obesity treatments while minimizing use of pharmaceuticals that carry a high potential risk of abuse.

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APPENDIX A: SUPPLEMENTARY MATERIAL

S1

2way ANOVA	LF		HF30		HF60	
Cytokine x Sex	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
Interaction	F (9, 108) = 0.5313	P=0.8489	F (9, 45) = 0.9121	P=0.5233	F (9, 108) = 0.5299	P=0.8500
IL-6 [5nM]	F (2.172, 26.06) = 2.8	P=0.0777	F (1.452, 7.259) = 0.3	P=0.7098	F (2.617, 31.40) = 0.7	P=0.5426
Sex	F (1, 12) = 0.1638	P=0.6928	F (1, 5) = 0.08381	P=0.7838	F (1, 12) = 0.05111	P=0.8250
Interaction	F (9, 54) = 0.2226	P=0.9899	F (9, 45) = 1.900	P=0.0765	F (9, 81) = 1.442	P=0.1842
IL-6 [10nM]	F (1.550, 9.301) = 0.7	P=0.4650	F (2.206, 11.03) = 2.8	P=0.0994	F (1.940, 17.46) = 9.6	P=0.0017 **
Sex	F (1, 6) = 0.05115	P=0.8286	F (1, 5) = 8.259	P=0.0348 *	F (1, 9) = 1.662	P=0.2294
Interaction	F (9, 81) = 0.1807	P=0.9956	F (9, 54) = 0.4685	P=0.8893	F (9, 90) = 0.1764	P=0.9960
TNFα [30nM]	F (2.553, 22.97) = 2.9	P=0.0663	F (1.508, 9.048) = 3.6	P=0.0796	F (3.109, 31.09) = 0.6	P=0.5972
Sex	F (1, 9) = 0.04217	P=0.8419	F (1, 6) = 0.05032	P=0.8299	F (1, 10) = 0.09989	P=0.7585
Interaction	F (9, 90) = 1.021	P=0.4296	F (9, 45) = 1.463	P=0.1911	F (9, 63) = 1.234	P=0.2911
TNFα [300nM]	F (2.159, 21.59) = 3.3	P=0.0516	F (1.785, 8.924) = 0.9	P=0.4450	F (2.140, 14.98) = 4.9	P=0.0220 *
Sex	F (1, 10) = 1.781	P=0.2116	F (1, 5) = 2.033	P=0.2132	F (1, 7) = 3.087	P=0.1223

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