TEMPORAL CONTROL OF ODOR-INDUCED MOTIVATIONAL STATE IN A *DROSOPHILA* MODEL

by

SHELBY THOMAS

(Under the Direction of Ping Shen)

ABSTRACT

Motivation and goal-directed behaviors are associated with prevalent human conditions such as addiction, ADHD, and depression, but the mechanisms mediating how motivational states are temporally regulated are not fully understood. *Drosophila melanogaster* larvae have a numerically simplified central nervous system that is organized into processing centers similar to mammalian brains and serve as a good model for determining neural pathways involved in motivational state. Here, I define a behavior paradigm to determine genes, receptors, and neurons required for the maintenance and termination of odor-aroused motivation in *Drosophila* using genetic and cellular tools. I show that dopamine release from the DL1 cluster onto Dop1R2 receptors in a higher-order odor-processing center, the mushroom body, is required for normal termination of the motivational state. I will outline a three-step model of the temporal control of motivational state through a NPF, dopamine, and mushroom body circuit.

INDEX WORDS: *Drosophila melanogaster*, Dopamine, Neuropeptide F, Motivation, Olfaction

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview

The neural circuits and mechanisms driving how humans interact with the sensory world are of widespread interest, however are largely unknown. While the numerical complexity of the human brain makes addressing behavioral circuits challenging, many of the proteins, cells types, and physiological functions seen in the human nervous system are conserved in lower-order, simplified model organisms. By taking advantage of conserved sensory systems, genetic tools, and behavioral output paradigms in lower-order organisms such as the fruit fly, *Drosophila melanogaster*, researchers have begun to elucidate the fundamental mechanisms regulating complicated behaviors such as motivation. Motivation is an internal state that involves integration of past and present sensory information to influence behavioral output in a manner that is not fully understood. In addition to its highly-recognized involvement in addiction, motivation drives behaviors essential to survival, such as eating and responding to danger. In this review, I will further define motivational states, outline multiple sensory systems that aid in our understanding of internal motivational states, and describe major findings that have guided our understanding of the mechanisms underlying these behaviors.

Motivational States

Definition and Significance

Motivational states fall within the scope of a broader concept on the frontier of neurobiology, the internal state. An internal state is the cognitive context under which an organism interacts with its surroundings, and encompasses a variety of statuses such as sleep, arousal, hunger, and aggression (Sayin et al., 2018; Anderson 2016). Here, we focus on an internal state of motivation, which broadly encompasses a cognitive drive to perform a behavior, typically for an incentive or anticipated reward (LeDoux 2012; Botvinick and Braver, 2015; Sayin et al., 2018). Motivational states can be assessed by observing the presence or absence of goal-directed behavior, for example increased lever-pulling for a drug reward in rodents or increased rate of eating a sugar-rich food in *Drosophila* larvae. The induction of a motivated behavior can be metabolically based, for example eating due to hunger, or hedonically based, such as consuming a food for pleasure despite no nutritional need for it. The research presented in the following chapter will focus on hedonic based motivation, which is often initiated in response to an external factor, such as an appetitive odor or visual stimulus.

Motivational states are essential from a survival standpoint in order for a species to seek food, mates, and safety. Without motivational states, the cognitive and behavioral functions required to make decisions based on the available information would be hindered. However, motivational states also cannot persist indefinitely, as eventually attention needs to be redirected from one task onto another to account for the organism's current needs and surroundings. This introduces the possibility that there is not only a pathway to initiate motivational state, but also to actively terminate it when necessary. While motivational states are required for basic functioning, the circuits involved in this phenomenon are still not understood. Therefore, it is

essential to further characterize motivational states through identification of the neurons, receptors, and circuits required to initiate, maintain, and terminate motivated behavior.

An Animal Model for Studying Motivational State

Drosophila melanogaster larvae serve as a strong model for studying motivational state due to our ability to genetically target knockdown of neurons and receptors of interest and observe any resulting shifts in motivated behavior. *Drosophila* experience three primary sensations: smell, taste, and touch. The senses of smell and taste can be employed to measure motivation by stimulating *Drosophila* larvae with an appetitive odor, such as the banana-like scent pentyl acetate (PA), then measuring larvae eating rate by counting their mouth hook contractions (MHC) in a sugar-rich food over a 30-second measuring period. It has been shown that without odor stimulation, satiated larvae eat sugar-rich food at a rate of approximately 30 MHCs per 30-seconds, and after brief exposure to an appetitive odor, satiated larvae display a 20% increase in feeding rate (Wu et al., 2013). This odor-aroused increase in eating behavior can serve as a behavioral readout for a motivational state, and therefore throughout this work satiated larvae are considered to be in a motivational state when driven to eat a sugar-rich food at a faster rate. To better understand the cellular mechanisms potentially mediating this internal motivational state, I will review the following key systems: the olfactory system, the dopamine system, and hunger signaling neuropeptide NPY(F).

The Olfactory System

Mammals

Odors are perceived in the periphery by olfactory receptor neurons in the nasal olfactory epithelium. The olfactory epithelium consists of 6-10 million olfactory sensory neurons (OSNs) with approximately 1000 types of olfactory receptors. Odor molecules stimulate distinct sets of OSNs, which relay the signal to a small number of glomeruli in the olfactory bulb. Here, OSNs synapse onto the output neurons of the olfactory bulb, mitral cells, which relay the information onto higher brain regions such as the cortex, hippocampus, hypothalamus, and amygdala (Firestein, 2001; Lledo et al. 2005; Figure 1.1). While the initial steps of the odor-perception pathway are well understood, the numerical complexity of the mammalian olfactory system makes it difficult to isolate how higher-order brain systems integrate and use olfactory information to influence behavior and internal states such as motivation.

Drosophila

The olfactory system in *Drosophila* larvae is quite simplified compared to that of adult flies and mammals. Odors are perceived in the larval dorsal organ, where 21 types of olfactory receptor neurons (ORN) pick up the odor signal and relay it to the primary olfactory processing center, the antennal lobe (AL), a region analogous to the mammalian olfactory bulb (Oppliger et al., 2000; Kreher et al., 2005; Python and Stocker, 2002; Heimbeck et al., 1999). Here, neurons synapse in a 1:1 fashion onto one of the 21 glomeruli of the AL (Ramaekers et al., 2005). ORNs relay information onto the secondary olfactory neurons, projection neurons (PN), which parallel mitral cells in the mammalian system (Vosshall and Stocker, 2007). PNs connect the AL to two distinct secondary olfactory processing centers, the lateral horn (LH) or the mushroom body

(MB) (Stocker 1994; Marin et al., 2005; Ramaekers et al., 2005; Figure 1.1). The lateral horn is known to be involved in innate reactions to odors, and it has been shown that knockdown of PNs projecting to this region results in a loss of odor response (de Belle and Heisenberg, 1994; Heimbeck et al., 2001; Parnas et al., 2013). Prior data from our lab has indicated that the LH is required for odor-aroused feeding behavior in satiated larvae, suggesting that the LH is necessary for the induction of odor-aroused motivational state.

Previously, it was hypothesized that the MB was not involved in innate odor perception, as loss of all MB neurons had no effect on odor response, however more recent studies have found that subsets of MB neurons do seem to regulate innate odor responses (Parnas et al., 2013; Cohn et al., 2015; Owald et al., 2015; Tsao et al., 2018; Sayin et al., 2018). The MB is largely considered the primary brain center for olfactory learning and memory (Gerber and Stocker, 2007; McGuire et al., 2005; de Belle and Heisenberg, 1994), as it has been shown that MB activity and output are required for olfactory associative learning and memory consolidation and retrieval (Owald and Waddell, 2015; Das et al., 2016; Busto et al., 2010; Davis 2005). The larval mushroom body can be broken down into seven primary distinct anatomical regions: the medial lobe, vertical lobe, pedunculus, spur, calyx, medial appendix, and lateral appendix (Selcho et al., 2009). There are approximately 400 MB intrinsic cells, called Kenyon cells (KC), in third instar larvae, which receive input from PNs in the calyx in a sparse and random manner and output onto the vertical and medial lobes (Campbell and Turner 2010; Technau and Heisenberg, 1982; Yao et al., 2012; Aso et al., 2014). KC output on the vertical lobe are onto cholinergic MB output neurons (MBONs) and are associated with aversive/avoidance stimuli (Sejourne et al., 2011; Pai et al., 2013; Plaçais et al., 2013), while KC output on the medial lobe are onto glutamatergic and GABAergic MBONs and are associated with appetitive/approach stimuli (Liu

and Wilson, 2013). It has been found that a subset of cholinergic MBONs project to the LH, allowing for lateral communication between the two secondary odor processing centers, however most MBON output is confined to 5 neuropils surrounding the MB, where it is believed signals are further processed an integrated (Aso et al., 2014). Additionally, some MBONs have presynaptic terminals in the MB, potentially enabling a feedback-loop mechanism. The connection between KCs and MBONs has been found to be mediated by dopaminergic input, and it has been suggested that dopamine relays a moment-by-moment update of external stimuli and tunes MB output accordingly (Aso et al., 2014; Waddell 2016; Cognigni et al., 2018).

The Dopamine System

Mammals

As the most predominant neurotransmitter in the brain, dopamine (DA) has been associated with many higher-order behaviors and cognitive functions, such as motivation, sleep, arousal, learning, and addiction, while dysregulation of DA has been associated with numerous clinical conditions including Parkinson's Disease, Huntington's Disease, schizophrenia, ADHD, and depression (Baik 2013; Colombo 2013; Kienast and Heinz 2006). DA involvement in many of these emotional and motivational behaviors has been attributed to its role in the mesolimbic dopaminergic pathway, often referred to as the reward pathway. In this pathway, DA neurons originate in the ventral tegmental area (VTA) and project to regions including the nucleus accumbens (NAc), prefrontal cortex, hypothalamus, and amygdala (Baik 2013). It is well received that synaptic activity within the mesolimbic DA pathway is modified by not only addictive drugs, but also food reward, and that this pathway is required for food cravings (Baik 2013; Volkow et al., 2011; Wise 2006). It has been shown that mice unable to synthesize

dopamine display low motivation to eat (Zhou and Palmiter 1995), and that DA release increased in the NAc after rats were exposed to sugar (Hajnal et al., 2004), exhibiting the mesolimbic DA response to rewarding stimuli and role in motivated behaviors.

DA is synthesized in the substantia nigra and the VTA via the hydroxylation of tyrosine to L-DOPA by rate limiting enzyme tyrosine hydroxylase (TH), followed by the decarboxylation of L-DOPA into dopamine (Molinoff and Axelrod, 1971; Baik 2013). There are two classes of dopamine receptors, D1-like, which consists of receptor subtypes D1 and D5, and D2-like, which consists of receptor subtypes D2, D3, and D4 (Niznik 1987; Kebian and Calne 1979; Spano et al., 1978). Dopamine receptors are G-protein coupled receptors (GPCR), with D1-like receptors coupled to $G\alpha_s$ to stimulate adenylyl cyclase (AC) activity, downstream cAMP accumulation, and thereby PKA activity to potentially drive neuron excitability, while D2-like receptors are coupled to $G\alpha_i$ to inhibit the AC-cAMP-PKA signaling pathway (Gingrich and Caron 2003; Neve et al., 2004).

Drosophila

Third instar *Drosophila* larvae house 120 dopaminergic neurons, a significantly simplified system in comparison to vertebrates (Rohwedder et al., 2016). In the brain lobes, DA neurons are divided into three main clusters: DL1, DL2, and DM, with each DA cluster containing 6-8 neurons (Selcho et al., 2009; Monastirioti 1999; Figure 1.2). Primarily, the DL1 cluster innervates various regions of the MB, DL2 innervates the dorsolateral protocerebrum region, which includes the LH, and DM neurons primarily innervate the MB medial lobe (Selcho et al., 2009).

Similar to mammalian systems, two classes of GCPR dopamine receptors have been identified in *Drosophila* larvae: D1-like and D2-like. D1-like receptors utilize the $G\alpha_s$ pathway to activate adenylyl cyclase and downstream cAMP, and can be broken down into two main subtypes: Dop1R1 (dDA1) and Dop1R2 (DAMB) (Gotzes et al., 1994; Feng et al., 1996; Han et al., 1996; Monastirioti 1999). Both of these receptors are highly expressed in the MB and mediate calcium influx (Han et al. 1996; Kim et al. 2003; Cohn et al., 2015). *Drosophila* only express one D2-like receptor, Dop2R (DDR2), which uses the $G\alpha_i$ pathway to down regulate adenylyl cyclase and cAMP signaling (Hearn et al., 2002). A fourth type of receptor, DopEcR, has been identified as a potential homolog of γ-adrenergic receptors in vertebrates (Srivastava 2005).

The *Drosophila* dopamine system has been associated with behaviors such as memory, learning, motivation, addiction, motor control, and arousal (Yamamoto and Seto, 2014; Waddell 2013, 2016; Schwaerzel et al., 2003; Schroll et al., 2006; Kim et al., 2007; Claridge-Chang et al., 2009; Wise 2004; Krashes et al., 2009; Lusher and Malenka 2011; Joshua et al., 2009; Kume et al., 2005; Andretic et al., 2005). Dopamine also plays a role in development as DA levels increase at certain transitional points during the life cycle of *Drosophila*, including during larval molts, pupation, and adult emergence (Wright 1987). Dopamine has been shown to play a role in odor perception, as the DL2 clusters are required in the LH for odor perception, and is believed to relay the reward value of odors on to higher-order processing centers (Wang et al., 2013; Waddell 2016; Tsao et al., 2018). Activation of all dopaminergic neurons drives aversive behavior, however, it has been suggested that more discrete stimulation of DA sub-clusters relays reward or punishment value at a more precise level (Liu et al., 2012; Rohwedder et al., 2016; Berry et al., 2012; Schroll et al., 2006; Claridge-Chang et al., 2009; Tsao et al., 2018).

NPY/F Signaling

Mammals

In mammals, NPY acts as a hunger signal to regulate metabolic and hedonic feeding (Currie, 2003; Pandit et al., 2014). During hungry states, NPY levels rise in the hypothalamus, particularly the paraventricular nucleus, arcuate nucleus, suprachiasmatic nucleus, and the dorsomedial nucleus (Chronwall et al., 1985; Sahu et al., 1988; Sanacora et al., 1990). It has been shown in rats that direct administration of NPY to the paraventricular nucleus induces hyperactive eating and over time can lead to obesity (Levine and Morely 1984; Stanley and Leibowitz, 1985). In contrast, loss of NPY neurons has led to starving in mice (Bewick et al., 2005; Gropp et al., 2005; Luquet et al., 2005). NPY administration also drives motivated eating behavior in satiated animals (Jewett et al., 1992) and drives food seeking behavior (Gruninger et al., 2007).

NPY is synthesized primarily in the arcuate nucleus, locus coeruleus, nucleus tractus solitarii, and the septohippocampal nucleus and is expressed in the amydala, hippocampus, nucleus accumbens, periaqueductal grey, basal ganglia, cortical neurons, and thalamus, importantly in a fibre tract connecting the arcuate nucleus with the paraventricular nucleus. Four NPY receptor types are functional in humans, all of which are GPCRs and coupled to the $G\alpha_i$ pathway to downregulate adenylyl cyclase and cAMP signaling (Reichman and Holzer 2016; Gehlert 2004) or to the $G\alpha_q$ pathway to induce PLC activity and stimulate calcium flux into the neuron (Gehlert et al., 1996; Larhammar et al., 1993; Blomqvist and Herzog 1997; Gehlet 2004).

Drosophila

The primary *Drosophila* hunger signaling peptide, NPF, is structurally analogous to NPY but has a C-terminal phenylalanine instead of tyrosine (Brown et al., 1999). NPF has been shown to mediate metabolic and reward motivated feeding (Larhammar, 1996). NPF levels regulate eating behavior during larval development, indicated by high NPF levels during frequent feeding activity in early third instar larvae that transition to lower NPF levels during the wandering behavior larvae display as they prepare for pupation. Additionally, NPF over-expression delays this transition from feeding to wandering (Wu et al., 2003). NPF activity is stimulated by appetitive odor and sugar, and activation of NPF is sufficient to increase eating behavior in satiated larvae even in the absence of an appetitive odor-stimulus (Shen and Cai, 2001; Zhang 2017; Pu 2016).

The *Drosophila* brain lobes contain three pairs of NPF neurons: DM, DL, and SOG (Shen and Cai, 2001; Figure 1.3). NPF signals through the NPFR1 receptor, a GPCR paired with the $G\alpha_i$ pathway (Garczynski et al., 2002). Knockdown of NPFR1 abolishes motivated eating behavior and increased expression of NPFR1 drives fed larvae to consume bitter-tasting food (Wu et al., 2005, 2013). Similar to NPY in mammals, it has been suggested that NPF promotes eating behavior by suppressing feed-inhibiting pathways in the brain (Krashes et al., 2009).

Objective and Applications

Our understanding of how the olfactory, dopamine, and NPF/Y systems interact to facilitate higher-order behaviors such as motivation is still preliminary. As we acquire more thorough knowledge of the mechanisms contributing to motivational states, we can apply it to

treatments and therapies for clinical conditions associated with motivation, such as depression, addiction, ADHD, and eating disorders. Thus far, the focus of many studies has been on illuminating the systems involved in the initiation of motivated behaviors. The goal of this work is to elucidate neural circuits, neurotransmitters, and receptors temporally controlling motivational state. As we gain a better understanding of factors contributing to the duration of motivated behavior, we potentially could manipulate this circuit pharmaceutically to increase the ability to maintain motivated behavior or drive the extermination of unwanted motivated behaviors.

In the following chapter, I will identify a three-part circuit involving the olfactory, dopamine, and NPF signaling systems to regulate odor-aroused motivational state in *Drosophila* larvae. These findings highlight certain brain regions, neurons, and receptors that are relevant to the temporal control of motivational states and could pave the way for a more thorough understanding of the pathways mediating motivation as a whole.

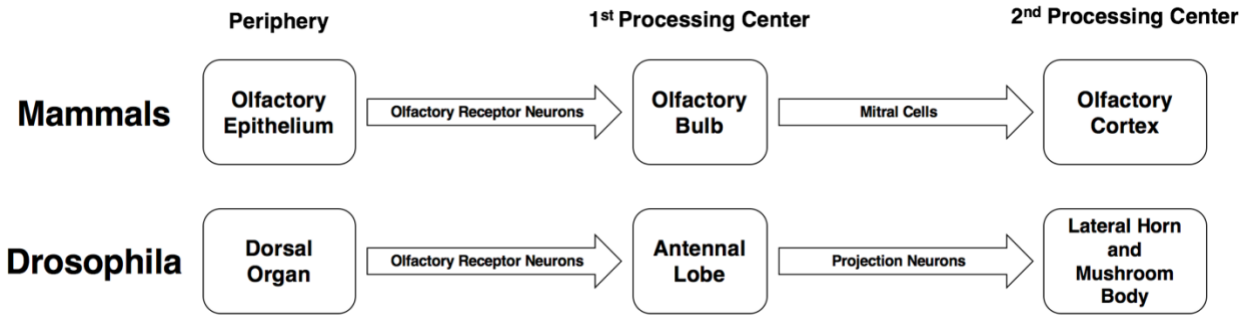


Figure 1.1. The Olfactory Pathway in Mammals and Drosophila Larvae.

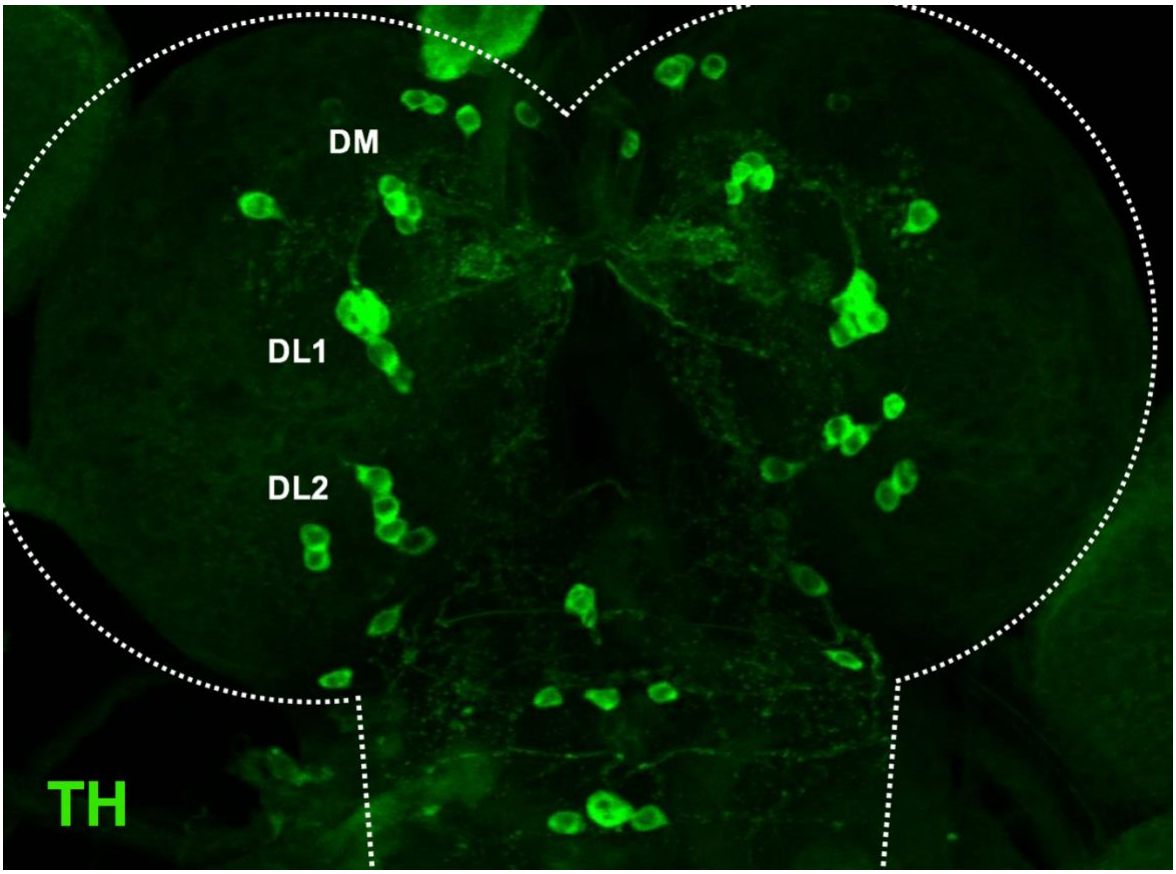


Figure 1.2. Three Paired Dopamine Clusters in Drosophila Larvae Brain Lobes.

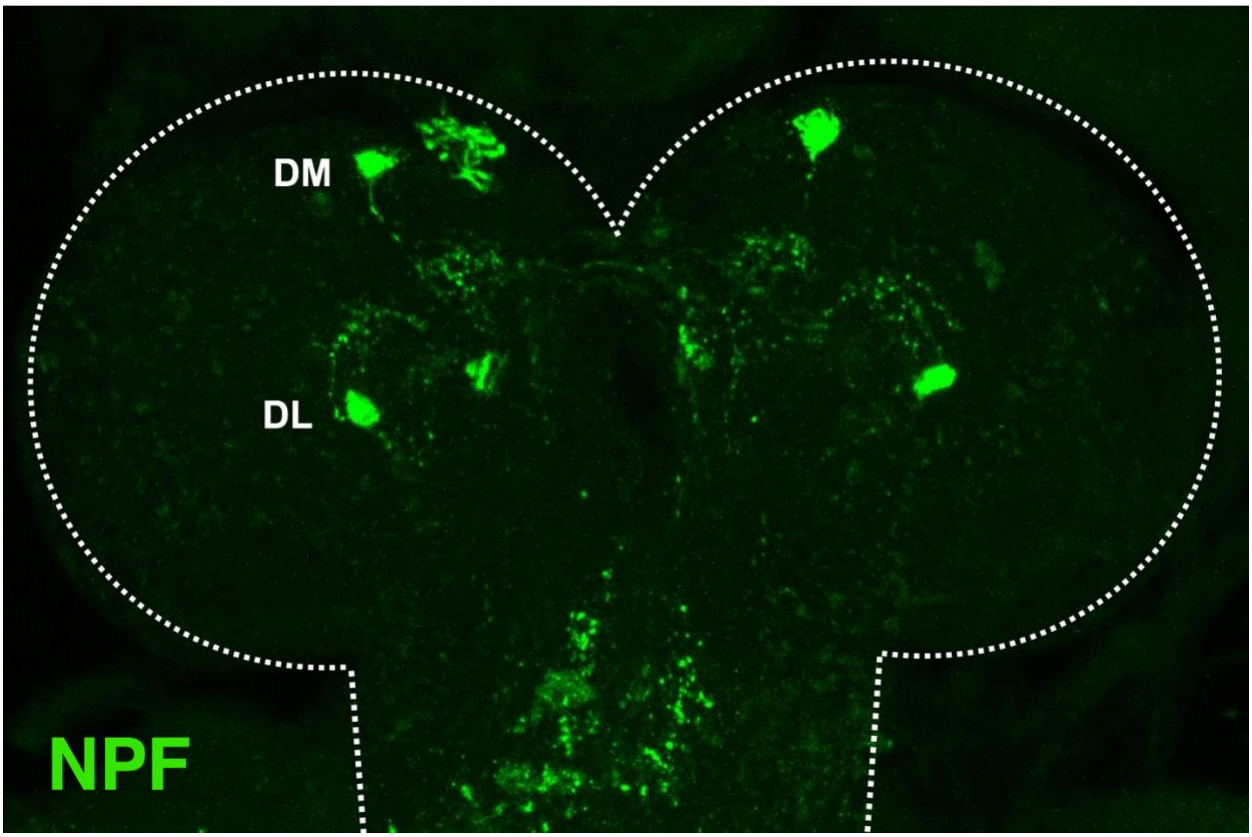


Figure 1.3. Two Paired NPF Neurons in Drosophila Larvae Brain Lobes.

CHAPTER 2

TEMPORAL CONTROL OF ODOR AROUSED MOTIVATION[1]

[1]Thomas, S., Wang, Y., Shen, P. To be submitted.

Introduction

Internal states, such as those of motivation and attention, are the current cognitive contexts under which an organism functions as a result of their needs and environment, past and present (LeDoux 2012; Sayin et al., 2018). Here we focus on motivational states, which drive a variety of essential behaviors such as searching for food or shelter. Motivational states often drive a goal-directed behavior, or behaviors that an organism will only perform in anticipation of a reward, such as rodent lever-pulling with the goal of receiving a food or drug reward. Dysregulation of motivational states has been associated with conditions such as addiction and eating disorders, and prior studies have outlined the role that reward systems play in driving motivated behavior (Baik 2013; Kienast and Heinz 2006; Barbano and Cador 2006). However, while significant focus has been placed on factors that induce motivated behavior, little is known concerning the mechanisms controlling the duration or termination of a motivational state.

Drosophila melanogaster larvae can serve as a useful model for delineating these mechanisms. The larval CNS shares a fair amount of homology to the mammalian nervous system but is numerically simplified, and despite their more rudimentary neuroanatomy, larvae are capable of performing higher-order tasks such as associative learning, memory, and motivationally driven behaviors (Selcho et al., 2009; Pauls et al., 2010; Krashes et al., 2009). It has previously been described that satiated larvae briefly exposed to an appetitive odor will display a significantly increased eating rate, indicative of an odor-aroused motivational state. This phenomenon persists for 10 minutes post appetitive odor exposure before feeding rate returns to a baseline level. Notably, this increase in eating rate is not metabolically based, as the larvae are well fed prior to odor stimulation, and thus serves as a behavioral model for hedonic odor-aroused motivation.

Multiple mammalian and *Drosophila* studies have highlighted the role of dopamine in reward systems and motivated behaviors (Baik 2013; Yamamoto and Seto 2014; Landayan and Wolf 2015). The dopamine system in *Drosophila* larvae consists of three paired clusters, DL1, DL2, and DM, of which the DL2 cluster has been implicated in motivated feeding response, as lesioning of this cluster resulted in loss of odor-aroused feeding increase (Wang et al., 2013). Hunger signaling neuropeptide NPF, the *Drosophila* homolog of mammalian NPY, has also been identified as a regulator of motivated feeding behavior (Krashes et al., 2009). It has been shown in mammalian and insect models that NPF/Y is required for odor-aroused eating behavior and is known to be a major regulator of both metabolic and hedonic feeding (Currie, 2003; Pandit et al., 2014; Jewett et al., 1992; Larhammar, 1996; Wang et al., 2013). The findings that both dopamine and NPF are required for odor-aroused motivation guided our investigation of the mechanism temporally controlling odor-induced motivation to eat.

Here we show a three-part system that regulates the duration of odor-aroused motivational state. First, we show that two dopaminergic neurons from the DL1 cluster induce the termination of odor-aroused eating by acting on Dop1R2 receptors in the mushroom body, a region of the brain traditionally associated with olfactory processing, learning, and memory. Second, we show that a subset of Kenyon cells, neurons intrinsic to the mushroom body, are required to induce temporally-normal termination of the odor-aroused eating behavior. Third, we show that this system is regulated by hunger-signaling peptide NPF, which promotes feeding behavior by inhibiting this termination system. These findings are the first to show a higher-order brain system directly involved in the termination of an odor-aroused motivational state.

Results

Motivational State is Regulated Via a Dopamine-Mediated Pathway

Previous studies have shown that appetitive odors, such as a banana-like scent, can arouse impulsive-like overeating in *Drosophila* larvae even under well-nourished conditions (Wang et al. 2013). Such odor-evoked feeding motivation involves a subset of dopamine (DA) neurons and DA-responsive neuropeptide F (NPF) neurons that underlie perception of olfactory inputs and discriminate assignment of appetitive values to the inputs (Figure 2.1A). The odor-aroused feeding activity of individual larvae can be quantified by measuring their mouth hook contraction (MHC) rate. For example, a 20% increase in MHC rate corresponds to a 50-100% increase in the ingestion of dyed food (Wang et al. 2013). A major characteristic of the odor-aroused feeding behavior is that a stimulated larva typically remains in an aroused motivational state for 10 minutes after odor withdrawal before the behavior decays (Wang et al. 2013, seen in Figure 2.1B). However, the molecular and circuit mechanisms underlying the observed decay profile remain completely unknown.

To exploit this neural circuit, we initially focused on those neurons responsive to stimulation by an appetitive odor (e.g., pentyl acetate or PA), including two paired clusters of DA neurons (DL1 and DL2) in the larval brain (Wang et al. 2013). Targeted laser lesioning analysis showed that PA-evoked DA release from DL2, but not DL1, neurons was required for the initial induction of increased feeding motivation (Wang et al. 2013). However, when examining the decay profile, we found that the DL1 cluster is required for a normal duration of odor-aroused eating. In wild type larvae, PA-aroused feeding increase was detectable for 10 minutes after odor stimulation, and when all eight neurons of the DL1 cluster (Selcho et al.,

2009) were laser lesioned the length of the aroused state roughly doubled to 20 minutes (Figure 2.1B).

To identify which DL1 neurons are required for this phenotype, we turned to those Gal4 drivers that label subsets of the clustered DL neurons. One of the drivers, c061-Gal4, directs GFP expression in two DL1 neurons that project to the mushroom body (MB), a hippocampus-like region in the fly brain (Figure 2.1C, 2.S1) (Krashes et al., 2009; Farris 2011). Targeted laser lesioning of the two DA neurons extended the duration of motivated eating, indicating these DL1 neurons are required for a normal duration of the odor-aroused state (Figure 2.1D). We then discretely suppressed neurotransmission of the c061-Gal4 neurons by expressing shibire, a temperature-sensitive, dominant-negative form of dynamin (Kitamoto 2001), in c061-Gal4/ UAS-shi^{ts1} larvae at a restrictive temperature of 31^{o}C immediately after odor treatment. Inhibition of c061-Gal4 synaptic release after odor exposure significantly extended the duration of the PA-aroused motivational state (Figure 2.1E). Together, these findings suggest that a MB-associated DA circuit, defined by two c061-Gal4-labeled DL1 neurons, underlies a higher-order neural mechanism in the larval brain that actively restricts the duration of odor-induced motivational state after appetitive odor exposure.

Dopamine Innervation of the Mushroom Body

In previous studies, we found that the induction of appetitive odor-aroused feeding motivation involves a lateral horn (LH) associated DA-NPF circuit that appears to be functionally independent from the MB (Wang et al., 2013). To better understand how DA activity in the MB contributes to the termination of the odor-aroused state, we examined which DA receptor(s) in the MB mediates the activity of the two c061-Gal4-labeled DA neurons. We

turned to OK107-Gal4, a driver broadly expressed in the MB (Martini et al., 2000), to screen the four *Drosophila* DA receptor types using an RNAi approach. D1-like receptor Dop1R2 was found to be essential for a normal duration of the odor-evoked eating behavior (Figure 2.2A). We then screened genetic mutants of this receptor and found that heterogeneity of this gene is sufficient for normal termination of odor-induced motivational state, but loss of both Dop1R2 alleles extended the motivational state (Figure 2.2B). Overall, these results suggest that dopamine innervation of the mushroom body may act as a timing mechanism for odor-aroused eating behavior.

The third instar larvae MB consists of approximately 400 Kenyon cells, cells that are intrinsic to the MB (Technau and Heisenberg, 1982). To determine if a more discrete subset of MB neurons is involved in regulating the duration of motivational state, we performed RNAi knockdown of the Dop1R2 receptor in a collection of MB intrinsic driver lines. One driver, R80H07-Gal4, labels approximately 50 of the 400 MB intrinsic neurons in each lobe of early third instar larvae (Figure 2.2C). The functional knockdown of Dop1R2 in R80H07-Gal4 neurons doubled the odor-evoked eating behavior to 20 minutes after odor withdrawal (Figure 2.2D), suggesting that DA innervation of a subset of MB neurons is required for a normal duration of motivational state.

To better understand the role that activity of MB subset R80H07-Gal4 plays in the timing mechanism of motivational state decay, we turned to shibire to inhibit synaptic activity at a restrictive temperature of 31^{o}C for three different heat-shock treatments: (1) Immediately after odor stimulation until the feeding test; (2) For only the first 10 minutes after odor stimulation; and (3) For only the 10 minutes before the feeding test (Figure 2.3A). To interpret our findings, we will focus on the 15-minute after odor time point (Group B in Figure 2.23), the time point at

which we can evaluate if the duration of the motivational state is normal or extended. We found that at 15 minutes after odor stimulation, both heat-shock treatments that restricted R80H07-Gal4 synaptic transmission during at least the first 10 minutes after odor stimulation (heat-shock treatments 1 and 2) resulted in high eating rates, indicating an extended motivational state (Figure 2.3B). This suggests that R80H07-Gal4 neurotransmission is required within the first 10 minutes after odor stimulation for a normal duration of the odor-aroused motivational state. Under heat-shock treatment 3, the 15-minute group displayed a baseline feeding rate, indicating a normal duration of motivational state. In this group, R80H07-Gal4 synaptic transmission was permitted for only the first 5 minutes after odor exposure yet normal termination of motivational state commenced, suggesting that R80H07-Gal4 neurotransmission in the first 5 minutes after odor exposure is sufficient for normal duration of the motivational state (Figure 2.3B). All together, our findings suggest that DA release from two DL1 neurons onto the Dop1R2 receptor in a subset of MB neurons, and the activity of these MB neurons, drives the termination of the odor-aroused motivational state in a time-sensitive manner.

We have shown that knockdown of R80H07-Gal4 leads to an extended odor-aroused motivational state, which led us to hypothesize that activity from this subset of MB neurons would actively terminate odor-aroused motivational state in an accelerated manner. To test this hypothesis, we activated synaptic release from the R80H07-Gal4 neurons using temperature sensitive TRP family cation channel dTrpA1 (Hamada et al., 2008). Larvae were incubated at the permissive temperature of 31ºC for the 30 minutes prior to odor stimulation, at which point they were transferred to the odor chamber and maintained at 25ºC until their feeding test. Activation of R80H07-Gal4 neurons prior to odor stimulation resulted in a shorter motivational state, with larvae returning to the baseline MHC rate by 10 minutes after odor (Figure 2.4A). This suggests

that activity from a subset of MB neurons actively drives termination of odor-aroused motivational state.

Our findings have suggested that upstream signaling from dopaminergic c061-Gal4 onto Dop1R2 in the MB mediates the durational of odor-induced motivated eating. Therefore, based on our result that R80H07-Gal4 stimulation shortens the duration of motivated feeding, we tested if activation of c061-Gal4 would also lead to a shortened motivational state. Indeed, dTrpA1 activation of c061-Gal4 neurons prior to odor stimulation also shortened the motivational state, with MHC rate returning to baseline levels by 10 minutes after odor stimulation (Figure 2.4B). Together, these findings suggest that the duration of motivational state is regulated by a subset of dopamine and mushroom body neurons in a bidirectional manner.

NPF has an Inhibitory Effect on the Dopamine-Mushroom Body Circuit

Thus far, we have found that DA release onto Dop1R2 receptors in R80H07-Gal4 promotes the termination of odor-aroused motivational state. However, the upstream mechanism modulating this behavior is still unknown. Prior studies have shown that NPF activity acts as an upstream inhibitor of c061-Gal4 neurons to promote appetitive memory in adult *Drosophila* (Krashes et al., 2009). Therefore, we investigated if a c061-Gal4-NPF circuit could be at play to regulate the duration of motivational state. We first verified that the DL1 cluster of neurons are NPF receptor positive by expressing NPFR1-Gal4 with dopamine indicating marker TH and found that five out of eight DL1 neurons (Selcho et al., 2009) were NPFR1 positive (Figure 2.4A). To determine if NPF innervation of c061-Gal4 does have an effect of the termination of motivational state and to further define the contributing DL1 neurons as the two present in c061-Gal4, we knocked down NPFR1 receptors in c061-Gal4. This resulted in a shorter motivational

state, with MHC rate returning to baseline by the 10 minutes after odor mark (Figure 2.4B). This suggests that a NPF-DA circuit is involved in the temporal control of odor-aroused motivational state and that specifically NPF activity has a maintaining effect on motivational state by inhibiting c061-Gal4 neurons.

Our findings suggest that NPF release onto DL1 neurons mediates the duration of odor-aroused motivational state, however the source of NPF has not been targeted at a cellular level. It is known that there are two pairs of NPF releasing neurons in *Drosophila* larvae brain lobes, the dorsal medial (dmNPF) pair and dorsal lateral (dlNPF) pair. We turned to targeted laser lesioning to determine which pair(s) of NPF neurons is mediating the termination of odor-aroused motivational state. We expected that knockdown of any pair interacting with DL1 neurons would have a shortening effect on the motivational state. It has previously been shown that lesioning of the dmNPF pair completely abolishes odor-aroused motivated eating behavior (Pu 2016). Therefore, we were unable to examine the effect lesioning dmNPF has on the duration of motivational state. However, we found that targeted laser lesioning of the dlNPF pair did not result in a shorter motivational state (Figure 2.5C), indicating that this pair alone is not a sufficient source of NPF to mediate the termination of odor-aroused motivational state. This suggests that NPF mediating the termination of the odor-aroused motivational state may come from only the dmNPF pair or a combination of dlNPF and dmNPF input.

Together, our findings suggest that a NPF-DA-MB neuron-mediated pathway regulates the duration of odor-aroused motivational state (Figure 2.6). Our results suggest that stimulating the activity of NPF neurons or inhibiting activity of DL1 or MB neurons will extend the motivational state. Inversely, our findings also suggest that inhibition of the NPF neurons or stimulation of DL1 or MB neurons will shorten the motivational state. The downstream

components of this circuit are still unknown, and the aim of future work is to identify how this mechanism connects to the motor neurons required for eating behavior.

Discussion

Two MB-Bound DL1 Neurons Terminate Motivational State

Here we show that two neurons from the DL1 cluster of dopaminergic neurons, labelled by driver line c061-Gal4, mediate the duration of odor-aroused eating behavior in satiated larvae. Anatomical analysis of c061-Gal4 suggests that axon projections from the two dopaminergic neurons innervate the MB, specifically in the spur and peduncle regions. Both targeted laser lesioning of the two dopaminergic c061-Gal4 neurons and temporal synaptic inhibition of this line resulted in extension of the motivational state. Inversely, temporal activation of these neurons resulted in a shorter motivational state, suggesting that these DA neurons regulate motivated eating behavior in a bidirectional manner.

Dop1R2 Receptors in the MB Mediate the Temporal Control of Motivational State

We report that loss of the Dop1R2 receptor, either via genetic mutation or RNAi approaches, resulted in longer motivational state. Notably, heterozygous expression of the Dop1R2 gene is sufficient for a normal duration of motivational state while loss of both alleles results in extension of motivational state.

Two types of dopamine receptors (Dop1R1/dDA1 and Dop1R2/DAMB) are known to be heavily expressed within the mushroom body, with Dop1R2 primarily expressed in the spur and peduncle regions, the target area of the c061-Gal4 dopaminergic neurons (Selcho et al. 2009). We verify that the role of Dop1R2 in the termination of motivated behavior is mushroom body specific through RNAi knock-down of receptor expression in driver lines marking both the full MB as well as a subset that includes approximately $1/8^{th}$ of total MB neurons and observed that

Dop1R2 is required in a subset of MB neurons for a normal duration of odor-aroused motivational state.

The MB Drives Termination of Motivational State in a Time-Dependent Manner

Our results suggest that the precise timing of the activity of MB neurons after odor exposure is pertinent. When synaptic transmission of MB neurons, driven by R80H07-Gal4, was inhibited within the first 10 minutes after larvae were odor stimulated, the duration of motivational state was extended. However, when R80H07-Gal4 neurotransmission was permitted within only the first 5 minutes after odor stimulation, this was sufficient for a normal duration of motivational state. This suggests that the activity or inactivity of these neurons, and perhaps others involved in this circuit, operate in a time sensitive manner to control the termination of motivational state. The MB's role in this mechanism does not seem to follow an "all or none" strategy in which activity at any point would result in a normal duration of the motivational state, but instead the timing of MB activity appears to be crucial.

NPFR1 Regulation of the DA-MB circuit

Previous work has shown that NPF is required for the initiation of motivated feeding behavior in fed larvae, however our findings are the first to exhibit NPF's role in the duration of motivated eating (Wang et al., 2013). When NPF receptors were knocked down exclusively within c061-Gal4, the duration of odor-induced motivated eating was shortened, indicating that NPF mediates the duration of motivated behavior by downregulating DA activity. When combined with our understanding of the role that DA and the MB play in termination of

motivated eating, which is one of motivated feeding suppression, we can infer that NPF acts to maintain motivated eating behavior by inhibiting the downstream DA-MB circuit.

Motivated Behavior is Mediated by Two Higher-Order Processing Centers

Prior work has defined a dopamine-mediated circuit in the lateral horn that is required for the induction of odor-induced motivated feeding behavior (Pu 2017, 2018). Here we have presented a second dopamine-mediated pathway involving the other secondary olfactory processing center, the mushroom body. Therefore, a novel conclusion from our findings is that dopamine signaling communicates at long distances and to discrete local processing centers in the larval central nervous system to mediate both the induction and termination of olfactory-stimulated motivated behavior.

This concept that different aspects of a behavior are facilitated in distinct local processing units is an important one, as it adds an additional layer of parallel between the nervous system organization seen in fly and mammalian models. Striking features of the *Drosophila* system that make it an appealing model system to study neuroscience include the conservation of neuro-molecules such as DA and NPF/Y, the organization of specific neuron-types into clusters, the integration of different classes of neurons into local processing centers, and finally the communication between processing centers at longer distances within the brain. Therefore, our findings that two dopamine-mediated circuits involving distinct processing centers regulate odor-aroused motivated behavior contributes to the increasingly observed notion that the numerical simplicity of the *Drosophila* system lends an accessible approach to address the neuro-circuitry and communication between the higher-order processing centers underlying behavior.

Methods

Fly Stocks and Larval Growth

All flies are in the w^{1118} background. Larvae were reared at 25°C until approximately 74 hours after egg laying and fed before behavioral experiments as previously described (Wu et al., 2013). Transgenic flies UAS-nlsGFP, TH-Gal4 (Friggi-Grelin et al., 2003), co61-Gal4 (BL30845), UAS-mcd8GFP, UAS-shibire (Kitamoto, 2001), OK107-Gal4 (BL854), Dop1R2 -/- (BL51098), R80H07-Gal4 (BL47078), UAS-TrpA1 (Hamada et al., 2008), UAS-NPFR1 RNAi (BL27237), and NPF-Gal4 were obtained from the Bloomington Drosophila Stock Center. Transgenic lines UAS-Dop1R1 RNAi (V107058), UAS-Dop1R2 RNAi (V105324), UAS-Dop2R RNAi (V11471), and DopEcR RNAi (V103494) were obtained from the Vienna Drosophila RNAi Center.

Behavioral Experiments

Fly larvae odor stimulation was performed as previously described with slight modification (Wang et al., 2013). 7.5µl of pentyl acetate (PA) (Sigma-Aldrich, 628-63-7) was incubated for two minutes in the odor stimulation chamber before synchronized, satiated early third instar larvae were stimulated in the chamber for 5 minutes. After rinsing with water, larvae were transferred to either the middle of a feeding media plate to have their feeding responses recorded or to a yeast paste plate for various minutes of delay before transfer to a feeding media plate. Feeding media consisted of 6g agar (US Biological, A0940) in a 45mL 10% glucose solution. Quantification of mouth hook contraction rate in feeding media was performed as previously described (Wu et al., 2005). UAS-shi^{ts1} was expressed by allowing larvae to feed in a pre-warmed yeast paste plate in a 31°C incubator for

defined periods after odor stimulation. UAS-dTrpA1 was expressed by allowing larvae to feed in a pre-warmed yeast paste in a 31°C incubator for 30 minutes before odor stimulation.

Immunostaining

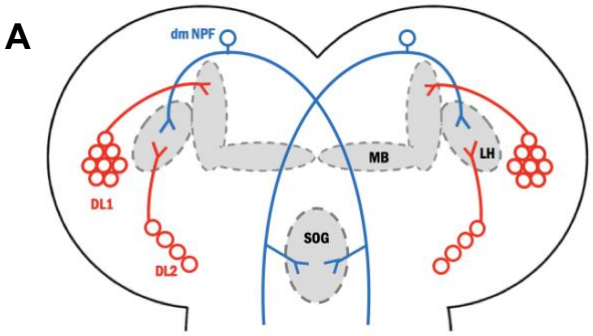
Tissue dissection, fixation, and antibodies were described previously (Wu et al., 2013). Images were collected using Zeiss LSM 710 META and LSM 880 Confocal Microscopes.

Targeted Laser Lesioning

The 337 nm nitrogen laser unit was calibrated as previously described (Xu et al., 2008). Larvae were lesioned as described previously with slight modification (Wang et al. 2013). To prepare for lesioning, five 2nd instar larvae were transferred onto a microscope slide with 0.5 mL of water. A coverslip was placed on top and larvae were allowed to orient themselves for one minute before excess water was slowly absorbed with a towel until larvae were immobilized. Mock larvae were handled in the same way except without laser treatment. DL1 neurons were identified morphologically using TH-Gal4/UAS-nlsGFP larvae, c061 dopamine neurons were identified using c061-Gal4/UAS-mcd8GFP larvae, and dlNPF neurons were identified using NPF-Gal4/UAS-nlsGFP larvae.

Statistical Analysis

Statistical analyses for behavioral experiments were performed using Two-way ANOVA followed by the Tukey test.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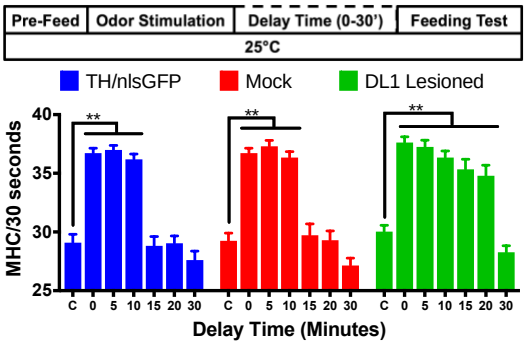
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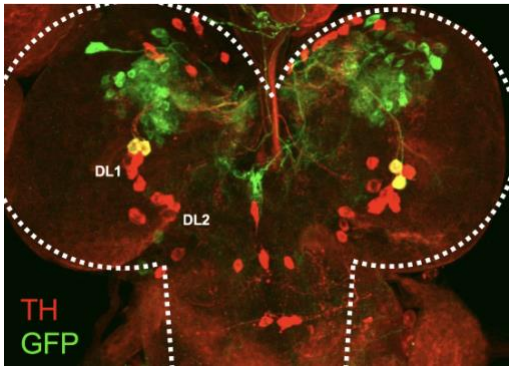
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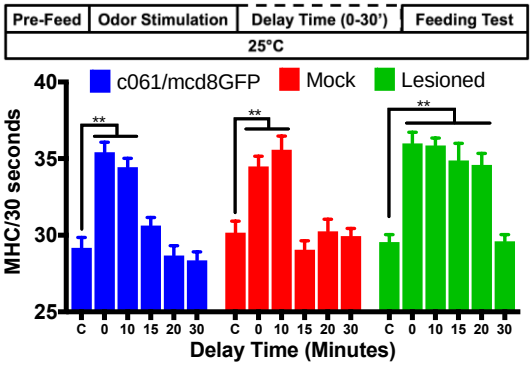
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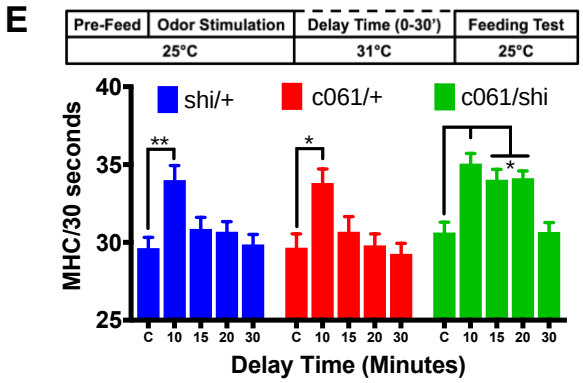
E
Pre-Feed
Odor Stimulation
Delay Time (0-30')
Feeding Test
25°C
31°C
25°C
shi/+
c061/+
c061/shi
MHC/30 seconds
Delay Time (Minutes)

Figure 2.1. Termination of Odor-Aroused Motivational State Involves a Dopamine-Mediated Pathway.

(A) Summary diagram of the odor-aroused eating induction pathway. Dopamine is released by DL2 neurons onto dmNPF neurons in the lateral horn. NPF then relays this odor arousal signal to the (subesophageal) SOG region to initiate motivated eating behavior. **(B)** Larvae were exposed to 7.5μL PA odor for 5 minutes. Larval mouth hook contraction (MCH) rate in a 10% glucose agar paste was measured during a feeding test various minutes after odor exposure (See Methods for details). C: control larvae, not exposed to odor. Laser lesioning of DL1 neurons extended the motivational state. Unless indicated otherwise, all behavior assays were quantified under blind conditions and statistically analyzed using Two-way ANOVA followed by a Tukey's multiple comparison test in all figures. (**$p < 0.01$, $n \geq 19$). **(C)** Immunofluorescence of c061-Gal4 (green) and dopamine marker TH (red). c061-Gal4 overlaps with two larval dopaminergic neurons in the DL1 cluster and its axons project to the mushroom body **(D)** Larvae were laser lesioned at the 2nd instar stage and allowed to recover before odor exposure and feeding test at early 3rd instar. Targeted lesioning of the two DA neurons in c061-Gal4 extended the duration of the motivational state. (**$p < 0.01$, $n \geq 15$). **(E)** Larvae were incubated at the restrictive temperature of 31°C after odor treatment until their feeding test at various minutes of delay. Inhibition of c061-Gal4 neurotransmission leads to extension of the motivational state. (**$p < 0.01$, *$p < 0.05$, $n \geq 15$).

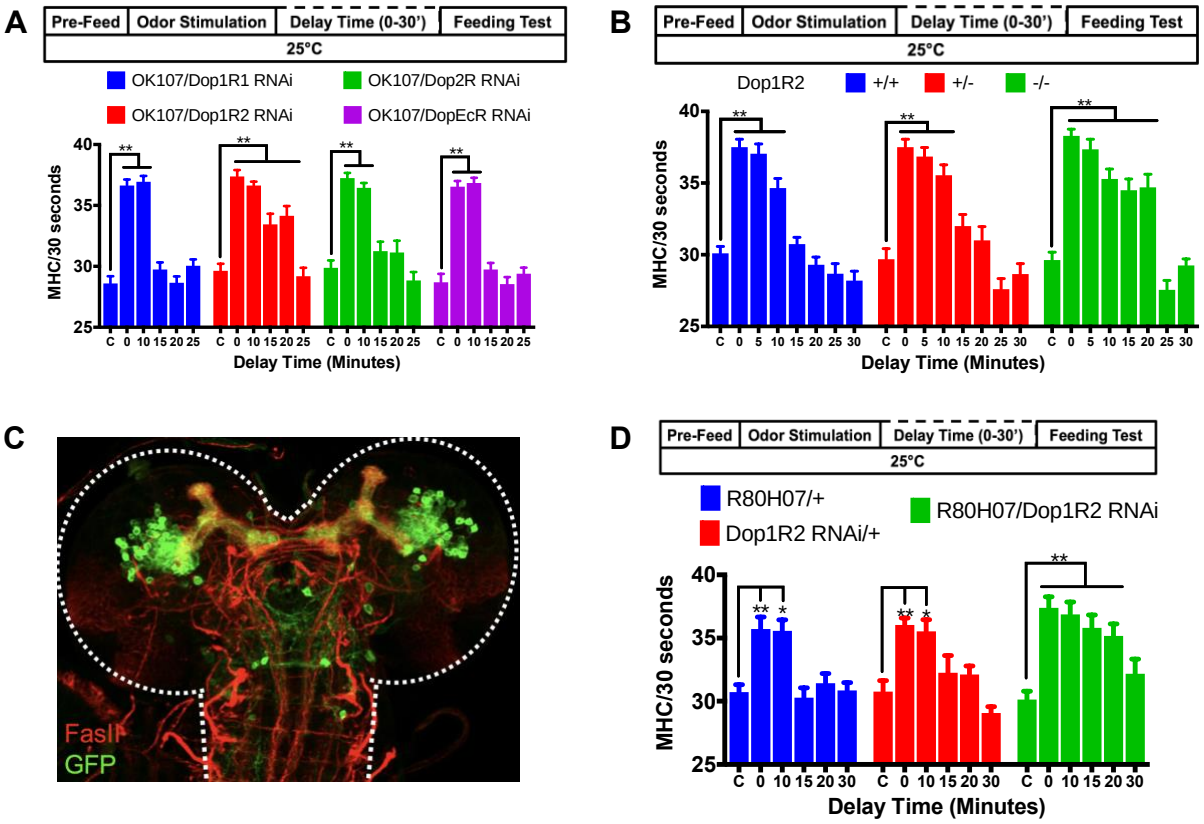

Figure 2.2. Dopamine Receptor Dop1R2 in the Mushroom Body Acutely Regulates the Decay of Motivational State.

(A) The four types of larval dopamine receptors were knocked down in mushroom body line OK107-Gal4. Only Dop1R2 knockdown significantly extended the motivational state. (**$p < 0.01$, $n \geq 19$). **(B)** Homozygous genetic mutation of the Dop1R2 receptor gene extended the motivational state. (**$p < 0.01$, $n \geq 19$). Heterozygous mutation of this receptor did not significantly extend the motivational state. **(C)** Immunofluorescence of R80H07-Gal4 (green) with mushroom body marker FasII (red). R80H07-Gal4 labels about 1/8 of total mushroom body intrinsic neurons. **(D)** Knockdown of the Dop1R2 receptor in mushroom body subset line R80H07-Gal4 extends the motivational state. (**$p < 0.01$, *$p < 0.05$, $n \geq 15$).

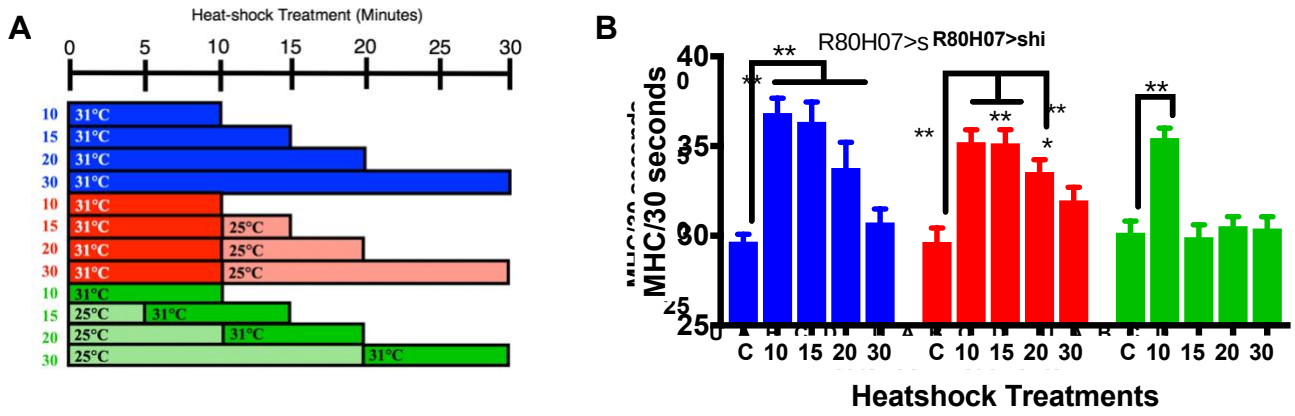

Figure 2.3. Activity of a Subset of Mushroom Body Neurons Immediately After Odor Exposure is Required for Normal Termination of Motivational State.

(A) Heat-shock paradigm in which larvae were incubated at the restrictive temperature of 31°C for different time periods to inhibit R80H07-Gal4 neurotransmission: Immediately after odor exposure until the feeding test (blue), for the first 10 minutes immediately after odor exposure (red), or for the 10 minutes before the feeding test (green). **(B)** Neurotransmission from this subset of mushroom body neurons within the first 5 minutes after odor exposure is sufficient for a normal duration of motivational state. R80H07-Gal4 inhibition for the full delay time (blue) or for only the first 10 minutes after odor (red) extended the motivational state, while R80H07-Gal4 inhibition for only the 10 minutes before the feeding test (green) exhibited a normal decay of the motivational state. C: control larvae, not exposed to odor. (**$p < 0.01$, $n \geq 15$).

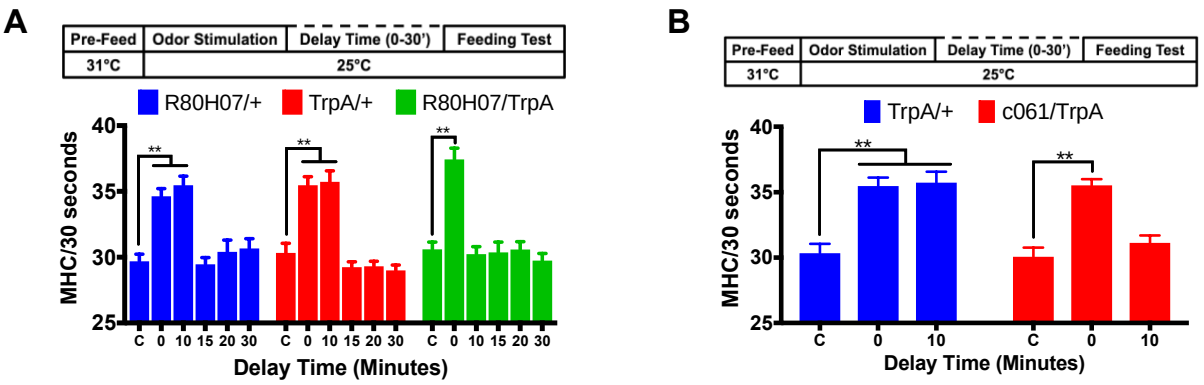

Figure 2.4. Mushroom Body Intrinsic Neurons and Dopamine Control the Termination of Motivational State in a Bidirectional Manner.

(A) Larvae were incubated at permissive temperature of 31°C for 30 minutes before odor exposure to proliferate R80H07-Gal4 neurotransmission. Increased activity of these neurons before odor exposure shortened the motivational state. (**$p < 0.01$, $n \geq 15$). **(B)** Larvae were incubated at permissive temperature of 31°C for 30 minutes before odor exposure to proliferate c061-Gal4 neurotransmission. Increased activity of these neurons before odor exposure shortened the motivational state. (**$p < 0.01$, $n \geq 15$).

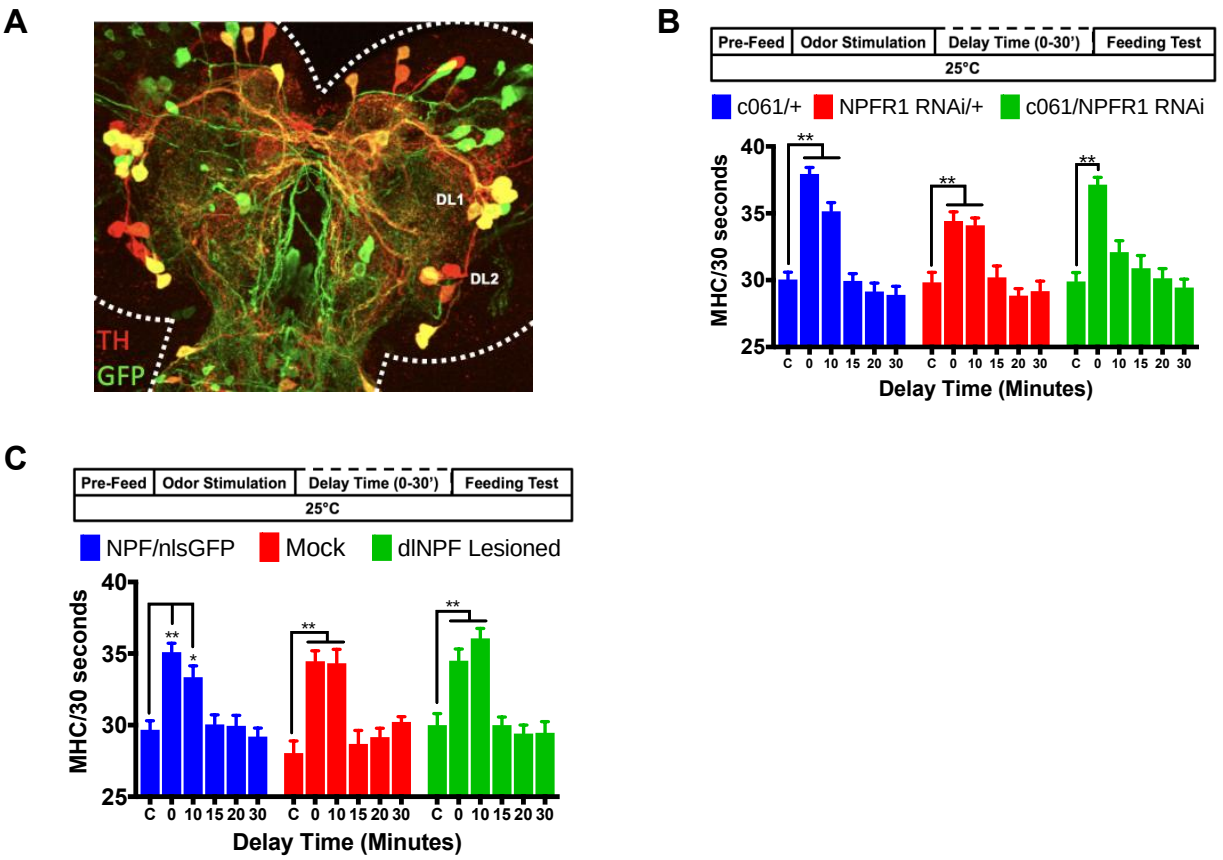

Figure 2.5. NPF Neurons Maintain Motivational State by Inhibiting Dopamine Activity.

(A) Immunofluorescence of NPFR1-Gal4 (green) with dopamine marker TH (red). Five neurons in the DL1 cluster are NPFR1 positive. **(B)** Knockdown of the NPF receptor in dopaminergic c061-Gal4 neurons shortens the duration of motivated eating. (**$p < 0.01$, *$p < 0.05$, $n \geq 15$). **(C)** Dorsal lateral NPF neurons were laser lesioned in 2nd instar larvae and larvae were recovered until odor exposure and feeding test at early 3rd instar. Targeted lesioning of the two dlNPF neurons in NPF-Gal4 has no effect on the duration of the motivational state. (**$p < 0.01$, *$p < 0.05$, $n \geq 15$).

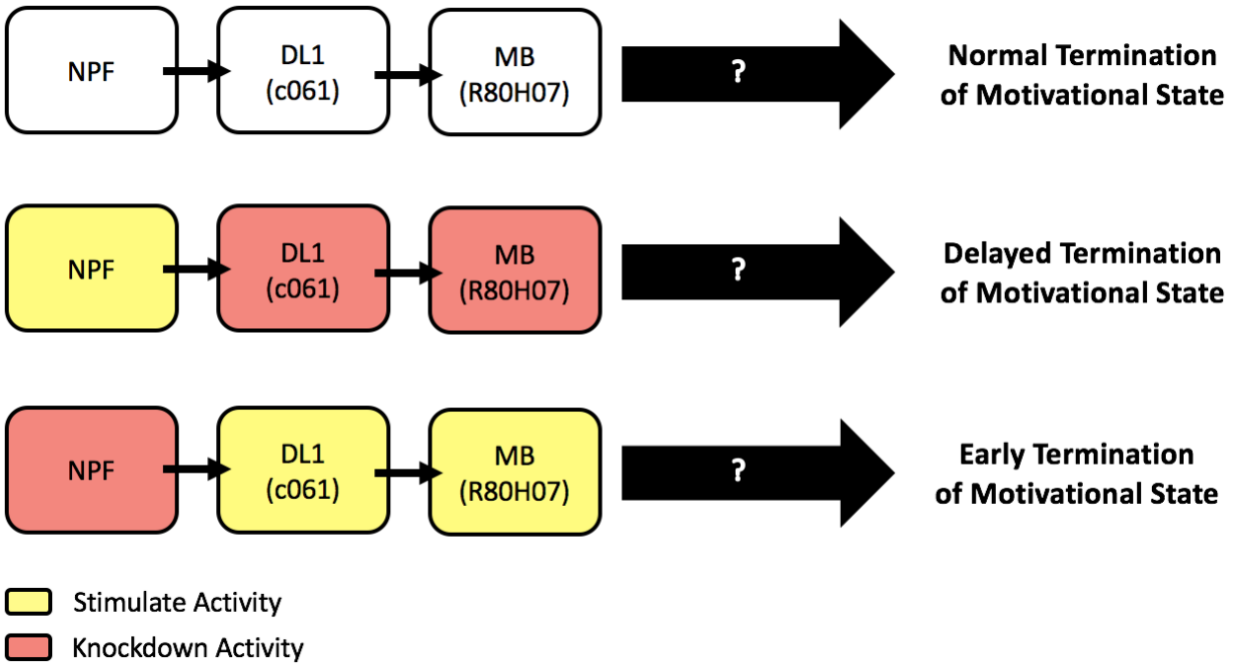

Figure 2.6. Temporal Control of Odor-Aroused Motivation

Top: Characterization of the proposed NPF-dopamine-mushroom body neuron circuit in wildtype conditions. Under wildtype conditions, this circuit mediates the normal duration of odor-induced motivational state. Middle: When NPF activity is stimulated, or, when downstream DA or MB neuron activity is suppressed, the duration of the motivational state is extended. Bottom: When NPF activity is inhibited, or, when downstream DA and MB neuron activity is stimulated, the duration of the motivational state is shortened.

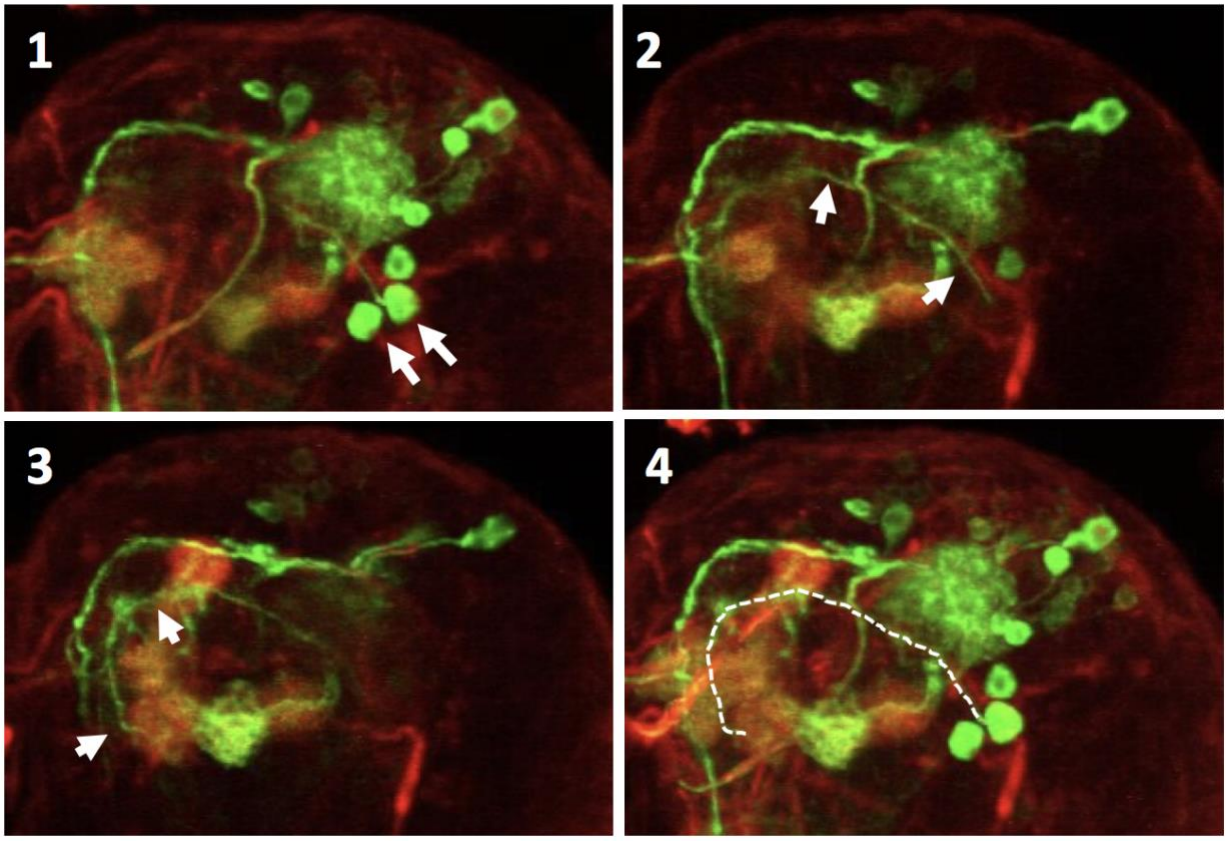

Figure 2.S1. c061-Gal4 Neurons Innervate the Mushroom Body. Immunofluorescence of c061-Gal4 (green) and mushroom body marker FasII (red). Panels 1-3 follow c061 axon progression, panel 4 displays a trace of the axon. The two dopaminergic c061-Gal4 neurons project to the mushroom body heel and peduncle.

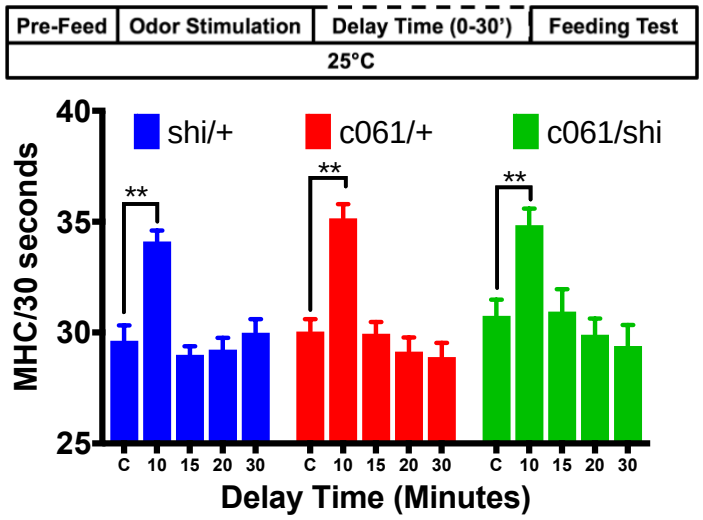

Figure 2.S2. The Duration of Motivational-State is Normal in c061/shi^{ts1} at the Permissive Temperature. Decay profile for control larvae held at 25°C, a non-restrictive temperature, after odor treatment until their MHC rate was measured in a feeding test at various minutes of delay (**$p < 0.01$, $n \geq 15$).

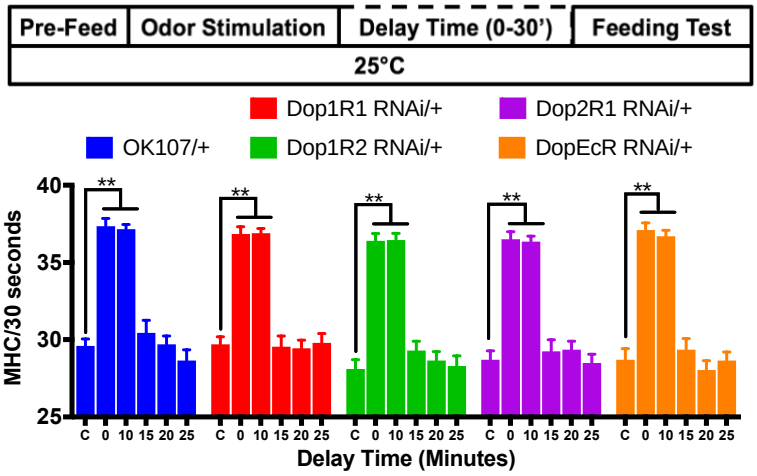

Figure 2.S3. The Duration of Motivational-State is Normal in Dopamine Receptor Screen Controls. Decay profile of heterozygous controls for the dopamine receptor RNAi screen (**$p < 0.01$, $n \geq 19$).

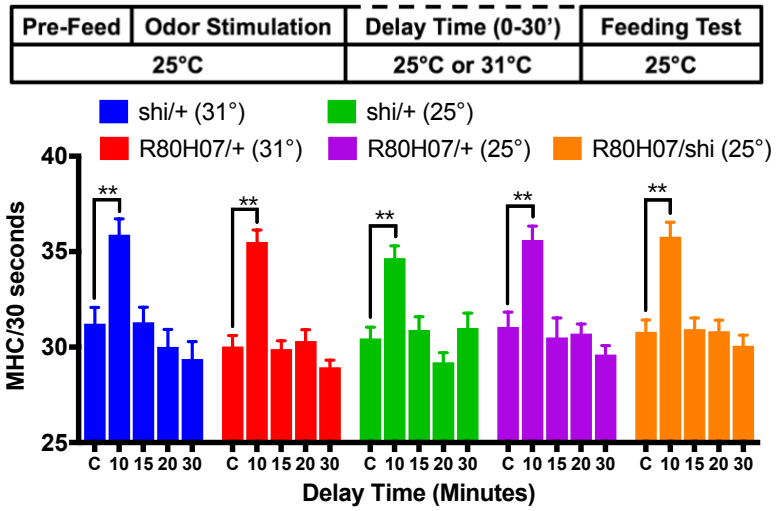

Figure 2.S4. The Duration of Motivational-State is Normal in R80H07/shi^{ts1} at the Permissive Temperature. Decay profiles for control larvae held at either non-restrictive temperature 25°C, or restrictive temperature 31°C, after odor treatment until their MHC rate was measured in a feeding test at various minutes of delay (**$p < 0.01$, $n \geq 16$).

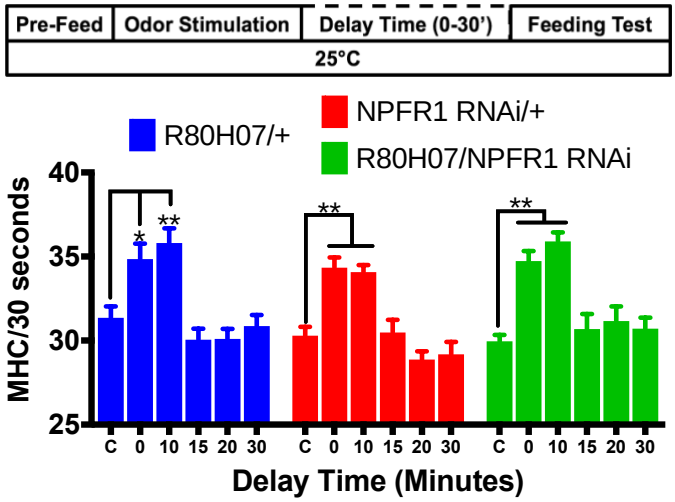

Figure 2.S5. NPF Receptor Knockdown in a Subset of Mushroom Body Neurons.

Knockdown of NPF receptors in R80H07-Gal4 does not significantly alter the decay profile (**$p < 0.01$, *$p < 0.05$, $n \geq 15$).

CHAPTER 3

CONCLUSION

Further Implications of Our Findings

The Decay of Motivational State is Likely Mediated by Multiple Circuits

It should be noted that while this DA-MB circuit temporally controls motivated eating behavior, it is not required for decay of the motivational state overall. Even with the loss of DA, Dop1R2, or MB activity, the motivational state eventually decays within 30 minutes after odor stimulation. This suggests that another mechanism exists to induce the end of motivated behavior when this mechanism is not functional. The involvement of multiple circuits would highlight the complexity of motivated behaviors as a whole and the importance of a reliable system to terminate motivational states.

A Novel Biological Function of NPF

Our lab has previously shown that appetitive odor-stimulation directly increases NPF activity, that NPF stimulation is sufficient to drive motivated feeding behavior, and that loss of the dmNPF neuron pair leads to loss of appetitive odor-aroused motivation to eat (Zhang 2017; Pu 2016). Together, these results suggest that NPF expression drives odor-aroused motivated feeding behavior. The findings presented in this work are the first to propose that NPF not only acts to induce motivated eating, but also mediates the duration of feeding behavior.

Additional Biological Functions of this Circuit

Our research is not the first to suggest that a NPF-DA-MB circuit regulates behavior. A previous study found that DA release from c061-Gal4 has an inhibiting effect on odor-associated memory and suggested that this DA-MB connection is responsible for the effect of a satiated state on odor-associated memory (Krashes et al., 2009). Furthermore, this study showed that NPF mediates dopamine output via NPFR1 receptors, and these receptors are required specifically in MB-bound dopaminergic c061-Gal4 neurons for appetitive memory performance in hungry flies and suppressed appetitive memory performance in fed flies (Krashes et al., 2009). Together with our findings, these results suggest that a NPF-DA-MB circuit could act as a regulator in multiple behaviors.

Other research has highlighted the role of DA in forgetting odor-associated memories (Berry et al. 2012; Plaçais 2017; Himmelreich et al., 2017). One group found that loss of Dop1R2 receptors extended the duration of aversive memory in adult flies and proposed that there is an active forgetting mechanism based on DA activity (Berry et al., 2012). In our work, we show that both Dop1R2 and DA synaptic activity are required for normal duration of odor-aroused motivated feeding. While memory duration is a notably distinct behavioral activity than that of our model, motivated feeding duration, together these findings suggest a broad function of the DA system in temporally mediating different MB-associated behaviors.

Mushroom Body Regulation of Innate Behavior

Significant studies involving the fly mushroom body have associated this processing center with learning and memory and, until recently, few studies found a role of the MB in innate behaviors. However, it is now recognized that the MB also plays a role in naïve odor response

and is required for food-seeking behavior (Owald et al., 2015; Tsao et al., 2018). Our results contribute to this pool of findings that suggest the MB plays a role in innate behavior, as we propose that the MB is required for temporal control of odor-aroused motivated feeding in naïve larvae.

Significance

Evolutionary Importance of the Termination of Motivation

As discussed, multiple circuits are capable of mediating the termination of an odor-induced motivational state. Therefore, the redundancy of this mechanism suggests that reliable termination of motivated behavior is likely highly biologically relevant to organism functioning. For example, eating behavior is tightly associated with larvae development. As larvae reach the end of their larval stages they switch over to wandering behavior and move away from food to prepare for pupation. If all mechanisms regulating this transition are lost it could prevent a successful transition from third instar larvae to pupae. Therefore, in this scenario appropriate temporal regulation of eating motivation is vital for survival.

A second requirement for organism survival is to respond and adapt to changes in the environment. The ability to redirect behavior from one focal point onto another is essential. Therefore, motivation to execute goal-directed behavior needs to be updated to reflect incoming stimuli, and termination of one motivated state is required to initiate a new behavior. Furthermore, once the goal an organism is attempting to accomplish is fulfilled, continuing the motivated behavior is no longer useful and a potential waste of the organism's energy and resources. Larvae in particular use odor as a motivating stimulus to help them locate sustainable food sources, and once this goal has been accomplished it is beneficial from a nutritional and

developmental standpoint if motivation to seek food ceases. Therefore, while odor-aroused motivated eating behavior is vital for survival, its termination is equally as important.

Clinical Implications

Understanding how interactions between neurotransmitter, neuron populations, neural circuits, and local processing centers facilitate the induction, maintenance, and termination of motivational state is vital for addressing motivation-related afflictions, such as addiction and eating disorders. A stronger knowledge of the factors temporally mediating motivational behavior will guide efforts to develop drug targets and other therapies for these conditions, and therefore continuing this work is imperative.

Future Directions

Integration of the Gustatory and Olfactory Systems

This work has focused on the role of the olfactory system on motivated behavior, however our behavior paradigm is also dependent on the integration of the gustatory system. It has been shown that larvae display motivated eating behavior only when presented with a particular concentration of both an appetitive odor stimulus and sugar rich reward (Wang et al., 2013). Therefore, we can infer that gustatory signaling is interacting with our proposed mechanism. Details on the higher-order processing of gustatory signals are not well understood in larvae, and therefore further research on how gustatory signals might be integrated into this circuit could be useful from multiple vantages.

Potential Downstream Pathways

While here we have identified the MB as a second high-order processing center involved in the regulation of motivational state, the downstream pathway involved in this system remains unknown. Previous studies have found that activity in certain subsets of MBONs can directly result in odor-approach or odor-avoidance behavior, indicating that a pathway exists downstream from the MB to regulate motor behavior (Owald et al., 2015). Further analysis to identify downstream constituents of R80H07-Gal4 could help elucidate which subsets of MBONs are involved in promoting motivated eating or satiated behavior. Additionally, we suspect that an even smaller subset of MB neurons than that marked by R80H07-Gal4 are required for regulation of motivated feeding, and targeting these neurons could help guide a more focused analysis of pertinent MBONs.

It is expected that MB output will lead to neurons in the subesophageal region, the hindbrain region associated with the execution of feeding motor movements, however there is a gap in our current model for how MB signaling connects to the motor circuits associated with feeding. It is possible that this system loops back to involve NPF, which is known to directly modulate eating activity via a DL2-NPF-SOG neuron pathway, thus creating a negative feedback loop (Pu 2016, Wu et al., 2013; Figure 3.1). Under this hypothesis, the temporal circuit outlined in this work would have an inhibiting effect on the motivational state induction pathway at the level of NPF. If this is the case it could be regulated in two different ways: NPF could be downstream of MBONs and receive signals to either increase eating rate or maintain the steady baseline rate. Or, NPF could serve as MBONs and receive input from R80H07-Gal4 neurons directly. In both cases NPF could be an essential piece in this circuit and complete a full feedback loop to regulate motivated eating behavior.

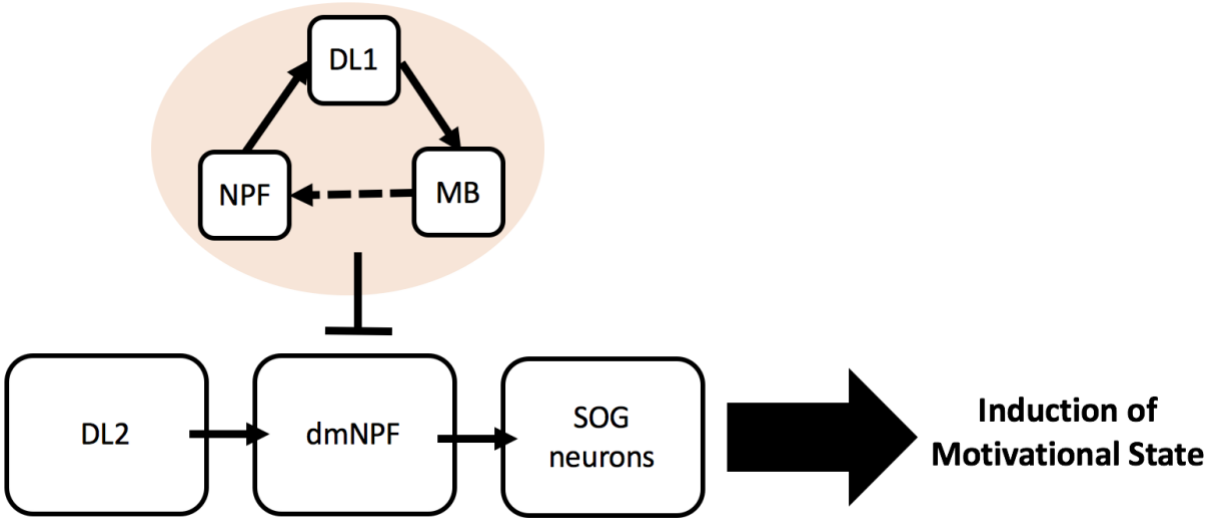

Figure 3.1. Working Model of the Termination of Odor-Aroused Motivation

The proposed NPF-DA-MB neuron circuit could act to initiate termination of the motivational state by inhibiting the induction pathway at the NPF level. There is a possibility that MB neurons signal to NPF neurons, creating a negative feedback loop in that could provide a mechanism to lower NPF levels, resulting in the termination of motivated eating behavior.

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