The Effects of Prolonged High Fat Diet Consumption on Feeding Behavior and Dopamine Signaling

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Abstract:

Obesity is a costly epidemic that claims an escalating number of lives with each passing year. Despite its complex etiology, one key tenet that is widely accepted in the development of obesity is the overconsumption of calorically-enriched foods. These foods are easily accessible, and their consumption is promoted by the brain's reward circuitry which consists primarily of the dopaminergic projections from the ventral tegmental area of the midbrain to the nucleus accumbens of the basal forebrain. This neural circuit, often referred to as the mesolimbic dopamine signaling pathway, is a key player in regulating various aspects of feeding behavior. However, the precise manner in which this circuitry and its attendant feeding behaviors respond to the prolonged consumption of energy-dense diets has yet to be fully elucidated. The work contained in this dissertation demonstrates that prolonged consumption of a high fat diet leads to food reward devaluation and alterations in preference that are reversible by dieting or exercise. In addition, we found that intact dopamine-1 receptor mediated signaling is essential for maintenance of food preference, and that high fat diet consumption desensitizes the response of the ventral tegmental area to palatable food reward. Our findings highlight a novel role for high fat diet consumption in the perturbation of both feeding behavior and relevant neural circuitry.

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Ch. 1: Introduction

One of the greatest medical challenges facing the developing world is the obesity epidemic. The World Health Organization (WHO) characterizes obesity as an unhealthy excess of body weight, manifesting as a body mass index (BMI) exceeding 30, while individuals with a BMI between 25 and 30 are considered overweight [1]. In 2015, 2.2 billion people worldwide were overweight, with more than 700 million of them being considered obese [2]. These individuals are at a dramatically increased risk for type 2 diabetes, cardiovascular disease, and some types of cancer [3]. Additionally, obesity is a costly epidemic, with its associated medical costs reaching a staggering $2 trillion in 2014 and rising with each passing year [4]. The obesity epidemic is therefore a multi-dimensional health crisis, claiming innumerable lives and mass quantities of financial resources every year.

Individuals in modern society are consuming more calories on a daily basis than is necessary for adequate health and survival, leading to excess weight gain. According to the Food and Agriculture Organization of the United Nations (FAO), daily calorie consumption has increased by nearly 25% over the past several decades [5]. Poor dietary choices are a significant determinant in this overconsumption and are often brought on by an overabundance of readily-accessible, highly-palatable, energy-dense foods [6,7]. The consumption of these foods is further reinforced by the brain's reward circuitry [8–11]. It is evident that poor dietary choices result in an increase in caloric consumption, leading to an increase in the prevalence of obesity and its associated cost and health risks.

However, a comprehensive understanding of the relationship between the consumption of energy-dense diets and the neurobiological basis for food choices is lacking. The insight gained from research on this topic will allow us to implement better strategies to combat the obesity epidemic before it costs us more lives and financial resources.

Feeding Behavior:

Feeding behavior is a significant determinant in the regular daily activity of organisms, as food is essential for survival. It encompasses all processing and actions necessary for an organism's acquisition and consumption of food, and as such, is goal-directed in nature. Unfortunately, since feeding behavior is comprised of a complex array of interconnected processes and components, it is difficult to study and understand as a whole. These individual components must first be fully characterized in order to understand how they shape an organism's overall feeding behavior.

Homeostatic and Hedonic Feeding: It is universally agreed that there are two synergistic motivational processes that mediate feeding behavior: the homeostatic and hedonic pathways. Homeostatic feeding refers to the drive to eat to maintain energy balance, while hedonic feeding refers to the drive to consume food because of its perceived palatability and rewarding properties [12,13]. The arcuate nucleus (ARC) of the hypothalamus is the site of key cell types that mediate homeostatic feeding [13–15]. The two most well characterized cell types within the ARC related to homeostatic feeding are the agouti-related peptide (AgRP) and the proopiomelanocortin (POMC) neurons, which act in

opposition to each other [14,16]. Specifically, the AgRP neurons stimulate appetite while the POMC neurons suppress it. Both cell types are notable for their expression of receptors that respond to circulating leptin, ghrelin, and insulin [13,15,16]. The importance of these hormones for regulating energy homeostasis and appetite is a key determinant in establishing the AgRP and POMC neurons as critical for homeostatic feeding. Hedonic feeding however is chiefly associated with the mesolimbic dopamine signaling pathway [12,13]. This pathway primarily consists of dopamine producing neurons of the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc) of the basal forebrain and is associated with conveying the rewarding properties of food [12–14]. Despite these two pathways possessing different neuroanatomical loci and thus being thought of as distinct and separable, they possess the ability to influence each other, thus acting in concert to shape feeding behavior. [12,13]. Though the focus of this dissertation will be primarily on hedonic feeding, it is important to note that the neural circuitry governing feeding behavior extends beyond one pathway.

Food Choice: The caloric content of various food substrates is a key factor in our decision to consume them from the perspective of both homeostatic and hedonic feeding. However, the precise manner in which caloric content shapes our dietary choices is poorly understood. It is known that an organism's ability to distinguish the caloric content between available foods guides subsequent foraging and consumption choices [17,18]. The effort and competition surrounding the acquisition of limited food supplies mandates the selection for the ability to direct food choices towards those of higher caloric content

(homeostatic) [19,20]. However, for people in modern society, calorically-enriched foods are plentiful and accessible without need for significant effort or competition to obtain. Unfortunately, these foods are typically low in nutritional content, while more nutritious food choices are less calorically-enriched, and thus not as preferable [21,22]. A major contributor to the preference for these calorically-enriched foods is their predisposition to contain higher amounts of sugar and fat, which contribute to their overall perceived palatability (hedonic) [23]. Furthermore, the hedonic valuation of these foods is reinforced by the mesolimbic dopamine signaling pathway, which serves as the brain's primary reward center [8–11]. A fully developed understanding of how this neural circuit responds to hypercaloric foods and influences subsequent feeding behavior is necessary in order to target the poor dietary choices that contribute to the obesity epidemic.

Mesolimbic Dopamine Signaling and Feeding Behavior:

Dopamine is a prominent neurotransmitter that is important for a variety of processes such as motor function, reward evaluation, and executive functions. Dopamine neurotransmission is used in distinct anatomical sub-regions for various purposes (See Box 1). One of these specialized pathways, the mesolimbic dopamine signaling pathway, consists primarily of dopamine released from ventral tegmental area (VTA) neurons in the midbrain onto the nucleus

Box 1. Dopaminergic Pathways

- **Mesolimbic –** Ventral tegmental area of the midbrain to the nucleus accumbens; functions in reward processing
- **Mesocortical –** Ventral tegmental area of the midbrain to the prefrontal cortex; functions in attention, higher order cognition
- **Nigrostriatal –** Substantia nigra to the caudate nucleus and the putamen; functions in motor control and learning
- **Tuberoinfundibular –** Arcuate nucleus to the pituitary gland; functions in regulating prolactin

accumbens (NAc) neurons of the basal forebrain and is critical for reinforcement learning, motivation, and reward processing [8]. This pathway is activated in the presence of food, leading to elevated NAc dopamine levels [13]. Though its precise role in mediating food choice and preference has yet to be explored, mesolimbic dopamine signaling has been implicated in being crucial for learning food-predictive stimuli, maintaining the motivation to seek a food reward, and conveying the hedonic value of a reward [9–11]. The involvement of mesolimbic dopamine signaling in this wide-array of feeding-related behaviors establishes it as a promising candidate for an investigation of the neurobiological basis of food preference and choice.

Food-Associated Cues: Mesolimbic dopamine signaling is a key player in facilitating the formation of associations between cues in our environment and the presence of food [24]. Presentation of a food reward is sufficient to cause dopamine release in the NAc. In animals taught to associate the delivery of food with a cue, subsequent presentations of the cue alone are sufficient to cause an influx of dopamine into the NAc, while presentations of a neutral cue are not [11,25]. Additionally, pharmacological blockade of dopamine-1 receptors (D1R) in the NAc interferes with an animal's ability to learn to associate a similar cue pairing with the delivery of a food reward [26]. Though these findings do not address food choice and preference specifically, they suggest that mesolimbic dopamine signaling could allow organisms to form an association between an energy dense food and its associative cues such as texture, smell, sight, or environmental indications of its presence. These learned associations are likely

used to establish food preferences and guide future feeding behavior towards specific food choices.

Food-seeking Motivation: Mesolimbic dopamine signaling is also important for conferring the motivation to seek a food reward, even if its acquisition is dependent upon effort. A common way researchers assess motivation in rodent models is via a progressive ratio schedule of reinforcement, where animals earn reinforcers by pressing levers a certain number of times. Depending upon the specific schedule of reinforcement, successful receipt of a reinforcer means that the animal will need to put in more work (i.e. more lever presses) to obtain the next one. Neurotoxic ablation of dopaminergic projections to the NAc leads to a decreased effort to receive a food reward [10]. Alternatively, enhancing dopamine release in the NAc by treatment with amphetamine enhances motivation to work for a food reward in a similar task [27]. Additionally, elevating NAc dopamine levels via pharmacological inhibition of the dopamine transporter (DAT) in the NAc produces a similar effect [28]. Transgenic knockdown of DAT in mice leads to increased daily food consumption, and an enhanced drive to seek out a novel food reward [29]. These findings further emphasize the importance of mesolimbic dopamine levels in controlling feeding behavior. Furthermore, they suggest that without it, it is impossible to exert the necessary effort to obtain food, which could cause individuals to continue to seek out convenient food choices such as fast-food.

Post-ingestive feedback: Mesolimbic dopaminergic neurons are capable of integrating post-ingestive information to influence feeding behavior [30]. An

example of this post-ingestive feedback involves learned flavor preferences based on nutritive value. Animals are capable of learning to prefer a flavored saccharin solution over a differently flavored saccharin solution when it is paired with an intragastric sugar infusion [31]. The role that mesolimbic dopamine signaling plays in mediating this association is made clear when D1Rs in the NAc are pharmacologically inhibited, resulting in an inability to develop a preference for the solution paired with sugar infusion. One way in which post-ingestive feedback can influence mesolimbic dopamine is through the release of ghrelin. Ghrelin is a hormone released from the gastrointestinal tract to promote hunger, and can bind to receptors in the central nervous system such as those located on dopaminergic neurons of the VTA [32]. Stimulation of ghrelin receptors in the VTA is sufficient to release dopamine into the NAc and induce feeding [33,34]. Conversely, blockade of these receptors attenuates the release of dopamine into the NAc, even following the administration of drugs of abuse [35,36]. Dopaminergic VTA neurons also express receptors for the hormones leptin and insulin, which are crucial for signaling satiety and for promoting the absorption of glucose, respectively [37]. Leptin is released from adipose tissue, and insulin is released from pancreatic beta cells. Insulin signaling in the VTA has been shown to decrease the consumption of palatable foods, preference for a location associated with the delivery of food, and has been shown to increase dopamine reuptake via the dopamine transporter [38,39]. Leptin in the VTA has been shown to decrease food intake, while knocking out its receptor in this region has the opposite effect [40]. Taken together, these findings emphasize the

widespread importance of mesolimbic dopamine in feeding behavior, as its activity is tightly regulated by multiple hormones that are released in response to ingestion of foods. This post-ingestive regulation of mesolimbic dopamine leads us to believe that it is a promising candidate for understanding how the brain encodes information about the caloric content and valuation of foods to shape preferences and dietary choices.

Reward Processing: Mesolimbic dopamine signaling contributes to arguably the most important aspect of feeding behavior: the evaluation of a food's rewarding properties. At a fundamental level, consumption of palatable foods leads to dopamine release into the NAc [41–43]. Conversely, consumption of aversive, non-palatable substances leads to a decrease in dopamine release into the NAc [44]. Even more compelling is the finding that rats consuming varying concentrations of a sucrose solution exhibit increases in NAc dopamine release that scale with the palatability [9]. Interestingly, systemic pharmacological blockade of dopamine 1, 2, or 3 receptors fails to affect rats' preference for a more palatable sucrose solution [45]. However, the sucrose solutions utilized in this study were of vastly different concentrations (5% vs 0.3%). This wide disparity in concentrations makes for a test that is lacking in sensitivity, making it difficult to detect subtle changes in preference related to reward magnitude. Taken together, these studies provide compelling evidence suggesting that mesolimbic dopamine signaling conveys information regarding the hedonic properties of food, thus providing major implications for how mesolimbic dopamine signaling may influence food preference.

Effects of High-Fat Diet on Mesolimbic Dopamine Signaling:

Gene-Expression: People in today's society have constant access to energy dense diets, which begs the question of how these diets are affecting our neural circuitry. Studies have demonstrated that prolonged high fat diet (HFD) consumption leads to decreased VTA expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine [46,47]. However, research examining HFD-induced changes in NAc dopamine receptor gene expression has yielded conflicting results. Studies have reported decreases in NAc dopamine 1 and 2 receptor mRNA levels or no change independent of the development of an obese-phenotype [48–50]. A similar disparity is observed in studies examining obese animals [51,52]. The issue of the inconsistency in these results is second only to the lack of methodological homogeneity across studies. Specifically, there is a lack of consensus on what constitutes "prolonged" exposure, with studies ranging anywhere from a few weeks to several months of hypercaloric diet access. Additionally, studies differ in terms of the animals used: some are obese, some don't differ in bodyweight from standard chow fed counterparts, and some are further characterized based upon their "obesity prone" nature. This lack of uniformity in the way the studies are conducted, coupled with the unclear results, makes it difficult to interpret the effects of energy dense diets on gene expression relevant to mesolimbic dopamine signaling. Further study is needed to give clearer insight into whether or not prolonged exposure to an energy dense diet can dysregulate gene expression related to mesolimbic dopamine signaling.

Dopamine Transmission: Despite the somewhat inconclusive nature of gene-transcript level investigations, other approaches have yielded definitive evidence demonstrating dysregulation of mesolimbic dopamine signaling in response to prolonged hypercaloric diet exposure. In rats, long-term HFD consumption leads to decreased extracellular dopamine levels in the NAc [53]. Additionally, prolonged exposure to a hypercaloric diet dampens dopamine release in the NAc during the presentation of a novel palatable food [49]. This HFD-mediated attenuation of dopamine release extends beyond food reward. Specifically, animals fed a HFD exhibit diminished dopamine release in response to amphetamine administration [54]. Under normal conditions, drugs of abuse such as amphetamine are capable of eliciting NAc dopamine release at levels almost four times as great as levels achieved by a food reward [55]. The aforementioned blunted dopamine response to amphetamine in HFD-raised animals suggests that not even a rewarding stimulus as potent as amphetamine is capable of overriding HFD-induced perturbations in mesolimbic dopamine signaling. Taken together, these findings suggest that energy dense diets are capable of disrupting mesolimbic dopamine signaling when consumed over a long period of time. Importantly, prolonged exposure to an energy dense diet leads to decreased dopamine turnover in the NAc, independent of the development of an obese phenotype [56]. This suggests that it is not obesity itself that is dysregulating mesolimbic dopamine signaling, but rather the mere exposure to the diet. The implications of this are alarming, as they suggest that

our neural circuitry is susceptible to perturbation from the foods we eat, regardless of how well we can maintain a healthy BMI.

Effects of High-Fat Diet on Reward-based Behavior:

The aforementioned studies clearly demonstrate how consumption of energy dense diets can perturb neural circuitry associated with mesolimbic dopamine signaling. However, these findings don't speak to the manner in which these diets may shape downstream behaviors. As the mesolimbic dopamine signaling pathway is a key mediator of reward-based behavior, it stands to reason that if HFD consumption can affect this pathway, it could also produce alterations in behavior as well. With regard to feeding behavior in rodents specifically, prolonged consumption of a hypercaloric diet has been shown to decrease the drive to seek out and consume a novel palatable food reward [50]. Prolonged HFD consumption has also been shown to lead to decreased motivation to exert effort to receive palatable food rewards [57–59]. In addition, long-term hypercaloric diet consumption leads to a decrease in the consumption of a standard lab diet, even if that diet is the only food option available [60,61]. Importantly, these behavioral alterations still persist when daily caloric intake and weight gain are restricted in animals consuming HFD [60,62,63]. This suggests that HFD-mediated behavioral alterations are not due to caloric satiation and decreased homeostatic drive. Taken together, these findings suggest that prolonged consumption of palatable energy dense diets leads to a devaluation of other food rewards.

Remarkably, this diet-induced devaluation phenotype extends to drugs of abuse as well. Specifically, prolonged consumption of a diet high in saturated fats leads to a decreased preference for a location associated with amphetamine delivery in a conditioned place preference assay [57,64]. Conversely, rodents with restricted food access exhibit a heightened preference for a location associated with amphetamine delivery and are willing to exert more effort to obtain amphetamine compared to animals give *ad libitum* access to standard lab diet [65,66]. Mice raised on a HFD also exhibit a diminished preference for a 1% sucrose solution over water in a two bottle sucrose preference test [58,67]. The sucrose preference test is a behavioral tool that is commonly used to assess anhedonia and reward devaluation in mouse models of depression [68]. Thus, the poor performance of HFD-raised mice on this assay suggests that prolonged energy dense diet consumption is inducing a state of overall reward dysfunction in these animals. Taken together, these findings indicate that diet-induced reward devaluation is not exclusive to food rewards, and may contribute to the remodeling of reward-based behaviors as a whole.

Exercise and Mesolimbic Dopamine Signaling:

Exercise is a regularly prescribed intervention strategy for combating obesity and its comorbidities such as high blood pressure and heart disease. However, the manner in which exercise affects neural circuitry, including the mesolimbic dopamine signaling pathway, has yet to be fully elucidated. It is known that exercise itself is a rewarding behavior; rodents will rapidly acquire wheel-running behavior and will actively seek situations that permit them to run

[69–71]. The reinforcing nature of exercise is promoted by mesolimbic dopamine signaling, as evidenced by the ability of VTA electrical stimulation to enhance treadmill running [72]. In vivo recordings from the VTA during wheel-running demonstrate enhanced burst firing of dopaminergic neurons at running onset and termination [73]. Treadmill running is also associated with elevated levels of dopamine and its metabolites in the striatum [74]. Additionally, treadmill running increases neuronal activity in the NAc as evidenced by increased c-Fos levels [75]. This effect is dependent upon dopamine-1 receptor (D1R) availability, and is blocked by systemic administration of the D1R antagonist SCH-23390. Exercise has also been shown to possess marked therapeutic potential in reversing phenotypes associated with several drugs of abuse [76,77]. This therapeutic potential, combined with exercise's engagement of mesolimbic dopamine signaling, makes it a worthy candidate in the investigation of ways to reverse potential diet-induced alterations in feeding behavior and reward circuitry.

Exercise's immediate utilization of mesolimbic dopamine signaling is clear, but the aforementioned diet-induced perturbations in dopamine signaling and reward-based behavior occur after prolonged exposure to a HFD. In order to fully understand the potential for exercise to attenuate these deficiencies, we must gain a better understanding of how continued exercise influences mesolimbic dopamine signaling in the long-term. Unfortunately, research on this particular topic is scarce and the studies that have addressed it have produced some contradictory findings. Studies examining changes in mRNA and receptor binding of both D1 and D2 receptors in the NAc following long-term exercise regimens

have demonstrated no change in D1R mRNA and reduced D2R mRNA [78] or decreased D1R binding and increased D2R binding [79]. Though these findings do appear in direct conflict of one another, it is important to keep in mind that mRNA levels are not necessarily indicative of relative protein expression levels, of which dopamine receptor binding assays are a better representation. Nevertheless, a better characterization of exercise's effects on NAc dopamine receptor expression is necessary for a complete understanding of how exercise can induce plasticity in the mesolimbic dopamine signaling pathway.

When looking upstream of dopamine receptors in the NAc, long periods of voluntary exercise has been shown to lead to increased TH mRNA expression in the VTA of rats [78,80,81]. This upregulation of TH in the VTA suggests that regular exercise could sensitize the mesolimbic dopamine signaling pathway by facilitating an increase in mesolimbic dopamine release. These exercise-induced alterations could function to counteract deficiencies in mesolimbic signaling brought on by prolonged consumption of energy-dense food. It has been demonstrated that a long-term exercise intervention in obese mice restores deficiencies in VTA tyrosine hydroxylase expression, as well as deficiencies in NAc dopamine levels and D2R expression [82]. These findings suggest that in addition to being effective for combating deadly comorbidities associated with obesity, exercise is an effective means to undo potential diet-induced changes in mesolimbic dopamine signaling.

When considering the current literature on dopamine signaling and HFD consumption as a whole, several key points are clear: (1) mesolimbic dopamine

signaling mediates various reward-related facets of feeding behavior, (2) HFD consumption leads to alterations in both mesolimbic dopamine signaling and reward-based behavior, and (3) exercise engages the mesolimbic dopamine signaling pathway and promotes plasticity therein. My graduate career sought to delve further into feeding behavior with a specific focus on food preference and choice, and the manner in which both prolonged HFD consumption and mesolimbic dopamine signaling affect these behavioral constructs. The effects of HFD consumption on food preference/choice and the neurobiological basis for these effects remains unknown, and the findings contained within this dissertation better characterize these relationships.

Ch. 2: Long-term High Fat Diet Consumption Reversibly Alters Feeding Behavior via a Dopamine-Associated Mechanism in Mice

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Abstract:

Obesity is a costly, global epidemic that is perpetuated by an unhealthy diet. A significant factor in the initial consumption and maintenance of an unhealthy diet is the abundance of highly palatable, calorically dense foods. The aim of the present study is to better understand the effects of high fat diet (HFD) consumption on food valuation and preference, and to elucidate the neurobiological mechanisms mediating these effects. By using a novel food preference assay, we found that prolonged consumption of a HFD diminishes preference for and consumption of the more calorically dense food choice when two lab diets are presented. Additionally, we demonstrated that prolonged HFD consumption dampens ventral tegmental c-fos induction during hedonic feeding, implicating the mesolimbic dopamine signaling pathway as a target of HFD. Notably, both the changes in food preference and this reduced c-fos induction were reversed during withdrawal from HFD. Further, HFD-induced alterations in food preference were attenuated by exercise. Our findings suggest that prolonged HFD consumption leads to anhedonia and altered feeding choices, and this is associated with changes in mesolimbic dopamine signaling.

Introduction:

According to the World Health Organization, obesity is a multifaceted health crisis stemming from overconsumption of calories relative to energy expenditure, leading to excess weight gain. Obese individuals are at a dramatically increased risk for type 2 diabetes, cardiovascular disease, and some types of cancer [3]. Dieting and exercise are the most commonly prescribed treatments for obesity. However, they are often ineffective as long-term solutions due to patients' frequent relapse into sedentary lifestyles [6,83]. Unhealthy diets are positively correlated with caloric overconsumption and are perpetuated by an abundance of readily accessible, highly-palatable, energy-dense foods [7,84]. Consumption of unhealthy diets is further promoted by prior experience with energy-dense foods [85,86]. Furthermore, the salience of palatable, high fat foods is conveyed via the mesolimbic dopamine signaling pathway, which serves as the brain's reward circuit [8–11]. This pathway consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and is activated in the presence of food, leading to elevated NAc dopamine levels [13]. Functionally, this pathway is critical for reinforcement learning, motivation, and reward processing [8]. Despite its necessity for food reward processing, the way mesolimbic dopamine signaling and its attendant feeding behaviors adapt to prolonged HFD consumption has yet to be fully characterized.

Feeding behavior involves an exceedingly complex array of processes that integrate information regarding energy homeostasis and incentive salience [12,13]. In rodents, the consumption of a HFD has been associated with

remodeling of reward-related behaviors, including feeding. Prolonged consumption of a HFD has been shown to lead to a diminished preference for sucrose over water in a two-bottle choice test, and decreased preference for a location associated with food rewards in a conditioned place preference assay [58,67,87,88]. It has been shown that prolonged HFD consumption leads to devaluation of standard chow even after HFD access has been ceased [60]. In addition, long-term HFD consumption has been associated with diminished motivation to attain food reward in progressive ratio and food seeking tasks [50,58,87]. HFD-induced reward devaluation extends to drugs of abuse as well, leading to a reduced conditioned place preference for amphetamine delivery [57,64]. Integrity of the mesolimbic dopamine signaling pathway is susceptible to HFD consumption [48–50]. For example, prolonged HFD consumption dampens food reward or amphetamine induced dopamine release in the NAc [49,89], independent of an obesity phenotype [57]. However, despite these established HFD-mediated effects, the capability of accessible behavioral interventions (ie. dieting and exercise) to reverse them is rarely investigated.

Because the mesolimbic dopamine signaling pathway serves as a nexus for many aspects of feeding behavior, consumption of a HFD has been shown to affect both this pathway as well as the evaluation of food rewards, and dieting and exercise are the most common means to achieve weight loss in humans, we investigated the following hypotheses: (1) prolonged consumption of a HFD alters feeding behavior via perturbations of the mesolimbic dopamine signaling pathway, and (2) these alterations are reversed via dietary changes and

exercise. To evaluate these hypotheses, we developed an assay that examines changes in preference between two different chows. We tested whether HFD changes food preference and amount of test food consumed. We then examined the effects of HFD on mesolimbic dopamine signaling during hedonic feeding. We discovered that HFD diminishes preference for a more calorically dense chow, and that this behavioral change can be reversed by withdrawing animals from HFD or by allowing them to engage in voluntary exercise. Additionally, we found that HFD consumption dampened c-fos induction in the VTA during palatable feeding, and that this effect was reversed in animals withdrawn from HFD. Our findings implicate a role for dopamine signaling following prolonged HFD consumption, which leads to reversible alterations in food preference.

Materials and Methods:

Animals: All experiments complied with NIH animal care and use guidelines, and were approved by the University of Virginia Institutional Animal Care and Use Committee. Male mice were housed on a 12-hour light/dark cycle (lights on between 7am and 7pm), in standard ventilated cages with corncob bedding, cotton nesting material (Nestlets™, Ancare, Bellmore, NY, USA) and a paper soufflé cup (product F400, Genpak, Charlotte, NC). C57BL/6 breeder mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Maintenance diets were furnished to both breeders and offspring except where acute dietary treatment is specified. All experiments took place using male mice, and all experimental mice were habituated to individual housing for at least 3

days prior to intake procedures. All behavioral tests and tissue collections took place at 14 weeks of age.

Diets Used: Control mice received *Envigo* (Huntingdon, United Kingdom) product 8664 *Teklad F6 Rodent Diet* (standard diet, 3.1 kcal/g; 19% fat, 31% protein, 50% carbohydrates). Control mice in the 2 bottle choice test (Fig 1A) were provided *LabDiet* (St. Louis, MO) product 5053 *PicoLab Rodent Diet 20* (standard diet, 3.07 kcal/g; 13% fat, 25% protein, 62% carbohydrates) due to discontinuation of *Envigo* product 8664. Mice on high fat diet (HFD) received: *Research Diets Open Source Diets* (Research Diets, New Brunswick, NJ, USA) product D12451 (OS red, 4.73 kcal/g; 45% fat, 20% protein, 35% carbohydrates*)*.

Food Preference Test: Mice were habituated to 2 novel test chows for 3 days prior to the preference test. During this habituation period, test chows were provided on the cage floor and maintenance diet remained in the wire cage top. At the start of the preference test, mice were weighed, the maintenance diet was withdrawn, and the test chows (~20 g of each) were weighed and added to the cage floor. Remaining test chow and animal body weights were recorded again 24 hr later. Consumption of each test chow for every animal was divided by that animal's body weight corresponding to the start of the 24 hour test period. Preference scores were calculated as the portion of total test chow consumed corresponding to the more energy enriched test chow. Novel test chows used during 24 hr food choice tests were as follows: *Bio-Serv (Flemington, NJ, USA) Product F3028 Rodent Diet Grain-Based Control* (BS F3028, 3.35 kcal/g; 13% fat, 29% protein, 58% carbohydrates) versus *Bio-Serv (Flemington, NJ, USA)*

Product F3156 Rodent Diet AIN-93G (BS F3156, 3.74 kcal/g; 17% fat, 19% protein, 64% carbohydrates). Both of these diets were selected because they are contrasting in color with respect to the animals' bedding, and are lower in energy and fat content compared to the diet the HFD animals were raised on. Bedding was thoroughly searched for test chow crumbs when weighing test chows at the conclusion of the test.

2-bottle choice test: Mice were habituated to our 2 bottle-choice setup for 2 days prior to the sucrose preference test. This habituation period was followed by two test days. During the habituation period, mice were housed in specialized cages (Columbus instruments DM-8 Drinking Event Monitor) with constant access to maintenance diet on the cage floor, as well as one bottle with water and a second bottle with a 1% sucrose solution. Bottle location (left vs. right side) alternated daily following each habituation and test day. Animals and bottles were weighed before and after each day. Fresh water and sucrose solutions were provided daily. Preference scores were calculated as the portion of total fluid intake attributable to the sucrose solution. In order to control for a potential side bias, the results from the two test days were averaged together for the final analysis.

Exercise: At 10 weeks of age, control mice and mice raised on HFD were provided with Bio Serv Mouse Igloos® equipped with either Fast-Trac accessory running discs or nothing. After four weeks of access to the Igloos with or without running discs, mice underwent the food preference test described previously (section 2.3). In order to quantify the amount of exercise achieved by control and

HFD-raised animals, a separate cohort of animals raised on either diet were individually housed with ad libitum access to drinking water and food (control diet or HFD). Each cage was also provided with a Bio Serv Mouse Igloo® equipped with a Fast-Trac accessory running disc. A magnetic sensor was attached to the igloo and two permeant magnets were attached to the running discs to allow the recording and analysis of disc turns through the ClockLab collection and analysis system (Actimetrics, Wilmette, IL). Seven days of recoding was collected for each animal and data from days 4 and 5 were averaged for the final comparison.

C-fos immunohistochemistry: Mice were habituated to a novel HFD, D12492 (OS aqua, 5.24 kcal/g; 60% fat, 20% protein, 20% carbohydrates), on the floor of their home cages for 2 days. Additional cohorts were habituated to a novel SD, 7912 (Teklad LM-485, 3.1 kcal/g; 17% fat, 25% protein, 58% carbohydrates). During habituation, animals had 30 minutes (starting at 2pm) to freely consume the novel chow. Maintenance diet was available *ad libitum* in the wire cage top. A single test day followed the two habituation days and was identical in procedure to habitation. 90 minutes after cessation of the 30-minute feeding period on test day, animals were euthanized via a ketamine/xylazine cocktail (280mg/kg; 80mg/kg) and intracardially perfused with cold 0.01M phosphate buffer solution (PBS) followed by fixative solution (4% paraformaldehyde (PFA) in PBS at a pH of 7.4). Following perfusion, brains were dissected and post-fixed in PFA overnight at 4 degrees C. The next day, fixed brains were rinsed in PBS and placed in 30% sucrose in PBS for 24 hours before being frozen on dry ice. Coronal sections (30 mm) containing the VTA were

collected with a cryostat (Microm HM 505 E). Sections were permeabilized with 0.5% Triton X-100 in PBS (PBS-T) and blocked with 3% normal donkey serum (Jackson ImmunoResearch) in PBS-T (PBS-T DS) for 60 min at room temperature. Sections were then incubated overnight in primary antibodies for c-Fos (rabbit, 1:1000, synaptic systems) and tyrosine hydroxylase (chicken, 1:500, Millipore AB9702) diluted in PBS-T DS. For visualization, sections were washed with PBS-T and incubated with appropriate secondary antibodies diluted in the blocking solution for 3 hours at room temperature. Secondary antibodies (Jackson Immunoresearch) were Cy-2 conjugated donkey anti-rabbit (1:250) and Cy-3 conjugated donkey anti-chicken (1:250). Sections were washed three times with PBS and mounted using DAPI Fluoromount-G (Southern Biotech). Images were captured on a Zeiss Axioplan 2 Imaging microscope equipped with an AxioCam MRm camera using AxioVision 4.6 software (Zeiss).

Food and water intake, and fat mass measurements: A separate cohort of SD and HFD-raised animals were monitored for daily consumption of water and home chow (SD or HFD) intake for four days. Each day the amount of water consumed was recorded, as was the amount of home chow. Home chow intake was corrected for body mass daily. These measures were averaged across four days for each animal. At the conclusion of the four days, animals were euthanized via cervical dislocation, and their brown (BAT), gonadal white (GWAT), and subcutaneous (SCAT) adipose tissue masses were measured.

Statistics: Statistical analysis was performed using *Prism GraphPad* software. Data are presented as means ± standard error of the mean (s.e.m.).

All statistical analyses were performed using ANOVA with Bonferroni *post-hoc* for multiple comparisons. Complete statistical results are included within the text.

Results:

Prolonged consumption of a high fat diet diminishes preference for and consumption of a more palatable liquid or solid food choice: To confirm previous studies demonstrating that HFD consumption leads to anhedonia [58,67,88], we first tested animals in a two-bottle sucrose preference assay. Two cohorts of animals, one raised on standard lab (SD) diet and one raised on HFD [Fig. 1A], were assessed on their preference for a 1% sucrose solution over water at 14 weeks of age. We discovered that animals that had been consuming HFD demonstrated a diminished preference for sucrose compared to water [$t=3.240$, $p<0.01$, $df=10$; n=9/group; Fig. 1B], and diminished overall liquid consumption [$t=3.593$, $p<0.01$, $df=10$; n=9/group; Fig. 1C] during test days. This reduced consumption was due to dampened consumption of sucrose specifically [$F(1,32)=11.44$; $p<0.01$; n=9/group; Supplemental Fig. 1A]. These data reaffirm the development of HFD-induced anhedonia in these animals [58,67,88]. However, the two-bottle preference assay utilizes liquids, and its findings cannot necessarily be generalized to feeding behavior and food preference. To overcome this limitation, we developed a novel food preference test that assesses animals' preference for a more palatable food choice over a less palatable one.

The two chows we used to assess food preference differed by 0.39 kcal/g (3.35 vs. 3.74 kcal/g) in caloric content. Importantly, these two chows are lower in

both fat and overall energy density than the diet consumed by the HFD group (4.73 kcal/g). Animals that were raised on either SD or HFD were assessed on their preference between these two novel chows during a 24-hr period [Fig. 1D]. Similar to what we observed during the sucrose preference test, animals raised on HFD exhibited diminished preference for a more energy dense chow [$t=5.802$, $p<0.0001$, $df=18$; n=10/group; Fig.1E], and consumed less test chow overall [$t=6.181$, $p<0.0001$, $df=18$; n=10/group; Fig. 1F] during testing when compared to SD fed controls. Comparable to our observations from the sucrose preference test, the HFD-raised animals' reduced test chow consumption is due to the decreased intake of the preferred choice specifically [$F(1,36)=47.83$; $p<0.0001$; n=10/group; Supplemental Fig. 1B]. Interestingly, HFD-raised animals were not significantly heavier than their SD-raised counterparts during both the sucrose preference test [$t=2.002$; $p=0.0626$; $df=16$; n=9/group; Supplemental Fig. 1C] and the food preference test [$t=1.312$; $p=0.2060$; $df=18$; n=10/group; Supplemental Fig. 1D]. In agreement with this finding, HFD-raised and SD-raised animals consumed similar amounts of maintenance diet [$t=1.211$; $p=0.2459$; $df=14$; n=8/group; Supplemental Fig. 2A] and had similar total fat mass (GWAT, BAT, SCAT) [$t=0.866$; $p=0.4008$; $df=14$; n=8/group; Supplemental Fig. 2B]. In addition, we observed no significant correlation between body weight and food preference [SD-raised: $R^2=0.001530$; $p=0.9146$; n=10/group; HFD-raised: $R^2=0.3569$; $p=0.0682$; n=10/group; Supplemental Fig. 3A] or body weight and total test chow consumption [SD-raised: $R^2=0.07323$; $p=0.4495$; n=10/group; HFD-raised: $R^2=0.02586$; $p=0.6572$; n=10/group; Supplemental Fig. 3B] in either group.

Taken together, these findings suggest that the devaluation of the more palatable food choice occurs independent of an obese phenotype. Next, we wanted to determine if a shorter period of access to HFD could produce the same results [Fig. 2A]. Indeed, when animals consumed a HFD for only four weeks prior to testing, the same effects on preference [$F(2,27)=26.40$; $p<0.0001$; n=10/group; Fig. 2B] and consumption [$F(2,27)=78.70$; $p<0.0001$; n=10/group; Fig. 2C] were observed. This includes decreased consumption of the preferred test chow specifically [$F(2,54)=72.86$; $p<0.0001$; n=10/group; Supplemental Fig. 4A]. These findings suggest that HFD consumption can affect feeding behavior over a shorter period, and with the first exposure not occurring until later in life.

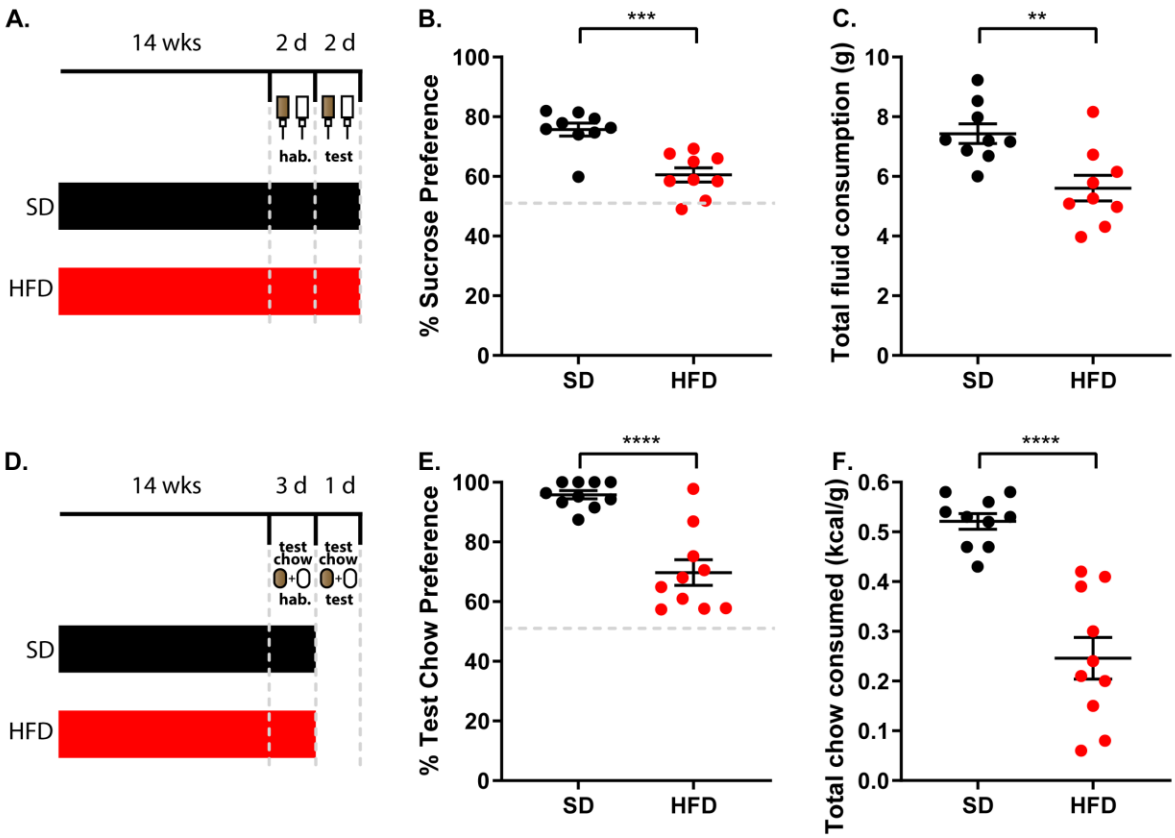
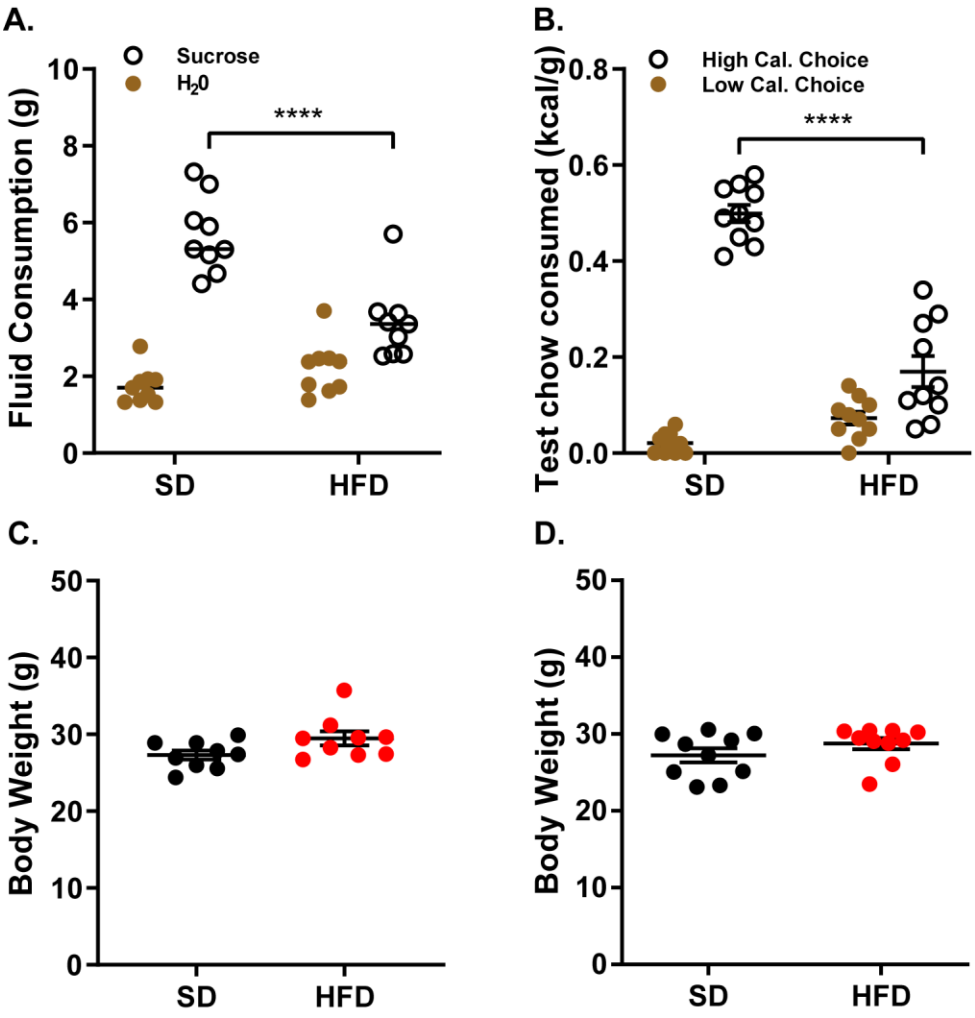
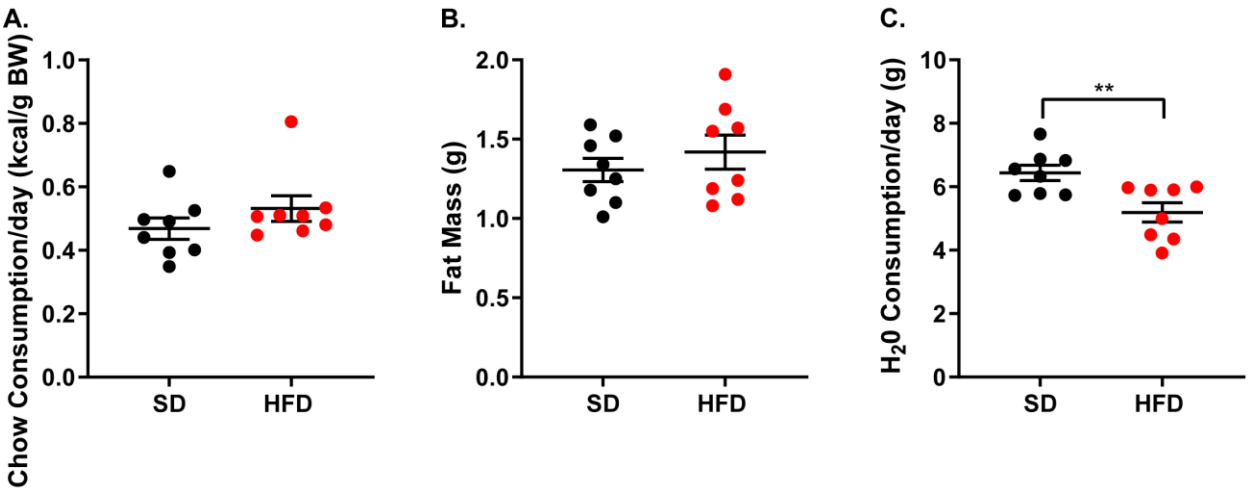


Fig.1. Prolonged consumption of a high fat diet diminishes preference for and consumption of a more palatable liquid or solid food choice. (A) Schematic illustrating the 2-bottle experimental setup. (B) Preference for sucrose over water in a two-bottle choice test. Student's two-tailed t-test; n=9/group. (C) Total fluid consumed (sucrose and water) during the two-bottle choice test. Student's two-tailed t-test; n=9/group. (D) Schematic illustrating food preference experimental setup. (E) Preference for the more energy dense test chow during the food preference test. Student's two-tailed t-test; n=10/group. (F) Total amount of both test chows consumed during the food preference test corrected for body weight. Student's two-tailed t-test; n=10/group. Data are represented as mean ± SEM. **p < 0.01; ***p<0.001; ****p < 0.0001. See also Supplemental Figures 1 and 2.

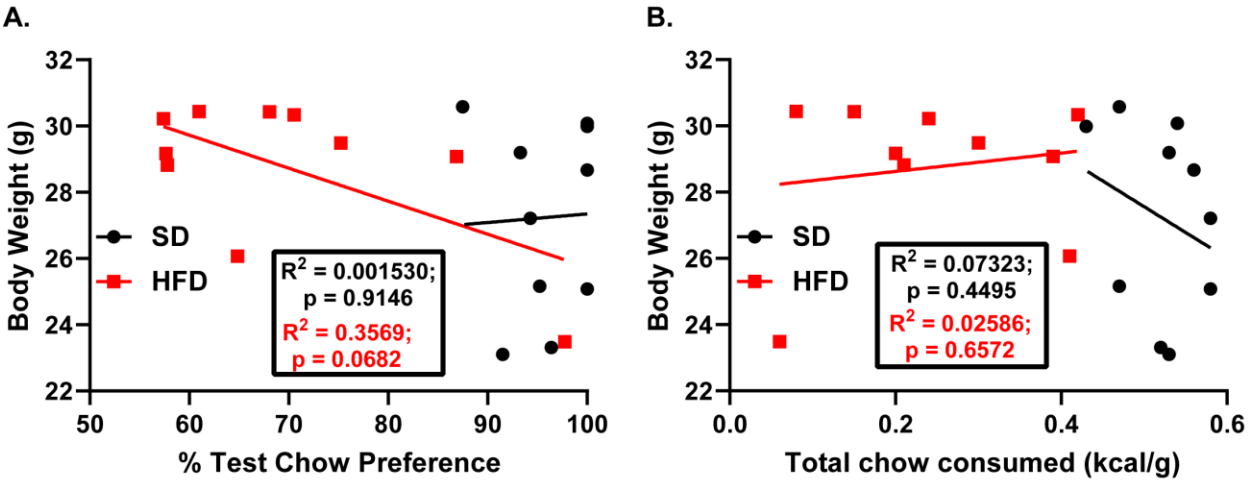


Supplemental Fig.1. Prolonged HFD consumption has no effect on body weight during the sucrose or food preference tests, and leads to decreased consumption of the preferred choice in both sucrose and food preference tests. (A) Amount of sucrose or water consumed during the sucrose preference test. Two-way ANOVA with Bonferroni *post-hoc* comparison; n=9/group; $F_{choice}(1,32)=89.63$, $p<0.0001$; $F_{diet}(1,32)=11.44$, $p=0.0019$. (B) Amount of either test chow consumed during the food preference test. Two-way ANOVA with Bonferroni *post-hoc* comparison; n=10/group; $F_{choice}(1,36)=206.1$, $p<0.0001$; $F_{diet}(1,36)=47.83$,

p<0.0001. (C) Body weight during the sucrose preference test. Student's two-tailed t-test; n=9/group. (D) Body weight during the food preference test. Student's two-tailed t-test; n=10/group. Data are represented as mean ± SEM. ****p < 0.0001.



Supplemental Fig.2. HFD-raised animals consume less water than SD-raised animals at baseline, but consume an equivalent amount of calories and do not differ in total fat mass. (A) Amount of home-cage chow (SD or HFD) consumed during baseline corrected for body weight. Student's two-tailed t-test; n=8/group. (B) Total fat mass (GWAT+SCAT+BAT) at baseline. Student's two-tailed t-test; n=8/group. (C) Amount of water consumed at baseline. Student's two-tailed t-test; n=8/group. Data are represented as mean ± SEM. **p < 0.01.



Supplemental Fig.3. Body weight is not correlated with test chow intake or preference during the food preference test. (A) Correlation between body weight and preference for the more energy-dense test chow during the food preference test. Simple linear regression; n=10/group. (B) Correlation between body weight and total amount of both test chows consumed during the food preference test corrected for body weight. Simple linear regression; n=10/group. R^2 and corresponding p-values are plotted for each slope.

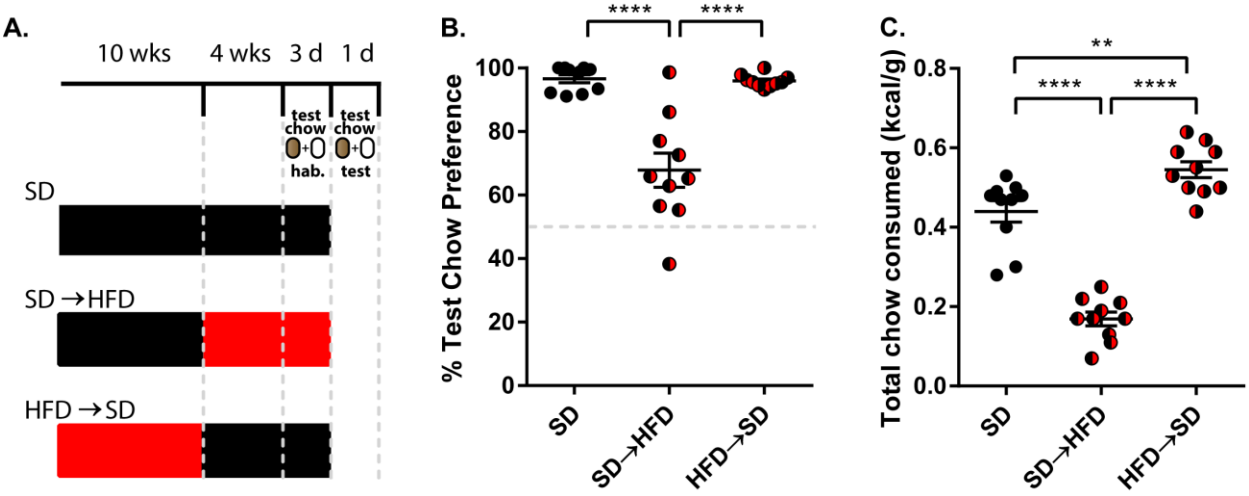
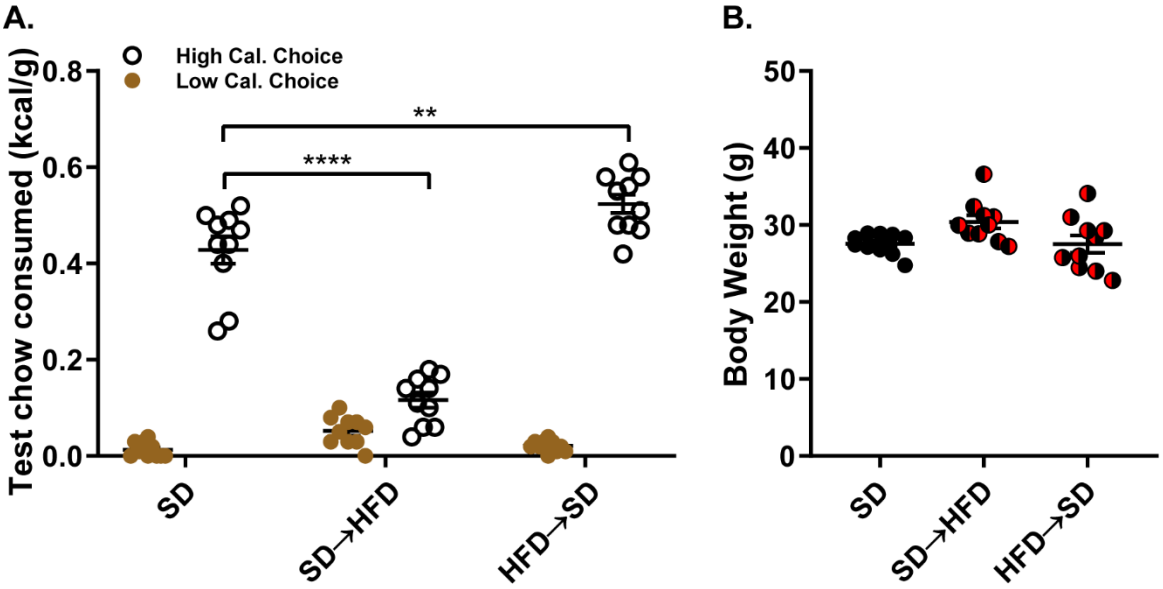


Fig.2. HFD-induced alterations in food preference are reversed by dieting. (A) Schematic illustrating the experimental setup. (B) Preference for the more energy dense test chow during the food preference test. One-way ANOVA with Bonferroni *post-hoc* comparison; n=10/group; F(2,27)=26.40; p<0.0001. (C) Total amount of both test chows consumed during the food preference test corrected for body weight. One-way ANOVA with Bonferroni *post-hoc* comparison; n=10/group; F(2,27)=78.70; p<0.0001. Data are represented as mean ± SEM. **p < 0.01; ****p < 0.0001. See also Supplemental Figure 4.



Supplemental Fig.4. Switching animals' diet for 4 weeks has no effect on body weight, but alters their consumption of the preferred choice in the food preference test. (A) Amount of either test chow consumed during the food preference test. Two-way ANOVA with Bonferroni *post-hoc* comparison; n=10/group; $F_{choice}(1,54)=617.7$, p<0.0001; $F_{diet}(1,54)=72.86$, p<0.0001. (B) Body weight during the food preference test. One-way ANOVA with Bonferroni *post-*

hoc comparison; n=10/group; F(2,27)=3.841; p=0.0486. Data are represented as mean ± SEM. **p<0.01; ****p < 0.0001.

HFD-induced alterations in food preference are reversed by dieting or exercise: We next sought to determine if behavioral interventions could reverse the alterations in feeding behavior that are produced by prolonged HFD consumption. We first chose to examine if withdrawing HFD-raised animals from HFD prior to the preference test could reverse their reduced preference and intake during the assay. To test this, we switched HFD-raised animals to SD at 10 weeks of age and assessed their preference at 14 weeks of age [Fig. 2A]. Following this dietary switch, we found that these animals no longer exhibited dampened preference for the more energy dense test chow [F(2,27)=26.40; p<0.0001; n=10/group; Fig. 2B] or attenuated test chow consumption [F(2,27)=78.70; p<0.0001; n=10/group; Fig. 2C]. Animals withdrawn from HFD weighed the same as animals raised on SD for 14 weeks [SD=27.57±0.4292 vs. HFD->SD=27.52±1.113; p=0.9991, Bonferroni multiple comparisons test; n=10/group; Supplemental Fig. 4B]. This was unsurprising considering that our HFD-raised mice failed to gain significant weight compared to SD-raised control mice [t=1.312, p=0.2060, df=18; n=10/group; Supplemental Fig. 1C]. Interestingly, these animals consumed more test chow than their SD raised counterparts [SD=0.44±0.02708 vs. HFD->SD=0.5450±0.02029; p<0.01, Bonferroni multiple comparisons test; n=10/group; Fig. 2C]. This heightened consumption is specifically due to an increased intake of the preferred food choice [F(2,54)=72.86; p<0.0001; n=10/group; Supplemental Fig. 4A]. One

possible explanation for this is that the animals withdrawn from HFD are exhibiting a sensitized behavioral response to the more palatable test chow, much like what is observed in animal models of drug abstinence and relapse [90]. Taken together, these findings demonstrate that switching to a less calorically enriched diet is sufficient to reverse HFD-induced alterations in food preference. These data demonstrate that the influence of HFD over food preference and consumption is not permanent.

We next assessed whether exercise could reverse the HFD-induced behavioral changes in preference and intake. To this end, we raised additional cohorts of animals on either SD or HFD. All groups were provided Bio Serv Mouse Igloos® for 4 weeks prior to the preference test [Fig. 3A]. One group of each diet received these igloos with Fast-Trac accessory running discs attached to them [Supplemental Fig. 5A]. When given these running discs, both SD and HFD raised animals run on them in equivalent amounts daily [$t=1.782$, $p= 0.0900$, $df=20$; n=11/group; Supplemental Fig. 5B]. Access to a running disc in HFD-raised animals reversed HFD-induced alterations in food preference [$F(1,35)=7.401$; $p<0.05$; n=8-14/group; Fig. 3B] without restoring total test chow consumption amount [$F(1,35)=1.516$; $p=0.2264$; n=8-14/group; Fig. 3C] or consumption of the preferred test chow specifically [$F(1,70]=1.455$; $p=0.2318$; n=8-14/group; Supplemental Fig. 5C]. This behavioral response is strikingly different than what we observed in response to dietary intervention (Fig. 2C) and suggests that food preference and consumption are independently modulated components of feeding behavior. Additionally, we were surprised to observe that

HFD-raised control animals with access only to the igloo weighed more than the SD-raised igloo controls [F(1,35)=20.11; p<0.0001; n=8-14/group; Supplemental Fig. 5D] in contrast to the lack of body weight gain in HFD-raised animals [t=1.312, p=0.2060, df=18; n=10/group; Supplemental Fig. 1C]. We suspect this to be due to the potential extra warmth provided by the igloo, allowing those HFD-raised animals to conserve more energy [91]. Taken together, our findings implicate relatively simple and accessible means by which HFD-induced behavioral modifications can be reversed.

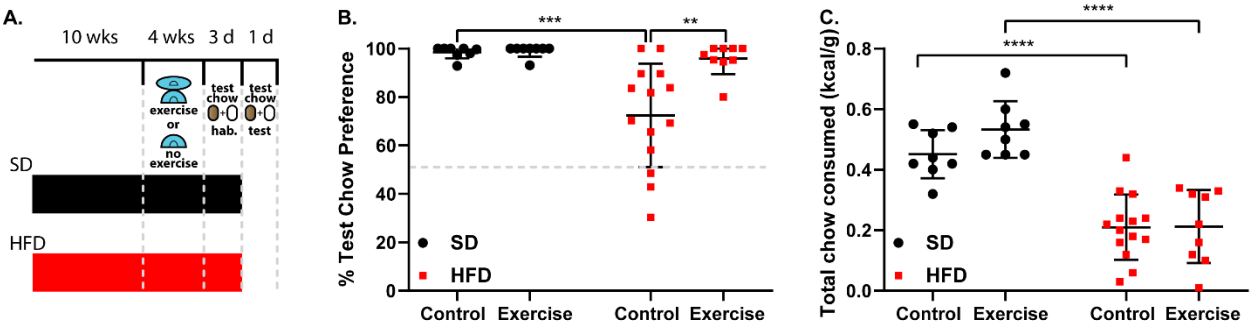
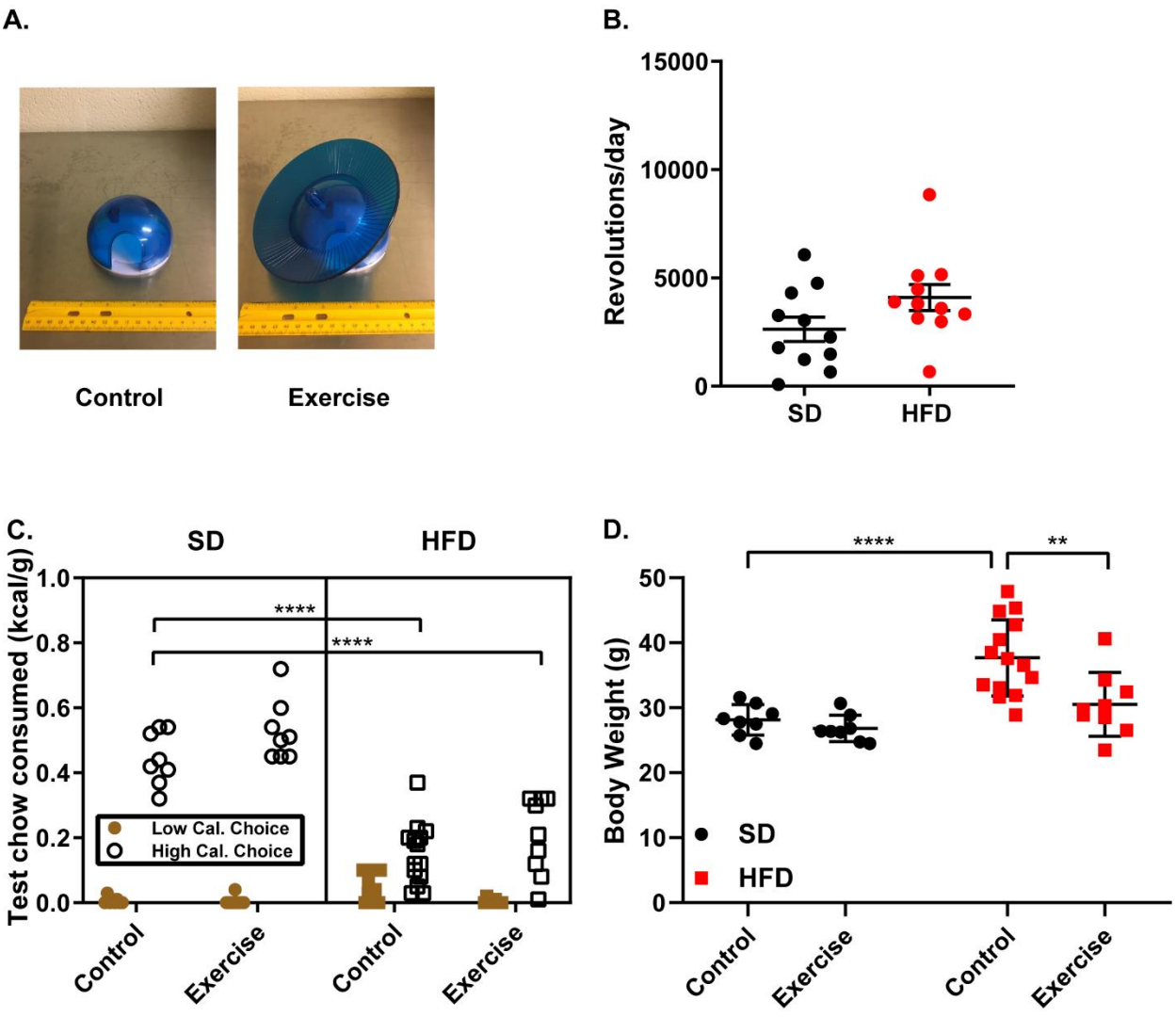


Fig.3. HFD-induced alterations in food preference are reversed by exercise. (A) Schematic illustrating the experimental setup. (B) Preference for the more energy dense test chow during the food preference test. Two-way ANOVA with Bonferroni *post-hoc* comparison; n=8-14/group; $F_{exercise}(1,35)=7.401$, p=0.0101; $F_{diet}(1,35)=11.01$, p=0.0021. (C) Total amount of both test chows consumed during the food preference test corrected for body weight. Two-way ANOVA with Bonferroni *post-hoc* comparison; n=8-14/group; $F_{exercise}(1,35)=1.516$, p=0.2264; $F_{diet}(1,35)=68.63$, p<0.0001. Data are represented as mean ± SEM. **p < 0.01; ***p < 0.001; ****p < 0.0001. See also Supplemental Figure 5.



Supplemental Fig.5. Non-exercising HFD-raised animals are heavier than SD-raised animals and exercising HFD-raised animals. SD and HFD-raised animals exercise in equivalent amounts. Both exercising and non-exercising HFD-raised animals consume less of the preferred choice in the food preference test. (A) Images of a Bio-Serv Igloo both with (exercise) or without (control) the Fast-Trac accessory running disc attached. (B) Mean number of exercise wheel revolutions per day. Student's two-tailed t-test; n=11/group. (C) Amount of either test chow consumed during the food preference test. Three-way ANOVA with Bonferroni *post-hoc* comparison; n=8-14/group; $F_{exercise}(1,70)=1.507$, $p=0.2237$; $F_{diet}(1,70)=71.46$, $p<0.0001$; $F_{choice}(1,70)=352.5$,

p<0.0001. (D) Body weight during the food preference test. Two-way ANOVA with Bonferroni *post-hoc* comparison; n=8-14/group; $F_{exercise}(1,35)=8.262$, p=0.0068; $F_{diet}(1,35)=20.11$, p<0.0001. Data are represented as mean ± SEM. **p<0.01; ****p < 0.0001.

Prolonged HFD consumption leads to a failure of the VTA to respond to a novel food reward: We next investigated the potential neurobiological impact of prolonged HFD consumption. We focused on the mesolimbic dopamine signaling pathway because of its established role in hedonic feeding and used c-fos immunohistochemistry to assess changes in activity of this pathway. Following stimulation in VTA neurons, the immediate early gene c-fos is rapidly expressed, allowing for examination of neural activity in this region [92,93]. To assess diet-induced changes in VTA activity during hedonic feeding, SD and HFD raised mice were allowed to consume a novel HFD for 30 minutes. This diet is more energy dense (5.24 vs. 4.73 kcal/g) and higher in fat content (60% kcal vs. 45% kcal) than the diet the HFD-raised animals consumed throughout their life. After consuming the novel 60% kcal HFD diet for 30 minutes, we examined differences in c-fos and tyrosine hydroxylase (TH) expression in the VTA of these animals. TH is the rate-limiting enzyme in the synthesis of dopamine and allows for the demarcation of dopaminergic cells. HFD raised animals exhibited dampened c-fos induction in the VTA in response to the novel 60% kcal HFD when compared to the standard chow raised controls [SD=61.56±3.705 vs. HFD=33.67±5.651; p<0.05, Bonferroni multiple comparisons test; n=9-12/group; Fig. 4A]. When compared to animals given a novel SD, SD raised animals given the 60% HFD exhibit marked VTA c-fos induction [SD given SD=16.90±4.868 vs. SD given

HFD=61.56±3.705; p<0.0001, Bonferroni multiple comparisons test; n=9-10/group; Supplemental Fig. 6A], whereas HFD raised animals do not differ in VTA c-fos activity when given either novel SD or HFD [HFD given SD=21.40±7.115 vs. HFD given HFD=33.67±5.651; p=0.3922, Bonferroni multiple comparisons test; n=10-12/group; Supplemental Fig. 6A]. These findings suggest that prolonged HFD consumption leads to a failure of the VTA to respond during hedonic feeding. We observed no effect of diet on the number of tyrosine hydroxylase (TH) positive cells in the VTA, suggesting that prolonged HFD exposure does not alter the number of dopaminergic cells in the VTA [F(2,28)= 1.892; p= 0.1696; n=9-12/group; Fig. 4B]. Interestingly, the observed HFD-induced reduction in VTA activity during palatable feeding is reversed by withdrawing animals from HFD for 4 weeks prior to testing [HFD=33.67±5.651 vs. HFD->SD= 61.70± 10.53; p<0.05, Bonferroni multiple comparisons test; n=10-12/group; Fig. 4A]. Additionally, this reversal appears to be associated with the hypersensitization of VTA dopaminergic cells specifically [F(2,28)=14.18; p <0.0001; n=9-12/group; Fig. 4C]. These findings show that prolonged HFD consumption blunts the response of the mesolimbic dopamine signaling pathway to rewarding food, but that dieting can restore the response via sensitization of dopamine neurons.

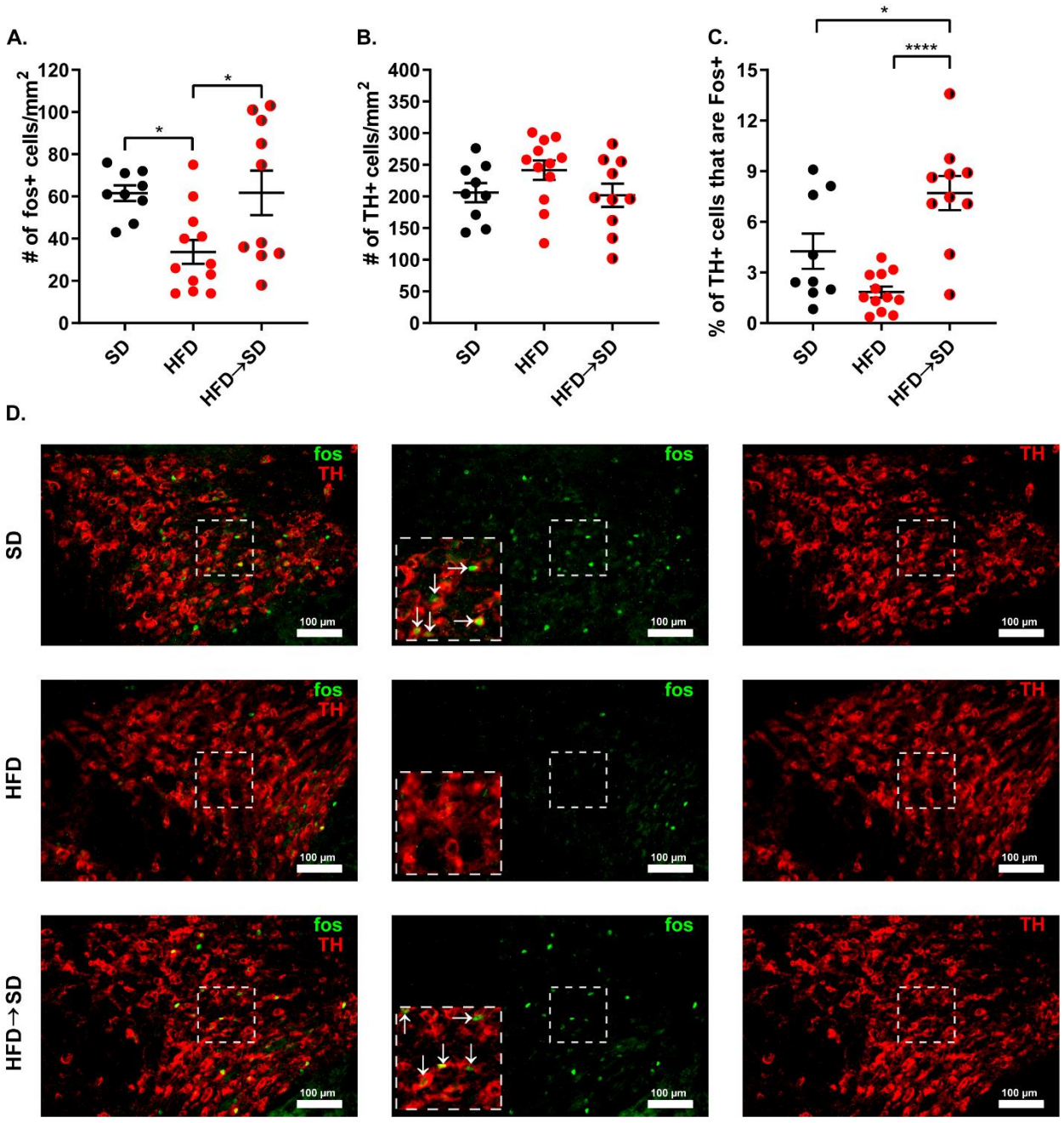
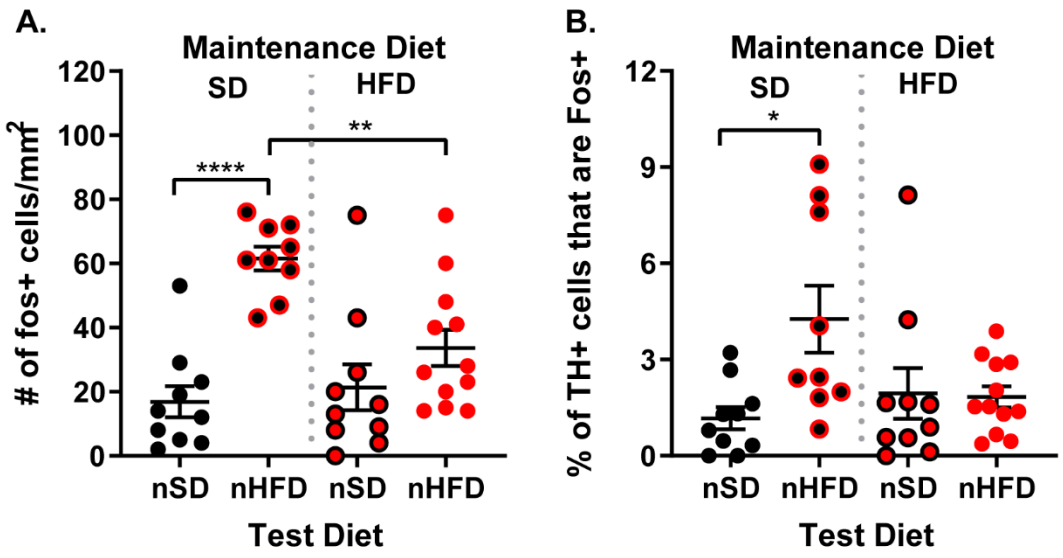


Fig.4. Prolonged HFD consumption leads to a failure of the VTA to respond to a novel food reward. (A) Total number of VTA c-Fos positive cells. One-way ANOVA with Bonferroni *post-hoc* comparison; n=9-12/group; F(2,28)=5.302; p=0.0111. (B) Total number of VTA TH positive cells. One-way ANOVA with Bonferroni *post-hoc* comparison; n=9-12/group; F(2,28)=1.892; p= 0.1696. (C)

Percentage of VTA TH positive cells that are also c-Fos positive. One-way ANOVA with Bonferroni *post-hoc* comparison; n=9-12/group; F(2,28)=14.18; p <0.0001. (D) Representative images of VTA sections stained for c-Fos and TH. Dashed boxes represent corresponding areas of the same section. Arrows indicate c-Fos immunoreactivity. Data are represented as mean ± SEM. *p<0.05; ****p < 0.0001. See also Supplemental Figure 6.



Supplemental Fig.6. Prolonged HFD consumption leads to a failure of the VTA to respond to a novel palatable food reward. VTA dopaminergic neurons respond to a novel palatable food reward in SD raised animals only. (A) Total number of VTA c-Fos positive cells in SD and HFD raised animals given a novel SD (nSD) or novel 60% HFD (nHFD). One-way ANOVA with Bonferroni *post-hoc* comparison; n=9-12/group; F(3,37)=11.70; p <0.0001. (B) Percentage of VTA TH positive cells that are also c-Fos positive in SD and HFD raised animals given a novel SD (nSD) or novel 60% HFD (nHFD). One-way ANOVA with Bonferroni

post-hoc comparison; n=9-12/group; F(3,37)=3.942; p=0.0155. Data are represented as mean ± SEM. *p<0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Discussion:

Using a novel food preference assay, we have discovered that prolonged consumption of a HFD leads to alterations in feeding behavior and devaluation of a more palatable food choice. Furthermore, we investigated the neurobiological effects of HFD exposure and demonstrated that animals raised on HFD exhibit dampened VTA activity during hedonic feeding. Moreover, we also found that diet-induced behavioral alterations could be reversed via both exercise and dietary change.

Previous work has demonstrated that diet affects feeding behavior, but effects on food preference specifically are poorly understood. Two-bottle choice tests, where animals can freely consume two different solutions (alcohol, water, sucrose, sucralose, etc.), are the most frequently used measure of preference, and are a staple behavioral assessment for anhedonia in rodents [94,95]. Rodents consistently prefer sucrose solutions over water in these tests, and diminished preference for sucrose is widely accepted as a manifestation of anhedonia. HFD consumption has specifically been shown to reduce this preference [58,67,88]. Our study has yielded the same finding (Fig. 1B), however, two-bottle tests do not directly address food preference because they utilize liquids. We designed a food preference assay that would serve to similarly address preference and anhedonia but using solid foods. Animals raised on a HFD exhibited both a reduction in preference for the more calorically dense test

chow, as well as decreased consumption of the test chows. These findings echo what has been shown in studies examining the effects of HFD consumption on sucrose preference. Additionally, a recent study demonstrated that prolonged HFD-consumption leads to devaluation of standard chow [60]. Specifically, this study found that when HFD-raised animals were withdrawn from HFD, their intake of the now available SD plummeted compared to SD raised controls. Taken together, these data suggest that prolonged consumption of a HFD leads to anhedonia and subsequent alterations in feeding behavior.

We were surprised to observe that all of our HFD raised cohorts failed to achieve significant weight gain when compared to their SD raised counterparts. This finding appears to contradict what has been demonstrated by other labs using the same HFD [96–99]. However, these labs use SD-fed breeders, and withhold HFD from the offspring until a predetermined time post-weaning. Our mice have access to HFD immediately after birth, and they are born from HFD-fed breeders. We suspect this distinction may be at least partially responsible for the lack of statistically significant weight gain in our animals, and that perhaps our animals are conferred some degree of protection against weight gain during prenatal development. Our lack of significant differences in maintenance diet consumption and fat mass also help explain the lack of significant gain in body weight among our HFD raised cohorts. These findings suggest that the behavioral and neurobiological differences we observe in HFD raised mice can occur independent of an obese phenotype.

Studying mice after 14 weeks of HFD consumption does not address the possibility that shorter periods of exposure to HFD could produce the same behavioral effects. Interestingly, when we restricted the time of access to HFD to just weeks 10-14, the animals exhibited the same behavioral changes during the food preference assay. This suggests that the behavioral effects of HFD can manifest after a shorter period of exposure later in life. Additionally, the effects of HFD on food preference are not permanent and can be reversed via behavioral interventions. We found that switching mice to SD reversed HFD-induced alterations in food preference and test chow intake, while exercise reversed changes in food preference, but not intake. Previous studies have suggested that exercising regularly produces changes in the mesolimbic dopamine signaling pathway [78,81,82]. One such study showed that 8 weeks of treadmill running in mice was sufficient to restore dampened VTA tyrosine hydroxylase and NAc dopamine levels following prolonged HFD consumption [82]. We suspect that similar neurological alterations are responsible for our observed behavioral findings. The ability of exercise to reverse changes in food preference independent of intake is noteworthy because it suggests that the two behavioral phenotypes, diminished food preference and food intake, are separable. Specifically, HFD-induced dampening of preference for the more calorically dense test chow may not simply be due to reduced intake of the test chows.

In our efforts to elucidate the potential neurobiological mechanism underlying the effects of HFD consumption on food choice, we found that the neural response of the VTA of HFD-raised animals was dampened during

consumption of the novel HFD chow compared to the VTA of animals raised on standard lab chow. Previous work has shown that HFD-raised animals exhibit blunted dopamine release into the NAc during exposure to a novel palatable food, which parallels these findings [49]. Interestingly, prolonged HFD consumption did not appear to influence the activity of the VTA dopaminergic neurons specifically. However, the VTA also contains non-dopaminergic glutamatergic and GABAergic neuronal subpopulations [100]. These subpopulations are likely where we are observing the bulk of the changes in VTA activity. Interestingly, we found that dieting resulted in a sensitized VTA response to the novel HFD, and this response occurred specifically in the dopaminergic neurons. Such a finding mirrors sensitization and relapse responses to drugs of abuse in the same neural circuit [90]. In summary, our results suggest that prolonged HFD consumption dampens the activity of the mesolimbic dopamine signaling pathway, but dieting can reverse this through sensitization of VTA dopaminergic neurons.

In conclusion, our research indicates that prolonged consumption of a HFD leads to alterations in food preference that are associated with HFD-induced changes in reward circuitry. These alterations in food preference are reversible via dieting and exercise. Further study is needed to elucidate the neurobiological mechanisms by which dieting, and exercise reverse HFD-induced changes in food preference. Based on the dampened neuronal activity in the VTA of HFD-raised mice, we suspect remodeling of mesolimbic dopamine signaling is the responsible mechanism. Additionally, our data suggest HFD-

induced changes in food preference and consumption are separable. Specifically, exercise restored food preference but not the dampened test chow consumption. This disparity suggests that prolonged HFD consumption could be exerting its effects on food preference through one mechanism, while altering intake through another. This distinction demonstrates how multifaceted the influence of HFD over feeding behavior is, but also suggests there may be multiple avenues to target therapeutically.

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Author Contributions:

Everett Altherr, Aundrea Rainwater, and Ali D. Güler conceived and designed all experiments. Everett Altherr performed the two bottle choice, exercise, and c-Fos experiments. Aundrea Rainwater performed the original preference experiment, as well as the HFD withdrawal/4 week access experiments. Darian Kaviani assisted with all experiments. Qijun Tang devised the exercise quantification methodology and assisted with both that experiment and statistical analysis. Everett Altherr wrote the manuscript with input from all co-authors.

Ch. 3: Midbrain Dopamine-1 Receptor Overexpression Fails to Restore Diet-Induced Perturbations in Food Preference

The following chapter is comprised of unpublished data collected throughout my graduate career.

Abstract:

In the previous chapter, I demonstrated that long-term HFD consumption diminishes preference for and consumption of more calorically dense food in a novel food preference assay. In addition, I discovered that HFD consumption perturbs mesolimbic dopamine signaling by causing the VTA to fail to respond to a novel food reward. The aim of this chapter is to better characterize the role of dopamine signaling in mediating food preference, and to determine if modulation of that signaling can reverse HFD-induced alterations in food preference/choice. Here I find that a global, but not NAc specific, knock-out of D1R leads to a decreased preference for the more palatable food choice in the food preference assay. In addition, I demonstrate that overexpression of NAc D1R in mice raised on a HFD fails to reverse the diet-induced alterations in food preference and consumption discussed in the previous chapter. These findings suggest that D1R is crucial for intact food preference, but insufficient for reversing HFD-induced impairments in food choice.

Introduction:

Obesity is a costly epidemic that claims an escalating number of lives every year [101]. Despite its complex etiology, a universally accepted factor in the development of obesity is the daily overconsumption of calories stemming

from dietary choices that are high in fat and sugar [3,102]. The consumption of these foods is promoted by the mesolimbic dopamine signaling pathway, which functions to convey the salience and rewarding properties of these foods [8–11]. The mesolimbic dopamine signaling pathway plays a major role in many other components of feeding behavior, such as facilitating the formation of learned associations between food and cues in the environment, as well as motivating organisms to seek out food [11,45]. Despite its widespread role in mediating several aspects of feeding behavior, the manner in which mesolimbic dopamine signaling influences food preference remains to be elucidated.

Mesolimbic dopamine signaling has long been understood to play a key role in reward processing, and food reward is no exception. Dopamine exerts its effects by binding to one of five dopamine receptor subtypes. These receptors are expressed throughout the brain to facilitate movement, learning and memory, and impulse control [103]. Of the five receptor subtypes, types 1 (D1R) and 2 (D2R) are the most well characterized in relation to feeding, and both are expressed in the NAc [104,105]. For example, blockade of either subtype has been shown to disrupt food intake, and activation of NAc D2 receptors is associated with cessation of binge-eating [104]. Additionally, studies have demonstrated that dampened D2R levels are associated with obesity, but this is typically attributed to changes in energy expenditure instead of appetitive/consummatory processes [106,107]. Lastly, NAc D1R expression is regulated in response to HFD consumption [48–50,52]. Despite the connection

between HFD consumption and D1R, the potential for D1R modulation to reverse diet-induced perturbations in feeding behavior has yet to be elucidated.

Because of the established role of D1R in feeding behavior in general, and the potential for it to be dysregulated by HFD, I investigated the following hypotheses in this chapter: (1) knocking out D1R in mice raised on SD disrupts preference for the more calorically enriched food choice in our novel food preference assay, and (2) overexpression of D1R in the NAc of mice raised on HFD reverses the diet-induced perturbations in food preference demonstrated in the previous chapter. To test these hypotheses, I used a combination of transgenic mouse lines and viral vectors to manipulate D1R levels under different conditions. In addition, I assessed behavior using the food preference assay introduced in the previous chapter to examine the preference between and consumption of two novel food substrates. I discovered that a global KO of D1R leads to a diminished preference for the more palatable choice in the food preference assay, but a NAc specific KO of D1R fails to replicate this change. In addition, selectively restoring D1R expression to the NAc of global D1R KO animals fails to restore preference to wild-type levels. Lastly, overexpressing D1R in the NAc of animals raised on a HFD fails to reverse the diet-induced perturbations in food preference and test chow consumption demonstrated in the previous chapter. These findings demonstrate that D1R expression as a whole, but not specifically in the NAc, is essential for intact food preference, implicating a novel role for dopamine signaling in feeding behavior. A summary table of all findings can be found at the end of the chapter.

Materials and Methods:

Animals: All experiments complied with NIH animal care and use guidelines, and were approved by the University of Virginia Institutional Animal Care and Use Committee. Male mice were housed on a 12-hour light/dark cycle (lights on between 7am and 7pm), in standard ventilated cages with corncob bedding, cotton nesting material (Nestlets™, Ancare, Bellmore, NY, USA) and a paper soufflé cup (product F400, Genpak, Charlotte, NC). C57BL/6 breeder mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). In addition to wild-type C57BL6 mice, the following mouse lines were used: *D1R*$^{Cre/Cre}$ (D1R-KO), *D1R*$^{+/+}$ (WT), and *Floxed-D1R*$^{lox/lox}$ (FD1R). Experimental transgenic mice were bred in-house using heterozygotes obtained from the University of Washington (*D1R*$^{Cre/+}$) or Jackson Laboratory (*Floxed-D1R*$^{lox/+}$) that were then backcrossed to C57BL/6 mice for at least 20 generations. Maintenance diets were furnished to both breeders and offspring except where acute dietary treatment is specified. All experiments took place using male mice, and all experimental mice were habituated to individual housing for at least 3 days prior to intake procedures. All behavioral tests and tissue collections took place at 14 weeks of age.

Diets Used: All mice with the exception of HFD-raised animals in the D1R overexpression experiment received *Envigo* (Huntingdon, United Kingdom) product 8664 *Teklad F6 Rodent Diet* (standard diet, 3.1 kcal/g; 19% fat, 31% protein, 50% carbohydrates). Mice on HFD in the D1R overexpression experiment received: *Research Diets Open Source Diets* (Research Diets, New

Brunswick, NJ, USA) product D12451 (OS red, 4.73 kcal/g; 45% fat, 20% protein, 35% carbohydrates*)*.

Food Preference Test: Mice were habituated to 2 novel test chows for 3 days prior to the preference test. During this habituation period, test chows were provided on the cage floor and maintenance diet remained in the wire cage top. At the start of the preference test, mice were weighed, the maintenance diet was withdrawn, and the test chows (~20 g of each) were weighed and added to the cage floor. Remaining test chow and animal body weights were recorded again 24 hr later. Consumption of each test chow for every animal was divided by that animal's body weight corresponding to the start of the 24 hour test period. Preference scores were calculated as the portion of total test chow consumed corresponding to the more energy enriched test chow. Novel test chows used during 24 hr food choice tests were as follows: *Bio-Serv (Flemington, NJ, USA) Product F3028 Rodent Diet Grain-Based Control* (BS F3028, 3.35 kcal/g; 13% fat, 29% protein, 58% carbohydrates) versus *Bio-Serv (Flemington, NJ, USA) Product F3156 Rodent Diet AIN-93G* (BS F3156, 3.74 kcal/g; 17% fat, 19% protein, 64% carbohydrates). Both of these diets were selected because they are contrasting in color with respect to the animals' bedding, and are lower in energy and fat content compared to the diet the HFD animals were raised on. Bedding was thoroughly searched for test chow crumbs when weighing test chows at the conclusion of the test.

Stereotaxic Surgery and Viral Expression: Prior to and during surgery, mice were anesthetized using isoflurane (induction 5%, maintenance 2%–2.5%;

Isothesia) and placed in a stereotaxic apparatus (Kopf). Ocular lubrication was applied to both eyes to aid in preventing desiccation. Throughout the surgery and during recovery mice were placed on a heating pad to maintain body temperature. For the D1R-KO rescue and HFD overexpression experiments, bilateral injections of adeno-associated viruses (AAV) that cre-dependently express hemagglutinin (HA)-tagged D1R or green-fluorescent protein (GFP)-tagged ChR2 (control group) were delivered to the NAc. For the NAc specific D1R-KO experiment, bilateral injections of AAV containing cre-recombinase or GFP (control) were delivered to the NAc. AAV was injected using a 10 mL syringe (Hamilton) and 26-gauge needle (Hamilton) at a flow rate of 100 nl/min driven by a microsyringe pump controller (World Precision Instruments, model Micro 4). Following injections, the needle was left in place for 10 min and was completely withdrawn 20 min after delivery of the virus. After surgery, mice were given ketoprofen (3 mg/kg) subcutaneously as an analgesic. Animals were behaviorally assessed at four weeks post viral administration to ensure optimal transgene expression. All surgical procedures were performed in sterile conditions and in accordance with University of Virginia IACUC guidelines.

Statistics: Statistical analysis was performed using *Prism GraphPad* software. Data are presented as means ± standard error of the mean (s.e.m.). All statistical analyses were performed using student's t-tests. Complete statistical results are included within the text.

Results:

Global dopamine-1 receptor knock out diminishes preference for a more palatable food choice: *Note: this specific experiment was initially conceived by Aundrea Rainwater, and the data/findings contained herein were collected by myself as a replication of her original experiment.*

To investigate the role of D1R-dependent signaling in mediating food preference, I used SD-raised mice in which both D1R alleles were replaced by cre-recombinase, resulting in knockout of D1R (Dopamine receptor type $1^{cre/cre}$; $D1R^{cre/cre}$) [Fig 1A]. These mice were assessed on their preference between the two test chows in the food preference assay. Similar to what was observed in HFD-raised animals in the previous chapter, D1R-KO mice exhibited diminished preference for the more calorically-enriched test chow [$t=3.353$, $p<0.01$, $df=16$; n=8-10/group; Fig.1B] and a trend towards decreased overall test chow consumption normalized for body weight [$t=2.083$, $p=0.0537$, $df=16$; n=8-10/group; Fig.1C] compared to wild-type controls. D1R-KO mice were also significantly lighter than their wild-type counterparts [$t=3.150$, $p<0.01$, $df=16$; n=8-10/group; Fig.1D], however this observation is unsurprising since this mouse strain has previously been characterized as smaller than wild-type controls [108]. Taken together, these findings demonstrate that D1R-dependent dopamine signaling is critical for intact food preference.

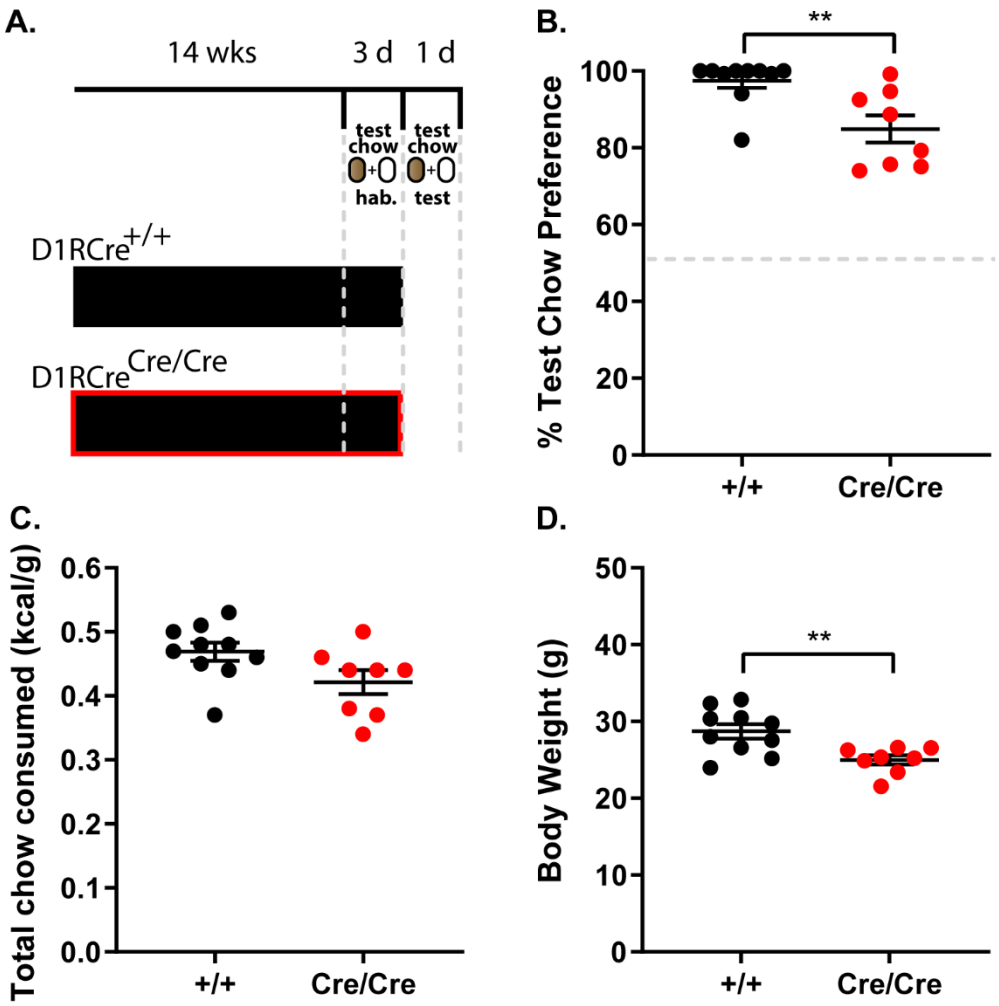


Fig.1. Global dopamine-1 receptor knock out diminishes preference for a more palatable food choice. (A) Schematic illustrating food preference experimental setup. (B) Preference for the more energy dense test chow during the food preference test. Student's two-tailed t-test; n=8-10/group. (C) Total amount of both test chows consumed during the food preference test corrected for body weight. Student's two-tailed t-test; n=8-10/group. (D) Body weight during the food preference test. Student's two-tailed t-test; n=8-10/group. Data are represented as mean ± SEM. **p < 0.01.

NAc-specific D1R-KO has no effect on food preference, and NAc D1R rescue fails to restore food preference in global D1R-KO mice: To further characterize the role of D1R-mediated signaling in food preference and choice, I used the cre-lox system to knock out D1R in the NAc of FD1R mice. FD1R mice were injected with cre-recombinase-expressing AAV or GFP-expressing AAV (control) targeted to the NAc and given four weeks of recovery to allow adequate viral expression before partaking in the food preference assay [Fig. 2A]. Unlike the global D1R-KO mice, NAc-specific D1R-KO animals did not exhibit any impairments in food preference [$t=0.3733$, $p=0.7138$, $df=16$; n=9/group; Fig.2B] or total test chow consumption [$t=0.5020$, $p=0.6225$, $df=16$; n=9/group; Fig.2C] in the assay. Additionally, NAc-specific D1R-KOs do not exhibit the diminished body weight observed in global D1R-KO mice [$t=0.1049$, $p=0.9178$, $df=16$; n=9/group; Fig.2D]. These findings suggest that while D1R signaling as a whole is important for food preference, dampening of that signaling in the NAc specifically is insufficient to induce impairments in preference.

In order to continue to investigate the role of NAc D1R in food choice, I further utilized the cre-lox system to attempt a viral restoration of D1R to the NAc of the global D1R-KO mice. D1R-KO mice were injected with adeno-associated viruses that cre-dependently express hemagglutinin (HA)-tagged D1R, or ChR2-GFP (control) to the NAc. Mice were given four weeks of recovery to ensure adequate viral expression before engaging in the food preference assay [Fig. 3A]. Re-expression of D1R to the NAc of global D1R-KO mice failed to restore preference for the more energy-dense test chow in the food preference assay

[t=0.5798, p=0.5713, df=14; n=8/group; Fig.3B]. These mice also did not differ from global D1R-Kos in terms of total amount of test chow consumed during the food preference test [t=1.078, p=0.2994, df=14; n=8/group; Fig.3C]. or body weight [t=1.119, p=0.2819, df=14; n=8/group; Fig.3D]. This failure to restore food preference in these animals suggests that NAc D1R on its own is not sufficient for intact food preference. When considered alongside the fact that NAc-specific KO of D1R failed to impair food preference, these findings together suggest that NAc D1R signaling individually is not necessary for mediation of food preference.

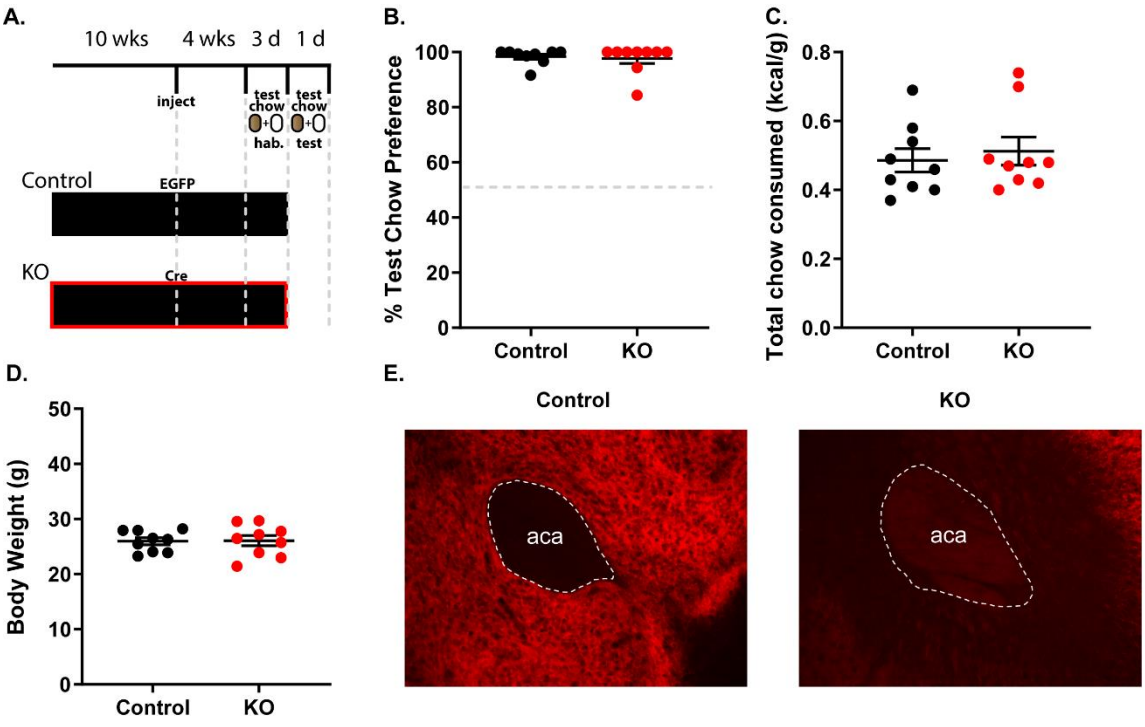


Fig.2. NAc specific dopamine-1 receptor knock out fails to diminish preference for a more palatable food choice. (A) Schematic illustrating food preference experimental setup. (B) Preference for the more energy dense test chow during

the food preference test. Student's two-tailed t-test; n=9/group. (C) Total amount of both test chows consumed during the food preference test corrected for body weight. Student's two-tailed t-test; n=9/group. (D) Body weight during the food preference test. Student's two-tailed t-test; n=9/group. (E) Representative immunohistochemistry images of NAc D1R expression in control animals (left) and animals receiving cre-recombinase injections (right). Red staining is indicative of D1R expression using D1R-specific antibodies. aca=anterior commissure. Data are represented as mean ± SEM. **p < 0.01.

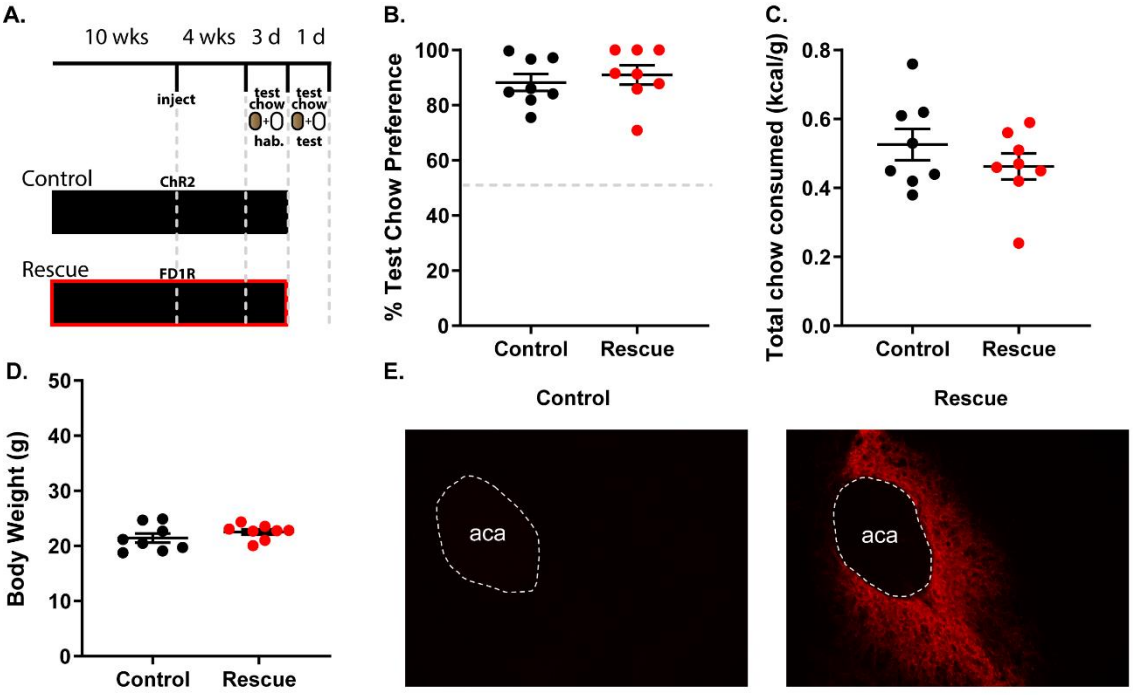


Fig.3. Viral restoration of D1R to the NAc of global D1R-KO mice fails to rescue preference for a more palatable food choice. (A) Schematic illustrating food preference experimental setup. (B) Preference for the more energy dense test chow during the food preference test. Student's two-tailed t-test; n=8/group. (C) Total amount of both test chows consumed during the food preference test corrected for body weight. Student's two-tailed t-test; n=8/group. (D) Body weight during the food preference test. Student's two-tailed t-test; n=8/group. (E) Representative immunohistochemistry images of NAc D1R re-expression in control animals (left) and animals receiving FD1R injections (right). Red staining is indicative of viral D1R re-expression using HA-D1R-specific antibodies. aca=anterior commissure. Data are represented as mean ± SEM. **p < 0.01.

NAc D1R overexpression fails to reverse diet-induced alterations in food preference in HFD-raised mice: In the previous chapter, I demonstrated that prolonged HFD consumption leads to a dampened VTA response to novel food reward as well as a diminished preference for and consumption of the more palatable test chow in the food preference assay. In order to further investigate the link between prolonged HFD consumption, mesolimbic dopamine signaling, and food preference, I utilized the cre-lox system to virally overexpress D1R in the NAc of HFD-raised D1R-Cre heterozygous animals [Fig. 4A]. I was surprised to observe that HFD-raised mice with NAc D1R expression exhibited further impaired food preference compared to control injected mice [t=2.432, p<0.05,

df=20; n=8-14/group; Fig.4B]. In addition, NAc D1R overexpression failed to reverse HFD-induced alterations in test chow consumption during the food preference assay [t=0.007230, p=0.9943, df=20; n=8-14/group; Fig.4C]. These findings indicate that modulation of mesolimbic D1R expression is insufficient for reversing HFD-induced alterations in feeding behavior. FD1R injected HFD-raised mice additionally did not differ from controls in terms of bodyweight [t=0.9660, p=0.3456, df=20; n=8-14/group; Fig.4D].

In addition to conducting the experiment in HFD-raised mice, I repeated these manipulations in mice raised on SD in order to determine if midbrain D1R modulation could affect food preference under “baseline” (non-HFD background) conditions [Supplemental Fig. 1A]. NAc D1R overexpression had no effect on preference for the more palatable test chow [t=1.829, p=0.0832, df=19; n=10-11/group; Supplemental Fig.1B], total test chow consumption [t=0.5749, p=0.5721, df=19; n=10-11/group; Supplemental Fig.1C], or bodyweight [t=1.564, p=0.1343, df=19; n=10-11/group; Supplemental Fig.1D]. When considered in conjunction with the lack of effects on preference and consumption observed in the NAc D1R KO experiment, these findings indicate that modulation of NAc D1R alone is insufficient to alter food preference under typical dietary conditions.

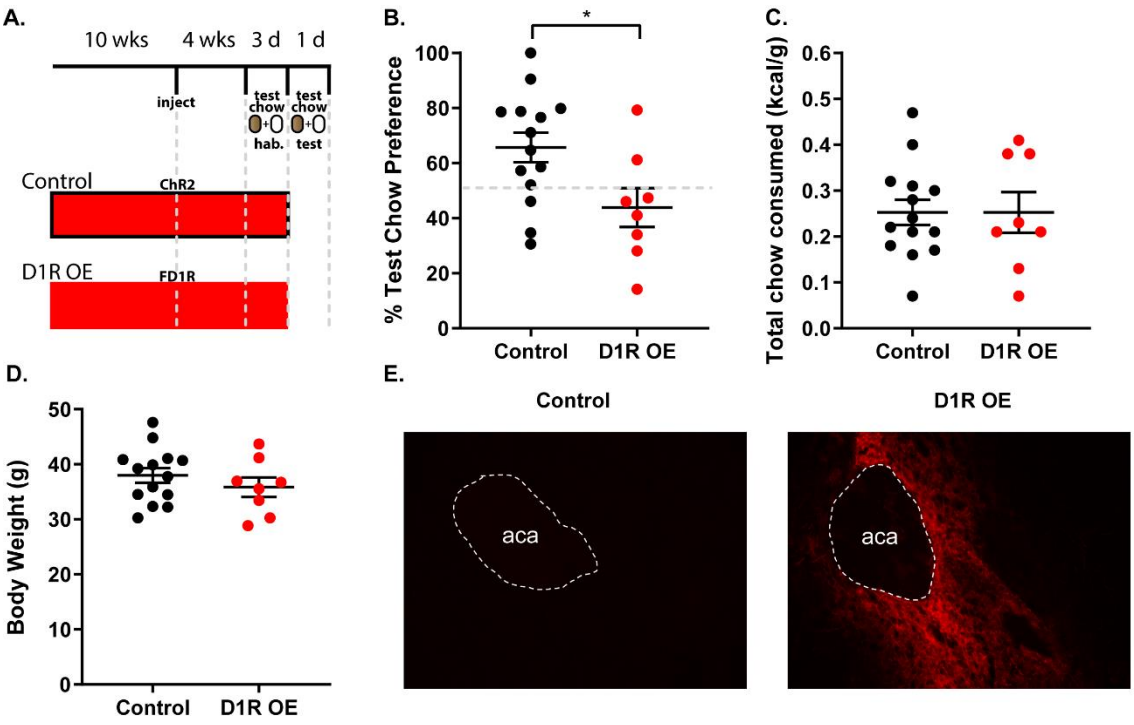
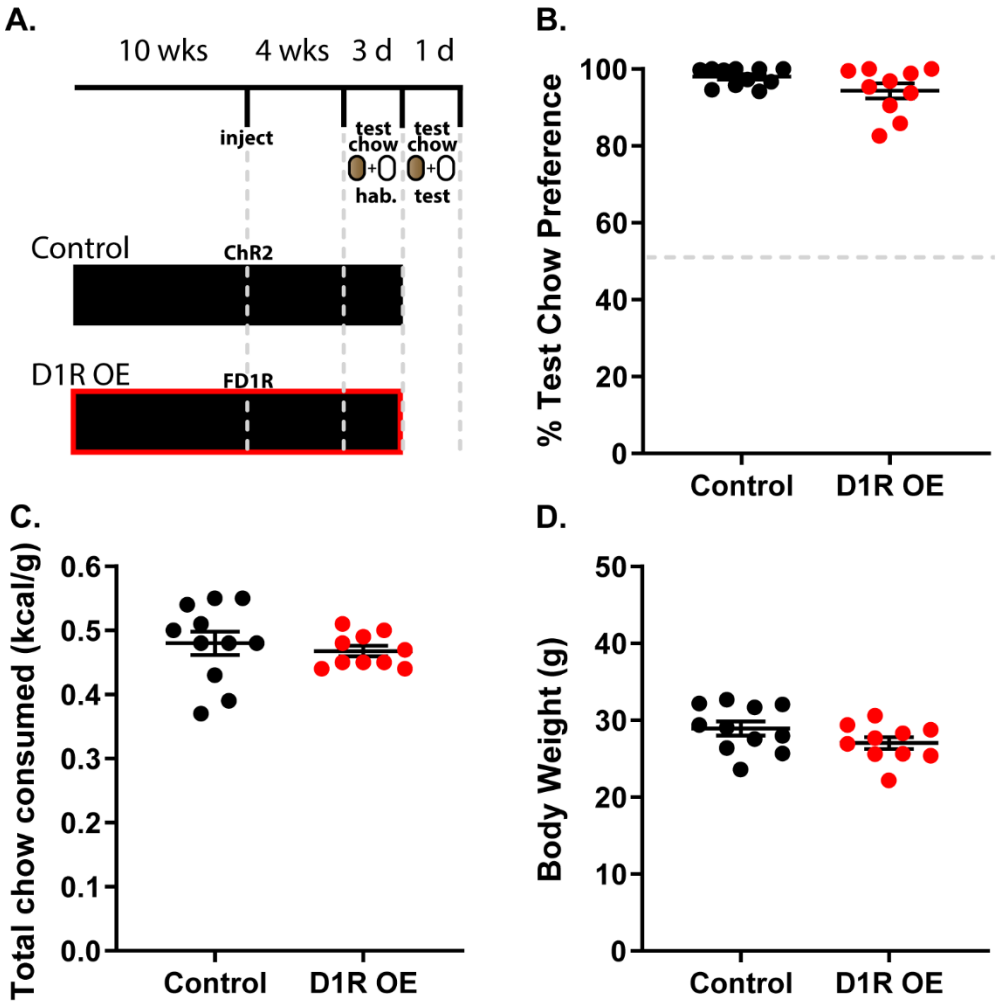


Fig.4. Viral overexpression of D1R to the NAc of HFD-raised mice fails to rescue preference for a more palatable food choice or total test chow consumption. (A) Schematic illustrating food preference experimental setup. (B) Preference for the more energy dense test chow during the food preference test. Student's two-tailed t-test; n=8-14/group. (C) Total amount of both test chows consumed during the food preference test corrected for body weight. Student's two-tailed t-test; n=8-14/group. (D) Body weight during the food preference test. Student's two-tailed t-test; n=8-14/group. (E) Representative immunohistochemistry images of NAc D1R overexpression in control animals (left) and animals receiving FD1R injections (right). Red staining is indicative of viral D1R overexpression using HA-D1R-specific antibodies. aca=anterior commissure. Data are represented as mean ± SEM. *p < 0.05. See also Supplemental Figure 1.



Supplemental Fig.1. Viral overexpression of D1R to the NAc of SD-raised mice has no effect on preference for a more palatable food choice or total test chow consumption. (A) Schematic illustrating food preference experimental setup. (B) Preference for the more energy dense test chow during the food preference test. Student's two-tailed t-test; n=10-11/group. (C) Total amount of both test chows consumed during the food preference test corrected for body weight. Student's

two-tailed t-test; n=10-11/group. (D) Body weight during the food preference test. Student's two-tailed t-test; n=10-11/group. Data are represented as mean ± SEM.

Discussion:

In this chapter I used transgenic and viral techniques in combination with my labs' novel food preference assay to further investigate the role of mesolimbic dopamine signaling and HFD consumption in food choice. Using a global D1R-KO mouse, I discovered that D1R signaling as a whole is critical for intact food valuation, but that modulation of NAc D1R expression specifically fails to affect food preference or consumption (Table 1). In addition, I found that NAc D1R overexpression fails to reverse the HFD-mediated alterations in feeding behavior demonstrated in chapter 2 (Table 1).

Dopamine signaling has been implicated in mediating and influencing multiple facets of feeding behavior such as food-related learning, hedonic evaluation, and food-seeking [8–10]. However, its role in mediating food preference and choice remains a mystery. To further investigate the role of dopamine signaling in food preference, I chose to focus on D1R because of this receptor's specialized function in mediating learned associations between rewarding food and its associated stimuli and cues [26,31,109]. I opted to conduct this experiment in SD-raised animals in order to focus on the role of D1R in food preference independent of background dietary effects and to establish a better understanding of how this circuitry works under baseline conditions. I discovered that mice lacking D1R demonstrated diminished preference for the more palatable chow in the choice assay. Because of this, I can infer that the

ability to associate rewarding food with its respective sensory properties is likely critical for the establishment of the food preferences and choices that lead to overindulgence in calorically dense foods. These data suggest that dopamine signaling plays a key role in food preference, and this is at least partially mediated by D1R.

I was surprised to discover that knocking out D1R in the NAc alone failed to elicit the same effect on food choice as a full D1R knockout. In addition, it was equally perplexing when my attempt to selectively restore expression of D1R to the NAc of global KO mice failed to rescue food preference. In spite of these findings, there are several possible explanations for why these manipulations fail to implicate NAc specific D1R expression on its own as critical for food preference. An important consideration is the fact that D1R is expressed widely throughout the brain, including in the prefrontal cortex, basolateral amygdala, hypothalamus, and dorsal striatum [110,111]. Moreover, D1R expression in the neurons of several of these regions has been implicated in affecting feeding behavior [112,113]. Therefore, it is possible that in the event of perturbation of D1R in one brain region (in this case the NAc), subsequent effects on food preference could be masked by compensatory mechanisms mediated by D1R elsewhere in the brain. This possibility would explain why a global D1R-KO is able to manifest a behavioral phenotype and a NAc-KO is not. Additionally, restoring D1R to just one of these regions (i.e. the NAc) may be insufficient in reversing the impaired preference in the global D1R-KO mice. Relatedly, it is impossible to determine how much D1R I was successfully able to remove from

the NAc using the chosen methodology of a viral cre-recombinase injection. It is possible that only a small amount of NAc D1R need be expressed for intact food preference, as the dynamics between specific D1R expression levels and maintenance of specific feeding behaviors such as preference are largely unknown.

In the previous chapter, I demonstrated that prolonged HFD consumption disrupts food preference and consumption during the food preference assay, and perturbs mesolimbic dopamine signaling by desensitizing the response of the VTA to a novel food reward. Prior studies have demonstrated that long-term HFD consumption is capable of altering NAc D1R levels [48–50,52]. In an attempt to further elucidate the mechanism by which HFD disrupts food preference, and to investigate a potential way to reverse it, I overexpressed D1R in the NAc of HFD-raised mice. I was initially surprised to discover that this manipulation failed to restore the HFD-induced perturbations in food preference and test chow consumption, and worsened preference even further. However, these findings are reinforced by my discovery that prolonged HFD consumption leads to an overall blunted response of the VTA to a novel HFD, and that the dopaminergic neurons of the VTA in these animals fail to respond to a novel HFD compared to a novel SD. Namely, if consuming a HFD leads to a failure of the VTA to respond to a food reward, then it follows that amplifying D1R downstream would not be sufficient to ameliorate feeding deficits since the upstream signal is no longer intact. It is also reasonable to suspect that increasing NAc D1R levels could lead to an additional diminishment of VTA dopamine signaling as a feedback

response. This could explain the enhanced deficit in food preference exhibited by these mice.

In conclusion, my research in this chapter indicates that D1R expression is critical for intact food preference. In addition, a deficit in NAc D1R alone is not sufficient to recapitulate the disturbances in food choice that a global D1R-KO elicits. Similarly, NAc D1R signaling on its own is insufficient for restoring intact food preference to global D1R-KO mice. This disparity suggests that there may be D1R-mediated compensatory mechanisms that can accommodate for D1R signaling deficiencies in the NAc or elsewhere. However, it remains clear that intact D1R expression throughout the brain is essential for maintaining food preference/valuation. In addition, overexpression of D1R in the NAc fails to reverse HFD-mediated deficits in food choice. When examined in tandem with the findings from chapter two, this suggests that the most efficacious avenue for targeting these diet-induced deficiencies could be via bolstering VTA activity upstream in the mesolimbic dopamine signaling pathway.

Genotype	**Experiment**	**Diet**	**Injection**	**Preference**	**Consumption**
C57BL6J	Ch.2: Fig.1	SD	-	-	-
C57BL6J	Ch.2: Fig.1	HFD	-	↓	↓
$D1RCre^{+/+}$	Ch.3: Fig.1	SD	-	-	-
$D1RCre^{Cre/Cre}$	Ch.3: Fig.1	SD	-	↓	-
$D1RCre^{Cre/Cre}$	Ch.3: Fig.3	SD	ChR2	↓	-
$D1RCre^{Cre/Cre}$	Ch.3: Fig.3	SD	FD1R	↓	-

$FD1R^{lox/lox}$	Ch.3: Fig.2	SD	EGFP	-	-
$FD1R^{lox/lox}$	Ch.3: Fig.2	SD	Cre	-	-
$D1RCre^{Cre/+}$	Ch.3: Fig.4	HFD	ChR2	↓	↓
$D1RCre^{Cre/+}$	Ch.3: Fig.4	HFD	FD1R	↓↓	↓
$D1RCre^{Cre/+}$	Ch.3: S.Fig.1	SD	ChR2	-	-
$D1RCre^{Cre/+}$	Ch.3: S.Fig.1	SD	FD1R	-	-

Table 1: Summary of findings from chapter 3. Arrows are indicative of deviations from phenotypes observed in C57BL6 mice raised on SD (first row).

Ch. 4: Conclusions and Future Directions

The obesity epidemic currently poses one of the greatest challenges to researchers and healthcare providers due to its complex etiology, prevalence, associated costs, and potential for lethality. It is well established that a significant factor in both the etiology and perpetuation of an obese phenotype is the overconsumption of palatable, calorically-enriched foods. The role of the mesolimbic dopamine signaling pathway in promoting the overconsumption of these foods is continually under investigation. However, the manner in which mesolimbic dopamine signaling responds to continued energy-dense diet consumption and how this alters future behavior remains poorly understood. The goal of this dissertation has been to better understand this link between diet, neural circuitry, and subsequent behavior.

Attempts to characterize feeding behavior and its underlying neural circuitry are considered daunting tasks. This is due to the complex nature of both the behavioral processes comprising “feeding” and the instantiation of those processes within the brain. In order to study feeding behavior, researchers have grown accustomed to focusing on studying individual facets of feeding using specific assays. Common examples of these assays include operant chambers with progressive ratio schedules of food reinforcement for the study of the motivation to work for food, or the conditioned place preference task for assessing food-associated memory formation [114,115]. In this dissertation I have focused primarily on assessing food choice and preference, an often neglected component of feeding behavior. To this end, our lab has designed a

novel food preference assay for assessing changes in food choice under a variety of conditions.

In chapter 3, we illustrated how critical intact D1R-mediated dopamine signaling is for mediating food preference. However, we were surprised to find that knocking D1R out of the NAc specifically or attempting to selectively re-express D1R in the NAc of global D1R-KO mice failed to implicate NAc D1R in food preference. As discussed in chapter 3, there are several possible explanations for these findings. The explanation that provides the most investigatory potential is the notion that D1R expression in other brain regions has been implicated in feeding behavior [112,113]. D1R is highly expressed in the prefrontal cortex (PFC), and the VTA projections to this region (mesocortical dopamine signaling pathway, see Box 1) are known for their role in mediating executive function and working memory [116–119]. Although its role in food preference hasn't been addressed, PFC D1R has been implicated in food intake, palatable food seeking, and learned associations between food and specific stimuli [113,120–122]. In addition, D1R is expressed in the amygdala and receives dopaminergic innervation from the VTA [123,124]. Food consumption has been shown to lead to elevated intra-amygdala dopamine levels, and D1R therein has been shown to play a role in mediating food seeking and food-related learning [125–127]. Although these examples don't explicitly address D1R and food preference specifically, they demonstrate that D1R expression in other brain regions exerts control over other food-related behaviors. This suggests that there

may exist the potential for D1R outside the NAc to both play a role in mediating food preference, and to compensate for any NAc-specific D1R deficits.

We have demonstrated that in the context of HFD consumption, our food preference assay functions similarly to sucrose preference tests. These tests are commonly used to assess anhedonia and reward devaluation in rodents [58,67,88,128]. Our lab's HFD-raised mice exhibit diminished preference for the more palatable test chow, and an overall reduction in test chow consumption during the food preference assay (chapter 2). Because these mice also exhibit an analogous reduction in preference for sucrose and blunted fluid consumption in the sucrose preference test, it could be inferred that mice consuming HFD for an extended period of time are experiencing some degree of anhedonia that shapes food choices/preference. Indeed, in both humans and rodents, obesity and depressive symptomology (such as anhedonia) are often comorbid [129,130]. Furthermore, anhedonia on its own is associated with reward circuitry dysfunction in both rodents and humans [131,132]. In chapter 2 I demonstrated that HFD-raised mice exhibit a dampened VTA response to food reward, implicating HFD in disrupting the mesolimbic dopamine signaling pathway. Additionally, chapter 3 illustrates how critical intact D1R expression is for food preference. Therefore, it is plausible that prolonged HFD consumption promotes anhedonia via perturbations in reward circuitry, which then manifests as alterations in food preference and choice.

In an effort to attempt to reverse the diet-induced alterations in feeding behavior observed in the food preference assay, we tried several approaches.

These included modulating D1R levels, switching animals' diets, or letting them exercise. I attempted to modulate dopamine signaling by overexpressing D1R in the NAc of HFD-raised mice. As discussed in chapter 3, this manipulation failed to reverse HFD-induced alterations in feeding behavior during the preference assay. I suspect that these findings are due to the fact that HFD raised animals have impairments in VTA activity during hedonic feeding (chapter 2). Namely, if the VTA is not functioning properly during feeding, then it stands to reason that altering the signal downstream in the NAc could be insufficient to restore intact behavior. Therefore, future work should focus on VTA manipulations in food preference, as it may be a more promising locus for therapeutic potential.

Although overexpressing NAc D1R failed to restore food preference following HFD exposure, allowing animals to access a running disc for 4 weeks successfully reversed HFD-induced alterations in food preference. As discussed extensively in chapter 1, exercise has been shown to promote plasticity of the mesolimbic dopamine signaling pathway, including in animals consuming a HFD [78,81,82]. These studies implicate VTA function as a key target in exercise-mediated neural plasticity. When considered alongside the fact that the HFD-raised animals examined in this dissertation exhibit perturbed VTA activity, I believe that exercise-mediated plasticity in the VTA is responsible for the behavioral reversal observed in chapter 2. Specifically, I believe allowing HFD-raised animals to exercise for four weeks leads to a restoration of VTA sensitivity to food reward, which in turn is responsible for the observed rescue of preference for the more palatable food chow in the preference assay. My lab is currently

following up on this by examining c-fos induction as described in chapter 2, but in HFD-raised animals that have been allowed to exercise. In addition, although this dissertation has established D1R-mediated signaling as crucial for food preference, future work will need to focus on explicitly establishing the activity of the VTA as a key mediator of preference during the assay.

There are several avenues to investigate the role of VTA dopamine signaling in food preference that could be pursued by our lab alone or in collaboration with others. First, we could ablate VTA dopaminergic projections to the NAc using 6-hydroxydopamine in SD raised mice and test their performance in the food preference assay. 6-hydroxydopamine is a synthetic toxin that targets dopamine neurons by entering the dopamine transporter, and when used to destroy VTA to NAc dopamine projections, it has been shown to disrupt rodents' motivation to work for food reward [10]. I believe that this manipulation would also disrupt preference for the more palatable test chow in the food preference assay – much like what we observe with our global D1R-KO animals. Alternatively, we could utilize an optogenetic or chemogenetic approach to silence VTA to NAc dopamine projections during the food preference assay. Once these methods have been utilized to establish the importance of VTA dopamine signaling for food preference specifically, they can be applied to a HFD context to attempt to rescue diet-induced perturbations in food preference. Specifically, optogenetic and chemogenetic approaches could be utilized to amplify VTA dopamine neuron activity in HFD-raised animals during the food preference assay. The experiments in chapter 2 established that VTA activity is

diminished in HFD-raised animals during consumption of a palatable food reward. Thus, if we establish the VTA as essential for intact food preference, I expect that amplifying VTA dopamine signaling in HFD raised animals would be sufficient to restore their food preference. These findings would explicitly implicate VTA dysfunction as a key mediator of HFD-mediated deficiencies in food preference.

The work contained within this dissertation provides a novel account of the manner in which HFD consumption can alter neurobiological function and subsequent feeding behavior. Our findings implicate VTA functionality as a target of HFD as evidenced by reduced VTA activity during hedonic feeding in HFD-raised animals. In addition, by demonstrating that HFD-induced alterations in food preference are reversible via both dieting and exercise, this dissertation provides several avenues for future study of interventions with therapeutic potential.

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