The Role of Food Memories on Feeding Behavior

by

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Dedication

I dedicate all of my achievements to my mother, father, and brother.

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Preface

For chapter 2 author contributions are as follows: TRPG: Conducted behavioral assays, imaging, and triglycerides experiments and analyses. KG: helped with triglycerides experiments. MD and TRPG designed the project, interpreted the data, and wrote the manuscript, which was read by all other authors. MD supervised the project and secured funding for research.

For chapter 3 author contributions are as follows: TRPG: Conducted behavioral assays and triglycerides experiments and analysis. AG performed some triglyceride experiments. KG: conducted imaging and helped with triglycerides experiments. TRPG designed the project, interpreted the data, and wrote the manuscript. MD supervised the project and secured funding for research.

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Abstract

The circuit and cellular mechanisms that allow us to effectively interact and navigate our food environment are still elusive. These interactions depend on our ability to form associations between sensory information, such as olfactory, visual or taste, and the rewarding properties of food. Therefore, how our brain processes this rewarding information will impact food intake. Our food environment is mostly composed of foods high in sugars, which have been shown to change the rewarding and motivational properties of sugar as well as lead to obesity and overeating. Additionally, these changes are correlated with dysregulated dopaminergic system in mammals and humans. Therefore, our objective is to establish a causal relationship between the mechanisms controlling reward-motivated learning and overeating that are disrupted on a sugar diet (SD). Thus, we hypothesized that a SD decreases dopamine signaling to impair reward-motivated learning, promoting overeating and obesity. Using behavioral assays, in vivo 2-photon imaging and metabolic measurements, our results suggest that a subset of glutamatergic Mushroom Body Output Neurons regulate feeding behavior through learning and memory activity, and that a SD disrupts this mechanism through changes in dopamine neuromodulation. Together, my experiments have uncovered a novel circuit and cellular mechanisms through which the food environment promotes overconsumption and weight gain by affecting reward-motivated learning.

Chapter 1 Introduction

1.1 The relationship between diet and obesity

The 21st century has seen an explosive disparity in both the overabundance, as well as a under abundance of food. Expectations of food production and availability in developed countries only keeps growing, while the policies regulating the quality of these foods is lagging. Even more lagging is our understanding of how our dietary environment shapes our eating behavior. Individually, this relationship is complicated and requires a certain degree of attention and well defined feeding parameters to ensure appropriate choice and amount of consumption of foods or it could lead to complications such as obesity, defined by high amounts of body fat per the Body Mass Index> 30 (BMI>30) (CDC 2021b). Diseases such as neurodegeneration, diabetes, and cancer, as well as reduced life expectancy and health, are among the complications that exist due to obesity and diet (Oleson et al. 2017; Zamroziewicz and Barbey 2016). The effects of diet on disease comes from the nutritional composition of the foods we eat and the mechanisms we have developed to utilize these in an efficient manner, to the point that it has become a double-edged sword. The macronutrients of food production have not changed significantly compared to the past (Lásztity et al. 2009). On the other hand, the nutritional range consumed has increased significantly, and our eating behaviors have changed due to food availability and ironically, the advances of food science; development of high calorie foods rich in sugar, salt, and fats (Alkerwi et al. 2014), as well as readily available, cheap, and highly advertised (CD C2021a).

The technique of chemically refining sugar has been recorded to be as old as 2,500 years, beginning in India and subsequently spreading towards China, the west of Persia and the early Islamic worlds, finally reaching the Mediterranean in the 13th century (Adas 2001, p. 2341; Denham T 2011; Schwartz B 2004). Today, examples of added sugars include lactose, sucrose, corn syrup, honey, fructose, fruit nectars, glucose, maple syrup, molasses, and high-fructose corn syrup (Scientific Report of the 2020 Dietary Guidelines Advisory Committee, n.d.). Most of these sugars did not exist before the 13th century and those that did exist were not used in excess, and instead, were used in very specific circumstances at low volumes (Sato 2015). Today, we have seen a correlation between amount of refined carbohydrates consumed and the obesity epidemic. A great example of the detrimental impact of sugar diets is the introduction of these added sugars to groups who recently were isolated from civilization, in most cases leading to metabolic disease (Funk 2016). It's no secret that the rise of these added sugars was a means to capture and entice more consumers, especially as the concerns of fat in foods became the number one topic in the food industry in the 20th century. Together with poor standards for the amount of sugar and type of sugar in food, as well as the effects on our brain chemistry, we have an added sugar epidemic, mainly through approximately 80% of grocery store foods (Ng, Slining, and Popkin 2012). This issue is aggravated in countries where there is food scarcity given that many of the help received comes in the form of non-perishable, highly processed foods, prolonging the suffering due to the lack of nutritional benefits of these foods (Johnson et al. 2009; Malik, Willett, and Hu 2013; Haddad et al. 2016; Popkin, Adair, and Ng 2012).

1.2 Dietary environment and eating behavior

The availability of highly processed foods containing high amounts of sugar, salt and fat are associated with increased palatability. Specifically, these foods become high caloric meals

that promote weight gain, potentially leading to obesity, and diseases at the metabolic, cardiovascular and neurological level (Johnson et al. 2009; Avena, Rada, and Hoebel 2008; Faruque et al. 2019; Morenga et al. 2012; Hall et al. 2019; Freeman et al. 2018); Not to mention the societal and mental health impact. The economic burden of such feeding behavior can be felt throughout the system, from loss of productivity at work to billions of dollars of burden on the healthcare system. Over the past 2 decades alone, mainly with the adoption of high amounts of added sugar on our foods, 70% of North Americans report being overweight or obese (CDC 2021a).

When the concerns for trans-fat on foods became a major concern for production of foods by major companies, it was decided to remove them completely from their products: 0 trans-fat, per advertisement. However, very quickly they realized that removing these made their foods not very appetitive. Hence, they began switching to the addition of sugar. Through time, neuropsychologists reported noticing a correlation between highly palatable foods and consumer demand (Cairns et al. 2009). Researchers began to understand that added sugars had the potential to "hook" consumers on their companies' products. Later on, animal models and humans showed that sugars had the ability to promote food intake and skew preference away from naturally occurring sugars – i.e. apples – towards highly processed foods (Bertino, Beauchamp, and Engelman 1982, 1986; Heinze et al. 2018; Weiss et al. 2019; Maliphol, Garth, and Medler 2013; Appleton et al. 2018). As research continued to advance, correlations between satiation and sugar diet began to surface as a possible explanation for why individuals overconsume these highly palatable foods, and called it sensory-enhanced satiety (Chambers, McCrickerd, and Yeomans 2015; Yeomans 2017; May et al. 2020). In addition, data suggest that the ability to learn from

our experiences with food, such as the nutritional value, meal pleasantness, adverse or positive effects, are skewed on a sugar diet, rendering itself to overconsumption (Kroemer et al. 2016).

1.3 The interplay between learning & memory and eating behavior

Learning & memory are a fundamental process that are essential for survival. Declarative memory is focused on facts and events, while nondeclarative encompasses many more types of memories, and of particular importance to eating behavior is associative learning and memory. This type of learning and memory consists of creating connections/associations between more than one event. A classical comparison would be that of Pavlov's dogs. By pairing or creating an association between a stimulus – called the conditioned stimulus or CS – with a substance that automatically and naturally triggers a specific response – called the unconditioned stimulus or UC – you can change the valence of the CS depending on the UC. Ultimately, the goal is not to change the valence of a compound but to create a strong association that leads to the subject to think that the CS now represents the UC. In Pavlov's dogs, both the sound (cue or CS) and reward (food or UC) are sequentially necessary to establish the associated memory of "if bell, then food". However, if after creating the associative memory the bell rings but there is no food, the memory should be refined so that the CS (bell) is no longer predictive of the food. On the other hand, if the process of reward learning is perturbed, the memory is not updated, and the cue remains a symbol of reward, i.e., the dogs will keep showing up for food but never finding any. This is a classic example of the power of food on creating associations, also called food associations. This begs the question of what else can food be paired to. The answer is anything if the food is relevant enough to be associated to the conditioned stimulus. Even an aversive stimulus can become appetitive if the food it's paired to is rewarding enough (Keller and Dunsmoor 2020). The appetitiveness of foods that elicit strong reward responses in our society

comes from the high concentrations of sugar, which makes it highly palatable and strongly stimulates our senses (Chambers, McCrickerd, and Yeomans 2015; Yeomans 2017; May et al. 2020).

The incentive salience model postulates the existence of a wanting vs liking neurobiological process that contributes to food intake. It postulates that the experience with a rewarding substance begins with liking, or the pleasurable experience with food, and then turns into wanting, were the motivational value elicited by a substance through cues is increased and potentiates cravings (Berridge 2009; Berridge et al. 2009). Individual differences in food-cue reactivity, which could be genetic, learned or due to obesity, can be related to variations in sensitivity to stimuli that directly impact the reward center and that predict the occurrence of a reward, thereby it follows that the self-control is truly not a matter of will but it is a matter of neural wiring that predisposes us to such behavior (Johnson and Kenny 2010; Tetley et al. 2010; Babbs et al. 2013; Schiff et al. 2016).

Due to the limiting factor of the animal model utilized, several questions surrounding the order of events in overeating remain. In addition, the cellular and molecular pathways that contribute to overeating in the context of appetitive associative learning and memory (food association/memory) are also poorly understand. For example, we still do not understand how foods can alter brain circuits and cellular mechanisms to cause shifts in the reward centers of the brain? Furthermore, to what extent does the reinforcing properties of food play in dysregulating the food associations that allow us to update past experiences with food and adjust to new circumstances? Another unanswered question that has not been answered due to the complicated layout of the mammalian brain is whether it's the diet or the obesity that leads to changes in the reward pathway and to dysregulation of the food association centers? Research on this has thus

far been correlation, and while substantially important in advancing our understanding of our feeding behavior, it does not answer the question of who comes first. Hence, utilizing the right animal model with a more simple but still similar circuit at the functional level might shed more light into these questions and potentially open new avenues of research and therapies. Interestingly, the *D. melanogaster* fly is such an animal model; It possesses the similar appetitive behavior towards high caloric foods and its processing of food is similar. These flies can incorporate caloric value, sensory information, and reward in a manner similar with mammals and leading to food associations (Mason et al. 2012; Landayan and Wolf 2015).

1.4 Dopamine and obesity

Dopamine is one of the most studied neurotransmitters in the brain. It is known by the common reader as the "pleasure chemical", causing feelings of reward when presented with a positive experience. Recently it's been hypothesized that impairments in food associations are responsible for the current obesity epidemic (Volkow et al. 2017; Matikainen-Ankney and Kravitz 2018). Data to back this hypothesis comes from work in obese humans, where their ability to learn from negative outcomes is impaired when compared to healthy weight group. For example, on a learning task that relies in probabilities, obese individuals had a deficit in this type of learning when compared to the healthy weight group, however, no changes were observed for the positive outcome learning (Coppin et al. 2014; Mathar et al. 2017). An important question that arises from this is whether it is the diet or the obesity that leads to impairments in reward learning. Mammalian experiments have been unable to answer this question due to the complexity of the animal model. However, one can hypothesis that the impairment in food associations might be coming from a dysfunction in the dopaminergic system given that food

associations depend on the faithful transmission of reward signals conveyed as dopamine neurotransmission.

1.4.1 Dopamine in feeding behavior

Dopamine is well known to regulate food intake (Palmiter 2007; Stice et al. 2008; de Macedo et al. 2016; May et al. 2020), the alteration of energy expenditure, and the integration of metabolic signals into brain reward circuits (Davis et al. 2011; Domingos et al. 2011; King et al. 2011; Beeler et al. 2012 and 2016; Cone et al. 2014 and 2015). These processes can be broadly divided into two categories, short-term and long-term modulation of dopaminergic neurons. An example of such a process is that sugar exerts its strong reinforcing effects by activation of the gustatory pathways – short -term modulation - and the post-ingestive pathways – long-term modulation. These two pathways can be attributed to the caloric value and hedonic value of food, as well as the nutritional value of food, respectively. At the cognitive level, and in the same way, these two are also part of the short-term and long-term learning & memory functions that rely on the reinforcing properties and the nutritional value of food (Swithers and Davidson 2008; Davidson et al. 2011; Babbs et al. 2013; Veldhuizen et al. 2017). In mammals, the different inputs are then segregated into distinct pathways that culminate in dopamine release in the ventral striatum for caloric/hedonic information, and dorsal striatum for the nutritional value of sugar (Tellez et al. 2016). It has been proposed that the inability to update memories about the rewarding properties of food drives increased food intake. This idea is based on a large body of evidence showing that the region of the vertebrate brain involved in food associations, such as the striatum, has lower activity in obese humans and mice fed high nutrient diets. Specifically, many have observed defects in dopamine transmission from worms to humans (Wang et al. 2001; Colantuoni et al. 2001; Small et al. 2003; Bello et al. 2002; Horstmann et al. 2015;

Kroemer and Small 2016). It has been hypothesized that the defects in dopamine transmission are due to multiple reasons. One of these hypotheses is that dopamine transmission is decreased due to decreased taste information (Swithers and Davidson 2008; Davidson et al. 2011; Babbs et al. 2013; Veldhuizen et al. 2017). High amounts of sugar have been reported to lead to decreased taste (Davidson et al. 2011; Maliphol et al. 2013; May et al. 2019) In addition, with the advent of artificial sweeteners, most of these sweeteners have sweet information but no caloric value encoded at the pre-ingestive level, the disjunctive between these two becomes clearer. Through the process of food associations, we learn to associate the caloric value with the sweetness of the food. This allows us to understand at the short-term how much of the food should we consume before terminating the meal (Liu et al. 2016; Kroemer and Small 2016; Landayan et al. 2018). Because artificial sweeteners have no short-term caloric value it creates confusion in our brain, and in order to match our expectations of our previous caloric value we increase the amount of sweet information to try and match the previous experience. Obese subjects experience a similar phenomenon, where caloric information is decreased while sweet taste receptor availability for sweet information decreases. As a result, obese individuals increase their food intake to match those previous expectations (Swithers and Davidson 2008; Davidson et al. 2011; Babbs et al. 2013; Kroemer and Small 2016; Veldhuizen et al. 2017).

Another hypothesis is that either diet or obesity can lead to alterations in the go/no-go dopamine interplay. Specifically, there is an increase in the Dopamine – type 1 Receptor (D1R, Go signal, $G_{s/q}$ coupled) availability or activation in the pre-synaptic neuron of the striatum (Colantuoni et al. 2001; Bello et al. 2002; Frank et al. 2004; Frank and Hutchison 2009; Cox et al. 2015), while there is a decrease in the dopamine – type 2 Receptor (D2R, no-go signal, G_i coupled) in the post-synaptic neuron of the striatum in the mammalian brain (Kuo 2002; Thanos

et al. 2008; Jonhson and Kenny 2010; Eisenstein et al. 2013; Guo et al. 2014; Karlsson et al. 2015; Beeler et al. 2016). This leads to a behavior that reinforces positive outcomes of an experience while not integrating the negative consequences of the same experience. In the context of obesity, it translates into the experience with sugar as being one of increased food intake regardless of the negative consequences, which could range from a disconnect between sweetness and caloric value to withdrawal-like symptoms (Kroemer and Small 2016). There is much evidence of the role of D2R in negative outcome learning. D2R is the higher affinity receptor when compared to D1R and are sensitive to smaller dips in dopamine release that occur after unexpected reward omission, or the negative outcome (Frank and Hutchison 2009). In this way, low dopamine tone would represent punishment-related learning event while high dopamine tone would favor the reward-related event (Cools et al. 2009). Between D1R and D2R, the latter has consistently been linked to learning from reward feedback, furthermore, this reinforcement learning framework can be applied to any design, making the study of D2R in obesity attractive to other animal models.

1.5 Drosophila melanogaster as a model

While there is a lot of circumstantial evidence for the idea that defects in reward learning occur with obesity, how they arise and how they affect food intake is still unknown. On one hand, impairments in food associations could be a consequence of the obese state but, on the other hand, they could be the result of the dietary environment that typically leads to obesity. Further, the circuit and cellular mechanisms through which the inability to update memories influences food intake are still largely a mystery. These gaps in knowledge are in large part due to the complexity of the mammalian brain and the lack of good genetic tools to uncouple diet from obesity. Furthermore, there is a lack of precise neural tools to define specific populations of

neurons involved in food memories alone. The Drosophila *melanogaster*, or vinegar fly, shares many of the genes involved in the regulation of body fat, such as the Adipose protein, which showed structural and functional similarities with that of plants (Ducos et al. 2017), mice (Suh et al. 2007) and humans (Lai et al. 2009). Furthermore, the anti-obesity function of WDTC1, the ortholog of the adipose gene in flies was first observed in fly and when impaired leads to adipose mutant-like phenotype (Baumbach et al. 2014a), followed by in mice and humans (Groh et al. 2016). In addition to these shared proteins, the fruit fly is also a convenient model for obesity and metabolic disease due to a variety of reasons. For example, the analogy between the systems, tissues and organs involved or affected by obesity and linked to metabolic disease are present in flies and in humans (Musselman and Kuhnlein, 2018; Brankatschk and Eaton, 2010; Gronke et al., 2003; Lazareva et al., 2007; Lee and Park, 2004; Colombani et al., 2003; Asha et al., 2003; Fischer et al.,1988). Additionally, a majority of those genes that are important for their participation in metabolic disease are conserved in both flies and humans. Also, flies fed a diet high in calories have significant increase in body fat and are considered obese, similar to humans (Reiter 2001). Furthermore, they develop metabolic complications such as insulin resistance (Musselman et al. 2011). These similarities coupled with the accessibility to genetically manipulate the fly genome utilizing a variety of cutting-edge approaches, coupled with the decreased redundancy and consolidation of functionality in cells, as well as the high turnover of generations makes an ideal model to study the complications associated with obesity and metabolic disease (Musselman and Kuhnlein 2018).

In addition, food intake is also similarly controlled in flies as it is in humans. Food intake is a trait that is essential for survival and is needed to maintain proper energy balance. In humans, understanding the genetic and environmental contributions to variations in food intake

can be very challenging given that human behavior can vary depending on emotional or environmental circumstances, and quantifying food intake without relying on the information provided by the participant can be very difficult (Kim et al. 1984; Basiotis et al. 1987; Schoeller 1990; Schoeller 1995; Kaczkouski et al. 2000; Seale et al. 2002; Bray et al. 2008; Champagne et al. 2013). To understand and quantify food intake in flies, there exists a number of tools that have been produced to study the genetic, neural and environmental factors modulating food consumption in the fruit fly. For example, we can measure feeding frequency utilizing the proboscis extension as a proxy of the interactions humans have with food, providing an estimate of the total food intake (Wong et al. 2009). Furthermore, more sensitive assays that measure food intake to the millisecond and through several days has also allowed to study the patterns of food consumption over extended periods of time with minimal human or environmental disturbances (Ro et al. 2014).

In addition to obesity and metabolic disease, the vinegar fly is also well suited for studying food associations. Flies possess a simplified version of the striatum, where reward signals are integrated together with sensory stimuli to create an association (Tully and Quinn, 1996). Specifically, they possess dopaminergic neurons, and sensory neurons that can transmit orosensory or visual stimuli that can be paired with a rewarding or aversive substance. The advances in genetic manipulation, as well as imaging tools have made it much easier to study the genetic, neural and environmental factors that can either affect or be affected by food consumption and its relation to reinforcement learning (May et al. 2020). Exploring questions related to the role of D1R and D2R in learning & memory are facilitated by the use of genetic tools to target fly homologues, Dopamine-like Type 1 Receptor 1 and 2, as well as the Dopamine-like type 2 receptor, respectively. Hence, the Drosophila *melanogaster* animal model

offers the possibility to answer questions that were thought to be impossible or at the very least, difficult to answer.

1.6 The fly mushroom body

The valence held by stimuli are normally encoded in the striatum in mammals. This is the area of the brain that is mostly affected by drugs of abuse and has been the source of extensive research on how reward information is encoded in the brain and the effects of food intake on neurochemical transmission as it relates to behavior (Stice et al. 2008; Stice et al. 2010; Smeets et al. 2011; Coppin et al. 2014; Stice and Yokum 2016). The analogous in flies is the mushroom body. The mushroom body contains the projections of dopaminergic neurons that relay reward and aversive information. In addition, the mushroom body also holds the projections of neurons that relay different types of sensory input such as visual and odor information. These two then converge on the mushroom body to allow for the association of information that can dictate the valence of a stimulus. For example, giving flies sugar while exposing them to an odor that would usually elicit aversion, leads to the odor acquiring a temporary appetitive valence that is encoded in the mushroom body (Huetteroth et al. 2015; Owald et al. 2015; Handler et al. 2019).

1.6.1 The fly dopaminergic system

In flies, there are two major categories of dopamine neurons that act in reinforcement learning. The first set of neurons are called the Protocerebral anterior medial (PAM) neurons. These dopaminergic neurons are extensively involved in the relay of reward information and mainly innervate the horizontal lobe of the mushroom body (Huetteroth et al. 2015). These are the neurons that are mainly activated by food intake of substances with high amounts of sugar. In addition, they also provide other types of information related to reward such as the caloric value

of foods, as well as the nutritional value of food. To further break this down, dopaminergic neurons innervating the β' compartment of the mushroom body are typically associated with the caloric value of foods. On the other hand, the dopaminergic neurons that innervate the γ compartment of the mushroom body relay the nutritional value of foods (Huetteroth et al. 2015; Yamagata et al. 2015). It is further possible to pinpoint the exact subcompartments of the β' and the γ regions that are most associated to these types of information. For example, caloric value is mostly represented in the β'2a and mp compartments, while the nutritional value is mostly represented in the γ5 compartment (May et al. 2019 and 2020). Hence, the pattern of innervation, as well as the dopaminergic neurons responsible for encoding specific types of behaviors that are translated into behavioral outcomes can be easily delineated in flies in part because of the great availability of genetic tools and the structural organization of the fly brain.

1.6.2 The fly olfactory system

In flies, the cells of the olfactory system that innervate the mushroom body are called the Kenyon Cells. Their high-density projection into the mushroom body was first described to be organized into 3 Kenyon Cell classes based on their inputs, morphology and neural activity properties (Strausfeld et al. 2003; Sjöholm et al. 2005), and subsequently further characterized by its innervation pattern and connection with other neuropil of the mushroom body (Lin et al. 2007; Aso et al. 2014a). The 3 main olfactory Kenyon Cell classes are represented by hundreds of neurons per hemisphere and have their dendrite in the main calyx. These 3 classes of olfactory Kenyon Cells will form the γ, α′/β′, and α/β lobes of the mushroom body, and they have varying degrees of response to different types of odors. These cells have a negative feedback circuit that allows for the storage of odor-specific memories that lead to more refined behavioral outputs such as discriminating the fine details between similar but distinct odors (Lin et al. 2014). Their

involvement in food associations has several angles. First, Kenyon cells relay olfactory information onto the mushroom body to allow for the association of an odor with a stimulus relayed by the dopaminergic system. Depending on the sequence of events between Kenyon cells and dopaminergic neurons, the assigned value of the CS might vary and switch from appetitive to aversive or vice versa (Handler et al. 2019). Second, dopaminergic projections onto Kenyon cells modulate the finer details of the pattern of neurotransmission release from these neurons so as to refine the food associations that lead to a behavioral output that is representative of the expected association (Lyutova et al. 2019; Handler et al. 2019). Furthermore, dopaminergic and cholinergic input, the main neurotransmitter of olfactory Kenyon Cells, are configured into a axoaxonic reciprocal synapses (Cervantes-Sandoval et al. 2017). In the context of food intake, the experiences that humans have with sugar involve multiple sensory information such as that of smell. When presented with food and a pleasant smell, it can increase our appetitiveness and lead to more consumption, or it can also be a deterrent if it's an unpleasant smell. These smells that serve to create associations with food can remind us of previous experiences with food that might trigger motor outputs to move towards and to forage for these foods (Wells and Wenner 1971; Finkelstein and Amdam 2018; Zjacic and Scholz 2022). Hence, the olfactory system is being leveraged to study the reinforcing properties of food and being utilized to further dissect the consequences of foods high in sugar on the dopaminergic system

1.7 Mushroom Body Output Neurons

The convergence of sweet and olfactory input into the mushroom body through PAMs and Kenyon Cells, respectively, happens at the mushroom body output neurons (MBONs). These neurons have a variety of function that span from sleep to thermo-sensation to associative learning and memory, as well as pre-motor coordination (Aso et al. 2014b; Lei et al. 2022).

MBONs are subdivided into the compartments that they have dendrites on which correlates with the type of PAM and Kenyon cell labeling and modality. In addition, they are also divided into the neurotransmitter they release, i.e. glutamatergic, cholinergic, and GABAergic. Each one of these types of MBONs has different intrinsic neural properties as well as mechanisms of action towards specific behaviors (Aso et al. 2014b). For example, glutamatergic MBONs are characterized by having lower depolarization threshold and show higher frequencies of depolarization, meaning that they are in an active state. It is thought that this active state encodes for the aversive properties of the environment and work to keep the fly safe from environmental risks. When flies are presented with an appetitive substance that activates the dopaminergic pathway innervating the mushroom body, these neurons have decreased calcium neural responses, signaling the motor output neurons to engage approach behavior (Owald et al. 2015; Felsenberg et al. 2018; Ichinose et al. 2021). Hence, MBONs can also be considered pre-motor neurons that signal motor neurons to move towards or away from foods.

On a sugar diet, the dopaminergic connections onto a specific subgroup of glutamatergic MBONs, *MB011B* , are seen to be weakened, with another subgroup of dopaminergic PAM neurons, *MB301B*, having decreased calcium responses to sweet taste (May et al. 2020). Given that MBONs rely on the connections of PAMs and Kenyon cells to perform food associations, it suggests that proper integration of information is hindered on a sugar diet. In mammalian literature, it's been shown that obese subjects have changes in the dopaminergic system, specifically, they experience decreased dopamine tone, increased D1R at the presynaptic neuron and increased D2R at the post-synaptic neurons, although whether it's the diet or the obesity still remains unknown (Bello et al. 2002; Horstmann et al. 2015; Kroemer and Small 2016). As previously mentioned, in flies, we showed that a sugar diet leads to decreased neural activity of

the dopaminergic neurons *MB301B*, which are necessary and sufficient for modulating food intake (May et al. 2020). However, whether this decreased neural activity also causes any impairments in food associations and how this causally modifies feeding behavior is still unknown. In addition, *MB301B* and *MB011B* possess dopaminergic receptors on their axons and dendrites, respectively that span from the Dopamine-like type 1 receptor 1 and 2 (Dop1R1 and Dop1R2) to the dopamine-like type 2 receptor (Dop2R) (Handler et al. 2019). Dop1R1 and 2 are coupled to G_s and G_q proteins while the Dop2R is coupled to Gi/o, similar to mammalian D1R and D2R, respectively. Whether dopaminergic receptor expression changes on a sugar diet in flies and how this causes changes in food associations that then leads to over feeding, is also still unknown. Hence, our objective is to elucidate the circuits and neural mechanisms by which a sugar diet modifies food intake. We hypothesized that a sugar diet promotes feeding behavior by inhibiting food association-mediated satiation through decreased dopamine transmission and increased Dop2R expression.

We found that a sugar diet leads to impairments in food associations through decreased MB301B dopaminergic transmission that reflects in the inability of *MB011B* to encode for new learned information. These changes are due to the diet and not to the obesity given that genetically lean flies still exhibit the same phenotypes in food associations while on a sugar diet. In addition, *MB011B* are necessary and sufficient for modulating food intake in addition to their classical role in food associations. Furthermore, *MB301B* are necessary for modulating food associations on a sugar diet. Food associations are abolished after a brief meal period followed by conditioning, suggesting a satiation-dependent mechanism controlled by a feedback loop between MB301B and *MB011B* (Chapter 2). In addition, we demonstrate that Dop2R, but not Dop1R2, is necessary for diet-induced obesity. In addition, it is also necessary for food

associations on a sugar diet, as well as food intake. The main reason for its contributions to the aberrant behaviors is its overexpression on the dendrites of the *MB011B* (Chapter 3).

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Chapter 2 Food Memories Regulate Eating and Energy Balance

2.1 Abstract

Diet composition has a profound influence on brain physiology and behavior, but the mechanisms through which the central processes of the brain modulate satiation throughout the feeding cycle are still elusive. Here, we uncover how the appetitive associative learning and memory (food associations) pathway transforms information about the dietary environment into satiation signals. We show that in the fly *D. melanogaster*, the dietary environment can directly modulate short-term memory Protocerebral antero-medial (stmPAM) neurons and Mushroom Body Output Neurons (MBONs) to modulate feeding behavior. Specifically, stmPAMs and MBONs regulate a series of steps in the feeding cycle that are coded by dopamine transmission intensity. Diet directly disrupts dopamine transmission, which then leads to the degradation of associative learning and memory, thereby hindering the integration of satiation signals that stop food consumption. Together, our findings have uncovered a novel role for food memories: regulating food consumption.

2.2 Introduction

Foods high in sugar elicit dopaminergic responses that correlate with its sugar concentration. Furthermore, there is a direct relationship between the sugar concentration of these foods and its reinforcing properties (Small et al. 2003; Hajnal et al. 2004; Rada et al. 2005). These responses are part of a circuitry that regulates appetitive associative learning and memory

(food association/memory), forming the basis of the adaptive and maladaptive behaviors towards food, like substances of abuse (Johnson and Kenny 2010; Tang et al. 2012; Alhadeff et al. 2019).

In obese humans, changes to dopamine receptor availability (Colantuoni et al. 2001; Wang et al. 2001; Stice et al. 2008), and decreased dopamine neural response, seem to correlate with the inability to form and recall past experiences to update the present value of food (Stice et al. 2008; Hartmann et al. 2020). Consequently, the ability to inform future decisions towards the foods we eat might become impaired, leading to a series of cascades that might result in overfeeding and obesity. In mammalian studies, a diet high in sugar also leads to decreased dopamine pathway activity input onto their food association center, the striatum (Bello et al. 2002; Johnson and Kenny 2010). This decrease can be associated with a lessening of the reinforcing properties of sucrose which involves but does not begin with the dopaminergic neurons that relay this information (Comings and Blum 2000; Szczypka et al. 2001; Wang and Wang 2002). In humans and mammals, these signals are usually responsible for mediating go or no-go signals that modulate a range of behaviors such as drug and food seeking, and approach behavior (Babbs et al. 2013; Veling et al. 2013; Love et al. 2020). Hence, altering this circuitry might lead to either too much positive reinforcement or too little negative reinforcement, possibly modulating food intake and satiation. At different organism levels, satiation involves dopaminergic signals at the short and long-term, that regulate food intake (Papageorgiou et al. 2016; Crossley et al. 2018; May et al. 2020; Han et al. 2021). Hence, satiation might be one aspect by which diets high in sugar reprogram our brain to increase satiation threshold and promote food seeking behavior (May et al. 2019; May et al. 2020; Vaziri et al. 2020).

Like humans and rodents, *D. melanogaster* flies exposed to palatable diets rich in sugar or fat overconsume, gain weight, and become at-risk for obesity and develop phenotypes associated with metabolic syndrome (Musselman and Kühnlein 2018). In addition, they possess an food association/memory circuitry that functionally acts in a similar manner to humans and mammals to allow flies to create associations that enable them to navigate their environment (Aso et al. 2014). In addition, anatomically, this circuitry is composed of dopaminergic neurons and peripheral sensory information that are conveyed onto the food association/memory center, the mushroom body (Yamagata et al. 2015; Huetteroth et al. 2015). These dopaminergic neurons contribute to a variety of food related behavior such as food seeking, satiation and satiation, which enable flies to determine when to initiate or halt their feeding based on reward signals. These neurons then relay the coded reward onto the mushroom body, where subsequent neurons integrate other signals for a more complex decision (Landayan et al. 2018). One such neuron that integrates these signals within the mushroom body to perform food association is the Mushroom Body Output Neurons (MBON)(Owald et al. 2015). These neurons are also involved in a range of behaviors from sleep and thermoregulation to learning & memory, and the regulation of approach and avoidance behavior (Chia and Scott 2020).

Our previous work showed that blunted sensory input of sugar sensing neurons that results from a diet high in sugar leads to the impairment of the neural activity of the dopaminergic neurons, stmPAMs. In addition, these neurons are also necessary and sufficient for feeding behavior. These neurons are part of a subgroup of dopaminergic neurons MB301B, that are specifically important for short-term learning and memory, as well as satiation. Furthermore, stmPAMs innervate a subgroup of glutamatergic neurons MBONs (May et al. 2019; May et al. 2020) that have been identified to be necessary and sufficient for food associations, and that have

dendrites in the β' compartment, *MB011B* (Aso et al. 2014). Therefore, we hypothesized that diets high in sugar promote feeding behavior by inhibiting food associations-mediated satiation. Here we show that diet impairs food associations through changes in dopamine transmission to the *MB011B* that are diet- and not obesity dependent. These dopaminergic inputs to these *MB011B* are necessary for food associations. In addition, we report that this circuit is also necessary and sufficient for feeding behavior on a high sugar diet. Together, our results argue that diet-dependent decreased reinforcement of sucrose impairs the formation of food memories that regulates satiation.

2.3 Results

SD impairs associative learning independently of fat accumulation and internal state

We previously showed that a subset of dopaminergic short-term memory Protocerebral Antero-Medial (stmPAM) neurons innervating the β' compartment of the mushroom body had decreased activity on a SD when fed a rewarding substance, sucrose. In the mushroom body, reward stimuli, together with and olfactory stimuli, are key for the formation of food memories (May et al. 2020; Aso et al. 2014b). Given the decrease in stmPAM activity at the mushroom body when given sucrose, our overall hypothesis was that on a SD, flies are unable to form food associations due to a decrease in reward input. However, we first needed to test the possibility that a SD might also affect olfactory responses. To test this, we utilized the T-maze assay (Fig. 1A), which streams air through two different sides and allows flies to choose which side to move towards. We found that flies on a CD and SD responded equally to a novel aversive odor, 4-methylcyclohexanol (MCH) and 3-Octonal (OCT), when matched against paraffin oil, an odorless substance (Fig. 1B), suggesting that a SD does not affect the behavioral response to novel aversive odors. Next, we tested whether a SD leads to a decrease in food memories at three

different fasting lengths, the latter to control for motivational states. Flies on a SD had a preference index of around zero, regardless of fasting length (Fig. 1C, Supplement 1A), suggesting that flies on a SD have impaired food memories regardless of fasting length.

A big unanswered question in the field of obesity is whether it is the SD or the obesity state that lead to impairments in food associations (Eric Stice et al. 2008; Johnson and Kenny 2010; Tellez et al. 2013). We found that Perilipin $2^{-/-}$, which are unable to store fat, on a SD were also unable to form food associations (Fig. 1C), consistent with wild-type flies on a SD (Fig. 1C). This suggests that it is the diet and not the obesity state that impairs food associations. Together, these data suggest that a SD impairs food associations by decreasing stmPAM activity.

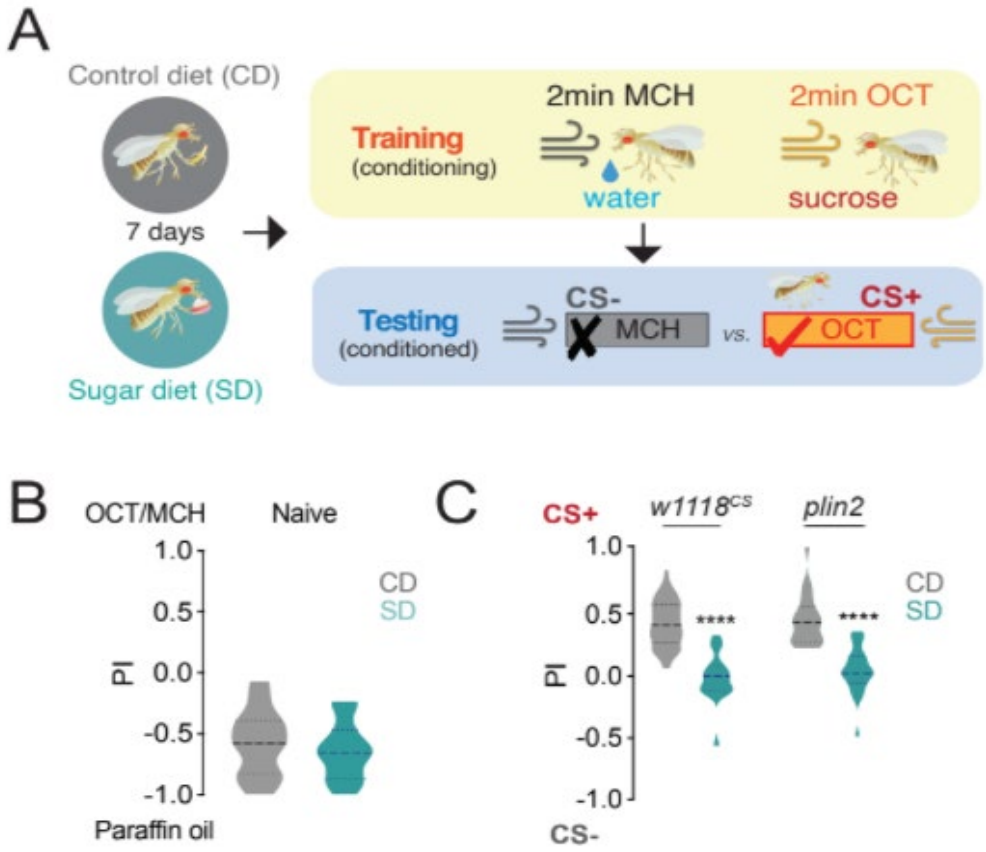

Figure 2.1 A high sugar diet impairs the formation of food memories independently of fat accumulation.

A) Schematic of appetitive conditioning: flies were fed a Control (CD) or Sugar diet (SD) for 7 days, trained to pair an odor with either water (CS-) or sucrose (CS+), and then tested for the preference between the CS- or CS+. **B)** Performance index (PI) for naive olfaction between paraffin oil and MCH or OCT in *w1118*^{CS} flies fed a CD (Gray) and SD (Teal). Data consist of a combined set with half of the flies tested with OCT and the other half with MCH. n=24, 25 flies per n, Mann-Whitney; **C)** The PI of control *w1118*^{CS} (n = 53 [24 hrs CD], 17 [24 hrs SD]) and obesity-resistant *plin2* mutant flies (n = 22) on CD (gray) and 7 days of SD (teal). Data shown as +/- SEM. Kruskal-Wallis with Dunn's multiple comparison test, ****p<0.0001. Thicker dotted line in the violin plot shows the mean.

The neural signatures of food associations are absent in SD flies

Food associations are encoded in the mushroom body, specifically in the mushroom body output neurons (MBONs), and as previously mentioned, relies on reward and olfactory input. Since we previously showed that on a SD stmPAMs innervating the β'2 region of the mushroom body have decreased neural activity, we focused on MBONs with dendrites in the β'2 compartment. Specifically, we selected the glutamatergic *MB011B* MBONs (Figure 2A) due to their prominent and specific role in food associations (Aso et al. 2014).

A hallmark of food associations in these MBONs is the decrease in neural activity when presented with a sucrose-paired odor, CS+ (Owald et al. 2015). Hence, we hypothesized that the behavioral impairment of food associations on a SD is due to the absence of a dendritic neural trace of food associations in *MB011B* . To this end, we subjected flies to a 3-step protocol and performed two-photon *in vivo* calcium imaging at the beginning and end (Figure 2B). Specifically, we performed neural assessment of responses to naïve aversive odors (pre-training) (Figure 2C and D, left side), followed by a training session, and finalized by a testing session (Figure 2C and D, right side) that ended with the presentation of a novel odor as a control (Supplement Figure 2C). We found that on a CD and SD, the calcium responses to two novel odors, 4-MCH and 3-OCT, was the same (Figure 2C and D, left side). This suggests that a SD has no significant role in modifying neural responses to aversive odors, like the behavioral responses previously observed (Figure 1B). In addition, the calcium response to the CS+ was lower than the CS- on a CD (Figure 2C, right side), demonstrating the validity of this assay. However, on a SD, the CS+ failed to induce a decrease in the calcium responses (Figure 2D, right side), suggesting that a SD abolishes the ability of flies to form food associations.

Next, to determine whether the calcium responses previously observed are not odor specific but diet specific, we grouped the dendritic calcium responses of *MB011B* by odor and compared between diets (Figure 2E and F). We found that both novel aversive odors elicited similar calcium responses between CD and SD (Supplement 2A, B), suggesting that these diets don't have different overall calcium responses to these novel aversive odors. The CS- was also not significantly different between diets (Figure 2E,). On the other hand, the CS+ was significantly higher on a SD (Figure 2F), suggesting that the absence of an food associations on a SD is diet-dependent, and not odor-dependent. Together, these data suggest that the behavioral impairment of food associations previously observed on a SD are a consequence of the inability of *MB011B* MBONs to form food associations, most likely due to decreased reward stimulus.

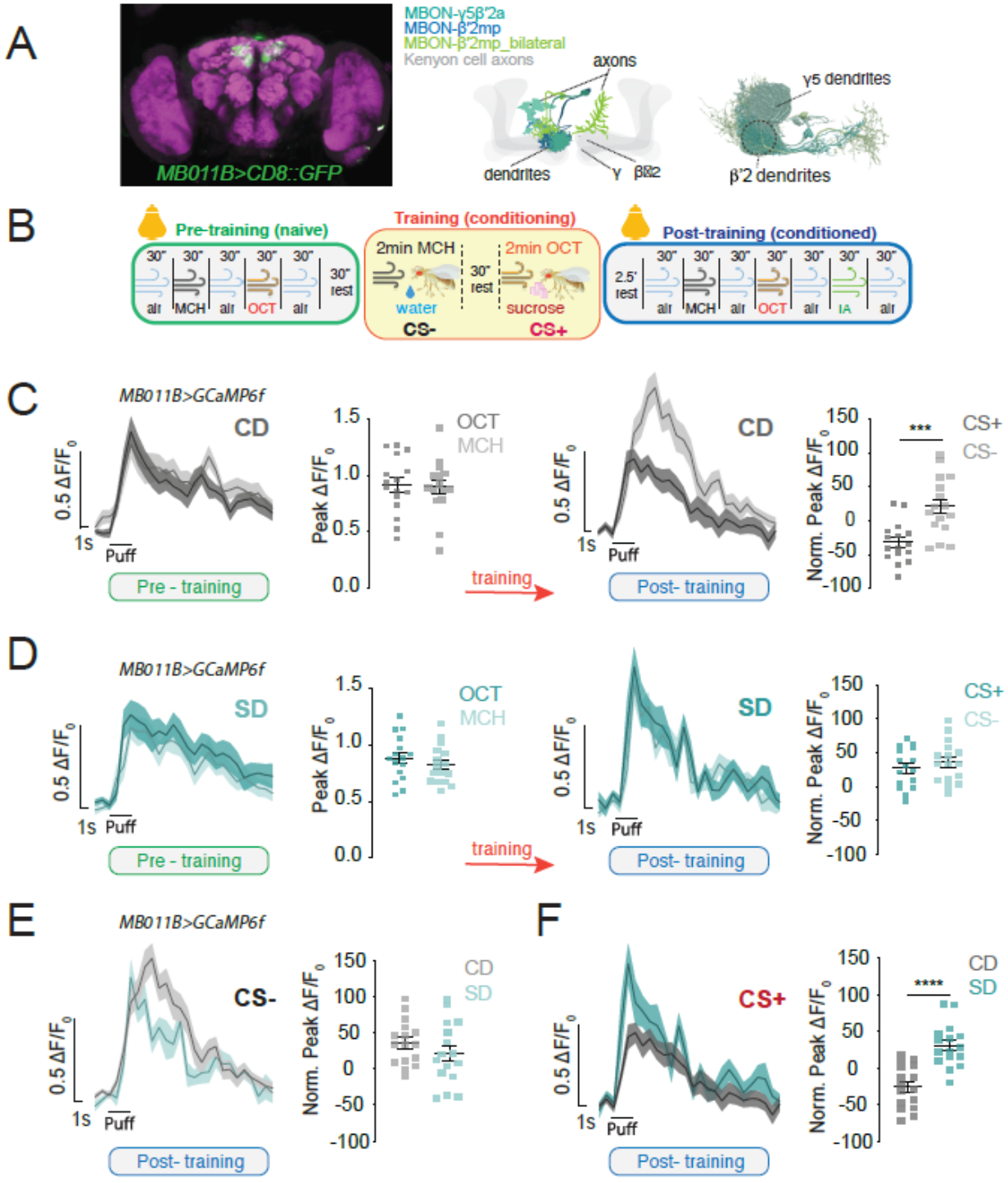

Figure 2.2 Exposure to a high sugar diet abolishes the neural signatures of food associations

A) *(left)* Confocal maximum intensity projection showing the expression of *MB011B >CD8::GFP* (green) in the fly brain, co-labeled with the antibody nc82 to label the presynapses (magenta). *(Mid)* Diagram of the different classes of MBONs labeled by the *MB011B -GAL4* in shades of blue (MBON-γ5β′2a, MBON-β′2mp and MBON-β′2mp_bilateral) and the MB Kenyon Cells (KCs) axons in gray. (*right*) Connectome reconstruction of *MB011B* + MBONs with region used for imaging experiments shown inside the dotted circle. Cells in only one brain hemisphere are shown for clarity. **B)** Schematic of the appetitive conditioning protocol under the 2-photon microscope in pre-training (naive, green), training (yellow), and post-training (testing, blue) phases; bulbs represent imaging time; reverse pairing not shown for clarity. **C-D)** The calcium responses to MCH and OCT (puff) in the β′2 dendrites of *MB011B >GcAMP6f* neurons before (green) and after (blue) training (orange arrow) in CD (C) and SD (D) flies. **E, F)** Comparison of CS- (E) and CS+ (F) responses in the β'2 dendrites of CD (gray) and SD (teal) *MB011B >GcAMP6f* flies after training (data from C and D). Data are shown as mean +/- SEM, $\Delta F/F_0$ traces and quantified as maximum peak $\Delta F/F_0$ response (pre-training) or normalized to naïve responses (post-training). n=16 (n=8 with OCT as the CS+, and n=8 as MCH as the CS+); Student's t-test; ***p<0.001, ****p<0.0001.

Flies on a SD have decreased DA signaling onto MBONS

Since stmPAM but not olfactory responses change on a SD (Supplement 3A, B), we wanted to determine whether this decrease directly impacts *MB011B 's* ability to form food associations. Specifically, we hypothesized that a decrease in stmPAM responses to sucrose leads to decreased dopamine transmission to the dendrites of *MB011B* . To this end, we utilized the dopamine sensor, $GRAB_{DAm1}$ (Sun et al. 2018), and imaged the dendrites of *MB011B* on the β'2 compartment (Figure 3A). Results showed that sucrose taste leads to less dopamine transmission on a SD (Figure 3B), which is not due to a decrease in taste since flies on a SD have normal proboscis extension reflexes when presented 30% sucrose (May et al. 2019). This suggests that the absence of a food memory is the result of a decrease in the reinforcing properties of sucrose on a SD.

To test this hypothesis, we used the $GRAB_{DA\mathrm{m1}}$ system, and observed dopamine transmission at the β'2 compartment dendrites of *MB011B* before and after training, using the same protocol established on Figure 2B. Results showed that the naïve aversive odors had similar dopamine responses whether on a CD (Figure 3C, left side) or on a SD (Figure 3D, left side), suggesting that the odor-induced dopamine responses are not altered by a SD. In addition, the CS+ elicited higher dopamine responses than the CS- on a CD (Figure 3C, right side), highlighting the reinforcing properties of sucrose. Interestingly, the CS+ showed a decreased dopamine response compared to the CS- on a SD (Figure 3D, right side), suggesting a shift in valence of the CS+ from appetitive to aversive.

Next, to determine whether the dopamine responses previously observed are not odor specific but diet specific, we grouped the dopamine responses of *MB011B* by odor and compared between diets (Figure 3E and F, Supplement 3C and D). We found that both novel

aversive odors elicited similar dopamine responses between the CD and SD (Supplement 3E and F), suggesting that these diets don't change the overall dopamine responses to these novel aversive odors. On the other hand, the CS- elicited an increased dopamine response on a SD (Figure 3E), suggesting that its acquiring some of the properties of a reinforced odor. In addition, the CS+ elicited a decreased dopamine response on a SD (Figure 3F), suggesting that the decrease in dopamine transmission on a SD is diet-dependent, and not odor-dependent. Together, these data reinforce the idea that flies on a SD are not able to form food associations due to a significant decrease in the reinforcing properties of sucrose that are the results of decreased dopamine transmission. In addition, it also suggests that in the absence of an expected rewarding stimulus, the valence of expectation shifts towards the CS-. We previously showed that correcting the neural activity of stmPAMs with an inhibitor of the metabolic-signaling enzyme O-GlcNAc-Transferase (OGT), rescues their calcium responses to sucrose stimulus in flies on a SD (May et al, 2020). Hence, we utilized this inhibitor, OSMI-1, to test the hypothesis that rescuing dopamine transmission to the *MB011B* re-establishes food associations. Test this hypothesis, we performed two-photon *in vivo* dopamine imaging on flies expressing $GRAB_{DAm1}$ and fed OSMI-1, and repeated the same protocol as previously shown (Figure 4A). Both CD and SD flies did not differ in their dopamine responses to naïve aversive odors (Supplement 4A and B), suggesting that OSMI-1 does not interfere with odor-evoked dopamine transmission to the *MB011B s*. In addition, dopamine responses were higher on the CS+ on a CD (Figure 4B) suggesting that OSMI-1 had no visible repercussions on the reinforcing properties of sucrose. On the other hand, on a SD, flies fed OSMI-1 had significantly higher dopamine responses to the CS+ compared to the CS- (Figure 4C). This suggests that restoring the reinforcing properties of sucrose is sufficient for food associations.

To test this hypothesis, we did similar experiments with *MB011B >GcAMP6f* flies fed OSMI-1 (Figure 4D and E; Supplement 4D and E). Results showed that the calcium responses to naïve aversive odors were the same on a CD and SD (Supplement 4D and E,), suggesting that OSMI-1 does not alter olfactory input. In addition, OSMI-1 did not alter the formation of food associations on a CD (Figure 4D). On the other hand, OSMI-1 treated flies on a SD had decreased CS+ elicited calcium responses (Figure 4E), suggesting the formation of food associations. Together, these data point to the significant role that stmPAM-*MB011B* signaling have on a SD in food associations.

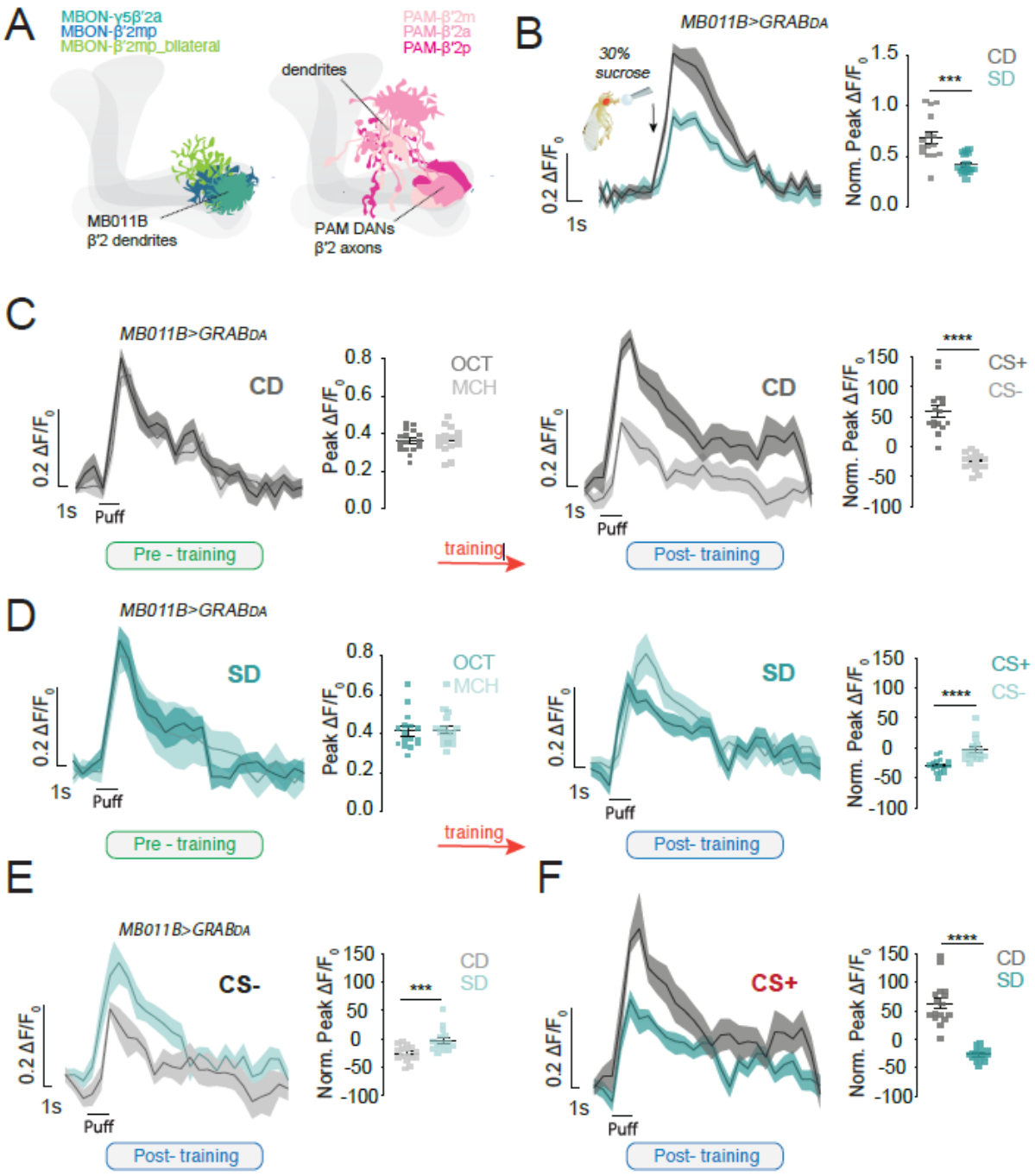

Figure 2.3 A sugar diet decreases dopamine-mediated plasticity onto MBONs during associative learning.

A) Graphics showing the dendrites of *MB011B* + MBONs (blue) and the axons and dendrites of β'2 PAM DANs (pink). **B)** Mean $\Delta F/F_0$ traces and quantification of maximum peak $\Delta F/F_0$

response to stimulation of the proboscis with 30% sucrose (arrow) in the β'2 dendrites of CD (gray) and SD (Teal) *MB011B >GRAB-DA* flies. n = 15-16, Mann-Whitney test, ***p<0.001. **C, D)** The mean $\Delta F/F_0$ traces and quantification of maximum or normalized peak $\Delta F/F_0$ response to OCT or MCH in the β'2 dendrites of CD (C, gray, Student's t-test) and SD (D, teal; Student's t-test and *right* Mann-Whitney test) *MB011B >GRAB-DA* flies before training (*left*, naive, green) and after training (*right*, conditioned, blue); ****p<0.0001. **E, F)** Comparison of CS- (E, Mann-Whitney Test) and CS+ (F, Student's t-test, ****p<0.0001) responses in the β'2 dendrites of CD (gray) and SD (teal) *MB011B >GRAB-DA* flies after training (data from C and D). n=16, ****p<0.0001. Half of the flies experienced OCT=CS+ and half MCH=CS+. Data shown as mean +/- SEM.

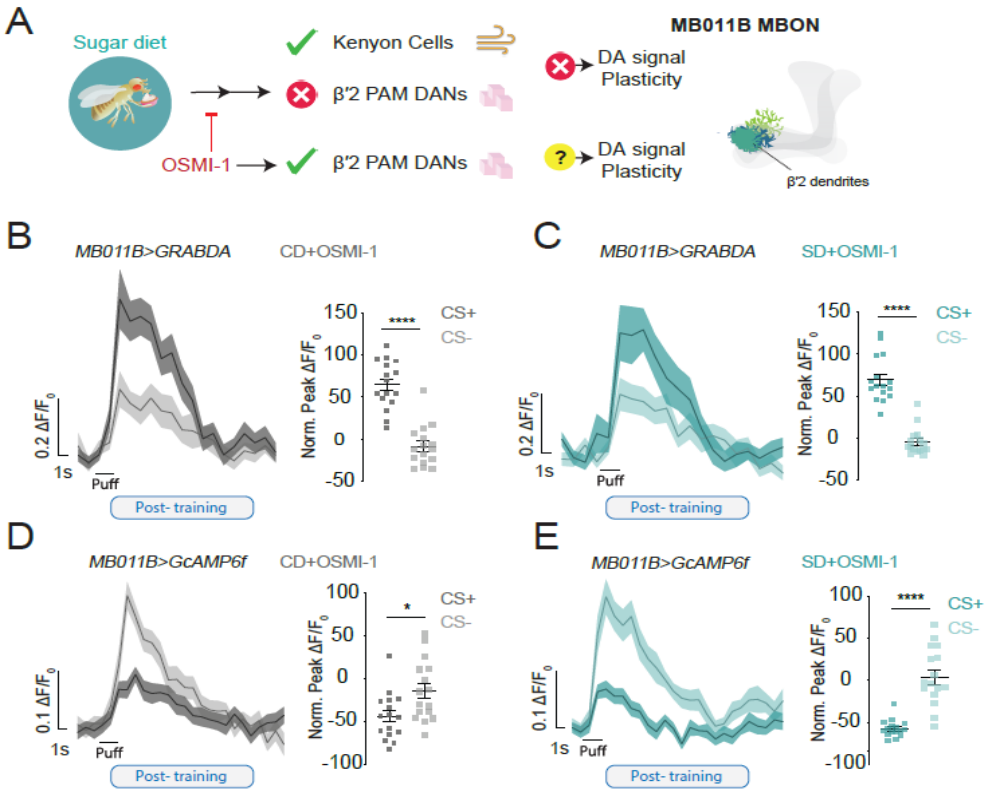

Figure 2.4 Lower DA-induced plasticity impairs appetitive learning in SD flies

A) *(left)* Schematic representation of the action of OSMI-1 on the reinforcing signal carried by β'2 PAM DANs and (*right*) a diagram showing the dendrites in the β'2 region where imaging occurs. **B, C)** The mean $\Delta F/F_0$ traces and quantification of normalized peak $\Delta F/F_0$ response to CS+ or CS- in the β'2 dendrites of CD (B, gray, Student's t-test, ****p<0.001) and SD (C, teal, Mann-Whitney test, ****p<0.001) *MB011B >GRAB-DA* flies after training. **D, E)** The mean $\Delta F/F_0$ traces and quantification of normalized peak $\Delta F/F_0$ response to CS+ or CS- in the β'2 dendrites of CD (D, gray, Student's t-test, *p<0.01) and SD (E, Student's t-test, ****p<0.001) *MB011B >GCaMP6f* flies after training. n=16, (half of the flies experienced OCT=CS+ and half MCH=CS+).

Food associations shape the output of MBONs during eating

We have shown that the lack of dopamine transmission to the *MB011B* leads to impaired food associations on a SD. However, we have yet to determine whether this disjunction consequently leads to impaired neural output. To this end, we performed two-photon *in vivo* calcium imaging at the axons of *MB011B* (Figure 5A) utilizing the same protocol as previously described (Figure 5B). Both CD and SD fed flies did not differ in their odor-induced calcium responses (Figure 4C and D, left side), suggesting that the neural output of these MBONs in the presence of a naïve aversive odor is not changed on a SD. In addition, on a CD, calcium responses to the CS+ were significantly lower than the CS- (Figure 5C, right side), reflecting the neural trace of food associations. On the other hand, on a SD, calcium responses between CS+ and CS- were not significantly different (Figure 5D, right side), suggesting that these neurons are unable to relay food association information onto downstream neurons.

To determine whether the calcium responses previously observed are not odor specific but diet specific, we grouped the calcium responses of *MB011B* by odor and compared between diets (Supplement 5A-D). We found that both novel aversive odors elicited similar calcium responses between CD and SD (Supplement 5A and B), suggesting that these diets don't change the overall calcium responses to these novel aversive odors. In addition, both diets have the same CS- calcium response (Supplement 5C). On the other hand, flies on a SD have significantly higher CS+ calcium responses than on a CD (Supplement 5D), suggesting that flies on a SD do not relay food associations. Together, these data suggest that on a SD not only is there an absence of food associations at the dendrites but there is also the absence of a neural trace of memory being relayed to downstream neurons.

Next, we tested whether feeding lead to changes in the ability of these neurons to retain food memories on a CD. We imaged from the axons of *MB011B* at pertaining to the β' group, before feeding (fasted) and after 30min of feeding (fed) (Figure 6A). Results showed that while on a CD the CS+ induced a decrease in the neural response of the fasted group, flies fed 2M sucrose for 30min had the same neural responses to CS+ and CS- (Figure 6B). On a SD however, given that food associations were already abolished, no differences were seen between fasted and fed groups (Figure 6C). This suggests that feeding sucrose on a CD degrades the food memory. Likewise, comparing CD and SD shows that only the fasted group puffed the CS+ retained the food memory (Supplement 6B, left side), while all other groups did not (Supplement 6A, B right side). Furthermore, the observed abolishment of the food memory was not due to a decrease in the motivation to eat given that both CD and SD flies at the same amount (Supplement 6C).

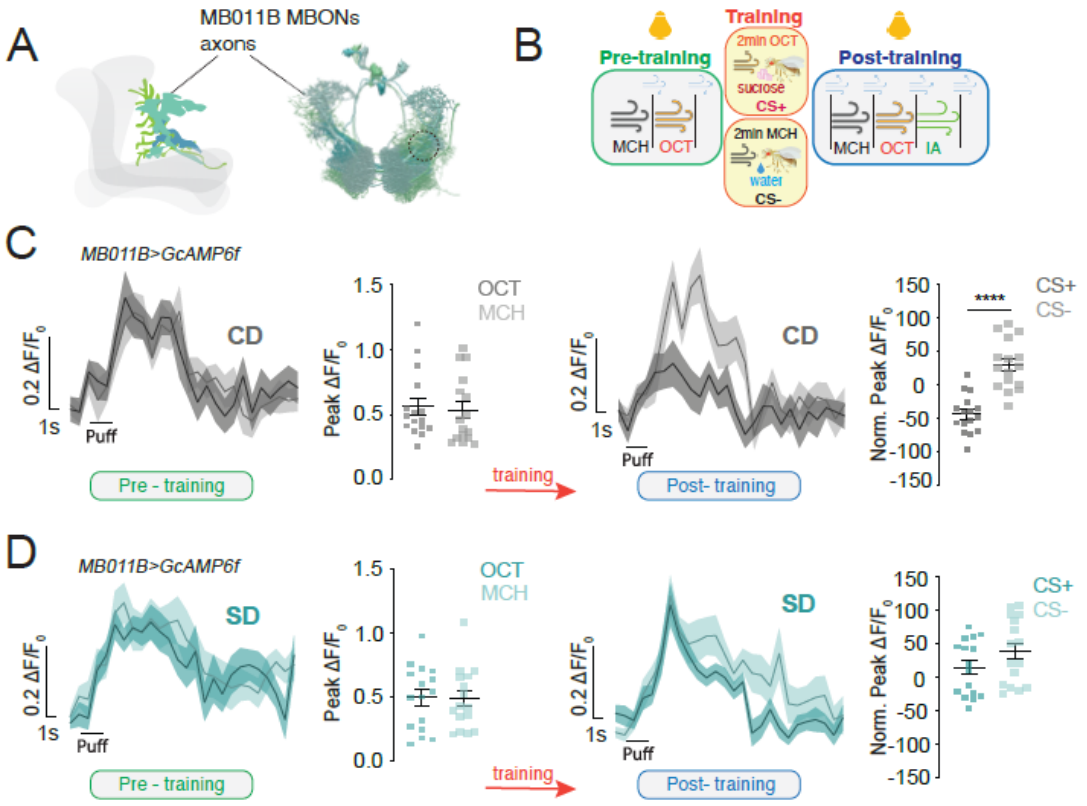

Figure 2.5 A SD changes the presynaptic responses to cues of *MB011B* + MBONs

A) Graphic (left) and connectome reconstruction (right) of *MB011B* β'mp axons; in the graphic only cells in one hemisphere are shown; dotted circle shows the region imaged. **B)** Schematic of the appetitive conditioning protocol under the 2-photon microscope in pre-training (naive, green), training (yellow), and post-training (testing, blue) phases; yellow bulbs represent imaging; blue puffs are air. **C-D)** The calcium responses to MCH and OCT (puff) in the β′2mp axons of *MB011B >GcAMP6f* neurons before (green) and after (blue) training (red arrow) in CD (C) and SD (D) flies. Data are shown as mean +/- SEM $\Delta F/F_0$ traces and quantified as maximum peak $\Delta F/F_0$ response (pre-training) or normalized to naive responses (post-training). C) *left* Mann-Whitney and *right* Unpaired T-test, D) *left* Unpaired T-test and *right* Mann-Whitney test; ****p<0.0001.

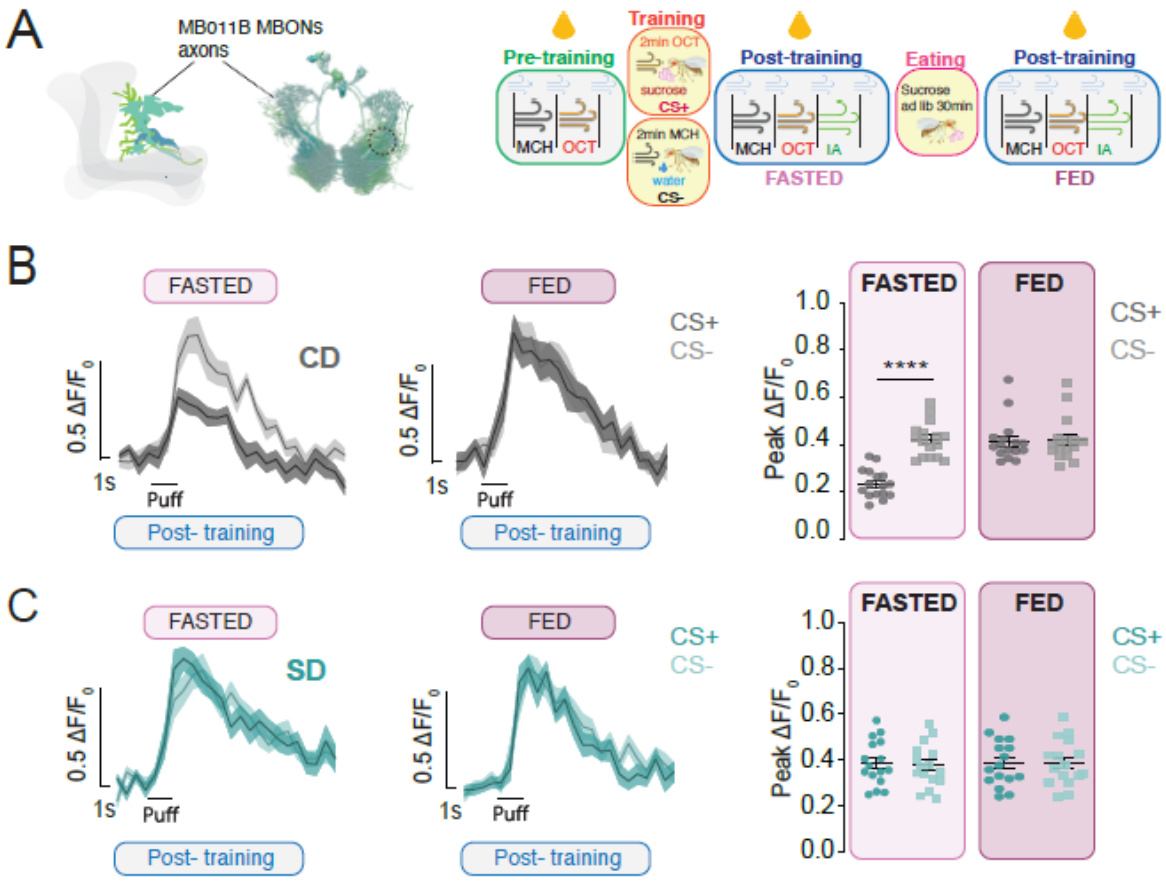

Figure 2.6 Food associations shape the output of MBONs during eating

A) *Left,* Graphic and connectome reconstruction of β'mp axons of *MB011B* MBONs; in the cartoon only cells in one hemisphere are shown; dotted line shows the region imaged. *Right,* Schematic of the appetitive conditioning protocol under the 2-photon microscope in pre-training (naive, green), training (yellow), and post-training (testing, blue) phases, 30 minutes apart, one before (light pink) and one after eating 2M sucrose (dark pink); yellow bulbs represent imaging time and blue puffs are air. **B-C)** The calcium responses to MCH and OCT (puff) in the β′2mp axons of *MB011B >GcAMP6f* neurons after training before (light pink, fasted) and after (dark pink, fed) consuming sucrose in CD (B) and SD (C) flies; n=16, data are shown as mean +/- SEM, B) *left* Student's t-test and *right* Mann-Whitney test, C) Student's t-test; ****p<0.0001.

The activity of *MB011B* + MBONs controls eating and energy balance

Previously, we showed that stmPAMs with inputs in the β' compartment of the mushroom body are necessary and sufficient for normal feeding behavior on a SD (May et al. 2020). In addition, activation of some MBONs with dendrites in the β' compartment inhibits food seeking behavior (Chia and Scott 2020). Hence, we hypothesized that *MB011B* also regulates feeding behavior, and that this regulation is dependent on food associations. Increase or decrease in body fat could be due to changes in metabolic rate and/or feeding behavior. Hence, we next tested whether *MB011B* modulation can regulate feeding behavior. To do so, we used the OptoFLIC, a feeding frequency assay (Ro, Harvanek, and Pletcher 2014) modified for closed-loop optogenetic stimulation (May et al. 2019) to provide temporal resolution to the activation or inhibition of *MB011B* while feeding. *MB011B >CsChrimson* flies that were not pre-fed (-) with Retinal overeat, while flies that were pre-fed (+) with Retinal did not overeat on a SD (Figure 7A). On the other hand, the control group, *UAS-CsChrimson/w118CS* overate on both pre-treatment (Figure 7B). We then hypothesized that inhibition of *MB011B* with *GtACR1*, an anion channel, would lead to increased feeding behavior on a CD of 10% sucrose. Light stimulation of (+) Retinal *MB011B >GtARC1* led to significantly higher mean licks compared to (-) Retinal group (Figure 7C). These data suggest that *MB011B* are necessary and sufficient in the control of food intake. Next, we tested how these changes to feeding behavior affected triglyceride levels. To do so, we first tested whether activation of *MB011B* using Nachbac rescued diet-induced obesity. Results showed that after 7-days on diet, SD *MB011B >Nachbac* flies were as lean as the CD group (Figure 7E), suggesting that activation of *MB011B* leads to sucrose avoidance. In addition, at baseline, *MB011B* neurons provide avoidance signals to the fly until an appetitive stimulus is present (Owald et al. 2015). Hence, we hypothesized that

inhibition of *MB011B* using *GtACR1* would lead to increased triglyceride levels. Results showed that after 7-days on diet, CD *MB011B >GtACR1* flies are as obese as the SD group (Figure 7F), suggesting that inhibition of *MB011B* leads to sucrose attraction on a CD. Together, these data suggest that decreased sweet taste information on a sugar diet leads to decreased dopamine transmission that inhibits proper food association and subsequent control of food intake through the *MB011B* MBONs (Figure 7G).

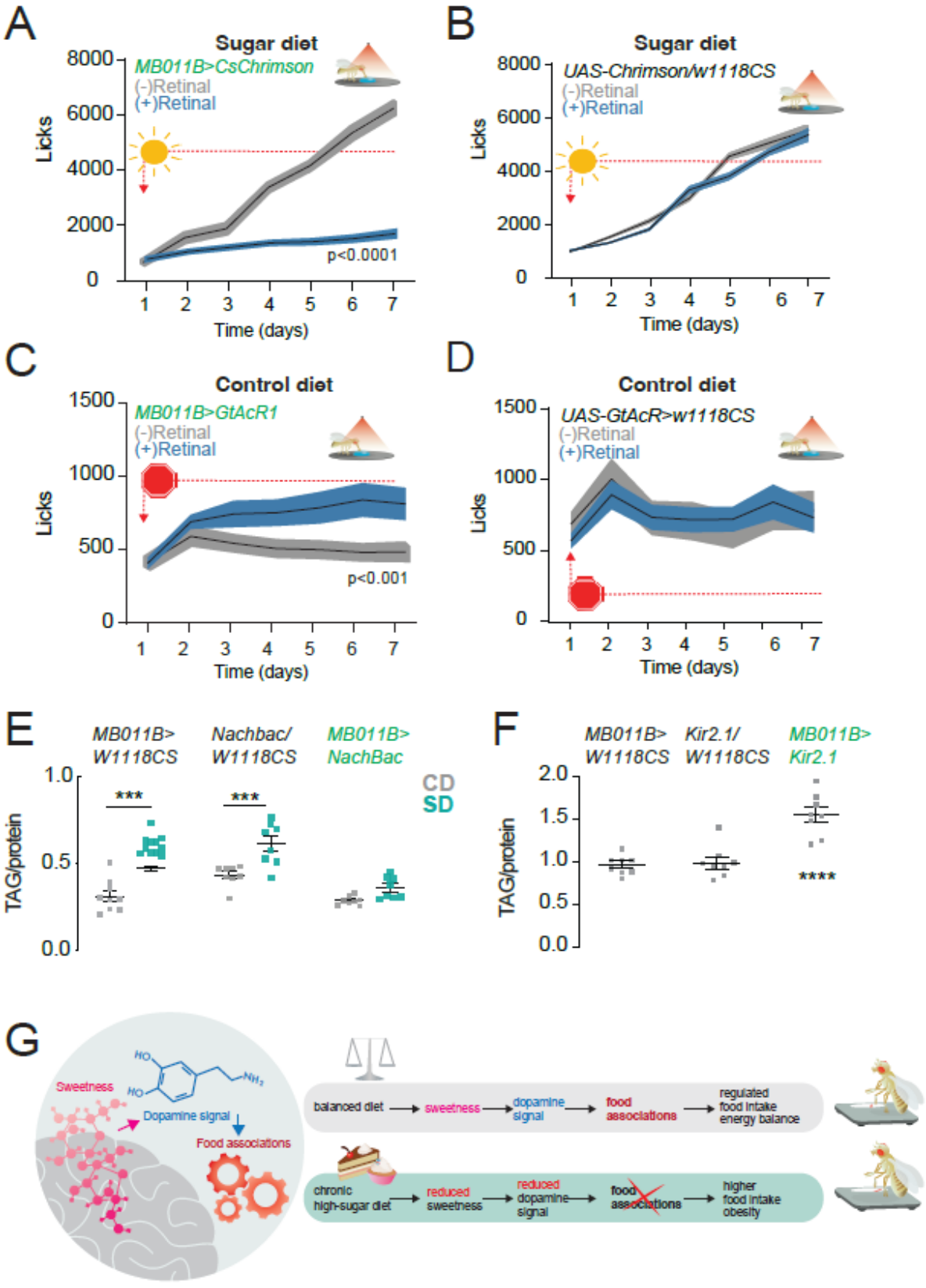
A
Sugar diet
MB011B>CsChrimson
(-)Retinal
(+)Retinal
Licks
Time (days)
p<0.0001
B
Sugar diet
UAS-Chrimson/w1118CS
(-)Retinal
(+)Retinal
Licks
Time (days)
C
Control diet
MB011B>GtAcR1
(-)Retinal
(+)Retinal
Licks
Time (days)
p<0.001
D
Control diet
UAS-GtAcR>w1118CS
(-)Retinal
(+)Retinal
Licks
Time (days)
E
MB011B> W1118CS
Nachbac/ W1118CS
MB011B> NachBac
CD
SD
TAG/protein
***
***
F
MB011B> W1118CS
Kir2.1/ W1118CS
MB011B> Kir2.1
TAG/protein
****
G
Sweetness
Dopamine signal
Food associations
balanced diet
sweetness
dopamine signal
food associations
regulated food intake energy balance
chronic high-sugar diet
reduced sweetness
reduced dopamine signal
food associations
higher food intake obesity

Figure 2.7 The activity of *MB011B* + MBONs controls eating and energy balance

A) The number of food interactions (licks) per day on a high sugar diet in experimental *MB011B >CSChrimson* +retinal (blue) flies or control n *MB011B >CSChrimson* - retinal (gray) flies. n=27 flies, Two-way Repeated Measure ANOVA with Sidak's test, ****$p<0.0001$. **B)** The number of food interactions (licks) per day on a high sugar diet in control *CSChrimson>w1118CS* +retinal (blue) or - retinal (gray) flies. n=27 flies, Two-way Repeated Measure ANOVA with Sidak's test. **C)** The number of food interactions (licks) per day on a control diet in experimental *MB011B >GtAcR1* +retinal (blue) flies or control n *MB011B >GtAcR1*- retinal (gray) flies n=24-25, Two-way ANOVA with Sidak's test, **$p<0.001$. **D)** The number of food interactions (licks) per day on a 20% sucrose diet in control *GtAcR1>w1118CS* +retinal (blue) or - retinal (gray) flies. n=24 flies, Two-way Repeated Measure ANOVA with Sidak's test. **E)** Triglyceride levels normalized to protein in age-matched male *MB011B >NaChBac* and control flies on CD (gray) or SD (teal). **F)** Triglyceride levels normalized to protein in age-matched male *MB011B >Kir2.1* and control flies on a CD (10% sucrose in food). n=8-11, one-way ANOVA with Tukey's test, comparisons to control diet; ***p<0.001, ****p<0.001. **G)** A model for how food associations regulate eating behavior and energy homeostasis, and the effects of a high sugar diet on this process.

2.4 Discussion

In this study, we found that the reinforcing properties of sucrose are down on a sugar diet. Downstream effects include the loss of food association, which we found is a critical component of feeding behavior. Specifically, it hinders satiation-dependent neural adaptations to control the quantity and size of a meal. We show that these adaptations turn into maladaptation when dopamine transmission is not reflective of the sweet taste experience due to the decrease in reinforcement from sucrose. The reciprocity of dopamine transmission initiation and termination between *MB301B* and *MB011B* might determine the short-term engagement that instructs the course of a meal. These changes are encompassed within the β' compartment of the mushroom body, which is known for mediating short-term sweet taste memory formation (Huetteroth et al. 2015; Yamagata et al. 2015). Presumably, we believe that exposure to a diet high in sugar triggers a strong food memory formation towards these foods that remains even after the circuit's response to sugar is dampened due to decreased sweet taste and dopamine transmission. Consequently, the learning center does not update its current memory, disrupting the learning process that leads to satiation (Figure 7G, summary). These changes are independent of the obesity state and pinpoint to a direct cellular and molecular intervention by the sucrose itself, most likely beginning at the sweet taste loci. Furthermore, it underscores how weight is not a direct nor reliable measurement of healthy eating habits in lean individuals.

Food association is traditionally viewed as a neural mechanism that allows animals to come back to specific scenarios that result in advantageous outcomes and promote survival (Allen 2012; Sonnenberg et al. 2019; de Vries et al. 2020). In the food environment, these served to remind the animal that food was found when certain conditions were met, such as a smell or a visual cue, and even prioritized certain memories in order for survival to be maximized (Plaçais

and Preat 2013). Furthermore, the degree to which the particular food elicited a reinforcing response, the stronger the association and the more likely the animal would come back for food (Di Ciano and Everitt 2004; Zhang et al. 2013). Previously, we had found that the neural activity of stmPAM neurons was down on a sugar diet (May et al. 2020) but we had not determined whether this decrease led to any potential behavioral phenotypes that suggested a decrease in the reinforcing properties of sucrose. In this study, we used food associations as a tool to measure the reinforcing properties of sucrose given that generally the degree of reinforcement of an unconditioned stimulus would correlate with the intensity of the CS+ seeking behavior in an associative learning and memory assay(Tully and Quinn 1985; Kaun et al. 2011; Petruccelli et al. 2018). It would serve as a measurement for potential impairments in the neuronal correlates of reward and food associations due to the sugar diet. Our data suggests that the reinforcing properties of sucrose are decreased on a sugar diet. Specifically, we found a decrease in preference index on the T-maze assay after food associations training while on a sugar diet, consistent with the idea that the dietary environment has an effect on behaviors that promote or hinder survival (Savage et al. 2007; Musselman et al. 2011; Stinson et al. 2018; Lee et al. 2019; May et al. 2020; Vaziri et al. 2020). The fact that the neural circuit of food associations are disrupted through decreased reward input further highlights the challenges imposed by an unhealthy dietary environment. Taking these findings and diving deeper into the neural circuits reveals that indeed, the neurons that are known for supporting the functions of food associations are impaired on a sugar diet, and their activity might directly be correlated with the aforementioned decreased reinforcing properties of sucrose. Specifically, *MB011B* and a subset of other glutamatergic neurons are mostly in an active state. It is thought that through the input of sensory and reward/punishment, they direct avoidance and thus, by being active, they create a

neuronal state of avoidance that promotes caution over riskier behaviors (Aso et al. 2014; Owald et al. 2015; Bouzaiane et al. 2015; Owald and Waddell 2015; Yamagata et al. 2015; Huetteroth et al. 2015). This would serve as a baseline that dependent on the environment can change to further increase the state of avoidance or decrease the activity so that approach is favored. In our assays, we promote approach by pairing an odor to sucrose. This pairing causes a decrease in the neural activity of these neurons that then promotes approach and is the hallmark food associations. That flies on a sugar diet do not exhibit this trait, suggests that sucrose is directly dampening its own signal to the brain, most likely through decreased sensory input that then reflects on decreased reward input (May et al. 2020; May et al. 2019; Vaziri et al. 2020). It is not due to decreased odor-evoked responses nor sucrose dampening this pathway given that flies on a sugar diet can smell aversive odors to a similar extent as it's control diet counterpart (Supplement 3A and B). It's possible to point directly to the diet as the relevant protagonist of this maladaptive behavior with an underlying circuit dampening component given that flies that are genetically programmed to stay lean and not store fat, are still not able to have increased preference index on a sugar diet (Figure 1C), which suggests that the decrease food associations is due to diet and not obesity. In addition, when representing data to showcase effects of diet on neural activity we see that depending on the diet, the neural response will be different post-training. Specifically, we see that flies on a sugar diet have increased calcium responses to the CS+ than the control diet, while similar calcium responses to the CS-, suggesting diet-specific effects rather than being odor-specific (Figure 2E, F). Furthermore, control diet flies and sugar diet flies exhibit the same calcium response to a novel appetitive odor post-training, suggesting that the neural pathways controlling naive responses to odors are not changed due to the training regime. A different explanation for the decrease in food associations on a sugar diet is that flies

are simply not motivated to create these associations due to either their energy reserves not being reduced enough after 30hrs of fasting. However, this is highly unlikely since flies fasted for 48hrs still exhibited no preference index, and at this point, survival was of 10 - 30%, most likely due to extreme conditions of starvation - flies were kept well hydrated through the procedure. Hence, lack of motivation is not behind the observed decrease in preference index. Another possible explanation is that these flies are not interested in sucrose anymore because of its high presence in the food during the 7 days of sugar diet. However, this is also unlikely given that flies on 7 days of sugar diet that are tested on the proboscis extension reflex, where 0 means no interest and 1 means maximum interest, exhibit numbers around 1, or maximum interest, towards 20% sucrose solution (May et al. 2019). Hence, the behavioral and circuit level analysis point to a decrease in the reinforcing properties of sucrose as the main reason why there's a decrease in food associations. There are other types of memories that could be affected by a sugar diet. We also tested long-term memory (data not shown) and similarly found a decrease in the preference index after 48hrs post-training. There is also aversive associative learning and memory, which could also be affected by a sugar diet. However, given that the utilization of this assay was aimed at evaluating the reinforcing properties of sucrose through associative learning and memory, it is evident that the reasonable choice was the use of sucrose and hence, the use of an assay that reflects appetitiveness.

But is it really sucrose reinforcement that is down? Not only is reward input down on a sugar diet, but the sensory inputs of Gr64f neurons that may be connected to the stmPAMs, MB301B, are also down (May et al. 2019). MB301B are stmPAMs with axons in the β' compartment of the mushroom body. This region has been seen to be involved in caloric sensing (Huetteroth et al. 2015), which ties with its connectivity to Gr64f neurons that transmit sweet

information. Together, they deliver sweet information that is altered on a sugar diet, suggesting that the caloric value of these foods by being dampened, also decreases the reward input that is ultimately transmitted to the *MB011B* . Calories are a form of reward in itself, especially during circumstances of starvation (Frank et al. 2008; Chambers et al. 2009; Smeets et al. 2011). On a sugar diet, caloric value is not fully transmitted to the brain, and therefore the interpretation of these reward signals as dopamine transmission are also not encoded on the *MB011B s*. There are two main ways that this was measured. First, we looked at whether taste information in the presence of sucrose in flies on a sugar diet was transmitted to the *MB011B s* through measurements of dopamine levels at the *MB011B* dendrites in the β' compartment (Figure 3B). We observed that ultimately, the layout of the circuitry represents an overall dampening of the reward signal from beginning, sensory input, to the pre-motor neurons, *MB011B* . This begins to create a picture that demonstrates how decreased dopamine transmission is the main culprit behind the lack of food associations. Taking it a step further, it was prudent to then test whether CS+ evoked dopamine transmission was also down on a sugar diet (Figure 3D). Even though dopamine transmission was decreased when given sucrose to taste, it still lacked clarity on whether this would translate into decreased reinforcement of sucrose. Paring the odor to sucrose and measuring dopamine transmission proved that the inability to perform food associations comes from the decreased reinforcing properties of sucrose, as observed by no changes in dopamine transmission when presented the CS+ in flies on a sugar diet. Furthermore, they are diet-dependent and not odor-dependent. Further evidence to support the role of the decrease in this sensory to reward transmission in impaired food associations is that utilizing OSMI-1 restores dopamine transmission and calcium responses to the CS+. We previously showed that OSMI-1 directly rescues MB301B neural activity and food intake/ satiation (May et al. 2020).

The extent of this involvement is beyond scope, but it again underscores how dampening of this sensory to reward pathway is behind the decreased reinforcing properties of sucrose that lead to the impairment of food associations. To further show that a sugar diet decreases the reinforcing properties of sucrose, we tested the necessity of MB301B by coupling optogenetics with the T-maze. Utilizing light as a complementary reward stimulus to sucrose taste and intake, we showed that on a sugar diet, the baseline dopamine transmission in the presence of sucrose is not enough, and that these dopaminergic neurons required activation for proper food associations. Together, these show that taste-*MB301B*-*MB011B* pathway is compromised on a sugar diet, which consequently hinders food associations. The result of such a behavior most likely is the inability to update coded information about the experiences with the food environment, leading to detrimental consequences such as obesity. *MB301B* and *MB011B* could also be forming a feedback loop, where *MB011B* signals MB301B about the status and progression of a meal to fine tune dopamine transmission (Ichinose et al. 2021). Since sucrose is a powerful natural reward, it makes sense that it would create a strong memory at the beginning of exposure to this dietary environment. However, as this exposure to sucrose becomes a diet, sucrose becomes less reinforcing, and there is an inability to perform food associations. Furthermore, the lack of feedback from *MB011B* to *MB301B* might exacerbate this disjunction. This in turn, brings about detrimental consequences such as obesity, that can ultimately lead to death, given that the negative consequences of sucrose are not registered, impeding the updating of the previous strong sucrose memory formed during the first days.

It is no secret that food and dopamine are largely intertwined. High calorie foods, if not always, result in changes in the dopamine system that have been traced specifically to changes in dopamine-type 1 and type 2 receptor availability in the reward center of mammals, the striatum

(Wang et al. 2001; Szczypka et al. 2001; Bello et al. 2002; Volkow et al. 2008; Johnson and Kenny 2010; Stice et al. 2010; Stice and Yokum 2016; Han et al. 2021). In flies, little to no data exists on the impact of a sugar diet on the dopaminergic system. Furthermore, in flies, the broader role of both of these receptors is also largely unknown with D2R being the most elusive. In mammalian studies, dopamine can be linked to food-related behaviors such as meal pleasantness, go/no-go signals, food cues, intake and satiation, among others (Bello et al. 2002; Johnson and Kenny 2010; Love et al. 2020; Stice and Yokum 2016; Han et al. 2021). This broad range of behaviors makes it particularly difficult to dissect the different stages of the food behavior cycle. However, the role of dopamine in learning is evident by its reinforcing properties in the presence of cues and has been extensively studied in drug addiction and to a lesser extent in food. Furthermore, across organisms, the absence of dopamine completely abolishes the ability to create food memories (Szczypka et al. 2001; O'Carroll et al. 2006; Busto et al. 2010; Rossato et al. 2009; Plaçais et al. 2012; Musso et al. 2015; Takeuchi et al. 2016). In the context of food, decreased dopamine tends to render meals unpalatable and inhibit food seeking and food ingestion behaviors (Szczypka et al. 2001; Liu et al. 2016; de Macedo et al. 2016; DiFeliceantonio and Small 2019). The process of food seeking and food ingestion most often is coupled to the process of learning, given that food and cue associations allow for encoding of prediction errors that update the decision-making process and provides context for a behavior that repeats and leads to survival. Hence, food association is an important component of eating behavior and depends on dopamine and the subsequent activation of motor pathways that drive the action and the consumption of foods. We found that in flies exposed to a sugar diet, the reinforcing properties of sucrose are down. This decrease means that the ability of flies to perform food associations is also down. Furthermore, this is not only reflected in the in-coding of

food information but also in the out-coding and subsequent transmission of food-related information to motor outputs innervated by the *MB011B* (Figure 5). Since glutamatergic MBONs are necessary for motor output of feeding mechanisms such as proboscis extension, it is reasonable to believe that the reflection of poor food memories in the output of *MB011B* ultimately reflects on feeding motor behaviors. However, this is specifically for the conversion of new information into a behavioral action, not to the updating of a memory that allows for a change in behavior. In this study, at the initial exposure of flies to the sugar diet, having a system intact means that the rewarding properties of sucrose at a high concentration are deeply ingrained and converted to the expected motor output of food intake until mechanoreceptors in the stomach signal satiation. Hence, the subsequent decay of reward information at the later days of the sugar diet means that the inability of *MB011B* to code for new food association and create new memories impedes the updating of signals to the motor outputs that under normal circumstances would serve as satiation signals to stop eating (Landayan et al. 2018; Chia and Scott 2020). This can be appreciated by the necessity and sufficiency of *MB011B* in feeding behavior during a 7 days sugar diet, and points to the direct role of the appetitive associative neurons *MB011B* , in food intake (Figure 7). Under normal conditions, feeding leads to the initiation of satiation signals that stop the consumption of food (Landayan et al. 2018; Chia and Scott 2020). These satiation signals that are induced by the consumption of food, directly degrade the previous food memories established before and at the beginning of the meal cycle, hence engaging in the termination of meal. Therefore, it is most likely that on a sugar diet, satiation signals are not engaged to degrade the initial food association that contributes to the increase in activity of *MB011B s*, signaling aversion and the termination of the meal.

In conclusion, the neural mechanisms of reward-based satiation signals to terminate meal consumption such as the use of food associations are non-existent on a sugar diet due to decreased dopamine transmission, and instead promote food consumption that exceeds their normal quantities, leading to obesity (Figure 7G). The aversive state of *MB011B s* that predominates in a satiation-dependent manner are diminished on a sugar diet. stmPAM to *MB011B* dopamine transmission might act as a sensor of the timing within the course of a meal to serve as a feedback loop that decreases dopamine transmission to trigger satiation signals. The lack of dopamine transmission to *MB011B* then suggests that there isn't an opportunity for updating the food memories that would then trigger a feedback signal to decrease dopamine transmission and terminate the meal. It is specifically the delta in neural activity of *MB011B* that triggers these changes more so than the input signal, which is why even when stmPAM neural activity is decreased in the presence of sucrose and on a sugar diet, this new baseline amount of dopamine transmission doesn't elicit the satiation responses necessary to trigger *MB011B s* aversive states for terminating the meal. This feedback loop might be orchestrated by possible differences in the types of dopamine receptors on the *MB301B* and the *MB011B* , similarly to the go/no-go signal mediated interplay within the striatum that allows for a flexibility in behavior and the appetitive or aversive nature of a particular stimuli. It would be then interesting to investigate whether the dopamine-type 1 and type 2 receptors in flies also have similar intracellular mechanisms in these two neurons and whether they are changed on a sugar diet. Perceivable changes might pinpoint to a dysfunctional feedback loop that would corroborate our findings of a satiation-based dopamine transmission decay. It's hence necessary to understand at the finer points the basis of overeating to serve as educational resources to lift the stigma behind obesity as a matter of choice and more as a disease that specifically hijacks the neural basis of

meal termination. Furthermore, refining existing policies on what the daily sugar servings as well as how we regulate the generous amount of sugar in foods might lead to a curving in the obesity epidemic.

2.5 Method Details

Fly lines and preparation:

Obesity All flies were maintained at 25°C in a humidity-controlled incubator with a 12:12 hr light/dark cycle. For all experiments, males were collected under CO2 anesthesia, 2–4 days following eclosion, and housed in groups of 20–30 within culture vials to age until 4-5 days old. The stocks used are listed in the Table 1. As a control, we used w1118Canton-S flies (gift from Anne Simon, University of Western Ontario), which were obtained by backcrossing a w1118 strain (Benzer lab, Caltech) to Canton-S (CS) (Benzer lab, Caltech) for 10 generations.

Dietary manipulations:

Flies were transferred to vials containing respective diets 4-5 days after eclosion and left on their food for 7 days; fresh food was provided every other day.

The composition and caloric amount of each diet were as follows:

- 'Control Diet/CD' was a standard cornmeal food (Bloomington Food B recipe), with approx. 0.6 cal/g.
- 'Sugar Diet/SD' was 30 g of table sugar added to 89 g Control Diet for 100 mL final volume of 30% sucrose w/v, with approx. 1.4 cal/g.
- For diets supplemented with OSMI-1, the inhibitor was added to CD or SD food at a concentration of 10 μM.

- For diets supplemented with all-trans-retinal, the final concentration was 200 mM in food.
- The sucrose for the optoFLIC was dissolved in 4 mg/L MgCl2 and consisted of 10% (CD) or 20% (SD).

Appetitive conditioning on the T-maze

Adult age-matched male flies, following 7 days of CD or SD, were fasted on a wet Kimwipe for 24hrs or 36hrs, respectively, before the behavioral assay. Flies were then allowed to acclimatize for 2 hours in a dark behavior room at a temperature of 24°C and humidity of 50%. We utilized a horizontal T-maze described in (73). Following acclimatization, appetitive training was performed as described (130). Briefly, flies were exposed to 0.1% of the CS- for 2 min followed by 30 seconds of air and then to 0.1% of the CS+ in the presence of 2M dry sucrose for 2 min. All behavioral experiments were performed in reciprocal (averaged between alternative order of odors) testing dietary conditions and genotypes in parallel. For testing flies were given 2 min to choose between the CS+ and CS- in the T-maze. Performance index (PI) was calculated as the number of flies approaching the conditioned odor, minus the number of flies going the other direction, divided by the total number of flies in the experiment (CS+) - (CS-)(CS+) + (CS-). A single PI value is the average score from flies of the identical genotype tested with the reciprocal reinforced/ non-reinforced odor combination, as shown in (130, 131).

To assay naïve responses to odors, fasted flies were exposed to two 0.1% of MCH or 0.1% OCT vs paraffin oil in the absence of conditioning, the arms in which the odors were presented were switched in half of the experiments. Preference index was calculated as the number of flies

approaching the odor minus the number approaching paraffin oil, divided by the total number of flies in the experiment (Odor A/B) - (Paraffin oil)/(Odor A/B) + (Paraffin oil).

Two-Photon in vivo imaging:

Adult age-matched male flies on 7 days of CD or SD exposure were fasted on a wet Kimwipe for 24hrs or 30hrs, respectively, before conducting the imaging. Flies were prepared for imaging with their head tilted forward under a small Petri dish with an empty space on top of the head cuticle to expose the brain to the artificial hemolymph and to access the antennae and proboscis for odor and sucrose delivery as described in (132). The fly's legs were removed to avoid interference with odor and sucrose delivery. Artificial hemolymph consisted of 51.5 mM NaCl, 2 mM MgCl2, 13 mM NaHCO3, 0.5 mM NaH2PO4, 0.75 mM CaCl2 -2H20, 1.5 mM KCl, 2.5 mM HEPES, pH to 7.00 - 7.10, prepared 24 hrs before and allowed to reach room temperature before use.

Conditioning: Flies were placed under the objective and presented with a modified conditioning protocol based on (71, 91): 1) Testing Pre-training responses: head-fixed, naive flies were exposed to 2" of 0.1% 4-methylcyclohexanol (MCH) and then to 2" of 0.1% 3-octanol (OCT) (with 30" of air stream in between); 2) Training for Appetitive Conditioning: After 30" of rest from the end of sequence in 1), one odor was presented for 2min (CS-); after 30" of rest, the second odor was presented for 2min while 2M sucrose (US) was offered (CS+); odor pairing was reversed in half of the animals; 3) Testing post-training responses: After 2.5min of rest, the post-conditioning calcium responses to the two odors were measured while animals were exposed to the same odor sequence as 1, with the addition of a new odor (0.1% isobutyl acetate, IA) as

control. The odor presentation before and after conditioning was done twice and responses averaged when available, except for refeeding assay, where, due to limited time, it was done once. To test responses to cues after feeding, flies were conditioned as described above; after the last odor presentation after training, they were fed 2M sucrose for 30 minutes as AHL was replaced manually. After 30 minutes, the odor sequence (1) was presented again. Imaging data were acquired with a two-photon microscope, 20x water immersion objective, and at a rate of 15Hz, with a resolution of 1024 x 1024 utilizing a resonant scanner. The odor delivery and imaging system was as (70, 132).

KCs responses: Same method as described above under "testing pre-training responses".
Sweet responses: Under similar preparations before training, flies were placed under the two-photon microscope and were given 30% sucrose to taste for 1 second using an electronic manipulator.

Imaging data analysis

Fiji was used to manually draw regions of interest around the β′2 *MB011B* dendrites or axons in both hemispheres and quantify the relative difference in fluorescence intensity (ΔF/F0) before (F0 5 frames) and after stimulus onset (F). To measure the difference in CS+ and CS- responses with conditioning (Norm peak ΔF/F) we normalized the peak responses after conditioning to those of naive flies [(ΔFpost/F0- ∑ΔFnaive/F0)/∑ΔFnaive/F0 *100].

Closed-loop optogenetic stimulation in Fly-to-Liquid-food Interaction Counter (OptoFLIC)

The feeding behavior was measured using the Fly Liquid-Food Interaction Counter (FLIC), described previously (133). Briefly, adult flies were placed on +/- retinal food and kept in the dark for 5 days until the start of the experiment. The feeding behaviors were recorded inside a 12-hour dark/dark cycle incubator with 40-50% humidity and 25°C. The LED activation protocols were as follows and activated only when the fly interacted with the food: For experiments with *MB110B>CsChrimson*, 100 ms of red (~627 nm) light pulsing at frequency 60 Hz and with a pulse width of 11 ms was triggered by every food interaction signal over 10. For experiments with *MB011B >GtACR1*, 100ms of green (~530 nm) light pulsing at a frequency of 20 Hz and with a pulse width of 11ms was triggered by every food interaction signal over 10. Analysis of daily food interactions was as previously described in (62); the R code used can be found on Github (https://github.com/chrismayumich/May_et_al_optoFLIC; copy archived at https://github.com/elifesciences-publications/ May_et_al_optoFLIC).

Triacylglyceride (TAGs) Assay

The levels of TAG and protein were measured as previously described in (134), n=1 equals 2 flies.

Immunofluorescence

The expression of *MB011B >CD8::GFP* was visualized as previously described (135).

Statistics

Prism GraphPad was used to analyze the normal distribution (Shapiro-Wilk test) and significance of data. Statistical tests and sample size can be found in each figure legend.

2.6 Supplemental Figures

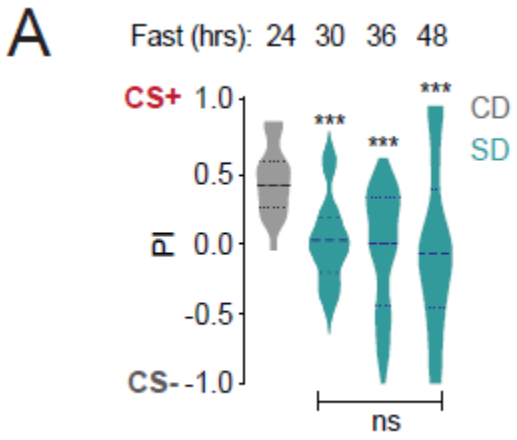

Figure 2.8 Changes to food associations at different diets and fasting times. Related to Figure 1

A) The preference index (PI) of *w1118*^{CS} flies on a CD (Gray) or SD (Teal) diet under different fasting times. n= 22 (30 hr SD), n=38 (36 hrs SD), n=26 (48 hrs SD), and n= 20 (24 hrs CD), 25 flies per n. Kruskal-Wallis with Dunn's multiple comparison test, ***p<0.0001.

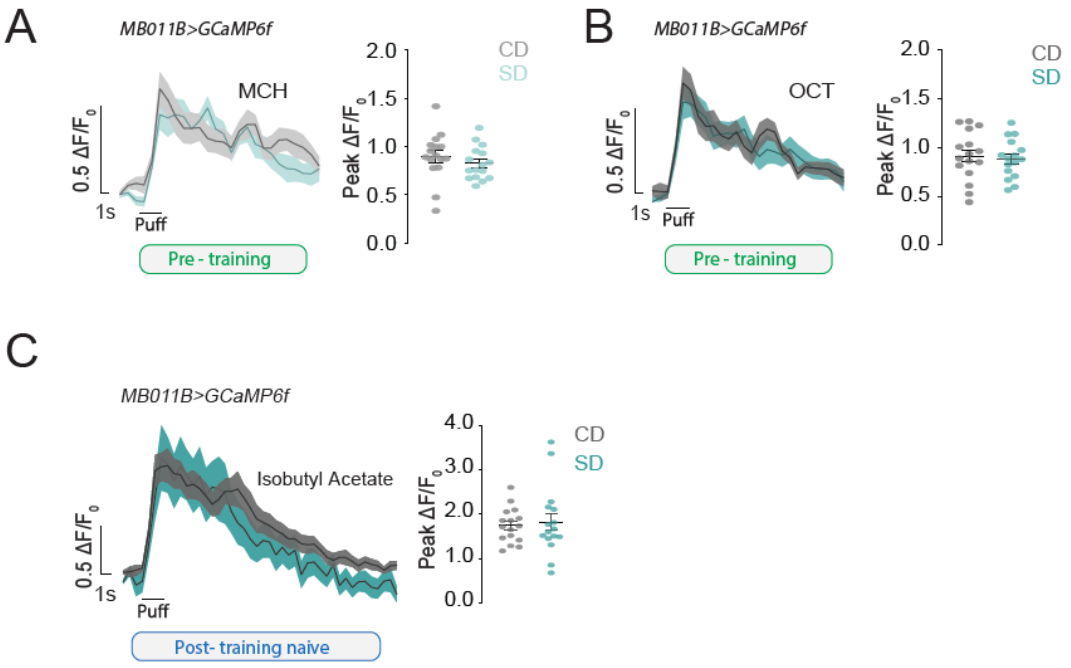

Figure 2.9 Calcium responses comparing CD vs SD. Related to Figure 2.

A, B) The naive calcium responses to MCH and OCT (puff) in the β′2mp dendrites of *MB011B >GcAMP6f* neurons before training in CD (gray shades) and SD (teal shades) flies; data are shown as mean +/- SEM, $\Delta F/F_0$ traces, and quantified as maximum peak $\Delta F/F_0$ response. Data from Figure 2C and D. **C)** The calcium responses to a novel odor (Isobutyl acetate) post-training shown as mean $\Delta F/F_0$ traces and quantified as maximum peak $\Delta F/F_0$ in CD (gray) and SD flies (teal). n=16; Student's t-test. Data shown as +/- SEM.

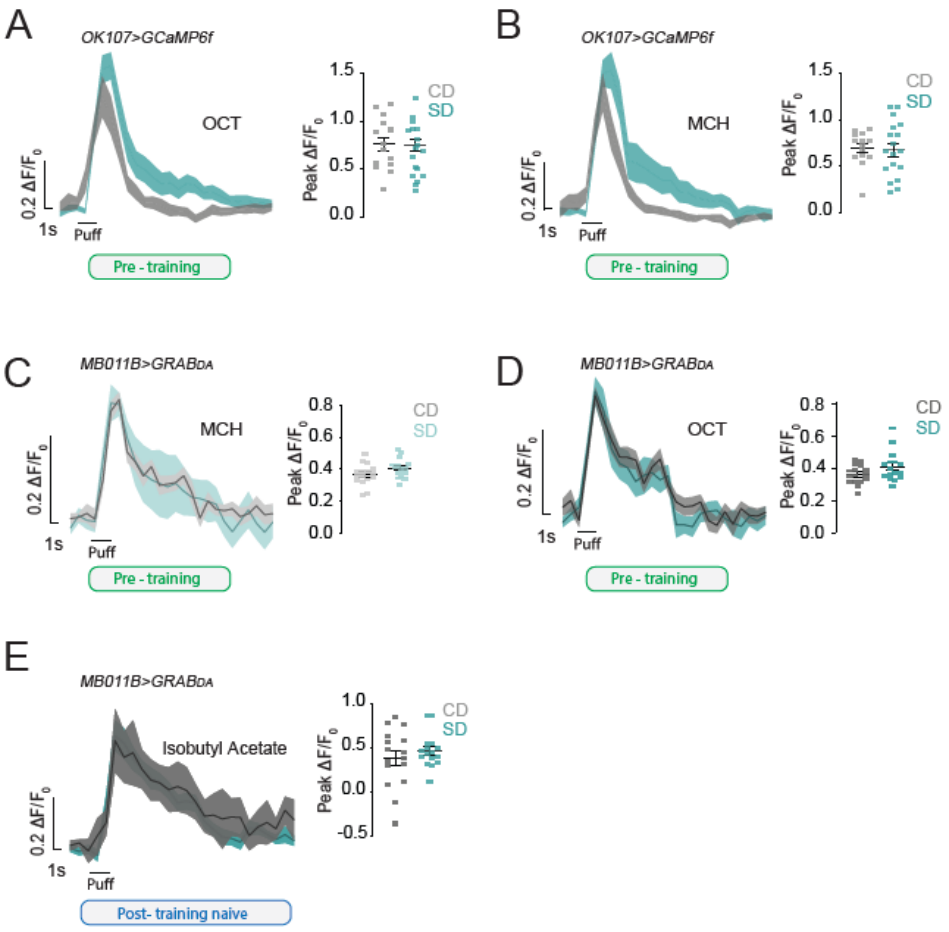

Figure 2.10 Naïve odor responses and dopamine transmission between CD vs SD. Related to Figure 3.

A-B) The calcium responses to OCT (A) and MCH (B) (puff) in the β′2 axons of Kenyon Cells of naive *OK107>GCaMP6f* flies fed a CD (gray) or SD (teal) shown as mean $\Delta F/F_0$ traces and quantified as maximum peak $\Delta F/F_0$ response. n=14-18, A) Student's t-test, B) Mann-Whitney Test. **C-E)** The mean $\Delta F/F_0$ traces and quantification of maximum peak $\Delta F/F_0$ response to CS- (C) and CS+ (D) pretraining, or a novel odor post-training (E) in the β'2 dendrites of CD (gray) and SD (teal) *MB011B >GRAB$_{DA}$* flies before training. Data from Figure 3C and Figure 3D. n=16; Student's t-test. Data shown as mean +/- SEM.

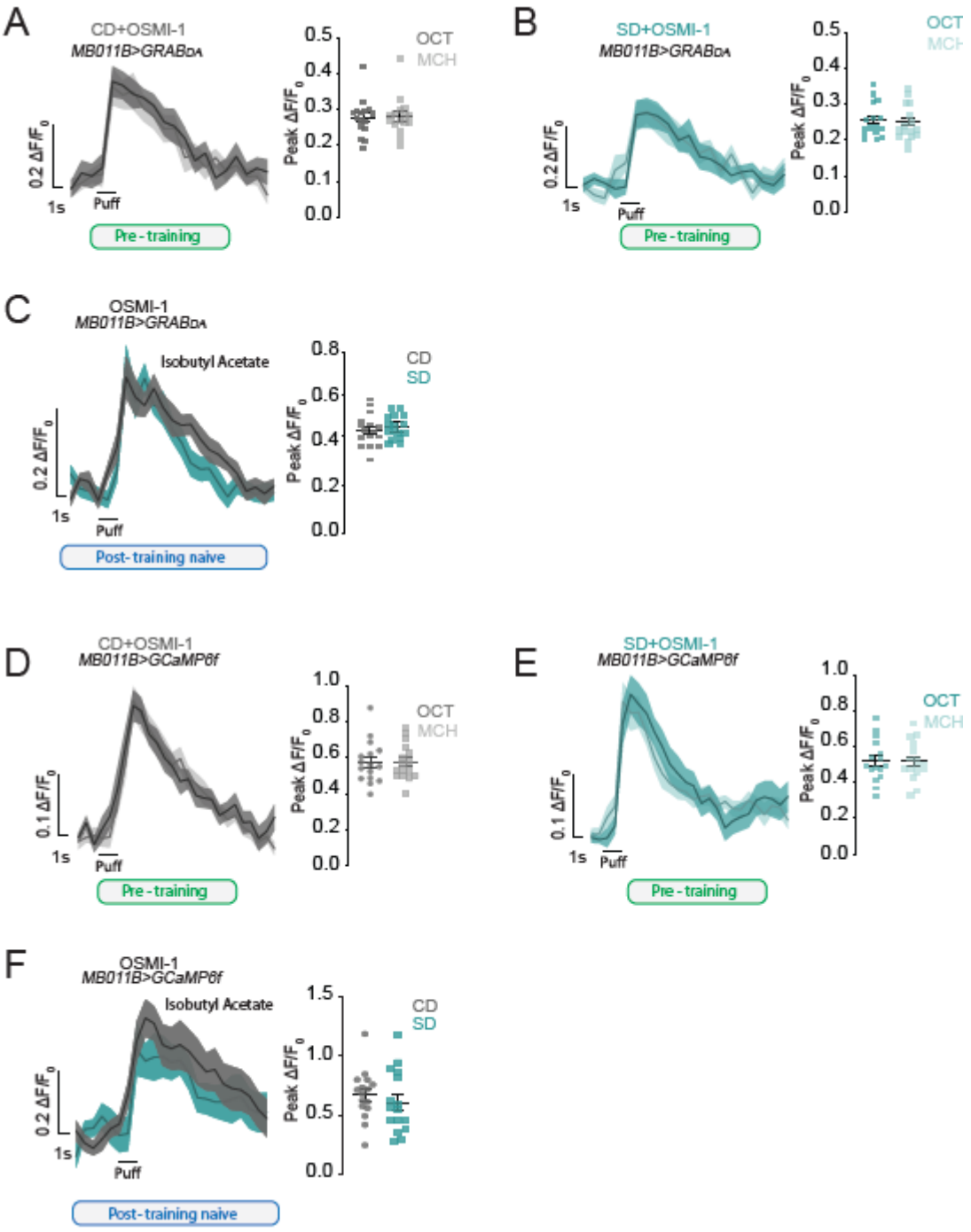

Figure 2.11Comparison between CD and SD of flies on OSMI-1. Related to Figure 4.

A-C) The mean $\Delta F/F_0$ traces and quantification of maximum peak $\Delta F/F_0$ response to MCH and OCT pretraining (A-B), or to a novel odor post-training (C) in the β'2 dendrites of CD+OSMI-1 (A, gray, Mann-Whitney), SD+OSMI-1 (B, teal, Mann-Whitney), and CD vs SD both with OSMI-1 (C, Student's t-test) in *MB011B >GRAB-DA* flies. n=16

flies, Data shown as mean +/- SEM. **D-F)** The mean $\Delta F/F_0$ traces and quantification of maximum peak $\Delta F/F_0$ response to MCH and OCT pretraining (D, E), or to a novel odor post-training (F, Student's t-test) in the β'2 dendrites of CD+OSMI-1 (D, gray, Student's t-test), SD+OSMI-1 (E, teal, Student's t-test), and CD vs SD both with OSMI-1 (F) in *MB011B >GCaMP6f* flies.. n=16 flies. Data shown as mean +/- SEM.

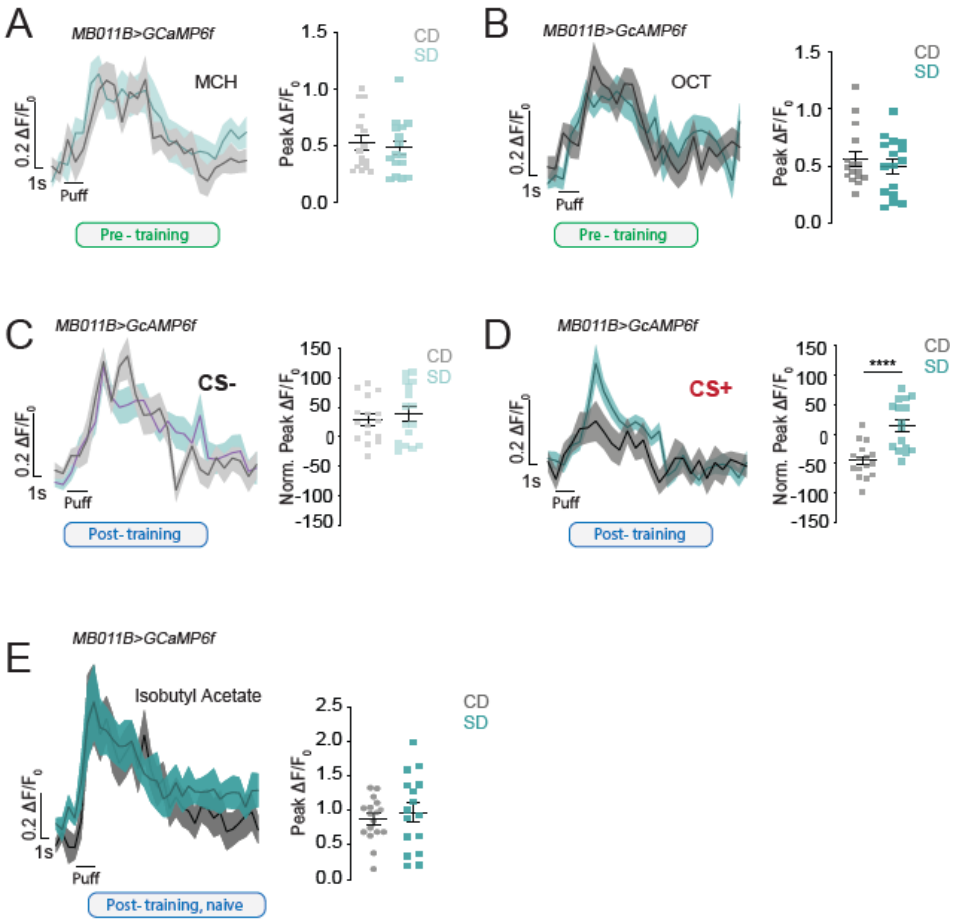

Figure 2.12 Output of *MB011B* MBONs between CD and SD flies. Related to Figures 5.

A-B) The pre-training mean calcium $\Delta F/F_0$ traces and quantification of maximum peak $\Delta F/F_0$ response to CS- and CS+ in the axons of CD (gray) and SD (teal) in *MB011B >GCaMP6f* flies. n=16 flies, Data shown as +/- SEM, Mann-Whitney. **C-E)** The post-training mean calcium $\Delta F/F_0$ traces and quantification of maximum peak $\Delta F/F_0$ response to CS- (C, Mann-Whitney) and CS+ (D, Student's t-test), or to a novel odor post-training (E, Student's t-test) in the axons of CD (gray), and SD (teal) *MB011B >GCaMP6f* flies. Data from Figure 5C and Figure 5D. n=16; Data shown as +/- SEM.

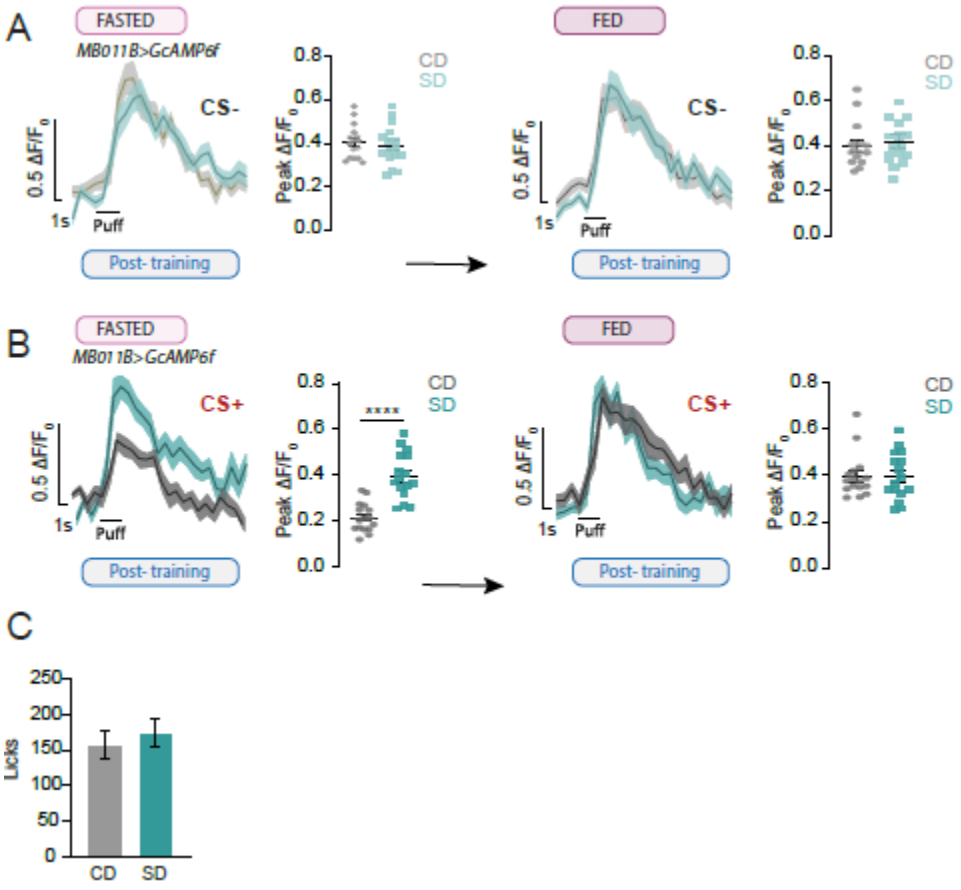

Figure 2.13 Fasted and fed flies on a CD vs SD, as well as feeding on 2M sucrose . Related to Figure 6.

A-C) The presynaptic calcium responses to the CS- (A) and CS+ (B) in *MB011B >GCaMP6f* CD (gray) and SD (teal) flies after appetitive conditioning, before (left, fasted) or after (right, fed). Data from Figure 6B and Figure 6C. n=16; Data shown as +/- SEM, A) left Unpaired T-Test, right Mann-Whitney, B) left Unpaired T-test, right Mann-Whitney test; ****p<0.0001. **C)** The number of feeding interactions (licks) for flies feeding on 2M sucrose during a 30 min period; CD (gray) and SD (teal). n=16-24, Student's t-test.

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Chapter 3 Dop2R Modulation of Food-Related Behaviors

3.1 Abstract

The dopaminergic system modulates many of our everyday activities such as food related behaviors. It allows for the formation of food memories that in turn modulate food intake via integration with other pathways that allow associations to be formed. The goal of this study is to determine the role of the fly dopamine-type 2 receptor (Dop2R) in food intake and food associations. We hypothesize that a sugar diet leads to homeostatic upregulation of Dop2R and lead to diet-induced obesity and impaired food associations. Using genetics, biochemical, and behavioral assays, results showed that Dop2R is necessary for diet-induced obesity and for food memories. Our results demonstrate that a sugar diet dysregulates energy balance and food associations through upregulation of Dop2R. In conclusion, our results confirm the dysregulation of Dop2R in mammals and advances the neurobiological substrates of obesity.

3.2 Introduction

Food intake relies on a feedback loop between energy intake and energy expenditure [1–4]. This process is highly dependent on dopamine [5,6] and can be broken down into several components that together add up to mediate this process. One of these underlying mechanisms regulated by dopamine is reward motivated behavior [7–12]. Upon presentation of a new and unexpected rewarding food, the brain secretes dopamine with the purpose jumpstarting the process of appetitive associations [13,14], a process called liking [5,15] but through repeated exposure, becomes paired to a cue - e.g. smell - instead of responding directly to the food itself [16,17]. These food

memories have the ability to shape the animals response at the short term and long-term level, providing a mechanism for survival in an ever changing food environment to meet its energy needs [18–20]. It's been hypothesized that the inability to modulate food memories in response to new information is in part responsible for the current obesity epidemic [21,22]. Indeed, data suggests that in obese humans, their ability to modulate food memories in face of a negative outcome is impaired [18,23,24].

Dissecting the dopaminergic system reveals the role that the interplay between the Dopamine-like type-1 (D1R) and Dopamine-like type-2 receptors (D2R) have on modulating food memories and subsequently food intake. D1R serves as a go signal (drives a behavior) [25,26], while D2R serves as a no-go signal (inhibits a behavior) in food intake [27,28]. Human and mammalian studies have shown that obese humans have an increase in D1R [25,26,29–31] and a decrease in D2R [27,28,32–36] in the striatum, the reward center of the mammalian brain. Closely knitted is evidence to suggest that decreased taste information leads to decreased dopamine transmission [37–40]. Decreased D2R levels in obese and mammalian subjects suggests the lack of learning from negative outcomes, given its role in curving increased food intake [25-28,33-36]. However, it is still unclear whether decreased taste information directly results in decreased D2R levels [18,37,39]. In addition, it is still unknown whether it's the D2R changes that lead to obesity [18,23–25,31]. These questions have been complicated to answer due to the anatomical and functional complexity of the animal model, where dopamine plays a wide range of roles and there exists a multitude of brain projections. To better tackle these questions, it is necessary to look for an animal model that allows for precise intervention, which will facilitate the advancement of our understanding of the role of D2R in food intake and how this is affected by diet. We used the D.

melanogaster to dissect the relationship between the fly analogues of D2R (Dop2R), food memories, food intake, and obesity and how these are deregulated by diet.

Flies, like vertebrates, will ingest increased amounts of food, gain weight, and develop the classic traits of chronic disease and metabolic syndrome on a sugar diet [41–43]. In addition, they possess a reward circuitry for food memories that functionally acts like in humans and mammals, allowing flies to create food memories [44]. Furthermore, food memories are also encoded utilizing dopaminergic neurons and peripheral sensory information that are conveyed onto the food memory center of the fly, the mushroom body (MB) [45,46]. Previously, we showed that a sugar diet leads to decreased neural responses to sucrose by dopaminergic neurons innervating the β' compartment of the MB [47]. Hence, we focused on the neurons that form food memories, the mushroom body output neurons (MBONs) and found that indeed decreased dopamine transmission leads to impairments in food memories (Pardo-Garcia et al. 2022, *in review*). However, the molecular mechanisms of the decrease in dopamine signaling remained elusive. Hence, we sought to determine the molecular mechanism of decreased learning plasticity and increased feeding, as well as fat accumulation on a SD. We showed that disruption to food memories is not only due to decrease in dopamine-induced plasticity (Pardo-Garcia et al. 2022, *in review*) but also due to decreased Dop2R in the MBONs. We also demonstrate that increased weight gain due to a SD is the result of decreased Dop2R in these MBONs. Together, our experiments unravel the neural substrates of how weight gain is modulated by dopamine-receptor specific changes and determine the mechanisms through which diet dysregulates food memories to promote obesity.

3.3 Results

Dopamine causes further inhibition of *MB011B* MBONs on a SD.

To investigate the effects of a high caloric diet on the dopaminergic system, we first performed RNA sequencing utilizing whole fly heads. Research in mammals has shown that a SD can lead to downregulation of D2R in mammals [30]. In addition, obese humans have also been shown to have decreased D2R availability correlating with body mass index (BMI) [48]. We previously had shown that a SD leads to decreased neural activity of PAM DANs when flies were exposed to sucrose taste [47]. In addition, dopamine availability at the dendrites of the *MB011B* MBONs was also down on a SD to be down following exposure to sucrose (Pardo-Garcia et al. 2022, *in review*). Hence, we hypothesized that changes in dopamine transmission due to a SD would result in a decrease in Dop2R availability. To our surprise results showed that a SD leads to increased Dop2R in the whole brain (Fig. 1A). The mushroom body holds the most amount of dopamine inputs than any other part of the fly brain [45,46,49]. In addition, dopamine lowers *MB011B* MBONs activity to induce food memories [44,50,51]. Based on these findings, we performed *ex-vivo* calcium imaging to determine the neural responses to dopamine infused into the bath holding the brain of a fly on a SD. We hypothesized that increased Dop2R will lead to further inhibition of the *MB011B* MBONs. When measuring dendritic calcium responses in *MB011B >GcAMP6f* flies, those on a SD were further inhibited by dopamine than the controls (Fig. 1B). In addition, adding dopamine to the *ex-vivo* preparation also further inhibited the output of *MB011B >GcAMP6f* flies on a SD (Fig. 1C), which is in line with the idea that Dop2R is inhibitory based on the G-protein it's coupled to, $G_{i/o}$[52]. Together, these results suggest that a SD potentially affects food memories by increasing Dop2R levels.

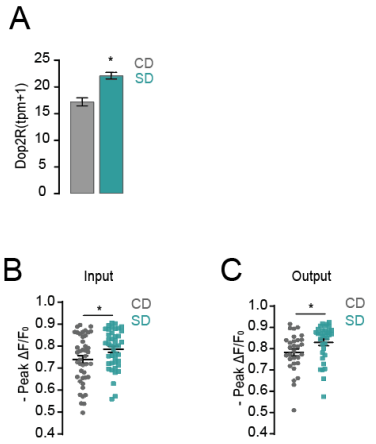

Figure 3.1. Dop2R **read counts and neural activity on a SD**

A) Number of reads for Dop2R after performing RNA Seq of whole fly heads of CD (gray) and SD (Teal). n = 2-3, each n = 300 fly heads; Data represented as +/-SEM, student t-test. **B, C)** The negative peak $\Delta F/F_0$ of the dendrites (B, n = 42-43, student t-test) and axons (C, n = 30-33, Mann-Whitney) of *MB011B >GcAMP6f* fly brains on a CD (gray) or SD (Teal) after applying dopamine to solution. D'Agostino & Pearson normality test, data represented as +/-SEM, *p<0.05.

MB011B -D2R KD rescues impaired food memories

The dopaminergic system is required for the formation of food memories as it relays information about the rewarding properties of the foods we eat [45,46,49]. During the formation of food memories, olfactory information is relayed by Kenyon Cells to allow for associations between the environment to be possible. A group of Mushroom Body Output Neurons (MBON) integrate the information to create a food memory that will guide the animal's food choices [44,49,53]. Among these MBONs there is a subgroup of 6 glutamatergic MBONs that are well defined in their contributions to appetitive olfactory conditioning, and they form the γ5, β′2a, β′2mp, β′2mp_bilateral subcompartments of the Mushroom Body [44,49]. We previously showed that the PAM DANs that innervate the β′ had decreased calcium responses on a SD to sucrose taste [47]. Furthermore, we also showed that a SD disrupts food memories by decreasing dopamine transmission (Pardo-Garcia et al. 2022, in review). Based on the data shown on Fig. 2, we hypothesized that the impaired food associations are further exacerbated by increased Dop2R. To test for this, we employed the T-maze [54]. Briefly, flies were put on a CD or SD for 7 days, followed by 24 or 36 hrs of starvation, respectively. Afterwards, flies were exposed to two different aversive odors. The first odor, which could be 3-Octanol or 4-Methylcyclohexanol (0.1%), would be paired with water and became the CS-. The subsequent exposure would be to whichever odor they were not exposed the first time at a concentration of 0.1% and would become the CS+ after being paired with 2M of sucrose. Flies were then gently pushed onto the middle of the T-maze and allowed to make a choice between two arms with the CS- and CS+ streaming on each side; Learned behavior would mean the flies moved towards the CS+ [44,45,50,51,55]. From the results shown in Pardo-Garcia et al. 2022 (in review), it is shown that flies on a SD can still smell these two odors at these concentrations. As a macro approach, we first

tested whether *Dop2R*$^{-/-}$ led to rescued food memory deficit on a SD (Fig. 2A) and compared our results to the *w1118*^{CS} flies on a CD only since based on Pardo-Garcia et al. 2022 (in review) we know that on SD these flies have a PI close to zero. Results showed that a global Dop2R knock-out led to a partial rescue of the SD (teal, Dop2R$^{-/-}$) when compared to the control groups (Fig. 2A). Given this partial rescue we further hypothesized that in order to have a full rescue, we would need to knock down Dop2R specifically from the dendrites of the *MB011B* MBONs. Hence, our next step was to utilize a *Dop2R-RNAi* to further test this on the T-maze. Results showed that knocking down Dop2R on the dendrites of the *MB011B* MBONs led to a full rescue of the group on a SD when compared to the controls, the *MB011B >w1118*^{CS}, and the *Dop2R-RNAi>w1118*^{CS} (Fig. 2B). Together, these data suggest that the food associations deficits seen on a SD are due in part to the upregulation of Dop2R receptors on the dendrites of the *MB011B* MBONs.

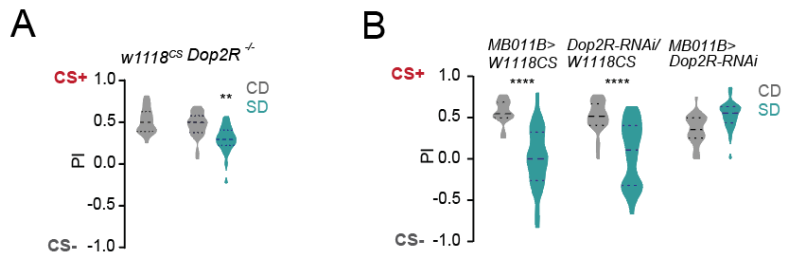

Figure 3.2 Dop2R **is necessary for food associations**

A, B) Preference Index (PI) of CS+ or CS- of Dop2R-/- (A) or *MB011B >Dop2R-RNAi* (B) flies on a CD (gray) or SD (teal). n=17-25, Data shown as +/- SEM. Ordinary One-way ANOVA with Tukey's test. *p<0.05, **p<0.01, ****p<0.0001.

***MB011B* -D2R KD rescues diet-induced obesity**

We then sought to determine the role of increased Dop2R expression on *MB011B* MBON dendrites in diet-induced obesity. To this end we performed a biochemical assay to measure the amount of triglycerides and protein levels in whole flies [56]. In mammals, D2R is known to be involved in food intake by regulating motivation, anticipatory reward, energy expenditure, among others [33,57–60]. Furthermore, diet and obesity have a direct and indirect contribution to the levels of D2R in the reward center of the mammalian and human brain [30,48]. Hence, we hypothesized that increased Dop2R levels on *MB011B* MBONs also contributes to diet-induced obesity in flies. We began by utilizing $Dop2R^{-/-}$ to test whether whole brain absence of Dop2R could rescue diet induced obesity (Fig. 3A). The $w1118^{CS}$ showed that on a SD the triglycerides normalized to protein were higher than the CD, which is consistent with our previous findings [41]. On the other hand, the $Dop2R^{-/-}$ showed that global disruption of this receptor rescues diet-induced obesity. To test whether rescued diet-induced obesity resulted specifically due to the Dop2Rs at the dendrites of the *MB011B* MBONs, we utilized an RNAi. Knocking down Dop2R specifically on the *MB011B* MBONs (*MB011B >Dop2R-RNAi*) did not affect food consumption on a CD. However, flies on a SD did not gain weight and instead had the same triglyceride/protein ratio than the CD. In addition, the control groups performed as expected, the *MB011B > w1118*^{CS} and the *Dop2R-RNAi> w1118*^{CS} on a SD gained significantly more weight than the CD (Fig. 3B). Because there is also mammalian and human literature suggesting that a SD or obesity lead to increased expression of D1R, we tested for the possibility that knocking down the G_q associated fly dopamine receptor Dop1R2 would increase the triglyceride/protein ratio on a SD significantly more than the control groups. Results showed that *MB011B* >Dop1R*2-RNAi* does not significantly increase or decrease triglyceride/protein ratios

on a SD nor a CD compared to the control groups *MB011B > w1118*^{CS} and the *Dop1R2-RNAi> w1118*^{CS}. Together, these results show that Dop2R is necessary for diet-induced obesity. D2R has an important role in modulating gene expression [61–64], and while studied to a lesser degree, so does the Dop2R in flies [64]. Hence, we next sought to understand the potential impact on transcriptional factors on a SD mediated by Dop2R. Specifically, we looked at cAMP response element-binding protein (CREBb) as it is a downstream target of Dop2R [65,66] and it is highly relevant in long-term memory in different fly neuronal populations [67,68]. Utilizing the same biochemical assay to measure the triglyceride/protein ration, we knocked down CREBb utilizing an RNAi and tested whether it rescued diet-induced obesity. Results showed that knocking down CREBb (*MB011B >CREBb-RNAi*) leads to rescued diet-induced obesity when compared to the CD and the other controls, *MB011B > w1118*^{CS} and the *CREBb-RNAi> w1118*^{CS} on a SD (Fig. 4). This suggests that CREBb might function in the same cellular pathway to regulate gene expression and obesity.

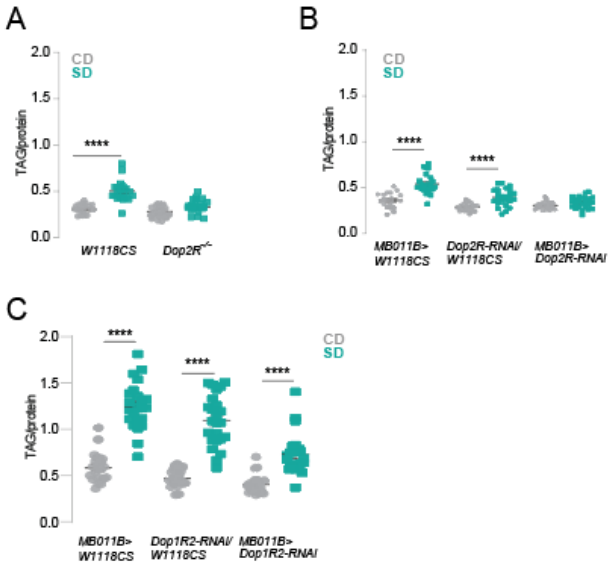

Figure 3.3 Dop2R **and not** Dop1R**2 is necessary for diet-induced obesity.**

A, B, C) Triglyceride levels normalized to protein of *Dop2R*$^{-/-}$ (A, n=19-24, ordinary one-way ANOVA with Tukey's test), *MB011B > Dop2R-RNAi* (B, n=20-21, ordinary one-way ANOVA with Tukey's test) and *MB011B > Dop1R2* (C, n=24, Kruskal-Wallis with Dunn's test) flies and their controls, on a CD (gray) and SD (teal). Data shown as +/- SEM, ****p<0.0001

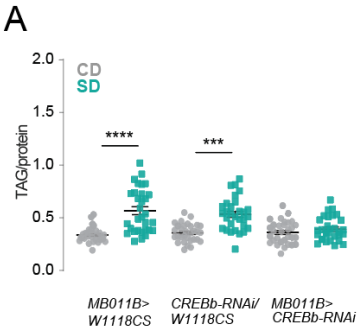

Figure 3.4 Diet-induced obesity is in part regulated by CREBb

A) Triglyceride levels normalized to protein of *MB011B >CREBb-RNAi* flies on a CD (gray) or SD (teal). n = 30-31, Kruskal-Wallis with Dunn's test. Data shown as +/- SEM, ***p<0.001****p<0.0001.

3.4 Discussion

The dopaminergic system has been at the center of discussion on the extent to which it contributes to diet-induced obesity [6,21]. Furthermore, the degree of contribution of each of the components of this system are even more elusive. In this study, the D. *melanogaster* provided a simple base to build on this story, and allowed us to demonstrate the role of Dop2R in diet-induced obesity and learning and memory on a SD. We show that Dop2R, and not Dop1R2, is necessary for diet-induced obesity. We then further showed how Dop2R is also necessary for maintaining dynamic synaptic plasticity that is abolished on a SD. In addition, Dop2R might have downstream consequences that also alter the activity of other transcriptional factors such as CREBb on a SD. Hence, we propose that the lack of food associations on a SD (Pardo-Garcia et al. 2022, *in review*) fits into the dopamine-mediated dysregulation through not only lack of synaptic transmission but maladaptive homeostatic plasticity that drive Dop2R to work against food associations.

It had previously been shown in mammalian and human studies that D2R goes down on a SD for mammals and in obese humans, specifically in the reward center of the mammalian brain [30,48]. Our results showed that in flies, a SD leads to an increase in Dop2R (Fig. 1A), which is the opposite as in the mammalian literature. This points to a certain degree of biological difference in the dopaminergic system between models. Nevertheless, it still corroborates how diet or obesity can lead to changes in the levels of this receptor. Unfortunately, we were not able to perform an Immunohistochemistry to pinpoint the exact location where the Dop2R was increased on a SD, given that this data was from fly whole heads. Dop2R in flies and mammals is known to be coupled to a $G_{i/o}$ protein – similar to D2R in mammals- leading to decreased calcium availability inside the cell in the presence of dopamine [52]. *Ex vivo* calcium imaging on

the dendrites and axons of *MB011B* showed further inhibition on a SD which correlates with whole brain increase in Dop2R on a SD and suggests that this increase might be localized to the *MB011B* MBONs. A caveat of this experiment is that we were not able to find a trustable Dop2R antagonist to test whether application of dopamine and subsequent decrease in neural activity was due to the action of dopamine on Dop2R or the other receptors. However, it is known that Dop1R1 and Dop1R2 are coupled to G_s and G_q, respectively, and these are usually considered excitatory, rather than inhibitory, similar to D1R in mammals. On the other hand, given that Dop2R is increased on a SD in flies while decreased in mammals, it is reasonable to suspect that the mechanisms of action downstream might elicit a different response. One way to bypass the lack of an antagonist would be to use a $Dop2R^{-/-}$ recombined with *GcAMP6f* and expressed on *MB011B* MBONs and determine whether addition of dopamine into the bath would still elicit further inhibition on a CD and SD. Further research on the distribution of Dop2R and the neural responses elicited by dopamine through this receptor is needed to draw a causal relationship between Dop2R levels and calcium activity on a SD.

Flies on a SD are not able to form food associations, which stems from a decrease in dopamine transmission (Pardo-Garcia et al. 2022, *in review*). The increase in Dop2R suggests a compensatory mechanism in face of the decrease in dopamine transmission. However, the fact that the flies still cannot learn suggests a lack of plasticity that could be due to this increase in Dop2R. Hence, we tested the role of Dop2R in food associations by first utilizing a $Dop2R^{-/-}$ and found that this improved the preference index towards the CS+ but not fully (Fig 2). This is not surprising given its high concentration in the mushroom body but also in other parts of the brain which includes in other types of MBONs with different behavioral outputs [44,49,69]. Therefore, we employed the power of the D. *melanogaster* genetic repertoire to test whether specifically

decreasing the levels of Dop2R on the dendrites of the *MB011B* MBONs could rescue dysregulated food associations. The PAM DANs that innervate the β' compartment of the mushroom body are those that relay the rewarding properties of sucrose. Furthermore, the *MB011B* MBONs are glutamatergic neurons that receive input from these PAM DANs to modulate food associations [44–46,49,55]. In addition, dopamine transmission at this synapse is disrupted on a SD (Pardo-Garcia et al. 2022, *in review*) due to decreased taste transmission [41] that reflects in decreased sucrose taste response of these PAM DANs [47]. What seems to be the result of such a decrease is the upregulation of Dop2R, and while we do not know if this is specific to the *MB011B* MBONs, the fact that these MBONs are dysregulated on a SD, and it is due to dopamine transmission strongly suggests that knocking down *Dop2R* in these neurons would potentially lead to rescued food associations. Indeed, *MB011B >Dop2R*-RNAi flies on a SD had the same preference index as the CD in the *MB011B* > $w1118^{CS}$ and the *Dop2R-RNAi*> $w1118^{CS}$ and the *MB011B >Dop2R-RNAi,* while the SD in both the first two controls were significantly lower. This suggests that the Dop2R in the *MB011B* is dysregulated on a SD. If Dop2R is truly upregulated on a SD and this is the reason why knock down in these MBONs leads to rescued preference index, then it would be appropriate to test whether knocking of Dop2R on a control diet mimics the behavioral phenotype seen on a SD. As a future direction, it would be important to specifically test whether Dop2R knockdown can also rescue the abolishment of the neural responses of learning and memory on a SD, like how restoring PAM DAN activity also restores these neural traces (Pardo-Garcia et al. 2022, *in review*). This would allow us to confirm the hypothesis that the abolishment of the neural trace of food associations on a SD is in part due to increased Dop2R in the *MB011B* MBONs that does not allow for proper synaptic plasticity through the change in delta of the activity of these neurons.

The role of D2R in feeding behavior and diet-induced obesity has been explored to a good degree [27,30,33–36,57–60,70], while in flies Dop2R is still widely unknown. Given the role of D2R in the mammalian brain and some of the conserved molecular pathways with Dop2R, we also expected to see Dop2R influence diet-induced obesity in flies. However, since it was not known whether the increase in whole brain Dop2R seen on a SD was primarily in the mushroom body and in the *MB011B* MBONs, we first tested whether Dop2R knockout on a SD led to rescued diet-induced obesity. When measuring for the triglyceride/protein ratio in Dop2R knockout flies, we observed that this knockout led to a full rescue in diet-induced obesity, different from the partial rescue seen when testing food associations. However, our main interest was to determine whether the Dop2R on the dendrites of the *MB011B* MBONs were necessary for diet-induced obesity on a SD. Hence, we utilized a Dop2R knockdown to test this hypothesis. The *MB011B >Dop2R-RNAi* flies on a SD did not differ significantly from those on a CD. Furthermore, they were also not significantly different from the CD of the other controls, the *MB011B > w1118*^{CS} and the *Dop2R-RNAi> w1118*^{CS}, corroborating the importance of Dop2R for diet-induced obesity, like D2R although in opposing directions. Since Dop1R1 is co-localized with Dop2R, we sought to test whether knockdown of Dop1R1 could increase diet-induced obesity, however, results were inconclusive, and we were not able to come to an agreement as to its role in diet-induced obesity. On the other hand, we also tested Dop1R2 because of its role in the olfactory system. Dop1R2 is highly expressed on Kenyon cells [53,69,71] and could influence feeding behavior. However, Dop1R2 knockdown did not have a significant effect on diet-induced obesity, where the SD group significantly ate more than the CD group but not more than the *MB011B > w1118*^{CS} and the *Dop1R2-RNAi> w1118*^{CS} SD groups, suggesting that it does not

have a role in diet-induced obesity. However, given its relevance to Kenyon cell modulation, it is highly likely that if this were to be tested for food associations, we would see a significant change on a SD. We were also interested in determining the role of Dop2R in feeding behavior utilizing the Fly to Liquid-Food Interaction counter [72] but the data was inconclusive and we were not able to reach a conclusion on the role of *MB011B >Dop2R-RNAi* in feeding behavior. Hence, our next steps are to further investigate the hypothesis that knocking down Dop2R in the *MB011B* MBONs will rescue feeding behavior, possibly through a combination of modulation of energy expenditure, anticipatory food reward, and food memory [36,58,60,63]. The cellular pathway of Dop2R in flies is poorly understood, however, there are studies that have been able to show that Dop2R possibly activates CREBb [67,68]. CREBb is important in the formation of long-term memory [67] and it has been shown to be important in peripheral and brain metabolism [73]. Furthermore, flies are on 7 days of HSD, giving rise to the potential involvement of long-term memory modulation of diet-induced obesity. To test the role of CREBb in diet-induced obesity, we knocked down CREBb and observed that *MB011B >CREBb-RNAi* led to a full rescue of diet-induced obesity in SD flies when compared to the CD and the other controls, suggesting that CREBb is necessary for diet-induced obesity and potentially plays a major role in the dysregulation of energy intake and expenditure, as well as memory related tasks on a SD. In the future we would test necessity of CREBb on the FLIC assay. The Dop2R upregulation remains a mystery and could be tested through several methods that would first require understanding whether there is an increase in the binding of the Dop2R gene transcription factor that promotes its transcription, or whether it might also be the alternative splicing being affected by the SD environment, similar to what also happens in flies exposed to alcohol [65]. Regardless of the reason, it is important to determine what causes this upregulation to better understand the overall

role of the dopaminergic system in promoting obesity through learning and memory deficits that translate into aberrant feeding behavior.

3.5 Method Details

Fly lines and preparation:

Every fly was kept in an incubator that had temperature set at 25°C with the humidity controlled and around 50% and kept in a 12:12 hr light/dark cycle.

In all of the experiments the males were collected via the use of pads releasing CO_2 for anesthesia, and this was done 2–4 days after they had eclosed. They were kept in vials that consisted of a maximum of 20-30 flies and aged until they were all 4-5 days old. The main control group consisted of *w1118Canton-S* flies (which was a generous gift from Anne Simon, from the University of Western Ontario), made from the backcrossing a *w1118* strain (from the Benzer lab, from Caltech) to the *Canton-S (CS)* (from Benzer lab, from Caltech) for 10 generations.

Dietary manipulations:

After eclosion, the flies were allowed to grow for 4-5 days in new vials followed by transfer to their respective diets for 7 days, where they were transferred to new vials every two days.

The breakdown of composition and caloric amount for the diets can be found here:

- The control diet consisted primarily of standard cornmeal prepared from Bloomington Food B recipe and consisted of approx. 0.6 cal/g.
- To prepare the sugar diet we mixed 30 g of table sugar to every 89 g of control diet, leaving us with a final volume of 100ml of 30% sucrose and a w/v, which is approx. 1.4 cal/g.

Appetitive conditioning on the T-maze

As described in Pardo-Garcia et al. 2022

Ex vivo Calcium Imaging

Imaging of *MB011B >GcAMP6F* fly brains was performed by extracting the fly brain and placing it on a custom-made dish with artificial hemolymph (as described in Pardo-Garcia et al. 2022) flowing fresh through the dish. 30mM of dopamine was flown through the artificial hemolymph delivery system 5 seconds after the beginning of recording and calcium responses were recorded. ROIs were then placed on the dendrites or the axons of the *MB011B* MBONs.

Triacylglyceride (TAGs) Assay

The steps to perform the reading of TAG levels and protein quantification can be found in [74], n=1 equals 2 flies.

Statistics

To analyze the normal distribution (Shapiro-Wilk test, except Fig. 1B and C where the test performed was D'Agostino & Pearson Test) and significance of the data we utilized GraphPad Prism. For more details on the type of statistical test and the sample size for each experiment please refer to the figure legends.

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Chapter 4 Discussion

The ability to effectively navigate our food environment comes in part from the associations with appetitive substances that promote our wellbeing, as well as being able to discriminate from those that do not. In this dissertation, we set out to answer the following questions: How does a sugar diet alter appetitive associative learning and memory (food associations)? What is the role of food associations in food intake? What are the molecular mechanisms of food associations that shape food intake? And is it the diet or the obesity that alters food associations?

In Chapter 2 we show that in the vinegar fly, one of the main factors that contributes to increased food intake leading to obesity on a sugar diet is the dysregulation of the food associations process. This dysregulation is due to a decrease in the reinforcing properties of sucrose due to decreased activity in the dopaminergic neurons conveying this information. These changes in food associations are specific to diet-induced and not obesity-induced. Correcting for the decrease in the reinforcing properties of sucrose prevents increased food intake and obesity, drawing a causal link between diet-induced changes in food associations and obesity.

In Chapter 3 we found that the decrease in the reinforcing properties of sucrose due to a sugar diet are due to homeostatic plasticity mechanisms compensating for the decrease in dopamine transmission. Specifically, flies on a sucrose diet have increased Dop2R protein availability on the dendrites of *MB011B* that contribute to the maladaptive, increased food intake that leads to obesity. Knocking down Dop2R on *MB011B* leads to increased

reinforcement of sucrose that restores food associations. In addition, it also rescues food intake and prevents obesity.

4.1 Circuits of diet induced obesity

The prevalence of obesity and metabolic diseases has been linked to advances in science that allowed for processed foods with high amounts of sugar, and that have become more and more affordable (Small et al. 2008; Volkow et al. 2011). Across a variety of organisms such as flies, mammals and humans, foods with added sugar leads to increased food intake (Avena et al. 2008). Although there are still many questions surrounding how these foods promote food intake, as well as challenges to elucidate the mechanisms, changes to the reinforcing properties of sucrose have been examined as a possible mechanism (Stice et al. 2010; Kroemer and Small 2016; Volkow et al. 2017). However, causally drawing a connection between the degree of reinforcement of sucrose and food intake has always been a challenge, mostly due to the complexity of the animal model being utilized. In the first part of our work, we identified a decrease in the reinforcement of sucrose on a sugar diet. Our lean and obese mutant animal experiments suggest that changes to the reinforcing properties of sucrose are due to diet and not to the obese state. The combination of circuit and behavioral assays with genetic and optogenetic manipulations determined that a sucrose diet leads to changes in the reinforcing properties of sucrose that is causally linked with decreased food associations. In addition, high throughput feeding measurements with also genetic and optogenetic manipulation establish that a change in the reinforcing properties of sucrose and food associations are causally linked to feeding on sugar diet. While sucrose has the ability to dampen food associations, we have yet to determine whether non-caloric sweeteners, such as sucralose, as well as other high caloric diets, such as fats, also contribute to increased food intake through these mechanisms. This is an interesting

question as both of these lead to increased food intake through most likely altered cellular mechanisms directly or indirectly connected to the reward center of the fly or mammal (Wang et al. 2016).

4.2 Mechanisms of diet induced food association defects

Flies fed a sugar diet have decreased dopamine transmission onto the *MB011B* glutamatergic MBONs. We specifically observed this decrease in the β' compartment of the Mushroom Body where *MB011B* MBONs have part of their dendrites. The β' compartment has previously been linked to caloric sensing, most likely through taste pathways that for sucrose are initiated in the *Gr64f* neurons of the fly's proboscis (May et al. 2019). Most likely, these neurons then connect to a relay zone in the fly brain before connecting to the *PAM* neurons, which then transform taste information into dopamine neurotransmission (May et al. 2020). The differentiation between caloric sensing and nutrient sensing aligns with the observed divergence in the functionality of the β' and the γ compartments. Given that flies have a smaller number of neurons than mammals, redundance as well as more generic set of neurons are an important anatomical and functionally distinction for proper encoding of information. In this case, the anatomical and spatial distribution of caloric and nutrient sensing pinpoints to a strong need to separate these components and avoid any overlaps at this level. This is also seen when comparing short-term and long-term memory, given that short-term memory is encoded primarily in the β' compartment while long-term memory is encoded in the γ compartment (Huetteroth et al. 2015, Yamagata et al. 2015). Hence, caloric value would most likely be used by the fly brain to encode for short-term food memories, while nutrient value would most likely be utilized by the fly brain to encode for long-term food memories given that nutrient value comes 20 – 30 min after the ingestion of food. In this study we were able to demonstrate that

short-term caloric value that is then encoded into short-term food associations is disrupted on a sugar diet given the finding disruption to the β' compartment. On the other hand, the γ compartment was not fully explored on sugar diet. Before flies are evaluated for disruptions of food associations, they are on a sugar diet for 7 days. This means that if we were to test the γ compartment on a short-term food memory paradigm we would most likely still see responses on the γ compartment. The main reason is that the flies experience with sucrose is not new, and hence, they most likely have a potentiation mechanism to keep the memory from degrading as the days go by. This potentiation mechanism is most likely also furthered by short-term food memory that then translates to a long-term food memory. Hence, dissecting the long-term memory specific changes due to a sugar diet might be more difficult than dissecting short-term memory, however, it is equally necessary for understanding how long-term food memory plays a role in food intake, and what is the interplay between short-term and long-term food memories. Ultimately, the reinforcing properties of sucrose are most likely not only tied to short-term events but also to long-term events that shape the degree of sucrose response in order to shape survival. Other types of memories that might also be of interest to explore in the future are aversive associative memories. Aversive associative memories are known to be potentiated when a particular event that is usually tied to a positive outcome result in a lesser degree of dopamine stimulation, and vice versa (Handler et al. 2019; Wang et al. 2021). It is possible that the decrease in the reinforcing properties of sucrose during the 7 days of sugar diet might lead to the development of aversive associative memories that might contribute to exacerbating the increased amount of food intake and subsequently obesity. However, given that flies increase their food intake even when there's a decrease in the reinforcing properties of sucrose suggests that the aversiveness might be for other stimulus that are normally not considered as highly

palatable, such as vegetables and high fiber diets. This is most likely a result of an increased baseline in dopamine secretion to these foods due to the sugar diet. Hence, a sugar diet might also be promoting the aversiveness of other foods that are not as dopamine-stimulating as added sugars but most likely also includes other aspects such as insulin receptor desensitization, among others.

Lastly, exposure to 7 days of sugar diet might be desensitizing the flies to the sucrose itself as being a rewarding compound; Meaning that their motivation to seek a source of reward that they have been constantly exposed to is diminished because its salience is diminished through repetition. Hence, the deficits in food associations observed on a sugar diet might be due to sucrose during the conditioning period not being motivating enough given the fact that they have already been exposed to high concentrations of sucrose for 7 days. A method of dissecting this problem is to utilize another rewarding compound during the training phase, such as fat, while feeding flies sucrose during the 7 days of diet. This way, we can separate the sucrose in the diet to the rewarding compound to which the odor is being paired to.

The mechanisms by which food associations is impaired on a sugar diet seems to be due to a decrease in dopamine transmission through decreased neural activity of the PAM DANs. On the other hand, this is not the only mechanisms through which food associations is impaired. Increased Dop2R on a sucrose diet and its subsequent knockdown suggest that Dop2R is also necessary for food associations and even if dopamine transmission were to remain intact, this increase might also still lead to impaired food associations. Hence, two different mechanisms are observed to be impaired on a sugar diet and to be impairing food associations. However, the question still remains as to whether decreased dopamine transmission might be eliciting increased transcription or translation of the Dop2R gene through a mechanisms still not

understood. Furthermore, alternative splicing might also be another mechanisms by which Dop2R levels are increased on a sugar diet. Hence, future studies should dissect the relationship between decreased dopamine transmission and increased Dop2R levels.

4.3 Modulation of dopaminergic circuits on a sugar diet

A key question that arises from this dissertation is what are the mechanisms of dopamine regulation and modulation that are altered on a sugar diet and promote food intake? Multiple studies in mammals have pinpointed to the dopamine receptors as key players in regulating food intake. Specifically, they have suggested that the interplay between D1R and D2R is key for maintaining appropriate regulation of dopamine transmission (Cox et al. 2015). In studies evaluating dopamine receptor availability, D1R has been seen to be upregulated, while D2R has been seen to be downregulated within the reward center of the mammalian brain (Eisenstein et al. 2013; Guo et al. 2014; Cosgrove et al. 2015; Horstmann et al. 2015; Beeler et al. 2016). In flies, much less is known about the basic distribution and function of the Dop1R1/2 and Dop2R in the mushroom body. We therefore explored the hypothesis of changes in dopamine receptor availability on a sugar diet in Drosophila *melanogaster* utilizing genetic and behavioral assays. Our experiments not only showed that Dop2R is increased on a sugar diet but showed that Dop2R mediates the maladaptive changes to food intake and hinders food associations. To our surprise, it seems that to some degree, the Dop2R of the fly does not anatomically distribute at the same typical synaptic level, nor does it most likely have similar downstream cellular effects as the mammalian version. Specifically, because Dop2R knockdown on the pos-synaptic cell, MBON, leads to the rescue of food intake, and appetitive associative learning and memory, and it's upregulated and not downregulated like in the mammalian brain. Sequence comparison between the mammalian D2R and the fly Dop2R reveals an amino acid identity ranging from 29

– 32%, and a Dop2R with an N-terminal sequence much longer than the D2R. Furthermore, in mammals, the long-isoform of the D2R is specifically localized to post-synaptic sites (Ford 2014), similar to what we have observed in the fly Dop2R, suggest more similarities between these two than with the short isoform that acts as an auto-receptor in the mammalian brain. Moreover, some known D2R agonists such as 1uinpirole failed to significantly sitmulate Dop2R mediated signaling, while others such as bromocriptine did stimulate Dop2R (Hearn et al. 2002). Hence, while is seems that some of the biochemistry is conserved, nonetheless there exists differences that seem to be affecting the anatomical and functional capacity of Dop2R, leading to the observed consequences. It would be an interesting avenue to fully explore the molecular mechanisms of the effects of a sugar diet on Dop2R and how this receptor balances dopamine-induced responses with the Dop1R1/2 on the dopaminergic neurons.

4.4 Theories of obesity

Obesity has been shown to be a complex disease; there is no one single reason for why an individual might become obese, and instead, depends on a variety of factors such as genetics and environmental factors, which have further ramifications. Several theories have been proposed to affect the way we perceive and eat different kinds of highly palatable foods. Reward deficiency syndrome comes from genetic and epigenetic factors that impair the ability of the brain reward circuitry to grasp the rewarding properties of a substance. For example, there has been several mammalian and human studies showing that individuals with the Taq1 A1 allele of the D2R gene do not respond with adequate increases in dopamine when exposed to rewarding substances (Blum et al. 1996; Stice et al. 2008), this is called the reward deficit syndrome. Not much further attention has been given in the last decade, and instead other theories have been more thoroughly explored.

Incentive salience does not mean that they cannot both happen at the same time. Using this model, one of the main problems with obesity is that there could be a disjunction between liking and wanting, where the food doesn't elicit the same liking response but due to changes in the neurobiology of the reward circuits of the brain, the animal does not modify its wanting, leading to excessive motivational state guided by subconscious and rudimentary processing.

In this dissertation, our findings do not fit one specific model. Instead, we believe that a sugar diet promotes a daily change in the neurobiochemistry of the brain that encompasses all of these theories as the days progress. The gradual increase in food intake where no genetic predispositions are present, might be due to the reward surfeit theory at the beginning of the diet. In this context, at the beginning of the diet the rewarding properties of sucrose elicit a strong liking, that with appetitive associative learning and memory develops into a significant wanting that drives behavior towards eating. However, as sugar directly and indirectly begins to change the neurobiochemistry of the brain, events such as loss of taste (May et al. 2019), decreased dopaminergic activity (May et al. 2020) in the presence of sucrose, dysregulation of appetitive associative learning and memory (Chapter 2) and redistribution of levels of dopamine receptors (Chapter 3) begin to shape and incorporate other elements. Due to decreased taste and dopaminergic transmission, liking is decreased, however, the disjunction between dopaminergic circuits and the associative neurons does not allow to update the wanting that arose in the first few days, hence the animal is still driven to consume. The lack of a proper go and no-go circuit exacerbates the problem by eliciting activity through the Dop2R that further contributes to the lack of memory update. Therefore, reward deficit and reward surfeit are both integrated into this model but have different degrees of active participation depending on the days on a sugar diet.

4.5 Concluding remarks and future directions

We perceive our food environment utilizing a multitude of modalities that converge on the reward center of the brain and are encoded into food memories. By effectively extracting the necessary information at the right time, we are able to determine the amount and length of engagement with our foods. Given the extensive involvement of the dopaminergic system on other diseases and conditions, it is necessary to explore the role of food memories on food intake beginning from a simpler model organism like the D. *melanogaster*, where the anatomy and functionality disrupted by a sugar diet can be explored with more precision and detail. It is imperative that we understand the mechanisms of food intake and how this is affected by diet given its extensive and detrimental impact on our health. The rising cases of obesity and metabolic syndrome, signal the need to commit to understanding these mechanisms since the health complications extend into neurodegeneration, diabetes, and cancer.

The current obesity epidemic signals the need to further explore less taken paths but with sound science that will allow us to inform future policy changes as well as educate the public. Efforts have been made to understand the consequences of obesity on health, but less have been taken to understand the etiology of obesity from a central brain processing perspective. With the advent of new techniques, research approaches and a multidisciplinary approach, tackling questions about the effects of diet on obesity will become less cumbersome and a holistic approach will be more feasible in higher brain order animals. However, it is important to practice retrospection and contemplate on the history, geographic, and cultures on the prevalence of obesity. The western diet has been a staple of what the world considers unhealthy. Fueled by corporate greed and public ignorance, the obesity epidemic began in the USA and is now spreading bit by bit throughout the world; As corporations continue to supply us with foods rich

in fats and/or sugars, stimulating our reward center and propelling us to consume more, no amount of research will subvert the consequences on this generation. The problem must be cut at its roots.

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