Central Neural Circuitry of Food and Water Seeking in *Drosophila melanogaster*

By

Dan Landayan

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Quantitative Systems Biology

In the

Graduate Division

of the

University of California, Merced

Committee Members:

Professor Mike Cleary, Chair
Professor Fred Wolf
Professor Xuecai Ge
Professor Ramendra Saha

Summer 2019

The dissertation of Dan Landayan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____
Professor Fred Wolf

_____
Professor Michael Cleary

_____
Professor Xuecai Ge

_____
Professor Ramendra Saha

University of California, Merced
2019

I would like to dedicate this to my mother, father, and sister for their perennial encouragement and support.

Thank you for always being there for me.

Acknowledgements

This research was supported by intramural funds from the University of California, Merced Graduate Division and Quantitative and Systems Biology Program, NIAA, and Genentech.

Thank you to my advisor Dr. Fred Wolf for the years of dedicated mentorship and support. My research and scientific training would not have been possible without the years of freedom you have given me to explore the unknown and enjoy the journey of scientific inquiry.

I would also like to thank all post-docs, graduate and undergraduate students of the Wolf Lab from 2013-2019 in chronological order: Greg Engel, Sarah Parkhurst, Ariel Legendre, David Feldman, Pratik Adhikari, Sharukh Syed, Jean-Christophe, Alex Lebeau, Maurice Jackson, Winnie Wu, Natalie Banh, Brandon Hyunh, Lawrence Fung, Alexiane Luc, Kenneth Mata, Jennifer Zhou, Anthony Lange, Donobon Orozco, Sammy Villa, Clarisse Macaset, and Brian Wang. You have all shaped the scientist I am today, through both scientific and non-scientific discourse. I am grateful to share my entire graduate experience with you all.

CURRICULUM VITAE

EDUCATION

University of California, Merced, CA
Ph.D. in Quantitative and Systems Biology (QSB) **2013-2019**

University of Missouri, Columbia, MO
PREP (Post-baccalaureate) Scholars Program **2011-2013**

University of California, Davis, CA
B.S. in Neurobiology, Physiology, and Behavioral Science **2007-2011**

AWARDS

Carl Storm Underrepresented Minority (CSURM) Fellowship	**2012**
QSB Travel Award	**2014-2015**
QSB Summer Fellowship	**2014-2016**
QSB Retreat – Outstanding Oral Presentation Award	**2014**
Society for the Advancement of Chicanos and Native Americans in Science (SACNAS) Travel Award	**2014**
Cold Spring Harbor Laboratory - Helmsley Scholarship	**2015**
Dean's Distinguished Scholar Fellowship Award Scholarship	**2016**
Molecular Cell Biology Outstanding Graduate Student Award	**2016**
UC President's Dissertation Year Fellowship	**2017-2018**

RESEARCH EXPERIENCE

University of California, Merced, CA
Wolf Lab –Ph.D. Student **2013-2019**
Advisor: Dr. Fred Wolf

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Course Rotation –Ph.D. Student **2015**
Advisor: Dr. Karla Kaun

University of Missouri, Columbia, MO
NIH PREP Scholar – Post-baccalaureate Research Assistant **2011-2013**
Advisor: Dr. Michael Garcia

University of California, Davis, CA
McAllister Lab – Undergraduate Research Assistant **2010-2011**
Advisor: Dr. Kimberley McAllister

University of California, Davis, CA
Kopp Lab – Undergraduate Research Assistant **2009 – 2010**
Advisor: Dr. Artyom Kopp

PRESENTATIONS

University of California Davis
Annual Neuroscience Graduate Group Retreat **2014**
Title: Neural Representation of Motivated Food Seeking in Flies

Los Angeles Convention Center, CA
SACNAS **2014**
Title: Neural Representation of Motivated Food Seeking in Flies

University of California, Merced
Interdisciplinary Foraging Workshop – Cognitive and Information Science Graduate Group **2013**
Title: Motivational Food Seeking Screen

Annual QSB Graduate Group Retreat **2014**
Title: Neural Representation of Motivated Food Seeking in Flies

Cold Spring Harbor Laboratory

Neurobiology of Drosophila **2015**
Title: Central Neural Circuitry for Motivated Water Seeking in Flies

University of California Davis
Annual Neuroscience Graduate Group Retreat **2016**
Title: Central Neural Circuitry Promotes Naïve Water Seeking

Cold Spring Harbor Laboratory
Neurobiology of Drosophila **2017**
Title: Water Seeking Neural Circuitry in Adult Drosohila

POSTERS

Cold Spring Harbor Laboratory
Neurobiology of Drosophila **2015**
Dan Landayan[1], Winnie Wu[1], Brandon Huynh[2], David Feldman[1] Lawrence Fung[1], Jovana Navarrete[1], Natalie Banh[1], Fred W. Wolf. (2015) Central Neural Circuitry Promotes Naïve Water Seeking.

Cold Spring Harbor Laboratory
Neurobiology of Drosophila **2017**
Dan Landayan[1], Jennifer Zhou[2], Fred W. Wolf [12]. (2017) Water Seeking Neural Circuitry in Adult Drosophila.

Cold Spring Harbor Laboratory
Brains and Behavior: Order and Disorder in the Nervous System **2018**
Dan Landayan[1], Jennifer Zhou[2], Fred W. Wolf [12]. (2018) Circuitry for Water Seeking Motivation in Drosophila.

MEMBERSHIPS

2013-Present
SACNAS –
Graduate Advisor **2013-2019**
SACNAS – Member **2011-2013**
Nexus Graduate Association - Treasurer

PUBLICATIONS AND PAPERS

Published:

Dan S. Landayan, Fred W. Wolf. (2015) Shared neurocircuitry underlying feeding and drugs of abuse in Drosophila. Biomed J. 2015 Dec;38(6):496-509. doi: 10.1016/j.bj.2016.01.004. PMID

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ABSTRACT

Central Neural Circuitry of Food and Water Seeking in *Drosophila melanogaster*

By

Dan Landayan

Doctor of Philosophy in Quantitative Systems Biology

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Professor Fred Wolf, Dissertation Advisor

The role of homeostatic hormones in the control of ingestive behaviors is well established, however the understanding of how cortical and subcortical reward systems (like the dopaminergic reward pathways) integrate with hormonal signals and other brain regions to regulate motivational seeking is incomplete. To better understand the neuronal circuitry underlying the neurobiology of obesity and motivation, it is essential to address the pre-ingestive phase of motivated homeostatic seeking behavior, when individuals are actively seeking reward.

To understand the fundamental neural processes underlying basic behaviors, like food, water, and drug seeking, it is critical to evaluate the potential interactions between common and diverse neural substrates known to mediate complex behaviors, like food and drug addiction. In the first part of this dissertation (chapter 1) I review distinct and overlapping neural motifs underlying motivated food and drug-related behaviors in *Drosophila melanogaster* to better understand the circuit logic underlying motivational survival behavior hierarchies (hunger, thirst, fear avoidance, sleep, copulation).

In the second part of this dissertation (chapter 2), I provide evidence that dopaminergic wiring within the fly brain is necessary and sufficient to promote food-seeking behavior in a satiation-state dependent manner. Here, we use sophisticated genetic tools to reversibly activate and inactivate neuronal ensembles and have categorized the function of discrete dopaminergic clusters of neurons. I demonstrate their ability to promote or inhibit pre-ingestive food seeking behaviors by using a novel food seeking assay. More importantly, we show that expression of the D1 receptor, DopR, is necessary in the mushroom bodies to promote food seeking in starved animals.

In the final part of this dissertation (chapter 3) I show that a persistent state of thirst is evoked by the precise activation of six central brain neurons in adult Drosophila. In a neuronal activation screen, we identified a subset of GABA and AstA-expressing neurons that evoke robust thirst-related behaviors, including water seeking and intake; we named these neurons Janu, the Estonian for thirsty. These central brain neurons function downstream of sensory input and internal osmotic sensors to drive seeking to either open or inaccessible water. Importantly, activation of Janu neurons overrides food seeking in water replete but hungry flies. We also identified neuropeptide F receptor (NPFR)-expressing neurons that appear to function as a water seeking homeostat. Neurons expressing NPFR, the invertebrate homolog of the NPY receptor, also promote insatiable hunger and voracious feeding. Like Janu neurons but independent of

them, activation of NPFR neurons overrides food seeking in water replete but hungry flies. Thus, neural circuit elements that regulate hunger and thirst are tightly integrated. These studies provide an entry point for mapping the fundamental homeostatic thirst neurons and the hierarchical wiring of neural circuits that encode opposing motivational states.

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Chapter 1: Shared neurocircuitry underlying feeding and drugs of abuse in Drosophila

Summary

The neural circuitry and molecules that control the rewarding properties of food and of drugs of abuse appear to partially overlap in the mammalian brain. This has raised questions about the extent of the overlap and the precise role of specific circuit elements in reward and in other behaviors associated with feeding regulation and drug responses. The much simpler brain of invertebrates, including the fruit fly *Drosophila*, offers an opportunity to make high resolution maps of the circuits and molecules that govern behavior. Recent progress in *Drosophila* has revealed not only some common substrates for the actions of drugs of abuse and for the regulation of feeding, but also a remarkable level of conservation with vertebrates for key neuromodulatory transmitters. We speculate that *Drosophila* may serve as a model for distinguishing the neural mechanisms underlying normal and pathological motivational states that will be applicable to mammals.

This work has been previously published: Dan S. Landayan, Fred W. Wolf. (2015) Shared neurocircuitry underlying feeding and drugs of abuse in Drosophila. *Biomed J*. 2015 Dec;38(6):496-509.

Introduction

In all animals, hunger drives the motivation to seek out food. Peripheral hormones directly regulate food seeking, and the targets of these peripheral hunger and satiety signals have been mapped to distinct hypothalamic and hindbrain nuclei in mammals. (Morton et al., 2014) Satiety signals and homeostatic brain circuits that limit feeding can be overridden by highly palatable food irrespective of the animal's nutritional state. (Kenny, 2011a) For example, remote manipulation of feeding circuits in mice and the fruit fly *Drosophila* promotes voracious eating in lieu of satiety signals. (Aponte et al., 2011; Wu et al., 2005a) In other words, organisms as distinct as mammals and invertebrates may have evolved common and hard-wired central feeding circuits in the brain.

Drugs of abuse have the capacity to evoke highly motivated and goal-directed behavior with an intensity that can eclipse even that of a very hungry animal. (Kenny, 2011b) Addictive drugs, like cocaine and alcohol, have reinforcing properties similar to food, and their pleiotropic actions are mediated in part by highly complex reward circuitry, such as the drug and feeding-engaged mesolimbic dopaminergic pathways. (Volkow et al., 2013) Despite some commonalities in behavioral states and implicated brain circuitry, direct functional overlap of specific circuit elements has been difficult to prove, partly because of the ever-more appreciated complexity of the brain, but also because the quality and interpretation of behavioral measurements are rapidly improving. (DiLeone et al., 2012a)

Drosophila is an attractive model organism for conjoining behavioral, neuroanatomical, and genetic studies, because of its genetic tractability, the development of precise and high throughput assays, and the availability of tools to manipulate neuronal properties in a spatio-temporally accurate manner. (Venken et al., 2011) Remarkably, homeostatic metabolic systems and neurochemical circuit motifs in mammals and *Drosophila* appear to be largely conserved. (Kaun et al., 2012; Smith et al., 2014) Circuit and neuron-specific manipulation in fruit flies has permitted the investigation of genetic and molecular targets that underlie the complex actions of addictive drugs (Kaun et al., 2012) as well as the homeostatic signals that regulate feeding. (Smith et al., 2014)

Here we review recent findings indicating that the regulation of feeding and the neural mechanisms of drugs of abuse in fruit flies may have significant overlap. We limit our scope to common neuromodulators and circuitry, including dopamine, the amines tyramine and octopamine, the NPY-like NPF, the insulin-like DILPs, and the neuropeptide corazonin. We include molecular and circuit-level descriptions for some drug-related behaviors that may be distinct from reward and motivation but that appear to share some common elements with feeding. More comprehensive reviews on the regulation of feeding (Pool and Scott, 2014) and on the molecular and behavioral actions of drugs of abuse in *Drosophila* were published recently (Devineni and Heberlein, 2013; Kaun et al., 2012).

Dopamine

Dopamine is a pleiotropic modulator of behavior in mammals and in fruit flies: depending on the behavioral context, dopamine in *Drosophila* affects sleep, mating, learning and memory, locomotion, feeding, and the effects of drugs of abuse (Keleman et al., 2012; Kong et al., 2010a; Ueno et al., 2012; Waddell, 2013; Wang et al., 2013a). There are approximately 280 dopaminergic neurons in the adult fly brain that are subdivided into eight major clusters based on their cell body location, and each cluster sends projections to distinct brain regions (Figure 1) (Mao et al., 2009; Nässel and Elekes, 1992). Dopamine signaling is detected by four receptors that are distributed broadly in the brain: the D1-like receptors DopR1 (DA1, DopR) and DopR2 (DAMB), the multiply spliced D2-like receptor D2R, and the DopEcR receptor that is also gated by the insect hormone ecdysone (Yamamoto and Seto, 2014). Emerging evidence indicates that particular dopamine clusters and even individual neurons likely form valence-specific circuit motifs that are engaged by conditioned (Waddell, 2013) or innate values of a stimulus (Azanchi et al., 2013; Lin et al., 2014a), and whose function can be modified by internal state (Berry et al., 2012; Plaçais et al., 2012).

Dopamine in Feeding Behaviors

Feeding behaviors are subdivided into six distinct phases: foraging/seeking, cessation of locomotion, meal initiation, consumption, meal termination, then finally food disengagement (Pool and Scott, 2014). The feeding behaviors we discuss are complex and can overlap between two or more of the respective aspects of feeding. A portion of our focus will encompass behavioral assays that assess goal-directed approach or avoidance behavior in the context of both unconditioned and conditioned food-related stimuli. The study of goal directed approach or avoidance is a method to evaluate the relationship between valence-specific circuit motifs and innate/learned feeding motivation (Waddell, 2013).

Protocerebral Anterior Medial Neurons

Most fruit fly dopamine neurons, about 130 per hemisphere, are located in the Protocerebral Anterior Medial (PAM) cluster. The PAM neurons densely innervate the mushroom bodies, prominent brain structures implicated in associative learning and memory and other behaviors. The mushroom bodies are composed of ~2,500 Kenyon cells per hemisphere that are named α/α', β/β', and γ based on anatomical division (Figure 1). The PAM presynaptic terminals contact discrete regions in the β, β', and γ lobes that comprise the horizontal lobes (Burke et al., 2012a; Liu et al., 2012a). Functionally, there exist distinct classes of PAM neurons that can impart positive (for example, the 15 MB-M8 neurons labeled in the *0279-Gal4* strain) and negative (for example, the 3 MB-M3 neurons labeled in the *NP5272-Gal4* strain) valence, and they innervate distinct parts of the mushroom bodies.

Classic associative learning assays, where flies are taught to associate a stimulus (for example sugar or electric shock) with a neutral cue (usually an odor), are commonly used to assess neural coding of reward and aversion. Genetic inactivation of most PAM neurons (with *R58E02-Gal4* or *0104-Gal4* transgenes that express the yeast transcriptional activator GAL4 in specific PAM neurons to facilitate their genetic manipulation) (Table 1) blocks appetitive learning with sucrose (Burke et al., 2012a; Liu et al., 2012a). Moreover, *R58E02* neurons increase activity in response to sucrose ingestion, responding more strongly following food deprivation (Liu et al., 2012a). These results suggest that the PAMs encode the rewarding value of sucrose. Conversely, inactivation of the MB-M3 neurons (*NP5272-Gal4*) blocks aversive learning (Aso et al., 2012). Importantly, activation of either the MB-M8 or MB-M3 neurons substitutes for the unconditioned stimulus (sugar or shock), and is sufficient for appetitive or aversive reinforcement, respectively (Aso et al., 2012; Perisse et al., 2013). The activation of both the positive and negative valence populations of all 130 PAM dopaminergic neurons promotes appetitive

reinforcement (Burke et al., 2012a; Liu et al., 2012a). Further investigation is needed to delineate the precise profile of the broadly targeted PAM neurons. For example, the profile of the subpopulations of neurons in the PAM cluster (other than the MB-M8 and MB-M3) is still largely uncategorized and have unknown functions.
Recent findings suggest that innate behaviors critical for survival, such as seeking food or even water, may be modulated by dopaminergic neurons that are also implicated in appetitive reinforcement learning paradigms. A group of approximately 55 PAM neurons (*R48B04-Gal4*) that include neurons that project to the γ4/5 lobe are necessary and sufficient for promoting water reward memory in a thirst-dependent manner (Lin et al., 2014a). Moreover, these neurons are activated by water intake in thirsty flies. This finding indicates that water, like sucrose, may be encoded in similar reward pathways. Activity in a non-overlapping set of β'-projecting PAM neurons (also from the *R48B04* pattern) is necessary for innate water seeking in thirsty flies, and, importantly, the γ and β'-projecting dopaminergic PAM neurons are exclusively involved in thirst-dependent learned and innate water seeking, respectively (Lin et al., 2014a). These results suggest that PAM neurons involved in other positive reward-seeking may be further categorized into innate and learned subdivisions.

Paired Posterior Lateral 1 Neurons

The 12 Paired Posterior Lateral 1 (PPL1) dopamine neurons synapse onto areas of the mushroom body that are largely distinct from the PAMs, including the medial (MB-MP1) and vertical (MB-MV1) lobes of the mushroom bodies (Claridge-Chang et al., 2009). The PPL1 MB-MP1 neurons, like the PAM MB-M3s, are involved in negative valence assignment (Aso et al., 2012; Waddell, 2013). PPL1 neurons integrate the satiety state (hungry or well-fed) of the fly in the context of learning and memory; well-fed flies form appetitive associations poorly, however inactivating MB-MP1 neurons (*c061-Gal4*) allows retrieval of appetitive memory (Krashes et al., 2009a). Conversely, activation of the MB-MP1 neurons can block appetitive memory retrieval in hungry flies (Krashes et al., 2009a). In vivo calcium imaging shows that the PPL1s are tonically active in the fed state, but are greatly attenuated in the food-deprived state (Berry et al., 2012; Plaçais and Preat, 2013). Together, these results suggest that in well-fed flies, the dopaminergic PPL1 neurons send tonic inhibitory signals to the mushroom bodies to suppress appetitive feeding behavior.

Ventral Unpaired Medial Neurons in Sensing Sugar

Dopamine also tunes the sensory perception of appetitive cues. Fruit flies, like blow flies, extend their proboscis upon detection of palatable gustatory cues through taste sensilla located on the proboscis or on the distal tarsal leg segment (Dethier, 1976). Taste reception is largely mediated by independent populations of sugar-sensing and bitter-sensing gustatory receptor neurons that send axonal projections to the subesophageal ganglion (SOG) in a modality (e.g. sweet/bitter) and organ-specific (e.g. labellum/tarsal segment) arrangement (Wang et al., 2004). A single dopaminergic neuron located in the SOG, the ventral unpaired medial neuron (TH-VUM), is necessary and sufficient to promote proboscis extension to sucrose and, further, its tonic activity is increased in starved flies (Marella et al., 2012). The TH-VUM makes synaptic connections broadly throughout the SOG. Additionally, dopamine acts directly on sugar-sensing taste neurons to enhance taste reactivity in starved flies (Inagaki et al., 2012a), however the specific dopamine neurons responsible for this sensory tuning need to be identified.

Larval Dopamine Neurons in Feeding Motivation

There are approximately 90 dopaminergic neurons in the 3rd instar larval central nervous system. Notably, three bilateral clusters of dopamine neurons called the DM, DL1, and DL2 project to higher brain regions in the protocerebrum including the mushroom bodies (Selcho et al., 2009). Larvae exhibit appetitive mouth hook contractions that scale with satiation state,

sucrose concentration, food source accessibility (easy to eat soft vs. more difficult to eat agar-embedded food), and with exposure to food-like odors (Wang et al., 2013a). Laser ablation of DL2 neurons that project to the larval lateral protocerebrum abolish the food-like odor enhanced mouth hook contractions, and their genetic activation is sufficient to increase mouth hook contractions (Wang et al., 2013a). Moreover, food-like odors increase DL2 neuronal activity, indicating that specific dopamine neurons in larvae react to appetitive cues to promote feeding behavior.

Mushroom Bodies

The activity of mushroom body Kenyon cell neurons that are postsynaptic to the PAMs and PPL1s is necessary for both appetitive and aversive conditioning (Perisse et al., 2013), and distinct regions of the mushroom bodies have valence-specific roles. Appetitive-encoding PAM neurons specifically innervate the β lobe surface and core neurons, whereas the aversive-encoding PAM neurons exclusively innervate the β surface neurons (Perisse et al., 2013). In particular, the α/β surface neurons are necessary for both appetitive and aversive conditioning, whereas the α/β core neurons are specific for appetitive conditioning (Perisse et al., 2013). In a differential aversion conditioning paradigm, flies are trained to choose between a 30V or 60V electric shock-conditioned odorant: the flies avoid the 60V-paired odorant, but also actively approach the 30V-paired odorant. In this paradigm both the α/β core Kenyon cells and appetitive dopamine neurons are necessary for the flies to approach the less “hazardous” odor (Perisse et al., 2013). These and other experiments argue that the PAM to mushroom body appetitive neural pathway encodes positive valuation even when the positive value is simply “less bad” rather than “good”. Because aversive conditioning is impaired in starved flies, it would be interesting if the PPL1-MP1 and PPL1-MV1 also gate relative aversive conditioning, similar to appetitive conditioning in well-fed flies.

Downstream of the Kenyon cells are 34 mushroom body output neurons (MBON) comprising 21 cell types and that are glutamatergic, GABAergic, or cholinergic. MBONs elaborate zonal dendritic innervation patterns along the vertical and horizontal stalks of the mushroom bodies. Interestingly, the dendrites of glutamatergic and GABAergic MBONs are largely restricted to the β, β’ and γ horizontal regions, whereas cholinergic fibers predominately occupy the α and α’ vertical stalks. The dendrites of select MBONs and pre-synaptic terminals of PAM and PPL1 dopaminergic neurons overlap, likely forming relays at the mushroom bodies (Aso et al., 2014a; Owald et al., 2015). Many MBONs elaborate presynaptic endings in close proximity to dopamine neuron dendrites, implying that the MBONs may form a feedback loop to modify the dopamine to mushroom body circuit.

A recent study methodically characterized the role of each MBON cell type for a spectrum of behaviors, including both innate and learned appetitive and aversive responses (Aso et al., 2014a). Inactivation of specific glutamatergic MBONs that innervate the tips of the β and γ lobe impair appetitive and aversive conditioning (Aso et al., 2014a; Owald et al., 2015). The requirement for activity of specific cholinergic neurons varies with the appetitive conditioning paradigm being tested (Owald et al., 2015). Interestingly, some of the same glutamatergic MBONs (β’2 and γ5 innervating) display decreased or increased activity when exposed to odors previously paired with a reward or punishment, respectively (Owald et al., 2015). In the context of innate behavior, remote activation of the β and γ lobe tip MBONs with the red-shifted channelrhodopsin Chrimson, promotes innate avoidance of red light (Aso et al., 2014a). Intriguingly, blocking the output of the same MBONs changes naïve odor avoidance to attraction (Owald et al., 2015). Current models argue that the MBONs bias selection of behavioral actions and this selection bias is modified by appetitive or aversive associations (Aso et al., 2014a; Owald et al., 2015).

Dopamine and Drugs of Abuse

Behavioral responses to drugs of abuse that can be easily measured in model organisms can be categorized as unconditioned and conditioned. Unconditioned behaviors include drug sensitivity, attraction or aversion, and locomotor effects such as hyperactivity and stereotypies. Conditioned behaviors arising from prolonged or repeated drug intake include the development of drug tolerance and sensitization, preference, withdrawal, and reinstatement following a period of abstinence. As with feeding behaviors, these responses are complex and are likely coded by multiple neural circuits acting simultaneously. The drugs of abuse that are most well-studied in *Drosophila*, ethanol and cocaine, elicit behavioral responses that remarkably parallel those in vertebrates. For example, ethanol stimulates locomotion at low doses, and causes incoordination and sedation at higher doses (Kaun et al., 2012). Flies also show dose dependent attraction and aversion to ethanol. Flies develop preference for ethanol, find it rewarding, show signs of withdrawal, and reinstate intake following a period of abstinence.

Drug Sensitivity and Tolerance

A role for dopamine in the acute sensitivity to drugs of abuse was first described using pharmacological and genetic techniques that affect all or many dopamine neurons simultaneously. Cocaine binds to the plasma membrane dopamine transporter, blocking dopamine reuptake following its release at synapses, resulting in higher and more sustained extracellular dopamine. Volatilized (crack) cocaine provided at moderate doses increases locomotor activity (hyperactivity) and causes stereotypies, or repeated motor behavioral patterns (Bainton et al., 2000; Li et al., 2000; McClung and Hirsh, 1998). Moderate doses of ethanol and nicotine also cause hyperactivity, and larvae fed amphetamine show dopamine-dependent hyperactivity (Pizzo et al., 2013). Adult flies made dopamine deficient become resistant to the acute behavioral effects of ethanol, cocaine and nicotine (Bainton et al., 2000), suggesting that dopamine is a common target for drugs of abuse in flies, as it is in mammals.

The dopaminergic step of the circuitry for acute ethanol promotion of hyperactivity is known. Genetic inactivation of either most, or even just a pair of dopamine neurons decreases ethanol-induced hyperactivity, whereas selective acute activation of the same pair of dopamine neurons promotes hyperactivity (Kong et al., 2010a). The pair of neurons are in the protocerebral posterior medial 3 (PPM3) cluster of dopamine neurons, and they make presynaptic contact with a circular structure termed the ellipsoid body that is part of the central complex in the fly brain. Moreover, postsynaptic D1-like dopamine receptors (DopR1) located in the ellipsoid body intrinsic neurons promote ethanol-induced hyperactivity. The central complex is a group of four highly interconnected brain structures that appear to integrate sensory and internal states to coordinate behavioral responses, including locomotion (Wolff et al., 2014).

Circadian control of arousal state involves dopamine and is affected by methamphetamine and cocaine. Arousal is heightened in the daytime (except when flies partake in a midday “nap”) and suppressed in the nighttime (Hendricks et al., 2000; Shaw et al., 2000). Dopamine promotes wakefulness: dopamine deficient flies sleep more and flies genetically manipulated to acutely activate dopamine neurons sleep less (Andretic et al., 2005; Liu et al., 2012c; Ueno et al., 2012). Methamphetamine, which binds to dopamine and other monoamine transporters and results in higher extracellular dopamine levels, decreases nighttime sleep (Andretic et al., 2005). Similarly, cocaine, when provided in the flies food, decreases sleep and increases arousal state (Lebestky et al., 2009). Cocaine heightened arousal works through the D1-like dopamine receptor DopR1: flies lacking DopR1 show increased nighttime sleep, and are resistant to cocaine. A second form of arousal is induced by repeated environmental stress and is also dopamine dependent and affected by cocaine (Lebestky et al., 2009). The wake promoting effects of methamphetamine, like cocaine, depends on the DopR1 receptor, and interestingly this function localizes in part to the mushroom bodies (Andretic et al., 2008). Consistent with this, methamphetamine, as well as exogenously supplied dopamine, restores a form of mushroom body-dependent aversive learning

that is compromised by sleep deprivation (Seugnet et al., 2008). Finally, we note that ethanol sedation sensitivity varies with circadian time (and so likely with arousal state), and circadian genes regulate ethanol sedation tolerance (Linde and Lyons, 2011; Pohl et al., 2013), however the role of dopamine in circadian regulation of these and other ethanol responses is not yet known.

Dopamine neurons that project to a region of the central complex called the fan-shaped body promote wakefulness (Liu et al., 2012c; Ueno et al., 2012). Further, DopR1 functions in the ellipsoid body for stress-induced arousal (Lebestky et al., 2009). Taken together with dopaminergic promotion of ethanol-induced hyperactivity mapping to the ellipsoid body (Kong et al., 2010a), it is possible that the highly interconnected central complex is a site of motor control connected to different forms of behavioral arousal.

Drug Preference and Reward

Dopamine is also critical for more complex ethanol-related behaviors, including a form of ethanol preference and also ethanol reward. Female flies given a choice between food with and without added ethanol will lay their eggs on the ethanol food: ethanol is present in decomposing fruit, the preferred food source and gathering place for *Drosophila* in the wild (Azanchi et al., 2013). Dopamine neurons in the PAM and PPM3 (the same neurons that promote ethanol hyperactivity) clusters promote egg-laying preference, whereas dopamine neurons in the PPL1 cluster inhibit egg-laying preference. Importantly, blocking neuronal activity in the PAMs labeled by *R58E02-Gal4* is ineffective, distinguishing egg-laying preference for ethanol food from appetitive learning with sucrose (Burke et al., 2012a; Liu et al., 2012a). Both the PAM and PPL1 neurons tested are presynaptic to the mushroom bodies, and genetic inactivation experiments show that the α'/β' mushroom body neuropil promotes egg-laying preference for ethanol food.

Ethanol is rewarding to *Drosophila*. The presence of reward is shown by a positive association between ethanol intoxication and co-presentation of a neutral odor cue: the neutral cue becomes attractive when later presented alone (Kaun et al., 2011). Importantly, flies perform work (they tolerate an aversive electric shock) to approach the previously ethanol-paired odor. Blocking either dopamine synthesis or dopamine synaptic transmission completely disrupts this ethanol conditioned preference. Blocking dopamine synaptic transmission is, perhaps surprisingly, not effective during either the pairing or consolidation phase as one might expect from mammals (Bromberg-Martin et al., 2010a), but is effective when the fly is asked to remember the odor:ethanol intoxication pairing. The lack of effect during learning about ethanol reward may be due in part, however, to tools used: all but the PAM neurons were inactivated. Finally, we note that appetitive valuation of ethanol is evident only after an initial period of conditioned aversion, highlighting the complexity of behavioral encoding for this and other addictive drugs (Kaun et al., 2011).

Drug targets downstream of dopamine

The mushroom bodies and the central complex, innervated by dopaminergic and other types of neurons, are critical for a broad spectrum of ethanol and other drug-related behaviors. Our understanding of the role of the central complex in ethanol behaviors is still rudimentary, however specific classes of ellipsoid body neurons are important for ethanol-induced hyperactivity and ethanol sedation tolerance (Ghezzi et al., 2013; Kong et al., 2010a; Urizar et al., 2007). The mushroom bodies promote ethanol-induced hyperactivity (King et al., 2011), ethanol preference (Azanchi et al., 2013; Xu et al., 2012), ethanol reward (Kaun et al., 2011), and recovery from sedation induced by the related benzyl alcohol (Ghezzi et al., 2013). Functional mapping of the mushroom body for ethanol behaviors, while still preliminary, suggests use of specific neuropils for simpler behaviors, and sequential use of distinct neuropils for more complex behaviors. For example, sequential use of the γ, α'/β', and α/β neuropils supports acquisition, consolidation, and

retrieval of ethanol reward memory, respectively (Kaun et al., 2011).

The circuitry for ethanol behaviors extends to both cholinergic and glutamatergic MBONs (Aso et al., 2014a). Intriguingly, the dendritic arborization patterns of ethanol reward and aversion MBONs largely overlap with the presynaptic terminals of PAM and PPL1 dopaminergic clusters (Aso et al., 2014a; Owald et al., 2015). For example, activity in the cholinergic MBON-α'2 is required for the expression of the appetitive response to alcohol conditioned odorants (Aso et al., 2014a). The PPL1s are currently the only known MB extrinsic neurons to project into the α'2 region of the vertical lobe, suggesting that the PPL1 to MBON-α'2 circuitry is critical for alcohol reward learning (Aso et al., 2014b; Kaun et al., 2011).

Dopamine: Food and Drugs

Distinct, valence-specific dopaminergic neurons that target the mushroom bodies seem to be engaged by the rewarding properties of both food and drugs.

Dopamine Neurons

The PAM cluster of dopamine neurons are necessary for ethanol preference (Azanchi et al., 2013), appetitive reinforcement (Burke et al., 2012a; Liu et al., 2012a), aversive reinforcement (Aso et al., 2012), and water attraction and reinforcement (Lin et al., 2014a). Importantly, non-overlapping sets of PAM neurons are critical for innate water attraction and water reinforcement, utilizing distinct yet anatomically related dopaminergic pathways. Similarly, a group of approximately 40 PAM neurons that is distinct from PAM sucrose and water reinforcement neurons is critical for ethanol egg-laying preference (Azanchi et al., 2013). These results indicate that the PAM cluster is a heterogeneous mixture of neurons that can drive both innate and learned behaviors. However, the valence-specific role of PAM subsets in the study of drugs is still unclear.

Investigations directly targeting the PPL1-MP1 neurons implicate their function in assigning negative valence (odor-shock) (Aso et al., 2012; Krashes et al., 2009a) and they are also necessary for ethanol aversion (Azanchi et al., 2013). These results suggest that stimuli with an aversive property such as electric shock and ethanol may converge onto a common dopaminergic pathway. Because an unconditioned stimulus like ethanol has simultaneous rewarding and aversive properties, it's possible that its behavioral actions are encoded by both the aversive (ex. PPL1-MP1, MV1; PAM-M3) and reward (ex. PAM-M8) circuits. Complex stimuli with both rewarding and aversive properties may be processed in parallel by separate valence-specific dopamine circuits. Interestingly, the PPL1 cluster may also code for appetitive functions: because the PPL1 to MBON-α'2 terminal endings and dendrites form a putative circuit, it is possible that PPL1 activity facilitates the transition from aversive to appetitive alcohol reward (Aso et al., 2014b; Kaun et al., 2011). Specific manipulation of PPL1 and other dopaminergic clusters is needed to verify the neuronal substrate mediating the appetitive alcohol response.

The locomotor stimulant effects of ethanol and innate ethanol preference are localized in part to the PPM3 dopamine cluster (Azanchi et al., 2013; Kong et al., 2010a). Similarly, the wake-promoting effects of dopamine utilize PPM3 neurons (Ueno et al., 2012). However, there is, as yet, no reported role of the PPM3s in food-associated behaviors. The PPM3 pathway may code for aspects of arousal or attention that underlie specific forms of motivated behavior (Salamone and Correa, 2012a).

Dopamine Neuron Postsynaptic Targets

Ethanol reward converges on some of the same pathways as sucrose reward because the γ, α'/β', and α/β mushroom body neurons are involved in similar phases of appetitive memory

acquisition, retrieval, and consolidation (Kaun et al., 2011; Krashes et al., 2007). Even more convincing is the remarkably similar set of MBONs required for two hour odor-sugar appetitive memory and odor-ethanol intoxication memory. Both forms of appetitive association require neuronal activity in the same glutamatergic MBONs innervating the β and γ lobes, and also the same cholinergic MBONs innervating the α and α' lobes (Aso et al., 2014a). Therefore, the reward circuits for appetitive conditioning of odors for sugar and ethanol converge at or just beyond the mushroom bodies.

Tyramine and Octopamine

Tyramine and octopamine, often called the trace amines, are synthesized in sequential steps from L-tyrosine. Tyrosine is converted into tyramine by tyrosine decarboxylase, which is encoded by functionally interchangeable products of the Tdc1 and Tdc2 genes; Tdc2 encodes the major neuronal form. Tyramine is converted into octopamine by tyramine β hydroxylase, encoded by the Tβh gene. Tdc2 mutations reduce levels of both tyramine and octopamine, whereas Tβh mutations reduce levels of octopamine but also increase tyramine by about ten-fold (Monastirioti et al., 1996). This interrelationship can complicate assignment of a particular trace amine to behavioral functions. Tβh (and so octopamine) is present in about 150 cells in the adult brain (Busch et al., 2009). Surprisingly little is known about the numbers and innervation patterns of tyraminergic cells. Similarly, the role of individual octopaminergic neurons, their innervation patterns, and their connectivity are just beginning to be explored. There are two classes of octopamine receptors in flies, including one α-adrenergic-like (OAMB) and three β-adrenergic-like (Octβ1R, Octβ2R, and Octβ3R), and three tyramine receptors (Evans and Maqueira, 2005; Kim et al., 2013). The trace amines tyramine and octopamine likely bestow vertebrate epinephrine and norepinephrine functions, respectively.

Tyramine and Octopamine in Feeding Behavior

Classic experiments in honey bees show that electrical stimulation of a single octopaminergic neuron (Hammer, 1993) or direct administration of octopamine to the olfactory antennal lobe or the mushroom bodies supplants the rewarding properties of sucrose in odorant conditioning assays (Hammer and Menzel, 1998). This led to the identification of the octopaminergic ventral unpaired median neuron 1 of the maxillary neuromere (VUMmx1) as the neuron that conveys the rewarding value of sucrose. It is located beneath the subesophageal ganglion (the first gustatory relay site), and it has dense ramifications onto the antennal lobe, lateral protocerebrum, and mushroom bodies (Hammer, 1993).

Octopamine is Necessary for Innate and Learned Appetitive Behaviors

Similar to the honey bee VUMmx1, in *Drosophila* there are three clusters containing 8-10 octopaminergic VUMs each that have widespread arborizations in the deutocerebrum and protocerebrum, including in the latter the antennal lobe and mushroom bodies (Sinakevitch and Strausfeld, 2006). Tβh mutant adults are unable to form short-term sucrose reward memories or extend their proboscis in response to tarsal stimulation with sucrose; both behavioral deficits can be rescued by feeding Tβh-deficient flies octopamine (Das et al., 2014; Scheiner et al., 2014; Schwaerzel et al., 2003a). Normal sucrose responsiveness to tarsal stimulation was restored in Tβh-deficient flies by expression of Tβh in 34 SOG and 11 antennal lobe neurons (labeled by *NP7088-Gal4*) (Busch et al., 2009; Scheiner et al., 2014).

Octopamine is Upstream of PAM Neurons for Learned Behaviors

Recent work using appetitive conditioning tests confirms that Tβh-deficient flies are unable to form appetitive memories; however appetitive memory can be acquired in flies that are Tβh-deficient when ~90 PAM dopamine neurons (*R58E02-Gal4*) are acutely activated (Liu et al.,

2012a). This result suggests that octopamine signaling may act upstream of or in parallel with the PAM neurons. Similarly, expression of an RNAi directed against the OAMB octopamine receptor in ~40 PAM neurons (*0104-Gal4*) blocks appetitive conditioning with the sweet but non-caloric sugar arabinose, and brain application of octopamine increases the activity of these same neurons (Burke et al., 2012a). Another group showed that OAMB is strongly and selectively expressed in the α/β mushroom body lobes where it promotes appetitive conditioning (Kim et al., 2013).

Octopamine Encoding of Sweet Palatability

Blocking the output of a subset of octopaminergic and tyraminergic neurons (labeled by *Tdc2-Gal4*) impairs appetitive learning with arabinose, but not with sucrose (both sweet and caloric) (Burke et al., 2012a). Two other independent studies show that flies exhibit enhanced appetitive reinforcement when an exclusively sweet sugar is supplemented with exclusively caloric sugars (Burke and Waddell, 2011; Fujita and Tanimura, 2011). Together, the results suggest that sucrose has two independent reinforcing properties, its sweet and nutritive value. Importantly, octopamine most likely encodes sweet palatability, whereas a caloric sensor, perhaps also octopaminergic, that is responsible for memory reinforcement remains to be identified. It is important to note that appetitive conditioning by direct activation of octopamine neurons is short-lived (reported to last for 30 minutes) compared to sucrose conditioning, and it does not depend on the satiation-state of the fly (Burke et al., 2012a).

Octopamine in Larval Feeding Behavior

Octopamine also promotes appetitive behavior in larvae. Tβh-deficient larvae have diminished, starvation-induced mouth hook contractions that can be rescued by feeding the larvae octopamine (Zhang et al., 2013a). Moreover, inactivation or activation of *Tdc2-Gal4* neurons showed that these neurons are necessary and sufficient to promote this appetitive response. Targeted laser ablations indicate that larval octopaminergic VUM1 and VUM2 neurons inhibit and promote the larval feeding response, respectively. Interestingly, the motivated feeding behavior is only observed when larvae are provided with soft liquid food as compared to agar-embedded sugar. This could mean that an aversive condition that requires work (extra energy expenditure) may prevent the expression of octopamine-dependent appetitive behaviors.

Tyramine and Octopamine and Drugs of Abuse

Ethanol sensitivity, ethanol tolerance, and ethanol preference are all regulated by the trace amines. Ethanol sedation sensitivity is decreased when synaptic output is blocked in a subset of Tdc2 neurons (*Tdc2-Gal4*), and feeding of tyramine but not octopamine to these synaptically silenced flies restores ethanol sensitivity, implicating tyramine in the sedative effects of ethanol (Chen et al., 2013). While ethanol sensitivity is unaffected in Tβh mutants, the development of ethanol tolerance is compromised, raising the possibility that initial sensitivity is tyramine-dependent and neuroadaptation to repeated exposures is octopamine-dependent (Scholz, 2005; Scholz et al., 2000). Similarly, sensitivity to acute crack cocaine is increased in Tdc2 mutants and when *Tdc2-Gal4* neurons are hyperpolarized, but is unaffected in Tβh mutants (Hardie et al., 2007), suggesting that, similar to ethanol, tyramine regulates cocaine sensitivity.

Flies are attracted to the smell of ethanol at low concentrations, when presented alone or mixed with food. This innate olfactory preference can be measured by trapping flies that come in proximity to the odor source (Schneider et al., 2012). innate olfactory preference for ethanol is lost in Tβh mutants and is regained when Tβh activity is restored to a small number of Tβh neurons that are likely to release acetylcholine in addition to the trace amines. While the individual neurons responsible for ethanol olfactory preference remain to be identified, the implicated cells are located in the subesophageal region of the fly brain.

Tyramine and Octopamine: Food and Drugs

In the context of drugs, Tβh-deficient flies' sensitivity is unaltered upon exposure with alcohol or cocaine which implies that sensitivity may be mediated by tyramine signaling. Moreover, octopamine activity may be important in mediating ethanol tolerance. Currently, there are no known feeding behaviors that are associated with tyramine.

Octopamine Promotes Innate and Learned Behaviors

The pioneering work in honey bees has implicated that octopamine is necessary for the rewarding value of sucrose (Hammer, 1993). Indeed, the notion that octopamine is an important transmitter of sucrose reward is consistent in flies. Two independent studies have shown that Tβh mutant flies are unable to form sucrose-reinforced memory or exhibit normal PER in response to sucrose stimulation (Scheiner et al., 2014; Schwaerzel et al., 2003a). These results suggest that octopamine is necessary for appetitive conditioning and innate responses to sucrose. Interestingly, the ventrally-located OA-VUMs are implicated in mediating innate alcohol approach and sucrose-induced PER (Scheiner et al., 2014; Schneider et al., 2012). Octopaminergic neurons responsible for appetitive learning for sucrose is attributed to the VUM –a6, a7, a8 and VPM 3 (labeled in *NP7088*) (Burke et al., 2012a). Thus, while direct overlap hasn't yet been proven, the OA-VUMs may have multiple modulatory roles in alcohol olfactory approach, gustatory sugar sensitivity, and appetitive conditioning.

Octopamine Encodes Sweet Value

Interestingly, it's been shown recently that blocking *Tdc2-Gal4* neuronal activity prevents appetitive short-term memory acquisition with arabinose but not sucrose, and this is because it is both sweet and caloric (Burke et al., 2012a). Compared with the earlier studies investigating the role of octopamine in learning, Tβh-deficient flies could not make appetitive associations with sucrose reinforcement (Schwaerzel et al., 2003a). It's important to consider that not all octopaminergic neurons in the *Drosophila* brain are labeled in the *Tdc2-Gal4* pattern, thus it may be possible that other octopaminergic neurons are mediating the calorie-dependent appetitive conditioning. Alternatively, Tβh-deficient flies also show reduced PER for sucrose, whereas silenced *Tdc2-Gal4* flies are unaffected (Marella et al., 2012; Scheiner et al., 2014). Another study shows that Tβh-deficient flies are able to form water reinforced memories in a novel water-reward learning paradigm, which is consistent with the model that octopamine encodes the sweet palatability of sugars (Lin et al., 2014a). Taken together, these results suggests that, depending on the internal motivational context (hunger, thirst, satiety), sweetness, nutritional content, and even water is rewarding to flies and may be ultimately encoded through dopaminergic reward pathways. Defining the role of octopamine in drug preference and reward in concert with refining the dopaminergic circuitry will be important for developing comparative circuit based-models of appetitive processes in feeding and addiction.

Neuropeptide F

Drosophila express neuropeptide F (NPF), which is evolutionarily related to mammalian neuropeptide Y (NPY), and the separately encoded short NPF (sNPF) that shares an RxRF C-terminal motif with NPF. NPF is present in only 20-26 neurons in the adult brain (10-13/hemisphere), whereas sNPF is expressed in approximately 280 neurons in the brain and in most or all mushroom body Kenyon cells (Nässel and Wegener, 2011). NPF and sNPF are co-expressed in four neurons. Similar to dopamine, NPF is implicated in a variety of motivated behaviors like learning and memory, feeding, drug seeking, and odorant attraction (Beshel and Zhong, 2013a; Krashes et al., 2009a; Shohat-Ophir et al., 2012; Wu et al., 2005a). sNPF regulates bitter taste responsiveness and also larval food intake (Inagaki et al., 2014; Lee et al.,

2004), but it's role in drug-related behaviors is not known.

Neuropeptide F in Feeding Behavior

Larval Feeding Behavior

NPF acts upstream of dopamine to promote appetitive behavior in larvae. The single NPF receptor (NPFR) is expressed in many dopaminergic neurons in larvae, including DL2 neurons, and RNAi against NPFR in dopamine neurons blocks both appetitive odor enhancement of DL2 neuronal activity and feeding behavior (Wang et al., 2013a). Furthermore, silencing NPF neurons not only blocks this appetitive odor enhanced feeding, but also in starved larvae decreases feeding behavior on solid (unpalatable) but not liquid (palatable) food (Wu et al., 2005b; Zhang et al., 2013a). NPF neuron silencing also decreases food intake on quinine-adulterated food (Wu et al., 2005a). Because NPF neuronal activity manipulation does not affect appetitive behaviors on more palatable food sources, the NPF system may be critical in situations that require risky behavior with aversive conditions (Lingo et al., 2007; Wu et al., 2005a; Zhang et al., 2013a).

Adult Feeding Behavior

Enhancement of NPF neuronal signaling increases food intake in food deprived adult flies, and it also increases sugar but not bitter taste reactivity when tested in fed adult flies (Hergarden et al., 2012a; Inagaki et al., 2014). NPF-enhancement of sugar taste reactivity is blocked in DopEcR-deficient flies, suggesting that NPF signaling is upstream of or in parallel with dopaminergic neurons that modulate sugar sensitivity. Importantly, the same manipulation of NPF neuron activity does not enhance tolerance of a bitter compound (lobeline) mixed with sucrose (Inagaki et al., 2014). These results suggest that NPF may not promote innate appetitive behavior under aversive conditions in the adult fly, and this distinction from NPF's role in larvae may be important in determining the shift from continuous feeding in larvae to selective feeding in adults.

Well-fed flies are much less able to form appetitive memories (Tempel et al., 1983). However, activation of NPF neurons during retrieval in well-fed flies allows the expression of a previously formed appetitive memory, indicating not only that appetitive memories are well-formed but suppressed in fed flies, but also suggesting that NPF activity mimics the state of hunger (Krashes et al., 2009a). Consistent with this notion, reduced expression of NPFR in the aversive-encoding PPL1-MP1 dopaminergic neurons blocks appetitive memory formation in hungry flies (Krashes et al., 2009a). This evidence suggests that NPF signaling is upstream of PPL1 neurons, perhaps keeping them turned 'off' in hungry flies to promote appetitive behaviors.

NPF also promotes innate attraction to appetitive odors in food-deprived flies (Beshel and Zhong, 2013a). Inhibition of NPF neurons decreases food odor attraction in starved flies, and, conversely, activation of NPF neurons promotes robust food odor attraction in fed flies. The activity of four NPF neurons in the dorsal protocerebrum is highly correlated with food odor attractiveness (Beshel and Zhong, 2013a). Intriguingly, NPF neuron activation in response to fruity odorants was high even in satiated flies, corresponding to robust behavioral attraction. Collectively, the evidence supports the role of NPF as a molecular signature encoding the motivational state of the fly. NPF activity functions in innate and conditioned contexts and signals upstream of dopaminergic (and likely other) neurons to mediate satiation-state dependent behaviors such as sugar taste reactivity and memory expression.

NPF and Drugs of Abuse

NPF regulates acute ethanol sensitivity, ethanol preference, and ethanol reward. Ethanol sedation sensitivity is reduced when NPF expressing cells are either ablated or synaptically silenced specifically during ethanol exposure (Wen et al., 2005). Conversely, NPF overexpression

in NPF neurons increases ethanol sedation sensitivity. Interestingly, NPF expression is increased following exposure to intoxicating levels of ethanol (Shohat-Ophir et al., 2012). Thus, NPF signaling actively promotes sensitivity to ethanol intoxication.

NPF expression is also regulated by mating history and the presence of predators. NPF levels are lower in sexually rejected males and higher in mated males, and rejected males show an increased preference for ethanol (Shohat-Ophir et al., 2012). Blocking NPF signaling by genetically reducing NPFR levels increases ethanol preference in mated males and, conversely, acute activation of NPF neurons in inexperienced males blocks ethanol preference. Importantly, both activation of NPF neurons and mating are rewarding to the fly since neutral odors paired with either manipulation become attractive when later presented alone. Finally, artificial activation of NPF neurons interferes with the ability of flies to find ethanol rewarding. Adult flies co-cultured with natural predator wasps lay more eggs on food containing ethanol concentrations (15%) that are toxic to the predators (Kacsoh et al., 2013). The visual presence of predators decreases NPF expression in the fan-shaped body region of the brain, and transgenic increases in NPF block the predator-driven egg-laying preference for ethanol. Taken together, these findings are consistent with NPF responding to rewarding and threatening stimuli to set the valuation of drug reward. It is not yet known if the role of NPF in ethanol sensitivity, reward, and preference are anatomically linked.

NPF: Food and Drugs

Neural Targets of NPF

NPF is an upstream modulator of satiation-state dependent behaviors such as odorant-enhancement of larval mouth hook contractions (Wang et al., 2013a), appetitive reinforcement (Shohat-Ophir et al., 2012), retrieval of appetitive memory (Krashes et al., 2009a), innate olfactory attraction (Beshel and Zhong, 2013a), sugar sensitivity (Inagaki et al., 2014), and motivated feeding in larvae and adult flies (Hergarden et al., 2012a; Wu et al., 2005a). Interestingly, the currently known downstream targets of NPF are dopaminergic neurons. For example, in larvae, appetitive odorant-induced mouth hook contractions require NPF signaling into DL2 neurons. In adult flies, NPF disinhibits the PPL1 neurons to allow starvation-dependent memory retrieval. More recently in adult flies, NPF promotes sugar sensitivity and may be upstream of the TH-VUM neuron located in the SOG (Inagaki et al., 2014; Marella et al., 2012).

Food and Drug Similarities in Learning and Memory

The activation of NPF neurons is sufficient for appetitive conditioning, similar to the functional role of the dopaminergic PAM cluster (Shohat-Ophir et al., 2012). In the context of alcohol preference, activation of NPF neurons during ethanol conditioning impairs 24 hour appetitive memory, but an immediate aversive memory (within 30 minutes post-training) that is formed during the same conditioning is intact (Kaun et al., 2011). Thus, transient NPF activity during the paired ethanol odorant phase seems to block the late stage ethanol attraction. In contrast, dopaminergic activity in neurons (labeled by *TH-Gal4*) is necessary only during the retrieval phase (Kaun et al., 2011). These two pieces of evidence suggests that NPF and distinct dopaminergic clusters must coordinate neural activity at particular phases of learning for proper expression of alcohol-conditioned appetitive memory. Moreover, the exact neuronal substrates that may be encoding the rewarding aspects of ethanol preference is still unknown.

*NPF Encodes Hunger, Reward Status, and Innate A*ttraction

In *Drosophila*, NPF has three distinct putative functions in hunger, reward status, and innate attraction. It's unclear if the entire NPF circuitry coordinates each of the putative functions in motivational context-dependent manner or if distinct NPF neurons assign value similar to the

valence-specific dopaminergic circuitry. Moreover, the involvement of NPF with other drugs of abuse like cocaine, nicotine, and amphetamines have yet to be explored.

Drosophila Insulin-like Peptides

In *Drosophila* there are eight insulin-like peptides (DILP1-8) and one insulin receptor (dInR) (Brogiolo et al., 2001; Colombani et al., 2012). Here we limit our discussion to the direct functions of the brain-derived DILPs expressed within the median neurosecretory cells of the pars intercerebralis, DILP2, 3, 5 (Nässel et al., 2013) and their effects on feeding and drug behaviors. The central neural mechanisms and systemic neurohemal modulators that may control the local secretion of DILPs into the central nervous system are covered extensively in other reviews (see (Nässel et al., 2013)).

Drosophila Insulin in Feeding Behavior

3rd Instar Larval Behavior

Ping Shen and colleagues showed that pan-neuronal misexpression of DILP2 significantly decreases larval mouth hook contractions on both unpalatable (solid or quinine-adulterated) and palatable (liquid) food (Wu et al., 2005a, 2005b). Importantly, expression of a dominant negative dInR in NPFR neurons increases mouth hook contractions in fed larvae, whereas expression of a constitutively active form of dInR in NPFR neurons significantly attenuates mouth hook contractions in starved larvae (Wu et al., 2005a, 2005b). These findings highlight that insulin is a potent modulator of feeding that can negatively regulate neurons downstream of NPF. Interestingly, manipulation of dInR activity in NPFR neurons only affected feeding on unpalatable substrates, however overexpression of DILP2 negatively regulated mouth hook contractions on both palatable and aversive substrates, suggesting the existence of NPFR-independent pathway for insulin in palatable feeding (Wu et al., 2005b).

Adult Fly Behavior

In capillary feeding preference assays, well-fed flies exhibit an initial preference for highly palatable sugars over less palatable yet more nutritious sugars. However, over time, there is a clear shift in preference towards substrates with greater caloric content (Stafford et al., 2012a). Therefore, adult flies have a preference for caloric sugar in a starvation-dependent manner. Well-fed DILP2 or DILP3-deficient adult flies prefer to consume a less palatable but caloric mixture of sucrose and mannose (1:4 ratio) versus the sweet but non-caloric L-fucose (Stafford et al., 2012a). Because these mutants behave like starved flies, the results imply that DILP2 and DILP3 encode a state of satiety. Perhaps surprisingly, then, genetically silencing DILP3 cells does not increase the probability of proboscis extension in response to sucrose (Marella et al., 2012). Moreover, activation or inactivation of DILP2 and DILP3 cells does not affect water consumption (Pool et al., 2014a). Finally, the transient activation of DILP2 cells during appetitive memory retrieval does not block approach to a sucrose-conditioned odorant (Gruber et al., 2013). All together, these results suggest that insulin activity may influence palatable versus nutritional food preference instead of satiety state.

Insulin and Drugs of Abuse

Insulin signaling is implicated in both adult ethanol sensitivity and in the long-term physiological effects of developmental ethanol exposure. A 50% reduction in dInR expression increases ethanol sensitivity without affecting other insulin-dependent processes, including nutrient signaling and organismal growth (Corl et al., 2005). Prolonged ethanol exposure during development does regulate these processes: flies raised on food with added ethanol are smaller and slower to develop, and show significantly suppressed cellular proliferation, concomitant with

reduced expression of DILP2 and the dInR in the brain (McClure et al., 2011). The effects of developmental ethanol exposure can be reversed by overexpression of DILP2, indicating that ethanol-induced decreases in insulin signaling mediate the developmental effects of ethanol exposure.

Insulin-like Peptides: Food and Drugs

Together the evidence in larvae and adult flies supports the notion that insulin encodes a state of repletion by negatively regulating potential targets such as NPFR neurons. Note that insulin is also a critical regulator of carbohydrate levels in the hemolymph (Broughton et al., 2005), thus manipulation of DILPs may mask direct or indirect effects of neuronal substrates sensitive to nutrients (Dus et al., 2013; Miyamoto et al., 2012). Studies of the effects of insulin manipulation on drug-related behavior is limited to alcohol. As mentioned in the NPF section, increased NPF activity is correlated with increased ethanol sedation. Insulin could potentially function upstream of NPF to influence alcohol sensitivity. Since insulin decreases feeding whereas NPF increases feeding, it will be interesting to determine if a singular neural pathway underlies both behaviors.

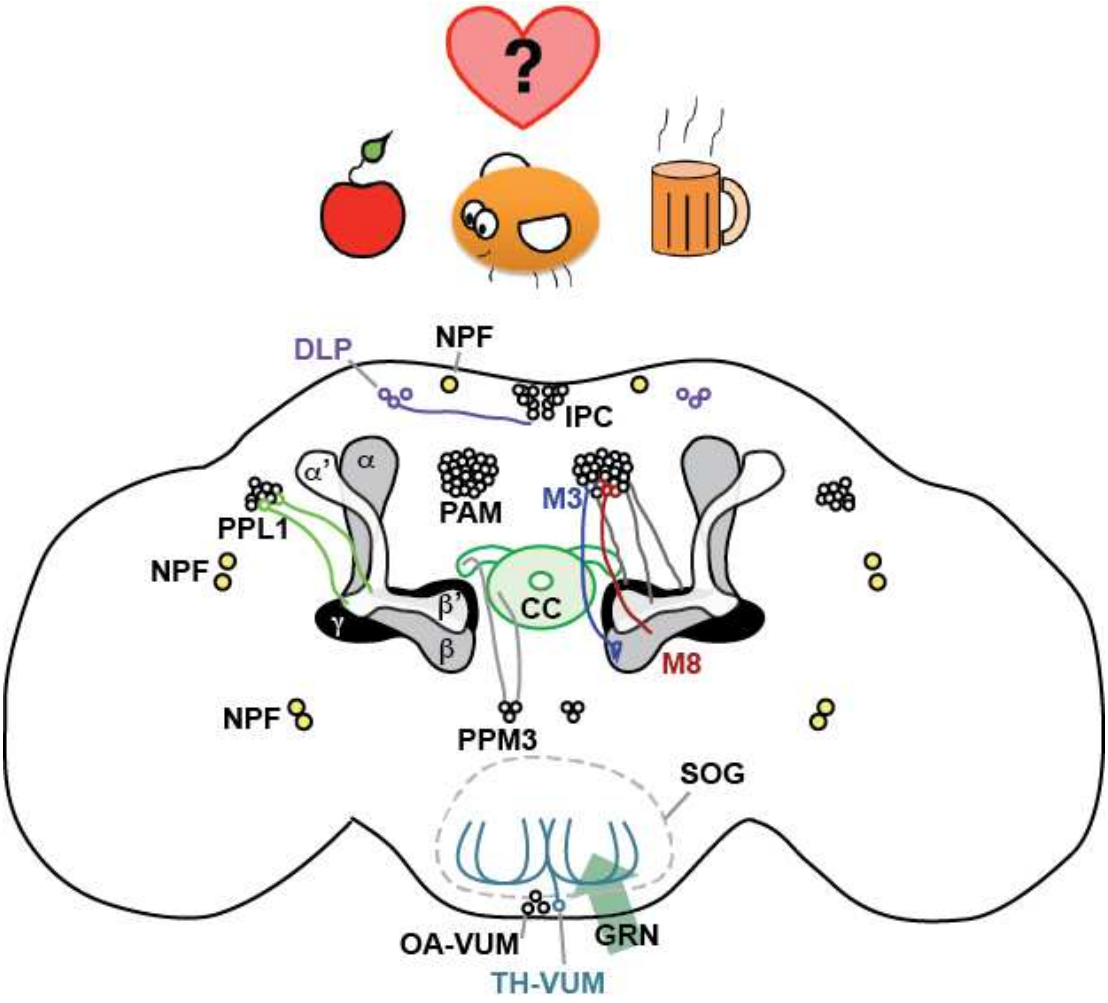
Corazonin in Feeding Behavior and Drugs of Abuse

Corazonin is a neuropeptide that is thought to be related to mammalian gonadotropin-releasing hormone. While less is known about corazonin, a couple of recent studies indicate that neuronal corazonin regulates behavior. Hergarden and colleagues showed that the activation of corazonin neurons, like NPF neurons, increases food intake in food-deprived flies (Hergarden et al., 2012a). Flies lacking neuronally-expressed corazonin or the cells expressing corazonin are resistant to ethanol sedation (McClure and Heberlein, 2013). Corazonin promotes sedation sensitivity specifically in adult flies, and activation of corazonin-expressing cells increases sedation sensitivity whereas synaptic silencing decreases it. Therefore corazonin signaling is engaged by ethanol exposure to regulate sedation sensitivity. Interestingly, the corazonin expressing cells implicated in ethanol sedation sensitivity likely project to the pars intercerebralis neuroendocrine organ that expresses the DILPs and other peptides (Kapan et al., 2012), and they also express Gr43a, a gustatory receptor that senses internal fructose levels and regulates feeding (Miyamoto and Amrein, 2014). Deletion of corazonin expressing cells or its receptor also causes a marked delay in recovery from sedation induced by pure ethanol (Sha et al., 2014). Interestingly, these manipulations of corazonin signaling also decrease the activity of acetaldehyde dehydrogenase (ALDH), an enzyme critical for ethanol metabolism. Ethanol is converted into acetaldehyde by alcohol dehydrogenase, and then into acetate by ALDH. Acetaldehyde accumulation in humans is likely the cause of many of the unpleasant and toxic effects of alcohol consumption, and this work in flies seems to tie neuroendocrine signaling to the regulation of metabolism.

Perspective

In this review we gather evidence for the behavioral actions of a limited set of neuromodulators in both feeding behaviors and drug-related behaviors. The overlap of molecules and neural substrates allows us to speculate that shared circuitry imparts shared functionality, as is similarly proposed in mammals. However, there remain important unanswered questions that preclude detailed analysis of each neuromodulator and their relationships that is critical in order to assign precise function. For most of the neuromodulators discussed single cell resolution has not yet been achieved. One exception is in the fruit fly dopamine system, where there is precedent for individual cells imparting specific functions. For example, ethanol-stimulated locomotion and the promotion of wakefulness map to specific PPM3 dopamine neurons. In another example, the tonic activity of three MB-MP1 neurons in the PPL1 cluster dictates the satiation state-dependent expression of appetitive behavior. Furthermore, because this type of comparative neuroanatomical/functional dissection of behavior is only recently possible, similar cellular resolution experiments between feeding and drug-related behaviors await future experimentation.

Fig 1 Schematic of the Drosophila adult brain



The diagram depicts the major neuropils and cell types discussed in this review, except for the mushroom body output neurons (MBON) that are excluded for purposes of clarity. All structures are bilaterally symmetric except for the ventral unpaired medial cells that are octopaminergic (OA-VUM) or dopaminergic (TH-VUM). Gustatory information is carried into the brain by gustatory receptor neurons (GRN) that terminate in the SOG. The TH-VUM makes an elaborate tree-like arborization in the SOG. The mushroom bodies are comprised of α/α′, β/β′, and γ lobes. The protocerebral anterior medial (PAM), protocerebral posterior lateral 1 (PPL1), and protocerebral posterior medial 3 (PPM3) clusters are all dopaminergic. The PAM and PPL1 neurons innervate distinct regions of the mushroom bodies and make both ipsilateral and contralateral (not shown) connections. The MBONs send dopamine/mushroom body information to protocerebral integration centers near the mushroom bodies. Individual PPM3 neurons innervate the ellipsoid body (doughnut) and fan-shaped body of the central complex (CC). The insulin-producing cells (IPC) of the pars intercerebralis neuroendocrine gland extend processes (not shown) medially to regions of the brain above the SOG and out of the brain to endocrine organs and other targets. The dorsal lateral protocerebral (DLP) cells express corazonin and extend processes to the IPC.

Table 1. Tools used to manipulate specific neurons

Type	Neuromodulator	Gal4 Driver	Cells	Functions	Reference
Biogenic Amines	Dopamine	Ddc	All DA	Appetitive Reinforcement Promotes Ethanol Preference Promotes Ethanol Reinforcement	[21, 26, 53]
		TH	All DA & 12 PAM (MB-M3)	Aversive Reinforcement Inhibits Food Intake Promotes Ethanol-Induced Locomotor Activity Promotes Ethanol Reinforcement Promotes Odorant-Induced Appetitive Behavior Promotes Sucrose Sensitivity	[14, 17, 21, 22, 26, 27, 29, 30, 34, 35, 53, 61, 71]
		0273	130 PAMs	Appetitive Reinforcement	[22, 25]
		R58E02	90 PAMs	Appetitive Reinforcement	[22, 26]
		R48B04	55 PAMs	Appetitive Reinforcement Promotes Innate Water Seeking	[22]
		0104	40 PAMs	Appetitive Reinforcement Promotes Innate Water Seeking	[22, 25, 28]
		0279	M8 PAMs	Appetitive Reinforcement	[28]
		NP5272	M3 PAMs	Aversive Reinforcement	[27, 61]
		NP1528	M3 PAMs	Aversive Reinforcement	[61]
		NP0047	MB-MP1 MB-MV1	Aversive Reinforcement	[27, 31]
		NP2758	MB-MP1	Aversive Reinforcement	[21, 30, 61]
		c061		Aversive Reinforcement	[25, 27, 30, 62]
		c259		Aversive Reinforcement	[27]
		kra		Aversive Reinforcement	[21, 30]
		5htr1b	MB-MV1	Aversive Reinforcement	[27]
		c346	PPM3	Promotes Ethanol-Induced Locomotor Activity Promotes Ethanol Preference	[14, 21]
	Octopamine	NP7088	VUMs, AL	Sucrose Sensitivity	[70]

		Tdc2	All	Appetitive Reinforcement Promotes Ethanol Attraction Promotes Odorant-Induced Appetitive Behavior Promotes Sucrose Sensitivity	[22, 25, 71, 75, 80]
Peptides	Neuropeptide F	NPF		Appetitive Reinforcement Inhibits Alcohol Preference Promotes Food Intake Promotes Odorant Approach Promtoes Odorant Attraction Promotes Odorant-Induced Appetitive Behavior Promotes Sucrose Sensitivity Promotes Willingness to Overcome Adversity	[4, 17, 30, 82, 83, 84, 86, 88]
	Insulin-like Peptide	DILP2		Food Preference Inhibits Innate Appetitive Behavior	[86, 95]
		DILP3		Food Preference	[95]
		DILP4		Inhibits Innate Appetitive Behavior	[86]
	Corazonin	crz		Promotes Food Intake	[88]

Chapter 2: Satiation state-dependent dopaminergic control of foraging in Drosophila

Summary

Hunger evokes stereotypic behaviors that favor the discovery of nutrients. The neural pathways that coordinate internal and external cues to motivate food seeking behaviors are only partly known. Drosophila that are food deprived increase locomotor activity, are more efficient in locating a discrete source of nutrition, and are willing to overcome adversity to obtain food. Here we developed a semi-naturalistic assay and show that two distinct dopaminergic neural circuits regulate food-seeking behaviors. One group, the PAM neurons, functions in food deprived flies while the other functions in well fed flies, and both promote food seeking. These satiation state-dependent circuits converge on dopamine D1 receptor-expressing Kenyon cells of the mushroom body, where neural activity promotes food seeking behavior independent of satiation state. These findings provide evidence for active food seeking in well-fed flies that is separable from hunger-driven seeking.

This work has been previously published: Dan S. Landayan, David S. Feldman, Fred W. Wolf. (2018) Satiation state-dependent dopaminergic control of foraging in Drosophila. Nature Scientific Reports. 2018 April; doi: 10.1038/s41598-018-24217-1.

Introduction

The neural mechanisms that regulate feeding motivation are ancient, fundamental for survival, and under complex regulation, and yet they remain partially defined and understood. Feeding motivation is classically divided into pre-ingestive and consummatory phases(Benoit and Tracy, 2008; Craig, 1917). In the pre-ingestive phase, nutritional deficits cause release of hormonal signals that act on the brain to bias behavioral states towards seeking food, including heightened attention to food-related environmental cues, increased locomotion, and suppression of incompatible behaviors such as sleep. Once a nutritional source is encountered, homeostatic mechanisms in concert with sensory and nutrient detectors cause a cessation of locomotion and engagement of motor programs for food intake. Both pre-ingestive and consummatory phase behaviors are motivated and goal-directed. However, the goals and the conditions for their completion are different, suggesting that the neural circuits controlling each phase are also different. Defining the neural mechanisms of feeding motivation is important in part because the dysregulation of feeding behavior is intimately tied to obesity and eating disorders, as well as to other pathological alterations of motivation, including drug addiction(DiLeone et al., 2012b; Kenny, 2011c).

Simpler organisms such as Drosophila hold promise for uncovering the neural circuit mechanisms for motivated feeding behavior. In Drosophila, feeding behavior studies have focused mostly on the consummatory phase, and have revealed satiation state-dependent effects on sensory(Jeong et al., 2013; Stafford et al., 2012b; Zhang et al., 2013b), motor(Flood et al., 2013a; Inagaki et al., 2012b; Mann et al., 2013), and central processing of feeding(Liu et al., 2017; Wang et al., 2013b; Wu et al., 2003, 2005c). Appetitive associative conditioning with feeding studies have defined detailed neural circuits implicated in reward learning(Burke et al., 2012b; Krashes et al., 2009b; Liu et al., 2012b; Placais et al., 2013). Drosophila behavioral studies of the pre-ingestive phase have focused mostly on sensory perception of appetitive stimuli, including odor tracking, satiation state-dependent olfactory acuity, and search strategies(Duistermars and Frye, 2008; Eriksson et al., 2017; Frye et al., 2003; Kim and Dickinson, 2017; Root et al., 2011). Here we report the development of a semi-naturalistic assay for innate pre-ingestive behaviors in Drosophila, in which flies search in an open arena for a discrete source of food. Semi-naturalistic assays may offer advantages over task-specific assays in defining how complex information is processed to drive behavior. We demonstrate specific roles for distinct dopaminergic neural circuits in the well-fed and food-deprived states for regulating food seeking behavior.

Results

Parametrics of Drosophila food seeking behavior

We developed a semi-naturalistic paradigm to measure various aspects of food seeking in freely behaving flies. Flies placed into a translucent arena (Fig 1A) are tracked with a video camera (Fig 1B). After a set acclimation period, a small volume of food is introduced at the center of the arena. Increasing lengths of food deprivation (wet starvation) increased the number of flies in contact with the food, the food occupancy rate (Fig 1C). Locomotor speed in the absence of food increased with increasing lengths of food deprivation time (Fig 1D). Introduction of food into the arena rapidly decreased the locomotor speed of food deprived flies in the arena. Food intake scaled with deprivation time, as measured in a separate assay that minimizes seeking time (Fig 1E). For subsequent experiments, 'food-deprived' indicates 16-20 hr of a water only diet, unless otherwise noted.

Sensory and nutritional inputs to food seeking

We tested for the role of olfaction, taste, and vision in food seeking in food-deprived flies (Fig 2A). Neither genetic nor surgical ablation of food odor-detecting neurons - olfactory coreceptor mutant *Orco*^{1} or removal of the third antennal segment - affected food seeking(Grosjean et al., 2011; Steck et al., 2012). Similarly, flies lacking sugar sensing taste receptors had no effect on food seeking for sucrose. These experiments suggested that flies may use more than one sensory modality when seeking nearby food. Flies with both ablated antennae and taste receptor mutations showed decreased food occupancy. Food seeking also remained robust in complete darkness. Taste receptor mutant flies showed reduced food occupancy in total darkness, and additionally removing olfactory input did not further reduce occupancy. These results indicate that flies use a combination of taste, olfactory, and visual cues to find and occupy a discrete food source.

Flies may seek one or more food constituents. Food deprived flies were most attracted to complete food, then sugars, and then protein (Fig 2B). When given a binary choice, flies preferred complete food over any other option, and preferred sugars over yeast (Supplementary Fig S1). Similarly, flies occupied sweet and nutritious sucrose more than either sweet-only sucralose or nutritious-only sorbitol (Supplementary Fig S1). Finally, nutrition appears to be important for switching the locomotor state of food deprived flies: flies slowed in the presence of sucrose or D-glucose, whereas they did not in the presence of sucralose or L-glucose (Fig S1D). These findings suggest that sweetness is a mechanism that captures flies on a food source, and that nutritional content of the food source is important for fully switching flies from the pre-ingestive to consummatory phase of food seeking.

A characteristic of motivated behavior is the willingness to overcome negative consequences. Flies will eat substantially less food when it is adulterated with bitter compounds, and this scales with satiation state(Wu et al., 2005c). In a binary choice competition, food deprived flies occupied quinine-containing food, but only if there was no better choice (Fig 2C). Furthermore, food intake was less suppressed with longer deprivation (Fig 2D). We used a sucrose food source for all subsequent experiments.

Role of dopaminergic neurons in food seeking

Dopaminergic neural circuits are critical for motivation, reward, and food seeking in mammals, and for many similar functions in flies(Bromberg-Martin et al., 2010b). To test the role of dopamine in food seeking in flies, we acutely inactivated and activated subsets of dopamine neurons in fed and food-deprived flies and assessed occupancy of sucrose. Dopamine neurons group into several discrete anatomical and functional clusters in the adult fly brain (Fig 3E). *TH-*

Gal4 labels most dopamine neuron clusters, but is largely absent from the PAM (protocerebral anterior medial) cluster of approximately 100 dopamine neurons. *0273-Gal4* labels most or all dopamine neurons in the PAM cluster but not other dopamine neurons. Acutely blocking transmitter release in *TH-Gal4* neurons with the temperature-sensitive dynamin Shibire (Shi^{ts}) had no effect on food occupancy in food deprived animals (Fig 3A). Food occupancy was decreased when TH-Gal4 neurons were transiently inactivated in fed animals. Conversely, inactivation of *0273-Gal4* neurons specifically decreased food occupancy in food deprived animals. *DAT-Gal80* (also named *R58E02-Gal80*) expresses the GAL4 inhibitor GAL80 exclusively in PAM neurons: *DAT-Gal80* blocked the *0273>*Shi^{ts} food occupancy phenotype (Fig 3A). Finally, chemical depletion of dopamine with 3-iodotyrosine also decreased food occupancy, indicating that dopamine is a neurotransmitter for food seeking (Supplementary Fig S2). Thus, dopamine neurons in the *TH-Gal4* pattern promote food occupancy in fed animals, and PAM dopamine neurons in the *0273-Gal4* pattern promote food occupancy in food deprived animals.

To test if dopamine neurons were permissive or instructive, we acutely activated them using the temperature-sensitive cation channel TrpA1. Consistent with an instructive role, activating *TH-Gal4* neurons in fed flies increased food occupancy (Fig 3C). Fed *0273>TrpA1* flies showed a marked decrease in food occupancy, and this was due to PAM dopaminergic activation in the *0273-Gal4* pattern. To identify the relevant neurons in the *TH-Gal4* pattern, we used transgenes that differentially label specific clusters of dopamine neurons (Fig 3F)(Liu et al., 2012b). Activation of patterns that included the PPL2ab and PAL but not the PPL1 or PPM3 dopamine neuron clusters increased food occupancy in fed flies (Fig 3C). To test if the identified dopaminergic neurons may regulate feeding motivation, we activated *TH-Gal4* neurons in mildly (4 hr) food-deprived flies. Under these conditions, activation of *TH-Gal4* neurons specifically increased consumption of quinine adulterated food (Fig 3D).

Taken together, these experiments are consistent with dual roles for dopamine in food-seeking behavior: a PAM dopamine neuron-mediated promotion of food seeking in the food-deprived state, and a *TH-Gal4* dopamine neuron-mediated promotion of food seeking in the fed state. In the fed state, PAM dopamine neurons can block food seeking.

Dopamine receptor regulation of food seeking

Dop1R1 encodes a D1-like dopamine receptor that functions in motivation-related behaviors, including arousal state, drug reward, and learning and memory. We tested flies with markedly reduced expression of Dop1R1 for food seeking behaviors. Food-deprived *Dop1R1* mutant flies were hyperactive and appeared to ignore food (Fig 4A). Moreover, Dop1R1 mutant food occupancy was reduced when fed or food deprived (Fig 4B). Loss of the dopamine D2-like receptor D2R did not affect food occupancy, but restored normal food occupancy to *Dop1R1* mutants. These data suggest that Dop1R1 promotes food seeking, and that an opposite role for D2R is uncovered in the absence of Dop1R1. Food intake was unaffected in food-deprived flies of these genotypes (Supplementary Fig S3).

The mushroom bodies promote food seeking independent of satiation state

We performed genetic rescue experiments to ask where Dop1R1 functions for food seeking in food deprived flies. To bias the rescue towards functionally relevant brain regions, we utilized *Dop1R1-Gal4* strains that expressed GAL4 under the control of short non-coding genomic DNA fragments cloned from the *Dop1R1* locus (Fig 4C). Food occupancy was partially rescued when *Dop1R1* was expressed with three different *Dop1R1-Gal4* strains in food-deprived *Dop1R1* mutants: *B07*, *B12*, and *C02* (Fig 4E). Anatomical analysis of the expression patterns for the rescuing *Dop1R1-Gal4* drivers revealed expression overlap. In the *B12* and *C02* strains, the mushroom bodies were prominently labeled, as were regions of the central complex, including the fan-shaped body and protocerebral bridge (Fig 4F,G). The *B07* strain prominently labeled the

ellipsoid body of the central complex (Fig 4H). We failed to rescue *Dop1R1* mutant food occupancy using GAL4 drivers that label the ellipsoid body, fan-shaped body, or the protocerebral bridge (not shown). By contrast, decreasing GAL4 activity with mushroom body-specific expression of GAL80 eliminated *B12* rescue of the *Dop1R1* mutant food occupancy phenotypes (Fig 4E). Moreover, restoring Dop1R1 with the mushroom body-specific driver *MB247-Gal4* rescued *Dop1R1* food seeking (Supplementary Fig S3). Thus, Dop1R1 expression in the mushroom bodies is sufficient to promote food seeking in food deprived animals.

We next tested the role of neurotransmission in Dop1R1-expressing mushroom body neurons in food seeking. Similar to loss of *Dop1R1*, acute blockade of synaptic output in *B12* neurons decreased food occupancy in both fed and food-deprived flies (Fig 4I). Importantly, this effect also localized to the mushroom bodies (Fig 4J). *B12>Shi^{ts}* flies also showed reduced locomotion, however this phenotype persisted when the mushroom body neurons were subtracted from *B12* (Supplementary Fig S3), suggesting that distinct Dop1R1 neurons control food occupancy and locomotion. Finally, acute activation of *B12* neurons in fed flies increased food occupancy (Fig 4K). Taken together, these results indicate that the activity of Dop1R1-expressing mushroom body neurons promote motivated food seeking in both the fed and food-deprived state.

Discussion

Distinct dopaminergic circuitry promotes food seeking under well fed and food deprived conditions. Dopamine neurons in the TH-C' pattern promote seeking in well fed flies, and dopamine neurons in the PAM cluster promote seeking in food deprived flies. The PAM neurons likely function directly upstream of Dop1R1-expressing neurons of the mushroom body that promote food seeking in both the fed and food-deprived states. These circuits function in food seeking under semi-naturalistic conditions, where flies can freely perform many steps of food seeking behavior. Understanding how these dopaminergic circuits contribute to discrete steps of feeding behavior, from local search through to repletion and disengagement from a food source, will help define how motivational states transition from task to task.

Roles of dopamine in appetitive behaviors

Dopaminergic neurons are critical for many appetitive and aversive behavioral responses across animal species. Dopamine may act as a salience, arousal, or attention signal that gives importance to specific valence information arriving from other circuit elements(Bromberg-Martin et al., 2010b; Kaun and Rothenfluh, 2017; Salamone and Correa, 2012b). In rodents, genetic, pharmacological, and lesioning studies indicate that striatal dopaminergic pathways can selectively function in the pre-ingestive phase to promote food seeking(Ilango et al., 2014; Palmiter, 2008; Salamone and Correa, 2012b). We found that acute activation of dopamine neurons in fed flies increased food occupancy, yet it did not cause increased food intake. Likewise, genetic elimination of the Dop1R1 receptor decreased food occupancy without affecting food intake. In contrast, inactivation of Dop1R1 receptor neurons decreased food intake in the food-deprived state, possibly reflecting their key role in integrating sensory and internal state information. These findings suggest that dopaminergic pathways promote pre-ingestive food seeking. However, the role of dopamine is more complex. For example, the PAM dopamine neurons are activated by ingestion of sugar, and their activation is greater in food-deprived flies, indicating that dopaminergic neurons are engaged during the consummatory phase of feeding, and they may be sensitized to responding to input during the pre-ingestive phase(Liu et al., 2012b).

Prior studies assigned dopamine to particular aspects of feeding behavior and also to motor functions that are critical to feeding(Eriksson et al., 2017; Liu et al., 2017). In particular, dopamine neurons in the *TH-Gal4* pattern are implicated in controlling motor output: *TH-Gal4*

neuron hyperpolarization, blocking synaptic input, interferes with motor performance and aspects of food seeking behavior in food deprived flies(Eriksson et al., 2017; Friggi-Grelin et al., 2003). We did not detect differences in unstimulated motor activity or in the magnitude of an olfactory-stimulated startle response when we blocked synaptic output from *TH-Gal4* neurons, indicating that flies exhibited grossly normal motor behavior in our assay(Kong et al., 2010b).

Which dopamine neurons are responsible for food seeking? In well-fed flies, neurons in the *TH-C'* pattern promote seeking. This pattern includes dopamine neurons in the PAL, PPM2, and PPL2 clusters, and was previously shown to promote female egg-laying preference on sucrose(Liu et al., 2012b; Yang et al., 2015). The neurons in these clusters project to many specific regions of the brain, and their individual functions remain largely unknown. The PAM neurons are also heterogeneous, sending projections that tile to well-defined regions of the mushroom body and to regions of the protocerebrum. Specific subsets of PAM neurons that are included in the *0273-Gal4* pattern have been implicated in various forms of appetitive learning and memory, however their inactivation did not impact food seeking in food deprived flies (not shown) (Aso et al., 2014b; Burke et al., 2012b; Liu et al., 2012b; Schwaerzel et al., 2003b; Yamagata et al., 2015). This suggests that there may be further segregation of PAM dopamine neuron function, possibly according to innate and learned appetitive responses.

Sensory tuning of food seeking motivation

Appetitive olfactory cues such as those emitted from palatable food elicit approach and can activate neurons important for feeding. Olfactory receptor neurons that respond to appetitive odors increase sensitivity through the actions of the neuropeptides sNPF and SIFamide (Martelli et al., 2017; Root et al., 2011). Further, neurons that release the neuropeptide NPF are activated to a greater extent in response to food odors in food-deprived flies; their activation promotes and inactivation inhibits odor attraction(Beshel and Zhong, 2013b). In well-fed larvae, the attractive odor pentyl acetate increases food intake through the actions of NPF and dopamine(Wang et al., 2013b). Therefore, food-related odors not only elicit approach behavior in a satiation state dependent manner, but also increase the activity of neurons expressing neuropeptides that regulate feeding behavior. Our results indicate that, under semi-naturalistic conditions, olfaction is important but apparently not crucial for food seeking in food-deprived flies: neither surgical nor genetic ablation of olfaction decreased food occupancy, and its role was only revealed by simultaneous partial ablation of taste responses. Further, flies were efficient in seeking odorless sucrose. Taken together, olfaction, hygrosensation, visual cues, and taste responses likely act in concert with internal cues to set the intensity of food seeking when flies are in relatively close proximity to a food source.

Methods

Strains and culturing

All strains were outcrossed for five generations to the Berlin genetic background prior to behavioral testing. Flies were raised on standard food containing agar (1.2% w/v), cornmeal (6.75% w/v), molasses (9% v/v), and yeast (1.7% w/v) at 25°C and 70% humidity unless otherwise indicated. *Dop1R1-Gal4* (*R72B03*, *R72B05*, *R72B06*, *R72B07*, *R72B09*, *R72B12*, *R72C01*, *R72C02*) strains were generated by the FlyLight project(Jenett et al., 2012), *TH-C'-Gal4* and *TH-D'-Gal4* were from Mark Wu, $Gr5a^{EP\text{-}5}$ and $Gr64^{a1}$ were from Anupama Dahanukar, *0273-Gal4* from Daryl Gohl and Thomas Clandinin, *MB-Gal80* from Hiromu Tanimoto, $Orco^{1}$ from Leslie Vosshall, and others from the Bloomington Stock Center.

Behavioral measurements

Groups of 21 males were collected 1-2 days prior to the experiment. For food deprivation flies were placed into empty culture vials containing water saturated Whatman filter paper. For 3-iodotyrosine treatment flies were cultured for 30 hr with 5% sucrose/2% yeast/10 mg/mL 3-iodotyrosine (3IY), and treated an additional 16 hr with 3IY in water for food deprivation. Standard fly food was used for all experiments except where indicated. Thin-walled Plexiglas behavioral chambers were designed with two side-by-side arenas, each arena measuring 45x75x10 mm, or 85x135x10 mm for experiments with *Shibire*^{ts}. Chambers were designed and built by IO Rodeo (Pasadena, CA). Flies were filmed from above at 10 fps with the arena placed on white light LED panel (Edmund Optics). Filmed flies were tracked with customized software(Wolf et al., 2002). For food occupancy, the number of flies off food was subtracted from the total number of flies and divided by total number of flies. Percent on food was calculated as the average of the last two measured time points. Locomotor activity was the average speed of all flies in 20 sec bins.

To measure food intake, 5 ml standard fly food with 2% erioglaucine (Sigma) with or without 3mM quinine was striped onto 1/4 of the inner surface of a wide fly vial, and condensation removed. 30-50 flies were introduced and the vial laid on its side so that the food edge was at the apex. After 30 min, the flies were homogenized in a volume adjusted to the number of flies and consumption was determined spectrophotometrically.

Statistical measurements were made with Prism 6.0 (GraphPad). Error bars are the SEM. Data is available upon request.

Immunohistochemistry

Adult fly brains were fixed and immunostained as described previously(Kong et al., 2010b). Antibodies were rabbit anti-GFP (1:1000, Life Technologies), rabbit anti-Dop1R1 1:1250 (Kong et al., 2010b), and nc82 (1:25, Developmental Studies Hybridoma Bank, Iowa).

Figure 1

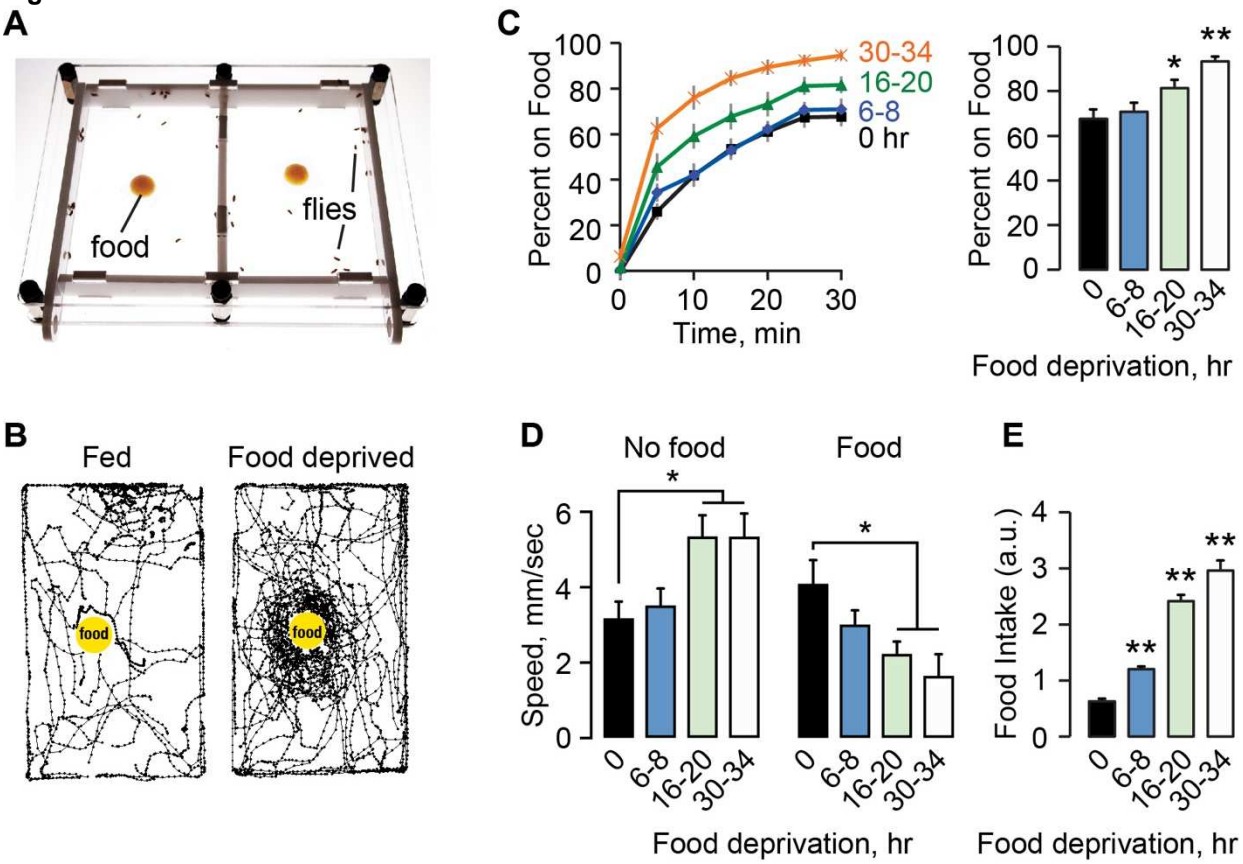


Figure 1. Food deprivation effect on food seeking behavior. **A**. Two-sided chamber for food seeking assays. Flies and 200 ul of cornmeal molasses food on Parafilm placed in each chamber via sliding side doors. The chamber is lit from below. Fly locomotion is recorded from above. **B**. 10 sec locomotor traces of 20 flies each filmed soon after addition of food (yellow dot). **C**. Left: The percent of flies on food over time for a food deprivation time course. Right, food occupancy averaged at 25-30 min. $P<0.0001$, ANOVA/Bonferroni comparison to 0 hr. n=17-18 groups. **D**. Locomotor speed. Left, speed at 20 min of acclimation, without food. Right, speed averaged over 0-10 min after food introduction. $P=0.0091$ no food, $P=0.0066$ food, ANOVA/Bonferroni compared to 0 hr. n=9-15 groups. **E**. Intake with increasing food deprivation time. $P<0.0001$, ANOVA/Bonferroni comparison to 0 hr. n=9 groups. *$P<0.05$, **$P<0.01$.

Figure 2

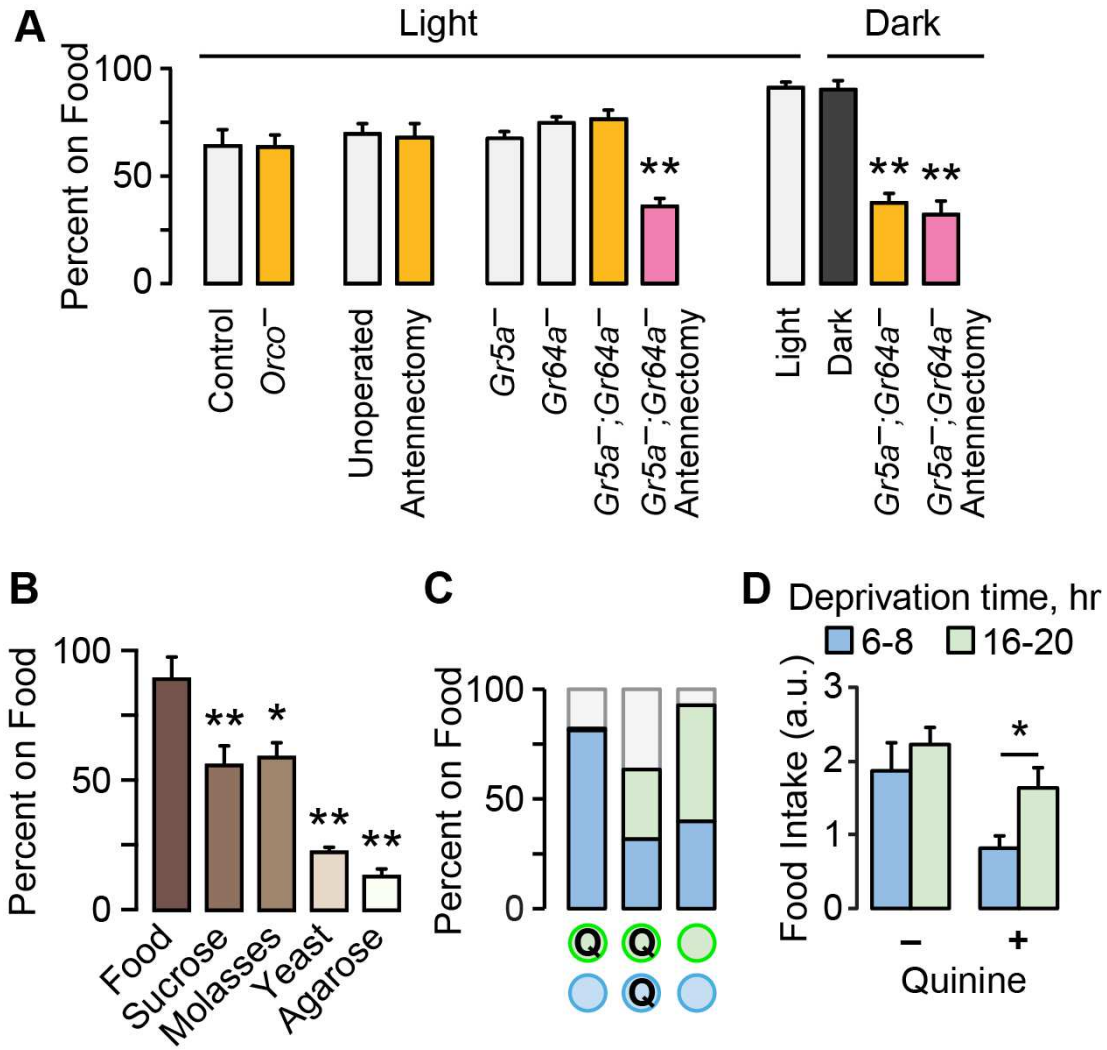


Figure 2. Environmental and sensory information in food seeking. **A**. Food occupancy following sensory ablations in 16-20 hr food deprived flies. Antennectomy is surgical removal of the third antennal segment. *Orco*⁻ flies lack the Orco olfactory coreceptor; *Gr5a*⁻ and *Gr64a*⁻ are taste receptor mutants. P<0.0001 for both Light and Dark, ANOVA/Bonferroni compared to control, n=8-12 groups. **B**. Occupancy of 16-20 hr food deprived flies to agarose with the indicated food component. P<0.0001, ANOVA/Bonferroni comparison to Food. n=4-5 groups. **C**. Two-choice tests with unadulterated (open circles) and 10 mM quinine food (Q). n=5 groups. **D**. Flies consumed greater quantities of quinine food (3 mM) when food-deprived for 16-20 hr (long) versus 6-8 hr (short). P=0.0251, Mann Whitney test, n=12. *P<0.05, **P<0.01.

Figure 3

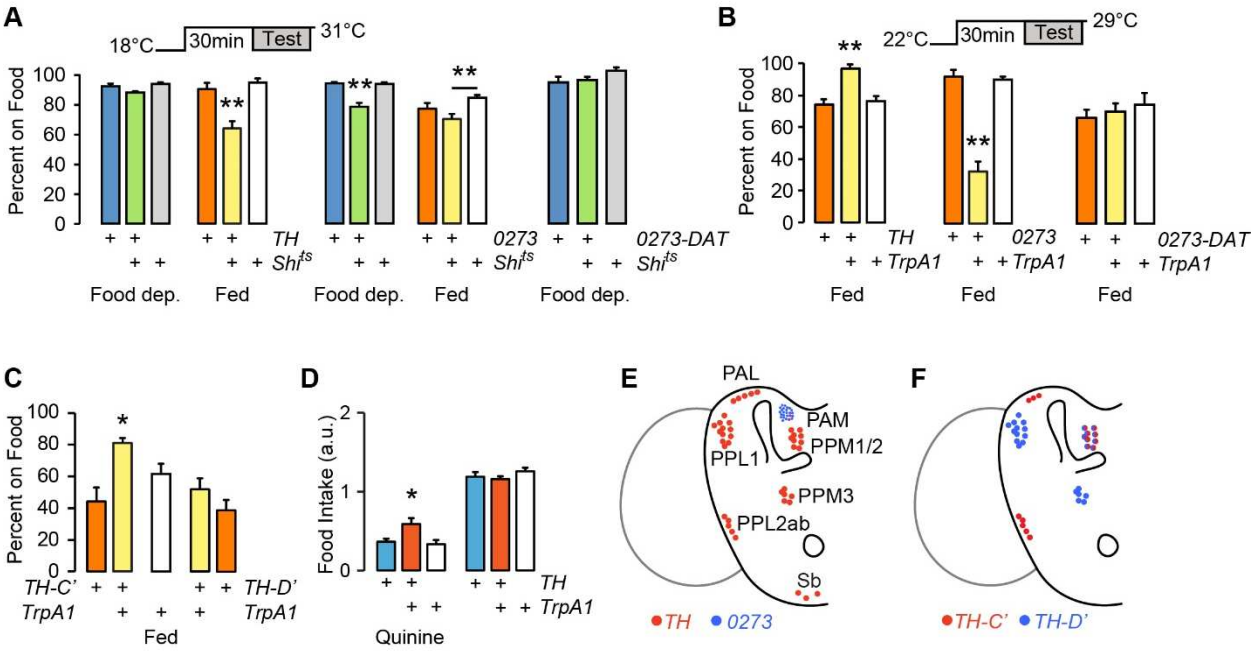
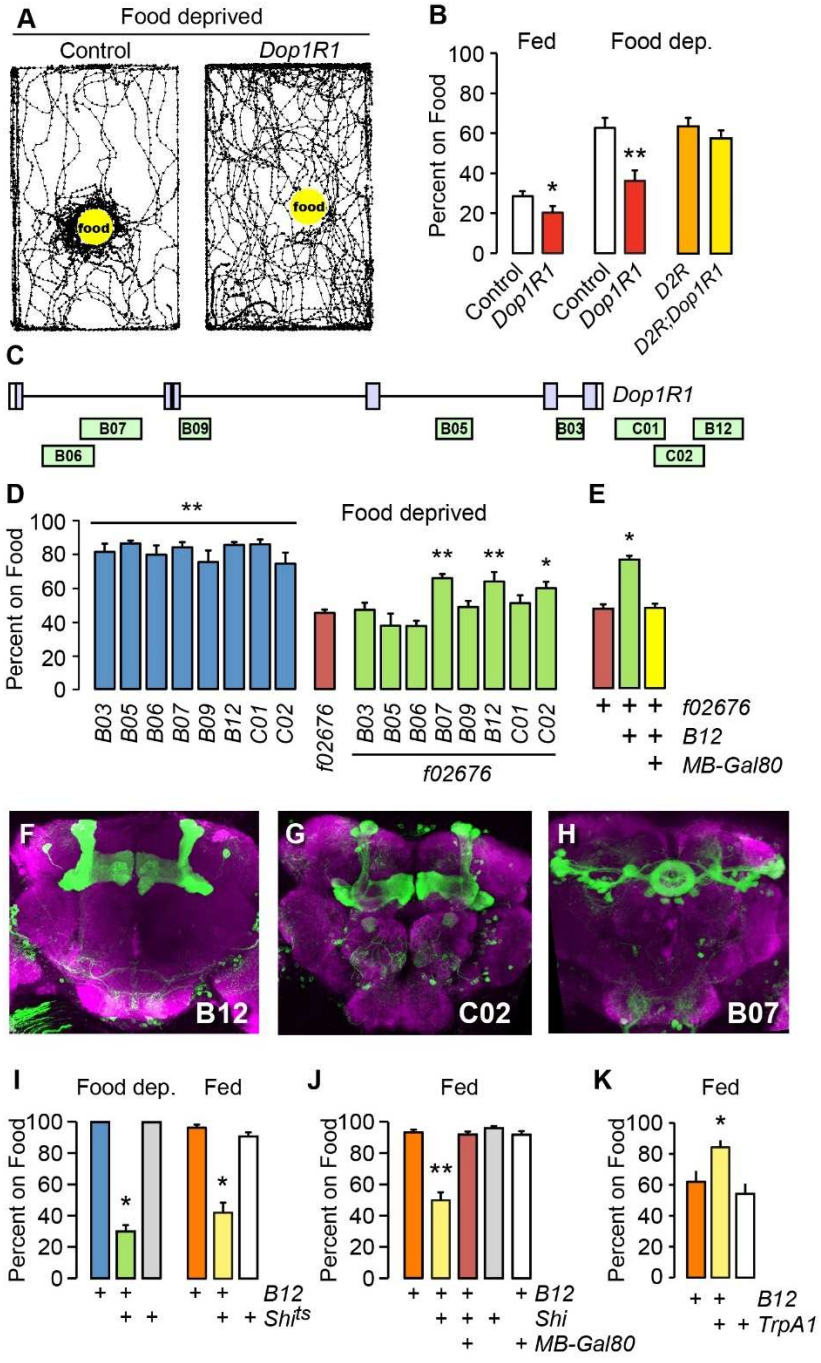


Figure 3. Satiation state-dependent effects of dopamine neuron activity on food seeking. **A**. Acute inactivation of dopamine neurons with Shibire^{ts} (*Shi^{ts}*), food occupancy in fed and 16-20 hr food-deprived flies. P=0.0012 ANOVA/Tukey's, n=8-11 groups with *TH-Gal4*. P=0.0001 Kruskal-Wallis/Dunn's, n=8-10 groups food deprived; P=0.0139 ANOVA/Tukey's, n=8-9 groups fed, with *0273-Gal4*. *0273-DAT* : *0273-Gal4* with *R58E02-Gal80* to specifically block Gal4 activity in the PAM cluster dopamine neurons. n=6 groups. **B**. Acute activation of dopamine neurons in fed flies, food occupancy. P=0.0002, ANOVA/Tukey's, n=8-11 groups with *TH-Gal4*. P=0.0002, Kruskal-Wallis/Dunn's, n=8 groups with *0273-Gal4*. *0273-DAT*: n=8 groups. **C**. Acute activation of subsets of *TH-Gal4* neurons, food occupancy in fed flies. P=0.0002, ANOVA/Tukey's, n=8-11 groups. **D**. Food intake in 4-6 hr food-deprived flies. P=0.0053, ANOVA/Tukey's, n=15-19 groups. **E**. Dopamine neuron clusters in the adult brain that express *TH-Gal4* and *0273-Gal4*. **F**. Dopamine neurons that express *TH-C'-Gal4* and *TH-D'-Gal4*. *P<0.05, **P<0.01.

Figure 4


A
Food deprived
Control
Dop1R1
food
B
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Percent on Food
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B07
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Figure 4. Dopamine receptor-expressing neurons in the mushroom body control food seeking. **A**. Locomotor traces of food-deprived flies 5 min after addition of food. *Dop1R1* mutant *f02676* vs. Berlin genetic background control. **B**. Food occupancy for the indicated genotypes that were fed or food deprived. t-test P=0.0492 fed (n=16-20 groups), P=0.001 food deprived (n=16-20 groups). *D2R*: the loss-of-function mutation *f06521*. **C**. Location of *Dop1R1* enhancer fragments. **D**. Genetic rescue of *Dop1R1* mutant food occupancy in 16-20 hr food deprived animals. *Dop1R1-Gal4* strains (blue) were made heterozygous in *f02676* homozygotes (rescuing configuration, green). P<0.0001 ANOVA/Bonferroni's comparison to *f02676*, n=8-16 groups. **E**. Inclusion of *MB-Gal80*, preventing GAL4 activity in the mushroom bodies blocks *B12* rescue. P<0.0001 ANOVA/Tukey's, n=10-19 groups. **F**-**H**. Expression pattern of *Dop1R1-Gal4* strains (CD8-GFP, green), and bruchpilot (magenta) to show the synaptic neuropil. **I**. Acute silencing of *B12 Dop1R1-Gal4* neurons with Shi^{ts}, food occupancy, food deprived and fed. Food deprived: P<0.0001 Kruskal-Wallis/Dunn's, n=4 groups. Fed: P=0.0002 Kruskal-Wallis/Dunn's, n=7-8 groups. **J**. Addition of *MB-Gal80* in *B12 Dop1R1-Gal4>*Shi^{ts} fed flies, food occupancy. P<0.0001 Kruskal-Wallis/Dunn's, n=6-10 groups. **K**. Activation of *B12 Dop1R1-Gal4* neurons in fed flies increased food occupancy. P=0.0054, ANOVA/Tukey's, n=7-9 groups. *P<0.05, **P<0.01.

Supplemental Figure 1

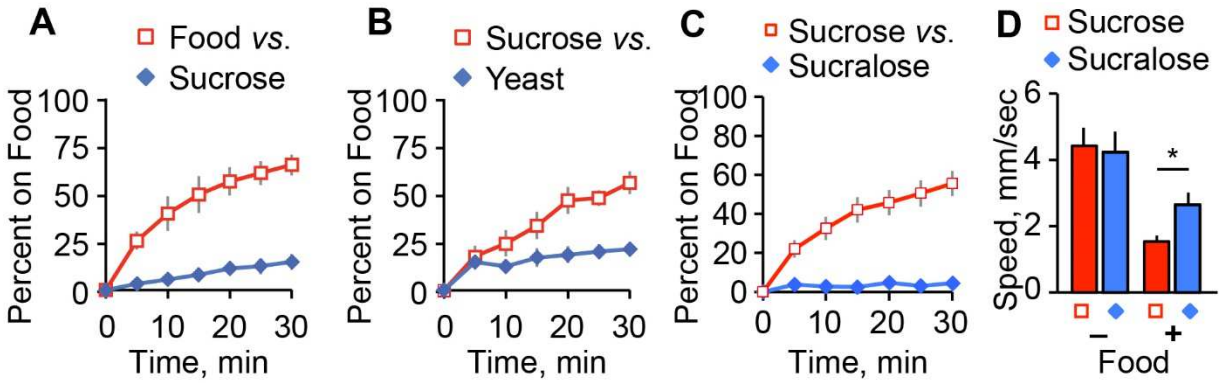


Figure S1, related to Figure 2. Food occupancy with binary choice. Percent of flies occupying **A**. food vs. sucrose, **B**. sucrose vs. yeast, and **C**. sucrose vs. sucralose. **D**. Locomotor speed of flies before and after addition of the indicated food source. t-test, $P<0.05$. n=6-10 groups.

Supplemental Figure 2

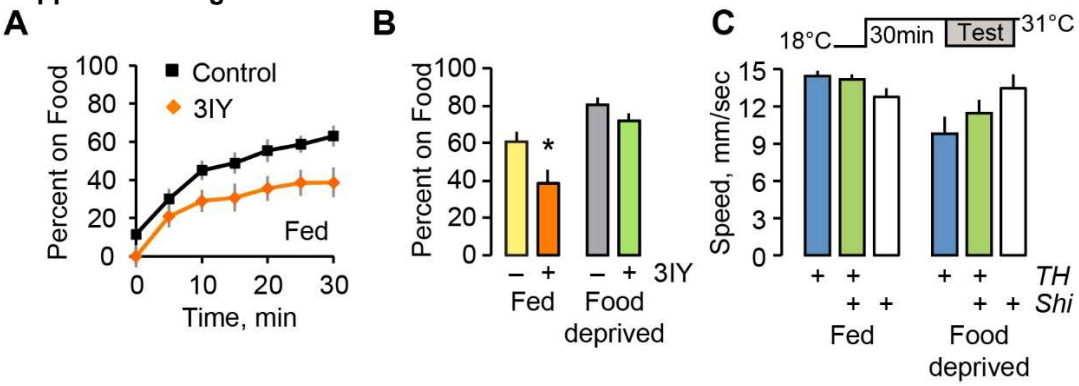


Figure S2, related to Figure 3. Food occupancy after overnight treatment with 1.25 mg/mL 3-iodotyrosine. **A**. time course. **B**. percent occupancy. T-test, $P<0.05$. n=8 groups. **C**. Locomotor speed with acute inactivation of *TH-Gal4* neurons. n=6-10 groups.

Supplemental Figure 3

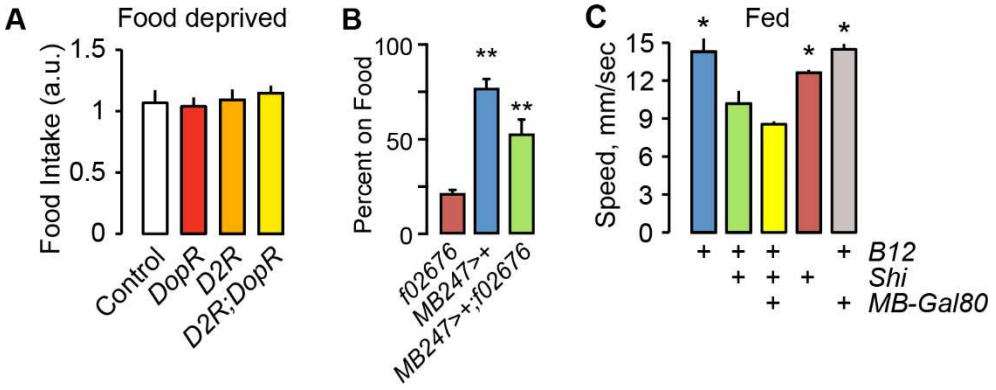


Figure S3, related to Figure 4. Dopamine receptor neuron manipulation. **A**. Food intake for the indicated genotypes. n=10 groups. **B**. Food occupancy for genetic rescue restricted to the mushroom bodies. P<0001, One-way ANOVA/Tukey's. n=12 groups. **C**. Locomotor speed in fed flies of the indicated genotypes. *P<0.0001 One-way ANOVA/Bonferroni compared to *B12-Gal4>UAS-Shi^{ts}*. n=8-12 groups.

Chapter 3: Janu Neurons Promote Naïve Water Seeking

Summary

Thirst is a fundamental internal state encoded by central brain neural substrates that detect water imbalances. Higher order thirst circuits that coordinate goal-directed water seeking while simultaneously repressing other competing internal states, like hunger, have not been identified. Through an unbiased neural circuit screen, we have identified a set of six, non-overlapping, GABA and AstA neurons that are both necessary and sufficient for contact-dependent water seeking in thirsty animals. The AstA subset projects dorsomedially and converges onto hunger-promoting NPF neurons. Our model suggests that Allatostatin A neurons have the capacity to bidirectionally promote water seeking and suppress food seeking through a novel AstA to NPF circuit pathway.

This work will be submitted to eLIFE.

Introduction

Thirst is an internal state that is critical for survival and is common amongst diverse animal phyla. Animals with an extremely large surface area to volume ratio, such as Drosophila melanogaster, are especially prone to water loss and have evolved robust neural circuits to rapidly detect internal salt and water imbalances to drive the appropriate goal-directed behavior.

In flies, dehydration engages osmolarity-sensing neurons (called ISNs) which are located in the subesophageal ganglion zone (SEZ) and can promote reciprocal consumption of food or water through drosophila insulin-like peptide (dILP) release. ISNs are regulated by adipokinetic hormone (AKH) signaling and changes in hemolymph concentration, respectively (Jourjine et al., 2016a). Similarly, the ion transport peptide (ITP) bidirectionally promotes thirst and hunger, but also controls water balance, through insulin-producing neurosecretory neurons in the central brain or the periphery (Gáliková et al., 2018).

Thirsty flies can rapidly detect olfactory humidity gradients in the environment to locate and drink water (Ji and Zhu, 2015). Humidity detection is mediated by two populations of humid (Ir68a positive) and dry-sensing (Ir40a positive) hygrosensory neurons that are housed within the sacculus, an invaginated structure located on the 3rd antennal segment (Knecht et al.). Water taste is facilitated by ppk28 positive gustatory neurons located on the labellum (Cameron et al., 2010) and is critical for water consumption in thirsty flies (Lau et al., 2017a). Interestingly, water consumption also requires expression of DopR1, but not water seeking; a subset of PAM dopaminergic neurons have been shown to be necessary for naïve water seeking in thirsty animals (Lin et al., 2014a).

In an unbiased screen, we identified a single Allatostatin A (AstA) neuron that is both sufficient and necessary for contact-dependent water seeking. We found that AstA signaling can bidirectionally promote water seeking and suppress dry sucrose seeking in a state-independent manner.

Results

Water deprivation drives water seeking and consumption

Thirsty flies are motivated to find a source of water and to drink until they reach repletion (**Figure 1A**). We developed a simple open field assay where we introduce a discrete source of water to a group of acclimated flies (**Figure 1B**). Flies can perform all steps of thirst, from seeking to repletion with a source of accessible water, or they can be limited to seeking only by placing a mesh grid over the water source. The longer flies are water deprived (on dry sucrose), the more quickly they find and occupy the water source (**Figure 1C,D**). Water deprivation also increases locomotor speed, albeit modestly until 12 hr of deprivation (**Figure 1E**). Thirst-driven seeking is water-specific: given a choice between water and dry sucrose, water deprived flies (thirsty but not hungry) will choose water and wet starved flies (hungry but not thirsty) will choose dry sucrose (**Figure 1F**). We next determined the sensory modalities that guide water deprived flies to water. Blocking access to the water source with a fine mesh grid had no effect on seeking, whereas removal of the third antennal segment that harbors hygrosensory neurons in the sacculus (antennectomy), with or without also removing the maxillary palps, reduced seeking (**Figure 1G**). Combining antennectomy with the mesh grid completely blocked seeking. Water deprivation also increased water intake; removing the third antennal segment hygrosensors had no effect, whereas blocking access with the mesh grid blocked intake (**Figure 1H**). Thirsty flies thus use a combination of environmental cues to locate and drink water, and we can separate hygrotaxis up a humidity gradient from other means of motivated water seeking.

Neuronal activation screen identifies water seeking neurons.

To identify neurons that promote seeking behavior, we activated neurons in 154 independent Gal4-driven patterns in well fed, water replete flies and tested seeking to standard fly food (containing agar, cornmeal, molasses, and yeast). We found that activation of specific patterns increased attraction or aversion, compared to the cognate *Gal4* driver alone (**Figure 2A**). One pattern, *Durstig-Gal4*, when activated resulted in markedly higher attraction to food. *Durstig-Gal4* is an *InSITE-Gal4* enhancer trap inserted into the first intron of CG4502 on chromosome 2 (Gohl et al., 2011). We next asked what *Durstig>TrpA1* flies were seeking. Well fed, water replete *Durstig>TrpA1* flies avidly occupied standard fly food, sucrose or yeast alone in 1% agar, 1% agar alone, and water alone, but they were not attracted to empty vessels (a Parafilm square or a 0.5 mL microcentrifuge tube cap) or to dry sucrose (**Figure 2B**). Hungry, water replete (16-20 hr food deprived on water) *Durstig>TrpA1* flies actively avoided dry sucrose, working against their internal state. Acute silencing of *Durstig* neurons in well-fed, water replete flies with temperature sensitive *Shibire* (*Shi*) also increased water seeking (**Figure 2B**). Thus, neurons in the *Durstig-Gal4* pattern promote water seeking in the water replete state and suppress food seeking in the food deprived state.

We next tested the sensory modalities used by *Durstig>TrpA1* flies to find and occupy water in the water replete state. Neither removing the antennae nor making the water source inaccessible blocked *Durstig>TrpA1* water seeking (**Figure 2C**). Combining antennal ablation with the mesh grid completely ablated seeking. Thus, *Durstig>TrpA1* flies use both hygrosensory and contact sensory information to locate water, and they use hygrosensory information to move up a humidity gradient. We also activated thirst sensory neurons, including the internal osmosensor ISNs and the hygrosensory neurons that express the receptors Ir40a and Ir68a, and observed no increase in seeking (**Figure 2D**) (Enjin et al., 2016; Jourjine et al., 2016b; Knecht et al., 2016). We conclude that *Durstig* driven water seeking is not due to direct activation of thirst sensory neurons.

Dopaminergic neurons modulate attraction to water vapor, and support water consumption and reward learning in water deprived flies (Lau et al., 2017b; Lin et al., 2014b;

Shyu et al., 2017). Activation of broad and largely non-overlapping groups of dopamine neurons in water replete flies did not affect or decreased water seeking (**Figure 2E**). We also activated neurons in the *R48B04-Gal4* pattern that contains dopamine neurons that are required for innate humidity preference in desiccated flies, and we found that water seeking increased in the water replete state (**Figure 2E**). However, *R48B04-Gal80* that expresses the GAL4 suppressor GAL80 in these same *R48B04* neurons was unable to repress the increased water seeking of *Durstig>TrpA1* flies, indicating that *Durstig* and *R48B04* label distinct thirst neurons (**Figure 2E**). Moreover, *R48B04* dopamine neurons are completely contained within the *R58E02* pattern and they also substantially overlap with the *0104* pattern in the protocerebral anterior medial (PAM) dopamine neurons, indicating that non-dopaminergic neurons in *R48B04* promote water seeking in our assay (Huetteroth et al., 2015; Lin et al., 2014b).
We asked if neurons previously implicated in the control of food intake affect water seeking in the water replete state. Of neurons implicated in feeding, two *Gal4* neuronal activation patterns increased water seeking: *NP883* and *R65D05* (**Figure 2A,F**). *NP883* contains a bilateral pair of SEZ interneurons, the Fdg neurons, that promote proboscis extension and ingestion of liquid sucrose (Flood et al., 2013b; Pool et al., 2014b). *R65D05* contains allatostatin A (AstA) peptidergic neurons whose activation inhibits food intake (Chen et al., 2016a; Hentze et al., 2015a; Hergarden et al., 2012b). These data indicate that water seeking and feeding may have shared and distinct circuit mechanisms. The Durstig water seeking circuitry may be represented in the *NP883* and *R65D05* patterns.

Durstig-Gal4 driven GFP revealed that many neurons are labeled in the central brain and in the ventral nervous system (**Figure S3A supplemental**). We used genetic intersectional techniques to isolate smaller groups of neurons in the Durstig pattern that promote water seeking. GAL80 driven by a neurotransmitter enhancers revealed Durstig neurons that promote seeking to accessible and to inaccessible water (**Figure 3A,B**). Cholinergic *Cha-Gal80* blocked *Durstig>TrpA1* seeking to both accessible and inaccessible sources, whereas glutamatergic *VGlut-Gal80* blocked seeking to inaccessible water only. This suggests that neurons shared between *Durstig-Gal4* and *VGlut-Gal80* promote hygrotactic water seeking. The same *VGlut* enhancer fragment driving *Gal4* is expressed in motor neurons; acute activation of *VGlut-Gal4* neurons causes severe locomotor incoordination (not shown). Therefore, we tested activation of neurons labeled by six shorter *VGlut* enhancer fragment-*Gal4* transgenes (**Figure S3B supplemental**). Of these, *R52A01>TrpA1* readily promoted water seeking to both accessible and inaccessible water, without causing locomotor incoordination (**Figure 3C,D**). Further, we used the same *R52A01* enhancer to express the LexA transcriptional activator. Like *VGlut-Gal80*, *R52A01-LexA>LexAOP-Gal80* blocked *Durstig>TrpA1* hygrotactic water seeking (**Figure 3D**). Therefore, *R52A01* contains water seeking neurons that are shared with *Durstig*. We performed a similar experiment with the AstA enhancer fragment *R65D05*, and we found that it too blocked *Durstig>TrpA1* water seeking, albeit partially (**Figure 3D**). Comparing publicly available expression patterns revealed that *R65D05* labeled fewer neurons than *R52A01*, and so we characterized it's behavioral functions more thoroughly. First, we asked if neurons in the central brain or the ventral nervous system promoted seeking, using *Otd-nls:FLPo*, *UAS>stop>TrpA1* to limit TrpA1 expression to the central brain (Asahina et al., 2014). Activation of *R65D05* central brain neurons was sufficient to drive hygrotactic water seeking (**Figure S3D**). Moreover, silencing *R65D05* neurons with either tetanus toxin light chain or acutely with Shibire^{ts} blocked hygrotactic water seeking in thirsty flies. R65D05 silencing also increased dry sucrose seeking in hungry flies, similar to previous findings (**Figure S3E-G**) (Hergarden et al., 2012b). Thus, *R52A01* and *R65D05* share neurons with *Durstig* that promote water seeking, and neurons in *R65D05* are both required and sufficient for hygrotactic water seeking.

We used the *split-Gal4* (*spGal4*) technique to ask if *R65D05* and *R52A01* harbor the same thirst neurons (Luan et al., 2006; Pfeiffer et al., 2010). We generated *Janu-spGal4* (the Estonian for thirst) by combining *R65D05-Gal4-DBD* (*Gal4* DNA binding domain) and *R52A01-Gal4-AD* (*Gal4* activation domain). *Janu>TrpA1* activation in water replete flies increased water

seeking to accessible water, but had no effect when the water source was inaccessible (**Figure 4A**). *Janu* inactivation in thirsty animals decreased seeking, specifically to inaccessible water (**Figure 4B,C**). Water intake was/was not affected (**Figure 4D,E**). Dry sucrose seeking was unaffected (**Figure 4F,G**). Thus, Janu neurons that are common between *R65D05* and *R52A01* are critical for water seeking, and they play a more specific role. Additional, as yet unidentified thirst neurons must exist in each of the progenitor *enhancer-Gal4* strains.

Janu>myristoylated-GFP immunohistochemistry revealed eight bilaterally symmetric neurons (**Figure 4H,I**). The morphology of individual Janu neurons was determined using the multicolor flip-out stochastic labeling technique (**Figure S4**). The four central brain Janu neurons all innervated the SEZ, that harbors neurons important for regulating feeding and water intake. In the SEZ, Janu neurons elaborated both presynaptic and postsynaptic arborizations, whereas Janu neurons innervating the dorsomedial region of the central brain elaborated presynaptic endings exclusively (**Figure 4J**). Ventral nervous system neurons included two ascending neurons that elaborated presynaptic endings in the SEZ, and two interneurons local to the mesoneuromere and the metaneuromere.

Discussion

AstA is a pleotropic modulator of behavior

AstA is an anorexigenic neuropeptide, known to suppress sugar consumption upon neuronal activation in traditional consumption-based assays such as dye based feeding, CAFÉ, and PER (Chen et al., 2016b; Hentze et al., 2015b; Hergarden et al., 2012a). AstA positive cells can be found in the central nervous system and the midgut (Hergarden et al., 2012a). AstA is also an important regulator of energy homeostasis through regulation of AKH in the corpus cardiacum and insulin producing cells in the pars intercerebrealis (Hentze et al., 2015b). Our unexpected results suggest that manipulation of AstA neuronal activity is both necessary and sufficient for naïve water seeking. Importantly, specific manipulation of only AstA neurons in the central brain is both sufficient and necessary for thirsty water seeking and direct activation of adipokinetic hormone and insulin-like peptide 2 and 3 does not promote naïve water seeking. Together, these results rule out potential water-promoting neuroendocrine pathways downstream of AstA.

What are the most likely secondary effector neurons post-synaptic to the AstA-MUP? Based on the synaptic termini localizing in the superior medial protocerebrum, the dopaminergic PAM neurons are a likely candidate (Aso et al., 2014c). In our screen of previously characterized appetitive neurons, we identified that R48B04 activation is sufficient to promote water seeking and is consistent with its original report that activity in the PAM- β'2 is critical for thirsty water seeking (Lin et al., 2014a). A neighboring subset of PAM- γ3 neurons express the cognate AstA receptor, DAR-1. AstA and has been implicated in the facilitation of reward learning by inhibition of a subset of PAM dopamine neurons called the PAM-γ3 (Yamagata et al., 2016). In future experiments, it would be interesting to address potential circuit connectivity between AstA and dopaminergic contributions to thirsty water seeking and learned behavior.
One sleep study showed that AstA neurons in the posterior lateral protocerebrum both promotes sleep and suppresses feeding (Chen et al., 2016b). Another sleep study revealed that a distinct set of AstA-expressing neurons in the dorsal fan-shaped body can also promote sleep through suppression of ellipsoid body helicon cells (Donlea et al., 2018). Taken together, these collective reports suggest that AstA contributes to pleiotropic roles involved in metabolic regulation, satiety, reward learning, and sleep.

Mammalian Homolog of AstA: Galanin

The mammalian homolog of AstA, galanin, is highly conserved across species and has been implicated in parental behavior (Kohl et al., 2018), anxiety (Möller et al., 1999), feeding, alcohol intake(Millón et al., 2019), and thirst. Interestingly, galanin is co-expressed with vasopressin in the hypothalamus and immunoreactivity for galanin is significantly reduced following dehydration and salt-loading in rats, suggesting that thirst may increase galanin signaling (Skofitsch et al., 1989). Galanin has also been reported to suppress vasopressin release and inhibit angiotensin II-sensitive neurons in the subfornical organ (SFO), a circumventricular organ known to facilitate water seeking(Kai et al., 2006; Kondo et al., 1991). Future mammalian studies will be necessary to deconstruct the vast amount of undefined neuromodulatory input onto the SFO (Oldfield and McKinley, 2015).

Competing Homeostatic Drive

In our unbiased screen, we discovered that activation of AstA neurons in the Durstig, R65D05, and Janu drivers are sufficient to promote water seeking. Activation of the R65D05 and Janu neurons in hungry, but water replete conditions with dry sucrose presentation does not suppress hunger. Conversely, inactivation of AstA neurons suppresses thirst-evoked water seeking. Remarkably, we found that inactivation of the same set of AstA neurons can also enhance hunger-driven dry sucrose seeking. The bidirectional drive of these neurons to promote

water seeking and suppress food seeking are similar to the previously characterized interoceptive sensory neurons (ISN) (Jourjine et al., 2016a). In our open field assay, direct manipulation of ISNs did not promote behavioral approach or avoidance, suggesting that these neurons may function in a highly context dependent manner.

Conclusion

Taken together, the Janu AstA-MUP and local GABAergic in the SEZ is both sufficient and necessary to promote naïve water seeking and can modulate competing internal states such as hunger-induced dry sucrose seeking. Our finding highlights that neuronal modulation of hunger and thirst are innately intermingled and provides an entry point to understand how competing homeostatic drives for food and water interact. It is crucial for future studies to examine homeostatic thirst and hunger in parallel to gain mechanistic insight into how specificity for a goal-directed behavior is achieved.

Figure 1

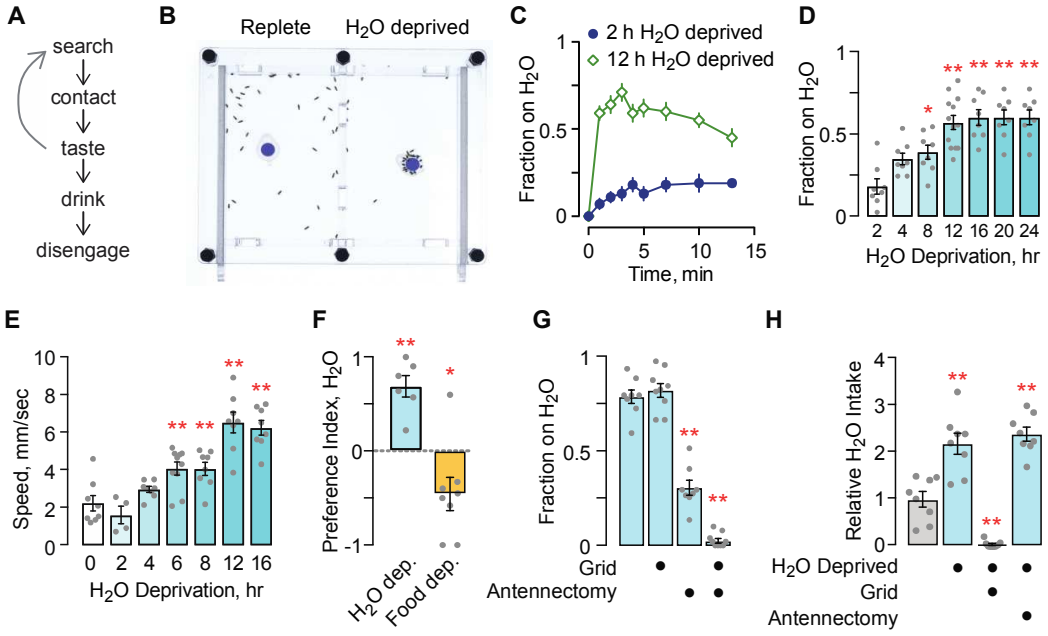


Figure 1. Thirst behaviors in Drosophila. **A**. Simplified thirst-induced behavioral sequence. **B**. Two-chambered open field assay for assessing water seeking behavior. Flies deprived of water on dry sucrose avidly seek and occupy a discrete water source (blue circle). **C**. Occupancy of an open water source over time by a group of 20 flies, n=8 groups. **D**. Occupancy increases with increasing water deprivation. One-way ANOVA/Dunnett's compared to 2 hr. Each dot is n=1, which represents a group of about 20 flies in this and all subsequent graphs. **E**. Water deprivation increases locomotor activity. **F**. Two choice preference for water (1) and dry sucrose (-1) depends on internal state. One-sample t-test, compared to 0 (no preference). n=8. **G**. Role of sensory input. A mesh grid atop the water source preserves humidity sensing and blocks water contact and ingestion. Antennectomy preserves contact and ingestion and blocks humidity sensing. One-way ANOVA/Tukey's. n=8-10. **H**. Role of sensory input in water ingestion. One-way ANOVA/Dunnett's compared to water replete (grey bar). n=8-9. *P<0.05, **P<0.01.

Figure 2

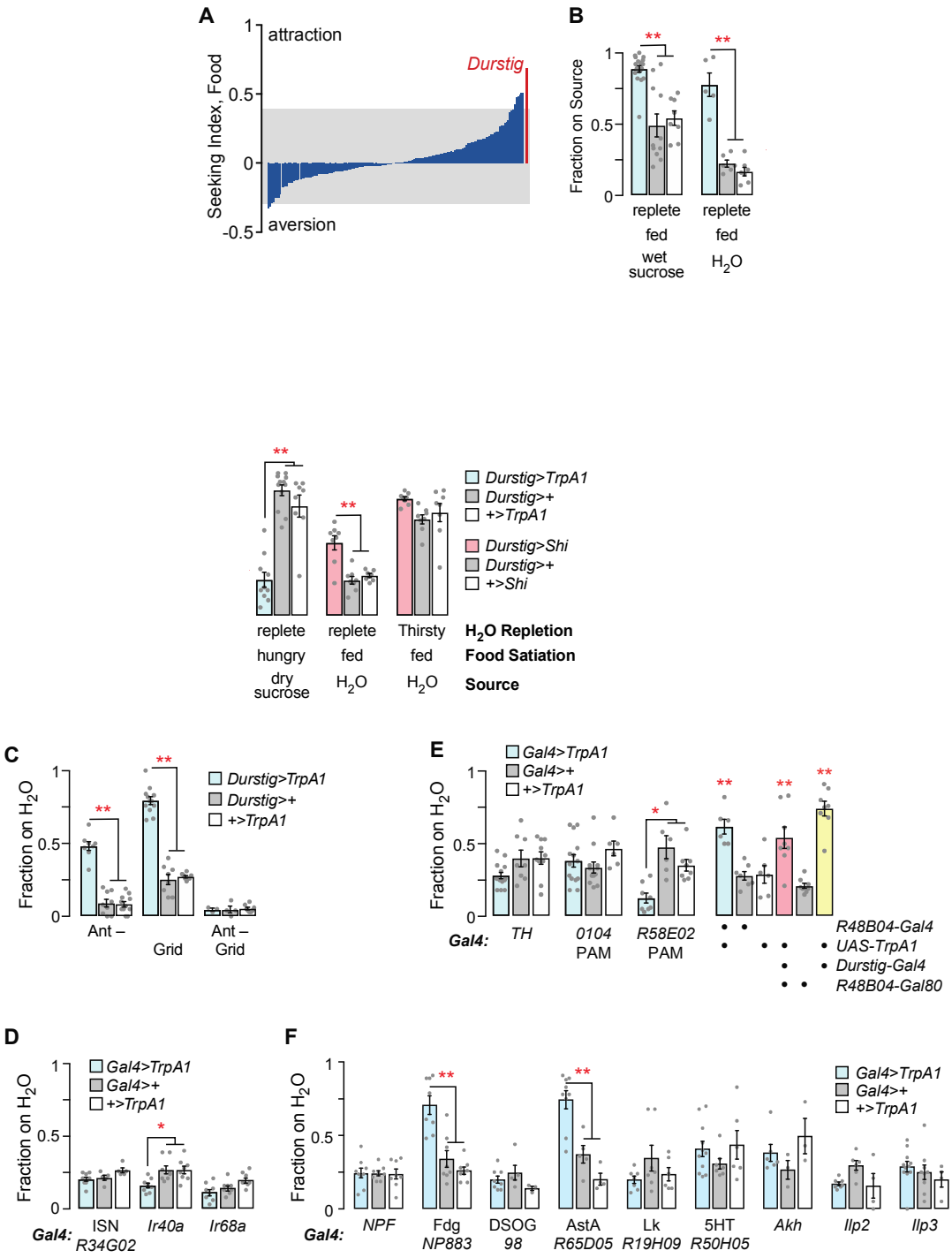


Figure 2. Neurons for appetitive behaviors. **A**. Neuronal activation screen for occupancy of standard fly food. Seeking Index is *Gal4>TrpA1* minus *Gal4>+* at 29°C. Positive index indicates greater occupancy in the open field assay. Grey area is 2 standard deviations from the mean. *Durstig-Gal4>UAS-TrpA1* most strongly increased food occupancy. **B**. Durstig neurons drive

thirst and suppress hunger. Water repletion and food satiation states were varied and the flies then given a source of sucrose or water. For each group of experimental and two controls: one-way ANOVA/Tukey's. **C**. Durstig activation promotes occupancy through hygrotaxis and contact-dependent mechanisms. One-way ANOVA/Tukey's. One-way ANOVA/Tukey's. **D**. Activation of osmosensory ISN and hygrosensory neurons did not promote water occupancy to an open source. One-way ANOVA/Tukey's. **E**. Activation of groups of dopamine neurons in water occupancy of an open source. One-way ANOVA/Tukey's. For *R48B04*, one-way ANOVA/Dunnett's compared to *+>TrpA1*. **F**. Activation of neurons implicated in the regulation of ingestion, occupancy of an open water source.

Figure 3

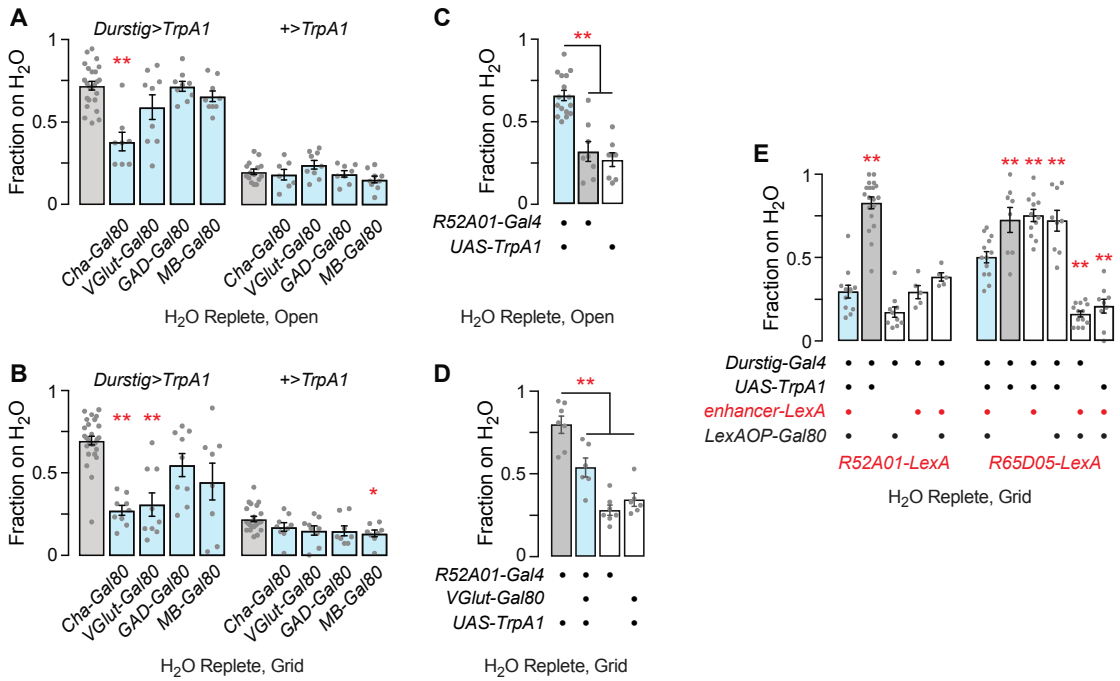
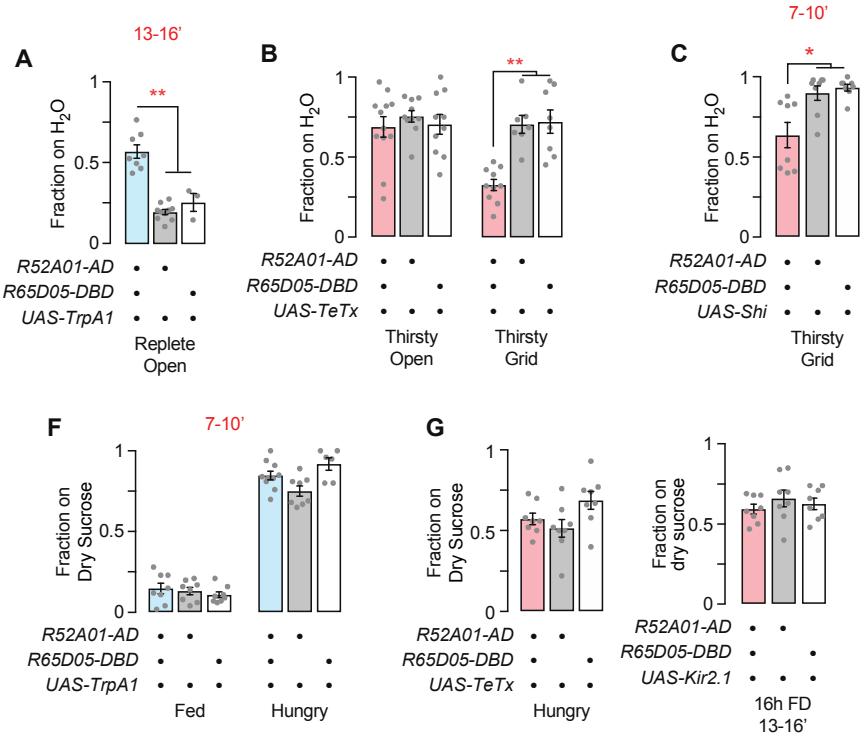


Figure 3. Neurons in the Durstig pattern that promote hygrotactic water seeking. **A**. Subtraction of subsets of neurons from Durstig>TrpA1 with patterns of Gal80 expression, occupancy of open water source. MB-Gal80 is MB247-Gal80 that expresses in the mushroom bodies. **B**. Same genotypes, occupancy of an inaccessible water source. Kruskal-Wallis/Dunn's compared to *Durstig>TrpA1* or *+>TrpA1*. **C**. VGlut locus enhancer-Gal4 *R52A01* activation promotes occupancy of an open water source. One-way ANOVA/Tukey's. **D**. *R52A01* activation promotes occupancy of a gridded water source; *VGlut-Gal80* partially blocks. One-way ANOVA/Dunnett's compared to *R52A01>TrpA1*. **E**. *Gal80* in the *R52A01* or the *R65D05* pattern blocks

Durstig>TrpA1 occupancy of a gridded water source. One-way ANOVA/Dunnett's compared to *Durstig>TrpA1,enhancer-LexA>Gal80*.

Figure 4



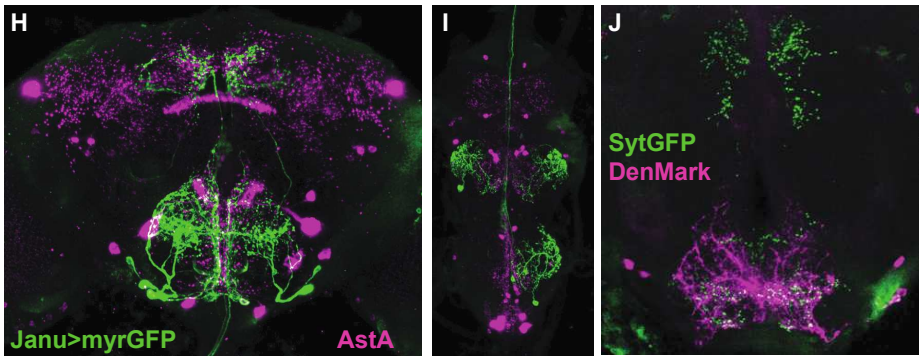


Figure 4. Janu neurons promote hygrotactic water seeking. **A**. *Janu* (*R65D05-DBD∩R52A01-AD*) spGal4 neuron activation promotes occupancy of an open water source. One-way ANOVA/Tukey's. **B**. *Janu* inactivation decreases hygrotactic water seeking. One-way ANOVA/Tukey's. **C**. *Janu* acute inactivation decreases hygrotactic water seeking. One-way ANOVA/Tukey's. **D,E**. Effects on water intake. **F**,**G**. Janu neuronal activity does not impact dry sucrose occupancy. **H**. Janu epression pattern in the adult brain. **I**. Janu expression pattern in the adult ventral nervous system. **J**. Janu neuron polarity by the presynaptic localized synaptotagmin-GFP (*UAS-sytGFP*, green) and dendrite localized *UAS-DenMark* (magenta). The SEZ is labeled by both sytGFP and DenMark, and the dorsal medial protocerebrum is labeled with sytGFP.

Figure S3

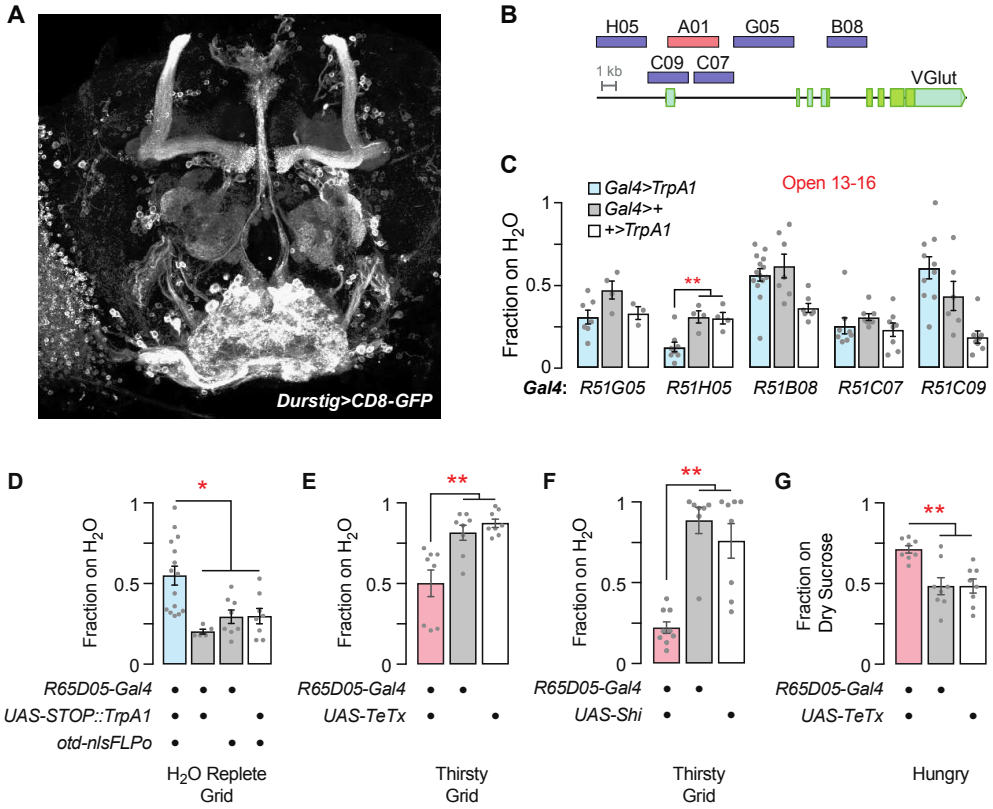


Figure S3. Characterization of *Durstig*, *VGlut* locus enhancers and *R65D05*. **A**. *Durstig* expression pattern in the central brain, maximum intensity projection. **B**. VGlut locus with location of enhancer fragments indicated. **C**. Effect on occupancy of an open water source of activation of *VGlut enhancer-Gal4* patterns. ANOVA/Tukey's for each group of experimental and controls. **D**. Selective activation of *R65D05* central brain neurons promotes occupancy of a gridded water source. ANOVA/Tukey's. **E**. Silencing of *R65D05* neurons with tetanus toxin light chain decreases occupancy of a gridded water source. ANOVA/Tukey's. **F**. Acute silencing of *R65D05* neurons with Shibire^{ts} decreases occupancy of a gridded water source. ANOVA/Tukey's. **G**. Silencing of *R65D05* neurons promotes dry sucrose occupancy. ANOVA/Tukey's.

Figure S4

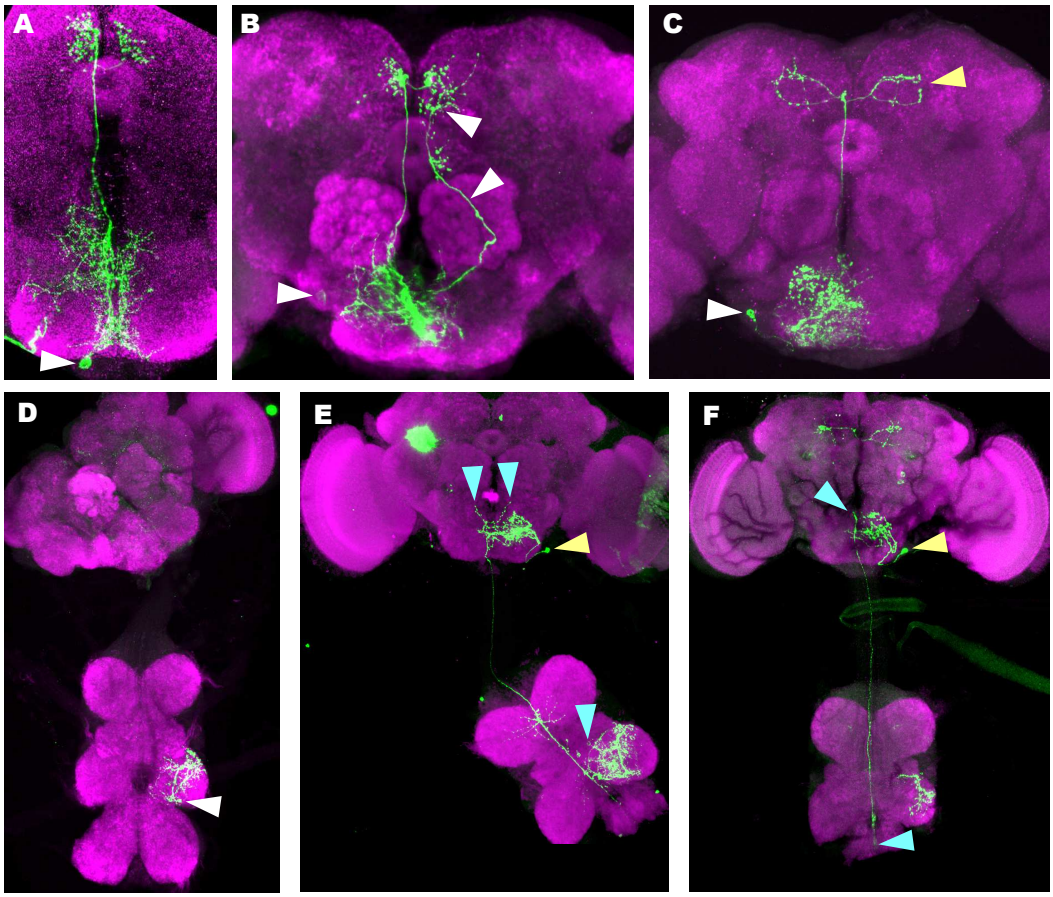


Figure S4. Stochastic labeling of Janu neurons using the multicolor flipout technique, detected with anti-FLAG (green) and counterstained with anti-VGAT (magenta). **A**. Janu-AstA, arrowhead points to cell body. **B**. Contralateral projecting neuron. Arrowhead on left points to cell body, and other arrowheads point to the axon and presynaptic endings. The neuron elaborates dendrites in the SEZ, partially hidden by co-labeled Janu-AstA neuron. **C**. Janu-GABA2 neuron (white arrowhead points to cell body), and the axon of a second neuron that elaborates presynaptic endings in the dorsal protocerebrum (yellow arrowhead). We were unable to locate the cell body for the latter neuron. **D**. Local interneuron in the mesoneuromere, arrowhead points to the cell body. **E**. Ascending interneuron with cell body and bifurcated presynaptic endings indicated by blue arrowheads. A Janu-GABA1 interneuron is also labeled (yellow arrowhead points to cell body). **F**. Ascending interneuron indicated with blue arrowheads, with a Janu-GABA1 neuron colabeled (yellow arrowhead points to cell body).

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