Modulation of Octopaminergic Signaling on a High Sugar Diet

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Abstract

Added sugar has become abundant in the modern dietary environment and excessive sugar consumption has been linked to obesity, diabetes, and other negative health outcomes. In flies, diets high in sugar have been shown to reduce sweet taste sensation, causing an increase in feeding behavior and subsequent accumulation of fat. However, the mechanisms through which alterations of sensation at the periphery affect a complex behavior such as feeding are unclear. Previous studies have shown that increasing the excitability of sweet taste reward neurons expressing the octopaminergic receptor OAMB prevents the overeating phenotype normally displayed on a high sugar diet and that knockdown of OAMB in this neuronal population eliminates sweet taste dependent short term memory.

Here we investigate the role of the neurotransmitter octopamine in the modulation of feeding behavior in flies fed a high sugar diet. Through both the activation and inhibition of octopaminergic neurons, we found that octopaminergic signaling does indeed play a role in mediating changes in behavior as a consequence of the dietary environment. However, knockdown of OAMB in the sweet taste reward neurons did not alter feeding behavior. Instead, our data suggests that octopamine influences feeding partially through activation of Octβ2R expressed in neurons involved in state dependent motivational learning. Our work here points to a more detailed understanding of the neural circuit connecting taste with behavior and lays the groundwork for further studies characterizing the role of octopamine in feeding behavior modification.

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Figure 1A	Experiment originally performed by Christina May, Replicated by Evan Dennis
Figure 1B	Experiment originally performed by Christina May, Replicated by Evan Dennis
Figure 1C	Experiment performed by Jen Gottfried
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INTRODUCTION

Introduction to the Field: Neuroscience in the Modern Dietary Environment

In complex organisms, the nervous system serves as the bridge that links the environment with behavior, allowing organisms to sense external stimuli at the periphery and respond in an appropriate manner. Often, discussion of the environment brings to mind images of climate, terrain, and temperature, but a major component of the environment that is sometimes overlooked is diet. Characteristic human responses to the dietary environment evolved to ensure that we obtain the nutrients and energy necessary for survival. However, the dietary landscape in which these behaviors arose has changed radically in recent years, and behaviors which once conferred an evolutionary advantage may now be detrimental in the abundance of modern society. Therefore, it is crucial to understand the neuroscientific principles underlying changes in behavior to orient human feeding behavior in context of the modern dietary environment.

Humans evolved in a setting where resources were scarce and there was no guarantee of regular access to food. In contrast, ultra-processed foods have come to dominate the dietary terrain of high and middle income countries in recent years and can be defined as cheap, ready-to-eat products made from processed substances extracted from whole foods (Monteiro et al., 2013). While low in nutrients, ultra-processed foods are energy dense and rich in dietary fats, salts, and especially added sugars. According to a nationally representative cross-sectional study, ultra-processed foods comprised 57.9% of the total energy intake of survey participants and 89.7% of the energy intake from added sugars (Steele et al., 2016). The most significant contributors to added sugar intake in the United States and the subjects of many studies are sugar sweetened beverages (SSBs), beverages that contain added caloric sweeteners such as sucrose and high-fructose corn syrup (Guthrie and Morton, 2000; Hu and Malik, 2010). With such a prominent position in the modern dietary environment, it is important to consider the health implications of diets high in added sugars.

The negative health risks associated with excessive consumption of added sugars are numerous. Many studies have specifically related consumption of SSBs to the growing obesity epidemic, often citing the low satiety to energy ratio of these drinks (Ludwig et al., 2001; Berkey et al., 2012; Luger et al., 2017). Recognized as a worldwide epidemic by the World Health

Organization in 1997, obesity affects 35% of the US adult population and about one third of US children (Smith and Smith, 2016). Obesity is the result of the positive energy balance that occurs when total energy consumption exceeds energy expenditure. The modern sedentary lifestyle combined with the availability of ultra-processed foods has made it very easy to reach such an energy surplus (Caballero, 2007). Added sugar has also been associated with type II diabetes. A report by Malik et al. found that individuals who regularly consume SSBs have a 26% greater risk of developing type 2 diabetes than individuals who did not (Malik et al., 2010). Additionally, regular SSB consumption has been linked to cardiovascular diseases, such as hypertension and coronary heart disease (Koning et al., 2012; Yang et al., 2014). The increased risk of type II diabetes and cardiovascular disease may partially be a consequence of weight gain caused by SSBs. However, added sugars also contribute to these conditions independent of weight due to their high glycemic load (GL) (Malik et al., 2010).

Evidence has also suggested that sugar can be addicting in much of the same way as drugs of abuse. Addictive drugs take advantage of the brain's natural reward circuitry that evolved to encourage certain behaviors important for fitness, such as feeding or sex. As one of the original activators of this system, it is plausible that sugar and its consumption could give rise to addictive behaviors. Indeed, the characteristic addictive behaviors and dependency signs of bingeing, withdrawal, craving, and cross-sensitization to amphetamine and alcohol are demonstrated in rats with intermittent access to sugar (Avena et al., 2008). Given its negative impact on health, the question as to how sugar elicits such behavior is of great interest. Through the systematic exploration of the neuronal circuitry of reward, we can begin to develop an understanding of this phenomenon on which to base treatments.

Before delving into the neuroscience of reward, a brief overview of the physiology of the nervous system is useful. In short, the nervous system allows organisms to sense and respond to the environment. Sensation begins when an environmental stimulus, like sugar, activates receptors on sensory neurons, causing them to fire an electrical pulse called an action potential. Action potentials stimulate the release of chemical signaling molecules known as neurotransmitters from axon terminals. These neurotransmitters diffuse across the gap between two neurons, or synapse, to bind to receptors on partnering neurons, effectively allowing the

signal to travel from cell to cell until it reaches the brain for processing. In the brain, neurons are organized in networks called neural circuits. These pathways allow specific neurons to respond to basic sensory signals and convey the information to other parts of the brain responsible for complex functions such as memory or emotion.

For example, in humans, the mesolimbic reward pathway is thought to be responsible for mediating the pleasurable sensation elicited by sugar or drugs. In this network, dopaminergic (DA) neurons from the ventral tegmental area (VTA) project to the nucleus accumbens (NAc) in the forebrain. It has also been suggested that the mesolimbic reward pathway is responsible for the development of incentive salience: the perception of a reward as a "wanted", or highly sought after goal (Kelley and Berridge, 2002). Regardless of the precise nature of the mesolimbic pathway's role in reward, evidence shows that there is an increase in both dopamine release and D_1 receptor binding in the NAc in response to sugar consumption (Hajnal and Norgren, 2001; Colantuoni et al., 2001). Furthermore, dysfunction of this reward circuit has been linked to obesity. Imaging studies reveal decreased D_2 receptor availability in obese subjects compared to controls (Volkow et al., 2011). It is suggested that overeating results from the need to compensate for this faulty circuitry.

Most of the studies referenced thus far were conducted in either humans or rodent models. Experiments conducted in humans are the most clinically relevant, potentially leading to influential medical breakthroughs. However, there are serious ethical limitations to using human subjects. While a great deal has been learned about the nervous system through non-invasive methods and experimental treatments, these are strictly regulated and rightfully so. Fortunately, much of the neurochemistry and neuroanatomy of humans has been phylogenetically conserved, allowing a wealth of knowledge to be gathered from studies with model organisms. Rats and mice are the most popular vertebrate model organisms and the ability of rats to demonstrate complex behaviors make them especially useful in behavioral paradigms. Additionally, since rodents are mammals, their neuroanatomy is proximal to that of humans. Still, no model organism is without its drawbacks, and the slow generation time of rats combined with their resistance to some current molecular neuroscience techniques render them difficult to manipulate genetically.

In contrast, the fruit fly *Drosophila melanogaster* is perhaps the most famous model organism for genetic studies and has many qualities that make it optimal for molecular neuroscience research. Perhaps the greatest advantage of fruit flies is the high degree of genetic tractability conferred by the abundance of transgenic fly lines made available through various stock centers throughout the world. By selectively crossing transgenic fly lines, scientists are able to alter gene expression in fruit flies with unparalleled flexibility. Additionally, flies are small, easy to keep, and have a high reproductive rate and a short generation time of approximately ten days. Furthermore, the entire fly genome has been sequenced and consists of only four pairs of chromosomes. The online resource, Flybase, is a remarkable database cataloging the *Drosophila* genome and publications relevant to each gene. Lastly, the relative simplicity of the fruit fly neurocircuitry compared to that of vertebrates can be useful in the investigation of the effects of the environment on neural pathways.

The genetic and molecular advantages of *Drosophila melanogaster* can be used to study their reward neurocircuitry and feeding behavior in response to sugar. The Dus Lab has previously demonstrated that flies fed a high sugar diet not only gain weight due to the increased caloric content of their food, but also increase feeding, interacting with their food more often and for longer periods of time (May et al., 2019). Many different factors are involved in this change of behavior and we have studied this phenomenon on both the genetic and neuroscientific levels. Our data has shown that when we use genetic techniques to neurogenetically correct the activation of sweet taste reward neurons in the *Drosophila* reward pathway, we see a rescue of the increased feeding behavior observed on a high sugar diet, suggesting the palatability of sugar is responsible for facilitating this change. These neurons express receptors for the neurotransmitter octopamine, the insect analogue of norepinephrine long known to be involved in reward signaling. Here, I specifically investigate the role of octopamine in this increased feeding behavior and whether or not octopaminergic signaling changes on a high sugar diet.

Background: A Critical Review of Relevant Literature

To begin to investigate the role of octopamine in feeding behavior, an understanding of the neurocircuitry of taste and reward is necessary. The chemosensory systems of taste and smell evolved to allow animals to navigate the chemical environment and distinguish beneficial food sources from potentially harmful substances. In Drosophila, tastants are sensed by gustatory receptor neurons (GRNs) housed in taste hairs called sensilla. While a majority of GRNs are located in the proboscis, the fly homolog to the human tongue, GRNs are also found in the wings, legs and female ovipositor. The external GRNs of the proboscis are found within sensilla on the labial palps and are tuned to either sugar, water, high salt, or low salt. Flies also have internal GRN's in the pharynx of the proboscis, allowing them to taste food after ingestion. (Vosshall and Stocker, 2007)

Tastants in the environment bind to gustatory receptors (GRs) expressed in GRNs, prompting them to fire action potentials. There are 68 different types of GRs in the fly genome with GRNs often coexpressing more than one receptor. A large number of GRNs are labeled by either one of two nonoverlapping receptor genes, *Gr5a* and *Gr66a. Gr66a* is thought to broadly label neurons that respond to bitter stimuli, whereas most if not all sweet-responsive neurons are *Gr5a* positive (Montell, 2009). Another family of receptors highly relevant to sweet taste are those encoded by the Gr64 locus. *Gr64f* is often coexpressed with *Gr5a* in sweet taste neurons, and does not overlap with *Gr66a*+ neurons (Dahanukar et al., 2007).

GRNs send their axons to the suboesophageal zone (SEZ), a central brain region located below the esophagus consisting of approximately 4,000 neurons. The SEZ is spatially segregated by taste organ, with inputs mapping in an anterior to posterior manner by organ (Wang et al., 2004). Additionally, sweet, bitter, and water neurons project to different cell populations, further segregating the SEZ by modality: most SEZ cells respond to only single modalities and these segregations are maintained in higher order regions of the brain (Harris et al., 2015). Indeed, fruit flies cannot distinguish between tastes of the same modality and are unable to discriminate between different sugars or different bitter compounds. Flies conditioned with fructose, were not able to distinguish it from glucose in a taste association paradigm (Masek and Scott, 2010). Rather, flies discriminate based on palatability or intensity within a modality.

Olfaction is the other chemical sense and is often studied in the context of learning. Similar to taste, olfactory receptor neurons (ORNs) enable flies to respond to the environment when chemical odorants bind to olfactory receptors (ORs) on the cell surface. Unlike GRNs, ORNs are restricted to the head and are housed in sensilla on the antennae or the maxillary palp. The neurocircuitry of the olfactory system is more characterized than that of taste, providing a useful context in which to study learning (Vosshall and Stocker, 2007).

Like most animals, flies can form sensory associations with both odorants and tastants. The site of associative learning in the fly brain is called the mushroom body (MB), a bilateral neuropil structure consisting of approximately 2,000 neurons termed Kenyon Cells. The dendrites of the Kenyon cells make up a segment of the MB called the calyx. The dendrites of Kenyon cells receive input directly from ORNs and much of what is known about the MB resulted from studies of its role in olfactory learning (Heisenber, 2003). Meanwhile, the axons of Kenyon cells form a bundle that is divided into three lobes: γ, α′/β′, and α/β (Aso et al., 2008). The neurons that make up these lobes are differentiated with respect to their morphology, gene expression, and function in behavior.

The lobes of the MBs are innervated by modulatory dopaminergic neurons (DANs) whose activity are necessary for olfactory learning (Liu et al., 2012). The projections of these dopaminergic neurons anatomically subdivide the MB lobes into 15 compartments. Different subsets of DANs project to different compartments and signal for either reward or punishment (Aso et al., 2014). For example, a group of DANs in the protocerebral anterior medial cluster (PAM) are responsible for assigning a positive predictive value to odors (Liu et al., 2012).

The sensory associations of taste are not as well characterized as those of olfaction, however, the mushroom body is still implicated in the integration of gustatory learning. While sugar stimulation activates the PAM DANs signaling reward, a discrete cluster of DANs in the paired posterior lateral (PPL1) region are activated by bitter substances. These PPL1 neurons are thought to encode punishment signals and mediate aversive learning in the fruit fly (Kirkhart and Scott, 2015). Despite these relations, there is still much to be learned about the relationship between taste and learning and the neurocircuitry relating the SEZ, MB, and DANs is far from fully understood. While the neural pathways connecting olfaction, taste, and reward presented

above may seem complex, it is relatively simple compared to that of a human and makes for a useful model to study feeding behavior.

However, perhaps the greatest advantage of *Drosophila* as a model organism is the genetic tractability provided by the GAL4/UAS system. The GAL4/UAS system is a binary system for conditional expression of transgenes in *Drosophila.* Developed in 1993 by Andrea Brand and Norbert Perrimon, the GAL4/UAS system makes use of transcriptional activation machinery utilized by yeast (Brand and Perrimon, 1993). By selectively crossing transgenic driver fly lines with transgenic responder lines, one can express a variety of different genes in almost any cell type. In one fly line, the gene encoding the yeast transcription factor GAL4 is inserted downstream of an endogenous promoter. In another line, GAL4's corresponding promoter, upstream activating sequence (UAS), is inserted in the fly genome along with the transgene of interest. When these two lines are crossed, in the resulting offspring GAL4 will be able to bind to UAS and drive expression of the transgene exclusively in cells that allow transcription to occur from whichever endogenous promoter is controlling GAL4. More nuanced expression can be achieved by creating recombinant fly lines that express GAL80 from an endogenous promoter which blocks the binding of GAL4 to UAS. Furthermore, temperature sensitive variants of these lines give the experimenter conditional temporal control of expression. Today, there are over 10,000 different driver lines and 15,000 different responder lines readily available from stock centers. Combined with the relative simplicity of their neurocircuitry, the GAL4/UAS system makes fruit flies an ideal model organism for the study of the effects of the dietary environment on neuronal signaling and behavior.

Like humans, flies suffer from a variety of negative health consequences when they consume a diet high in sugar. Studies have shown that flies fed a chronic sugar diet of 30% sucrose lasting several weeks develop both obesity and insulin resistance characteristic of Type II Diabetes (Musselman et al., 2011). Furthermore, a diet rich in sugary carbohydrates leads to heart disease in flies, causing arrhythmia and accumulation of fibrogen-like collagen (Na et al., 2013). On the other hand, flies fed a 30% sucrose high sugar diet (HSD) for only a week show fat accumulation without the accompanying insulin dysfunction (May et al., 2019). In addition to the direct health risks of a high sugar diet, overconsumption of sugar has been shown to lead to a

reduced sense of sweet taste in both humans and rodents as well as flies (Proserpio et al., 2016; Berthoud and Zheng, 2012; May et al., 2019). However, it is important to consider whether this loss of sweet taste is a consequence of obesity, or a consequence of the diet itself that contributes to the excess food intake and subsequent caloric surplus.

Flies fed a HSD for a week show a decreased taste response to sucrose stimulation of GRNs in both the proboscis and legs. This effect becomes more severe as the number of days on HSD increases. This phenotype was shown not to be due to a deficit in motor functioning or higher energy stores resulting from the caloric surplus. Importantly, this loss of sweet taste was not observed in flies fed a sweet, non-caloric diet or in flies fed a non-sweet, calorie rich lard diet, though lard supplemented flies still accumulated fat. This suggests that neither sweetness nor nutritional energy alone are sufficient to decrease sweet taste response. Furthermore, both genetically lean and genetically obese flies demonstrate sweet taste abilities comparable to control flies on both ND and HSD, indicating that obesity is neither necessary or sufficient to induce the sweetness insensitivity phenotype. (May et al., 2019)

On a physiological level, *Gr64f*+ sensory neurons that mediate sweet taste response decrease their activation in flies fed a HSD as measured using GCaMP. In vivo fluorescence of a vesicular release sensor in Gr64f+ neurons decreases as well on a HSD, indicating that their output is also dysfunctional. These physiological deficits also became more pronounced with increased time exposure to sugar, mimicking the pattern observed in taste response (May et al., 2019).

These sweet taste deficits on a HSD lead to a change in feeding behavior which can be analyzed in flies using the Fly to Liquid-Food Interaction Counter (FLIC) (Ro et al., 2014). The FLIC assay consists of an apparatus that houses flies in individual wells and sends an electric signal to a computer whenever their proboscis makes contact with a liquid food reservoir. Data collected using FLIC shows that flies on a 20% sucrose solution increase their feeding behavior over time. This was due to an increase in both the size and duration of their two characteristic "meals" a day, with size increasing more in proportion to duration, representing an increase in feeding rate. Again, results from genetically lean and obese flies were comparable to those of

controls, suggesting that the dietary sugar is responsible for the change in feeding behavior rather than obesity. (May et al., 2019)

Flies demonstrate a loss sweet taste when fed a HSD along with an overeating phenotype, but it is critical to consider whether or not these two phenomena are causally linked or simply two different ramifications resulting from the same diet. This distinction can be teased apart by taking advantage of the genetic tractability of *Drosophila.* NaChBac is a voltage gated sodium channel derived from bacteria that can be transgenically expressed in specific neurons using the UAS/Gal4 system to increase their excitability (Nitabach et al., 2006). Driving UAS-*NaChBac* with *Gr64f*-GAL4 resulted in a rescue of sweet taste response of HSD flies as measured by the Proboscis Extension Response (PER) assay. It also prevented fat accumulation and corrected feeding behavior. Moreover, artificially activating *Gr64f*+ cells exclusively during feeding using a closed loop optogenetic system prevented increased feeding behavior on the FLIC, further suggesting that a flaw in taste sensation is responsible for the overeating. (May et al., 2019)

Still, the mechanism by which a complex behavior such as feeding is modulated by alterations in sensation at the periphery is unclear. Insights into experience driven behavioral changes and their neural representations can be gained from studying learning and memory in the fruit fly. Memory is often studied in flies using two classic training paradigms. Repetitive pairing an otherwise neutral odor with an electric shock results in the formation of a long-term aversive memory. Likewise, pairing an odor with sugar forms an appetitive memory. After a training period, memory is assessed by measuring the avoidance or attraction to the previously conditioned odour when given a choice between two odours presented simultaneously. Transgenically manipulating different populations of neurons and measuring the result on memory using these paradigms have allowed scientists to discover the roles of certain clusters of neurons in reinforcement. (Waddel, 2010)

For example, it was through methods such as these that the MBs were identified as the sites of associative learning and then further characterized. Preventing synaptic output from different compartments of the MBs has provided evidence that neurons of the α′/β′ compartment are required during initial learning and early memory retrieval, while α/β are crucial for long term memory retrieval (Krashes and Waddel, 2008). These paradigms also revealed the function

of the aforementioned modulatory DA neurons and their role in reinforcement. It is believed that memories are written through activity of these DA neurons, with dopaminergic signaling leading to increases in intracellular cAMP levels and PKA activation, signals classically thought to be involved in LTM (Tomchick and Davis, 2009). DA was originally described as the neurotransmitter of aversive signals in the fly brain, with aversive DA clusters projecting to the α and α′ regions of the MB (Claridge-Chang et al., 2009). However, several studies have also shown DA to mediate motivation and reward (Liu et al. 2012; Yamagata et al., 2015).

The motivation to eat is closely tied to the central perception of the reward value of a food. A set of DA neurons in the protocerebral anterior medial (PAM DANs) cluster of the fly brain are responsible for signaling sugar reward and have been shown to play a role in olfactory appetitive reinforcement. These neurons are activated by sugar ingestion and project to the mushroom bodies (Liu et al., 2012). One subset of the PAM DANs represent the sweet tasting aspect of sugar reward and are responsible for mediating short term appetitive memory. Such neurons are labeled by the promoter R48B04. A distinct subset of PAM DANs signal the nutritious and energetic value of sugar reward and mediate long term appetitive memory (Yamagata et al., 2015). These two subsets are also spatially segregated in their projections to the mushroom body, with sweet taste reward neurons projecting to the β' lobe and nutrient reward neurons projecting to the β lobe.

Knowing that the R48B04 neurons mediate sweet taste reward and that a high sugar diet causes a loss of sweet taste and subsequent overeating, we previously questioned whether manipulating these neurons would lead to a modulation of feeding behavior. Indeed when *R48B04* + neurons were activated using *R48B04*-GAL4>UAS-*NaChBac*, we saw a decrease in feeding behavior in flies on both a HSD and ND (unpublished data). Interestingly enough, the R48B04 promoter fragment which labels dopaminergic neurons signaling the sweet taste reward value of sugar is part of the promoter for OAMB, a receptor for the neurotransmitter octopamine (OA). Knockdown of the OAMB receptor in these neurons using *R48B04*-GAL4>UAS-$oamb^{RNAi}$ results in a loss of appetitive olfactory STM (Huetteroth et al., 2015). OAMB is a GPCR that prompts an increase in intracellular Ca^{2+} upon the binding of OA and this result was observed in

PAM DANs in response to exogenous octopamine with expression of GCaMP3.0 (Balfanz et al., 2005; Burke et al., 2015).

There are approximately 30 known OA receptors, all belonging to the G-protein coupled receptor family (Evans and Mequeira, 2005; Blenau and Baldwin, 2001). Like all GPCRs, OA receptors have seven transmembrane domains and their activation is coupled to changes in intracellular secondary messengers, namely, Ca^{2+} and cAMP. These receptors were originally classified based on their pharmacological profiles using chemical agonists and antagonists, as well as their effect on changes in secondary messenger levels in tissue preparations. However, this method of classification was considered problematic due to different pharmacological results reported by different groups as well as the presence of more than one type of OA receptor in a given tissue sample. Fortunately, the onset of molecular cloning techniques allowed a new and more accurate classification system to emerge (Farooqui, 2012).

With the present system, OA receptors are divided into three different subclasses based on sequence homology and signaling properties: the α-adrenergic-like octopamine receptors (OctαRs), β-adrenergic-like octopamine receptors (OctβRs), and the octopaminergic/tyraminergic group. The OctαRs show structural similarities to vertebrate α-adrenergic receptors and have been shown to cause an increase in intracellular Ca^{2+} levels. Likewise, the OctβRs resemble the vertebrate β-adrenergic receptors, though their activation results in increased intracellular cAMP levels. Lastly, the octopaminergic/tyraminergic receptors resemble the OctαRs in sequence, but are preferentially stimulated by tyramine. The downstream effects of these receptors are more complicated and depend on the identity of the agonist. (Evans and Maqueira, 2005)

OA has a prominent role in the insect nervous system and modulates many peripheral physiological functions. Like norepinephrine in vertebrates, OA is thought to be a stress hormone that mediates the fight or flight response. It is proposed that OA allows peripheral insect muscles to adapt to different physiological demands (Roeder, 1999). For example, dorsal unpaired median (DUM) neurons in locusts supply OA to flight muscles stimulating glycolytic activity during rest and take-off phases, with a decrease in OA signaling a switch to fats as a metabolic source of energy for prolonged flights (Mentel et al., 2003). What's more, OA also

increases insect heart rate and ventilation rate, two physiological requisites for high energetic demand (Prier et al., 1994; Bellah et al., 1984). Octopamine is also required for ovulation with both mutations preventing OA synthesis and knockdown of the OAMB receptor in ovipositor muscles resulting in female sterility (Lee et al., 2003). Interestingly, OA has also been shown to play a critical role in the illumination of the firefly light organ (Nathanson, 1979).

Focusing more on the central nervous system, OA is involved in many learning and behavior processes. Studies in locusts have shown that OA mediates sensitization to novel stimuli as well as dishabituation (Sombati and Hoyle, 1984). Octopamine also mediates the division of labor in honeybee colonies, with increased levels of OA associated with foraging behavior (Schulz and Robinson, 2001). Another area of behavior affected by OA is aggression and mutant male fruit flies lacking OA show a decrease in aggressive behaviors (Hoyer et al., 2008). However, an area of particular interest is the role of octopamine in appetitive reward learning.

A series of studies have gone to show octopamine's involvement in reward conditioning, specifically in regards to sugar. Octopamine (OA) is thought to be the insect analog of the human neurotransmitter norepinephrine and like norepinephrine, it is synthesized from the amino acid tyrosine. Tyrosine is converted to OA in two steps catalyzed by the enzymes tyrosine decarboxylase (TDC) and tyramine β-hydroxylase (Tbh) (Monastirioti et al., 1996; Cole et al., 2005). There are two TDC genes in *Drosophila*, with *Tdc1* expressed non-neuronally and *Tdc2* expressed in neurons (Cole et al. 2005). When the gene encoding Tbh is mutated, flies show no detectable levels of OA and accumulate the intermediate tyramine (Monastirioti et al., 1996). Tbh mutant flies show impaired performance in a measure of sugar conditioning, however, they perform like controls in a measure of electric shock learning (Schwaerzel et al., 2003). These results point to OA being specifically involved in reward learning rather than global conditioning in general.

The role of octopamine in sugar reward has been further characterized, with studies suggesting that OA specifically mediates sweet-taste dependent short term memory. When the activity of octopaminergic *Tdc2*+ neurons is blocked, flies show impaired appetitive olfactory memory when conditioned with arabinose, a sweet but non-nutritious sugar. However, there is no

such impairment when these flies are conditioned with sucrose. These flies also display normal olfactory learning in response to conditioning with arabinose plus nutritious sorbitol. Such results indicate that the nutrient value alone of sugar is sufficient for appetitive learning and that OA mediates the sweet taste component of sugar reinforcement. Furthermore, artificial activation of *Tdc2*+ neurons paired with odor presentation resulted in formation of a robust appetitive memory when compared with controls. Increased performance indices were observed in these flies 30 minutes later, but the memory had dissipated after three hours, suggesting *Tdc2*+ neuron activation can only implant memory in the short term. (Burke et al., 2012)

OA has also been suggested to be involved in state-dependent memory formation in hungry flies. Artificial memory formation by *Tdc2+* neuron activation is impaired in sated flies with mutant Octβ2R octopaminergic receptors. However, this artificial learning is restored when flies are in a hungry state (Burke et al., 2012). State dependent learning in hungry flies is permitted through elevation of neuropeptide F (dNPF) levels in the hungry state that decreases activation of dopaminergic MB-MP1 neurons that project to the MB. Stimulation of the MB-MP1 neurons suppresses memory formation in hungry flies, while inhibition of the same neurons leads to an artificial memory formation in sated flies (Krashes et al., 2009). Knockdown of Octβ2R specifically in the MB-MP1 neurons once again blocked artificial memory formation in hungry flies, suggesting that OA input through Octβ2R modulates MB-MP1 activity (Burke et al., 2013). The MB-MP1 neurons are a subset of the PPL1 cluster of modulatory DA neurons that mediate aversive reinforcement signals and artificial activation of the MB-MP1 neurons leads to aversive memory formation (Aso et al., 2010). These findings suggest that the MB-MP1 neurons have a negative influence on appetitive memory formation, though the exact nature of this mechanism is unclear.

These studies demonstrate that OA is clearly involved in appetitive learning. However, while the MBs are the sites of associative learning, they are only sparsely innervated by OA neurons (Busch et al., 2009). Of the few OA neurons that do innervate the MBs, most have been shown not to be essential for olfactory conditioning in flies (Burke et al., 2012). Meanwhile, the previously discussed PAM DANs do synapse directly on the MB and can also be artificially activated to induce an appetitive memory (Liu et al., 2012). Double labelling of the PAM and

OA neurons reveal that their neuronal processes may be in direct contact. In regards to the directionality of this relationship, PAM DAN activation in Tbh mutants still results in formation of conditioned approach behavior despite the lack of OA (Liu et al., 2012). The *dumb^1* mutation in flies results in impaired appetitive memory thought to be caused by a deficiency of the dDA1 dopamine receptor and activation of OA neurons in *dumb^1* flies does not result in artificial memory formation (Kim et al., 2007; Burke et al., 2012). Taken together, these studies suggest that dopamine acts downstream of octopamine in the formation of olfactory memory and that OA neurons may directly modulate PAM DANs innervating the MBs.

Thesis Goals

The over-consumption of added sugars has been implicated in the development of obesity, diabetes, and other negative health risks (Ludwig et al., 2001; Berkey et al., 2012: Luger et al., 2017, Malik et al. 2010). Studies have shown that such levels of sugar consumption can lead to the loss of sweet taste sensation, prompting increased sugar feeding to compensate (Berthoud et al., 2012; Proserpio et al., 2016). In fruit flies specifically, the loss of sweet taste is mediated by decreased activation of *Gr64f*+ neurons in response to sweet stimuli, leading to increased feeding behavior and fat accumulation on a HSD (May et al., 2019). However, the exact neuroscientific principles allowing deficits in taste to alter behavior is unknown.

Many studies have suggested that sensation and behavior are linked via reward pathways in the central nervous system. In *Drosophila,* dopaminergic neurons of the PAM region labeled by *R48B04* represent the palatable aspect of sugar reward, mediating sweet taste dependent short term memory (Yamagata et al., 2015). Artificial activation of these R48B04 neurons using the bacterial voltage gated sodium channel NaChBac corrects feeding behavior on a HSD, pointing to their involvement in the development of an overeating phenotype. R48B04 neurons express the octopaminergic receptor OAMB and knockdown of OAMB in this neuronal population leads to a loss of short term memory formation when trained with sweet, but non-nutritious arabinose (Huetteroth et al., 2015). The goal of this study is to examine the role OA plays in modulations of fruit fly feeding behavior on a HSD to better understand the relationship between taste loss and behavioral changes. Specifically, we investigate the possibility that octopaminergic input to the R48B04 neurons is altered with exorbitant sugar consumption.

We hypothesized that knocking down OAMB receptors in the R48B04 neurons would cause flies fed a normal diet to behave in a manner comparable to that of flies fed a HSD. This reasoning was based on the previous findings that excitation of R48B04 neurons corrects feeding behavior and that OA activates these neurons to mediate sweet taste dependent short term memory. Here we show through both the activation and inhibition of octopaminergic neurons that OA indeed modulates feeding behavior. However, our data does not support the hypothesis that these changes are mediated through octopaminergic input to sweet taste reward neurons. Instead, our data suggests that octopamine influences feeding on a HSD partially through

activation of neurons involved in state dependent motivational learning that express the receptor Octβ2R. Still, knocking down Octβ2R in this neuronal population did not completely eliminate the overeating phenotype, leading us to conclude that a parallel octopaminergic pathway also influences feeding behavior on a HSD.

Materials and Methods

Fly Husbandry

All flies were grown on standard cornmeal-yeast-sucrose medium at 45%-55% humidity on a 12-hour light:dark cycle. Stock fly lines were kept at 25ºC while temperature sensitive crosses were reared at 20ºC with experiments conducted at a permissive 29ºC. Only male flies were used for experiments.

Fly Strains

Line	Source
W^{1118}*CS*	A. Simon, UWO
UAS-NaChBac	M. Nitabach, Yale
R48B04-GAL4	Bloomington Stock Center
UAS-$oamb^{RNAi}$	Vienna Drosophila Resource Center
Tdc2-GAL4	Bloomington Stock Center
UAS-Kir2.1, tub-$GAL80^{ts}$	Bloomington, Bloomington, Recombined by Anderson Lab
UAS-dTrpA1	Bloomington Stock Center
TH-D'-GAL4	Wu Lab, JHU
UAS-$Oct\beta2R^{RNAi}$	Bloomington Stock Center

Triacylglyceride (TAG) Assay

The triacylglyceride to protein ratio of flies on control and experimental diets was measured as described in Tennessen et al. (2014). Male flies were reared on either ND or HSD for seven days, with flies being flipped onto fresh vials every two to three days. At the end of the seven day period, flies were anesthetized on CO_2 and then frozen at -80ºC. Samples were prepared by homogenizing two whole flies in 250µL of lysis buffer with protease inhibitor. Lysis buffer consisted of 140mM NaCl, 50mM Tris-HCL pH 7.4, and 0.1% Triton-X and one Pierce Protease Inhibitor Mini Tablet, EDTA Free, for every 10mL. Samples were then centrifuged at 13,000 rpm for 15 minutes. 10µL per sample of diluted standards and supernatants were plated onto 96 well plates.

Protein concentration was determined using a Pierce BSA Assay Kit (PI-23225, Fisher). 200µL of reagent was added to each well and plates were incubated at 37ºC for 30 minutes. Absorbance at 562 nm was recorded using a Tecan Plate Reader Infinite 200.

Triacylglyceride concentration was determined using a Stanbio Triglyceride Kit ((2100-430, Fisher). 90µL of reagent was added to each well and plates were incubated at 37ºC for 15 minutes. Absorbance at 500 nm was recorded using a Tecan Plate Reader Infinite 200.

Fly to Liquid-Food Interaction Counter (FLIC)

Fly feeding behavior was measured using the FLIC as previously described in Ro et al. (2014). The FLIC apparatus consists of eight *Drosophila* Feeding Monitors (DFMs) signaling to a Master Control Unit (MCU). Each DFM houses twelve flies in individual wells where they have access to a liquid food reservoir. Data from every food interaction is sent from the DFM to the MCU. Normal liquid diet consisted of a 5% sucrose solution, while high sugar liquid diet consisted of a 20% sucrose solution. The FLIC was run in an incubator at 25ºC for normal fly lines and 29ºC for temperature sensitive lines with a 12 hour light cycle (Lights on from 7AM to 7PM). All FLIC experiments were done with male flies.

FLIC Analysis

Conversion of raw data files into excel files was done in RStudio using code available in R. Further analysis and visualization was done in GraphPad Prism. Data visualization was based on the optimal way to demonstrate the comparison that was made.

Statistics

GraphPad Prism was used for the creation of all graphs and for statistical analysis. All data are shown as Mean ± SEM,**** $p < 0.0001$, **** $p < 0.001$, **$p < 0.01$, *$p < 0.05$ for all figures.

Results

Preliminary Data: Feeding Behavior Increases on a HSD and is Corrected by Activation of R48B04 Neurons

Recent data from May et al. (2019) have shown that flies fed a HSD accumulate fat not only as a result of the increased caloric content of their food, but also due to an increase in feeding behavior (Figure 1A and 1B). That is, flies interact with their food more often and for longer periods of time on a HSD. This change of behavior as a consequence of diet was shown to be mediated by a loss of sweet taste as indicated by a decrease in activation of the *Gr64f*+ sweet taste neurons in response to sugar stimulus. However, the way alterations in the periphery go on to modulate a complex behavior such as feeding is unclear.

In complex organisms, sensations on the periphery are often centrally processed before a non-reflexive behavior is elicited. Naturally, it would follow that a loss of sweet taste would interfere with the integration of sensory information and that alterations of the central processing of sweet taste information may lead to a change in feeding behavior. Sweet taste reward in flies is mediated by the dopaminergic R48B04 neurons of the protocerebral anterior medial (PAM) region of the fly brain (Yamagata et al., 2015). Artificial activation of these neurons using UAS-NaChBac decreases the feeding behavior of flies fed a HSD, suggesting that alterations in taste sensation lead to deficiencies in reward processing and result in increased feeding behavior (Figure 1C and 1D) (unpublished data).

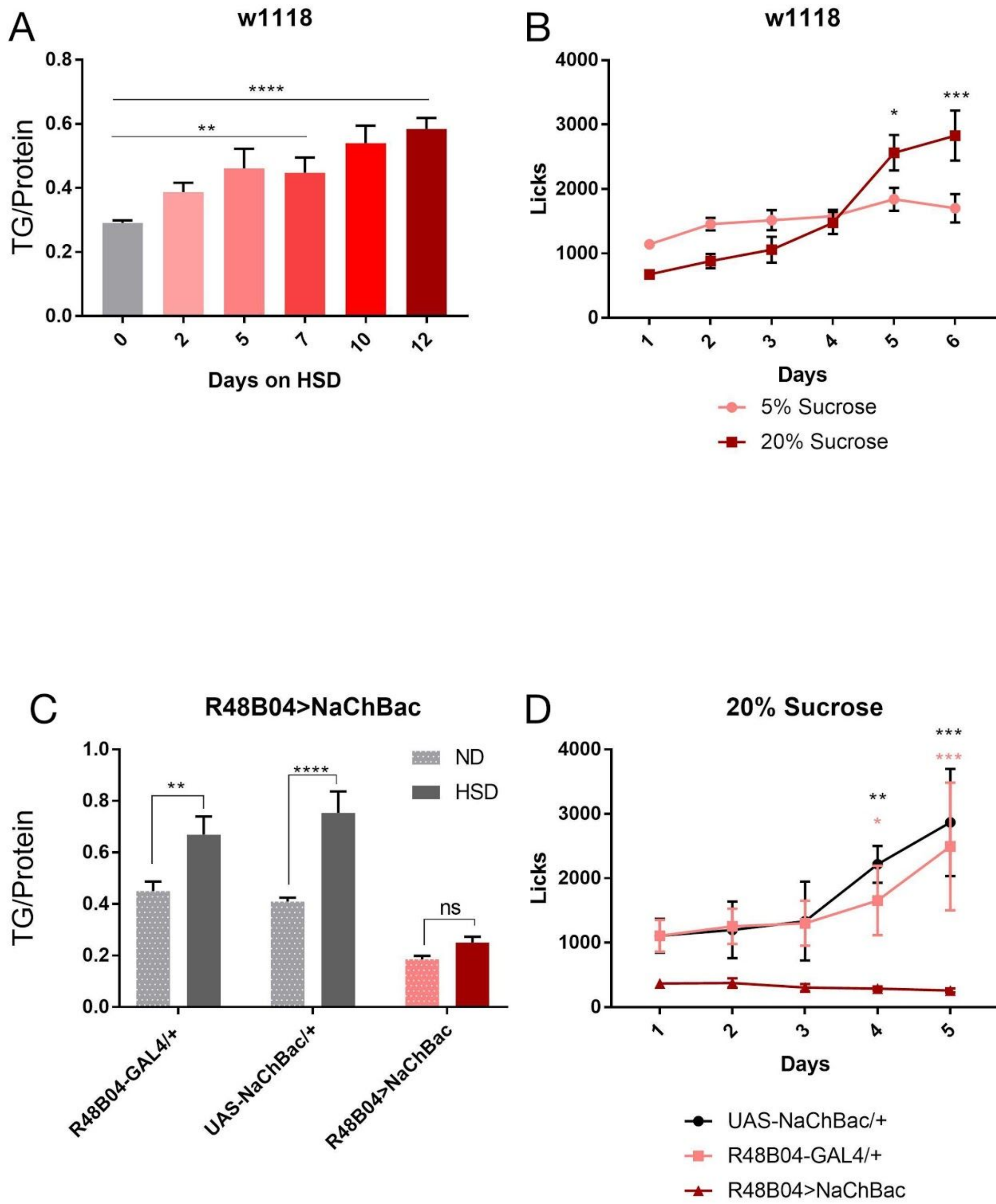


Figure 1: Activation of R48B04 Neurons Corrects Feeding on a HSD

A) Triglyceride levels normalized to protein in age-matched W^{1118}*CS* control flies fed a 30% sucrose HSD for different periods of time. n=8, unpaired t-test
B) Average licks per day of age-matched W^{1118}*CS* control flies fed either a 5% (salmon, n=96) or 20% (burgundy, n=72) sucrose diet. two-way ANOVA with Sidak's multiple comparisons test
C) Triglyceride levels normalized to protein in age-matched *R48B04>NaChBac* flies and appropriate genetic controls fed either a ND or HSD for 7 days. n=8, two-way ANOVA with Sidak's multiple comparisons test
D) Average licks per day of age-matched R48B04>NaChBac flies and appropriate genetic controls fed a 20% sucrose diet. n=24, two-way ANOVA with Tukey's multiple comparisons test

Knockdown of Octopamine Receptor OAMB in Sweet Taste Reward Neurons does not Increase Feeding on a ND

R48B04 is a fragment of the promoter for the octopaminergic receptor OAMB. Recently, octopamine has been shown to play a complex role in larval feeding motivation (Zhang et al., 2013). Furthermore, several studies have documented the involvement octopamine (OA) in appetitive olfactory short term memory, with a knockdown of the OAMB receptor in R48B04 neurons leading to a complete loss of STM when flies are trained with sugar (Huetteroth et al., 2015; Schwaerzel et al., 2003; Burke et al., 2013).

To investigate the role of OA in sweet taste reward signaling, we first knocked down the OAMB receptors in the R48B04 neurons with RNAi by crossing *R48B04*-GAL4 flies with a UAS-$oamb^{RNAi}$ line. As increasing the excitability of R48B04 neurons with NaChBac decreases feeding, we predicted that the OAMB knockdown would decrease the activation of these neurons and that *R48B04>*$oamb^{RNAi}$ flies would accumulate increased triacylglyceride levels on both a ND and a HSD diet. Additionally, we predicted that the transgenic flies would show an overeating phenotype on a 5% sucrose ND similar to WT flies fed a HSD. However, *R48B04>*$oamb^{RNAi}$ flies demonstrated no difference in fat accumulation as measured by the TAG

assay compared to genetic controls(Figure 2A). Feeding behavior as measured by FLIC also did not increase as predicted, but rather decreased slightly (Figure 2B).

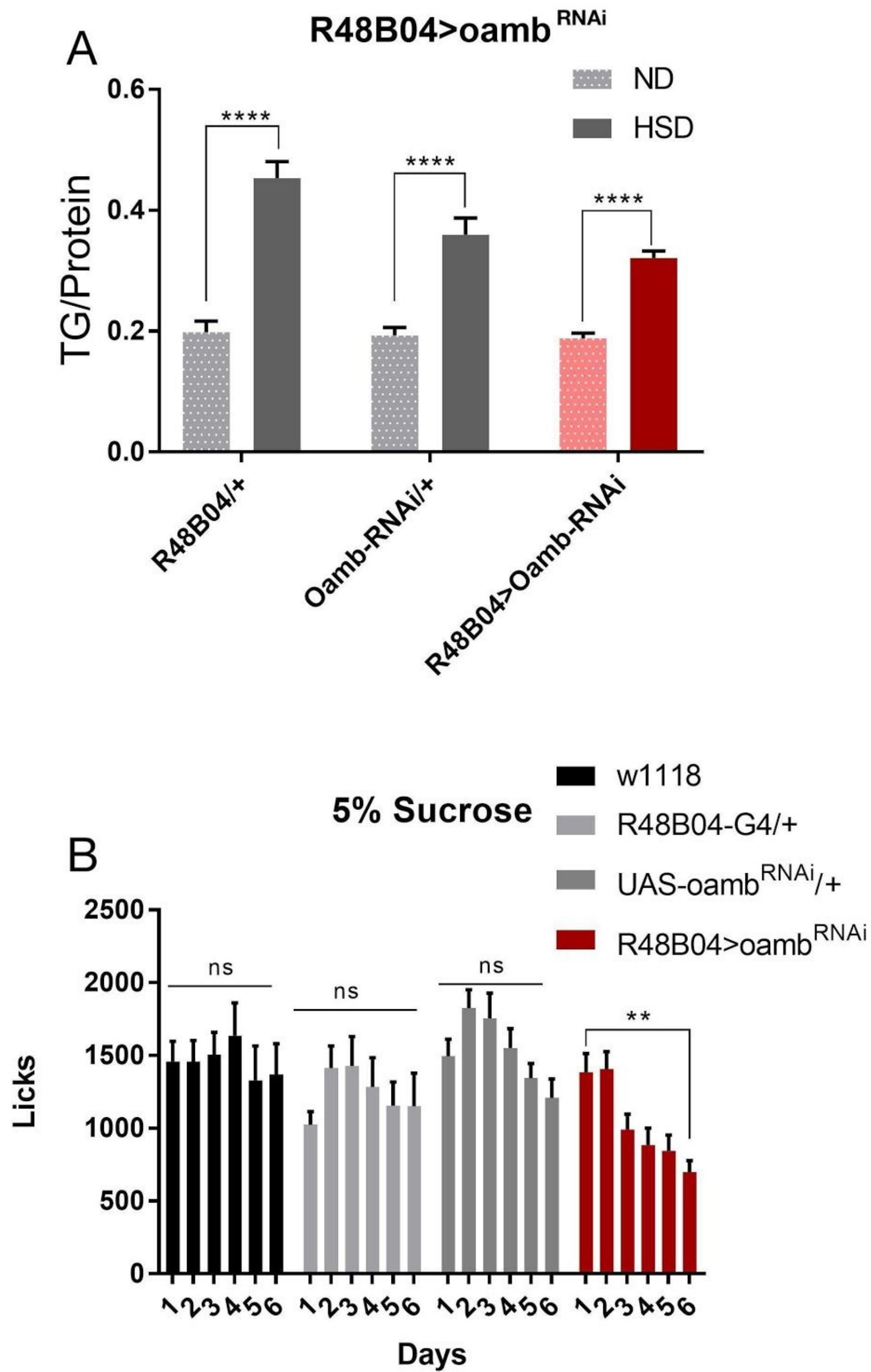


Figure 2: Knockdown of OAMB in R48B04 Neurons Does Not Increase Feeding

A) Triglyceride levels normalized to protein in age-matched *R48B04>oamb^{RNAi}* flies and the appropriate genetic controls fed either a ND or HSD for 7 days. n=9-16, two-way ANOVA with Sidak's multiple comparisons test

B) Average licks per day of age-matched *R48B04>oamb^{RNAi}* flies and the appropriate wild type and genetic controls fed a 5% sucrose diet. n=30, two-way ANOVA with Tukey's multiple comparisons test

Manipulation of Octopaminergic Neurons Influences Feeding Behavior on a HSD

To further explore the role of OA in feeding behavior, we manipulated octopaminergic neurons using GAL4 driven by TDC2, one of the two enzymes necessary for OA production (Cole et al., 2005). First, we inhibited *Tdc2+* cell activity using K_{ir}2.1, an inward rectifying potassium channel that stabilizes the resting potential of neurons closer to E_k (Fakler et al., 1994). This means a greater depolarization is required for cells expressing K_{ir}2.1to fire action potentials. K_{ir}2.1 was expressed by crossing *Tdc2*-GAL4 flies to UAS-*K_{ir}2.1, tub GAL80^{ts}* flies. The temperature sensitive GAL80 transgene allows for conditional expression of K_{ir}2.1. At lower temperatures (20°C), GAL80 binds to GAL4 and prevents transcription of K_{ir}2.1, while at higher temperatures (29°C), GAL80 is no longer functional, permitting K_{ir}2.1 to be expressed. This allowed us to rear flies at 20°C without interfering with development before keeping them at 29°C while conducting experiments.

We had predicted that if OA was modifying feeding behavior through input to R48B04 neurons, decreasing their activation would lead to increased fat levels and feeding behavior based on the same reasoning discussed in the previous experiment. However, we observed the opposite phenotype, with *Tdc2> K_{ir}2.1, tub GAL80^{ts}* flies demonstrating no significant increase in triacylglyceride levels on a HSD as compared to a ND (Figure 3A). Also, the characteristic increase in feeding behavior over time on a 20% sucrose HSD was not recapitulated in the experimental condition. Instead, the feeding level of flies expressing K_{ir}2.1 remained relatively constant over the course of the experiment (Figure 3B).

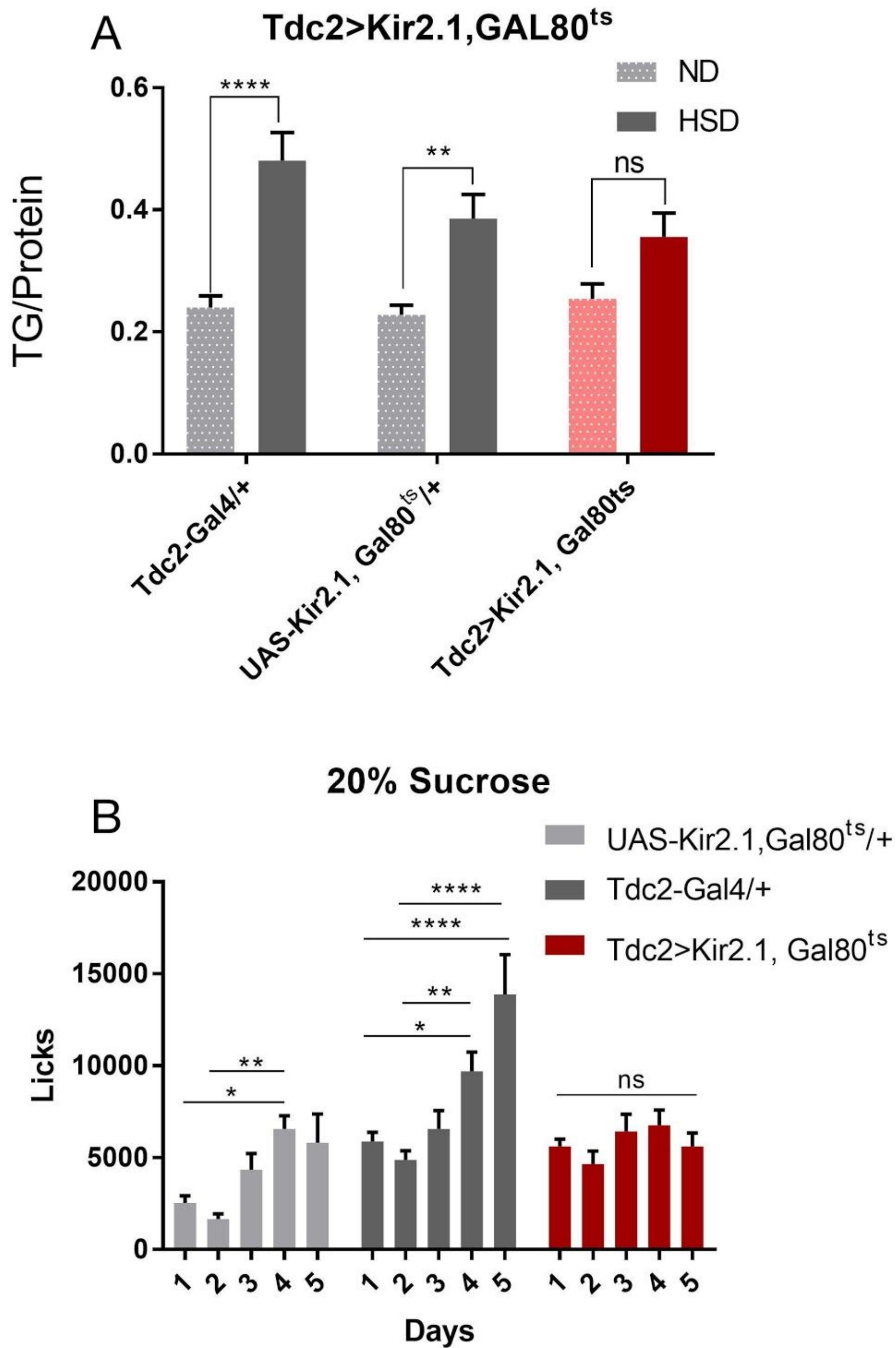


Figure 3: Reduction of Tdc2+ Neuronal Activity Prevents Increased Feeding Behavior on a HSD

A) Triglyceride levels normalized to protein in age-matched *Tdc2>Kir2.1, Gal80*^{ts} flies and the appropriate genetic controls fed either a ND or HSD for 7 days. n=8, two-way ANOVA with Sidak's multiple comparisons test

B) Average licks per day of age-matched *Tdc2> Kir2.1, Gal80*^{ts} flies and the appropriate genetic controls fed a 20% sucrose diet. n=24-30, two-way ANOVA with Tukey's multiple comparisons test

We also increased *Tdc2+* neuron activity by crossing *Tdc2*-GAL4 flies with a UAS-*dTrpA1* line. dTRPA1 is a transient receptor potential cation channel that is temperature sensitive. While normally used to regulate thermotactic behavior in flies, dTrpA1 can be transgenically expressed to activate neurons in a temperature dependent manner (Pulver et al., 2009). Once again, flies were reared at 20°C with experiments conducted at an activating 29°C.

Since dTRPA1 has the opposite effect of K_{ir}2.1 on neuronal excitability, we had expected to see an increase in fat accumulation of *Tdc2>dTrpA1* flies on a ND, as opposed to the decreased triacylglyceride levels observed in the *Tdc2>* K_{ir}*2.1, tub GAL80*^{ts} flies on a HSD. Instead, the triacylglyceride-to-protein ratio was down on a HSD once again in the experimental condition (Figure 4A). However, when feeding behavior was measured with the FLIC, *Tdc2>dTrpA1* flies showed an elevated number of licks per day relative to genetic controls on both 5% and 20% sucrose solution that remained constant over the course of the experiment (Figure 4B and 4C). As is normally observed, fly feeding behavior was elevated on a HSD. In the 5% FLIC experiment, feeding was slightly elevated across genotypes on the first day, but this is likely a result of increased fly activity as they adjust to the increased temperature. Due to technical difficulties, reliable data could only be collected for the first three days of the 20% sucrose FLIC assay, but despite the short time frame, the increased feeding phenotype is apparent.

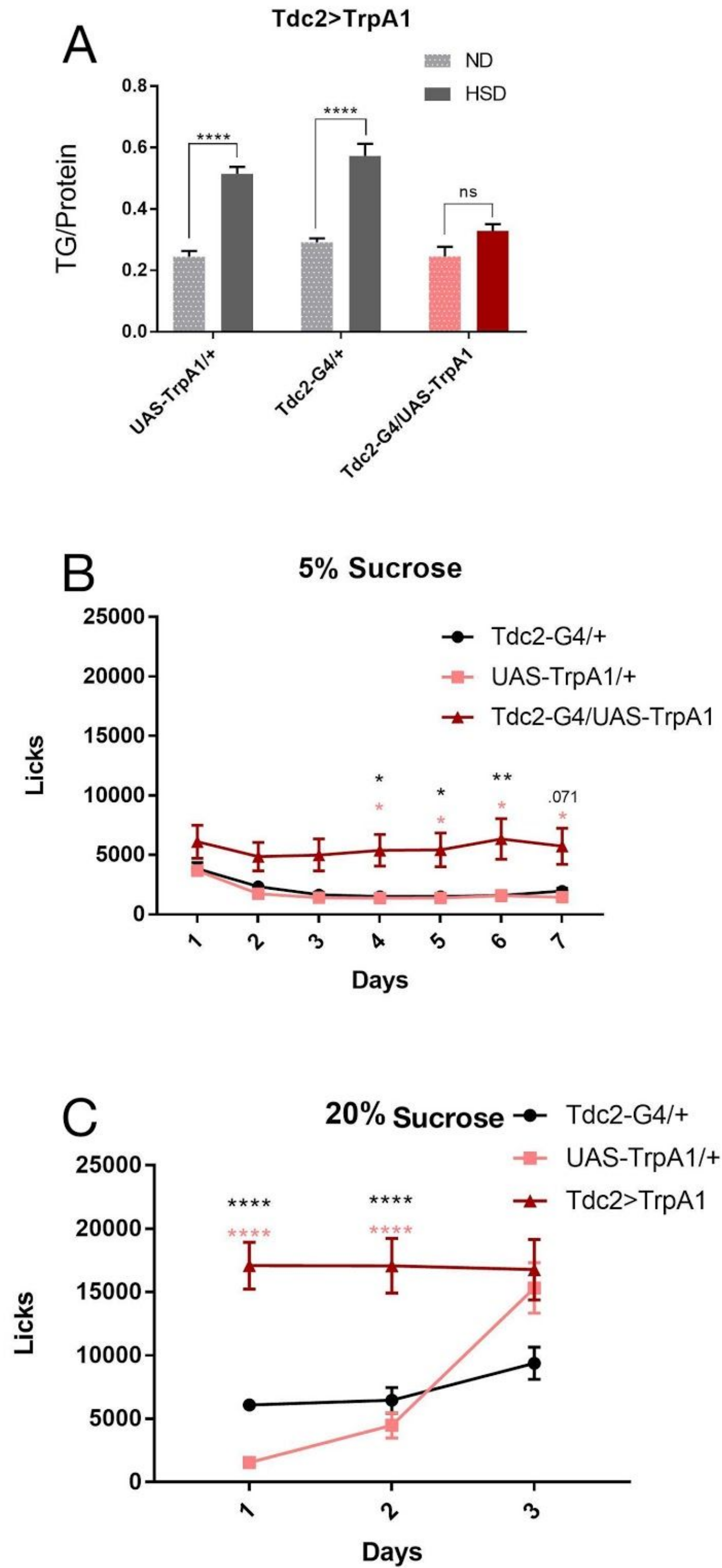


Figure 4: Increase of Tdc2+ Neuronal Activity Modulates Feeding

A) Triglyceride levels normalized to protein in age-matched *Tdc2>dTrpA1* flies and the appropriate genetic controls fed either a ND or HSD for 7 days. n=8, two-way ANOVA with Sidak's multiple comparisons test
B) Average licks per day of age-matched *Tdc2> dTrpA1* flies and the appropriate genetic controls fed a 5% sucrose diet. n=18-24, two-way ANOVA with Tukey's multiple comparisons test
C) Average licks per day of age-matched *Tdc2> dTrpA1* flies and the appropriate genetic controls fed a 20% sucrose diet. n=24-30, two-way ANOVA with Tukey's multiple comparisons test

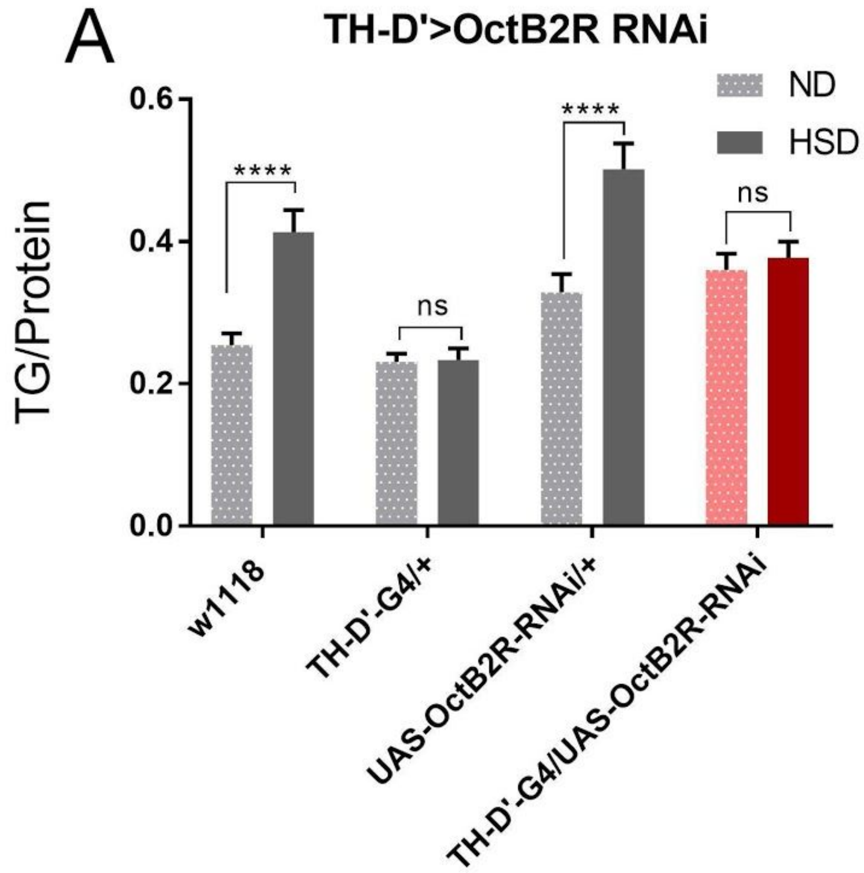
Knockdown of the Octopamine Receptor Octβ2R in Aversive Neurons Attenuates Feeding Behavior on a HSD

As seen in the two previous experiments, activation and inhibition of octopaminergic neurons modulates feeding behavior. However, based on the results of our knockdown of OAMB in R48B04 neurons, this effect does not seem to be mediated through the signaling of the sweet taste reward neurons. The question then becomes through which pathway is OA acting to bring about the changes in feeding behavior observed in *Tdc2>* K_{ir}*2.1, tub GAL80*^{ts} and *Tdc2>dTrpA1* flies. In addition to playing a role in sweet taste reward learning, OA has been shown to modulate state dependent learning in hungry flies through action on the Octβ2R receptors expressed in dopaminergic MB-MP1 neurons of the PPL1 cluster (Burke et al., 2012). MB-MP1 neurons confer the inhibitory satiety state in state-dependent motivational learning and also function in aversive memory conditioning (Krashes et al., 2009; Aso et al., 2010). We therefore investigated whether changes in feeding behavior observed due to the activation or inhibition of octopaminergic neurons are mediated through Octβ2R in this neuronal population.

The *TH-D'*-GAL4 driver line labels a fraction of DANs including the PPL1 cluster that houses the MB-MP1 neurons (Galili et al., 2014). We used *TH-D'*-GAL4 to drive UAS-*Octβ2R*^{RNAi} and measured fat accumulation (Figure 5A). Unfortunately, one of the genetic control lines, *TH-D'*/+, did not show increased triacylglyceride levels on a HSD, rendering the experiment inconclusive. This experiment was repeated multiple times with similar results,

though all the flies came from the same series of cross bottles. *TH-D'*/+ control flies had repeatedly demonstrated a fat accumulation phenotype in previous experiments conducted with a different UAS responder line, suggesting that the failure to display a phenotype in this case was not due to the genetic background of the flies, but rather some other external factor, such as a pervasive sickness in the cross bottle (Figure S1A and S1B). Interestingly, *TH-D'*-GAL4>UAS-$Oct\beta 2R^{RNAi}$ flies also did not accumulate excessive fat on a HSD. However, without working genetic controls, no conclusions can be drawn from this data.

Though the triacylglyceride assay failed to yield any usable data, a slight attenuation of feeding behavior as measured by FLIC was observed in the experimental condition, with *TH-D'*>$Oct\beta 2R^{RNAi}$ flies showing a reduced rate of increased feeding on a 20% sucrose diet (Figure 5B). This result supports the hypothesis that OA affects feeding behavior through action on the aversive MB-MP1 neurons rather than through activation of the R48B04 sweet taste reward neurons. However, since the experimental condition still shows some increase in feeding, there is likely a parallel mechanism through which behavior is modified. It is worth pointing out that in this experiment the feeding of the *TH-D'*/+ control flies was not significantly different from the other controls, and it should be noted these flies used in this experiment came from a different set of cross bottles than the one used in the TAG assay. Had the *TH-D'*/+ control worked in the TAG assay and we still observed no significant difference between ND and HSD in the experimental condition these results together would be consistent with previous studies showing that OA acts on Octβ2R in MB-MP1 neurons to modulate negative dopaminergic signals in the formation of appetitive reinforcement (Burke et al. 2012).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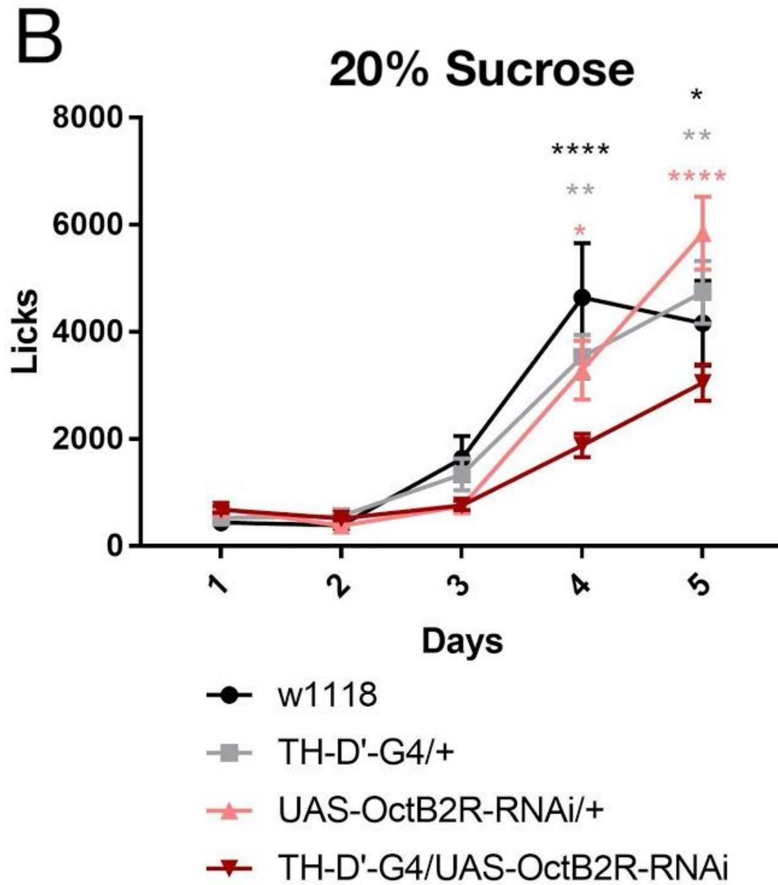
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Figure 5: Knockdown of Octβ2R in MB-MP1 Neurons May Attenuate Feeding

A) Triglyceride levels normalized to protein in age-matched *TH-D'>Octβ2R*^{RNAi} flies and the appropriate genetic controls fed either a ND or HSD for 7 days. n=8, two-way ANOVA with Sidak's multiple comparisons test

B) Average licks per day of age-matched *TH-D'>Octβ2R*^{RNAi} flies and the appropriate genetic controls fed a 20% sucrose diet. n=24-30, two-way ANOVA with Tukey's multiple comparisons test

Discussion

With the rise of the availability of ultra-processed food in modern society, added sugars have become a prominent fixture in the modern dietary environment. Overconsumption of added sugars has been associated with numerous health risks including obesity, diabetes, and heart disease. It is therefore essential to understand how sugar consumption affects feeding behavior. Flies, like humans and rodents, have been shown to lose sweet taste sensation when fed a high sugar diet (May et al., 2019; Proserpio et al., 2016; Berthoud et al., 2012). This loss of the ability to taste sugar in flies mediates an increase in feeding behavior, though the neural pathway through which changes on the periphery affect this central process is not well understood (May et al., 2019). Here we investigate the role of octopamine, a neurotransmitter shown to be involved in sweet taste reward, in the modulation of feeding behavior on a high sugar diet (Schwaerzel et al., 2003; Burke et al., 2012; Huetteroth et al., 2015).

We first explored the role of octopaminergic input to modulatory sweet taste reward neurons. Dopaminergic R48B04 sweet taste reward neurons of the PAM cluster express the octopamine receptor OAMB and our lab has previously shown that increasing the membrane excitability of these neurons prevents the overeating phenotype typically observed on a HSD (unpublished data). We knocked down OAMB in the R48B04 neurons using RNAi, predicting that the resulting decreased activation would lead to increased feeding behavior since exciting these cells prevents overeating. Instead, no phenotypic difference was observed in fat accumulation in *R48B04>oamb^{RNAi}* flies compared to controls (Figure 2A). Feeding behavior also did not increase as we had initially predicted, but rather decreased slightly in the experimental condition (Figure 2B). However, as the ND TAG levels looked comparable between the different genotypes and a downward trend was also seen in feeding was also seen in the UAS-oamb^{RNAi}/+ control, we did not take this to be due to the genetic background of the flies.

There are several different ways this data could be interpreted in context of our previous findings with the R48B04 neurons. Increasing the excitability of sweet taste reward neurons may merely be sufficient to rescue feeding behavior, while the root cause of the overeating phenotype is mediated by a different pathway or different neurons entirely. Later on, our lab had gone on to show that a distinct set of PAM DANs, the MB301B neurons, showed a reduced response to

sweet stimuli in flies fed a HSD and that optogenetic stimulation of this neuronal population restored normal feeding behavior (May et al., in press). It is also possible that a property of the R48B04 neurons other than sweet taste reward that is not dependent on OA is responsible. For example, one study demonstrated that R48B04 neurons convey the reinforcing effects of water and that OA is not required for water learning (Lin et al., 2014). Lastly, a knock down of the OAMB receptor may not impact feeding behavior if the phenotypic effects are caused by presynaptic deficiencies in neurotransmitter production or release.

To further investigate the effects of OA on feeding behavior on a high sugar diet, we both stimulated and inhibited octopaminergic cells expressing tyrosine decarboxylase, one of two enzymes required for OA synthesis. We originally hypothesized that inhibiting *Tdc2+* neurons with the inward rectifying potassium channel $K_{ir}2.1$ would result in an increase in feeding behavior based on the idea that decreased activation of R48B04 neurons by OA leads to increased feeding. Once again, our hypothesis was not supported. Instead, we obtained the opposite results, with *Tdc2> $K_{ir}2.1$, tub $GAL80^{ts}$* flies not accumulating triacylglycerides on a HSD (Figure 3A). Additionally, the experimental condition flies did not increase their feeding behavior over time (Figure 3B). This suggests that while OA does affect feeding behavior, it does not do so through action on OAMB.

Curiously enough, stimulating *Tdc2+* cells with TrpA1 also resulted in no significant change in fat accumulation when flies were fed a HSD (Figure 4A). However, these flies did show an increased food interaction level on both 5% and 20% sucrose that remained constant (Figure 4B and 4C). Given that TrpA1 has the opposite effects of $K_{ir}2.1$ on neuronal firing, these FLIC results are consistent with our findings in the previous experiment. The combination of these results also support the conclusion that OA release serves to increase feeding, rather than decrease feeding as was hypothesized based on the R48B04 data. The apparent discrepancy between triacylglyceride levels and feeding behavior on a HSD in *Tdc2>dTrpA1* suggests that somehow these flies do not accumulate excessive fat despite overeating. One possibility is that while stimulation of octopaminergic neurons promotes feeding on a HSD, it also causes flies to increase their energy expenditure, preventing excessive weight gain. For example, OA promotes wakefulness in *Drosophila,* with excitation of octopaminergic cells leading to sleep loss

(Crocker and Sehgal, 2008). If flies burn more calories due to spending more time awake, this phenotype might compensate for the increased energy consumption on a HSD and may be sufficient to prevent excessive fat levels.

Taken together, these results suggest that while OA affects feeding behavior, it does not do so through activation of R48B04 neurons. While knockdown of OAMB in the R48B04 neurons may abolish sweet taste dependent short term memory, OA must affect feeding through a separate pathway. However, this result is not entirely surprising. OAMB is a member of the OctαR subclass that is preferentially expressed in the *Drosophila* MBs and other brain regions and has been shown to increase both intracellular Ca^{2+} and cAMP levels (Han et al., 1998). Increased cAMP levels have been suggested to play a critical role in the molecular underpinnings of learning and memory (Menzel and Muller, 1996; Mayford and Kandel, 1999). Given the prominent role of the MBs in learning and memory in *Drosophila*, as well as the role of amine-receptors resulting in increases of cAMP in these same processes, it is not unreasonable to suggest OAMB may be involved in behavioral plasticity. However, other studies have shown that OAMB activation primarily affects Ca^{2+} levels, with only a small change in intracellular cAMP levels as a result of OA binding (Balfanz et al., 2005). Meanwhile, OctβRs are also primarily expressed in the brain and mediate their effects through activation of adenylate cyclase leading to increased intracellular cAMP levels (Balfanz et al., 2005). Therefore, it is possible that OA modulates feeding through activation of an OctβR.

As previously discussed, the beta octopaminergic receptor Octβ2R plays a function in state dependent appetitive learning mediated by the dopaminergic MB-MP1 neurons that innervate the MBs, similar to the R48B04 population. To research the possibility that OA mediates its effects on feeding behavior through influence on the negative dopaminergic MB-MP1 neurons, we used RNAi to knock down Octβ2R in this neuronal population. While no conclusions could be drawn from the TAG assay due to the failure of the *TH-D'-GAL4*/+ genetic control to display the expected phenotype, an attenuation of the increased feeding over time on a HSD was demonstrated in *TH-D'>Octβ2R*^{RNAi} flies (Figure 5A and 5B). These results suggest that octopamine affects feeding behavior through activation of *Octβ2R* expressed in MB-MP1 neurons involved in appetitive motivation.

Current research suggests that the MB-MP1 neurons serve as a gate on appetitive memory retrieval, tonically releasing dopamine to inhibit MB neuronal function in the sated state. When flies get hungry, dNPF levels rise and inhibit the MB-MP1 neurons allowing appetitive memory to form (Krashes et al., 2009). Artificial memory formation through *Tdc2+* stimulation is abolished by the knockdown of Octβ2R. However, the exact mechanism of how OA exactly affects learning is unclear since preventing the output of MB-MP1 neurons during *Tdc2+* stimulation also prevents artificial learning (Burke et al. 2013). This suggests that in this pathway, OA leads to appetitive memory through excitation of these cells, even though the current model proposes that the MB-MP1 cells are inhibitory. Given these seemingly contradictory findings, it becomes apparent that there is much to be learned about the nuances of this system.

To complicate matters further, stimulation of the MB-MP1 neurons leads to aversive memory formation, raising the possibility that OA activation somehow influences state-dependent motivation through this functionality (Aso et al., 2010). While the specifics of aversive memory formation are beyond the scope of this project, we had previously explored the possibility of aversive conditioning playing a role in the changes in feeding behavior in flies elicited by a HSD. Various studies have proposed that aversion to withdrawal serves as a major motivating factor in drug-taking behavior (Koob and Bloom, 1988; Bechara et al, 1998; Delfs et al., 2000). Other evidence has shown that intermittent sugar intake followed by removal of sweet stimuli leads to withdrawal-like symptoms (Avena et al., 2008; Mangabeira et al., 2015). To investigate whether aversion influences feeding behavior, we expressed both NaChBac and K_{ir}2.1 in the PPL1 cluster responsible for aversive reinforcement using the previously mentioned *TH-D'*-Gal4 driver line (Claridge-Chang et al., 2009). While we observed a slight decrease in the triacylglyceride levels of the *TH-D'>*K_{ir}*2.1,Gal80*^{ts} flies, *TH-D'>NaChBac* flies accumulated fat levels on both diets similar to controls (Figure S1A and S1B). The significant, but not absolute prevention of fat accumulation in the *TH-D'>*K_{ir}*2.1,Gal80*^{ts} flies is consistent with our results from the Octβ2R knockdown in these same neurons (Figure 5B)..

While the data presented here provides some insight into the role of OA in modulations of feeding behavior on a HSD, there are many future directions that can be taken to further

explore this subject. For one, the driver line used to express Octβ2R RNAi was not specific to the MB-MP1 neurons. *TH-D'*-GAL4 labels the entire PPL1 cluster as well as some other DANs in different brain regions (Galili et al., 2014). Considering that the MB-MP1 neurons are only a subset of the PPL1 cluster, it would be prudent to repeat the experiment using a driver line that exclusively labels MB-MP1. This would ensure that any phenotypes observed are due to the knockdown of Octβ2R in the MB-MP1 neurons and not some off target effects of the knockdown in other cells. When these experiments were conducted, *TH-D'*-GAL4 was the only driver line available in the lab that labeled the MB-MP1 neurons, and was used in the interest of time.

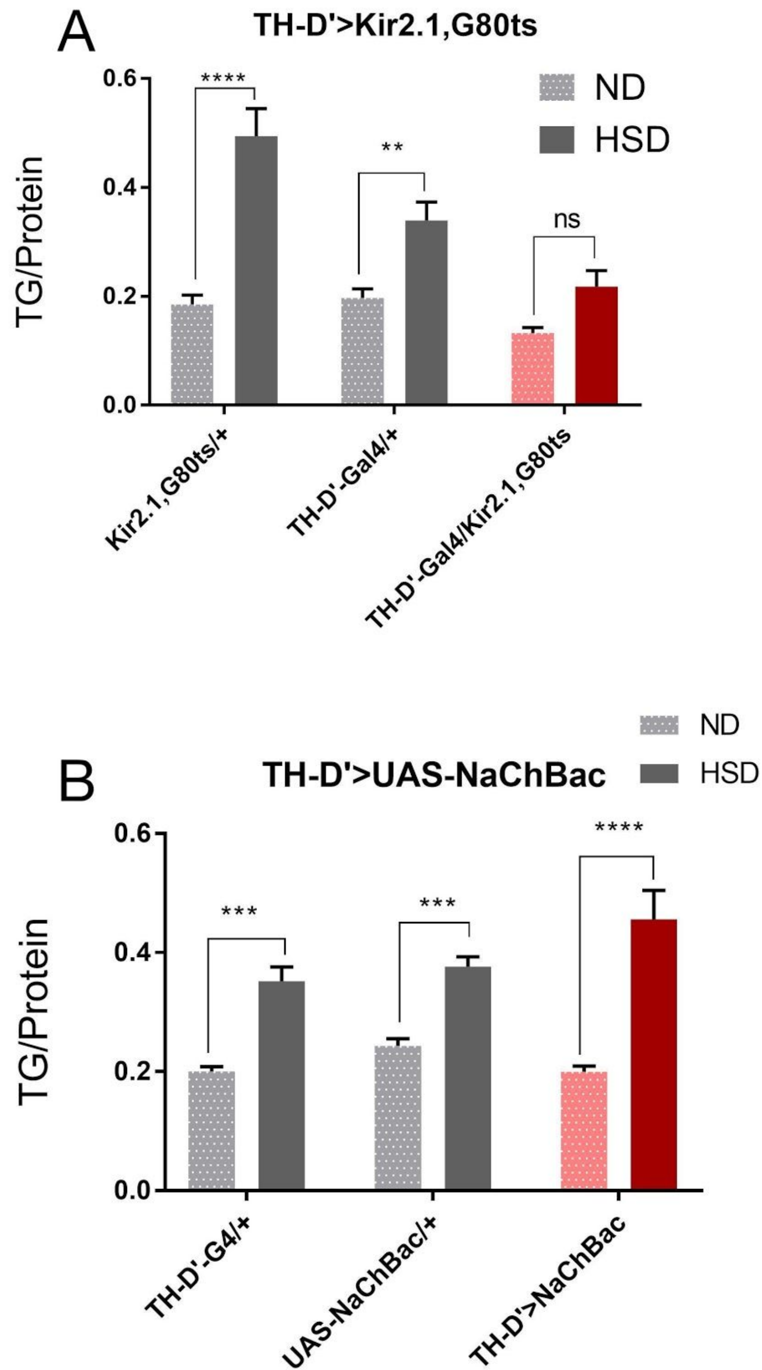
It should be pointed out that even though *TH-D'>Octβ2R*^{RNAi} flies ate significantly less than controls on days 4 and 5 of the FLIC, they still showed a modest increase in feeding over time on a HSD. In contrast, inhibition of octopaminergic neurons prevented the development of the increased feeding phenotype on a HSD, with *Tdc2>* K_{ir}*2.1, tub GAL80*^{ts} flies showing a constant number of licks per day over the course of the experiment (Figure 3B). This distinction indicates that either the knockdown of *Octβ2R* was incomplete or OA acts through a parallel pathway to affect feeding.

Therefore, another valuable follow up experiment would be to screen for the influence of other OA receptors on feeding behavior by systematically knocking down different receptors using a driver line that labels all neurons and seeing if feeding is affected. One such driver line is *nsyb*-GAL4, that drives expression from the promoter for neuronal synaptobrevin, a protein found in all neurons required for vesicular release of neurotransmitters. These experiments could reveal novel pathways involving OA that affect feeding behavior or could also point to existing circuits that utilize OA receptors in learning and memory.

For example, one study looking at larval feeding implicated Octβ3R in appetitive motivation (Zhang et al., 2013). Inhibition of *Tdc2+* neurons prevented a typical increase in feeding responses as measured by the rate of larval mouth hook contractions (MHC), while activation increased MHC rate. The increase in MHC rate due to stimulation of these neurons was abolished by knockdown of Octβ3R, but not any other OA receptors. Furthermore, this study suggested that larval feeding is differentially regulated by *Tdc2+* neurons in different

subsets of the ventral unpaired median neurons (VUM), with feeding being promoted by activity of the VUM2 neurons who are inhibited by neurons of the more anterior VUM1 region that acts to decrease appetite. It would therefore be reasonable to study Octβ3R and this neuronal population in the contexts of feeding on a HSD. (Zhang et al., 2013)

Our collective data suggests that octopaminergic signaling influences feeding behavior in *Drosophila* on a HSD, though not through input to sweet taste reward neurons expressing OAMB. Instead, our findings point to the possibility that OA influences feeding behavior through activation of MB-MP1 neurons involved in appetitive motivation and aversion. The data presented here lays the groundwork for future studies of the neurological path or paths that allows OA to affect feeding. Additional studies will lead to a more nuanced understanding of the cellular and molecular mechanisms through which modulation in sweet taste at the periphery lead to changes in behavior. Such an understanding of how the modern dietary environment can influence behavior is especially relevant in context of the obesity epidemic and other negative health risks associated with excessive sugar consumption.



Supplementary Figure 1: Excitation and Inhibition of Aversion Neurons

A) Triglyceride levels normalized to protein in age-matched *TH-D'>*K_{ir}*2.1,Gal80*^{ts} flies and the appropriate genetic controls fed either a ND or HSD for 7 days. n=8, two-way ANOVA with Sidak's multiple comparisons test

B) Triglyceride levels normalized to protein in age-matched *TH-D'>NaChBac* flies and the appropriate genetic controls fed either a ND or HSD for 7 days. n=8, two-way ANOVA with Sidak's multiple comparisons test

References

Aso Y, Grubel K, Busch S, Friedrich AB, Siwanowicz I, Tanimoto H (2009) The Mushroom Body of Adult Drosophila Characterized by GAL4 Drivers. Journal of Neurogenetics 23:156-U129.

Aso Y, Siwanowicz I, Bracker L, Ito K, Kitamoto T, Tanimoto H (2010) Specific Dopaminergic Neurons for the Formation of Labile Aversive Memory. Current Biology 20:1445-1451.

Aso Y, Hattori D, Yu Y, Johnston RM, Iyer NA, Ngo TTB, Dionne H, Abbott LF, Axel R, Tanimoto H, Rubin GM (2014) The neuronal architecture of the mushroom body provides a logic for associative learning. Elife 3.

Avena NM, Rada P, Hoebel BG (2008a) Evidence for sugar addiction: Behavioral and neurochemical effects of intermittent, excessive sugar intake. Neuroscience and Biobehavioral Reviews 32:20-39.

Avena NM, Rada P, Hoebel BG (2008b) Evidence for sugar addiction: Behavioral and neurochemical effects of intermittent, excessive sugar intake. Neuroscience and Biobehavioral Reviews 32:20-39.

Balfanz S, Strunker T, Frings S, Baumann A (2005) A family of octopamine receptors that specifically induce cyclic AMP production or Ca2+ release in Drosophila melanogaster (vol 93, pg 440, 2005). Journal of Neurochemistry 94:1168-1168.

Bechara A, Nader K, Van der Kooy D (1998) A two-separate-motivational-systems hypothesis of opioid addiction. Pharmacology Biochemistry and Behavior 59:1-17.

Bellah KL, Fitch GK, Kammer AE (1984) A CENTRAL ACTION OF OCTOPAMINE ON VENTILATION FREQUENCY IN CORYDALUS-CORNUTUS. Journal of Experimental Zoology 231:289-292.

Berkey CS, Rockett HRH, Field AE, Gillman MW, Colditz GA (2004) Sugar-added beverages and adolescent weight change. Obesity Research 12:778-788.

Berthoud HR, Zheng HY (2012a) Modulation of taste responsiveness and food preference by obesity and weight loss. Physiology & Behavior 107:527-532.

Berthoud HR, Zheng HY (2012b) Modulation of taste responsiveness and food preference by obesity and weight loss. Physiology & Behavior 107:527-532.

Blenau W, Baumann A (2001) Molecular and Pharmacological Properties of InsectBiogenic Amine Receptors: Lessons From Drosophilamelanogaster and Apis mellifera. Insect Biochemistry and Physiology:13-38.

Brand AH, Perrimon N (1993) TARGETED GENE-EXPRESSION AS A MEANS OF ALTERING CELL FATES AND GENERATING DOMINANT PHENOTYPES. Development 118:401-415.

Burke CJ, Huetteroth W, Owald D, Perisse E, Krashes MJ, Das G, Gohl D, Silies M, Certel S, Waddell S (2012) Layered reward signalling through octopamine and dopamine in Drosophila. Nature 492:433-+.

Busch S, Selcho M, Ito K, Tanimoto H (2009) A Map of Octopaminergic Neurons in the Drosophila Brain. Journal of Comparative Neurology 513:643-667.

Caballero B (2007) The global epidemic of obesity: An overview. Epidemiologic Reviews 29:1-5.

Claridge-Chang A, Roorda RD, Vrontou E, Sjulson L, Li HY, Hirsh J, Miesenbock G (2009) Writing Memories with Light-Addressable Reinforcement Circuitry. Cell 139:405-415.

Colantuoni C, Schwenker J, McCarthy J, Rada P, Ladenheim B, Cadet JL, Schwartz GJ, Moran TH, Hoebel BG (2001) Excessive sugar intake alters binding to dopamine and mu-opioid receptors in the brain. Neuroreport 12:3549-3552.

Cole S, Carney G, McClung C, Willard S (2005) Two Functional but Noncomplementing Drosophila Tyrosine Decarboxylase GenesDISTINCT ROLES FOR NEURAL TYRAMINE AND OCTOPAMINE IN FEMALE FERTILITY. The Journal of Biological Chemistry.

Crocker A, Sehgal A (2008) Octopamine regulates sleep in Drosophila through protein kinase A-dependent mechanisms. Journal of Neuroscience 28:9377-9385.

Dahanukar A, Lei YT, Kwon JY, Carlson JR (2007) Two Gr genes underlie sugar reception in Drosophila. Neuron 56:503-516.

de Koning L, Malik VS, Kellogg MD, Rimm EB, Willett WC, Hu FB (2012) Sweetened Beverage Consumption, Incident Coronary Heart Disease, and Biomarkers of Risk in Men. Circulation 125:1735-U1784.

Delfs JM, Zhu Y, Druhan JP, Aston-Jones G (2000) Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. Nature 403:430-434.

Evans P, Maqueira B (2005) Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. Invertebrate Neuroscience 5:111-118.

Fakler B, Brändle U, Glowatzki E, Zenner HP, Ruppersberg JP (1994) K ir 2.1 inward rectifier K + channels are regulated independently by protein kinases and ATP hydrolysis. Neuron 13:1413-1420.

Farooqui T (2012) Review of octopamine in insect nervous systems. Open Access Insect Physiology 4:1-17.

Guthrie JF, Morton JF (2000) Food sources of added sweeteners in the diets of Americans. Journal of the American Dietetic Association 100:43-51.

Hajnal A, Norgren R (2001) Accumbens dopamine mechanisms in sucrose intake. Brain Research 904:76-84.

Harris DT, Kallman BR, Mullaney BC, Scott K (2015) Representations of Taste Modality in the Drosophila Brain. Neuron 86:1449-1460.

Han K-A, Millar N, Davis R (1998) A Novel Octopamine Receptor with Preferential Expression in*Drosophila* Mushroom Bodies. Journal of Neuroscience 18:3650-3658.

Heisenberg M (2003) Mushroom body memoir: From maps to models. Nature Reviews Neuroscience 4:266-275.

Hoyer SC, Eckart A, Herrel A, Zars T, Fischer SA, Hardie SL, Heisenberg M (2008) Octopamine in male aggression of Drosophila. Current Biology 18:159-167.

Hu FB, Malik VS (2010) Sugar-sweetened beverages and risk of obesity and type 2 diabetes: Epidemiologic evidence. Physiology & Behavior 100:47-54.

Huetteroth W, Perisse E, Lin SW, Klappenbach M, Burke C, Waddell S (2015) Sweet Taste and Nutrient Value Subdivide Rewarding Dopaminergic Neurons in Drosophila. Current Biology 25:751-758.

Kelley AE, Berridge KC (2002) The neuroscience of natural rewards: Relevance to addictive drugs. Journal of Neuroscience 22:3306-3311.

Kim Y-C, Lee H-G, Han K-A (2007) D 1 Dopamine Receptor dDA1 Is Required in the Mushroom Body Neurons for Aversive and Appetitive Learning in Drosophila. The Journal of Neuroscience:7640-7647.

Kirkhart C, Scott K (2015) Gustatory Learning and Processing in the Drosophila Mushroom Bodies. Journal of Neuroscience 35:5950-5958.

Koob GF, Bloom FE (1988) CELLULAR AND MOLECULAR MECHANISMS OF DRUG-DEPENDENCE. Science 242:715-723.

Krashes MJ, Waddell S (2008) Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in Drosophila. Journal of Neuroscience 28:3103-3113.

Krashes MJ, DasGupta S, Vreede A, White B, Armstrong JD, Waddell S (2009) A Neural Circuit Mechanism Integrating Motivational State with Memory Expression in Drosophila. Cell 139:416-427.

Lee HG, Seong CS, Kim YC, Davis RL, Han KA (2003) Octoparnine receptor OAMB is required for ovulation in Drosophila melanogaster. Developmental Biology 264:179-190.

Lin SW, Owald D, Chandra V, Talbot C, Huetteroth W, Waddell S (2014) Neural correlates of water reward in thirsty Drosophila. Nature Neuroscience 17:1536-1542.

Liu C, Placais PY, Yamagata N, Pfeiffer BD, Aso Y, Friedrich AB, Siwanowicz I, Rubin GM, Preat T, Tanimoto H (2012a) A subset of dopamine neurons signals reward for odour memory in Drosophila. Nature 488:512-+.

Ludwig DS, Peterson KE, Gortmaker SL (2001) Relation between consumption of sugar-sweetened drinks and childhood obesity: a prospective, observational analysis. Lancet 357:505-508.

Luger M, Lafontan M, Bes-Rastrollo M, Winzer E, Yumuk V, Farpour-Lambert N (2017) Sugar-Sweetened Beverages and Weight Gain in Children and Adults: A Systematic Review from 2013 to 2015 and a Comparison with Previous Studies. Obesity Facts 10:674-693.

Malik VS, Popkin BM, Bray GA, Despres JP, Hu FB (2010a) Sugar-Sweetened Beverages, Obesity, Type 2 Diabetes Mellitus, and Cardiovascular Disease Risk. Circulation 121:1356-1364.

Malik VS, Popkin BM, Bray GA, Despres JP, Willett WC, Hu FB (2010b) Sugar-Sweetened Beverages and Risk of Metabolic Syndrome and Type 2 Diabetes. Diabetes Care 33:2477-2483.

Mangabeira V, Garcia-Mijares M, Silva MTA (2015) Sugar withdrawal and differential reinforcement of low rate (DRL) performance in rats. Physiology & Behavior 139:468-473.

Masek P, Scott K (2010) Limited taste discrimination in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 107:14833-14838.

Mayford M, Kandel ER (1999) Genetic approaches to memory storage. Trends in Genetics 15:463-470.

Mentel T, Duch C, Stypa H, Wegener G, Muller U, Pfluger HJ (2003) Central modulatory neurons control fuel selection in flight muscle of migratory locust. Journal of Neuroscience 23:1109-1113.

Menzel R, Muller U (1996) Learning and memory in honeybees: From behavior to neural substrates. Annual Review of Neuroscience 19:379-404.

Monastirioti M, Linn C, White K (1996) Characterization of Drosophila Tyramine β-HydroxylaseGene and Isolation of Mutant Flies Lacking Octopamine. The Journal of Neuroscience:3900-3911.

Monteiro CA, Moubarac JC, Cannon G, Ng SW, Popkin B (2013) Ultra-processed products are becoming dominant in the global food system. Obesity Reviews 14:21-28.

Montell C (2009) A taste of the Drosophila gustatory receptors. Current Opinion in Neurobiology 19:345-353.

Musselman LP, Fink JL, Narzinski K, Ramachandran PV, Hathiramani SS, Cagan RL, Baranski TJ (2011) A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. Disease Models & Mechanisms 4:842-849.

Na JB, Musselman LP, Pendse J, Baranski TJ, Bodmer R, Ocorr K, Cagan R (2013) A Drosophila Model of High Sugar Diet-Induced Cardiomyopathy. Plos Genetics 9.

Nathanson JA (1979) OCTOPAMINE RECEPTORS, ADENOSINE-3',5'-MONOPHOSPHATE, AND NEURAL CONTROL OF FIREFLY FLASHING. Science 203:65-68.

Nitabach MN, Wu Y, Sheeba V, Lemon WC, Strumbos J, Zelensky PK, White BH, Holmes TC (2006) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. Journal of Neuroscience 26:479-489.

Prier KR, Beckman OH, Tublitz NJ (1994) MODULATING A MODULATOR - BIOGENIC-AMINES AT SUBTHRESHOLD LEVELS POTENTIATE PEPTIDE-MEDIATED CARDIOEXCITATION OF THE HEART OF THE TOBACCO HAWKMOTH MANDUCA-SEXTA. Journal of Experimental Biology 197:377-391.

Proserpio C, Laureati M, Bertoli S, Battezzati A, Pagliarini E (2016a) Determinants of Obesity in Italian Adults: The Role of Taste Sensitivity, Food Liking, and Food Neophobia. Chemical Senses 41:169-176.

Proserpio C, Laureati M, Bertoli S, Battezzati A, Pagliarini E (2016b) Determinants of Obesity in Italian Adults: The Role of Taste Sensitivity, Food Liking, and Food Neophobia. Chemical Senses 41:169-176.

Pulver SR, Pashkovski SL, Hornstein NJ, Garrity PA, Griffith LC (2009) Temporal Dynamics of Neuronal Activation by Channelrhodopsin-2 and TRPA1 Determine Behavioral Output in Drosophila Larvae. Journal of Neurophysiology 101:3075-3088.

Ro J, Harvanek ZM, Pletcher SD (2014) FLIC: High-Throughput, Continuous Analysis of Feeding Behaviors in Drosophila. Plos One 9.

Roeder T (1999) Octopamine in invertebrates. Progress in Neurobiology 59:533-561.

Schulz DJ, Robinson GE (2001) Octopamine influences division of labor in honey bee colonies. Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology 187:53-61.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. Journal of Neuroscience 23:10495-10502.

Smith KB, Smith MS (2016) Obesity Statistics. Primary Care 43:121-+.

Sombati S, Hoyle G (1984) CENTRAL NERVOUS SENSITIZATION AND DISHABITUATION OF REFLEX ACTION IN AN INSECT BY THE NEUROMODULATOR OCTOPAMINE. Journal of Neurobiology 15:455-480.

Steele EM, Baraldi LG, Louzada MLD, Moubarac JC, Mozaffarian D, Monteiro CA (2016) Ultra-processed foods and added sugars in the US diet: evidence from a nationally representative cross-sectional study. Bmj Open 6.

Tomchik SM, Davis RL (2009) Dynamics of Learning-Related cAMP Signaling and Stimulus Integration in the Drosophila Olfactory Pathway. Neuron 64:510-521

Volkow N.D., Wang G.J., Fowler J.S., Tomasi D., Baler R. (2011) Food and Drug Reward: Overlapping Circuits in Human Obesity and Addiction. In: Carter C., Dalley J. (eds) Brain Imaging in Behavioral Neuroscience. Current Topics in Behavioral Neurosciences, vol 11. Springer, Berlin, Heidelberg

Vosshall LB, Stocker RE (2007) Molecular architecture of smell and taste in Drosophila. Annual Review of Neuroscience 30:505-533.

Waddell S (2010) Dopamine reveals neural circuit mechanisms of fly memory. Trends in Neurosciences 33:457-464.

Wang ZR, Singhvi A, Kong P, Scott K (2004) Taste representations in the Drosophila brain. Cell 117:981-991.

Yamagata N, Ichinose T, Aso Y, Placais PY, Friedrich AB, Sima RJ, Preat T, Rubin GM, Tanimoto H (2015) Distinct dopamine neurons mediate reward signals for short- and long-term memories. Proceedings of the National Academy of Sciences of the United States of America 112:578-583.

Yang QH, Zhang ZF, Gregg EW, Flanders D, Merritt R, Hu FB (2014) Added Sugar Intake and Cardiovascular Diseases Mortality Among US Adults. Jama Internal Medicine 174:516-524.

Zhang T, Branch A, Shen P (2013) Octopamine-mediated circuit mechanism underlying controlled appetite for palatable food in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 110:15431-15436.