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Layered Reward Signalling Through Octopamine and Dopamine in Drosophila: A Dissertation

Christopher J. Burke
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LAYERED REWARD SIGNALLING THROUGH OCTOPAMINE AND DOPAMINE IN *DROSOPHILA*

A Dissertation Presented By

Christopher J. Burke

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Friday, The Tenth of May, 2013

Program in Neuroscience

LAYERED REWARD SIGNALLING THROUGH OCTOPAMINE AND DOPAMINE IN *DROSOPHILA*

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The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

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The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

_____

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Program in Neuroscience
May 10th, 2013

Dedicatory

To Dominique, my wife, who stood by me, supported everything I do, and put up with more talk about science, flies and learning than any rational person should.

Acknowledgements

It's been often said that science doesn't occur in a vacuum. It's also been said that you get by with a little help from your friends. I believe this to be especially true for me in the completion of my Ph.D., as it truly took a village (or department) to raise a student.

First and foremost among those who made me the scientist I am today is my mentor Scott. From the beginning he's been a patient sensei, constant scientific advisor, my personal lack of athleticism shame-inducer, UK music resource, snowboarding pow pow seeking co-conspirator, and good friend. He has always made himself available for whatever help or advice I might need, whether from in is office with scotch on hand, or at 1:30 in the morning over email. At the same time, I've greatly appreciated that he's given me the freedom to explore what topics of learning and memory have interested me the most, with great success. Though I believe his greatest skill and contribution in my education both in and out of the lab lies in his ability to push me when I needed it. There have been countless times throughout my Ph.D. where I felt hesitation or doubt surrounding a particular experiment, or may have dismissed a theory because I felt I might not be able to prove it experimentally. One of Scott's lasting words of wisdom I'll take from my years in lab was that "the only way you'll really know is to try it." This was as true in lab as in several of our outings in search of the best powder

to snowboard in, often taking me down some horrific-looking backcountry slope because it looked cool and no one had ridden it yet. The man is utterly fearless, a trait that inspires and which has I hope has rubbed off on me somewhat over time. I believe it's made all the difference in my development as a scientist over the past 5 years as well as a person. Thanks man.

I would like to thank all members, past, present, UMassian and Oxfordite, of the Waddell lab for making my Ph.D. experience the friendliest, most supportive, collaborative and fun it could ever have been. Even now that I'm several thousand miles away from them, I can still feel their presence.

Wolf, the finest insect neuroanatomist around, eminent social butterfly wherever he goes and my Franconian brother from another mother. Wolf gets immense credit for putting up with all my German-related jokes, making up various Germanic sounding phrases to exclaim loudly around lab such as "FLIESENFRÜGGEN!," or just emphatically yelling "NEIN!" more so than was ever necessary. But it's not only his patience and constant optimism that makes Wolf such an excellent lab mate and friend, it's his willingness to go above and beyond to help anyone at anytime. Well before he officially became co-author on our paper, Wolf spent countless hours if not days researching the project, thinking of experiments and performing painfully detailed anatomical analysis to complement my behavioral studies just because he "thought it would be cool." It

is that optimism and wonder which make him such a successful scientist and good friend both in and outside the lab.

Manu, master of djembe, gecko of rock climbing and in a lot of ways, the French yang to Wolf's German yin. Manu was always able to put experiments in perspective and provide counter argument to over exuberance when needed. Though his scientific rigor and skeptical eye keep him focused, he still found the time to help me with whatever I needed from kicking ideas around to testing a mountain of flies that gave us nothing but negative data. He also may have spent more time in the rooms at 31 degrees than is considered healthy for humans and we're all a bit worried about him now.

Gaurav "60-cent" Das. A man who brings more ideas, theories and wild scientific speculation than anyone I've met. While some of his proposals involved more complex detail than my brain could handle at the time, he's also found some really creative ways to get at the questions underlying feeding behavior and memory. He also brings a great deal of laughter and entertainment to the lab, two things I feel are critical to fostering an environment in which to thrive and feel a part of.

Suewei and David, our two newest post docs (well as of a year ago or so), continue our tradition of excellence, are on track to put us all to shame with their work, and have fit in pretty much from day one. A quick shout out to Ruth, who, as everyone had mentioned for years prior to my finally meeting her, is in fact the nicest person ever to grace the scientific world.

To members of the days of Waddell past: Jena, you looked out for me in the early years and as bench mate, and attempted to keep me organized as best as possible. Sorry everything is still a bit of a mess. Ben, who has probably forgotten more science literature than I will ever know, and is now mastering the field of patent law, I thank you for our joint therapy sessions in the fly room on science, marriage and vigorous discussions on how and why music was intrinsically better in the early 90's. Yan, you gave me an example of dedication that I hope to match someday and I that hope all is well. Shamik, you taught me to never toss a fly stock as someday I would need it again. This has proven true too many times to count, as has other lab advice you've bequeathed to me over the years. Also, sorry the moustache creeped you out, I haven't grown it back since. And finally Mike, the man who taught me fly behavior and somehow got me hooked on it. The first taste was free, and now I'm dependent on the behavior rooms like a junkie. I think I still feel a bit separation anxiety if I'm out of there for more than 3 days at a time thanks to you. Thanks and best of luck at the NIH, you'll be great.

I have to give special acknowledgement to my brethren of Waddell Lab West, she who stayed behind, the other neurobiology orphan, Paola. First, I apologize for seemingly hiding in the behavior rooms for 6 months following the lab moving to Oxford. It was great to have someone else there in lab for support, to help lament bad news together and celebrate the good when it came along. She also

gets points for keeping on me to write this thesis asking each Monday morning "So how many pages did you write this weekend." Thanks Pao and I'll miss ya.

Even though the lab relocated to Oxford in the fall of 2011, by no means was I alone on the floor. The neurobiology department really came together to make sure that I still felt a part of everything, taking me under their collective wing to offer scientific advice, career counseling, and even just being there to grab a beer now and then. There are really too many of you to single out everyone, but I'd like to specifically acknowledge the members of the Freeman and Alkema labs. You guys brought me in to both your groups and really helped me stay connected with the floor. And, importantly checked in to make sure I hadn't "expired" in the behavior rooms unbeknownst to everyone. Thanks and I hope wherever I go next the department could be half as cool as ours is.

I'd like to thank the members of my thesis committee, Marc Freeman, Mark Alkema, Eric Baehrecke, and Mike Francis who have guided me scientifically, given me life advice and been there to chat whenever I needed. A special thanks goes to Ed Kravitz as my external examiner for being willing to read this rapidly expanding dissertation.

I must give a quick thanks to Tara, Colleen, Gail and Kristin for putting up with all my bizarre funding allocation requests, travel plan mishaps and for helping me along in the department as well.

I'd like to thank my friends and family for their love and support during my time in grad school. I'm thankful for my many relatives being rather accepting of the fact that I spent my days training fruit flies. You were always inquisitive and helped me put my work into perspective. My friends both in and out of grad school, you were there to help push me along, commiserate on the trials of grad coursework (typically via poker and beer) and later of lab work, and help me get through it all. I'd like to thank my parents, both Burke and Pradella side for being there to help out a poor grad student and support me. I probably never said it, but it was always good to hear that someone is proud of you for what you've done.

Finally, I thank my wife Dominique, for her constant love, support and great deal of patience during the past 5 years. She has motivated and inspired me in just about everything I do, from music (impress chicks) to brewing and fermentation science (impress cool chicks) to my career in lab. She has the uncanny gift of always making me realize what's important in life and for that and so much more, I am eternally grateful.

Thank you.

Abstract

Evaluating our environment by deciding what is beneficial or harmful, pleasant or punishing is a part of our daily lives. Seeking pleasure and avoiding pain is a common trait all mobile organisms exhibit and understanding how rewarding stimuli are represented in the brain remains a major goal of neuroscience. Studying reward learning in the fruit fly, *Drosophila melanogaster* has enabled us to better understand the complex neural circuit mechanisms involved in reward processing in the brain. By conditioning flies with sugars of differing nutritional properties, we determined that flies trained with sweet but non-nutritive sugars formed robust short-term memory (STM), but not long-term memory (LTM). However, flies conditioned with a sweet and nutritious sugar or a sweet non-nutritious sugar supplemented with a tasteless nutritious compound, formed robust 24 hour LTM. These findings led us to propose a model of parallel reinforcement pathways for appetitive olfactory conditioning in the fly, in which both sweet taste and nutrient value contribute to appetitive long-term memory. We followed this line of research by examining the neural circuitry in the fly brain that represents these parallel reward pathways. We found that the biogenic amine octopamine (OA) only represents the reinforcing effects of sweet taste. Stimulation of OA neurons could replace sugar in olfactory conditioning to form appetitive STM. Surprisingly, implanting memory with OA was dependent on dopamine (DA) signaling, which although being long associated with reward in

mammals, was previously linked with punishment in flies. We found that OA-reinforced memory functions through the α-adrenergic OAMB receptor in a novel subset of rewarding DA neurons that innervate the mushroom body (MB). The rewarding population of DA neurons is required for sweet and nutrient reinforced memory suggesting they may integrate both signals to drive appetitive LTM formation. In addition, OA implanted memory requires concurrent modulation of negatively reinforcing DA neurons through the β-adrenergic OCTβ2R receptor. These data provide a new layered reward model in *Drosophila* in which OA modulates distinct populations of both positive and negative coding DA neurons. Therefore, the reinforcement system in flies is more similar to that of mammals than previously thought.

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List of Abbreviations

5-HT	5 Hydroxytryptophan (Serotonin)
AKH	Adipokinetic Hormone
CS	Conditioned Stimulus
CPP	Conditioned Place Preference
CTA	Conditioned Taste Aversion
CXM	Cycloheximide
DA	Dopamine
dNPF	*Drosophila* Neuropeptide F
DPM	Dorsal Paired Medial
fMRI	Functional Magnetic Resonance Imaging
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
GR	Gustatory Receptor
GRN	Gustatory Receptor Neuron
GRASP	GFP Reconstitution Across Synaptic Partners
LTM	Long Term Memory
MB	Mushroom Body
MB-MP1	Mushroom Body-Medial lobe and Peduncle 1
MCH	4-Methylcyclohexanol
MTM	Middle Term Memory

NAc	Nucleus Accumbens
NPY	Neuropeptide Y
OA	Octopamine
OAMB	Octopamine Receptor in Mushroom Bodies
OCT	3-Octanol
PAM	Protocerebral Anterior Medial
PER	Proboscis Extension Reflex
PPL1	Paired Posterior Lateral 1
SOG	Sub Esophageal Ganglion
STM	Short Term Memory
TβH	Tyramine Beta Hydroxylase
TDC	Tyrosine Decarboxylase
TH	Tyrosine Hydroxylase
TRP	Transient Receptor Potential
US	Unconditioned Stimulus
VPM	Ventral Paired Medial
VTA	Ventral Tegmental Area
VUM	Ventral Unpaired Medial

Copyright Page

The chapters of this dissertation have appeared in separate publications or as part of publications:

Burke C. J., Waddell, S. (2011). Remembering nutrient quality of sugars in *Drosophila*. *Current Biology* 21: 746-750.

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Preface to Chapter I

…and in flies, you say?

In the later years of working on a Ph.D., one gains a certain perspective that comes only with time in the lab. The most fruitful example of this comes in describing one's work to anyone outside of science. A bright and sprightly first or second year student might proudly reply to an inquisitive mind asking about their work (big breath): "my Ph.D. is on looking at novel roles for the biogenic amine octopamine functioning though a subset of mushroom body horizontal lobe-innervating dopamine neurons via an α-adrenergic as well as β-adrenergic-like receptor in driving both short term and long term appetitive memory in *Drosophila melanogaster*, which, in case you were wondering is an excellent model organism to study this because……" After years of skeptical at best, disdainful at worst looks from my family members on holidays after describing what it was I did in lab, I found the gift of brevity to be a true ally in conversation:

Relative 1:	So what is it that you do in school?
Me:	I work on learning and memory
Relative 2:	Wow, like remembering and Alzheimer's and stuff? 'Cause last week I totally forgot where my keys were. Are you working to help me with that?
Me:	Sure.

However, while not mentioning our little friend the fruit fly as the subject of our work lends itself to a much smoother conversation, it also does the *Drosophila*

model an injustice. Throughout my thesis work and seen in numerous prior studies, every time we've challenged the fly with a new task to investigate just how complex its relatively small brain might be, every time we pushed what we thought the fly could learn or recall, it has stood up to the task and delivered with flying colors. Such a small brain is in fact, quite capable of complex memory tasks (Chittka & Niven, 2009), recalling the relative value of different reward signals (Burke & Waddell, 2011), punishment (Yin et al., 2009), and even is able to detect internal motivational states to gate expression of memory (Krashes et al., 2009).

While primate and rodent models have long been used in the analysis of behavior, disease and the brain, the fruit fly *Drosophila melanogaster* is an excellent organism for the study of the genes and neural circuits underlying the complex processes of learning and memory. Their cost effectiveness of maintenance, high fecundity, and ease of genomic manipulation has long made the fly an excellent model to investigate the genetics of development, immunology and neuroscience. But the great advantage the fly has over other models is the wide arsenal of ever expanding genetic tools we have at our disposal to manipulate specific neural populations in the intact fly brain. We have the power to selectively turn neurons of interest "on" or "off" with a pulse of light or burst of heat. We can knockdown a gene in a single neuron to test what role it might play in learning and memory, then return that gene to restore its normal

function. We can even image neural responses to external stimuli in an intact living fly brain, to create a functional road map of neuronal connections.

For all these reasons and many more, I've found working with fruit flies, in contrast to many people's reactions of "ew," quite a pleasurable experience. The fly has enabled me to explore questions underlying reward learning, how circuits are arranged in the brain and how an organism learns and remembers, endeavors difficult to investigate with such ease in any other system. For all that I believe I owe *Drosophila* and those who walked the path of the fly before me a debt of gratitude.

Thanks, and I'll remember to turns the lights off in the behavior rooms when I'm done.

Christopher J. Burke

May 10, 2013

Chapter I: An Introduction to Learning and Memory in the Fly

At their most basic level, our memories are what make us who we are, direct our behavior, and allow us to plan for the future based on past experience. We exist in a present that is in a constant flux of becoming our past and as the future lies before us, we move forward with our remembered experience to help guide us.

In the past it has been believed that while our physical selves and bodily actions rested in the physical world, our thoughts, emotions and memories resided in a more ethereal plane, inaccessible to us. Only in recent years have we begun to explore the corporeal nature of "the mind" and how it fits into our current understanding of the human brain.

But what *are* memories? How does a change in synaptic strength result in recalling the exact smell of a particular day at summer camp as a child? Is there really a physical representation in your brain for the words to your favorite song? While our progress in understanding the human brain has advanced by leaps and bounds, we are still left puzzled by some of the most basic questions of learning and memory. As we sit on the precipice of a new era of neuroscience research, soon to be characterized by the potential merits of the new BRAIN Initiative, it's tempting to wonder whether we'll ever fully understand our own brains, or if there's an aspect to us that really does lie outside our physical world.

Olfactory Learning and Memory in Drosophila

The concept that one could study learning and memory in a fruit fly is not a novel one, and the use of *Drosophila melanogaster* as a genetic model dates back over a century. However, with the rise of molecular biology and molecular genetics throughout the 1950's and 60's, it was debated just how much of ourselves our genes control. For while it had been known for years that particular genes encode information for certain physical traits such as hair and eye color and even predisposition for certain diseases, whether our genes directed our *behavior* such as forming memories was a much more tenuous concept, and laden with controversy.

Uncovering what genes might play a role in the processes of learning and memory seemed as much a daunting task as any endeavored in biology, yet there were some who weren't dissuaded by the vastness of the topic nor the critics in the field. From the hypothesis that genetics could be used to unravel the mystery of learning, scientists in the lab of Seymour Benzer began investigating new models for learning.

It was the seminal work of Chip Quinn, William Harris and Seymour Benzer which established the fruit fly, *Drosophila melanogaster* as a model organism in which to explore learning and memory. Employing what essentially amounted to operant conditioning, they were capable of training a fly to associate a neutral

odor (conditioned stimulus, CS) with the delivery of punishing electric shock (unconditioned stimulus, US) (Quinn et al., 1974). The flies formed an aversive memory to the shock-associated odor that was able to persist up to one day, and could be extinguished by repeated CS presentation in the absence of US. Furthermore, this learning was found to be a probabilistic phenomenon, as roughly only 30% of the trained flies learned to avoid the shock-paired odor. Yet re-training that "smart" population of flies would result once again in only 30% of those flies making the correct odor choice (Quinn et al., 1974). The establishment of this learning assay now enabled the group to test for genetic mutants defective in learning and memory. These early mutagenesis screens uncovered several key genes involved in the formation and consolidation of shock associated odor memory including *dunce*, a cAMP phosphodiesterase, *rutabaga*, a type 1 adenylyl cyclase and *amnesiac*, a putative neuropeptide required for the stability of memory (Dudai et al., 1976; Davis et al., 1989; Livingstone et al., 1984; Levin et al., 1992; Quinn et al., 1979; Feany & Quinn, 1995).

Future studies further refined this assay, yielding more reproducible and robust results through the use of a T-maze apparatus to test shock-associated memory performance (Tully & Quinn, 1985). Studies from Tully et al. defined what are considered to be 3 distinct phases of shock-associated memory: labile short-term memory, intermediate middle-term memory, and fully consolidated long-term

memory (Tully et al., 1994). The formation of these phases required specific training protocols. While STM could be formed with a single training trial pairing shock with odor, forming LTM required 6-10 spaced training trials with a 15 minutes inter-trial interval. LTM was found to be at least partially dependent on the synthesis of new protein products, as administering cycloheximide (CXM) to flies before training significantly disrupted spaced-training conditioned performance (Tully et al., 1994).

However, a key question still remained: could the flies learn just as well using a positive reward and might it involve the same genes and memory circuitry? While the electric shock conditioning paradigm is a robust assay in which to study the mechanics of associative learning, might a more ethologically relevant stimulus provide more insight into memory processes and integration of sensory information? After all, how often do fruit flies encounter a copper-wire grid conducting 90V shocks out in nature?

Appetitive Olfactory Conditioning

The studies of Tempel et al. suggested that hungry flies could associate odor with a sugar reward to form robust appetitive food reward memory (Tempel et al., 1983). Not surprisingly perhaps, the appetitive memory observed when

conditioning with sucrose differed to aversive shock memory in several ways. First, although the performance of sucrose-trained flies tested 2 minutes after training was similar in strength to that of shock-trained flies, sucrose reinforced memory persisted to 24 hours, while shock reinforced memory faded by 3 hours. Furthermore, Tempel et al. noted that the flies needed to be food deprived for 19-20 hours both prior to training as well as testing in order to see appetitive performance, whereas the internal hunger state did not effect shock based learning (Tempel et al., 1983). Finally, as it was shown that flies were able to simultaneously retain both aversive shock and appetitive sugar memories, Tempel hypothesized that in the fly, separate pathways for positive and negative reinforcement must exist, mechanistically distinct from each other. Yet it would be another two decades before a molecular and neural circuit model was proposed that accommodated the dichotomy between positive reward, and negative punishment-associated memory in the fly.

In 2003, Schwaerzel et al. investigated a molecular basis for associative learning in the fly, covering both paradigms of sugar and shock reinforced olfactory conditioning. Based on studies manipulating the modulatory amines octopamine (OA) and dopamine (DA) in the fly, it was concluded that sugar reward learning required octopamine, yet not dopamine signaling, while shock punishment learning required dopamine, but not octopamine (Schwaerzel et al., 2003). From these results, Heisenberg proposed a model which became modern dogma in

the field of *Drosophila* learning: that unlike in mammalian systems, DA coded for aversive reinforcement, while OA, found only as a “trace amine” in mammals (Burchett & Hicks, 2006), mediated appetitive reinforcement (Heisenberg, 2003). My studies presented in Chapter III reveal a more nuanced and complex view of reinforcement that implicates both OA and DA in reward learning.

Work form the Waddell lab refined the appetitive olfactory conditioning protocol from Schwaerzel et al, and further explored how appetitive memory relates to aversive shock reinforced memory. Keene et al. showed that synaptic output from the *amnesiac*-expressing Dorsal Paired Medial (DPM) neurons was required between training and testing to maintain 3hr memory performance of both appetitive as well as aversive memory, suggesting a common circuit to consolidate labile STM to stable LTM in the fly (Keene et al., 2004; Keene et al., 2006). Furthermore, it was found that both aversive and appetitive olfactory memory required output from mushroom body (MB) neuron subsets in a similar sequential manner: output from the α’β’ neurons was essential during and immediately after training, while αβ neuronal output was only required for retrieval of both aversive and appetitive memory (Krashes et al., 2007). However, despite using similar cellular processes and machinery to form, consolidate and retrieve olfactory memory, the nature of appetitive sugar-reward memory significantly differs from aversive shock memory in many ways.

Krashes and Waddell refined the appetitive olfactory conditioning paradigm using a T-maze into the protocol routinely used in the Waddell lab today (Figure I-1). Here, about 100 flies food deprived for 18-20 hours are loaded into the training arm of a T-maze lined with filter paper and exposed to one odor for 2 minutes, followed by 30 seconds of clean air. The flies are transferred into a second training tube lined with saturated sucrose dried on filter paper and exposed to a second odor for 2 minutes. The flies are then moved into the elevator of the T-maze to a choice point where they are given 2 minutes to choose between the two odors presented during conditioning (Krashes & Waddell, 2008). In typical experiments, about 65% of the flies make the correct odor choice, approaching the odor previously paired with sucrose reward. A numerical measurement of learning is then calculated as a performance index (PI) by subtracting the number of flies that chose incorrectly from the number choosing correctly and dividing by the total number of flies (Figure I-2).

One of the remarkable features of appetitive memory seen in (Krashes & Waddell, 2008) and consistent with previous observations from (Tempel et al., 1983) and (Schwaerzel et al., 2003) was that flies needed to be food deprived for at least 18 hours prior to conditioning with sucrose and also needed to be in a food deprived state at the time of testing to see memory performance. Flies could be fed in between training and testing to keep them alive, showing performance days later as long as they were food deprived again for at least 18

hours prior to testing (Krashes & Waddell, 2008). This suggested that there exists a motivational component to appetitive memory performance based on the internal nutritional state (either satiated or fasted) of the fly. A mechanism governing the state-dependence of appetitive memory retrieval was uncovered by (Krashes et al., 2009) and found to involve the fly equivalent of mammalian Neuropeptide Y (Neuropeptide F) inhibiting a subset of MB innervating dopamine neurons. This finding demonstrated a novel inhibitory role for DA neurons in the fly in gating appetitive memory performance. I will discuss the topic of the specific roles of dopamine in appetitive reward further in Chapter III.

Another striking feature of appetitive memory formed in this paradigm is that, unlike shock conditioning in which multiple spaced training trials are required to form LTM, a single two minute pairing of odor with the sugar sucrose enabled genuine appetitive LTM formation that could last for days (Krashes & Waddell, 2008). This finding suggested that there was something intrinsically different about how reward memory is encoded in the brain, and lead us to question what made sucrose such a potent reinforcer of memory. This project was my first in the Waddell lab and will be discussed in the following chapter.

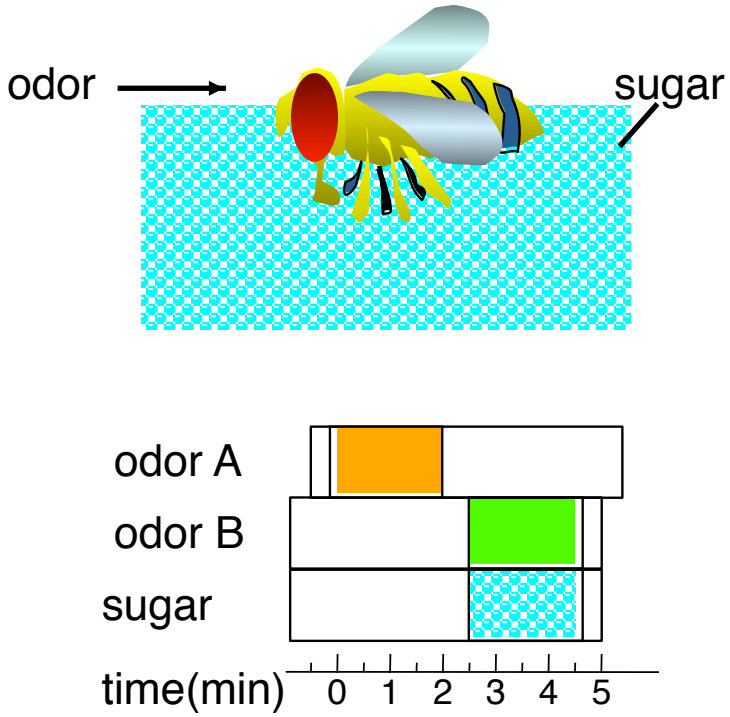
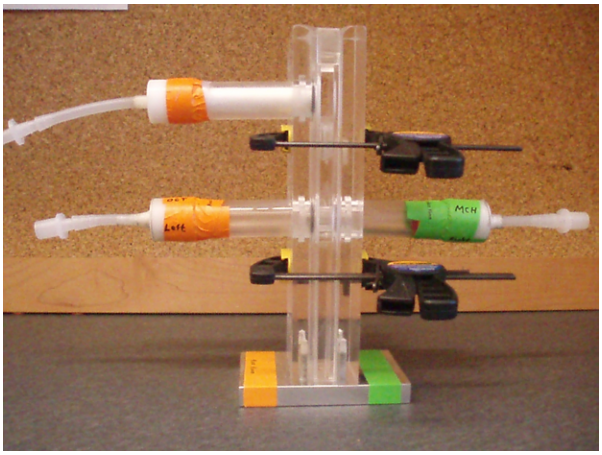


Figure I-1. Appetitive Olfactory Conditioning Paradigm. Food deprived flies are presented with odor A for 2 minutes in a T maze followed by 30 seconds of clean air. The flies are then presented with odor B for 2 minutes now paired with a sugar reward. Following training, the flies are transferred to the choice point of the T maze and given 2 minutes to choose between the 2 odors presented during conditioning



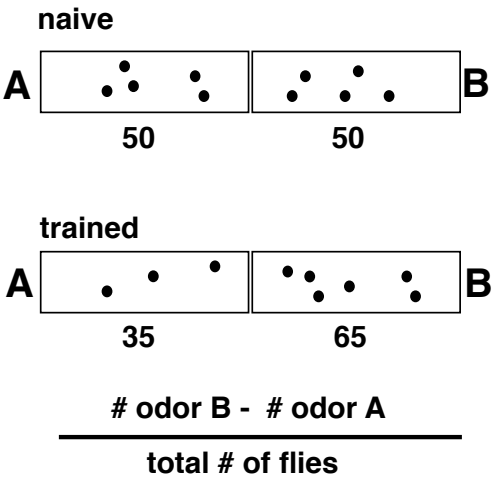


Figure I-2. T-maze olfactory training machine and performance index (PI) calculation. Flies are conditioned with sugar and odor in the top training tube, then transferred via the elevator to a choice point where they are given 2 minutes to choose between the conditioned odor (CS+) and a reference odor (CS-). Naïve flies will have formed no association with the odors and therefore show a 50:50 distribution approaching either odor. After conditioning, about 65% of the flies will approach the odor previously paired with sugar reward, yielding a PI score of 0.3.

Preface to Chapter II

In nature we find not only that which is expedient, but also everything which is not so inexpedient as to endanger the existence of the species – Konrad Lorenz

The work presented here was previously published in:

Burke, C. J., Waddell, S. Remembering nutrient quality of sugars in *Drosophila*. (2011). *Current Biology* 21: 746-750.

Burke CJ performed all experiments

Burke CJ and Waddell S designed the experiments

Waddell S and Burke CJ wrote the manuscript

Chapter II: Remembering Nutrient Quality of Sugars in *Drosophila*

Nutrient: It's what's for dinner

Food selection based on memories of past experience is a critical component of survival, as recalling a bitter tasting food that made you sick in the past might help prevent you from ingesting toxic substances in the future. On a more positive note, remembering the taste of a particularly good and nutritious food source is highly beneficial as well, enabling quicker and more efficient food selection and reducing vulnerability to predation. While there has been a great amount of progress in recent years uncovering the molecular and neuronal basis of taste detection in both mammals and insects (Yarmolinsky et al., 2009), the means and mechanisms by which nutrient value is encoded in the brain has remained more elusive.

The study of food selection and preference behaviors in a diverse cohort of organisms from humans to insects has shed light on how reward is processed in the brain. This work has lead to our current understanding of what makes nutritious and highly caloric food sources so highly desirable, and how nutrient content differs from sweet taste to act as potent reward. Functional magnetic resonance imaging (fMRI) studies in humans have revealed differential activity in

the upper hypothalamus in response to ingestion of a sweet and caloric versus a sweet but non caloric drink (Smeets et al., 2005). Whereas consumption of a nutritious glucose solution elicited a decrease in upper and lower posterior hypothalamic activity, consumption of highly sweet aspartame or nutritious but tasteless maltodextrin alone failed to have similar effect, suggesting the presence of both sweetness and nutrient value are required to drive changes in hypothalamic activity (Smeets et al., 2005), suggestive of differential reward value for hedonic sweetness and nutrient.

Furthermore, a more recent fMRI study found that patients given only a taste (without ingestion) of a solution of either the sweet and nutritious sugar glucose or a non-sweet carbohydrate maltodextrin showed preferential activation of the dorsolateral prefrontal cortex, striatum and singulate cortex, areas of the human brain associated with sugar reward, while those fed a saccharin solution of equal sweetness and taste did not (Chambers et al., 2009). The same study found that a mouth rinse of either nutritious solution (glucose or non sweet maltodextrin) was sufficient to significantly increase mean power output for patients in a cycling time trial exercise performance task, whereas a mouth rinse of saccharine solution did not (Chambers et al., 2009). These results suggest that in addition to the well characterized detection of sweet taste, there also may exist a specific nutrient sensor present in the human mouth which can rapidly evaluate nutritional value and weigh metabolic gain.

Food discrimination and taste preference studies performed in rats and mice showed similar results and advanced the theory of nutrient sensing independent of taste. It was shown in rats that intra-gastric injection of a nutritious carbohydrate could be used to form positive food reward association with various flavor compounds (Sclafani, 2001; Ackroff et al., 2009). In addition, mice that lack the transient receptor potential M5 (TRPM5) channel are considered to be "taste blind," and show no preference for a sweet solution (of either sucrose, glucose or non-caloric sucralose) over water across short time periods (de Araujo et al., 2008; de Araujo et al., 2010). However, if given access to nutritious sucrose over time, the TRPM5 knockout mice will develop a strong preference for a sucrose solution over water in a similar manner as wild-type mice. This preference was not observed when mice were exposed to non-nutritive but highly sweet sucralose, suggesting that the knockout mice were forming their preference based solely on the nutritive content of sugar solutions (de Araujo et al., 2008). Furthermore, it was found that the TRPM5 knockout mice showed normal changes in blood glucose levels and showed a significant increase in the level of dopamine release in the nucleus accumbens (NAc) in response to ingestion of sucrose, but not sucralose (de Araujo et al., 2008). Of important note is that increases in NAc dopamine levels were also observed in wild-type mice in response to ingestion of sweet sucralose alone, suggesting a role for dopamine in mediating the rewarding aspects of both sweet taste and post-ingestive nutrient content. While the concept of nutrient detection independent of taste

sounds rather complex, post oral sensing of nutrition content is not limited to vertebrates.

Experiments performed in locusts showed that complex mechanisms for specific internal nutrient detection also exist in insects. By depriving locusts of the marconutrients carbohydrate or protein, Simpson and White found that locusts showed a strong preference for a food source which could replenish protein over isocaloric food sources (Simpson & White, 1990). Furthermore, locusts can be trained to associate either visual or olfactory cues with presentation of nutrient-replenishing foods suggesting that not only is the insect able to internally sense specific nutrient deficiencies, but that consumption of those nutrients was sufficient to drive reward learning (Simpson & White, 1990; Raubenheimer & Tucker, 1997).

Analysis of *Drosophila* feeding behaviors, food preference and nutrition have a long history dating back to the 1940's and continue today. A recent study found that flies reared on certified organic foods showed significantly increased lifespan, fertility and stress resistance to those reared on conventional produce (Chhabra et al., 2013). Early on, Hassett (1948) determined the ability for fruit flies to survive on various sugars, carbohydrates, alcohols, proteins and other substances as the sole source of food, also observing the effects on egg laying ability and larval development (Hassett, 1948). The studies of Wigglesworth went

a step further, analyzing the ability of various sugars to re-initiate flight in an energy-depleted fly. He discovered that nutrient content, rather than just palatability of the sugar is critical for the fly's rapid use of energy to fuel flight (Wigglesworth, 1949). Furthermore, both proboscis extension reflex (PER) and ingestion experiments found that flies showed strong appetitive responses to natural nutritional sugars such as sucrose and fructose as well as artificial sweeteners like aspartame and sucralose, suggesting that flies prefer sweet tasting compounds like mammals (Gordesky-Gold et al., 2008).

How does a fly taste?

It is believed that the fly tastes its environment through a family of gustatory receptors (GRs) first found via structural prediction algorithms and characterized by *in situ* hybridization analysis of taste organs (Clyne et al., 2000; Scott et al., 2001). These putative 7 transmembrane receptors are found in gustatory receptor neurons (GRNs) housed in the hair-like sensillia present on the fly's forelegs, wing margins, genitalia, maxillary palps, tip of the proboscis and internal mouthparts (Wang et al., 2004; Thorne et al., 2004). In addition a select few are expressed more internally (Miyamoto et al., 2012) and have been hypothesized to play various roles involved with sugar sensing. The GRNs can be categorized based on the GRs they express, being mostly sensitive to sweet compounds, bitter, salt, or water with minimal overlap of modality (Wang et al., 2004; Thorne et al., 2004; Marella et al., 2006; Cameron et al., 2010; Dahanukar et al., 2007;

Slone et al., 2007). Gr5a and Gr64a were identified as the receptors primarily responsible for mediating response to various sweet compounds and are present in nearly all GRNs responsive to sugars (Dahanukar et al., 2001; Dahanukar et al., 2007). Conversely, Gr66a was identified as critical to mediating response to bitter compounds (Wang et al., 2004; Thorne et al., 2004). Furthermore, these differing modalities of sweet and bitter taste are represented in distinct non-overlapping sets of neurons, defined by their expression of Gr5a/ Gr64a and Gr66a, and project to discrete regions of the subesophageal ganglion (SOG) in the base of the brain (Wang 2004, Thorne 2004). The GRs were shown to be essential in mediating both electrophysiological (Dahanukar et al., 2007) and behavioral (Wang et al., 2004; Thorne et al., 2004; Slone et al., 2007) responses to sweet and bitter compounds. Additionally, artificial stimulation of sweet or bitter sensing GRNs was sufficient to drive appetitive or aversive behavior in a quadrant preference plate assay (Marella et al., 2006).

In contrast to the well characterized projection neurons present in the fly olfactory system, second order taste neurons have yet to be identified, and despite our detailed knowledge of sweet taste perception in the fly, only recently have we begun to unravel mechanisms and neural circuits underlying nutrient sensing.

Introduction

Taste is an early stage in food and drink selection for most animals (Yarmolinsky et al., 2009; Dethier, 1976). Detecting sweetness indicates the presence of sugar and possible caloric content. However, sweet taste can be an unreliable predictor of nutrient value because some sugars cannot be metabolized. In addition, discrete sugars are detected by the same sensory neurons in the mammalian (Zhao et al., 2003) and insect gustatory systems (Wang et al., 2004; Thorne et al., 2004), making it difficult for animals to readily distinguish the identity of different sugars using taste alone (Breslin et al., 1994; Dotson & Spector, 2007; Masek & Scott, 2010). Here we used an appetitive memory assay in *Drosophila* (Tempel et al., 1983; Krashes & Waddell, 2008; Colomb et al., 2009) to investigate the contribution of palatability and relative nutritional value of sugars to memory formation. We show that palatability and nutrient value both contribute to reinforcement of appetitive memory. Non-nutritious sugars formed less robust memory that could be augmented by supplementing with a tasteless but nutritious substance. Nutrient information is conveyed to the brain within minutes of training when it can be used to guide expression of a sugar-preference memory. Therefore we propose that flies can rapidly learn to discriminate between sugars using a post-ingestive reward evaluation system and they preferentially remember nutritious sugars.

Appetitive olfactory memory formation in hungry adult *Drosophila* is very efficient with a single two minute pairing of odorant and sucrose being sufficient to form memory lasting for days (Krashes & Waddell, 2008; Colomb et al., 2009). Although there is evidence that other insects can learn to associate visual and olfactory cues with specific nutrients, carbohydrates or proteins (Simpson & White, 1990; Raubenheimer & Tucker, 1997; Bitterman et al., 1983; Zhang et al., 2005) it is unclear how taste and the respective nutrient value of these components contributes to the processes of learning and memory. Here we investigated whether nutrient value of sugar contributes to the reinforcement of appetitive olfactory memory in fruit flies.

Adult *Drosophila* feed on soft rotting fruits that are rich in sucrose, fructose and glucose, eg. apples, peaches, grapes and pears. In a classic series of experiments, Wigglesworth (1949) determined that these sugars provide energy for adult flight. Flies that were depleted of muscle glycogen by flying them to exhaustion, resumed flight within two minutes when fed glucose, sucrose, fructose, mannose, maltose or trehalose. In contrast, some sugars such as arabinose were completely ineffective. A similar relative value of sugars was established by Hassett (1948) who evaluated survival when flies were provided with these sugars as the sole food source. Sucrose, glucose and fructose supported survival whereas arabinose and xylose were very poor.

Results

Fruit flies conditioned with palatable and nutritious sugars form robust persistent memory

Led by these prior studies (Wigglesworth, 1949; Hassett, 1948) we chose D-sucrose and D-fructose as nutritious sugars and D-arabinose and D-xylose, which are both abundant in fruits containing large amounts of pectin, as less nutritious sugars. We verified the relative nutritional value of each sugar for our wild-type Canton-S fly strain (Figure II-1A) by housing flies in food vials with either 1% agarose (as a source of water) or 1% agarose containing 3M sucrose, fructose, arabinose or xylose as their sole source of food. Consistent with previous studies (Wigglesworth, 1949; Hassett, 1948), the majority of flies housed on either sucrose or fructose remained alive for 4 days whereas most flies housed on water, arabinose or xylose were dead within 4 days. We also noted, as did Hassett (Hassett, 1948), that flies housed on arabinose died even quicker than those on water or xylose, suggesting that prolonged arabinose feeding may be detrimental to flies. We conclude that sucrose and fructose provide nutritional benefit in that they are capable of sustaining life, whereas arabinose and xylose do not.

Drosophila primarily sense sugars using gustatory receptor neurons (GRNs) on their tarsae and mouthparts (Amrein & Thorne, 2005). Tarsal contact with

desirable sugars drives proboscis extension whereas stimulation of sweet-sensing gustatory neurons on the labellum of the proboscis promotes food acceptance and ingestion. The physiological response of labellar gustatory receptor neurons to some of these sugars has been reported. Electrophysiological recordings from the L1 sensilla on the proboscis showed that sucrose more strongly activated sweet-sensing neurons than fructose and arabinose whereas xylose did not evoke a response (Dahanukar et al., 2007). Using Ca^{2+} imaging, sucrose, fructose and arabinose were shown to evoke similarly strong responses in sweet-sensing Gr5a-expressing neurons (Wang et al., 2004; Thorne et al., 2004) whereas xylose was not tested (Marella et al., 2006). We tested whether each sugar was detected as favorable using a proboscis extension reflex (PER) assay (Wang et al., 2004; Masek & Scott, 2010; Dahanukar et al., 2007; Shiraiwa & Carlson, 2007) (Figure II-1B). We applied sugar solutions to the front leg of restrained flies and determined the frequency of the PER. Whereas sucrose, fructose and arabinose elicited high levels of PER that were statistically indistinguishable, xylose-evoked PER was significantly lower. Sugars applied directly to the labellum evoked a very similar PER profile (Figure II-4). Therefore, published data (Wigglesworth, 1949; Hassett, 1948; Dahanukar et al., 2007; Marella et al., 2006) and those presented here suggest that sucrose and fructose are nutritionally beneficial sugars that strongly activate sweet-sensing GRNs whereas arabinose, and to a lesser extent xylose are detected as sweet but provide no obvious nutritional benefit.

We next used each of these sugars as reinforcement in an olfactory conditioning assay (Krashes & Waddell, 2008) and measured appetitive olfactory memory formation and persistence. Memory tested immediately after training revealed clear differences in performance between flies reinforced with the different sugars (Figure II-1C). Performance followed a similar rank order to the robustness of PER evoked by each sugar. Immediate memory performance of flies conditioned with sucrose was indistinguishable from those trained with fructose but was statistically greater than those trained with arabinose or xylose. The performance of arabinose conditioned flies was statistically indistinguishable from flies conditioned with fructose but was statistically different from those trained with xylose. Therefore short-term memory can be formed with non-nutritious sugars suggesting that sensation of sweetness is sufficient for memory formation. We also tested whether memory persisted 24hr after training. Strikingly, the nutritious sugars, sucrose and fructose, formed robust 24hr memory whereas the non-nutritious sugars, arabinose and xylose, did not (Figure II-1D). Memory formed with sucrose and fructose was statistically different from that formed with arabinose and xylose whereas memory formed with arabinose was statistically indistinguishable from xylose conditioned memory.

Published data from functional Ca^{2+} imaging registered a significant response for arabinose in bitter-sensing Gr66a-expressing neurons as well as in sweet-sensing Gr5a-expressing neurons (Marella et al., 2006). We therefore tested

whether the poor 24hr memory observed following conditioning with arabinose resulted from an integration of bitter and sweet signals. We arabinose-conditioned flies in which output from the Gr66a-expressing neurons (Wang et al., 2004; Thorne et al., 2004) was blocked prior to and during training with the dominant temperature-sensitive uas-*shibire*^{ts1} (*shi*^{ts1}) transgene (Kitamoto, 2001). *shi*^{ts1} blocks membrane recycling and thus synaptic vesicle release at the restrictive temperature of 31 ˚C. The 24hr memory performance of these flies was statistically indistinguishable from wild-type and all control genotype flies (Figure II-5A). Therefore, activation of bitter-sensing neurons is unlikely to be responsible for poor 24hr memory performance observed following conditioning with arabinose reinforcement. In addition, although prolonged exposure of flies to arabinose appears to be detrimental (Figure II-1A and (Hassett, 1948)), the exposure during the 2min training session does not impair short-term memory performance when compared to fructose (Figure II-1C), or alter longevity (Figure II-5B).

A reduced amount of sugar ingested during conditioning could also determine the strength of memory formation. To address this possibility, we added tasteless dye to each sugar and measured dye uptake using spectrophotometry (Figure II-1E). Flies were deprived of food as if to prepare them for conditioning, and given a mock 5min training session with each of the dyed sugars (the amounts ingested during the usual 2min training session were beyond the limits of

detection). Importantly, ingested material takes more than 5min to pass through the fly (Edgecomb et al., 1994). Despite differences in PER evoked by each sugar, the amount of dye ingested with each sugar in 5 minutes was statistically indistinguishable. These data suggest that arabinose is not aversive and that the amount of each sugar ingested during training is unlikely to account for differences in memory performance. We concluded that differences could result from an additional reinforcing effect of nutritional value with sucrose and fructose.

Conditioning with arabinose or xylose supplemented with nutritious sorbitol forms robust 24hr memory

To further test a role for nutritional value in memory reinforcement we trained flies with supplemented arabinose or xylose. Hassett (1948) reported that the polyhydric alcohol sorbitol and polysaccharides such as starch/maltodextrin were nutritionally valuable to *Drosophila*. He also demonstrated phagostimulation of these compounds by mixing them with low doses of simple sugars (Hassett, 1948). We therefore verified the nutritional value of these supplements and assayed their palatability using the PER. Survival on medium containing sorbitol or maltodextrin was statistically indistinguishable to survival on sucrose across four days (with the exception of a single time point, Figure II-2A). PER experiments revealed that flies respond significantly to maltodextrin but they

respond very rarely to sorbitol (Figure II-2B). We suspect strong maltodextrin driven PER results from the 10% contaminating simple sugars in maltodextrin that come from partial hydrolyzation of this polymer of glucose. We therefore favored the use of 'tasteless' sorbitol as a supplement in our memory experiments.

We conditioned flies using odorants and sucrose, arabinose, xylose or sorbitol alone or with arabinose or xylose mixed with sorbitol, as reinforcement. Training with arabinose, xylose or sorbitol formed little to no 24hr memory (Figure II-2C & D). Memory performance was statistically different to that formed with sucrose reinforcement. However, 24hr memory formed with sorbitol supplemented arabinose or xylose was very robust and was statistically indistinguishable from flies trained with sucrose. We also trained flies with maltodextrin supplemented arabinose (Figure II-6). As in previous experiments, training with arabinose alone did not form robust 24hr memory whereas arabinose supplemented with maltodextrin, exhibited 24hr memory that was indistinguishable from sucrose conditioned flies. We also tested the amount of dyed sorbitol ingested in 5min when either presented alone, or mixed with arabinose or xylose. Flies ingested significantly more dye mixed with arabinose+sorbitol or xylose+sorbitol than with sorbitol alone (Figure II-2E). These data are consistent with previous results (Hassett, 1948) and with the notion that flies ingest insufficient amounts of sorbitol to form 24hr memory when sorbitol is presented alone for 2min.

However, using non-nutritious sugars to stimulate sorbitol (or maltodextrin) ingestion apparently provides sufficient reinforcement to form robust 24hr memory. Therefore these data support a role for nutrient value in the reinforcement of persistent long term appetitive memory.

Nutrient information is rapidly coded and can be used to guide preference behavior immediately after training

Conditioned taste aversion learning in mammals is noteworthy for the long delay (up to 12hr) between the presentation of the tastant and the induction of nausea (Garcia et al., 1955). One might imagine a post-ingestive nutritive affect would also develop slowly. However, data from both rats and flies suggest otherwise. Intra-gastric injection of either glucose or fructose was sufficient to drive strong associative flavor preference in rats within 60 minutes (Ackroff et al., 2004). In the flying to exhaustion studies, Wigglesworth (Wigglesworth, 1949) observed that flies resumed flight 30-45sec after feeding with glucose and 60-90sec after feeding with sucrose. This work suggests that energy resources might be internalized and utilized extremely fast.

We therefore assessed the speed of nutrient detection in our memory assay by immediately testing flies for discrimination between odors following a differential

conditioning protocol pairing one odor with a nutritious sugar and the other odor paired with a non-nutritious sugar (Figure II-3). We first trained flies with sucrose versus arabinose as reinforcement, or sucrose versus fructose for comparison. Flies showed an immediate preference for the odor previously paired with sucrose rather than arabinose. However flies showed no preference when differentially trained with the two nutritious sugars, sucrose and fructose. We next trained flies with odor paired with arabinose versus arabinose plus sorbitol or with odors paired with arabinose plus sorbitol versus sucrose. Flies showed immediate preference for the odor previously paired with sorbitol-supplemented arabinose over arabinose alone.

Strikingly, flies showed no preference for either odor when trained with arabinose plus sorbitol versus sucrose. We therefore propose that nutrient value is very quickly assigned to memory processing so that flies can exhibit preference behavior to the nutrient associated odorant less than two minutes after training. These experiments also suggest that nutrient content contributes to immediate memory performance in this behavioral choice assay.

Discussion

In conclusion, we believe our work shows that efficient appetitive memory formation in Drosophila involves signals representing nutrient value. Whereas sweet taste is sufficient to form short-term memory, persistent memory appears to be preferentially formed with sugars that also provide some nutrient benefit. Strikingly, supplementing inadequate sugars with nutritious but tasteless sorbitol forms stronger 24hr memory suggesting that nutrition is a key element for memory persistence. This model is supported by a previous study in which bees only formed persistent memory if they were allowed to ingest sugar during conditioning (Wright et al., 2007). Using a post-ingestive mechanism overcomes the shortcomings of the gustatory system to discriminate between sugars (Masek & Scott, 2010) and of potentially being fooled by certain sweet- tasting substances that an animal may not be able to usefully metabolize.

The ability to assign nutrient information to sources of food, and remember them, seems very valuable for an animal to forage effectively. Our data suggest that nutrient information can be very rapidly and accurately assigned to a particular food source. This rapidity contrasts to conditioned taste aversion memory formation in mammals (Garcia et al., 1955) and the honeybee (Wright et al., 2010), both of which involve a post-ingestive mechanism that is delayed and appears to be less accurately assigned to food sources to be avoided. Although conditioned taste aversion has not yet been demonstrated in fruit flies, it seems

logical that an animal might benefit from accurately learning nutritious food sources while being more conservative in learning to avoid sources that are potentially dangerous.

Taste defective mice can develop preference for nutritious liquids and calorie content activates the dopaminergic reward system (de Araujo et al., 2008). Furthermore, fMRI studies in human subjects administered sucrose, saccharin or maltodextrin concluded a central brain response for the caloric content of carbohydrate that was independent of sweet taste (Chambers et al., 2009). Therefore, a system of post-ingestive sensing of the nutrient content of carbohydrate may be conserved. Finding that taste and nutrient value of sugar contribute to appetitive memory formation suggests there may exist parallel reinforcement pathways for appetitive memory in the fly. Strikingly, bitter taste and post-ingestive effects of toxin provide parallel reinforcement signals for aversive learning in honeybees (Wright et al., 2010). It will be important to identify the molecular nature of post-ingestive nutrient detection and the mechanisms through which it is broadcast to the brain. Our work here suggests that the neural circuits of reinforcement and perhaps memory consolidation in the fruit fly brain contain neurons that are receptive to the nutrient signals, or alternatively, receive signal input from the periphery conveying nutrient status of ingested foods.

Materials and Methods

Fly strains

Fly stocks were raised on standard cornmeal food at 25°C and 60% relative humidity. Mixed sex populations of wild-type Canton-S flies that were housed together were used throughout. To disrupt neurotransmission from bitter-sensing neurons (Figure II-5A) we crossed Gr66aGAL4 (Thorne et al., 2004) male flies to females harboring a double insertion of uas-*shibire*^{ts1} (Kitamoto, 2001). Heterozygous transgenic control flies were from crosses of each transgenic line to wild-type Canton-S flies.

Appetitive Olfactory Conditioning

All flies were food deprived before training by storing them for 16-20hr in glass milk bottles containing water dampened 3MM filter paper to prevent desiccation. The olfactory appetitive paradigm was performed essentially as described (Krashes & Waddell, 2008) with the following modifications. Sugars were prepared as 3M solutions and 2.5ml was pipetted onto a 2.25 inch X 3 inch piece of 3MM paper and allowed to dry. Following training, flies were either tested immediately or stored in vials with standard fly food for 3hr, then transferred into vials containing 1% agar until testing 21 hours later. The performance index (PI) was calculated according to (Krashes & Waddell, 2008). The odors used were 3-octanol and 4-methylcyclohexanol. Statistical analyses were performed using

KaleidaGraph (Synergy Software). Overall analyses of variance (ANOVA) were followed by planned pairwise comparisons between the relevant groups with a Tukey HSD post-hoc test. Unless stated otherwise, all data points in each conditioning experiments represent n≥14.

Survival Assays

Groups of 20 (10 male, 10 female) 24hr old wild-type flies were housed in vials containing either a 3M solution of each substance (sucrose, fructose, arabinose, xylose, sorbitol) or 1.25M maltodextrin in 1% agar or 1% agar alone as a water control. The number of flies still alive in each vial was counted every 12hr. Each data point represents the mean of 10 separate vials per condition.

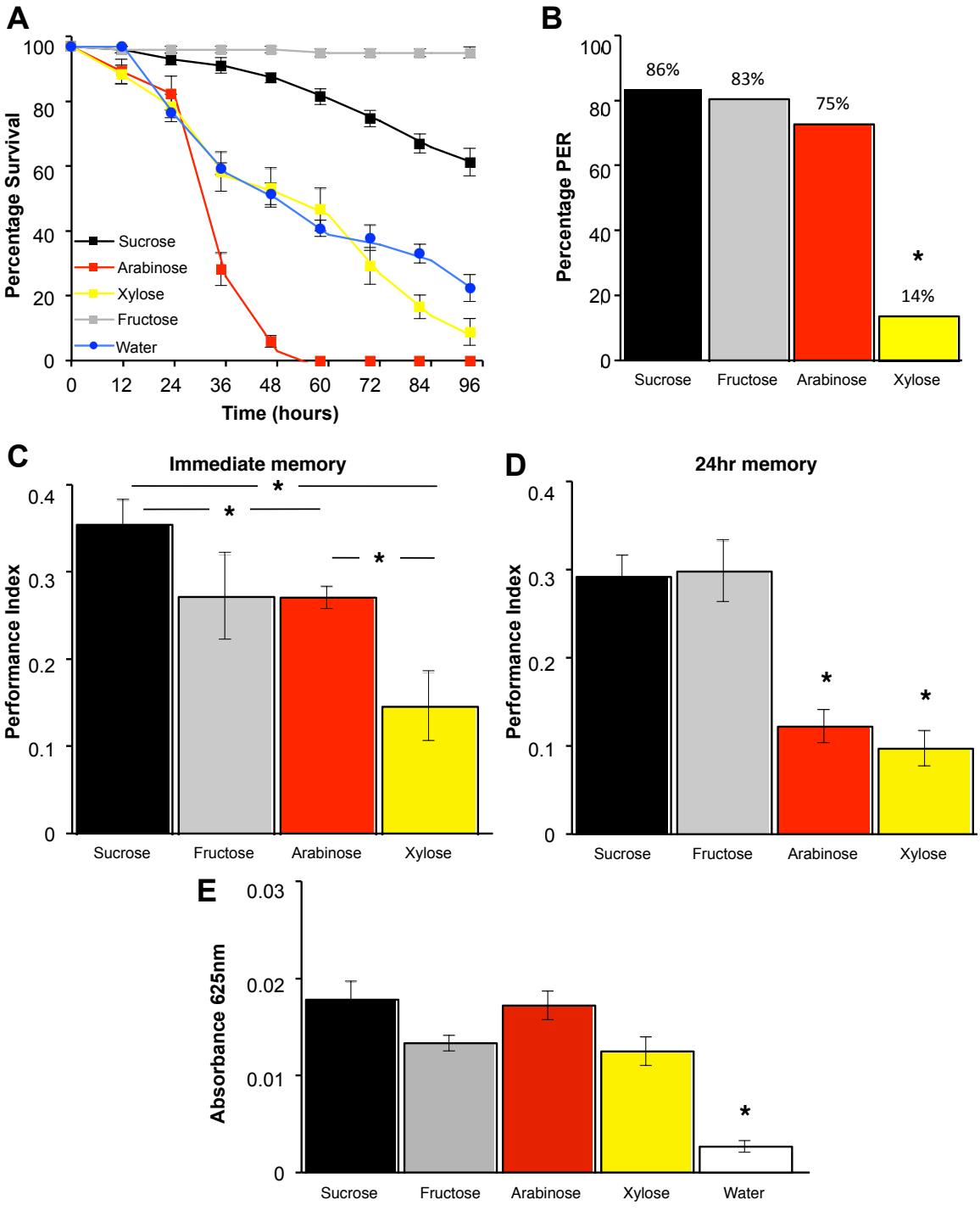
Proboscis Extension Reflex (PER) Assay

PER was performed similarly to as described in (Wang et al., 2004; Dahanukar et al., 2007; Shiraiwa & Carlson, 2007; Slone et al., 2007) with the following modifications. Groups of 3-7 day old wild-type flies were food deprived as described above for 24hr. The flies were then anesthetized for 1min by placing them in a cold test tube immersed in a 4 ˚C ice bath. Flies were stuck back-down onto non-toxic adhesive fly paper and left to recover for 1.5hr at 25 ˚C/ 60% relative humidity. To assay PER each fly was either presented to the fore-leg or labellum with the following regimen on a rolled Kim-wipe wick: water (negative control), test compound, 3M sucrose (positive control). Test compounds were

presented 3 times per fly and each fly was exposed to only one of the test substances flanked by the water and sucrose controls on either side. Data are presented as the percentage of presentations that elicited PER from the total number of presentations. Flies that extended their proboscis to water alone or that failed to extend to 3M sucrose at the end, were discounted from the analysis. Data were analyzed using the Yates' chi-square test.

Ingestion Assay

This assay was inspired by previous work (Tanimura et al., 1982). All flies were food deprived before testing by storing them for 16-20hr in glass milk bottles containing water dampened 3MM filter paper to prevent desiccation. Sugars were prepared as 3M solutions with 0.4% FD & C Blue No. 1 food dye (Spectrum Chemical) and 2.5ml was pipetted onto a 2.25 inch X 3 inch piece of 3MM paper and allowed to dry. The papers were inserted in the training chamber of the olfactory appetitive conditioning paradigm (Krashes & Waddell, 2008). 100 flies were loaded and given 5min to feed in the presence of airflow. Flies were then removed from the training chamber, immediately chilled to prevent excretion and homogenized in 1ml of Phosphate Buffered Saline (PBS; 1.86mM NaH_2PO_4, 8.41mM Na_2HPO_4 and 175mM NaCl). Following clearance of debris by centrifugation the dye in the supernatant was quantified by measuring the absorbance at 625nm. All $n \geq 8$.


A
Percentage Survival
100
80
60
40
20
0
0
12
24
36
48
60
72
84
96
Time (hours)
Sucrose
Arabinose
Xylose
Fructose
Water
B
Percentage PER
86%
83%
75%
*
14%
Sucrose
Fructose
Arabinose
Xylose
C
Immediate memory
Performance Index
0.4
0.3
0.2
0.1
0
*
*
*
Sucrose
Fructose
Arabinose
Xylose
D
24hr memory
Performance Index
0.4
0.3
0.2
0.1
0
*
*
Sucrose
Fructose
Arabinose
Xylose
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Absorbance 625nm
0.03
0.02
0.01
0
*
Sucrose
Fructose
Arabinose
Xylose
Water

Figure II-1. Fruit flies conditioned with palatable and nutritious sugars form robust persistent memory.

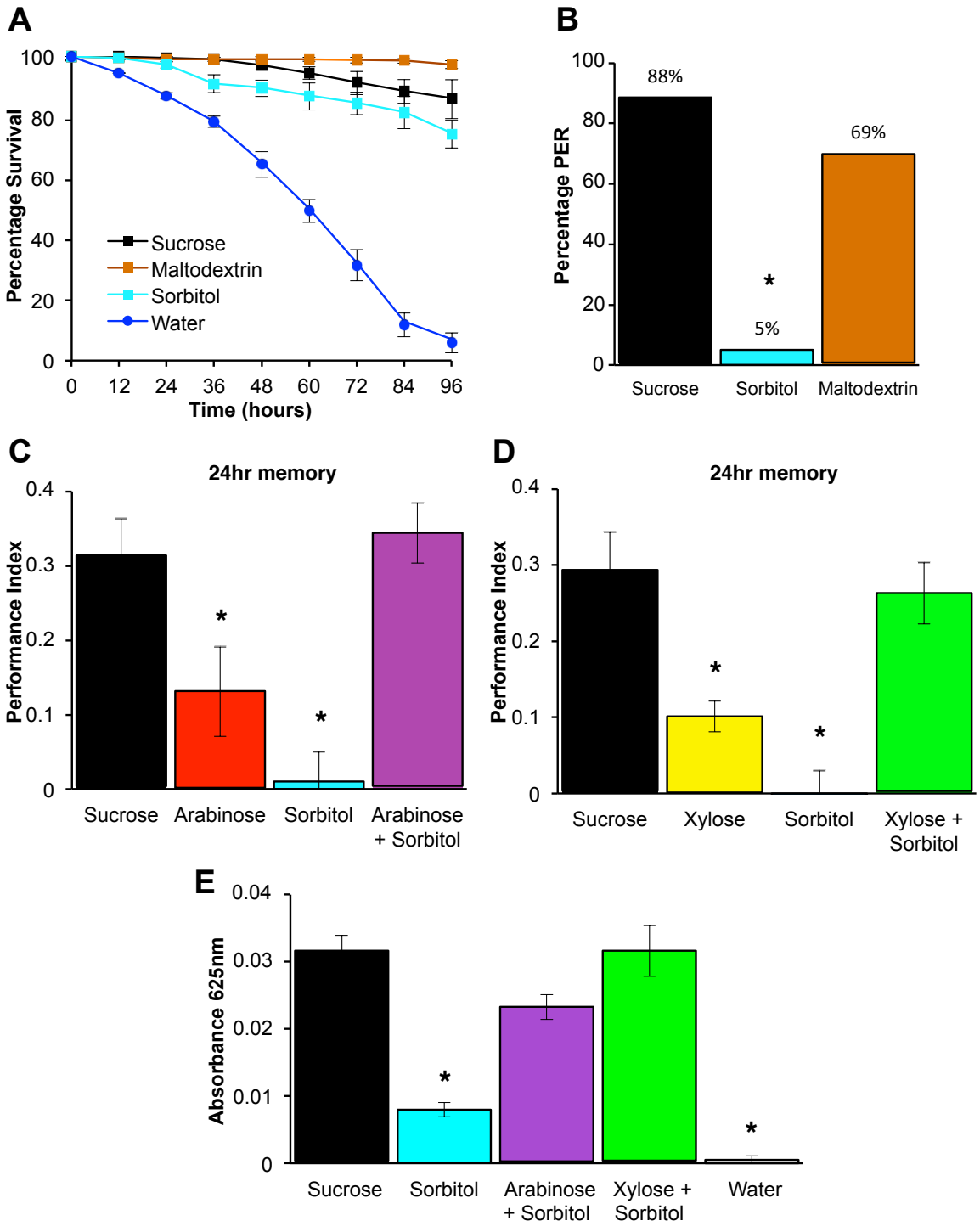
(A) Sucrose and fructose support fruit fly survival for several days but xylose and arabinose do not. Survival on fructose or sucrose was statistically different from water (1% agar) at all time points after 24hr. Xylose was not statistically different to water (all $p>0.13$) except at 84hr ($p=0.04$). Arabinose was statistically different to water at all time points after 24hr (all $p<0.0001$, T-test). All sugars were 3M in 1% agar. Data are mean ± standard error of the mean (SEM). n=10 for each data point.

(B) Sucrose, fructose, arabinose and to a lesser extent xylose, elicit proboscis extension behavior. Flies were presented with all sugars as 3M solutions to the front leg. Performance of xylose exposed flies is statistically different from all other groups (all $p<0.0001$, chi-squared test, marked by asterisk). n≥20 flies for each sugar.

(C) Short-term appetitive memory following conditioning with sucrose, fructose, arabinose and xylose. Performance of sucrose conditioned flies is statistically different from arabinose and xylose conditioned flies ($p<0.01$ and $p<0.005$ respectively). Arabinose performance is also statistically different to xylose ($p<0.04$, ANOVA). Data are mean ± SEM. n≥14 except xylose n=6.

(D) Sucrose and fructose form robust 24hr memory but arabinose and xylose do not. Asterisks denote significant difference between marked groups and all others (all $p<0.01$). There is no statistical difference between arabinose and xylose performance ($p=0.6$, ANOVA). Data are mean ± SEM. n≥16.

(E) A similar amount of each dyed sugar is consumed during a 5min mock training session. Each sugar was mixed with dye and presented dried on filter paper in the conditioning apparatus. No statistical differences were observed between sugars (all $p>0.05$, ANOVA) although all were statistically different to dye alone (water, $p<0.001$). Data are mean ± SEM. n≥8.


A
Percentage Survival
100
80
60
40
20
0
0 12 24 36 48 60 72 84 96
Time (hours)
Sucrose
Maltodextrin
Sorbitol
Water
B
Percentage PER
100
80
60
40
20
0
88%
*
5%
69%
Sucrose
Sorbitol
Maltodextrin
C
24hr memory
Performance Index
0.4
0.3
0.2
0.1
0
*
*
Sucrose
Arabinose
Sorbitol
Arabinose + Sorbitol
D
24hr memory
Performance Index
0.4
0.3
0.2
0.1
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Sucrose
Xylose
Sorbitol
Xylose + Sorbitol
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Absorbance 625nm
0.04
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Sucrose
Sorbitol
Arabinose + Sorbitol
Xylose + Sorbitol
Water

Figure II-2. Olfactory conditioning with arabinose or xylose supplemented with nutritious sorbitol forms robust 24hr memory.
(A) Sorbitol, maltodextrin and sucrose support fruit fly survival for several days. The number of flies alive on sorbitol or maltodextrin was statistically indistinguishable to those on sucrose at all time points ($p>0.07$) except at 84hr where maltodextrin was different to sucrose ($p=0.03$, asterisk). Survival on sorbitol, maltodextrin and sucrose was statistically different to on water at all time points (all $p<0.001$). Data are mean ± SEM. $n=10$ for each data point.
(B) Sucrose and maltodextrin elicit robust proboscis extension behavior but sorbitol does not. Flies were presented with 3M solutions of sucrose or sorbitol or 1.25M maltodextrin to the front leg. Performance of sorbitol exposed flies is statistically different from other groups ($p<0.0001$, chi-squared test, marked by asterisk). $n\geq20$ flies for each sugar.
(C) Training with sorbitol supplemented arabinose forms persistent memory. 24hr appetitive memory performance of flies trained with sorbitol supplemented arabinose is not significantly different to flies trained with sucrose ($p>0.6$). Asterisks denote significant difference between marked groups and others ($p< 0.05$, arabinose; $p<0.001$ sorbitol, ANOVA). Data are mean ± SEM. $n\geq14$.
(D) Training with sorbitol supplemented xylose forms persistent memory. 24hr appetitive memory performance of flies trained with sorbitol supplemented xylose is not significantly different to flies trained with sucrose ($p>0.6$). Asterisks denote significant difference between marked groups and others ($p <0.05$, xylose; $p<0.001$ sorbitol, ANOVA). Data are mean ± SEM. $n\geq14$.
(E) More dyed sorbitol is consumed in 5min when mixed with arabinose or xylose. Each substance or combination was mixed with dye and presented dried on filter paper in the conditioning apparatus. The amount ingested with arabinose+sorbitol or xylose+sorbitol was statistically different to sorbitol ($p<0.05$ and $p<0.001$ respectively) or water alone (both $p<0.001$, ANOVA). Consumption of dye with sorbitol was not statistically different from dye with water ($p>0.05$). Data are mean ± SEM. $n\geq8$.

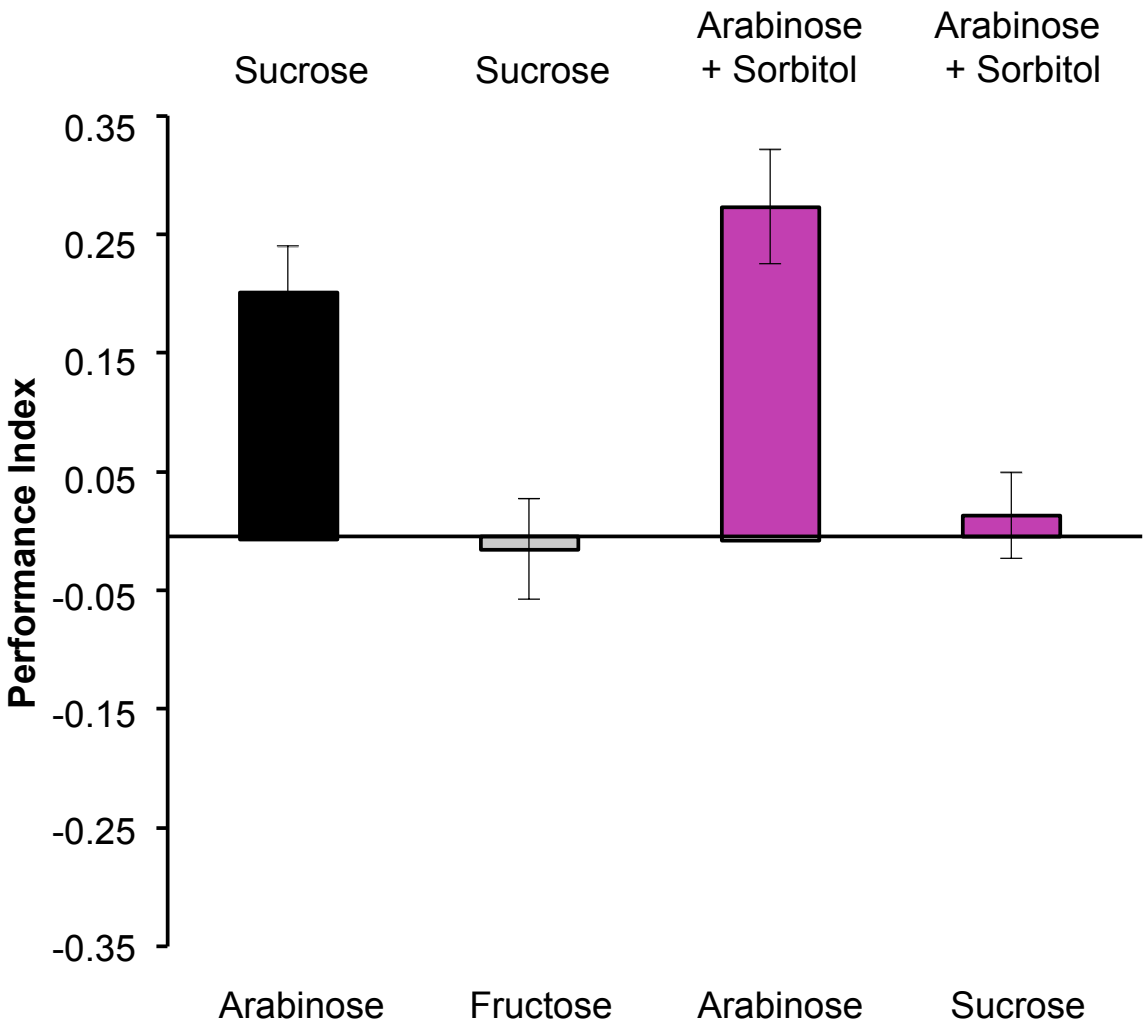


Figure II-3. Nutrient information is rapidly coded and can be used to guide preference behavior immediately after training.
Flies were differentially conditioned by pairing one odor with one sugar and the other odor with a different sugar, or supplemented sugar. They were then immediately tested for olfactory preference. Flies always exhibited preference for the odor that had been previously paired with a substance with nutrient value – sucrose over arabinose; arabinose+sorbitol over arabinose alone. They showed no preference when both odors were paired with nutritious substance – sucrose versus fructose; sucrose versus arabinose+sorbitol. Both of these scores were not statistically different from zero (both $p>0.6$, Mann Whitney U test). Data are mean ± SEM. $n\geq10$.

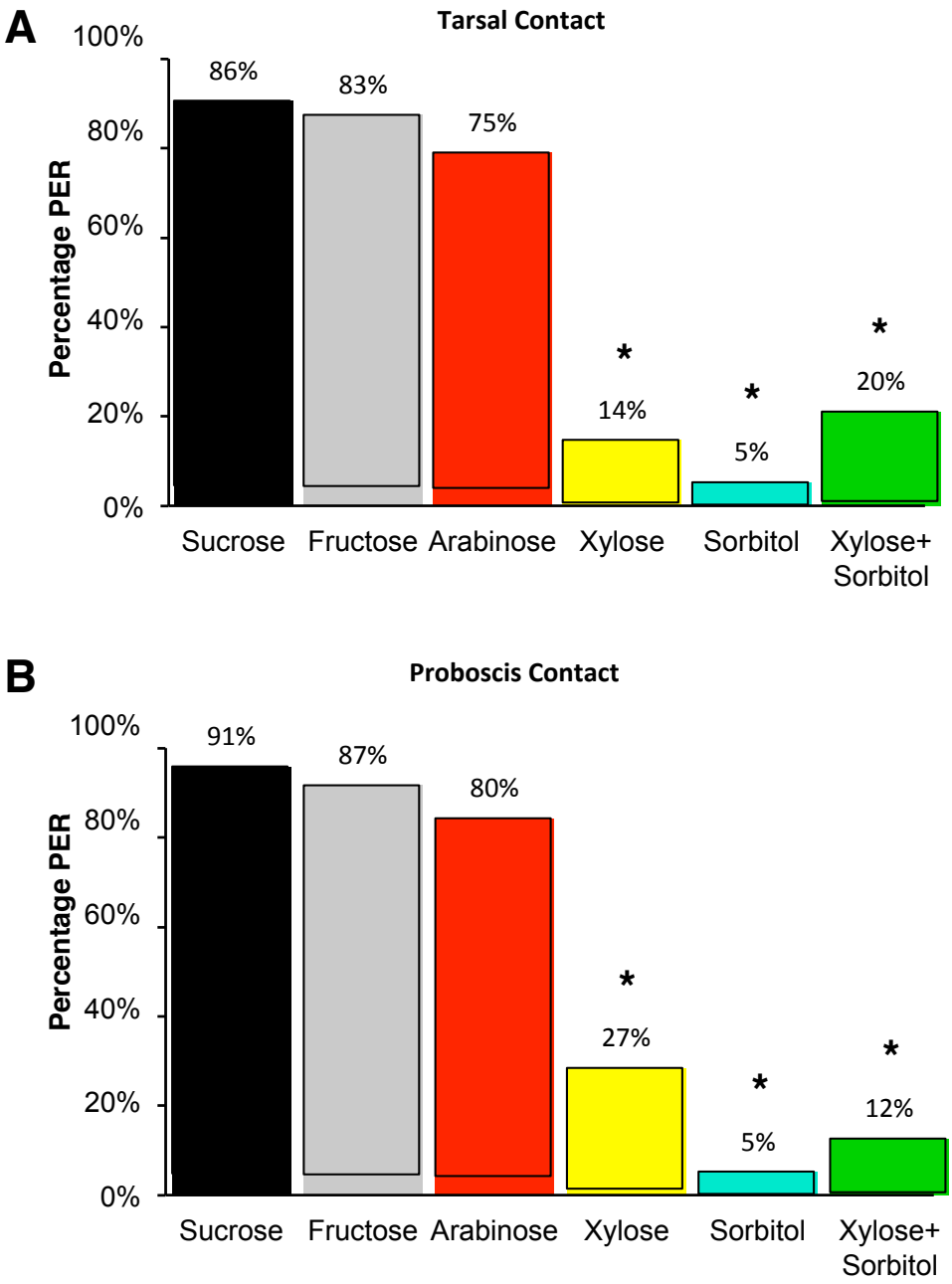


Figure II-4. Proboscis extension evoked by either tarsal or labellar contact. **(A)** Flies were presented with all substances as 3M solutions to the tarsus of the front leg. Performance of xylose and sorbitol exposed flies is statistically different from all other groups (all $p<0.001$, chi-squared test). n=16-48 flies for each sugar. **(B)** Flies were presented with all substances as 3M solutions to the labellum of the proboscis. Performance of xylose and sorbitol exposed flies is statistically different from all other groups (all $p<0.001$, chi-squared test). n=16-48 flies for each sugar.

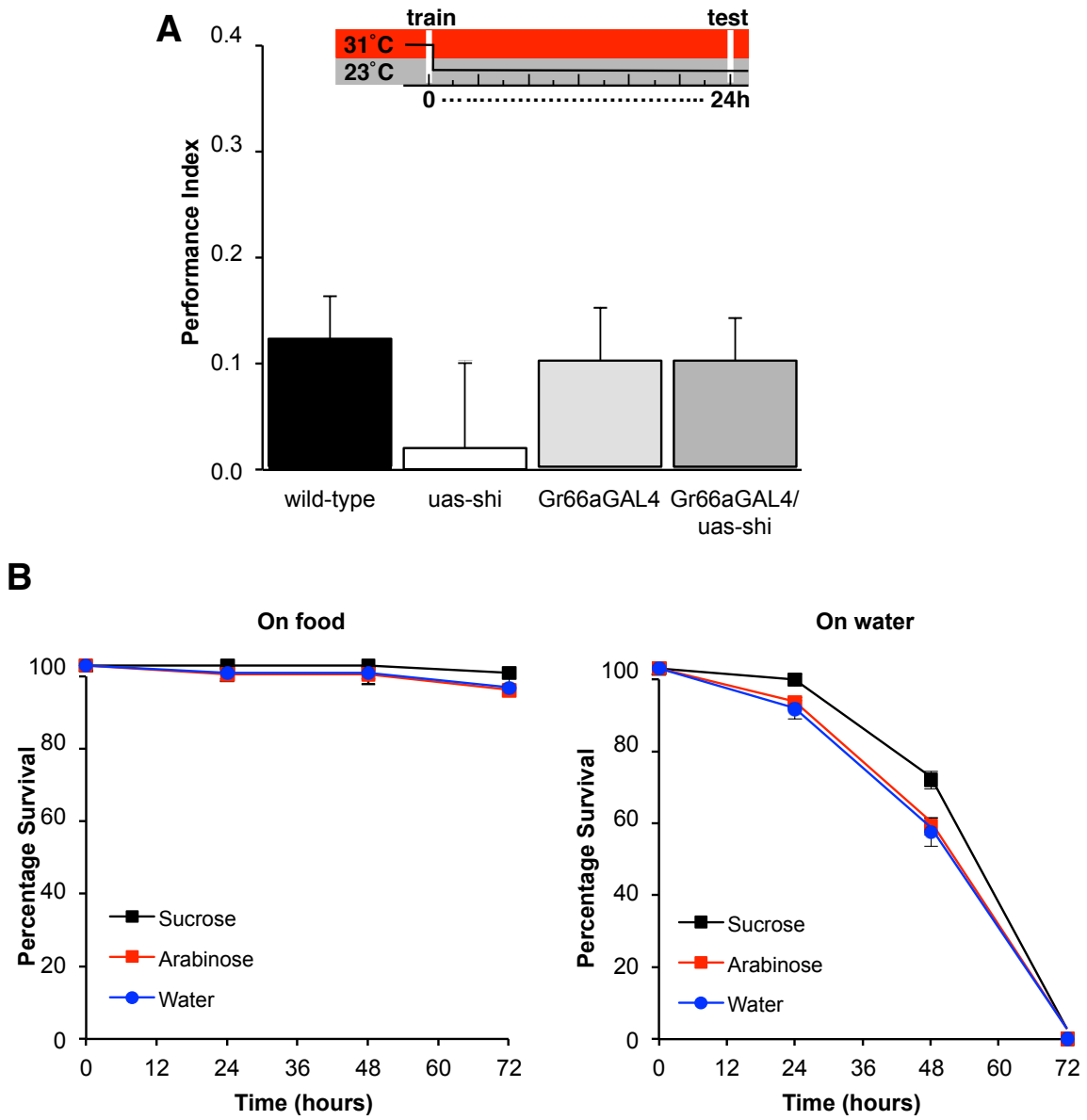


Figure II-5. Arabinose memory is not improved by blocking bitter taste neurons and brief arabinose exposure is unlikely to be detrimental.
(A) The temperature shift protocol is shown pictographically above the graph. Blocking bitter sensing neurons (Gr66aGAL4/uas-shi) before and during training does not impact 24hr appetitive memory formed by arabinose. Flies were incubated at 31℃ for 30min prior to and during training. Immediately after training they were returned to 23℃ and tested for 24hr memory. There were no statistically significant differences between the groups ($p>0.4$, ANOVA). Data are mean ± SEM. $n \geq 6$.

(B) A two-minute exposure to arabinose does not compromise viability. Food-deprived flies were kept for 2min in training tubes containing 3M sucrose or arabinose dried on a filter paper, or a blank filter paper; similar to during training. Each group was then split in two and maintained in vials containing either standard molasses fly food (left graph) or 1% agar for hydration (right graph). Survival was monitored daily and no differences were apparent between the 3 groups in the 'on food' (all $p>0.1$ on food) or between arabinose and water 'on water' (all $p>0.5$). Arabinose 'on water' is statistically different to sucrose at 24hr ($P<0.01$) and 48 hr ($p<0.004$, T-test). Data are mean ± SEM. n=5 experiments with 20 flies per group per experiment.

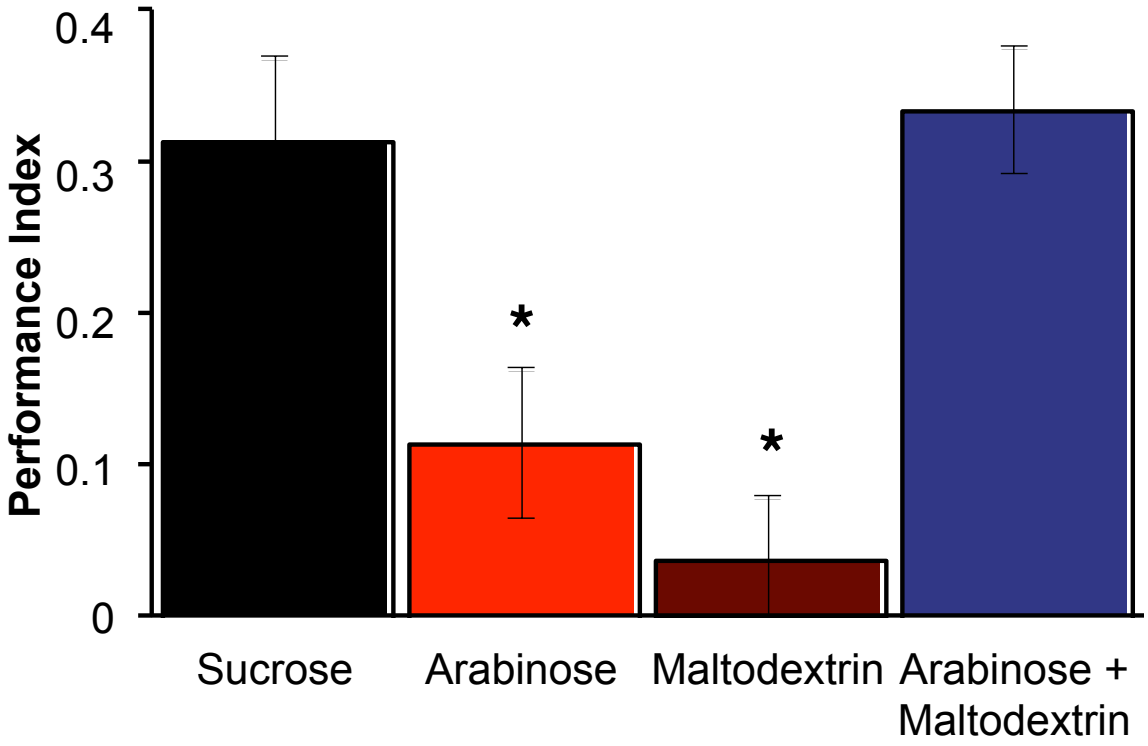


Figure II-6. Olfactory conditioning with arabinose supplemented with nutritious maltodextrin forms persistent memory.
Training with maltodextrin supplemented arabinose forms robust memory. 24hr appetitive memory performance of flies trained with maltodextrin supplemented arabinose is similar to flies trained with sucrose. Asterisks denote significant difference between marked groups and others. (all $p<0.01$, ANOVA). Data are mean ± SEM. $n \geq 9$.

Preface to Chapter III

Now I only told you that story so I could tell you this one – Bill Cosby

The work presented here was previously published in:

Burke, C. J.[*], Huetteroth, W.[*], Owald, D., Perisse, E., Krashes, M. J., Das, G., Gohl, D., Silies, M., Certel, S., Waddell, S. (2012). Layered reward signaling through octopamine and dopamine in *Drosophila*. *Nature* 492: 433-437.

Burke, C. J. performed all behavior experiments with exception to Fig III-1a performed by Krashes, M. J. and groups in Fig. III-2I which was performed by Perisse, E.

Huetteroth, W. did all imaging and performed all anatomical analysis with the exception of Fig III-5 by Burke, C. J.

Owald, D. and Huetteroth, W. performed all live imaging experiments

Das, G. constructed the lexAop-*dTrpA1* flies

Gohl, D. and Silies, M. generated and initially characterized the 0104-, 0273-, 0665-and 0891-GAL4 lines within the framework of the InSite collection.

Certel, S. generated and initially characterized the *Tdc2-LexA* flies

Burke, C. J., Huetteroth, W. and Waddell, S. designed the experiments

Waddell, S., Burke, C. J., and Huetteroth, W. wrote the manuscript

Chapter III: Layered Reward Signaling Through Octopamine and Dopamine in Drosophila

Biology can be considered the study of observing a phenomenon in nature and wondering how and why it is happening. While my observations explained in Chapter II revealed that flies process both sweet taste and nutrient value as rewarding stimuli, we wanted to better understand the molecular nature of these parallel pathways of appetitive memory reinforcement.

Systems of reward in mammals and insects

The neurotransmitter dopamine (DA) has long been linked with reward in mammals (Dayan & Balleine, 2002; Wise, 2004). Early studies in mice and rats found that pharmacological blockade of DA receptors greatly attenuates reward driven behaviors such as lever pressing for either food (Wise & Schwartz, 1981) or electrical stimulation of the lateral hypothalamus (Gallistel et al., 1974). Likewise, other associative reward tasks such as conditioned place preference (CPP) to either food reward or injection of cocaine (Spyraki et al., 1982; Spyraki et al., 1987) are also dependent on dopamine. Furthermore, studies have suggested that dopaminergic neurons in the ventral tegmental area (VTA) of the mammalian brain provide error-prediction in reward-based learning (Schultz et al., 1997). However, recent work in primates from Matsumoto and Hikosaka

showed that there exist distinct populations of midbrain DA neurons which are responsive to either appetitive or aversive cues during associative conditioning, revealing a more complex role for dopamine in valuation of varied stimuli (Matsumoto & Hikosaka, 2009b). When it comes to reward in the fly however, until very recently the prevailing thought in the field had been that evolution had taken quite a different turn.

Our understanding of reward in insects is dominated by seminal work performed in the honeybee by Hammer (1993). Through an anatomical analysis of neurons hypothesized to mediate the unconditioned stimulus (US) in classical conditioning, Hammer found that the Ventral Unpaired Medial (VUM) mx1 neuron in the SOG fired numerous action potentials in response to presentation of sugar to the proboscis or antenna of the bee (Hammer, 1993), suggesting it might play a role in mediating appetitive reward. He further showed that electrical stimulation of VUMmx1 could replace the presentation of sucrose during olfactory conditioning of the bee proboscis extension reflex (PER) (Hammer, 1993). This single neuron which projects to both the antennal lobes and MB calyces (sites essential in the formation of associative olfactory memories in bees and flies) was later found to contain the modulatory neurotransmitter octopamine (OA) (Hammer & Menzel, 1995). Further studies found that microinjection of OA into either the antennal lobe or MB calyx could replace presentation of sugar reward in olfactory conditioning (Hammer & Menzel, 1998).

Octopamine is believed to be the insect analog of mammalian norepinephrine, and has been found to play roles in a diverse set of behaviors in *Drosophila* ranging from aggression (Certel et al., 2007), to sleep and arousal states (Crocker & Sehgal, 2008; Crocker et al., 2010), to ovulation (Lee et al., 2003) and even synaptic plasticity at the neuromuscular junction (Koon et al., 2011). But it was the work of Schwaerzel et al. (2003) that first found a role for octopamine in mediating appetitive reward signals in the fly.

The approach taken by Schwaerzel et al. (2003) to investigate the roles of octopamine and dopamine in fly learning was a combination of behavioral analysis of genetic mutants and also the relatively new approach of functional neuronal circuit analysis. Behavioral analysis of the *Tβh* mutant defective in the production of octopamine (Monastirioti et al., 1996), revealed that flies which lack OA are unable to form sugar-conditioned memory, yet appeared to be normal for shock learning (Schwaerzel et al., 2003). Furthermore, temporal rescue of *Tβh* expression during acquisition or feeding OA to *Tβh* mutants directly prior to sugar training fully rescued this appetitive learning defect (Schwaerzel et al., 2003).
Anatomical analysis of octopamine neurons in *Drosophila* revealed widespread innervation across the brain. Though despite OA's role in mediating reward signals in appetitive learning, innervation of the mushroom body is relatively sparse (Sinakevitch & Strausfeld, 2006; Busch et al., 2009) with only 4 classes of OA neurons projecting from cell bodies in the SOG to the γ lobe, calyx and heel

regions (Busch et al., 2009). While these OA neuron subsets may allow us to identify the relevant circuits in reward signaling, a second approach analyzing the roles of OA receptors would give us even greater resolution.

To date, four octopamine specific receptors have been identified in the fly. A single α-adrenergic-like type whose activation gates Ca^{2+} release from intracellular stores is encoded by the *oamb* gene (Han et al., 1998; Balfanz et al., 2005). Three β-adrenergic-like receptors linked to cAMP production are encoded by the *octβ1R*, *octβ2R* and *octβ3R* genes (Maqueira et al., 2005). Roles for these receptors in appetitive learning in the fly has been only speculative to date, as it was shown that OAMB, as its name implies (octopamine receptor in mushroom bodies) appears to be preferentially expressed in the MB (Han et al., 1998).

Dopamine: Say goodnight to the bad fly

In addition to finding octopamine as critical to appetitive reward, Schwaerzel et al (2003) also analyzed dopamine signaling in learning using a temporally controlled block of neurotransmission approach. The host of genetic tools available in the fly allows one to identify functionally relevant neural circuitry by

selectively turning neurons “off” or “on” and assaying changes in behavior in transgenic flies.

We can assay the necessity of a particular set of neurons by expressing the dominant temperature-sensitive dynamin encoded by the uas-*shibire*^{ts1} (*shi*^{ts1}) transgene (Kitamoto, 2001), and testing sugar and shock learning in the fly at the restrictive temperature of 31 ˚C. At temperatures above 29 ˚C, shibire^{ts1} prevents vesicle recycling and it is believed to block transmission from neurons in which it is expressed. Importantly, this neuronal blockade is both fast acting and reversible by returning the flies to the permissive temperature of 23 ˚C, thus providing temporal control over neuronal function. Under spatial control using the GAL4/uas or LexA/ lexAop binary transcriptional systems (Brand & Perrimon, 1993; Lee and Luo, 2006), *shi*^{ts1} has been widely used in the *Drosophila* neurobiology field. It has been employed with great success in the Waddell lab to identify functional and temporal requirements for synaptic output from various neuronal circuits during distinct phases of learning and memory (Waddell et al., 2000; Keene et al., 2004; Keene et al., 2006; Krashes et al., 2008; Krashes et al., 2009; Pitman et al., 2011).

By expressing uas-*shi*^{ts1} in DA neurons using the *tyrosine hydroxylase* promoter *TH*-GAL4 transgene (Friggi-Grelin et al., 2003), Schwaerzel et al found that unlike in mammalian systems, output from dopamine neurons was not required

for the flies to form sugar reward memory, yet was critical for the formation of aversive shock memory at acquisition (Schwaerzel et al., 2003). A critical point to make however, is that the *TH*-GAL4 line expresses in *most* dopaminergic neurons throughout the fly brain, but not all, leaving the Protocerebral Anterior Medial (PAM) cluster of DA neurons which sends projections in the mushroom body horizontal lobes largely unlabeled (Mao & Davis, 2009).

Further studies identified specific subsets of dopamine neurons innervating the MB that convey negative value and whose direct activation can replace shock in olfactory conditioning (Claridge-Chang et al., 2009; Krashes et al., 2009; Aso et al., 2010; Aso et al., 2012). Together these findings comprised dogma in the field of olfactory learning in *Drosophila* in which appetitive reward is mediated by OA signaling, while aversive shock punishment signals through DA neurons.

Cracks in the pavement

Studies in recent years have begun to question the strict dichotomy of octopamine and dopamine. Led by the finding that the dDA1 dopamine receptor is enriched in the mushroom bodies (Kim et al., 2003), it was found that *dumb*^{1} flies mutant for *dDA1* were defective in aversive shock and surprisingly, were also moderately but significantly impaired in appetitive sugar learning (Kim et al.,

2007). This defect could be rescued by re-establishing expression of the receptor in the nervous system using the pan neuronal driver *elav*-GAL4 or specifically in the mushroom bodies using the MB247-GAL4 line (Kim et al., 2007). These data suggested that dopamine might be involved in coding appetitive reward.

The first work demonstrating a role for specific DA neurons in appetitive memory came from Krashes et al. (2009) who established a role for dopamine in mediating the state-dependence of appetitive memory retrieval. Dopaminergic mushroom body-innervating MB-MP1 neurons from the PPL1 cluster were found to provide the inhibitory control of satiety. They proposed that tonic dopamine released on the mushroom body while the fly was in a state of satiety prevented expression of appetitive memory (Krashes et al., 2009). This inhibition could be released by food depriving the fly (as shown in (Krashes & Waddell, 2008) or by stimulating neuropeptide F (dNPF) releasing neurons. dNPF inhibits MB-MP1 neurons through its receptor dNPFR1, blocking tonic inhibition and permitting retrieval of appetitive memory (Krashes et al., 2009). This finding revealed a more nuanced role for dopamine in gating the expression of reward memory.

Additionally, studies from Kaun et al. 2011 suggested that dopamine signaling may be necessary for the expression of rewarding ethanol reinforced memory (Kaun et al., 2011). It was also shown that dopamine modulates a fly's

acceptance of sucrose dependent on internal state (Marella et al., 2012). Here, neuronal blockade of *TH*-GAL4 neurons significantly reduced PER to a range of sucrose concentrations and stimulation of a single dopaminergic neuron in the SOG drove PER in hungry, but not satiated flies (Marella et al., 2012).

Recent studies found a population of DA neurons required for reward learning. These rewarding DA neurons have cell bodies in the PAM cluster, and they are not labeled in the previously used *TH*-GAL4 line. Rewarding DA neurons are labeled by the *DDC*-GAL4 or R58E02-GAL4 lines and they project to the horizontal MB lobes. Importantly they do not overlap with the previously described PPL1 or MB-M3 PAM DA neurons which signal negative value (Aso et al., 2010; Aso et al., 2012; Liu et al., 2012). Stimulation of PAM DA neurons could replace presentation of sugar reward during olfactory conditioning, forming appetitive memory that was dependent on *dDA1* expression in the MB (Liu et al., 2012). By performing *in vivo* Ca^{2+} imaging using GCaMP, Liu et al. showed that these rewarding PAM DA neurons were responsive specifically to ingestion of sucrose by the fly (Liu et al., 2012).

These data suggest complex actions of dopaminergic signaling in several aspects of appetitive behavior and helped guide us in our study of appetitive reward signals involved in learning and memory. While compelling, the model

proposed by Liu et al. did not integrate the prior findings of OA in providing appetitive reward in insects. The work I now present sought to unify the models.

Introduction

Dopamine is synonymous with reward and motivation in mammals (Dayan & Balleine, 2002; Wise, 2004). However, only recently has dopamine been linked to motivated behavior and rewarding reinforcement in fruit flies (Krashes et al., 2009; Liu et al., 2012). Instead, octopamine has historically been considered to be the signal for reward in insects (Hammer, 1993; Hammer & Menzel, 1998; Schwaerzel et al., 2003). Here we show, using temporal control of neural function in *Drosophila*, that only short-term appetitive memory is reinforced by octopamine. Moreover, octopamine-dependent memory formation requires signaling through dopamine neurons. Part of the octopamine signal requires the α-adrenergic-like OAMB receptor in an identified subset of mushroom-body-targeted dopamine neurons. Octopamine triggers an increase in intracellular calcium in these dopamine neurons, and their direct activation can substitute for sugar to form appetitive memory, even in flies lacking octopamine. Analysis of the β-adrenergic-like OCTβ2R receptor reveals that octopamine-dependent reinforcement also requires an interaction with dopamine neurons that control appetitive motivation. These data indicate that sweet taste engages a distributed octopamine signal that reinforces memory through discrete subsets of mushroom-body-targeted dopamine neurons. In addition, they reconcile previous

findings with octopamine and dopamine and suggest that reinforcement systems in flies are more similar to mammals than previously thought.

Prior work has implicated OA, the invertebrate analog of norepinephrine, as the reinforcing signal in appetitive conditioning in insects. Electrical stimulation of a single octopaminergic neuron, or injection of OA into the honeybee antennal lobes or MB calyx, can replace the presentation of a sucrose reward in olfactory conditioning of the proboscis extension reflex (Hammer, 1993; Hammer & Menzel, 1998). In the invertebrate nervous system OA is synthesized from the amino acid tyrosine via a two-step reaction catalyzed by tyrosine decarboxylase (TDC) and tyramine b-hydroxylase (Tβh) (Cole et al., 2005; Monastirioti et al., 1996). *Tβh* mutant *Drosophila* that lack octopamine cannot form appetitive memory (Schwaerzel et al., 2003). However, the precise role of OA release is currently unknown. The *Tdc2* gene encodes the neuronal TDC and a *Tdc2*-GAL4 can be used to label and manipulate many of the OA neurons in the fly (Cole et al., 2005). Expressing a light-activated trigger in OA neurons enabled replacement of reward with OA neuron activation in olfactory conditioning in larvae (Schroll et al., 2006). In contrast, activation of specific subsets of dopaminergic neurons in larvae and adult flies (Claridge-Chang et al., 2009; Aso et al., 2010) could substitute for negative stimuli in aversive memory formation. These findings led to the prevailing and somewhat surprising model that insect reinforcement signaling was unlike that in mammals, with DA mediating aversive

reinforcement and reward being signaled by OA (Schwaerzel et al., 2003; Heisenberg, 2003).

Recently this separatist view of reinforcement across phyla has been overturned. Although some mammalian midbrain DA neurons encode value-related reward signals (Schultz et al., 1997) others have been identified that are activated by rewarding and aversive stimuli (Matsumoto & Hikosaka, 2009a; Schultz, 2010). In *Drosophila,* mutations in the DopR dopamine receptor disrupt both aversive and appetitive memory (Kim et al., 2007). Furthermore, discrete subsets of DA neurons can convey aversive and appetitive reinforcement in addition to appetitive motivational control (Krashes et al., 2009; Liu et al., 2012; Claridge-Chang et al., 2009; Aso et al., 2010; Waddell, 2010). Therefore in this study we investigated the respective roles of OA and DA in appetitive reinforcement in *Drosophila*.

Results

Octopamine neurons can mediate the short-term reinforcing effects of sweet taste

We first tested whether output from the octopamiergic *Tdc2*-GAL4 expressing neuron was required for appetitive olfactory conditioning with sucrose

reinforcement. OA neurons were blocked throughout the experiment using *Tdc2*-GAL4 driven uas-*shibire*^{ts1} (uas-*shi*^{ts1}) (Kitamoto, 2001). We assayed *Tdc2*-GAL4;uas-*shi*^{ts1} flies in parallel with flies harboring the GAL4 driver or uas-*shi*^{ts1} transgene alone and wild-type flies for comparison (Figure III-1A). All flies were incubated at 31˚C to disrupt neurotransmission from OA neurons for 30min prior to being trained and tested for 3h appetitive memory performance at 31˚C. Surprisingly, no defects were apparent.

Bees form more robust appetitive memory if they are allowed to ingest sucrose rather than just taste it (Wright et al., 2007) and sweet taste and nutrient value both contribute to appetitive memory reinforcement in *Drosophila* (Burke & Waddell, 2011; Fujita & Tanimura, 2011). We reasoned that OA blockade might lack consequence if OA only represents sweet taste and nutrient value provides sufficient reinforcement. To test this model we blocked *Tdc2* neurons while training flies with arabinose, a sweet but non-nutritious sugar (Burke & Waddell, 2011)(Figure III-1B). All flies were incubated at 31˚C for 30min prior to and during training and testing for 3min memory. In this case, memory performance of *Tdc2*-GAL4;uas-*shi*^{ts1} flies was significantly impaired compared to all control groups. Importantly, no significant differences were apparent between groups trained with arabinose and tested at the permissive temperature (Figure III-1C). To further challenge a nutrient bypass model we blocked OA neurons when flies were conditioned with arabinose supplemented with nutritious sorbitol (Burke &

Waddell, 2011). No differences were apparent between the groups (Figure III-1D), similar to blocking OA neurons in flies conditioned with sweet and nutritious sucrose (Figure III-1A). These data are consistent with OA conveying the reinforcing effects of sweet taste only and that reinforcing signals representing nutrient value are sufficient for appetitive learning (Fujita & Tanimura, 2011).

OA neuron stimulation can replace sugar presentation during conditioning to form short-term appetitive memory

To determine whether OA provides instructive reinforcement we conditioned adult flies with odor presentation paired with artificial OA neuron activation, achieved by expressing uas-*dTrpA1* with *Tdc2*-GAL4. *dTrpA1* encodes a Transient Receptor Potential (TRP) channel that conducts Ca^{2+} and depolarizes neurons when flies are exposed to temperatures >25˚C (Hamada et al., 2008). *Ad libitum* fed wild-type, *Tdc2*-GAL4, uas-*dTrpA1* and *Tdc2*-GAL4;uas-*dTrpA1* flies were conditioned by presenting an odor with activating 31˚C, and immediately tested for memory performance (Figure III-2A). *Tdc2*-GAL4;uas-*dTrpA1* flies exhibited robust appetitive memory performance that was statistically different from all other groups (Figure III-2B). Significant memory remained at 30min (Figure III-2C) in satiated flies but performance was statistically indistinguishable from all other groups at 3h, even in hungry flies

(Figure III-2D). Therefore we found that appetitive memory implanted with OA neuron activation is short-lived. *Tdc2*-GAL4 expresses in neurons that contain and could plausibly release tyramine, either alone or together with OA (Cole et al., 2005)(Figure III-5). To confirm that artificial learning requires OA we stimulated *Tdc2* neurons in *Tβh* mutant flies (Monastirioti et al., 1996) that cannot synthesize OA from tyramine (Figure III-2E). No learning was observed suggesting that OA release from *Tdc2* neurons is required for artificial learning, however this does not completely rule out roles for numerous neuropeptides co-expressed in *Tdc2*-GAL4 neurons in mediating aspects of appetitive reward (Nassel, 2002).

Although OA neuron innervation of the MB is relatively sparse in the γ lobe, heel and calyx (Busch et al., 2009) (Figure III-5), prior work suggests that the MB neurons are the likely eventual destination of appetitive reinforcement signals (Liu et al., 2012; Schwaerzel et al., 2003; Heisenberg, 2003; Kim et al., 2007). We therefore used the NP7088, 0665-GAL4 and 0891-GAL4 (Gohl et al., 2011) lines to investigate the role of the four individual classes of OA neurons that innervate the MB (Busch et al., 2009): OA-VUMa2, OA-VPM3, OA-VPM4 and OA-VPM5 (Figure III-2F). NP7088 expression broadly overlaps with *Tdc2*-GAL4 neurons in the brain (Figure III-2G) but does not label OA-VPM5 neurons (Busch et al., 2009). 0665-GAL4 (Gohl et al., 2011) expression is even more restricted and labels the OA-VPM3 and OA-VPM4 neurons (Figure III-2H). Finally, 0891-

GAL4 (Gohl et al., 2011) is the most sparse and only labels OA-VPM4 (Figure III-2I).

Activating these more restricted populations of MB-innervating OA neurons during odor presentation did not form appetitive memory (Figure III-2J). Similarly, blocking any of these populations (NP7088, 0665-GAL4 or 0891-GAL4) using uas-*shibire*^{ts1} did not significantly impair arabinose-reinforced memory (Figure III-6). Importantly, these data suggest that the fly equivalent of the bee VUMmx1 neuron (Hammer, 1993), OA-VUMa2 (Busch et al., 2009), and the other MB-innervating neurons covered by these drivers, are neither sufficient nor essential for conditioned olfactory approach behavior in fruit flies. Instead the data support the notion that either the calyx-innervating OA-VPM5 neurons are critical, or a more distributed OA signal involving other non-MB innervating OA neurons is required for appetitive memory reinforcement, and possibly involve other neurons. This stands in contract to the findings that single DA neurons can mediate aversive signals (Aso et al., 2010; Aso et al., 2012).

Reinforcing DA neurons are functionally downstream of OA-dependent reinforcement

One study has implicated the DopR dopamine receptor in appetitive memory. Flies with the *dumb*^{1} mutation have impaired appetitive memory and the defect can be reversed by restoring DopR receptor expression to the MB (Kim et al., 2007). We therefore tested whether memory formation with OA neuron activation required DopR (Figure III-3A). No significant memory was observed in any group carrying the *dumb*^{1} mutation. Therefore a functional DA system is required to form appetitive memory with OA neuron activation, suggesting that DA is downstream of OA in appetitive memory processes.

A recent study implicated DA neurons in the PAM cluster in appetitive reinforcement (Liu et al., 2012). We independently identified GAL4 lines in the InSITE collection that express in subsets of rewarding PAM-DA neurons (Gohl et al., 2011). 0273-GAL4 and 0104-GAL4 label many neurons innervating the MB and whose cell bodies predominantly lie in the PAM cluster (Mao & Davis, 2009; Tanaka et al., 2008) (Figure III-3B-E, Figure III-7A). Staining 0273-GAL4; uas-mCD8::GFP brains with anti-tyrosine hydroxylase (TH) antibody revealed that 0273-GAL4 expresses in all the approximately 140 DA neurons in the PAM cluster (Figure III-3C). Importantly, 0273-GAL4 does not label DA neurons in the Paired Posterior Lateral 1 (PPL1) cluster that convey negative value (Claridge-

Chang et al., 2009; Aso et al., 2010; Mao & Davis, 2009) (Figure III-7A). 0104-GAL4 expression is more restricted and labels about 40 of the PAM-DA neurons included in 0273-GAL4 (Figure III-3E).

We tested whether 0104-GAL4 and 0273-GAL4 PAM neurons could provide appetitive reinforcement by activating them with uas-*dTrpA1* while presenting an odor during conditioning in satiated flies. Both 0104-GAL4;uas-*dTrpA1* and 0273-GAL4;uas-*dTrpA1* flies exhibited very robust appetitive memory that was statistically different from all control flies (Figure III-3F) and far greater than scores observed with a similar stimulation of OA neurons (Figures III-2, III-3A).

Since 0104-GAL4 more precisely labels reinforcing PAM-DA neurons than 0273-GAL4, we used uas-*shi*^{ts1} to test whether neurotransmission from PAM-DA neurons was required for appetitive learning with sugar reinforcement. The 0104-GAL4;uas-*shi*^{ts1} flies were tested in parallel with those harboring the GAL4 driver or uas-*shi*^{ts1}transgene alone and wild-type flies for comparison. All flies were incubated at 31˚C to disrupt neurotransmission from PAM-DA neurons for 30min prior to and during conditioning with arabinose or sucrose reinforcement. Blocking 0104-GAL4 neurons completely abolished memory performance in arabinose-conditioned flies (Figure III-3G). The initial memory performance of sucrose-conditioned flies was also significantly impaired (Figure III-3H). Moreover, sucrose-conditioned 24h memory was abolished if 0104-GAL4

neurons were only blocked during training (Figure III-7B). Importantly, training and testing the flies at the permissive temperature did not impair performance (Figure III-7C). Therefore PAM-DA neurons, like OA neurons, are critical for conditioning with arabinose but, unlike OA neurons, they also contribute towards the reinforcing effects of nutritious sucrose.

We used PAM-DA neuron activation to further challenge whether DA reinforcing signals are downstream of OA. We artificially conditioned *Tβh* mutant flies that lack OA. Appetitive memory formed in *Tβh* mutant flies with 0104-GAL4;uas-*dTrpA1* or 0273-GAL4;uas-*dTrpA1* was statistically indistinguishable from that formed in the wild-type background (Figure III-3I and Figure III-7D) confirming that DA-mediated reinforcement may be downstream, and can function independently, of OA.

OA-dependent reinforcement functions through discrete groups of DA neurons

To investigate a plausible direct link between OA and DA neurons we tested whether appetitive memory could be formed with OA neuron activation in OA receptor mutant flies. Artificial learning worked effectively in satiated *octβ1R* mutant flies (Figure III-8) but was impaired in hungry *oamb* mutant flies (Han et al., 1998) (Figure III-4A) suggesting a key role for OAMB in OA reinforcement.

We tested for a role of *oamb* in PAM-DA neurons by expressing a uas-*oamb*^{RNAi} transgene (Figure III-9A) with 0104-GAL4 and conditioning flies with arabinose (Figure III-4B). Memory performance of 0104-GAL4;uas-*oamb*^{RNAi} flies was significantly different to that of both control groups. We also tested the same flies conditioned with sucrose. Consistent with prior experiments with OA manipulation, no effect was observed with this nutritious sugar (Figure III-9B). Therefore the OAMB receptor is involved in mediating OA-dependent memory in the PAM-DA neurons.

The OAMB receptor couples to the release of calcium from intracellular stores (Han et al., 1998; Balfanz et al., 2005). We therefore expressed GCaMP3.0 (Tian et al., 2009) in PAM-DA neurons with 0104-GAL4 and assayed intracellular Ca^{2+} responses evoked by application of exogenous octopamine. Octopamine application drove a significant increase in Ca^{2+} signal in PAM-DA neurons that was abolished by pre-exposing the brain to the OA receptor antagonist mianserin (Maqueira et al., 2005; Crocker & Sehgal, 2008) (Figure III-4C and Figure III-10). It is worthwhile to mention however, that we saw the best response using bath concentrations of 5mM OA. Though well outside of physiological levels on the synaptic scale, such concentrations were necessary as one could imagine diffusion across the brain was somewhat hindered and the effective dose reaching receptors in the PAM DA neurons was most likely reduced, though not quantified. Still, these behavioral, anatomical and physiological data are

consistent with OA-dependent reinforcement involving OAMB-directed modulation of PAM-DA neurons.

Studies in *octβ2R* (Maqueira et al., 2005) flies revealed a more nuanced picture for OA-mediated reinforcement. Artificial olfactory learning with OA neuron activation was impaired in satiated *octβ2R/+* heterozygous flies (Figure III-4D) but was restored in *octβ2R/+* flies by food-deprivation (Figure III-4E). These data suggest that OA also integrates with systems that are responsive to hunger to provide instructive reinforcement. Such a role for OA is also highlighted by the observation of memory performance in all prior experiments using satiated flies (Figure III-2, III-3 and III-8).

Our prior work demonstrated that fly neuropeptide F (dNPF) modulates the MB-heel innervating MB-MP1 DA neurons to limit retrieval of appetitive memory performance to hungry flies (Krashes et al., 2009). Artificial learning with OA worked effectively in dNPF receptor mutant flies suggesting that OA functions independently of dNPF (Figure III-11). We therefore tested for a role of Octβ2R in MB-MP1 DA neurons. We used a new *Tdc2*-LexA (Figure III-12) to simultaneously express lexAop-*dTrpA1* in OA neurons and uas-*octβ2R*^{RNAi} in MB-MP1 neurons using c061-GAL4;MBGAL80 (Krashes et al., 2009) (Figure III-4F). Hungry *Tdc2*-LexA;lexAop-*dTrpA1* flies formed robust appetitive memory. However, *Tdc2*-LexA;lexAop-*dTrpA1* flies that also carried c061;MBGAL80;uas-

octβ2R^{RNAi} transgenes to knockdown *octβ2R* expression in MB-MP1 neurons did not display memory performance (Figure III-4F). We independently tested the role of MB-MP1 neurons in OA-mediated reinforcement by simultaneously stimulating OA neurons while disrupting output from MB-MP1 neurons with uas-*shi*^{ts1} (Figure III-13). Flies in which MB-MP1 neurons were simultaneously blocked during artificial conditioning showed no significant memory. Since MB-MP1 neurons can provide aversive reinforcement if artificially engaged during odor presentation (Aso et al., 2010), they likely provide negative influence to the system (Waddell, 2010). Therefore our data indicate that OA-dependent appetitive reinforcement requires Octβ2R modulation of negative DA signals from MB-MP1 neurons in addition to OAMB signaling in positive PAM-DA neurons.

The 0104-GAL4 DA neurons have presynaptic terminals in the tip of MB β´ and γ lobes and presumed dendrites in the anterior medial protocerebrum (AMPR, Figure III-4G, III-4H). We used GFP-reconstituted across synaptic partners (GRASP) (Feinberg et al., 2008; Gordon & Scott, 2009) to investigate plausible sites of synaptic contact between OA neurons and the PAM and MB-MP1 DA neurons. We expressed lexAop-mCD4::spGFP11 with *Tdc2*-LexA and uas-mCD4::spGFP1-10 with 0104-GAL4 or c061-GAL4;MBGAL80. Both of these combinations revealed strong GFP labeling in the AMPR (Figure III-4I and Figure III-14). In addition, MB-MP1 DA neuron:OA GRASP labeled the MB-heel region. The best candidates to bridge these two regions are the OA-VPM4 neurons,

which densely innervate the MB heel and γ lobes and the AMPR (Busch et al., 2009). However OA-VPM4 neurons are cleanly labeled in 0891-GAL4 (Figure III-2I) and included in the 0665 (Figure III-2H) and NP7088 (Figure III-2G) GAL4 lines, all of which were insufficient for appetitive conditioning (Figure III-2J). The rest of the AMPR innervating neurons, OA-VUMa6, OA-VUMa7, OA-VUMa8 and OA-VPM3, are also included in the NP7088 GAL4 labeled population (Busch et al., 2009). Lastly, the MB-calyx innervating OA-VPM5 neurons that are in *Tdc2*-GAL4 but not NP7088 do not have arbors in the AMPR or MB-heel (Busch et al., 2009) so cannot provide direct modulation of PAM or MB-MP1 DA neurons. Our data therefore suggest that reinforcing OA in the fruit fly is provided by a distributed set of neurons, some of which have arbors in the AMPR where they modulate reinforcing PAM and MB-MP1 DA neurons. We speculate that reinforcement through OA also requires simultaneous regulation of other unidentified DA neurons, or involvement of additional parallel modes of OA action.

Discussion

Although OA is a 'trace' amine in mammals and may have its own physiological role (Burchett & Hicks, 2006), OA is largely considered the functional insect analog of noradrenaline. Our data suggest that OA conveys reinforcement by differentially modulating discrete recipient neurons through the OAMB and

Octβ2R receptors. OAMB resembles α-adrenergic receptors being preferentially coupled to Ca^{2+} entry (Han et al., 1998; Balfanz et al., 2005). Studies in mammals have linked α-adrenergic signaling to rewarding DA neurons (Drouin et al., 2002). In contrast, the Octβ2R is most similar to mammalian β-adrenergic receptors acting through cAMP (Maqueira et al., 2005). β-adrenergic signaling has a documented influence on synaptic plasticity and memory in mammals and is a potential avenue for treatment of post-traumatic stress disorder (PTSD) following memory reactivation (Debiec & Ledoux, 2004; Debiec & LeDoux, 2006). The β-adrenergic like Octβ2R appears to play a similar role in the fly brain gating appropriate information flow and memory by modulating specific DA neurons.

Taken with prior work from others (Krashes et al., 2009; Liu et al., 2012; Claridge-Chang et al., 2009; Aso et al., 2010; Mao & Davis, 2009), our data demonstrate that flies have discrete populations of DA neurons representing negative or positive value. This antagonistic organization of populations of DA neurons appears to reflect that in mammals (Schultz, 2010). In addition, some mammalian rewarding DA neurons are also inhibited by aversive cues (Ungless et al., 2004), through neurons in the lateral habenula proposed to code negative prediction error (Matsumoto & Hikosaka, 2007). Our data are consistent with the balance between the two fly DA systems being critical to determine appetitive learning and memory-driven behavior (Waddell, 2010). We propose that OA provides appetitive reinforcement by coordinately regulating activity in the

positively and negatively acting fly DA neuron populations. It will be interesting to determine whether OA influences other behaviors (Crocker & Sehgal, 2008; Koon et al., 2011; Crocker et al., 2010; Certel et al., 2007; Certel et al., 2010) via a similar route and whether they are simultaneously influenced by reinforcing OA signals.

Materials and Methods

Fly strains

Fly stocks were raised on standard cornmeal/agar food at 25°C and 60% relative humidity. The wild-type strain is Canton-S. The *Tdc2*-GAL4, NP7088, uas-*shi*^{ts1} (carrying insertions on the first and third chromosome) and uas-*dTrpA1* flies are described (Cole et al., 2005; Kitamoto, 2001; Hamada et al., 2008; Busch et al., 2009). The uas-mCD8::GFP and uas-mCD8::mCherry strains are those in (Lee & Luo, 1999; Lai & Lee, 2006). The *Tβh*^{nM18}, *oa2*^{f02819}, *octβ2r*^{f05679}, *oamb*^{584} and *dumb*^{1} mutant strains are described (Monastirioti et al., 1996; Kim et al., 2007; Lee et al., 2003; Koon et al., 2011). The MB-MP1 expressing c061-GAL4:MBGAL80 flies are described (Krashes et al., 2009). The *Tdc2-lexA:VP16* transgenic line was generated by cloning the same regulatory region as described previously (Cole et al., 2005) into the pBS_LexA::VP16_SV40 vector (Lai & Lee, 2006). Transgenic flies were raised by standard procedures and lines were screened for those with appropriate expression. The GRASP reporters lexAop-mCD4::spGFP11 and uas-mCD4::spGFP1-10 are described (Gordon & Scott, 2009). The lexAop-*dTrpA1* was constructed by subcloning a *NotI* and *XhoI* restriction site-flanked *dTrpA1* cDNA from pOX-*dTrpA1* (Paul Garrity, Brandeis University) into the pLOT transformation vector (Lai & Lee, 2006). Transgenic flies were commercially generated (BestGene Inc., CA). The uas-*oamb*^{RNAi}(2861GD) and uas-*octβ2R*^{RNAi}(104524KK) were obtained from the

VDRC (Dietzl et al., 2007). The 247-LexA::VP16 flies are described (Pitman et al., 2011). 0104-GAL4, 0665-GAL4, 0891-GAL4 and 0273-GAL4 flies refer to PBac{IT.GAL4}0104, PBac{IT.GAL4}0665, PBac{IT.GAL4}0891 and PBac{IT.GAL4}0273 and were generated within the framework of the InSITE project (Gohl et al., 2011) The lexAop-rCD2::mRFP, uas-Brp::GFP, and uas-DenMark flies are described (Lee & Luo, 1999; Wagh et al., 2006; Nicolai et al., 2010). The uas-GCaMP3.0 flies are described (Tian et al., 2009).

Behavioral analysis

To generate flies to block or stimulate OA neurons we crossed uas-*shibire*^{ts1} or uas-*dTrpA1* female flies to *Tdc2*-GAL4 males. 6-8 day old flies were tested. To block refined subsets of OA neurons we crossed uas-*shibire*^{ts1} female flies to male NP7088/CyO, 0665-GAL4 or 0891-GAL4/TM3 males, and only flies negative for CyO or TM3 were assayed. To stimulate *Tdc2* neurons in the *Tβh* mutant background, *Tβh* ^{nM18}/FM7i-GFP;uas-*dTrpA1* females were crossed with *Tβh* ^{nM18};*Tdc2*-GAL4 males and only progeny negative for FM7i-GFP were assayed. To stimulate *Tdc2* neurons in the *dumb*^{1} mutant background, uas-*dTrpA1*; *dumb*^{1}/TM3 females were crossed with *Tdc2*-GAL4;*dumb*^{1} males and only *dumb*^{1} homozygous flies were assayed. To stimulate 0273-GAL4 or 0104-GAL4 PAM-DA neurons, uas-*dTrpA1* female flies were crossed to 0273-GAL4 or 0104-GAL4/TM6b male flies. To block 0104-GAL4 DA neurons uas-*shibire*^{ts1} females were crossed to 0104-GAL4/ TM6b males. To stimulate 0273-GAL4 or

0104-GAL4 neurons in the *Tβh* mutant background, *Tβh*^{nM18}/FM7i;uas-*dTrpA1* females were crossed with *Tβh*^{nM18};0273-GAL4/TM6b *or* *Tβh*^{nM18};0104-GAL4/TM6b males respectively and progeny negative for FM7i and TM6b were assayed. To stimulate *Tdc2* neurons in the *oa2*, *oamb* and *octβ2R* mutant backgrounds uas-*dTrpA1*; *oa2*^{f02819}/TM3 or uas-*dTrpA1*;*octβ2R*^{f05679}/TM6 or uas-*dTrpA1*;*oamb*^{584}/TM6 females were crossed to *Tdc2*-GAL4;*oa2*^{f02819}/TM3 or *Tdc2*-GAL4;*octβ2R*^{f05679}/TM3 or *Tdc2*-GAL4;*oamb*^{584}/TM3 males, respectively. Only flies homozygous for *oa2* and *oamb* flies were assayed. The *octβ2R* insertion is homozygous lethal so only heterozygous *octβ2R*/+ flies were assayed. To express *oamb*^{RNAi} in 0104-GAL4 neurons, we crossed uas-*oamb*^{RNAi}(2861GD) females to 0104-GAL4/ TM6b males and only flies lacking TM6b were assayed. To express *octβ2R*^{RNAi} in MB-MP1 neurons while stimulating*Tdc2* neurons, we crossed c061-GAL4;MBGAL80;*Tdc2*-LexA/TM3 females to uas-*octβ2R*^{RNAi}(104524KK);lexAop-*dTrpA1*/TM3 males. To express *shibire*^{ts1} in MB-MP1 neurons while stimulating *Tdc2* neurons, we crossed c061-GAL4;MBGAL80;*Tdc2*-LexA/TM3 females to uas-*shi*^{ts1};lexAop-*dTrpA1*/TM3 males. To stimulate *Tdc2* neurons in the *npfr1*^{c01896} mutant background female uas-*dTrpA1*;*npfr1*^{c01896} flies were crossed to *Tdc2*-GAL4;*npfr1*^{c01896} males. Heterozygous control flies were generated by crossing the respective uas-transgene flies with wild-type flies. For the *oa2*, *octβ2R*, *oamb*, *dumb*^{1} and *npfr1* experiments, *Tdc2*-GAL4;(mutant) or uas-dTrpA1;(mutant) flies were flies mutant at the same locus to generate heterozygous transgene controls within the

relevant homozygous mutant background. Controls for MB-MP1 neuron manipulation were generated by crossing c061-GAL4;MBGAL80;*Tdc2*-GAL4/TM3 or uas-*shi*^{ts1};lexAop-*dTrpA1*/TM3 or uas-*octβ2R*^{RNAi}(104524KK);lexAop-*dTrpA1*/ TM3 females to wild-type flies.

Mixed sex populations were tested together in all behavior experiments unless genotype required sorting single sexes. Hungry state experiments involved food depriving flies for 18–20h before training in milk bottles containing a damp filter paper. To test flies in the satiated state, flies were food-deprived 14-16h, then transferred into fresh bottles containing food to satiate 4h before training. The olfactory appetitive paradigm was performed as described (Krashes & Waddell, 2008) with the following modifications: For neural blockade experiments using uas-*shi*^{ts1}, flies were incubated at 31˚C for 30min prior to and during training and testing for 3min memory. For permissive temperature experiments flies were kept at 23˚C at all times. For memory implantation experiments using uas-*dTrpA1*, flies were presented with one odor at the permissive temperature 23˚C for 2min in filter paper-lined tubes. They were then transferred into a new prewarmed filter paper-lined tube and immediately presented with a second odor at the activating 31˚C for 2min. Flies were then returned to 23˚C and tested for immediate memory. To test 3h memory flies were trained as above and stored in plastic vials containing dampened filter paper until testing. For 24h memory experiments, flies were trained as above and stored in food vials for 3h followed

by 21h of food-deprivation before testing. Odors were 3-octanol (9.2 µl in 8 ml mineral oil) or 4-methylcyclohexanol (18 µl in 8 ml mineral oil). The performance index (PI) was calculated as the number of flies running toward the conditioned odor minus the number of flies running toward the unconditioned odor divided by the total number of flies in the experiment. A single PI value is the average score from flies of the identical genotype tested with each odor.

Statistical analyses were performed using PRISM (GraphPad Software). Overall analyses of variance (ANOVA) were followed by planned pairwise comparisons between the relevant groups with a Tukey HSD post-hoc test.

Imaging

To visualize native GFP, mRFP or mCherry adult female flies were collected 2-10 days after eclosion (1 day for GRASP flies) and brains were dissected in ice-cold 4% paraformaldehyde solution in PBS (1.86 mM NaH2PO4, 8.41 mM Na2HPO4, 175 mM NaCl) and fixed for an additional 60-120 min at room temperature under vacuum. Samples were washed 3X10 min with PBS containing 0.1% Triton-X100 (PBT), and 2X in PBS before mounting in Vectashield (Vector Labs).

For immunohistochemistry, brains were fixed and washed as described above, followed by overnight incubation on a shaker in 10% normal goat serum (NGS) at 4˚C. For staining using the Tβh antiserum (Koon et al., 2011), brains were dissected in ice-cold PBS, then fixed in undiluted Bouin's solution for 20 minutes.

Samples were washed 3X10 min with PBST as above. The anti- Tβh antiserum and anti-nc82 antibody were added to a final dilution of 1:200 (Koon et al., 2011). An anti-tyrosine hydroxylase (TH) antibody raised in rabbit (AB152, Millipore) was added to a final dilution of 1:200 and kept in same conditions for another three days. After washing with PBT, Alexa594-coupled goat anti-rabbit antibody (A-11037, Invitrogen) was added 1:200 for one more night, followed by washing and embedding as described before.

Imaging was performed on a Zeiss LSM 5 Pascal confocal microscope and a Leica TCS SP5 X. Images were processed in AMIRA 5.2 (Mercury Systems). In some cases, debris on the brain surface and/or antennal and gustatory nerves were manually deleted from the relevant confocal sections to permit construction of a clear projection view of the z-stack.

Live imaging

Up to 7 day old uas-GCaMP3.0;0104-GAL4 flies were anaesthetized on ice and waxed to a custom imaging chamber. The head capsule was opened under 800 µl of sugar-free HL3-like saline (Yoshihara, 2012), and the whole preparation transferred under a SliceScope microscope (Scientifica). Epifluorescence images were acquired using a Pike CCD camera (Allied) at a rate of 3 images/s at one set gain. The spontaneous baseline GCaMP3.0 response was imaged for 30s, then either 100 µl saline or 100 µl mianserin (12.5 mM, Sigma-Aldrich, filtered)

were added to the bath. After another 30 s, 100 µl octopamine (50 mM, Sigma-Aldrich) or 100 µl octopamine (50 mM) + mianserin (12 mM, filtered) were added as before, to reach a final bath concentration of 5 mM octopamine and 2.45 mM mianserin, respectively. After registration of images (StackReg plugin) a standardized region of interest (ROI) was centered within the area of the MB β‘ lobe tip (Figure III-4C). Image processing and analysis was performed with Fiji / ImageJ 1.4. Intensity tables were exported to Excel and the (ΔF – F) / F was calculated, with an F consisting of the averaged first 24 images. Traces were generated in Prism 6 (GraphPad Software). Respective peak intensities within 5 s after saline/octopamine/mianserin application were selected and compared to other groups for significant differences.

Real-Time PCR

Total RNA from adult fly heads was isolated with Trizol (Invitrogen) and cleaned with RNeasy Micro Kit (Qiagen) with DNAse I treatment. RNA (200 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and oligo(dT)12-18. The cDNA was used for quantitative real-time PCR with ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) with standard cycling parameters (2 min at 50 °C, 10 min at 95 °C, and 45 alternate cycles of 15 s at 95 °C and 60 s at 60 °C). The PCR mixture contained TaqMan® Gene Expression Master Mix and the appropriate Gene Expression Assay (Applied Biosystems). TaqMan qPCR assays were ordered for

OAMB (AB: Dm02150048m_1). GAPDH (AB: Dm01841185_m1) was used as an endogenous control for normalization (ΔCT value). The increase in expression (ΔΔCT value) was calculated and transformed to the exponential scale.

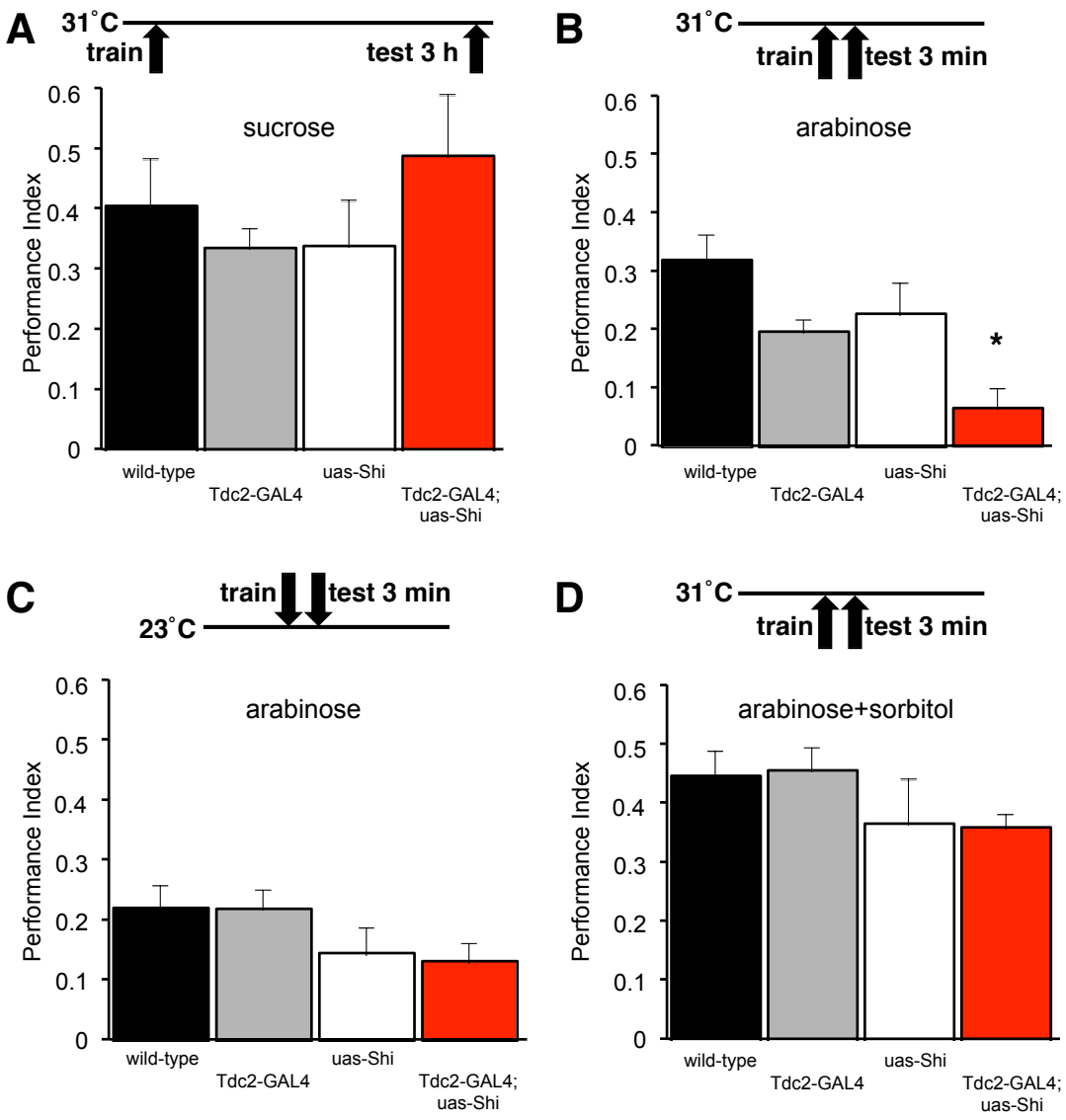


Figure III-1. Octopamine mediates the short-term reinforcing effects of sweet taste.

(**A)** Blocking OA neurons with *Tdc2*-GAL4/ uas-*shi*^{ts1} during conditioning with sucrose has no consequence on 3 h appetitive memory, (all $p>0.4$, $n\geq6$).

(**B)** Blocking OA neurons during conditioning with arabinose significantly impairs appetitive learning, ($p<0.05$, $n\geq10$).

(**C)** No significant defects are observed when flies are conditioned with arabinose at the permissive 23˚C, (all $p>0.1$, $n\geq8$).

(**D)**, Blocking OA neurons during conditioning with arabinose supplemented with nutritious sorbitol has no significant effect on appetitive learning (all $p>0.4$, $n=8$). All behavioral data are mean ± standard error of the mean (SEM). Asterisks denote significant difference between marked groups and all others (all $p<0.05$, ANOVA).

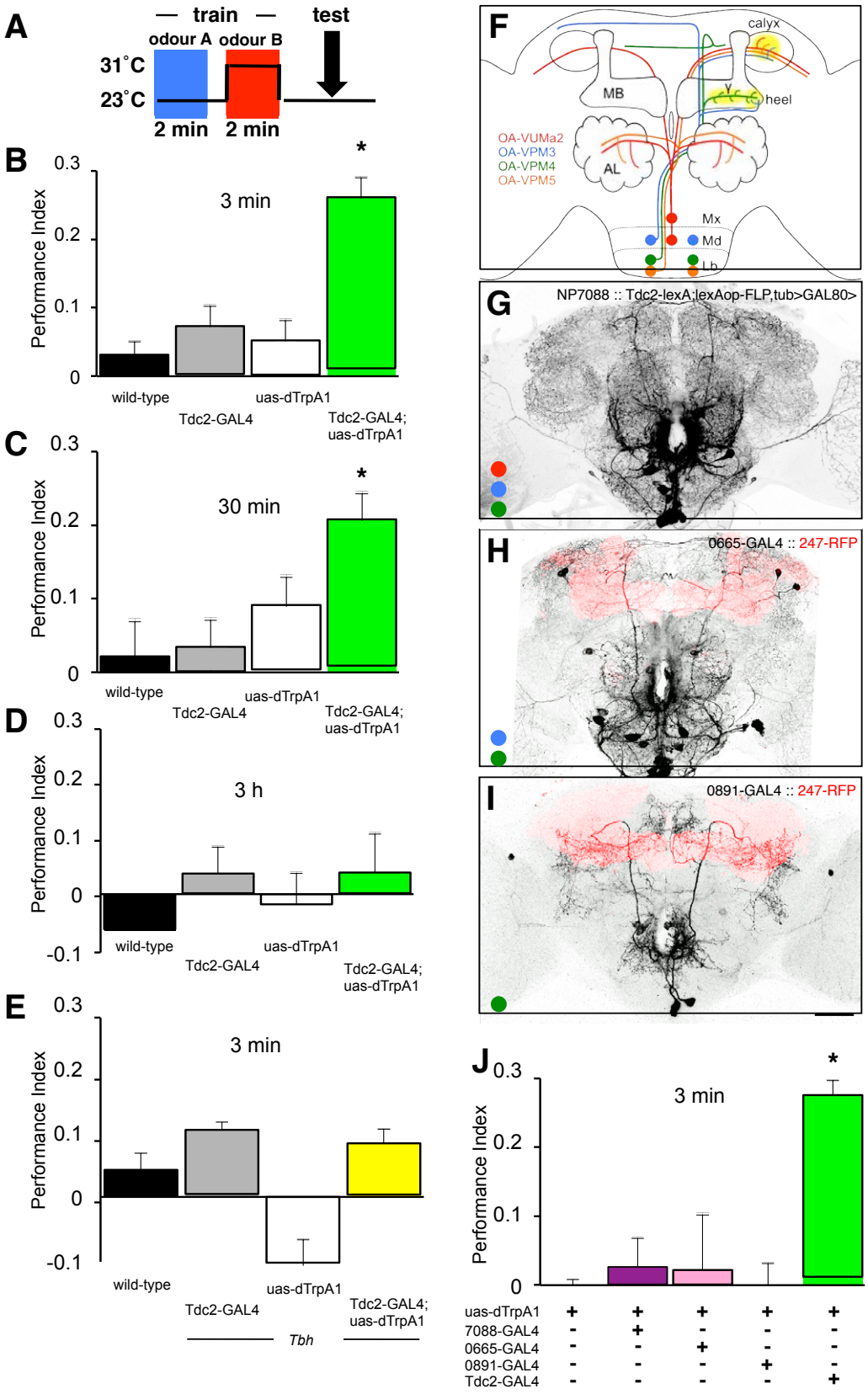

A
train
test
odour A
odour B
31˚C
23˚C
2 min
2 min
B
3 min
Performance Index
0.3
0.2
0.1
0
*
wild-type
Tdc2-GAL4
uas-dTrpA1
Tdc2-GAL4;
uas-dTrpA1
C
30 min
D
3 h
-0.1
E
Tbh
F
calyx
MB
γ
heel
OA-VUMa2
OA-VPM3
OA-VPM4
OA-VPM5
AL
Mx
Md
Lb
G
NP7088 :: Tdc2-lexA:lexAop-FLP,tub>GAL80>
H
0665-GAL4 :: 247-RFP
I
0891-GAL4 :: 247-RFP
J
uas-dTrpA1
7088-GAL4
0665-GAL4
0891-GAL4
Tdc2-GAL4

Figure III-2. OA neuron stimulation can replace sugar presentation during conditioning to form short-term appetitive memory.
(A) Conditioning protocol pairing a 2 min odor presentation with heat-activation (red) of uas-*dTrpA1* expressing neurons.
(B) *Tdc2*-GAL4/uas-*dTrpA1* driven OA neuron activation contingent with odor presentation forms appetitive olfactory memory in satiated flies ($p<0.001$, $n\geq14$).
(**C)** *Tdc2*-GAL4 neuron implanted appetitive memory remains significant 30 min after training in satiated flies ($p<0.05$, $n\geq8$).
(D) No appetitive memory is observed 3 h after training, even in hungry flies ($p>0.5$, $n=6$).
(E) Implanting memory with *Tdc2*-GAL4 neuron stimulation requires OA. Artificial conditioning does not form significant memory in hungry *Tβh* mutant flies ($p>0.05$, $n\geq8$).
(F) Schematic of all four types of OA neurons that innervate the mushroom body (MB) in either the calyx (OA-VUMa2, OA-VPM5, plus the antennal lobe, AL), heel (OA-VPM4, plus the MB γ lobe), or both calyx and heel (OA-VPM3). Neurons arise from either the maxillary (Mx), mandibulary (Md), or labial (Lb) neuromere in the ventral brain.
(G) NP7088-GAL4 expresses in many *Tdc2* positive OA neurons in the brain. Projected view of a brain showing OA neurons that are common to *Tdc2*-GAL4/*Tdc2*-LexA and NP7088-GAL4 revealed by genetic intersection. The MB-OA cell types included in each line are color-coded.
(H) 0665-GAL4 labels the MB-innervating OA-VPM3 and OA-VPM4 neurons in the brain; 247-RFP labeled MB is shown in red for reference.
(I) 0891-GAL4 specifically labels the MB-innervating OA-VPM4 neurons in the brain. Scale bar 50 μm.
(J) Stimulating subsets of OA neurons cannot replace sugar presentation in appetitive conditioning. ($p>0.05$, $n\geq6$).

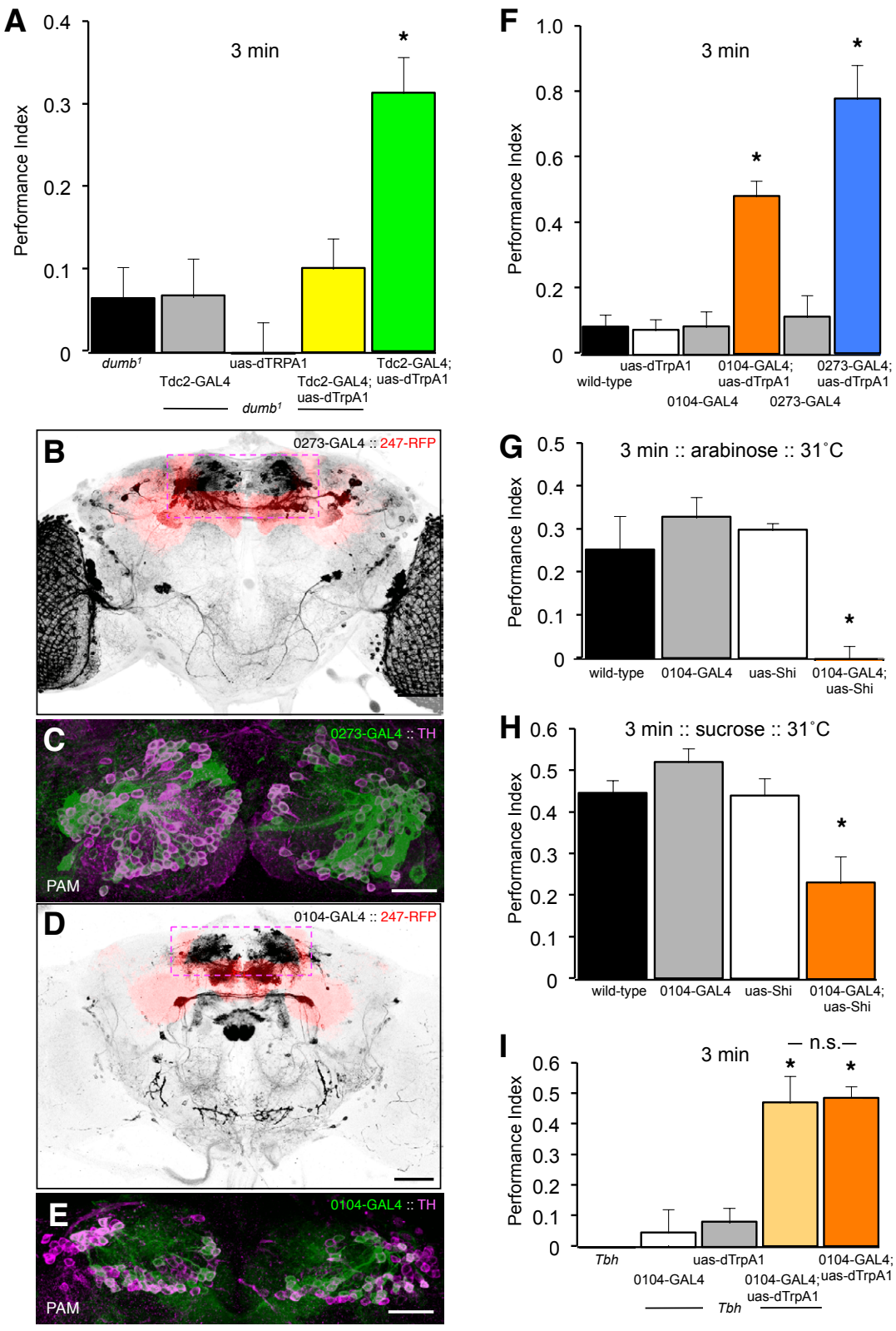

A
3 min
Performance Index
0.4
0.3
0.2
0.1
0
*
dumb1
Tdc2-GAL4
uas-dTRPA1
Tdc2-GAL4; uas-dTrpA1
dumb1
B
0273-GAL4 :: 247-RFP
C
0273-GAL4 :: TH
PAM
D
0104-GAL4 :: 247-RFP
E
0104-GAL4 :: TH
PAM
F
3 min
Performance Index
1.0
0.8
0.6
0.4
0.2
0
*
*
wild-type
uas-dTrpA1
0104-GAL4
0104-GAL4; uas-dTrpA1
0273-GAL4
0273-GAL4; uas-dTrpA1
G
3 min :: arabinose :: 31˚C
Performance Index
0.5
0.4
0.3
0.2
0.1
0
*
wild-type
0104-GAL4
uas-Shi
0104-GAL4; uas-Shi
H
3 min :: sucrose :: 31˚C
Performance Index
0.6
0.5
0.4
0.3
0.2
0.1
0
*
wild-type
0104-GAL4
uas-Shi
0104-GAL4; uas-Shi
I
3 min
— n.s.—
Performance Index
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
Tbh
0104-GAL4
uas-dTrpA1
0104-GAL4; uas-dTrpA1
Tbh

Figure III-3. Reinforcing DA neurons are functionally downstream of OA-dependent reinforcement.

(A) Memory cannot be implanted with *Tdc2* neuron stimulation in *dumb*^{1} (DopR) mutant flies (all $p>0.05$, except *Tdc2*-GAL4/uas-*dTrpA1* control $p<0.001$, $n\geq8$).

(B) 0273-GAL4 labels DA neurons in the PAM cluster (dashed box) that innervate the MB (red).

(C) 0273-GAL4 labels all ~140 TH-positive DA neurons in the PAM cluster.

(D) 0104-GAL4 labels DA neurons in the PAM cluster (dashed box) that innervate the MB (red).

(E) 0104-GAL4 labels ~40 TH-positive DA neurons in the PAM cluster. Scale bar 50 μm (b and d), 20 μm (c and e).

(F) Robust appetitive memory can be implanted with 0104-GAL4 and 0273-GAL4 neuron activation contingent with odor presentation. Three minute memory performance of 0104-GAL4;uas-*dTrpA1* and 0273-GAL4;uas-*dTrpA1* flies is significantly different from all other groups ($p<0.01$, $n\geq4$).

(F) Blocking DA neurons with 0104-GAL4/uas-*shi*^{ts1} during conditioning with arabinose abolishes appetitive learning ($p<0.001$, $n\geq8$).

(H) Blocking 0104-GAL4 DA neurons during conditioning with sucrose significantly impairs appetitive learning, ($p<0.05$, $n\geq6$). **i**, 0104-GAL4 neuron stimulation can form robust appetitive memory in satiated *Tβh* mutant flies ($p<0.001$, $n\geq6$).

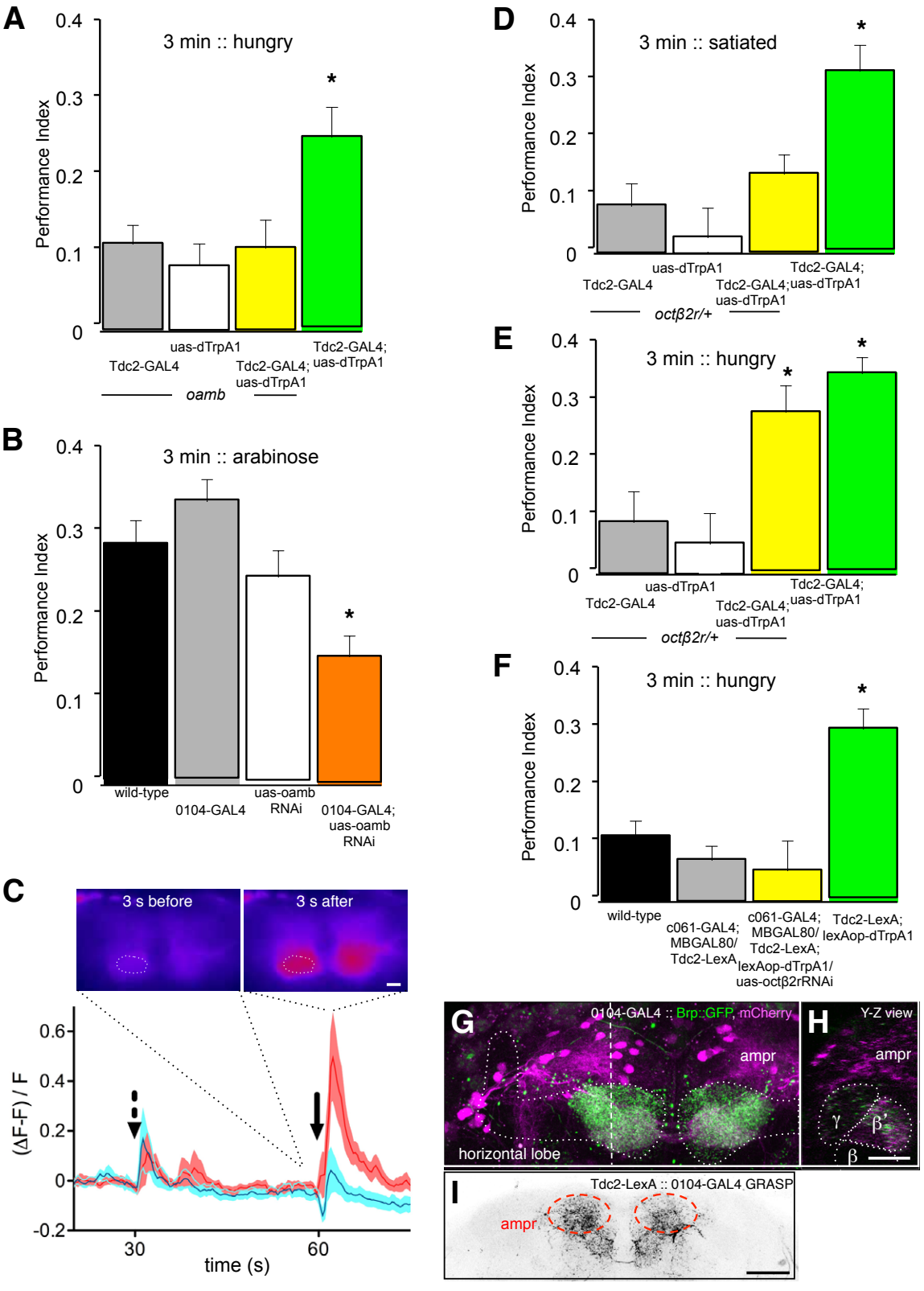

A
3 min :: hungry
Performance Index
0.4
0.3
0.2
0.1
0
*
Tdc2-GAL4
uas-dTrpA1
Tdc2-GAL4; uas-dTrpA1
Tdc2-GAL4; uas-dTrpA1
oamb
B
3 min :: arabinose
Performance Index
wild-type
0104-GAL4
uas-oamb RNAi
0104-GAL4; uas-oamb RNAi
C
3 s before
3 s after
(ΔF-F) / F
0.6
0.4
0.2
0
-0.2
30
60
time (s)
D
3 min :: satiated
Performance Index
Tdc2-GAL4
uas-dTrpA1
Tdc2-GAL4; uas-dTrpA1
Tdc2-GAL4; uas-dTrpA1
octβ2r/+
E
3 min :: hungry
Performance Index
Tdc2-GAL4
uas-dTrpA1
Tdc2-GAL4; uas-dTrpA1
Tdc2-GAL4; uas-dTrpA1
octβ2r/+
F
3 min :: hungry
Performance Index
wild-type
c061-GAL4; MBGAL80/ Tdc2-LexA
c061-GAL4; MBGAL80/ Tdc2-LexA; lexAop-dTrpA1/ uas-octβ2rRNAi
Tdc2-LexA; lexAop-dTrpA1
G
0104-GAL4 :: Brp::GFP, mCherry
ampr
horizontal lobe
H
Y-Z view
ampr
γ
β'
β
I
Tdc2-LexA :: 0104-GAL4 GRASP
ampr

Figure III-4. OA-dependent reinforcement functions through discrete groups of DA neurons.

(A) Memory cannot be implanted with *Tdc2* neuron stimulation in hungry *oamb* mutant flies. Only the *Tdc2*-GAL4/uas-*dTrpA1* flies display significant learning ($p<0.01$, $n\geq8$).

(B) Appetitive memory formation with arabinose reinforcement requires *oamb* in 0104-GAL4 neurons. Immediate memory of 0104-GAL4;uas-*oamb*^{RNAi} flies is significantly different to both control groups ($p<0.05$, $n\geq18$). The uas-*oamb*^{RNAi} causes an approximately 40% decrease in *oamb* transcript levels (Supplementary Fig. 5A).

(C) Application of 5 mM OA to the exposed living fly brain drives an increase in intracellular Ca^{2+}, measured using GCaMP3.0, in reinforcing 0104-GAL4 DA neurons. The OA-evoked response (red trace) is significantly decreased in brains treated with 2.45 mM of the OA antagonist mianserin (blue trace, see also Supplementary Fig. 6). First dotted arrow marks time of application of mianserin or vehicle. Solid arrow marks application of OA or OA together with mianserin. Traces are averaged (*n* = 11 animals each) with the solid line representing the mean and the shaded areas showing the s.e.m. Panels above show representative pseudocolored images of fluorescence intensity 3 s before application (left) and 3 s after OA application (right). The dotted circle represents the region of interest used for analysis. Scale bar: 10 µm.

(D) Memory cannot be implanted with *Tdc2* neuron stimulation in satiated *octβ2R*/+ heterozygous mutant flies ($p>0.05$ for all groups, except *Tdc2*-GAL4/uas-*dTrpA1*, $p<0.05$, $n=8$).

(E) Memory can be implanted with *Tdc2* neuron stimulation in hungry *octb2R*/+ heterozygous mutant flies. Performance of the *Tdc2*-GAL4/uas-*dTrpA1* flies and *Tdc2*-GAL4/uas-*dTrpA1*; *octβ2R*/+ flies is significantly different to all other groups ($p<0.05$, $n\geq6$).

(F) Memory cannot be implanted with *Tdc2* neuron stimulation in flies that also express uas-*octβ2R*^{RNAi} in MB-MP1 neurons (all $p>0.05$ except *Tdc2*-GAL4/uas-*dTrpA1* control, $p<0.001$, $n\geq8$). The efficacy of uas-*octβ2R*^{RNAi} has been reported previously[42].

(G) 0104-GAL4 driven co-expression of mCherry (magenta) and the presynaptic marker Bruchpilot::GFP (Brp::GFP, green) reveals exclusive presynaptic label in the tip region of the horizontal MB lobes. Structures not labeled by Brp::GFP in the anterior median protocerebrum (ampr) above the MB lobes are likely to be dendritic arbors of these PAM-DA neurons.

(H) Y-Z section (at the level of the dashed line in b) reveals Brp::GFP expression is refined to the b' and g lobe tips. Scale bar: 20 µm. i, GRASP indicates plausible synaptic contact between OA neurons and 0104-GAL4 DA neurons in the anterior medial protocerebrum (ampr, dashed circles). Scale bar: 50 µm

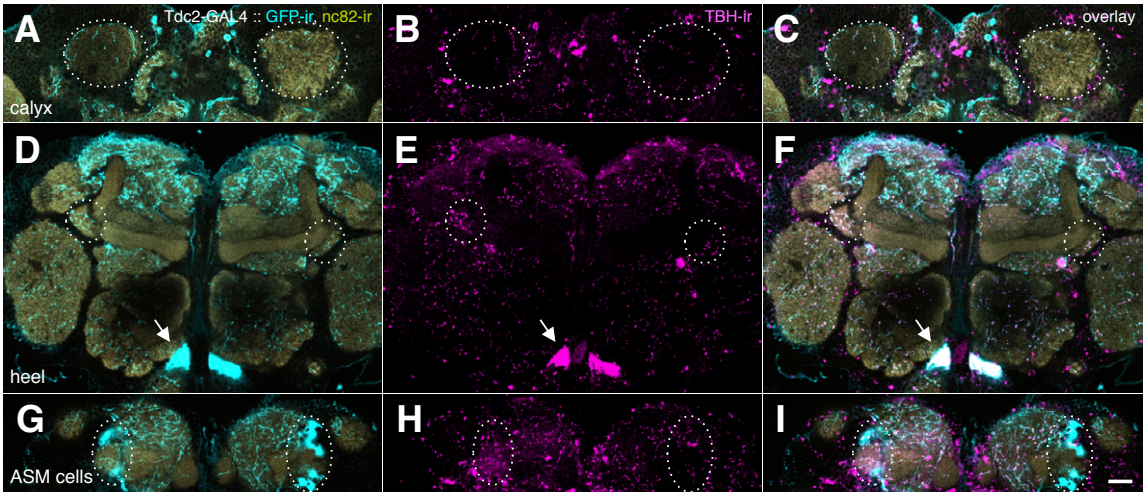
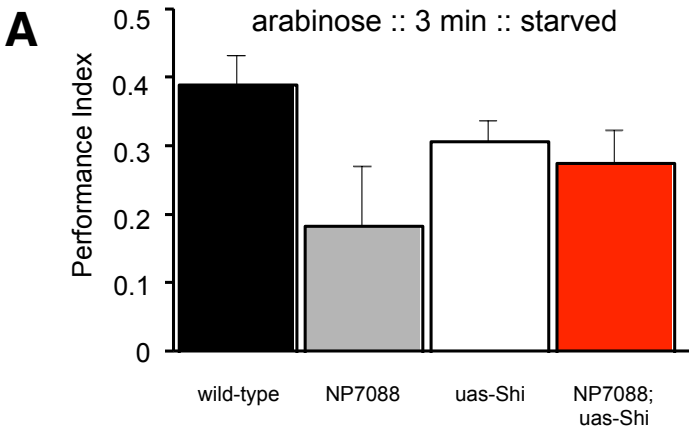
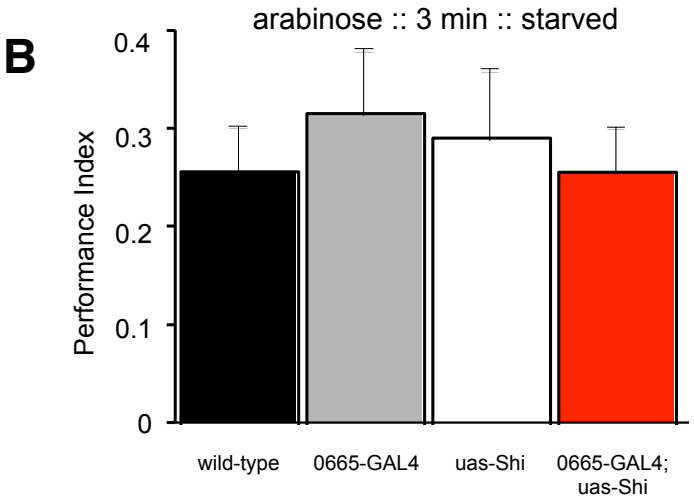


Figure III-5. Tdc2-GAL4 labels all OA neurons that innervate the MB.
Tdc2-GAL4: uas-CD8::GFP brain counter-stained with anti-GFP antibody (cyan), anti- Tβh antiserum (magenta), anti-nc82 antibody for neuropil reference (yellow), and overlay. GFP and Tβh staining patterns reveal very sparse labeling of the MB structure by OA neurons with innervation being restricted to the calyces (**a-c**, dashed circles), heels (**d-f**, dashed circles), and γ lobes (not shown). The absence of anti- Tβh label throughout the rest of the MB structure strongly suggests that no additional MB extrinsic neurons produce OA. Most described OA cells exhibit overlap with anti- Tβh labeling, as for example AL2 cells (**d-f**, arrows). Lack of overlap between Tdc2-GAL4 and Tβh is evident in a few cells consistent with them releasing tyramine but not OA. Of particular note in this regard are the cells in the ASM cluster (**g-h**). Scale bar: 20 μm.


A
arabinose :: 3 min :: starved
Performance Index
0.5
0.4
0.3
0.2
0.1
0
wild-type
NP7088
uas-Shi
NP7088;
uas-Shi


B
arabinose :: 3 min :: starved
Performance Index
0.4
0.3
0.2
0.1
0
wild-type
0665-GAL4
uas-Shi
0665-GAL4;
uas-Shi

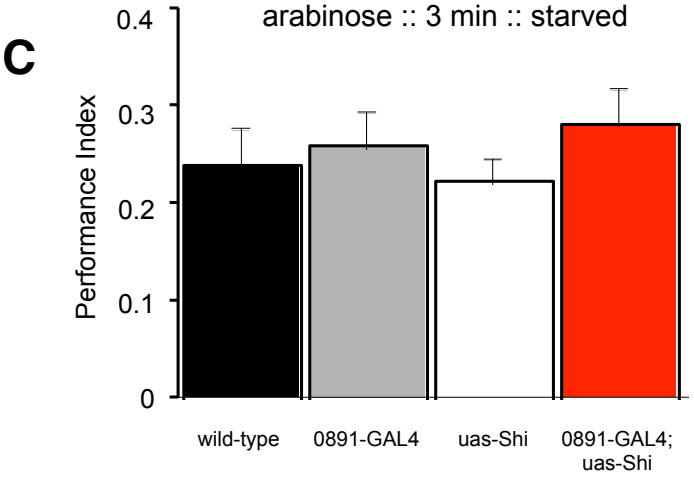
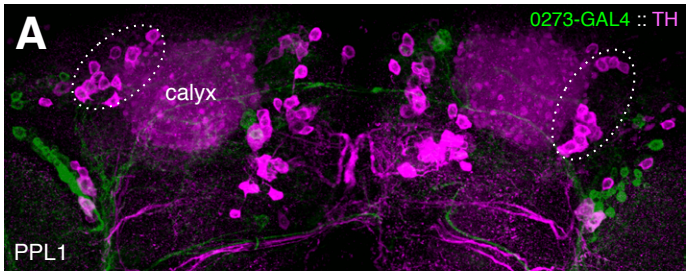
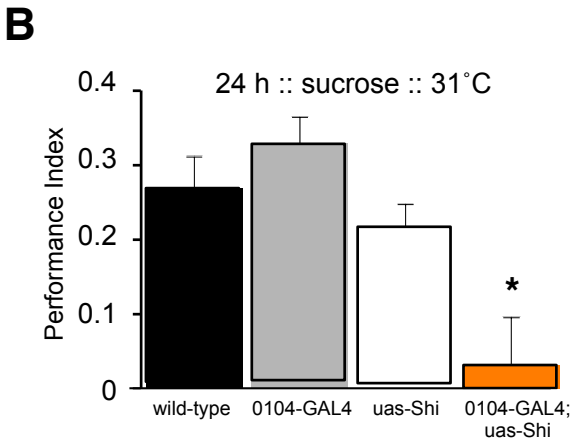
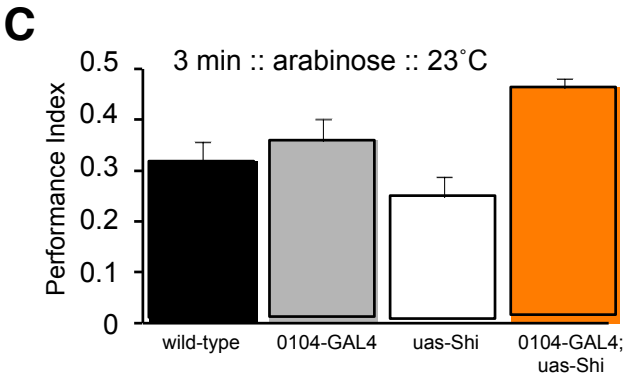

C
arabinose :: 3 min :: starved
Performance Index
0.4
0.3
0.2
0.1
0
wild-type
0891-GAL4
uas-Shi
0891-GAL4;
uas-Shi

Figure III-6. The NP7088, 0665-GAL4 and 0891-GAL4 subsets of OA neurons are not critical for arabinose reinforced memory. Blocking OA neurons with **(A)** *NP7088*-GAL4/ uas-*shi*^{ts1},**(B)** 0665-GAL4/ uas-*shi*^{ts1} or **(C)** 0891-GAL4/ uas-*shi*^{ts1} during conditioning with arabinose has no consequence on 3 min appetitive memory, (all $p>0.08$, $n\geq6$).


A
0273-GAL4 :: TH
calyx
PPL1


B
24 h :: sucrose :: 31˚C
Performance Index
0.4
0.3
0.2
0.1
0
*
wild-type
0104-GAL4
uas-Shi
0104-GAL4; uas-Shi


C
3 min :: arabinose :: 23˚C
Performance Index
0.5
0.4
0.3
0.2
0.1
0
wild-type
0104-GAL4
uas-Shi
0104-GAL4; uas-Shi

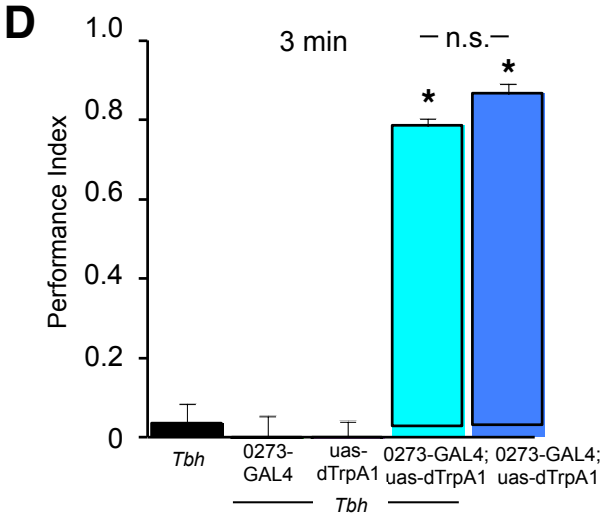

D
3 min
– n.s.–
*
*
Performance Index
1.0
0.8
0.6
0.4
0.2
0
Tbh
0273-GAL4
uas-dTrpA1
0273-GAL4; uas-dTrpA1
0273-GAL4; uas-dTrpA1
Tbh

Figure III-7. Detailed anatomy and additional behavioral analysis of neurons labeled by 0104-GAL4 and 0273-GAL4 lines.
(A) 0273-GAL4 (green) does not label TH-positive DA neurons (magenta) in the PPL1 cluster (dashed circles) that sit next to the MB calyx.
(B) 24h appetitive memory is abolished when 0104-GAL4/ uas-*shi*^{ts1} flies are conditioned with sucrose at 31˚C (all $p<0.05$, $n\geq8$).
(C) No significant defects are observed when 0104-GAL4/ uas-*shi*^{ts1} flies are conditioned with arabinose at the permissive temperature of 23˚C (all $p>0.1$, $n\geq8$).
(D) 0273-GAL4 neuron stimulation can form robust appetitive memory in satiated *Tβh* mutant flies ($p<0.001$, $n\geq6$).

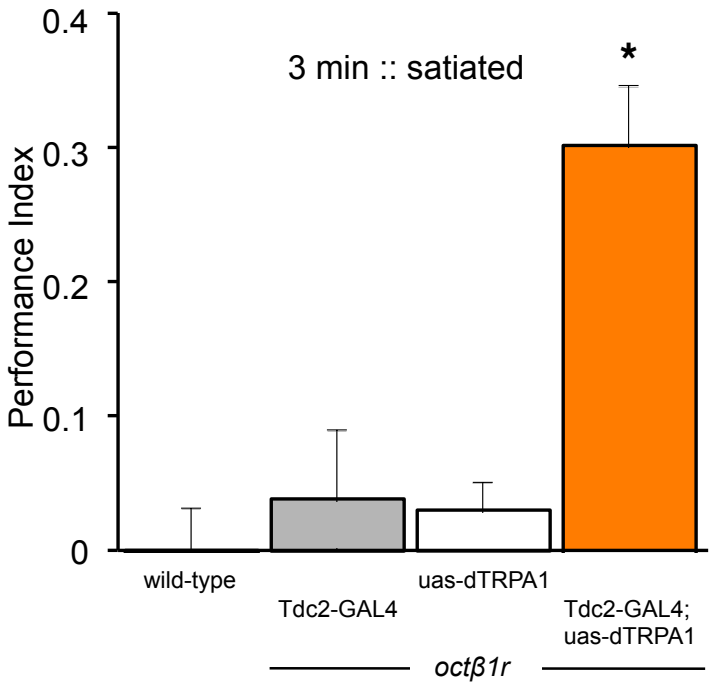


Figure III-8. Memory implantation with OA neuron stimulation does not require the *octβ1r* gene.
Tdc2-GAL4 neuron stimulation forms robust appetitive memory in satiated *octβ1r* mutant flies ($p<0.001$, $n\geq11$).

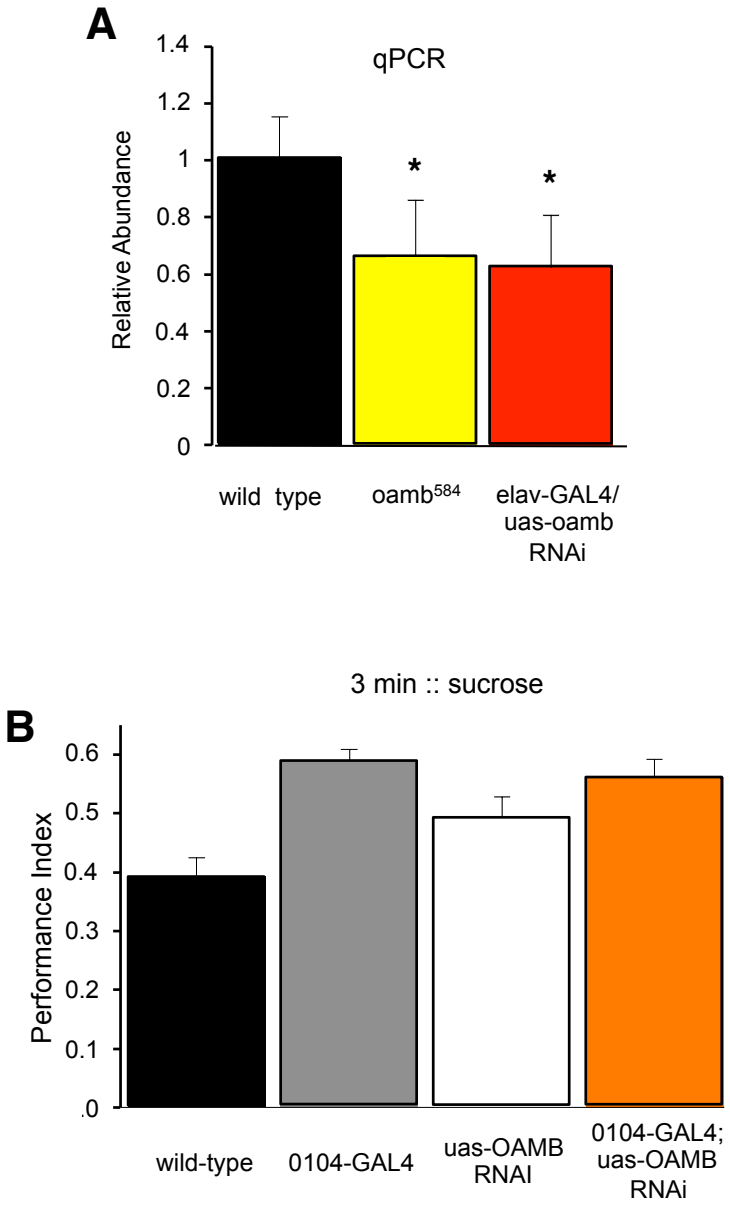


Figure III-9. Confirmation of the efficacy of the uas-oambRNAi transgene and sucrose control.

(A) Quantitative RT-PCR of OAMB transcripts from wild-type, *oamb*^{584} mutant and Elav-GAL4/ uas-*oamb*^{RNAi} fly heads. Values normalized to those from wild-type flies. Asterisks denote significant decrease in abundance from wild-type samples, ($p<0.01$, T-test, n=6).

(B) Knockdown of OAMB in 0104-GAL4 DA neurons has no consequence for appetitive learning when conditioning using nutrient-rich sucrose ($p>0.05$, n=6).

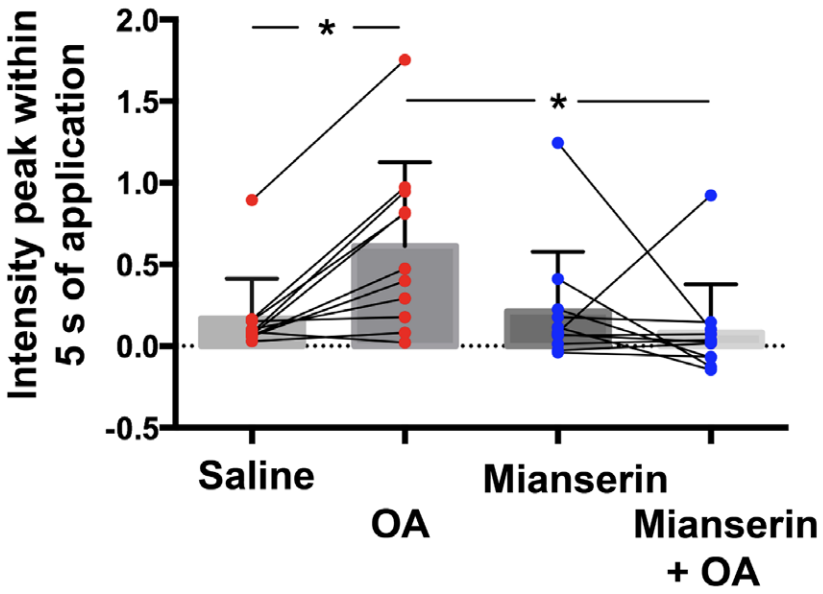


Figure III-10. Application of OA to the exposed living fly brain drives an increase in intracellular Ca^{2+}, measured using GCaMP3.0, in reinforcing 0104-GAL4 DA neurons.

More detail of the data represented in Figure III-4C. Application of 5 mM OA to the exposed living fly brain drives an increase in intracellular Ca^{2+}, measured using GCaMP3.0, in reinforcing 0104-GAL4 DA neurons. The OA-evoked response is significantly decreased in brains pre-treated with 2.45 mM of the OA receptor antagonist mianserin (mianserin + OA, $P < 0.01$, $U = 15$, Mann-Whitney U-test). OA application elicits different responses to saline application ($P < 0.05$, $U = 24$, Mann-Whitney U-test). Responses to mianserin and mianserin + OA ($P > 0.05$, $U = 37$, Mann-Whitney U-test) are not statistically different. Lines connect datapoints corresponding to the same individual fly ($n = 11$ each).

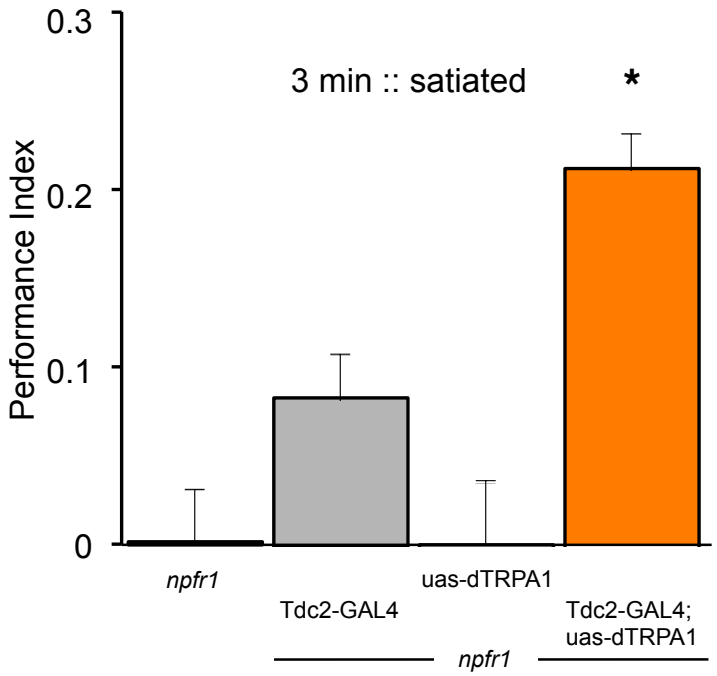


Figure III-11. Memory implantation with OA neuron stimulation does not require the *npfr1* gene.
Tdc2-GAL4 neuron stimulation forms robust appetitive memory in satiated *npfr1* mutant flies ($p<0.05$, $n\geq8$).

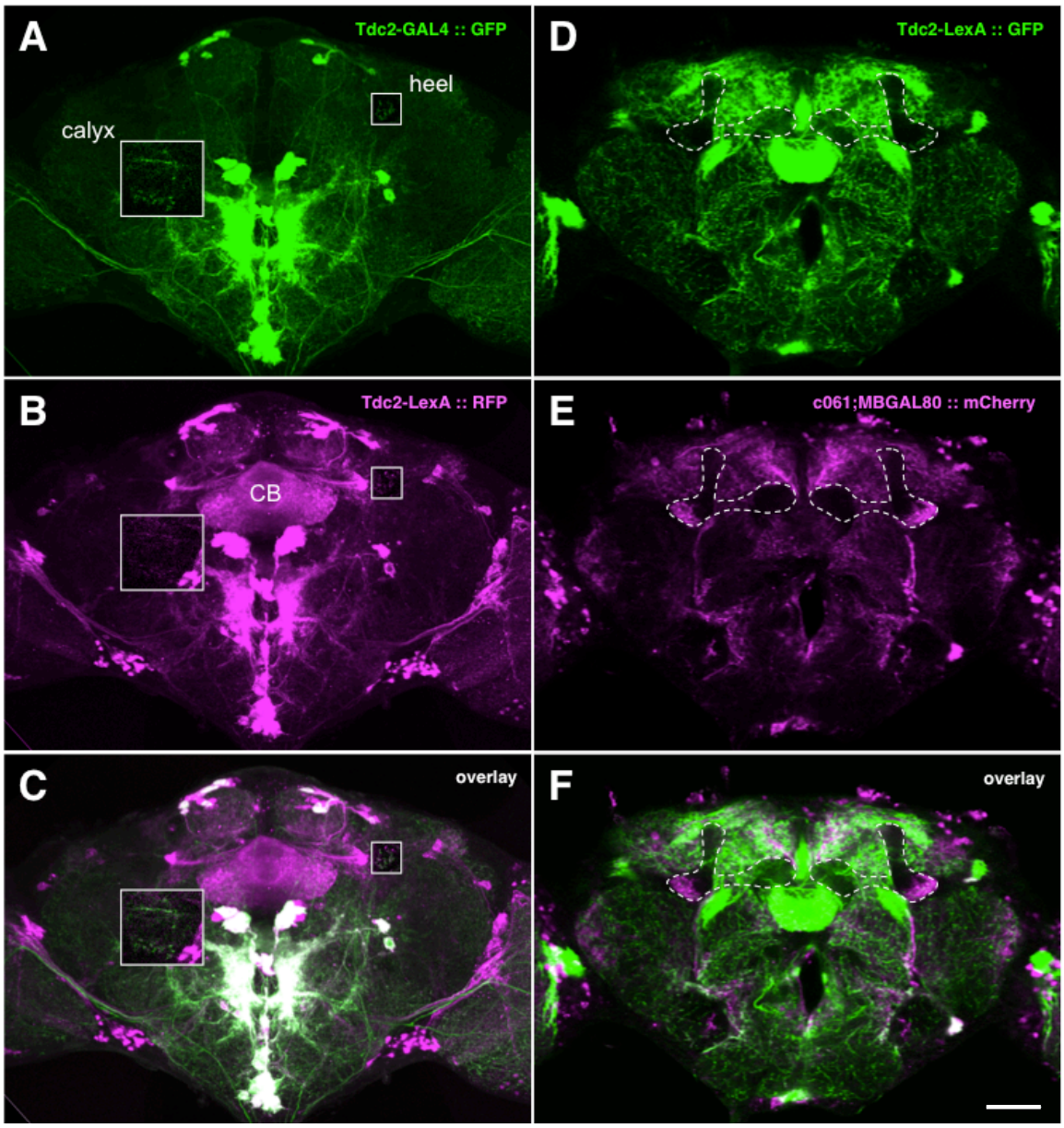

A
Tdc2-GAL4 :: GFP
heel
calyx
D
Tdc2-LexA :: GFP
B
Tdc2-LexA :: RFP
CB
E
c061;MBGAL80 :: mCherry
C
overlay
F
overlay

Figure III-12. Tdc2-LexA recapitulates Tdc2-GAL4 expression and allows simultaneous analysis of OA and specific DA neurons.
(A) Whole brain projection of *Tdc2*-GAL4 expression of uas-mCD8::GFP (green), including single sections at the level of the MB calyx and heel (insets).
(B) *Tdc2*-LexA expression of lexAop-rCD2::mRFP (magenta) resembles the *Tdc2*-GAL4 pattern, with notable additional labeling in posterior central complex (CB) neurons.
(C) merge of Tdc2-GAL4 and Tdc2-LexA patterns.
(D-F) OA and MB-MP1 DA neurons innervate the heel region of the MB lobes (dashed line). Single confocal sections at the level of the heel from a c061-GAL4;MBGAL80/lexAop-mCD8::GFP;*Tdc2*-LexA/uas-mCD8::GFP fly brain. **d**, OA neurons in the *Tdc2*-LexA channel (green), **f**, MB-MP1 neurons in the c061-GAL4;MBGAL80 channel (magenta), **e**, merge of **d** and **f** showing that OA neuron processes are intermingled with DA processes. Scale bar 50 µm.

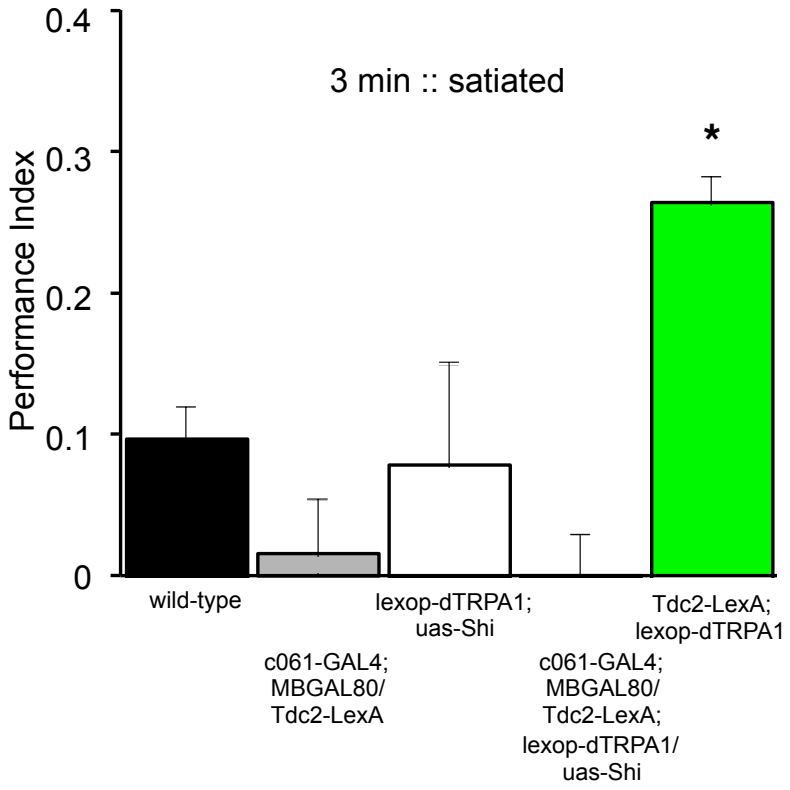


Figure III-13. Transmission from MB-MP1 DA neurons is required for OA-dependent appetitive memory formation. Memory cannot be implanted with *Tdc2*-GAL4 neuron stimulation in satiated flies if MB-MP1 neuron output is simultaneously disrupted with uas-*shi*^{ts1} (all $p>0.05$ except *Tdc2*-GAL4/uas-*dTrpA1* control $p<0.001$, $n\geq8$).

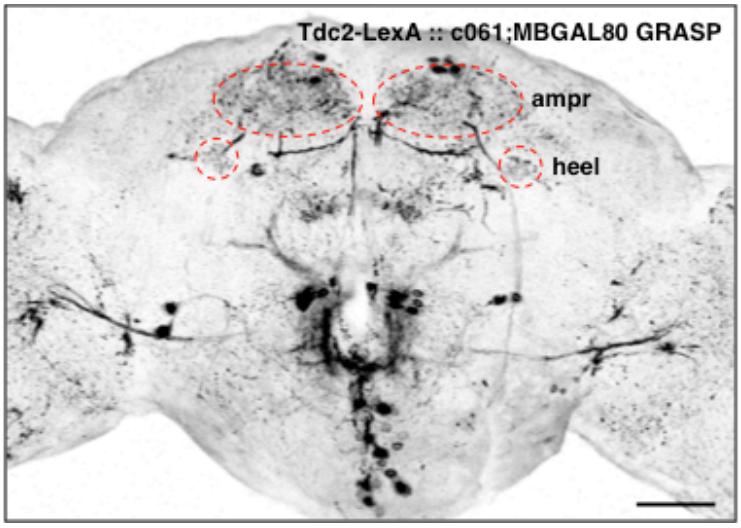


Figure III-14. GRASP reveals sites of putative contact between OA and MB-MP1 DA neurons.
OA and DA neuron processes make close contact in the heel (dashed circle) and the anterior median protocerebrum (ampr, dashed ellipse). A single confocal section at the level of the MB heel from a c061-GAL4;MBGAL80/lexAop-mCD4::spGFP11;*Tdc2*-LexA/uas-mCD4::spGFP1-10 brain showing GRASP between OA and MB-MP1 DA neurons. Scale bar 50 µm.

Chapter IV: Discussion

How we evaluate our experiences in the environment in which we live often determines how successful we will be within it. Being able to determine what's good, better and best from bad, worse and just downright terrible is essential to survival. Qualitative analysis of our world involves not only our immediate sensory perceptions on a moment to moment basis, but also evaluation over time, leading to a remembered experience of the benefit or harm of various stimuli. Were this not the case, we would probably never eat nutritious broccoli or Brussels sprouts, or consume high fat and sugar-rich foods without regard to the damage it does to our bodies in the long run. What might seem aversive at first may in fact have long-term benefit, while initial instant gratification may leave you unfulfilled in the future. Our internal systems of reward and punishment must be able to appropriately assign value to our actions and interactions. As not all aversive or attractive stimuli are of equal valance, developing separate and parallel methods to signal different aspects of reward or punishment is highly beneficial for efficient processing. Separate parallel systems of reward processing for both the hedonic sweet taste and the underlying nutritive value of sugars have been observed in humans, rats, mice and locusts (Smeets et al., 2005; Chambers et al., 2009; de Araujo et al., 2008; Sclafani, 2001; Ackroff et al., 2009; Simpson & White, 1990; Raubenheimer & Tucker, 1997). My own work

has shown that a similar system of organization is present in our model organism of choice, *Drosophila melanogaster*.

But what is the neurological nature of reward? With a host of genetic tools, techniques and technology becoming more sophisticated, it would at first seem reasonable to assume that we have the ability to shed light on what makes a particular stimulus rewarding in the brain. However, as soon as we take into account the complexity of human emotional response, personal preference, cultural and environmental influences, individuality and our prior experiences, it becomes clear that finding something as direct as how all pleasure is registered and evaluated in the human brain becomes a gargantuan task. For example, how might one reconcile individuals with what might be termed a “masochistic” inclination, in which pain, which by all natural means should be avoided at all costs, its registered as rewarding, and sought out? Likewise, in the case of addiction, individuals seek out rewarding actions or stimuli despite often (rather subjectively) disproportionally severe negative consequences. Moreover, how might something as subjective and varied as emotional pleasure or reward differ from physical pleasure or reward in how it is represented in the brain? In the studies presented in this thesis, we examined but one aspect of reward in response to food using the fruit fly. And while the findings were fruitful in aiding in our understanding of a relatively simplistic neural reward circuit, they can by no

means explain the intricate details of what we term pleasurable or rewarding in human behavior and in the brain.

Layered Reward in Drosophila, Part I: A Refined Palate

The study presented in Chapter II took a somewhat ethological approach to the question of how sugar reward learning is processed in the fruit fly brain. We sought to better understand how and ultimately why the sugar sucrose, long used in appetitive olfactory conditioning in the fly as reward (Tempel et al., 1983; Schwaerzel et al., 2003; Krashes & Waddell, 2008; Colomb et al., 2009), acted as such a potent reinforcer of memory both short and long term. By conditioning flies using sugars of differing nutritional properties, we found that the sweet tasting but non-nutritive sugars arabinose or xylose could drive only STM formation. Conversely, a post-ingestive nutrient signal provided by ingestion of sucrose, fructose or the nutritious but tasteless sorbitol or maltodextrin was required for the formation of appetitive LTM. Our definition of "sweetness" was determined by the flies' performance in the PER assay, which measures a fly's acceptance of a substance. Though a few studies have examined the fly's ability to discriminate between different sugars of similar nutritional profile (Masek & Scott, 2010), it remains to be seen whether there are other properties of these sugars being coded by the brain by unknown mechanisms. Are there subtle but distinct differences in how the fly "tastes" sucrose or fructose?

Likewise, our use of the term “nutritious” in this study refers only to the ability of each sugar or compound to sustain fly life as the sole source of food. There are inevitably more aspects to nutrition in the food selection of a fly such as the presence of amino acids, lipids, minerals and even water content. The systems of water sensation (Cameron et al., 2010) and a mechanism by which flies modulate preference for amino acids (Ribeiro & Dickson, 2010; Toshima & Tanimura, 2012; Itskov & Ribeiro, 2013) have been recently described in the fly, and it would be interesting to speculate whether appetitive conditioning using other macronutrients may show similar features as we observe using sugars.

Our finding that arabinose-trained flies performed poorly for 24hr memory was not dependent on amounts ingested during olfactory conditioning (Fig II-1E and II-2E), nor due to any negative effects on health (Fig. II-5B) but rather, appetitive LTM performance was based solely on the consumption of nutritional content by the fly. The practical implication of this finding being that in nature, it would seem more beneficial for an organism to accurately recall a food source that provided life-sustaining nutrient rather than just one that tasted sweet, but lacked nutrient completely. While a situation such as this might be exceedingly rare in nature, this mechanism might aide a fly in selecting a food source with the highest nutritional benefit over others.

Furthermore, flies can rapidly discriminate between sweetness alone and a sweet and nutritious compound based on nutrient content, showing strong preference behavior within 2 minutes. The rapidity with which the fly can sense the presence or absence of nutrient in two equally sweet compounds is quite remarkable, and might be of further advantage to the fly's foraging strategy, as less time spent evaluating and sampling food means less time being exposed and vulnerable to predation.

Layered Reward in Drosophila, Part 2: Octopamine and Dopamine

We next took an interventionist approach to explore what neural circuit mechanisms mediated these parallel systems of reward in the fly brain, building upon our previous findings. Blocking *Tdc2*-GAL4 OA neurons with *shibire*^{ts1} while training flies with either sweet or sweet and nutritious sugars revealed that OA represents the rewarding effects of sweet taste only, and that nutrient reward signals which drive appetitive LTM function independently of OA. Consistent with the finding that "sweet taste conditioning" forms only STM, stimulation of OA neurons was sufficient to drive robust 2 minute memory in the absence of sugar reward, but the memory did not persist, suggesting an alternate pathway for nutrient signaling. Analysis of OA subsets which innervate the MB revealed that unlike the DA system in which single neurons can drive aversive behavior, a more distributed OA signal was required for appetitive memory formation.

One key caveat of blockade experiments using *shibire* which must be mentioned is that shibire blocks vesicle recycling, and might have non-neuronal consequences for cells at the restrictive temperature. Crippling normal vesicular transport might significantly disrupt a host of metabolic functions and negatively affect the overall health of the cell. While our experiments blocking only during the acquisition phase reveals normal expression of memory post blockade, suggesting normal cellular function when returned to the permissive temperature, the possibility remains that our "blocked" neurons might still show some altered neuronal function not detected in our assay.

Furthermore, the initial studies characterizing the shibire flies (Koenig et al., 1983) were done at the neuromuscular junction. To date, there has not been an exhaustive study of a similar effect of prevention of vesicle recycling within an intact CNS. This may be why we don't necessarily see a complete disruption of appetitive memory performance when blocking certain subsets of neurons in the fly brain, suggesting incomplete blockade of neurotransmission. The dynamin protein acts as a tetramer whose assembly acts to pinch off budding clatherin-coated vesicles during endocytosis (Muhlberg et al., 1997). As such, it has been suggested that manipulations of dynamin such as those in our shibire experiements only disrupt transmission involving neurotransmitters packaged in such vesicles, and not dense core vesicles which may house larger neuropeptides (Nassel, 2002). Furthermore, activation of either 0273 or 0104-

GAL4 neurons by dTrpA1 stimulation could also plausibly lead to co-release of expressed neuropeptides alongside the neurotransmitter dopamine (Yew et al., 2009). As such, it is hard to completely rule out any secondary roles for these neuropeptides during appetitive conditioning.

In the manipulation of any system in an intact organism, compromises must be made, and no approach is without caveats, secondary effects and potential pitfalls for misinterpretation. Behavioral testing using populations of animals is inherently noisy and often slight day to day variation in performance scores was found to be quite common. To counteract this variation, we performed each experiment with side-by-side testing of age-matched positive (and negative where applicable) control flies. Rather than assaying absolute levels of learning, in this way we can determine behavioral performance relative to daily controls. This allows us to make more confident conclusions of our data when manipulating neural circuits to disrupt or drive appetitive behavior.

dTrpA1, while a very powerful tool to investigate the instructive role of neural circuits, is often regarded as a "sledgehammer" approach. Studies ectopically expressing uas-*dTrpA1* in the NMJ found firing rates of action potentials in excess of 100Hz when prep temperatures approached 29˚C (Hamada et al., 2008). This prolonged, sustained and high frequency stimulation of neurons by *dTrpA1* may result in neural activity outside of physiological levels, making

interpretation more complicated. However, despite this aberrant stimulation of the neurons, in our experiments, we do see what appears to be a specific response when stimulating during odor presentation. One approach we have considered but not yet investigated would be to perform a behavioral "dose response curve" monitoring appetitive learning performance scores as a function of increased temperature during conditioning. If we were to see increasing performance scores as temperature (and therefore, firing rate) increased, we could be more confident of our heat-activated stimulation results. Likewise, a rigorous analysis of coincident pairing and reverse training using neuronal stimulation (US) and odor (CS) during conditioning might also shed light on how closely direct stimulation can mirror the effects of sugar-odor conditioning

OA-implanted memory was found to be dependent on the dDA1 dopamine receptor, suggesting a link between OA and rewarding DA systems. Stimulation of DA neurons residing in the PAM cluster also produced robust appetitive memory that was able to persist well past 24hrs, even in *Tβh* mutant flies lacking OA. Therefore, rewarding DA is functionally downstream and can act independently of OA. Furthermore, blockade of PAM DA neurons using *shibire*^{ts1} significantly impaired both sweet as well as sweet and nutritive conditioned appetitive memory. Our finding that DA acts as a final arbiter of appetitive reward was both surprising given the previous literature on DA mediating punishment in the fly, but also made sense in light of years of work linking DA to

reward in mammalian systems. OA-implanted memory requires the α-adrenergic like OA receptor OAMB, and knockdown of *oamb* in PAM DA neurons significantly impaired sweet but not sweet and nutrient learning in flies, strongly suggesting that OA functions through DA to provide sweet taste reinforcing signals. Furthermore the β-adrenergic receptor OCTβ2R involvement is likely to intersect the motivational state element of appetitive memory, as we were able to implant memory with OA in starved, but not fed *octβ2r/+* heterozygous mutant flies. These divergent roles for two OA receptors provides an explanation for one of our initial findings, that memory could be implanted with OA stimulation in both food-deprived as well as satiated flies. This finding suggested that in addition to providing instructive reward information, OA stimulation acts to relieve the fly of the usual satiation based restraint on appetitive memory performance (Krashes et al., 2009).

Finally, a live-imaging approach using GCaMP showed that application of OA to the exposed fly brain evoked a significant increase in intracellular Ca^{2+} in rewarding PAM DA neurons. These data lead us to propose a new model for reward learning in *Drosophila* in which OA represents the reinforcing of sweet taste through the OAMB receptor in a subset of rewarding DA neurons that innervate the MB. In addition, these rewarding DA neurons integrate post-ingestive nutrient reward signals required to reinforce appetitive LTM (Figure IV-1).

While this system of parallel reward through OA and DA has only been demonstrated to mediate sugar driven reward, it is tempting to speculate whether the same circuitry is used to code for other rewarding stimuli. Could other nutrients such as water, protein, lipids or even specific minerals/ salts provide reward in nutrient-deprived flies using a similar mechanism? And what of the fly's other pleasurable pursuits? The act of mating in male flies has been shown to be highly rewarding and can be used as the US in associative olfactory conditioning of memory (Shohat-Ophir et al., 2012). Could a similar arrangement of OA and PAM DA neurons mediate this reinforcement, or might another set of DA neurons mediate these rewards?

OA has been implicated in modulating a host of other behaviors in the fly (Certel et al., 2007; Crocker & Sehgal, 2008; Crocker et al., 2010; Lee et al., 2003; Koon et al., 2011). It is not unreasonable to question whether OA also exerts its influence in these behaviors through various DA subsets, or perhaps through other modulatory systems such as serotonin (5HT). OA, 5HT and DA have been implicated in the selection/initiation, escalation and continuation of aggressive behaviors respectively, in male flies (Certel et al., 2007; Certel et al., 2010; Alekseyenko et al., 2010; Alekseyenko et al., 2013), though an interaction of these aminergic systems in mediating these behaviors has yet to be investigated.

Chasing the elusive nutrient reward signal

For all our knowledge of how fleeting sweet taste is mediated in both the periphery and in the brain, our understanding of the mechanism by which the post-ingestive nutrient reward signal remains largely incomplete. It is also a rather compelling subject, as typically the first question I'm asked after presenting this work is: "So what's this nutrient signal?"

While there is a vast and ever growing literature on the subjects of neuronal and humeral mechanisms underlying behaviors of feeding, food seeking, appetite and satiety, the exact mechanism by which the fly senses nutrient content to relay reward value is unknown. As our model for appetitive reinforcement integrates OA signaling through PAM DA neurons to mediate sweet taste, might another neuronal or peptidergic signal converge on these DA neurons to convey nutrient reinforcement?

Previous studies on larval feeding behavior found that overexpression of *Drosophila* neuropeptide F (dNPF) prolonged feeding in larvae and overexpression of its receptor *npfr1* could drive satiated larvae to consume bitter laced food typically avoided by WT controls (Wu et al., 2003; Wu et al., 2005). dNPF is considered the insect ortholog of mammalian neuropeptide Y (NPY), long associated with stimulation of feeding, and whose levels were found to increase in response to starvation (Sanacora et al., 1990). From this data, it

seemed plausible that inhibition of dNPF signaling might encode satiety and therefore represent ingestion of nutrient. A recent study has found quite the opposite effect, that stimulation of *dNPF*-GAL4 neurons is sufficient to drive appetitive LTM, but only if multiple spaced training cycles are employed (Shohat-Ophir et al., 2012). Stimulation of *dNPF*-GAL4 neurons was also found to disrupt ethanol-conditioned reward learning and NPF levels increased in response to ethanol consumption, suggesting that dNPF might not only gate food reward memory expression as in (Krashes et al., 2009), but also code for appetitive reward itself in ethanol conditioning (Shohat-Ophir et al., 2012).

Another neuropeptide candidate which may mediate nutrient information is that encoded by the gene *hugin* (Bader et al., 2007). *Hugin* is the insect analog of neuromedin U also associated with feeding in mammals, but having the opposite effect of NPY. Whereas injection of neuromedin U into the hypothalamus of rats results in a marked reduction in feeding and subsequent weight loss, *neuromedin U* knockout mice display hyperphagia and increased body weight (Ivanov et al., 2002; Hanada et al., 2004; Budhiraja & Chugh, 2009). Overexpression of *hugin* in flies using *tubulin*-GAL4 causes reduced food consumption in larvae and slows growth (Melcher & Pankratz, 2005). In adults, neuronal blockade of *hugin*-GAL4 neurons using tetanus toxin promotes increased initiation of feeding (Melcher & Pankratz, 2005). These data suggest that *hugin* neurons may convey nutritional information to the brain to inhibit feeding behaviors at a point of satiety.

However, stimulation of *hugin*-GAL4 neurons coincident with arabinose conditioning failed to drive LTM formation (data not shown), suggesting *hugin* signaling, though clearly involved in regulation of feeding, does not appear to convey nutrient reward information to the brain to guide appetitive memory.

Recent studies have identified internal molecular sensors for several sugars. Work from Miyamoto et al. revealed that a particular gustatory receptor, Gr43a, present in all major peripheral taste organs is also selectively expressed within 6 neurons in the adult brain, and these neurons are specifically responsive to the sugar fructose (Miyamoto et al., 2012). Furthermore, the investigators found that stimulation of Gr43a neurons during conditioning with odor was sufficient to drive mild appetitive memory formation in starved flies, whereas in fed flies an aversive response was seen (Miyamoto et al., 2012). Though the data points to Gr43a as an internal sensor of nutrient by way of monitoring hemolymph fructose, my own manipulations using this line showed it to be ineffective in driving LTM, nor could its knockout disrupt sucrose-conditioned LTM (data not shown).

Dus et al. found expression of the sodium/glucose co-transporter like *cupcake* in the R4 neuron of the ellipsoid body. *cupcake* expression, as well as output from R4 cells is required for flies to select food in a preference assay based on nutrient content independent of taste (Dus et al., 2013). However, as output from these neurons was dispensable for associative spatial learning, it is possible the

cupcake containing R4 neurons may not play a role in mediating nutrient reward signals required for associative olfactory memory, though future work is warranted.

Furthermore, our lack of identification of a neuronal nutrient signal may suggest that the signal itself, is non-neuronal in nature. It is therefore worth considering a possible role for *Drosophila* glial cells in providing a nutrient signal to reward centers in the brain. Based on the ability for glia to provide neurotrophic support during synapse development and maintenance in adult brains (Clarke and Barres, 2013), its possible a post-ingestive nutrient signal my be mediated though glial:neuron interactions.

Additional evidence points to adipokinetic hormone (AKH) as another candidate which might signal ingestion of a nutritious substance. A recent study showed that stimulation of AKH neurons only at the time of testing for 4hr sucrose-conditioned memory could suppress appetitive performance, effectively satiating the flies and reducing their motivational drive (Gruber et al., 2013). While an attractive candidate to pursue in mediating nutrient signals, it brings about a fundamental question of our search: will a satiating signal which prevents appetitive feeding behavior also be able to code for ingested nutrient reward? As seen from the study of Gerber et al. it is critical that flies be either food deprived or artificially brought to a proper motivated state by neural stimulation in order to

see appetitive performance. It is therefore quite possible that stimulation of a nutrient signal may satiate flies during training, masking associative learning and result in a lack of appetitive memory performance. As such, the search for the "nutrient reward neuron" is ongoing still.

Final Remarks

In conclusion, the work present here represents a significant contribution to the field of *Drosophila* learning and memory and to our understanding of the organization of the brain as well. A system of layered reward in flies that employs dopamine reconciles a long discordant theory of reward between mammals and insects; where OA signaled for reward while DA mediated punishment. The complexity with which the fly brain functions to process 2 stages of food reward, both sweet taste via OA and sweet and nutrient value through DA suggests that the *Drosophila* model will be of great importance to future endeavors of neuroscience and ultimately our understanding of our own brains.

As a final remark, while the work presented in the dissertation reveals a complex and nuanced model for reward learning in *Drosophila*, it is always worth remembering that it is but one facet of the complete picture of what drives motivated behavior. I'm confident that in the years to come, future studies will

build upon and elucidate these findings further still, coming one step closer to the truth of how things work. However, while our knowledge of the systems governing reward in flies and in humans grows with each new discovery, each model presented will inevitably fall short of explaining in full detail all that we are. Even if we crack the code of memory and can map out experience dependent changes in neuronal circuitry down to the last synapse, will it tell us why we are who we are? While seemingly pessimistic at first, this lack of knowledge and absence of definitive answer I believe to be actually hopeful, as it is what has always driven scientific pursuit in the first place and will continue to do so in the future.

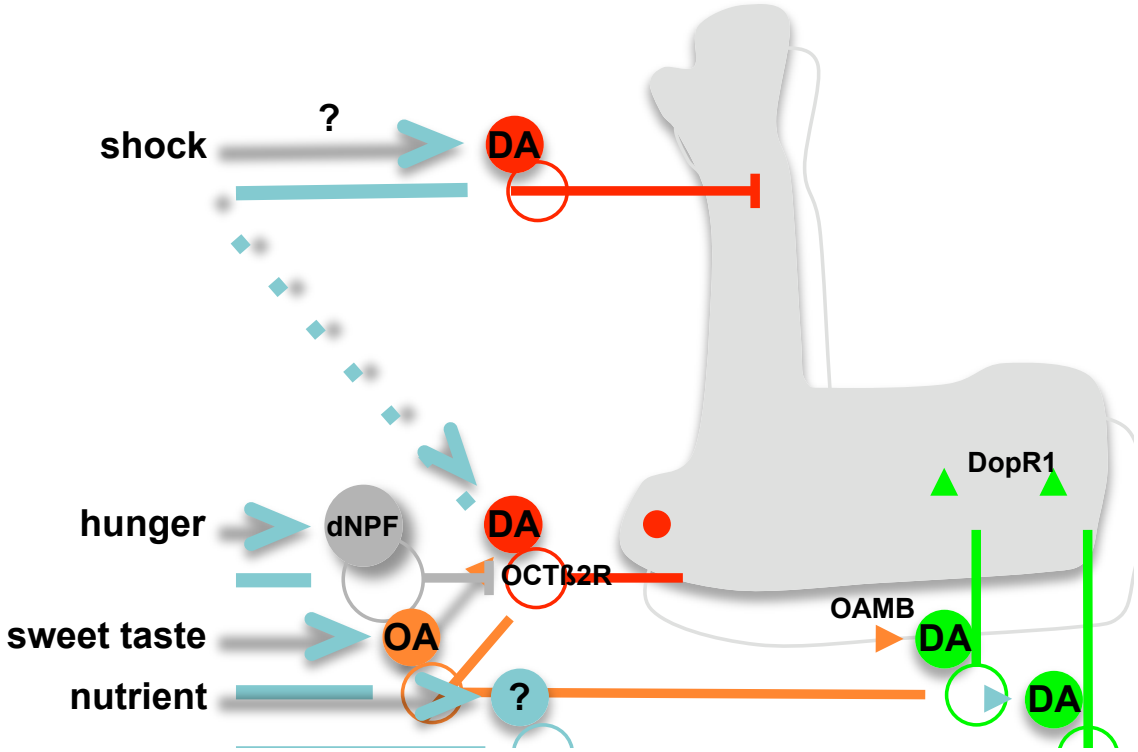


Figure IV-1. A New Model for Appetitive Olfactory Memory in Drosophila
OA (orange) functions through PAM DA neurons (green) via OAMB receptor to provide reinforcement of sweet taste alone and also through MB-MP1 DA neurons (red) via OCTb2R to modulate motivational state to the MB (grey). The rewarding PAM DA neurons also integrate post-ingestive nutrient reward signals of unknown source to drive appetitive LTM

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