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Olfactory modulation of visual object behaviors in *Drosophila melanogaster*

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Neuroscience

by

Yu-Chen Karen Cheng

2021

ABSTRACT OF THE DISSERTATION

Olfactory modulation of visual object behaviors in *Drosophila melanogaster*

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Yu-Chen Karen Cheng

Doctor of Philosophy

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Professor Mark Arthur Frye, Chair

Visual objects in the natural world convey many meanings to vinegar flies, signifying the presence of predators, food sources, or potential mates. How animals rapidly distinguish among these small objects remains unclear at the mechanistic level. It is thought that multimodal integration of sensory cues, likely via actions of neuromodulators, is one mechanism for this behavioral plasticity. In this thesis, we begin by reviewing the current understanding of neuromodulation of insect vision. Next, we describe innate object behaviors in a flight simulator paradigm in both freely rotating, magnetically-tethered and yaw-restricted, rigidly-tethered *melanogaster*. The experimental paradigm in rigidly-tethered flies is then modified to assess the effects of odor on object responses. In a paradigm in which visual stimuli positions were negatively coupled to the fly's steering effort, we find that appetitive odor reduces the probability that flies engage in aversive behaviors in response to encountering small visual objects. In a complementary paradigm in which visual stimuli position were restricted to the visual periphery, we

show that the presence of appetitive food odors reverses innate object avoidance to attraction, whereby flies begin to approach and track the small object. We term this behavior odor-induced visual valence reversal. Subsequently, we show through optogenetic activation studies that this modulation seems in part to be induced by the neuromodulator octopamine, the insect orthologs of norepinephrine, as well as small-field visual motion detectors, T4/T5 neurons. Efforts to assess whether octopamine and T4/T5 neurons mediate object valence reversal via the same neural circuit were inconclusive, likely due to off-target effects and genetic backgrounds. Separately, *in vivo* calcium imaging of T4/T5 responses to visual objects with pharmacological application of octopamine or its agonist, chlordimeform, suggest that variation in T4/T5 visual responses were likely due to the quiescent animal's internal state. Our results identify neural components involved in olfactory modulation of object vision and highlight the importance to further assess the contributions of locomotion in understanding neuromodulatory mechanisms.

The dissertation of Yu-Chen Karen Cheng is approved.

Elissa A Hallem

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University of California, Los Angeles

2021

To my parents

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One of the first things I heard upon starting graduate school was how hard it would be. But few people tell you that the journey gets much better when surrounded by intelligent, highly-achieving, but most importantly, kind and generous people. I'd like to think of the numerous people I have met and worked with in the last few years my "guay ren", which, loosely translated, refers to people who've had tremendous influence in your life to shape you into who you are today.

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2019 Saravanapandian, V., Sparck, E.M., **Cheng, K.Y.**, Yaeger, C., Hu, T., Suthana, N., Romero-Calderon, R., Ghiani, C.A., Evans, C.J., Carpenter, E.M., Ge, W. (2019) *Quantitative assessments reveal improved Neuroscience engagement and learning through outreach.* Journal of Neuroscience Research: jnr.24429

2018 Mongeau, J.M., **Cheng, K.Y.**, Aptekar, J., Frye, M.A. (2018) Visuomotor strategies for object approach and aversion in *Drosophila melanogaster*. Journal of Experimental Biology: jeb.193730

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1. NEUROMODULATION OF INSECT MOTION VISION

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ABSTRACT

Insects use vision to choose from a repertoire of flexible behaviors they perform for survival. Decisions for behavioral plasticity are achieved through the neuromodulation of sensory processes including motion vision. Here, we briefly review the anatomy of the insect motion vision system. Next, we review the neuromodulatory influences on motion vision. Serotonin modulates peripheral visual processing, whereas octopamine modulates all stages of visual processing tested to date. The physiological and behavioral states that elicit neuromodulation of motion vision include locomotion, changes in internal physiological state such as hunger, and changes in the external environment such as the presence of additional sensory cues. The direction of influence between these states and neuromodulators remains unknown. The influence of neuromodulators on motion vision circuitry has been revealed mostly through pharmacological application, which broadcasts widely with unnatural spatiotemporal dynamics. Thus, insight from this method are limited. Aminergic neurons likely act in local hierarchical fashion rather than globally as a group. As genetic tools advance in *Drosophila*, future work restricting the experimental focus to subpopulations of modulatory neurons will provide insight into the local functional modifications of visual circuits by interacting neuromodulators.

ABBREVIATIONS

Tdc2 — neuronal tyrosine decarboxylase 2, denotes a Gal4 line that labels octopaminergic/tyraminergic neurons

OA — octopamine

CDM — chlordimeform, octopamine receptor agonist

T4 and T5 — columnar retinotopic 'T' neuron classes with dendrites in the medulla (T4) and lobula (T5) and axon terminals in the lobula-plate

VPNs — visual projection neurons

LPTCs — lobula plate tangential cells, a class of VPNs

LCs — lobula columnar cells, a class of VPNs

INTRODUCTION

Insect behavior is astonishingly complex, despite the fact that it is driven by numerically compact brains [1]. Neuromodulation can enhance the computational capacity of small neural circuits via functional reconfiguration to allow animals to exhibit flexible, plastic, physiological responses and behaviors in response to context specific internal states and external stimuli, without the need for additional parallel hard-wired pathways. Behavioral plasticity in insects has been broadly attributed to the action of biogenic amines including dopamine, serotonin, histamine and octopamine, which can act as neurotransmitters, neuromodulators or neurohormones [2–6]. These derivatives of amino acids exert neuromodulatory effects on olfactory learning, aggression, feeding and egg-laying [7–17]. Visual processing is required for many of these behaviors. For example, in order to court a potential mate, an animal must visually identify, then follow and approach its paramour. In order to feed, it might visually identify, then approach and assess the nutritional value of the resource. During

locomotion, motion vision is used for many actions including stabilizing visual gaze, correcting external perturbations, avoiding obstacles or threats, and approaching desired objects.

The elemental computation for motion vision requires comparing luminance changes at two points in space over time [18,19]. Thus, the functional output of a motion detector ultimately transforms the spatiotemporal activity patterns of individual photoreceptors into visual signals used to program motor commands for an appropriate behavioral response. During flight, an animal must evaluate the visual scene to quickly determine whether to approach or avoid an object that could be a potential mate or deadly predator, and this determination might change with context, such as whether there is another sensory cue to disambiguate the two possibilities.

Among arthropods, insects and crustaceans have been the dominant groups for experimental vision research because they generate reliable, measurable visually-induced behavioral responses and are amenable to stable neurophysiological recordings over a long period of time. This review focuses on the neuromodulation of motion vision in insects, but occasionally ties in relevant findings from crustaceans. Many studies of insect vision have focused on locusts, blowflies, honeybees, and the vinegar fly *Drosophila melanogaster*.

THE INSECT OPTIC LOBE

Several recent in-depth reviews describe the neurobiology of motion vision in insects [19–22]. Here, we briefly review the relevant anatomy to facilitate an

understanding of the targets of neuromodulation. The insect visual system includes the compound eye and the optic lobe, the latter comprising several neuropils called the lamina, medulla, lobula and lobula-plate [23]. The eye is comprised of repetitive structures called ommatidia, which focus light through hexagonal lenses onto photoreceptors [21,24]. For each ommatidium, six photoreceptor neurons R1-R6 project to the first synaptic relay, the lamina (Figure 1), and two photoreceptors R7 and R8, involved in color vision and polarization vision, bypass the lamina and project directly to the second neuropile, the medulla [25,26]. Lamina monopolar cells receive histaminergic, hyperpolarizing signals from spectrally tuned photoreceptors (in fruit flies, R1 through R6 are green) and in turn supply the rest of the motion vision circuitry [27,28]. Lamina neurons temporally filter their inputs to enhance contrast [19,21].

Lamina columnar neurons split into parallel ON and OFF pathways and are thought to comprise the spatially separated inputs required for motion vision [19,29]. However, the first directionally selective motion signals emerge downstream, in columnar T4 and T5 neurons (Figure 1), each of which is broadly tuned for one of the cardinal directions of motion [30]. A substantive, recent body of literature in *Drosophila melanogaster* is now cracking the neuronal input circuitry for T4 and T5 motion detectors, each of which pools synaptic input from four columnar medulla neurons (Figure 1) [21,31–37]. T4 and T5 neurons project axons to the lobula-plate where their small receptive fields are postsynaptically pooled by the large dendrites of individual visual projection neurons (VPNs) that run tangential to the surface of the neuropil. Each lobula plate tangential cell (LPTC) samples many retinotopic columnar T4 and T5 inputs to form large and complex receptive fields. Historically, LPTCs had been categorized

into horizontal system (HS) and vertical system (VS) classes (Figure 1) depending on their average preferred motion axis, and have been characterized extensively in the blow fly *Calliphora vicina* and the vinegar fly *Drosophila melanogaster* [25,38–42].

Another class of VPNs that have received attention recently are the lobula columnar (LC) neurons (Figure 1). Unlike LPTCs that pool across layers and columns of the lobula plate, LCs are columnar neurons that 'tile' the visual field and have dendritic arbors restricted to specific lobula layers [25,43–45]. There are over twenty classes of LCs, and each type projects its population of columnar axons into a glomerulus structure in the central brain [44,45]. LC neurons have been implicated in behaviors such as jumping, avoidance of looming stimuli, backward and forward walking, and reaching [45,46]. This evidence, in addition to a previously identified motion vision neuron in the lobula of the honeybee [47], suggest that LCs form a feature detection pathway in parallel to the motion pathway projecting from the lobula plate.

VISUAL NEUROMODULATORS

Work on insects has found evidence for the neuromodulation of visual processing by serotonin and octopamine (there is no known role for dopamine), and the anatomical distribution of aminergic terminals and receptors support these physiological findings, to be presented later in this review. Serotonin-immunoreactive neurons are widespread in insect visual systems [48–51]. Less is known about the distributions of serotonin receptors in the optic lobe, although recent efforts using sequencing analysis are underway. Octopamine is a monohydroxylic analog of norepinephrine, sharing similar

chemical structure but missing a hydroxyl group [52]. Like its mammalian adrenergic counterparts, octopamine triggers fight-or-flight responses by modulating metabolic processes [53]. In invertebrates, octopaminergic neurons send projections to the optic lobe (vinegar fly: [54,55]; locusts: [56,57]) where they are both axonal and dendritic [55,58]. Notably, the highest density of octopaminergic receptors in the brain are found in the optic lobe [54,55,59]. Insect octopaminergic receptors are G-protein coupled receptors that are further categorized depending on the elicited downstream mechanisms [60–62].

To date, most studies examining the modulatory effects of octopamine have taken a pharmacological application approach. Whereas some experiments bath apply octopamine onto the recording site and throughout the brain, other studies use an octopamine agonist, chlordimeform (CDM). An insecticide in the formamidine family [63], CDM binds to octopaminergic receptors and few other aminergic receptors [64,65], and elicits modulatory effects similar to those of octopamine on light production and molecular activation of octopamine-sensitive adenyl cyclase in the firefly lantern, as well as potentiation of the locust slow extensor neuromuscular junction [64,66–69]. CDM likely converts into its demythelated form (DCDM) *in vivo* [70], and DCDM has a dose-dependence and binding affinity for octopaminergic receptors more similar to octopamine, and is sometimes even more potent than octopamine at lower concentrations [64,66]. However, unlike octopamine, CDM has a longer tissue solubility, tends to occupy the receptors for longer periods of time, and at higher concentrations, could act as an octopamine antagonist before inducing neurotoxicity

and lethality Despite the differences between octopamine and its agonist, the field tends to treat CDM modulation as synonymous with OA modulation.

Regardless whether OA or CDM is used, pharmacological approaches lack natural spatial and temporal specificity and intensity, which could bias experimental results by activating octopaminergic receptors in a non-physiological manner. Recent neurogenetic tools in *Drosophila melanogaster* have allowed for direct optogenetic or thermogenetic activation of aminergic neurons. *Tdc2* is a gene that encodes the neural tyrosine decarboxylase, an enzyme that catalyzes the first step of octopamine synthesis: the precursor tyrosine is decarboxylated into tyramine, which is then hydroxylated into octopamine [71]. Therefore, because Tdc2-Gal4 labels both octopaminergic and tyraminergic neurons, we will refer to these neurons as Tdc2 neurons instead of octopaminergic neurons.

NEUROMODULATION OF PHOTORECEPTORS

Across multiple insect species, photoreceptors are modulated on a diurnal rhythm to achieve the acuity and sensitivity state that accommodates changing light levels [72]. Throughout the day, locust photoreceptors have high acuity and low sensitivity, but switch to low acuity and high sensitivity at night. These day and night states are mediated by potassium (K+) channel conductances. In the day state, photoreceptors exhibit a fast activating, slow inactivating K+ current followed by a non-inactivating K+ current. Thus, day-state photoreceptors exhibit sustained rectification. By contrast, photoreceptors in the night state show transient, non-rectifying K+

conductance [73,74]. Photoreceptors release histamine as their neurotransmitter [75–77]. Intracellular recordings from photoreceptors show that bath-application of serotonin recapitulates the change from day to night state by modulating the conductance of potassium channels (vinegar flies: [78,79]; locusts: [74]. Serotonin's modulatory role in mediating circadian changes in photoreceptor sensitivity has also been shown in the mollusca Aplysia and Hermissenda [80,81].

In addition to serotonin, photoreceptors have been shown to be modulated by octopamine. Bath applications of octopamine to electrophysiological recordings in Drosophila increases photoreceptor sensitivity [82]. Octopamine increases cAMP levels and photoreceptor sensitivity in the horseshoe crab [83,84]. Interestingly, the effects of octopamine do not extend to *Limulus* motion vision [84]. Hence important differences exist between insects and other arthropods that likely reflect their different life history strategies.

NEUROMODULATION OF HIGHER ORDER VISUAL CIRCUITS

Downstream of photoreceptors, higher-order visual neurons are modulated by both serotonin and octopamine. Similar to their effects on photoreceptors, serotonin modulates lamina L1 and L2 neurons to mediate circadian rhythmic changes in the anatomical structure of these lamina columnar neurons. In *Musca* and *Drosophila*, serotonin injections increase the axon size of lamina L1 neurons, but have no effect on L2 neurons. By contrast a serotonin neurotoxin, 5,7-dihydroxytryptamine, decreases L2 axon size with no effect on L1 neurons [85]. Similar cellular effects are observed to occur

on the animal's circadian cycle. Hemolymph injections of 5-HT or pigment-dispersing factor peptide modulate the amplitude of transient responses of lamina-driven electroretinograms [86], but the functional consequences of axon diameter modulation are unknown. Bath applied serotonin reduces spontaneous activity and visually-evoked responses of a lobula neuron sensitive to vertical motion; this centrifugal neuron has dendrites in the protocerebrum and axon terminals in the lobula [47]. Recent findings suggest that serotonin modulates two neuron types involved in aggression, one GABA-ergic and one cholinergic [87]. These neurons are downstream of a feature-detecting visual projection neuron, lobula columnar neuron LC12 [88]. Bath applications of serotonin in brain explants results in increased calcium activity in both GABA-ergic 5-HT1A receptor expressing neurons, and cholinergic short neuropeptide F receptor and 5-HT1A receptor expressing neurons [87]. Although the mechanisms need to be worked out, for example to localize the source of endogenous serotonin release, these data nevertheless hold promise for elucidating the role of serotonin in modulating visual behavior.

Whereas evidence to date of serotonergic modulation is so far restricted to the early or late stages of visual processing, evidence of octopaminergic modulation can be found across all stages of the circuit. Across insect species, there is evidence of octopaminergic modulation of fourth-order LPTCs. LPTCs encode complex optic flow fields generated during self-motion [38,40,42,89,90]. Wide-field optic flow is required to estimate an animal's locomotion trajectory [91–93], and optogenetic activation and silencing has implicated one LPTC in steering control by flies [94]. The spontaneous spike rates of several LPTC types increase after bath application of octopamine, or its

agonist, chlordimeform (CDM) [93]. Similarly, results from patch-clamp recordings demonstrate that resting membrane potentials of vertical system LPTCs increase after either pharmacological application of octopamine or thermogenetic activation of Tdc2 neurons [95].

It is worth noting that CDM, a potent insecticide, has been shown in moths to better penetrate the perineurial sheath surrounding the brain and be more persistent than octopamine [69]. More recently, octopamine has been used roughly as frequently as CDM to explore visual neuromodulation, with no explicit rationale for choosing one or the other. To our knowledge, there has been no systematic comparison of the effects of octopamine and CDM on the same visual preparation. Thus, we will refer to the reagent that was used in each experiment.

In addition to increasing baseline activity, octopamine also modulates the amplitude of visually evoked neural responses. In honeybees, work from several decades ago showed that bath-application of octopamine increases the amplitude and kinetics of responses to moving gratings by wide-field lobula neurons [47]. In the lobula plate, after subtracting the elevated spontaneous spike rates, Longden & Krapp found that mean spike rates of vertically-tuned LPTCs show significantly increased responses to moving gratings after bath application of CDM [96]. Octopaminergic neuromodulation on LPTCs seems to change the sensitivity to a stimulus by dishabituation. In flies, the extent of the effect of CDM on visually evoked responses has been shown to depend on the temporal frequency of the visual stimulus (temporal frequency is the number of periodic pattern cycles moving past a fixed point on the retina per unit time). At low frequencies, the visual boost from the non-modulated to modulated state is small

[93,95,97]. At higher frequencies, results vary depending on the readout. When measuring mean steady-state activity of recorded neurons, the visual response amplitude boost from the octopamine agonist is stronger for high temporal frequencies [93,97]. The frequency specificity of octopamine action may be absent if the readout is instead the response onset transient measured soon after pharmacological application [95]. This discrepancy could be due to the motion-adapted state of an LPTC that elicits varying effects of CDM [98]. Motion detecting neurons quickly adapt [99] to continuous motion stimuli by becoming less excitable (for more in depth definitions and findings on adaptation, see [100–105]. Motion adaptation as well as contrast adaptation in LPTCs are attenuated by CDM (blowfly: [93,106]; hoverflies: [107]. Lüders and Kurtz [98] showed that the effects of CDM on an LPTC responses in an unadapted state is consistent across temporal frequencies between 0.5 to 32 Hz, but the effects on the adapted state is greater for higher temporal frequencies, which would explain discrepancies in the literature. Taken together, the field seems to concur that octopamine or its agonist indeed increases response amplitude by LPTCs, with stronger effects at higher temporal frequencies through the attenuation of adaptation (vinegar fly: [95,97]; blowfly: [93]). The result of attenuated motion adaptation would include dishabituation. In locusts, octopamine application dishabituates a visual collision-detecting circuit back to the response level to a novel stimulus [108]. Octopamine's role in dishabituation is further confirmed, as epinastine, an octopamine-antagonist, abolishes the observed dishabituation effects [57,109].

The wealth of evidence for neuromodulation of first-order photoreceptors and fourth-order LPTCs in flies, locusts and bees has been achieved due to the accessibility

to electrophysiological recordings at these two stages of the visual processing circuit. Are intermediate synaptic processing stages also subject to neuromodulation? With the advent of powerful genetic tools, it has become possible in recent years to examine neuromodulation of visual neurons upstream of LPTCs. In an *in vivo* calcium imaging experiment, responses from T4/T5 neurons in layer 3 of the lobula-plate, tuned to upward motion [30], show a shift towards higher temporal frequency after CDM application [110]. Interestingly, similar octopamine-evoked temporal response shifts are observed in each class of medulla columnar neurons presynaptic to T4/T5 [110,111]. Therefore, octopaminergic or agonist-driven neuromodulation is observed at three synaptic levels of motion vision processing: non-directional medulla neurons, directionally selective small-field T4/T5 motion detectors, and multi-columnar wide-field LPTCs (Figure 1). Many questions remain. We do not know to what extent the neuromodulation of downstream elements is due to neuromodulation of upstream nodes of the circuit. The cellular mechanisms of motion adaptation in LPTCs are not understood and may be dependent upon upstream pathways. Finally, since studies to date have predominantly used a pharmacological delivery approach, it remains to be determined whether octopamine release is targeted to specific levels of the processing hierarchy, or if it is indeed broadcast throughout the optic lobe.

OCTOPAMINE INCREASES GENERAL AROUSAL

As with the far-reaching physiological functions of norepinephrine in mammals, the complex multi-system influences of octopamine in insects helps to contextualize its role as a prevalent neuromodulator of motion vision. Octopamine increases general

arousal [53]. The term 'arousal' takes on a myriad of definitions. "Central arousal" has been used to describe the relative level of responsiveness an animal has to external stimuli [112,113]. Central arousal tends to be evoked by transitions in behavioral state, such as when an animal switches from quiescence to locomotion, or from fleeing to engaging. Central arousal may also be induced by changing internal states, such as becoming hungry, or by changing environmental states, such as cross-modal facilitation or arousal generated by concomitant input from another sensory modality. Octopamine is considered an important regulator of central arousal and stress responses in various insects [114–117]. Over a longer timescale, arousal may be equated with wakefulness. In this context, octopaminergic neurons within the anterior superior medial (ASM) protocerebral regions of *Drosophila* promote the flies to stay awake much longer than normal [118,119]. The timescale of sleep delay is long, much longer than the timescale for changing locomotor state, indicating that arousal can occur at different levels of organization operating on different timescales.

BEHAVIORAL STATE: LOCOMOTION

The shift from quiescence to locomotion either results from or causes arousal. It is perhaps not surprising, then, that octopamine is released during locomotor activity, particularly flight. Octopamine levels in the hemolymph increase when an insect is in flight or engaged in other motor activities such as aggression [113,120,121]. In *Drosophila* calcium activity in the terminals of Tdc2 neurons innervating the optic lobe increases significantly during flight, suggesting that flight directly activates at least some octopaminergic neurons [95]. Not only does flight induce the release of octopamine, but

octopamine also appears to be required for normal, sustained, flight behavior. Defects in the enzyme tyrosine beta-hydroxylase, involved in octopamine synthesis in *Drosophila melanogaster* leads to deficits in flight initiation and maintenance [122]. This is consistent with classical evidence from locusts in which octopamine released during flight recruits the action of proprioceptors and muscles, and triggers changes in the animal's physiology to serve the increased metabolic needs of this energy-taxing behavior [120,121]. Octopamine also plays a role in modulating the flight motor program. Injections of a high enough concentration of octopamine or CDM into the mesothoracic ganglion increases the duration of active flight bouts in adult moths *Manduca sexta* [69] and locusts [123]. Furthermore, fruit flies with inactivated octopaminergic neurons shows impaired ability to modulate their flight speed with changes in the speed of visual stimuli [124]. On the biophysical level, the flight state is required for locust flight interneurons 566 and 567, involved in generating flight motor patterns, to exhibit bursting properties [125] and rhythmic bursting is recapitulated by octopamine application in a quiescent recording preparation [126].

Given the apparently bi-directional relationship between flight coordination and octopamine, it seems reasonable that many of the neuromodulatory effects of CDM or octopamine application on physiological responses in the motion pathway should be recapitulated by the onset of flight, or even by walking. Indeed, the onset of flight as well as sustained flight behavior increases membrane potential and steady state visual responses while broadening temporal frequency tuning in motion detecting neurons [41,95,97]. Quite recently, it was shown that octopamine mimics the effects of flight onset by gating information flow by descending neurons in *Drosophila* [127]. Walking

behavior modulates visual responses by LPTCs in a manner similar to flight: during walking, the visual response gain of a horizontal system LPTC is shifted toward higher velocities. Furthermore, the animal's instantaneous walking speed influences the temporal tuning of LPTCs in a graded fashion. Essentially, faster walking results in higher speed sensitivity [128,129]. However, whether octopamine is involved in walking-related modulation of motion vision remains to be determined.

Remarkably, locomotor activity in the absence of visual input rapidly modulates cellular excitability within at least three levels of motion processing: one of the columnar inputs (Mi4) to the small-field T4 directional motion detectors [111], the downstream wide-field integrating LPTC neuron HS [130], and premotor descending neurons [127]. Furthermore, Mi4 neurons show rapid calcium responses upon optogenetic stimulation of Tdc2 neurons [111]. However, the neural circuitry that links locomotion-onset to the modulation of motion vision via octopamine is unknown, nor whether these are serial or parallel events.

INTERNAL STATE: HUNGER

Arousal levels are increased by changing internal states, such as the transition from satiation to hunger, and the resultant behavioral changes are octopamine dependent. A starved internal state alters an animal's behaviors and responses to external stimuli, presumably to increase foraging probability. Starved flies have decreased bitter sensitivity so that they can accept a broader selection of food sources [131] and choose nutritious foods rather than sweet-tasting ones [132]. Furthermore,

food deprivation increases locomotor activity in vinegar flies. This starvation-induced hyperactivity is dependent on the adipokinetic hormone (AKH) and is abolished in octopamine null mutants, as AKH receptor-expressing neurons are octopaminergic [133–135]. One study found that hungry flies show weakened visual responses by a spiking LPTC and reduced strength of optomotor responses by behaving animals [136], and these effects recovered upon feeding. However, anecdotally, for studies involving flight behavior, researchers have found that depriving flies of food for periods up to 24 hours improves the animals' motivation to perform [137]. Liquid chromatography-mass spectrometry analysis shows that hemolymph octopamine levels increase about five-fold in male vinegar flies that have been starved for 24 hours [138]. A subset of octopaminergic neurons is activated by a hormone released during starvation in the cockroach *Periplaneta americana* [139]. Furthermore, exogenous octopamine application changes sensitivity to food-related stimuli, modulating honeybee responses to brood pheromone that results in increased foraging behavior [117].

ENVIRONMENTAL STATE: MULTIMODAL INTEGRATION

There is abundant evidence for visuo-olfactory integration behavior that would be associated with foraging-like locomotor behavior, as a food resource or the environment containing it would emit cues for multiple sensory modalities. In a circular free flight arena with an odor source hidden in its floor and visual patterns projected on its walls, *melanogaster* require visual feedback from vertical edges in order to robustly localize the invisible odor source [140]. The static visual stimuli presumably generate robust self-motion cues for visual guidance that are biased by the changing odor gradient

during approach. For a tethered fly, an attractive odor cue increases the amplitude of steering optomotor responses [141,142], a motion-dependent behavioral reaction in which animals attempt to turn in the direction of a moving visual scene to minimize retinal slip and fly straight [142–144].

If using motion vision to stabilize flight heading in an odor plume is adaptive, so would an increase in forward flight speed within the plume. Flight motor responses, including changes in wingbeat amplitude and wingbeat frequency, show tonic increases during appetitive odor presentation, resulting in increased thrust and likely underpinning the upwind surge observed as a fly encounters an odor plume in free flight [137,145]. Similar wing kinematic responses are evoked by visual expansion cues, and responses to both cues presented together are similar to the sum of the responses to stimuli presented in isolation [146].

Odor modulation of optomotor behavior is required for active plume tracking. Flies suspended on a magnetic tether, which are free to rotate in flight, strongly track a narrow odor plume emanating from one side of the arena, but are unable to remain localized within the plume when the surrounding visual panorama is switched from high contrast stripes to uniform grayscale [147], and flies in the same arena in which the T4/T5 motion detectors are genetically silenced are unable to maintain their heading in the plume [148]. The visual response amplitude of one LPTC has been shown to be modulated by odor in *Drosophila* [148], whereas no changes were observable in the presynaptic T4/T5 cells in a quiescent imaging preparation. Theoretical modeling suggests that subtle modulation of optomotor responses by odor presentation could significantly bias the flight path of a fly toward an otherwise invisible odor source [149].

An attractive hypothesis is that octopamine plays a role in linking olfactory signaling to modulation of visual motion sensitivity and behavior.

The presence of an additional, cross-modal cue not only changes visual behavioral responses in a quantitative way, but has also been shown to alter visual behaviors qualitatively. Mosquitoes in a free-flight chamber begin approaching a small dark object in the presence of CO_2, but otherwise ignore the visual cue in clean air [150]. Similarly, freely flying fruit flies spend more time exploring a visually salient landing platform when the platform emits an odor indicating a food source [151]. In addition to increasing the salience of an otherwise neutral stimulus, the presence of an olfactory cue can also reverse the perceptual valence of a visual cue. An innately aversive small object that likely represents an approaching threat becomes attractive to flying fruit flies when paired with attractive food odors, but not an aversive odor [58]. Optogenetic activation of Tdc2 neurons in flying flies recapitulates this visual valence reversal behavior in the absence of odor, directly implicating octopaminergic neurons. This result also highlights the hierarchical nature of octopaminergic signaling, since the animals were already in the flight state, having engaged the locomotion-dependent neuromodulation of motion vision, and yet optogenetic activation of Tdc2 neurons further modulated visual processing to reverse the valence of a small object.

MOVING FORWARD

Some functional evidence has been presented to suggest that odor activates Tdc2 neurons; *in vivo* calcium responses of sparse Tdc2 processes within the optic lobe are

increased during the presentation of a food odor in adult *Drosophila* [148]. Similarly, optogenetic activation of olfactory neurons in *Drosophila* larvae cause calcium accumulation in Tdc2 neurons [152]. However, the mechanism by which Tdc2 is activated by the olfactory system is unknown. With the recently available drivers to target olfactory neurons within the lateral horn [153], in conjunction with the existing drivers targeting olfactory neuropils such as the mushroom body and antennal lobes, it will be possible to assess anatomical as well as functional connectivity between different classes of olfactory neurons and the Tdc2 system.

Indeed it is unknown how octopamine-releasing neurons are activated presynaptically, what is the spatial distribution of this neurochemical, nor how octopamine influences postsynaptic targets in the visual system or any physiological system. The dual roles of octopamine as a neurotransmitter or neurohormone makes any such exploration challenging. *Drosophila* is poised to bridge this gap, becoming an ever more productive research model, as increasing numbers of specific Gal4 drivers for identified visual circuitry have become available, and sequencing analysis of postsynaptic receptor and presynaptic transporter distribution are becoming more reliable, we shall be better able to study how Tdc2 neurons exert their effects on motion vision in response to internal, behavioral, and environmental state changes.

At this point we cannot experimentally separate central arousal with any putative local effects of octopamine on select visual circuits. Unlike in rodents and other mammals whose arousal levels are correlated with pupil dilations [129,154], there is no uniquely distinguishing readout for whether an insect is aroused other than increased or varied behavioral activity. Therefore, to date, effects due to increased general arousal

versus (e.g.) locomotor-related motor feedback, is difficult to decouple [129]. However, findings such as octopamine shifting LPTC responses specifically in the high temporal frequency domain might suggest that octopamine is not merely boosting the gain of responses to all stimuli equally. The more parsimonious explanation is that there are spatially local effects plus hierarchical control of octopaminergic modulation.

Hierarchical levels of octopaminergic modulation is aligned with the classical orchestration hypothesis [123], a modern interpretation of which would suggest that different subpopulations of Tdc2 neurons modulate separate behaviors. It seems plausible that a subpopulation of octopamine neurons is activated by general arousal, or hunger, and another, perhaps somewhat overlapping, by locomotor feedback. Experiments to tease apart global, arousal-dependent effects from local, modality-specific effects could involve the inactivation of individual octopamine receptor types while the animal is in a highly aroused mode, such as flight, and observe whether the experimental readouts, such as LPTC responses to visual stimuli of different temporal frequencies, remain intact.

Some challenges still remain for assessing subpopulations of Tdc2 neurons in mediating behavior. Approaches that have extended beyond bath application findings to opto- or thermogenetic activation have overcome the problem of overdriving the effects of octopamine release in a non-ethological manner. However, the Tdc2-driver used in these activation studies likely activates 100 or more Tdc2-positive neurons that ramify broadly throughout the insect nervous system [55]. Given the distributed physiological functions that octopamine serves, and the hierarchical nature of octopaminergic neuromodulation on visual behavior, it seems unlikely that the entire group of Tdc2

neurons operate at once. Furthermore, without careful determination of activation levels and patterns, it is easy to overdrive neurons opto- or thermogenetically beyond the ethologically relevant levels. Results obtained from these manipulations could thus be difficult to interpret, as elicited responses are a result of unnatural activation patterns.

In addition to modulating motion vision, octopamine is also involved in coordinating behaviors such as courtship, aggression, and egg-laying. By determining which Tdc2 subpopulations are involved in each behavior, we may start to gain insight into how a behavior switch takes place: how may a fly switch from being aggressive towards another fly or courting her, what overlap is there in the Tdc2 neurons involved?

Since octopamine is involved in a multitude of behaviors, many inactivation results to date have been difficult to interpret. This is partly due to additional defects that render flies too unhealthy for experiments. Spatially restricting Tdc2 driver expression can limit these defects. Flies with Tdc2 neurons inactivated since birth may also exhibit compensatory mechanisms that compromise experimental analysis. Since octopamine shares the same molecular precursor as tyramine and dopamine [71], blocking the octopamine synthesis pathway may increase levels of tyramine [155] and dopamine levels during development, thereby introducing complex confounds. Such artifacts can be limited by temporally restricting the onset and offset of Tdc2 inactivation using optogenetic approaches. Future experiments that manipulate subsets of Tdc2 neurons will provide more interpretable results than ablation studies and will thereby advance the field.

One notable caveat when considering the mechanisms of aminergic modulation of visual processing is interaction effects; the Tdc2-driver labels both octopaminergic and tyraminergic neurons. Most studies have considered the Tdc2-driver synonymous with octopaminergic. However, emerging evidence across invertebrates suggests that tyramine exerts an independent set of functions [52,156,157]; C. elegans: [158]) and tyramine and octopamine may be antagonistic modulators [159]. Going forward, we must tease apart the actions of these two neuromodulators and probe interactions with other neuromodulators. For example, foraging-related behavior involves both short neuropeptide F (sNPF) and octopamine. Two types of relay neurons downstream of an optic glomerulus within the ventrolateral protocerebrum generate aggressive motor behaviors. One of these descending neuron types is GABAergic and expresses 5-HT1A receptors. The other is cholinergic and expresses sNPFR and 5-HT1A receptors. Serotonin release from 5HT-PLP neurons inhibits the GABAergic neurons, which then disinhibits the parallel, cholinergic neurons that eventually lead to increased aggression behavior [87]. However, the functional importance of serotonin activation/inhibition of the cholinergic neurons, which are themselves 5HT1A-expressing, remains unknown. It is also unknown how sNPF activation on the same type of neuron affects its function in mediating aggression. This is a very recent representative example of the complex interactions at play. Many studies related to neuromodulation restrict their focus, by design, on one neuromodulator at a time. Moving forward, it will be vital to understand not just how neuromodulators change the state of a circuit, including for motion vision, but also how neuromodulators interact with each other to exert synergistic effects or sculpt specificity.

In conclusion, the cellular and circuit mechanisms of motion vision have become a widely studied subject with broader implications for highlighting general principles of neural circuit processing. The extensive neuromodulatory influences that this circuit receives is at some level profoundly surprising. It might seem that motion vision should operate at a very low level in the brain, robust at all times. But apparently this is not the case. Rather, motion vision provides a conceptual scaffold for further mechanistic insights of how synaptic circuits can be functionally altered to allow insects to adapt to changes in behavioral, internal, or environmental states. Motion vision is modulated somewhat by serotonin, and strongly by octopamine. A heightened arousal state that results from locomotion, cross-modal input, or hunger to put the animal in a more alert state, thereby paralleling attention-based modulation of visual neurons in mice and primates [129,160]. Future studies involving spatially restricted manipulation of Tdc2 neurons and the assessment of interactions among neuromodulatory substances will provide further insights of how multiple neuromodulators could come together to exert synergistic or antagonistic effects when needed to increase the dynamic range of circuit function. These neuromodulatory principles will further our understanding of how neuromodulation allows animals to react to a changing world.

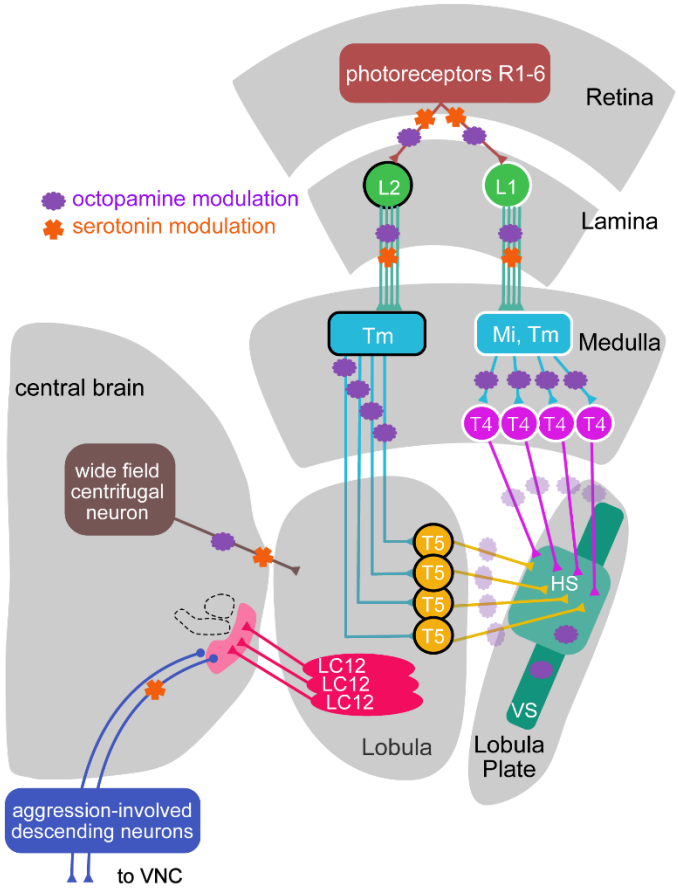

Figure 1. Schematic summary of neuromodulation at stages of motion vision

Sites that have been shown to be modulated by octopamine are indicated with a purple cloud. Sites shown to be modulated by serotonin with an orange asterisk. Light signals are processed by photoreceptors, which supply columnar lamina neurons L1 (ON) and L2 (OFF) pathways. L1 neurons project to medulla intrinsic (Mi) and transmedullary (Tm) neurons. L2 project to downstream Tm neurons. Columnar OFF-sensitive T5 neurons and ON-sensitive T4 neurons are directionally selective and sample the visual space in a retinotopic fashion, and project to four layers of the lobula plate, each layer represents a separate cardinal direction of motion. Wide-field lobula plate tangential cells (LPTCs) integrate the synaptic output many small-field T4 and T5 outputs to assemble large visual receptive fields that encode complex motion fields. Two groups of LPTCs encode roughly horizontal (HS) and vertical (VS) optic flow fields. A parallel system of lobula columnar (LC) neurons, with one example showing converging axons upon a single distinct glomerular structure in the central brain (glomeruli from other LCs outlined with dashes). A wide field sensitive centrifugal neuron (projecting from center to periphery) has dendrites in the medial protocerebrum and axon terminals in the lobula. The LC12 glomerulus supplies two types of neurons involved in aggression that descend into the ventral nerve cord (VNC)

2. VISUOMOTOR STRATEGIES FOR OBJECT APPROACH AND AVERSION IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Animals classify stimuli to generate appropriate motor actions. In flight, *Drosophila melanogaster* classify equidistant large and small objects with categorically different behaviors: a tall object evokes approach whereas a small object elicits avoidance. We studied visuomotor behavior in rigidly- and magnetically-tethered *D. melanogaster* to reveal strategies that generate aversion to a small object. We discovered that small object aversion in tethered flight is enabled by aversive saccades and smooth movement, which varies with the stimulus type. Aversive saccades to a short bar had different dynamics than approach saccades to a tall bar and the distribution of pre-saccade error angles were more stochastic for a short bar. Taken together, we show that aversive responses in *Drosophila* are driven by processes that elicit signed saccades with distinct dynamics and trigger mechanisms. Our work generates new hypotheses to study brain circuits that underlie classification of objects in *D. melanogaster*.

INTRODUCTION

Animals on the move must rapidly integrate and evaluate sensory information to generate appropriate motor actions. For instance, flies must distinguish between potential threats, a landing site or a food source while locomoting through spatiotemporally complex environments [161]. Often, a decision must be generated extremely quickly to avoid predation [162], leaving little time for sensorimotor processing [163]. A compelling hypothesis for rapid decision making is that feature-selective neurons relay salient information to trigger appropriate behavior [45,164]. For instance, a looming object generates stereotyped expanding optic flow that can be passed on to appropriate escape circuits to trigger backward walking, jumping and/or flying [164–166].

In *Drosophila melanogaster*, an equidistant long vertical object and a small object elicit visually evoked steering responses of opposite valence, implementing a simple object classification algorithm that enables flies to approach an elongated vertical bar, which likely represents a landscape feature [167], while avoiding a small object, which likely represents a threat or conspecific [168]. However, small object aversion does not generalize across all *Drosophila* species, suggesting ecology-dependent specialization [169]. At present, the behavioral strategy in *D. melanogaster* that generates approach and aversion is unclear. The object classification system that distinguishes between tall and small objects must link visual information to an appropriate motor action to orient the animal toward or away from the stimulus [168].

Previous work showed that tethered, flying flies that are free to rotate about the yaw axis perform body saccades toward a moving, tall vertical bar and the dynamics of these saccades can be predicted by integrating the angular position of the bar relative to the fly's forward axis over time. These results are consistent with a model in which the fly brain temporally integrates the angular position of the bar relative to its body axis over time, until this value reaches a threshold, to trigger (with noise) a saccade toward the bar [170]. Such an underlying visuomotor algorithm, characterized in tethered flight, is one way to explain why flies in free flight aggregate near tall vertical objects. It remains unknown whether small object aversion is also controlled by saccades and how saccades and smooth movement interact. Here, we hypothesized that small object aversion relies on a distinct behavioral strategy that generates larger, visually-guided saccades that could enable avoidance of a potential threat or conspecific. To test this hypothesis, we studied the behavioral strategy that underlies bar tracking and small-object aversion by studying flight in a rigid- and magnetic-tether paradigm. We discovered that aversion to a motion-defined object is mediated by saccades oriented away from the small object. Together, our results support the hypothesis that object classification and saccade-based behavioral algorithms for approach and avoidance are distinct.

MATERIALS AND METHODS

Animals

A wild-type *Drosophila melanogaster* strain was maintained at 25°C under a 12 h:12 h light:dark cycle with access to food and water ad libitum. This *Drosophila melanogaster* strain was reared from a wild caught iso-female line. All experiments were performed with 3- to 5-day-old adult female flies.

Visual stimuli

Most tethered behavioral experiments in virtual reality flight simulators have historically used stimuli composed of solid dark objects or black-and-white gratings superimposed on a uniform white background [143,171]. These visual objects, though convenient and intuitive, can be discriminated from the visual background by any combination of luminance, contrast, or motion cues. These visual cues may drive motion vision and feature detection differently. Figures composed of random texture superimposed upon a similarly random background are defined only by their motion relative to the background, yet nevertheless elicit robust figure-ground discrimination in flies rigidly tethered under virtual (experimentally coupled) closed-loop feedback conditions, even when the figure is defined by higher order statistical properties that are undetectable by a classical model of motion vision [172–174]. Furthermore, a motion-defined vertical bar elicits more robust saccadic tracking than a similarly sized dark bar against a uniform background [170]. The different experimental approaches used here were designed to show (1) saccadic steering responses (spikes in ΔWBA) by a rigidly tethered fly to sinusoidal object movement centered at a fixed azimuthal location

(Figure 2C, Fig. 4), (2) saccade orientation and amplitude tuning by rigidly tethered flies in response to varying object size at all azimuthal locations (Figure 2D-E), and (3) how saccade dynamics for object approach and avoidance map onto the behavior of flies operating in the more naturalistic magnetically tethered paradigm (Figure 3).

Rigid tether paradigm

After cold-anesthetizing flies, we glued a small tungsten pin onto the thorax using UV-activated glue. Flies were given at least one hour prior to experiments. Flies were then placed in the center of a cylindrical flight arena (Figure 2A). The arena has been described elsewhere [175]. The display consisted of a cylindrical array of 96 × 32 LEDs subtending 330° horizontally and 94° vertically. An infrared diode was used to project light onto the beating wings, casting a shadow unto an optical sensor. A wingbeat analyzer (JFI Electronics, Chicago, IL, USA) transformed the signal from the optical sensor into a signal that is proportional to the wingbeat amplitude of the left minus right wing. Changes in wingbeat amplitude (ΔWBA) signals from the optical wingbeat analyzer were acquired at 1000 Hz.

In Figure 2C, each fly was presented with 6 seconds of open-loop virtual object motion followed by 5 seconds of closed-loop bar fixation. The open-loop stimulus motion was sinusoidal; specifically the object oscillated at 1 Hz and moved 22.5° in each direction from its starting position at ±45° from visual midline (angular speed = $90°s^{-1}$). This 10 second stimulus epoch was repeated until each fly was presented with each bar height variation 12 times, resulting in approximately 4 minutes of stimulus per fly. Tall bar and short bar height were 94 and 15°, respectively, and width was kept constant at 30°. We show the averaged response for an object on the fly's right, in addition to

several raw traces. A subset of the full data set of raw trials is contained in Fig 4. The motion-defined (Fourier) object was composed of vertical stripes with an equal number of ON and OFF columns superimposed over a background with the same statistics, i.e. equal number of ON and OFF columns.

To quantify how bar height affects torque spike valence and amplitude, we employed the Spatio-Temporal Active Field (STAF) methodology, as described previously [172,176] Briefly, the path of a bar of variable height was prescribed by a pseudo-random, 15.6-second m-sequence. Thus, the bar "jittered" around a fixed azimuthal location and ΔWBA spikes were identified from the ΔWBA signal as described in prior work [172]. The initial bar position was set at 24 equally spaced azimuthal positions, therefore each fly went through 24 stimulus trials, one at each of 24 randomly shuffled azimuthal locations.

Magnetic tether paradigm

Animals were prepared for each experiment according to a protocol that has been described previously [177,178]. Briefly, flies were cold-anesthetized flies by cooling on a stage maintained at approximately 4°C. For the magnetic-tether, stainless steel pins (100 μm diameter; Fine Science Tools, Foster City, CA) were glued onto the thorax by applying UV-activated glue. The pins comprised less than 1 percent of the fly's moment of inertia about the yaw axis. Flies were allowed at least one hour to recover before running experiments.

The magno tether system has been described elsewhere (Bender and Dickinson, 2006; Duistermars and Frye, 2008). Briefly, the display consisted of an array of 96 × 16

light emitting diodes (LEDs, each subtending 3.75° on the eye) that wrap around the fly, subtending 360° horizontally and 56° vertically (Figure 3A). Flies were suspended between two magnets, allowing free rotation along the vertical (yaw) axis and illuminated from below with an array of eight 940 nm LEDs. The angular position of the fly within the arena was recorded at 160 frames s^{-1} with an infrared-sensitive camera placed directly below the fly (A602f, Basler, Ahrendburg, Germany).

After suspending the fly within the magnetic field, flies were given several minutes to acclimate. We began each experiment by eliciting sustained rotation of the fly by rotating a visual panorama either clockwise or counterclockwise for 30 s at 120° s^{-1}. This stimulus elicited a strong rotatory, yaw-based smooth co-directional optomotor turning response with occasional saccades. From these data, we estimated the fly's point of rotation by computing the cumulative sum of all camera frames and measuring its centroid. Flies that could not robustly follow the rotating panorama were not used in experiments.

To study flies' responses to tall and short bars, we rotated a motion-defined, 8-pixel-wide (30°) bar on a randomly-generated background of 'on' and 'off' pixels (Figure 3B). The bar's initial azimuth position in the arena was generated from a pseudo-random sequence. We rotated the bar at 113°s^{-1} and randomized the direction of motion (clockwise counterclockwise) and bar type (short and tall bar) to minimize habituation. We presented each stimulus for a period of 30 s, defining the duration of an individual trial. Between each trial, we presented a fixed visual landscape for 25 s for the fly to rest. If flies stopped flying during a trial, the trial was discarded. We ignored the first 1 s of a trial in order to avoid the inclusion of saccades which could be generated when the

stimulus first appears. We rejected saccades below 10° and above 180° in amplitude in order to exclude possible tracking error. The procedure to identify saccades from heading data has been described elsewhere [170]. Briefly, we modeled the fly as an ellipsoid and determined the heading by calculating the major axis of the ellipse in each video frame.

Statistical analysis

All statistical analysis was performed using Matlab (Mathworks, Natick, MA, USA) and JMP (SAS, Cary, NC, USA). Unless otherwise specified, we report mean ± 1 standard deviation. When displaying box plots, the central line is the median, the bottom and top edges of the box are the 25th and 75th percentiles and the whiskers extend to ± 2.7 standard deviations.

RESULTS AND DISCUSSION

To determine whether flies perform saccadic turns away from short visual objects, analogous to how they perform saccades to steer toward tall, narrow objects, we measured the steering effort of flies in response to oscillating bar motion in open-loop tethered flight. A randomly textured motion-defined tall bar or a short bar was presented at ±45° from the center of the arena (0°). The bar oscillated at 1 Hz and moved 22.5° in each direction from its starting position at ±45° (Figure 2A,B). Confirming the results of a previous study that used solid black bars on a uniform background [168], on average flies steered toward a tall textured bar and avoided a short bar moving across a static random background (Figure 2C). Averaging across trials

masks the dynamics of the behavior for fixation and aversion. The spikes in ΔWBA—which have been referred to as 'wing hitches' [179] or 'torque spikes' by direct torque measurements in tethered flight [180]—indicate attempted body saccades. ΔWBA spikes are readily observed within single trials that are generally oriented toward the tall bar and away from the short bar located 45-degrees from visual midline (Figure 2C and Fig. 4). ΔWBA spikes were superimposed upon a shift in mean ΔWBA toward the tall bar and away from the short bar (Figure 2C, Fig. 4), consistent with prior work [168].

The raw traces from multiple flies (Figure 2C) would seem to suggest that not only does the valence of ΔWBA spikes switch with bar size, but also that the short bar might elicit ΔWBA spikes with distinct dynamics. To explore the distribution of saccadic steering spikes across the full visual azimuth, and how saccade dynamics vary with object size, we used an experimental method in which a bar was randomly jittered at each of 24 azimuthal positions [172,176]. We randomly shuffled trials for bar vertical heights of 94° ("tall bar", full height of arena), 56°, 30°, and 15° ("short bar"). We measured the amplitude of individual ΔWBA spikes binned at 24 azimuthal position (Figure 2D). This analysis revealed a switch of sign and increased amplitude in ΔWBA spikes as the bar height decreased (Figure 2D, E). The overall ΔWBA spike rate was similar across object height (Figure 2E). However, the changes in ΔWBA spike amplitude in the rigid tether must be interpreted with caution as different tonic ΔWBA levels between short and tall bars could bias ΔWBA spike amplitude.

To test whether body saccades drive short bar aversion under more naturalistic feedback conditions, we recorded flight responses in a magnetic tether system [177,178]. This experimental paradigm allowed flies to freely rotate in yaw thereby enabling more

naturalistic flight dynamics and neural feedback conditions (Figure 3A). As in the rigid tether system, we presented an object rotating over a randomly-textured stationary background. We confirmed that a rotating tall bar elicited robust, attractive tracking saccades, i.e. saccades that bring the bar closer to visual midline [170]. We discovered that a rotating short bar elicited more aversive saccades, i.e. saccades that move the bar further away from visual midline (Figure 3B,C). Together, these results suggest that bar height has a strong effect on saccade valence, supporting results in the rigid tether paradigm.

Notably, there was little-to-no smooth pursuit between saccades during the presentation of a small motion-defined bar (Figure 3E). To reconcile the lack of smooth movement in the magnetic tether with previous studies in a rigid tether paradigm using a dark bar on uniform background that showed strong tonic steering responses [168], we performed an experiment in the magnetic tether where we revolved a motion-defined or dark bar at constant speed ($75°s^{-1}$). We found that the short dark bar generated robust smooth movement between saccades whereas a short motion-defined bar revolving at the same speed generated little-to-no smooth movement (Figure 3E). The smooth movement in response to a short, dark bar in the magnetic tether is co-directional, which is consistent with the in-phase oscillations when a small object oscillates at the fly's visual midline in the rigid tether [168]. Therefore flies can use saccades to perform orienting behavior, but they can also generate slower smooth pursuit, which varies with the stimulus type [181].

In some cases, short bars elicited bouts of co-directional saccades seemingly being chased by the object, whereas in other cases flies generated bouts of contra-

directional saccades away from the object (Figure 3B). To clarify whether flies generally saccade to avoid the small bar, we defined tracking saccades as sustained, co-directional saccades in the same direction as the bar for at least 180° around the arena (bout of 4–5 saccades), as defined in a previous study [170]. Using this operational definition, flies overall generated 36% tracking saccades in the presence of a tall bar (fly following bar) and 2% in the presence of a short bar (fly chased by bar), thus suggesting more robust, sustained tracking in the presence of a tall bar (Figure 3D).

The presentation of a tall bar generated a higher median rate of saccades than for a short bar (tall bar: 1.1 saccade s^{-1}, short bar: 0.63 saccade s^{-1}). The short bar saccade rate was higher than previously-reported spontaneous saccade rates in the magnetic tether (~0.4 saccade s^{-1})[170,177], suggesting that the short bar stimulus elicited visually guided saccades. However, we expect that some saccades we measured were spontaneous, triggered by endogenous processes [182,183]. Nevertheless, there was a significant association between the stimulus type and saccade valence (χ^2 test, $p<0.001$, DF = 1, n = 2,833 saccades). For a short bar, there were more aversive saccades than predicted by chance (χ^2 test, $p=0.001$, DF = 1, n = 877 saccades), whereas for a tall bar, there were more attractive saccades than predicted by chance (χ^2 test, $p<0.001$, DF = 1, n = 1956 saccades). The amplitude, duration and peak angular velocity of saccades in the magnetic tether were overall smaller for the tall bar compared to the short bar, which is consistent with the findings under open-loop (Figure 2D) (t-test, $p<0.001$, DF = 1, n = 2,833 saccades) (Figure 3F). The statistical outcome did not change when considering non-parametric distributions (Kruskal-Wallis test) or considering the possible effect of individuals (mixed-effect model). To determine if the pre-saccade error

angle could be influencing the saccade dynamics, we computed the pre-saccade error angle in azimuth for both tall- and short-bar experiments. For the tall bar, the pre-saccade error angle was centered at ~45–60° and correlated with saccade amplitude, consistent with our previous work (Figure 3G) [170]. In contrast, for a motion-defined short bar the pre-saccade error angle was more stochastic, with a wider distribution (Figure 3G), and similarly for a short, dark bar (Fig. 5). These data show that the error angles that generate saccades have substantially different distributions between a tall and short bar, suggesting different trigger algorithms. The difference in saccade dynamics and trigger suggest that saccades are highly adaptable, as discovered in avoidance and spontaneous saccades in free flight [162,184].

Together, the behavioral responses measured in the rigid and magnetic tether to the presentation of a tall bar and a short bar suggest that the approach and aversion flight orientation responses in *Drosophila* are driven by processes that elicit signed and saccades with distinct dynamics (Figure 2D–E, Figure 3). Prior work had revealed a simple visual algorithm by which the vertical size of an object controls a switch from visual approach to aversion [168]. The evidence for the size-dependent valence switch, which we confirm here, was that under open-loop tethered flight conditions, steering responses to an object oscillating in the visual periphery were tonically oriented toward an elongated bar and away from a small object, with phasic modulations in steering that track the sinusoidal oscillation of the stimulus (Figure 2C). Likewise, flies fixate a tall vertical bar but avoid a short bar in closed-loop tethered flight [168]. Our results go substantially further by demonstrating that (1) small object classification by the visual system outputs saccades as well as smooth movement, which depends on the stimulus

type, (2) a small object triggers more aversive saccades (Figure 2D,E, Figure 3C) and (3) small-object aversion saccades have significantly different dynamics and trigger mechanisms than bar-tracking saccades (Figure 3F,G). Thus, as with bar tracking [170], small object aversion behavior in flight is mediated in part by body saccades. Our study provides new hypotheses to interrogate the neural basis of object classification for decision making in insects.

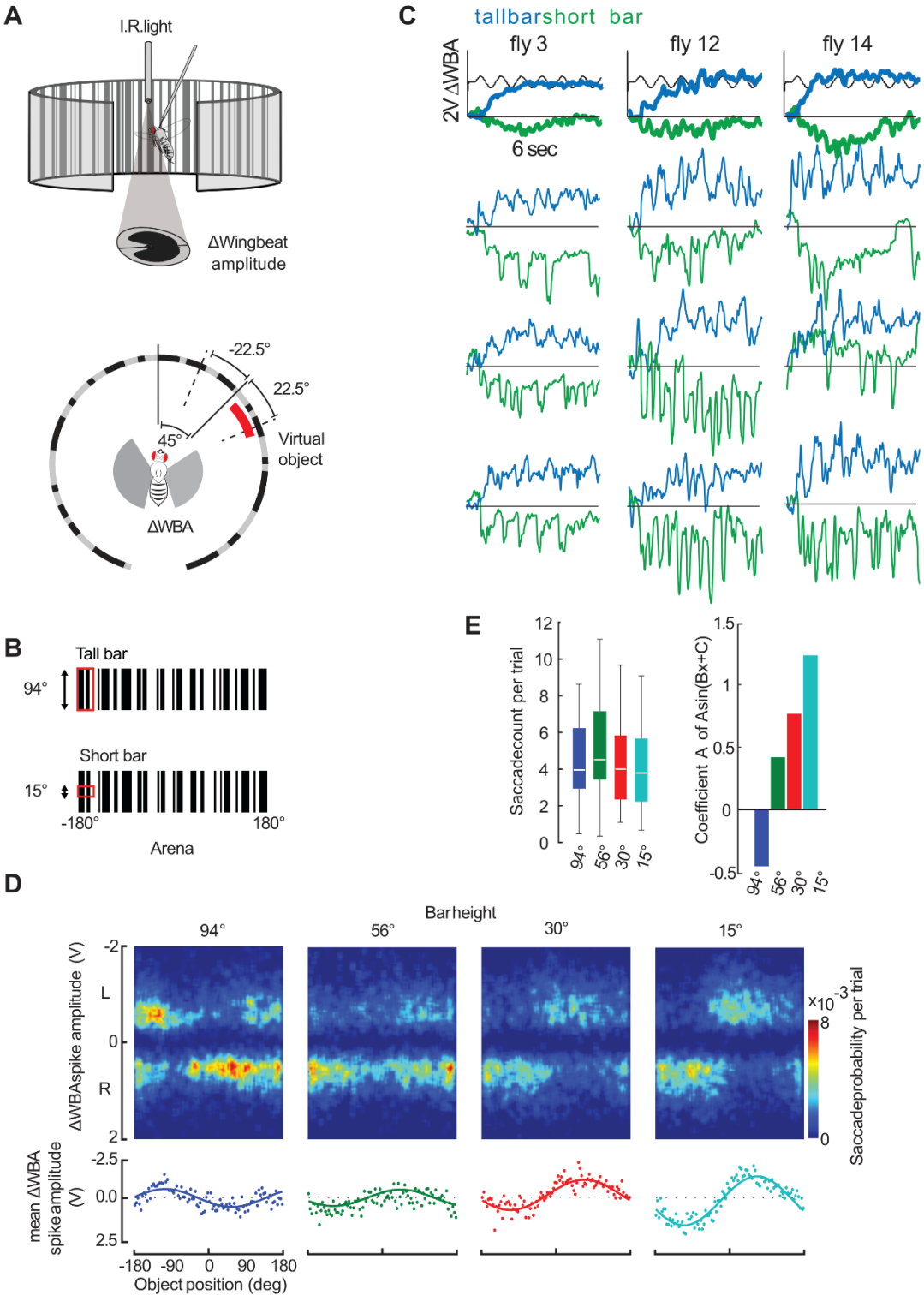
A
I.R.light
ΔWingbeat amplitude
-22.5°
22.5°
45°
Virtual object
ΔWBA
B
Tall bar
94°
Short bar
15°
-180°
180°
Arena
C
tallbarshort bar
fly 3
fly 12
fly 14
2V ΔWBA
6 sec
D
Barheight
94°
56°
30°
15°
ΔWBAspike amplitude (V)
L
R
mean ΔWBA spike amplitude (V)
-180 -90 0 90 180
Object position (deg)
x10^-3
Saccadeprobability per trial
E
Saccadecount per trial
Coefficient A of Asin(Bx+C)

Figure 2. Bar height influences saccade valence in open-loop tethered flight in rigid tether

A) Flies were rigidly tethered and their steering response—changes in wingbeat amplitude (ΔWBA)—measured by an optical wingbeat analyzer. A moving virtual object was presented at ±45° from the center of the arena (0°). The figure oscillated at 1 Hz and moved 22.5° in each direction from its starting position at ±45°. B) Top: Tall bar stimulus with height = 94°, spanning the full height of the display. Bottom: Short bar stimulus with height = 15°. Stimulus width = 30°. C) Top: average fly steering ΔWBA responses for n = 3 flies to tall and short objects displaced to the right of the fly. For the tall bar, the steering response is tonically oriented towards the position of the bar. For the short bar, the steering offset is oriented away from the bar position. Bottom panels: three exemplar individual trials showing ΔWBA spikes. Subset of data set from n = 18 flies is in Fig. S1. D) Top: surface histograms mapping ΔWBA spike amplitude (pseudocolor) oriented toward the left or right (vertical axis) for 24 different bar locations (horizontal axis) and different virtual object height. Bottom: same data as in top, average saccade amplitude at each azimuthal location with sinusoidal fits by azimuthal position. Bar width was kept constant at 30°, and bar height was varied between 94° ("tall bar"- full height of arena), 56°, 30°, and 15° ("short bar"). E) Saccade count per trial (left) and best fit amplitude coefficient of sinusoid in D) for different object heights. n = 30 flies, 24 trials per fly, for D, E.

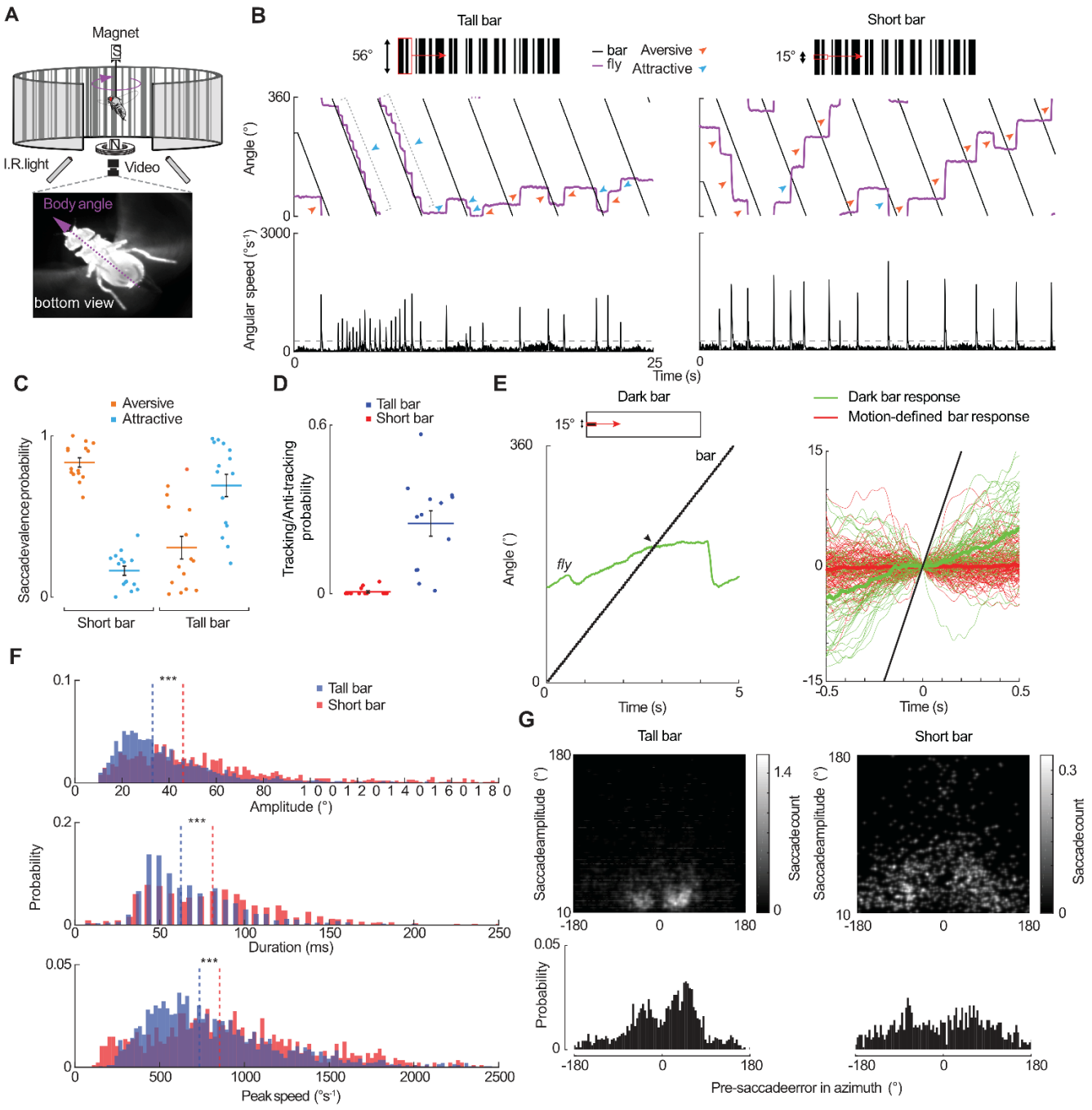
A
Magnet
I.R.light
Video
Body angle
bottom view
B
Tall bar
Short bar
56°
15°
bar
fly
Aversive
Attractive
Angle (°)
Angular speed (°s-1)
Time (s)
C
Aversive
Attractive
Saccade valence probability
Short bar
Tall bar
D
Tall bar
Short bar
Tracking/Anti-tracking probability
E
Dark bar
Dark bar response
Motion-defined bar response
bar
fly
Angle (°)
Time (s)
F
Tall bar
Short bar
Amplitude (°)
Probability
Duration (ms)
Peak speed (°s-1)
G
Tall bar
Short bar
Saccade amplitude (°)
Saccade count
Probability
Pre-saccade error in azimuth (°)

Figure 3. Bar height influences saccade tuning in magnetic tether

A) Flies were suspended magnetically and free to rotate about the yaw axis. We used high-speed video (160 frames s^{-1}) and offline image processing to track the fly's body angle. Flies were illuminated with infrared lights. B) Left: a fly fixates a moving tall bar (56° height, full vertical height of area) by generating bouts of attractive saccades and some aversive saccades. Right: flies generate primarily aversive saccade to a moving short bar (15° height). Virtual object width = 30°. Bottom: absolute value of angular velocity of fly body angle. Gray dotted line: computed saccade detection threshold. Bar speed: 113°s^{-1}. C) Distribution of saccade valence probability during the presentation of tall and short bar. Thick line: mean. Error bar: SEM. Individual dots are the mean for an individual fly. D) Probability of flies generating bouts of tracking saccades via sustained, co-directional saccades covering 180° of the arena. Thick line: mean. Error bar: SEM. Individual dots are the mean for an individual fly. E) Left: example response to motion of a small, dark bar *via* smooth movement and saccades. Arrow indicates midline crossing. Right: comparison of inter-saccade body angles during the presentation of short, motion-defined (red) and dark (green) bars. t = 0 s is the fly's visual midline. Thick line: median. Stimulus: black line. Stimulus speed = 75°s^{-1}. n = 5 flies, 80 trials. F) Histograms of saccade amplitude, duration and peak angular speed for tall bar (blue) and short bar (red). ***: $p \leq 0.001$ Dotted lines: median. G) Top: Colormap of pre-saccade error in azimuth vs. saccade amplitude for motion-defined tall and short bars. 0° is the fly's visual midline. Bottom: Probability histogram of pre-saccade error angles. For C,D,F, G: n = 14 animals, 150 trials total.

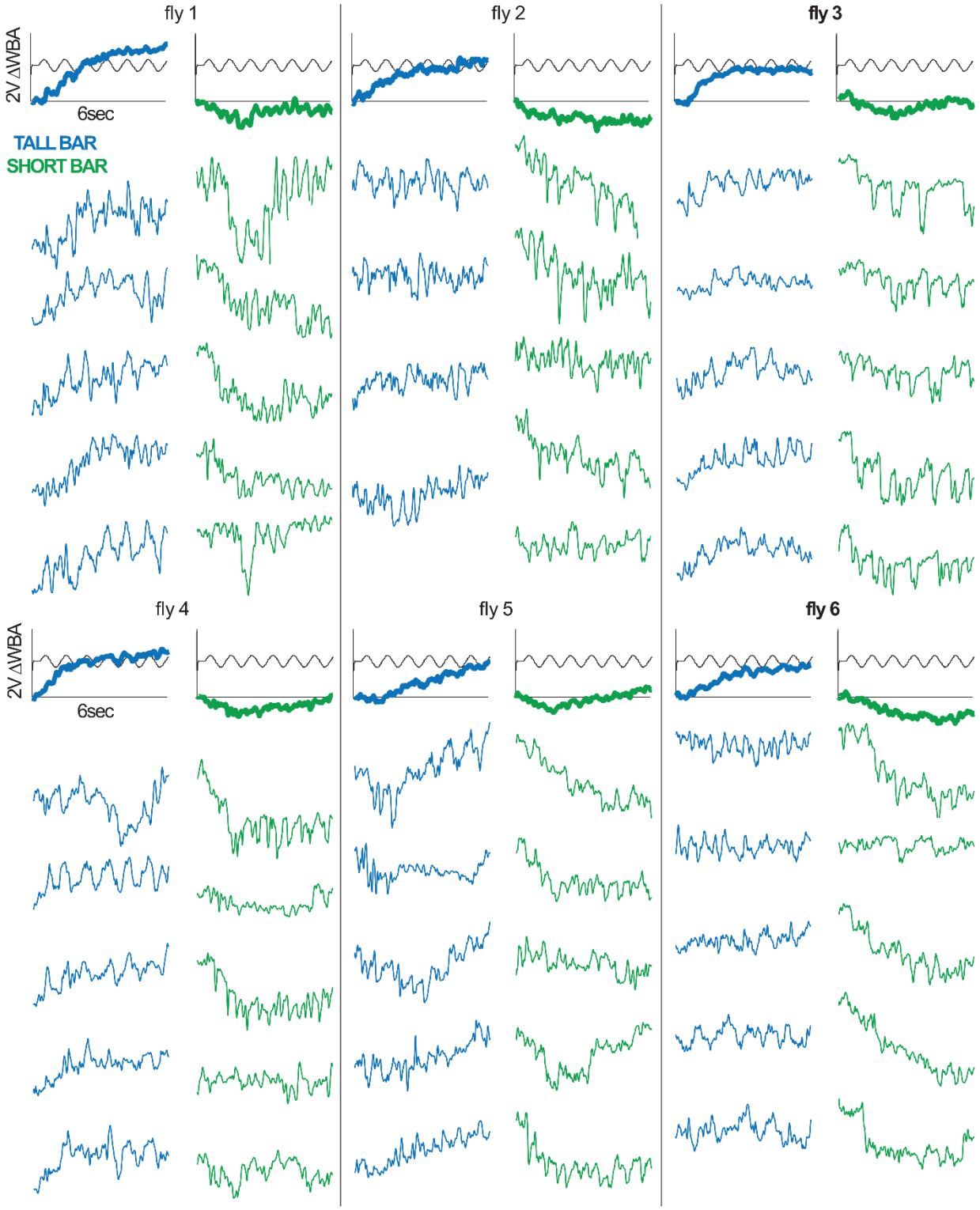
fly 1
fly 2
fly 3
2V ΔWBA
6sec
TALL BAR
SHORT BAR
fly 4
fly 5
fly 6
2V ΔWBA
6sec

Figure 4. Raw data traces for a subset of tall (blue) and short (green) motion-defined bar trials

The open-loop stimulus oscillated at 1 Hz and moved 22.5° in each direction from its starting position at ±45° from visual midline (angular speed = 90°s-1). Tall bar and short bar height were 120 and 30°, respectively, and width was kept constant at 30°. For each fly, we show the average response for an object for 12 trials on the fly's right (thick line), in addition to a subset of raw traces (thin lines).

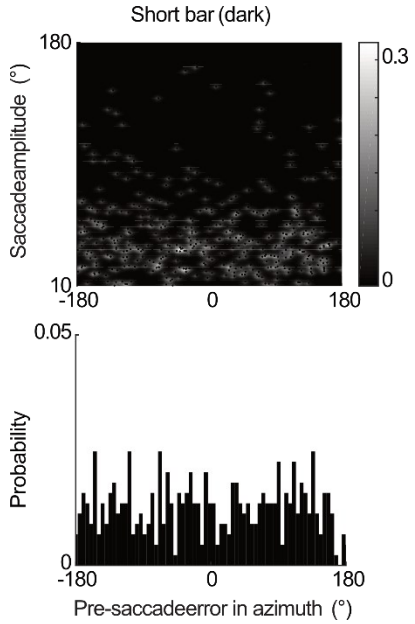

Figure 5

Top: Colormap of pre-saccade error in azimuth vs. saccade amplitude for a short, dark bar. 0° is the fly's visual midline. Bottom: Probability histogram of pre-saccade error angles. n = 5 animals, 80 trials total.

3. ODOR BOOSTS VISUAL OBJECT APPROACH

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ABSTRACT

Multisensory integration is synergistic - input from one sensory modality might modulate the behavioral response to another. Work in flies has shown that a small visual object presented in the periphery elicits innate aversive steering responses in flight, likely representing an approaching threat. Object aversion is switched to approach when paired with a plume of food odor. The 'open loop' design of prior work facilitated the observation of changing valence. How does odor influence visual object responses when an animal has naturally active control over its visual experience? In this study, we use closed loop feedback conditions, in which a fly's steering effort is coupled to the angular velocity of the visual stimulus, to confirm that flies steer toward or 'fixate' a long vertical stripe on the visual midline. They tend to either steer away from or 'anti-fixate' a small object or to disengage active visual control, which manifests as uncontrolled object 'spinning' within this experimental paradigm. Adding a plume of apple cider vinegar decreases the probability of both antifixation and spinning, while increasing the probability of frontal fixation for objects of any size, including a normally aversive small object.

INTRODUCTION

In flight, flies approach the vertically elongated edges of landscape features such as plant stalks, whereas they avoid threats posed by small moving objects [58,168,185]. This simple algorithm, based only on vertical object size, reduces the computational resources required for the brain to quickly make a crucial behavioral decision [168]. In

free-flight this behavioral decision happens during a single turn, within a fraction of a second, but the valence of a visual feature has been shown to persist far longer [58]. Under so-called 'open-loop' experimental conditions, in which the wing kinematics of a tethered fly are recorded in response to imposed visual stimuli but the animal cannot control its visual experience, flies steer towards a tall object projected into the visual periphery and away from a small object in the same location for seconds [58,168,186], an artificially elongated time frame. When provided with virtual 'closed-loop' feedback, in which the fly's steering effort controls the visual stimulus [143,168], persistent approach towards a bar manifests as centering the object on the visual midline. Under closed-loop control, object aversion manifests either as spinning, in which a fly seems to forego active control and instead steers constantly in one direction, or as antifixation, in which a fly actively avoids the stimulus, keeping it centered in the rear field of view [168].

For *Drosophila melanogaster*, the presentation of an attractive odor modulates the attractiveness of small objects [58,151]. Mechanistically, under open-loop tethered flight in which a peripheral object evokes tonic aversion, odor switches the steering valence from avoidance to approach [168,186]. However, under natural flight conditions, object position would vary with steering effort. How does food odor modulate visual object valence when the animal has active control over the trajectory of the object?

We sought to answer this question using a standard virtual closed-loop flight simulator. We compared how flies actively control the spatial location of three visual objects in odorless air and in a plume of the naturally appetitive odor apple cider vinegar

[187]. We measured the influence of odor on three visual control modes: fixation, spinning and antifixation. We confirm that for progressively taller objects flies show less antifixation, less spinning, and more fixation. We then show that odor further decreases both antifixation and spinning, while increasing frontal fixation of all objects.

METHODS

3-5 day old female wild-type flies (*Drosophila melanogaster*) reared from an iso-female line were used [146]. Flies were removed from food, rigidly tethered at the dorsal thorax (the head was not immobilized) onto a 0.1mm-diameter tungsten pin and allowed to rest for one hour. A tethered fly was suspended in the center of a circular display of 570nm light emitting diodes [175] with a separate infrared wingbeat analyzer to record wing beat amplitude and frequency (Fig. 6A). The steering effort, proxied as the difference between left and right wing beat amplitudes, ΔWBA [144], was negatively coupled to the angular velocity of the visual stimulus such that when the fly steered in one direction, the visual stimulus moved in the opposite direction to 'virtually' close the control loop. A mass flow regulated odor plume (40ml/min) was delivered through a 20µl pipette tip suspended 1cm fronto-dorsal of the fly's head [58,141] (Fig. 6A). 70% apple cider vinegar diluted in water (Ralph's Grocery generic brand) was interspersed with water vapor in a randomized fashion.

Visual stimuli were composed of solid dark objects set against a bright equiluminant background, sized 7.5°x 30° 'small object', 30°x 30° 'medium object', and 94°x 30° 'tall bar' (Fig. 6B right). Visual objects were presented randomly, appearing

behind the fly at 180° for each trial. The 20-second trials were repeated six times per odor condition at a closed loop gain of -20 frames/second per volt of ΔWBA. Trials were interspersed 8-second periods of closed-loop with a 94° x 15° bar at -10 frames/second gain. Experiments generally lasted two hours. All control, acquisition, and analysis was performed with custom MATLAB scripts.

Analysis was similar to that used previously [168]. Stimulus position was sampled at 1kHz from flies whose wing beat frequency did not dip below 100 cycles/second for more than two seconds during the experiment; 17 out of 19 flies prepared were used for analysis. The first 2 seconds of each trial were discarded while flies adjusted to the new random condition.

We calculated probability distributions (Fig. 6C) of the residence time at each azimuthal position for each visual object. Object position traces were averaged in one-pixel bins (1 pixel = 3.75° azimuth), and averaged across flies (n=17). We plotted azimuthal probability density in polar coordinates (Fig. 7B, 7C) using a sliding 2-second window analysis to compute mean resultant vector (θ), a measure of angular heading in the arena (Fig. 7A), and resultant vector length (r), a measure of circular spread of the heading values (Fig. 7A) [188]. r-values, radii along the unit circle, ranged between 0 and 1, with values closer to 1 indicating a narrower spread of unit vectors, or tighter visual control over the visual object, within the window. The probability of each bin of heading values (bin width = 3.75°) and r (bin width= 0.1) was averaged across trials and flies (n=17). Each binned measurement was classified for its behavioral mode based on θ and r. Frontal fixation is defined by $-90° < \theta < 90°$ (front hemifield) and $r > 0.6$ (Fig. 7A red zone). Antifixation is defined by $-90° > \theta > 90°$ θ (rear hemifield) and $r > 0.6$ (Fig.

7A, purple zone). Spinning is defined as any mean θ value with r <= 0.6 (Fig. 7A, cyan zone). Criteria were based on prior results [168]. From these values, we also calculated a preference index (PI = attraction responses - aversion responses / total responses). PI ranged from -1 to 1, with positive values denoting attraction tendency, and negative values denoting aversion tendency (data not shown).

RESULTS

We assessed how appetitive food odor (apple cider vinegar, ACV) influences flies' spatial control over three visual stimuli by computing residence probability of the visual object across flight arena azimuth under closed-loop feedback (CL) conditions. A clear peak in residence probability at midline was observed for all three visual objects in clean air (black traces), with peak probability proportional to object size (Fig. 6C,C',C"). Conversely, the residence probability of objects within the visual periphery was larger for the small objects than the tall bar. After switching from clean air to odor, and repeating the randomized object size trials, the probability of midline object positioning increased for all 3 visual objects (Fig. 6C,C',C" orange traces, * $p<0.05$ Student's paired t-test), accompanied by decreased probability at the visual periphery. The effect of odor was most pronounced for the small object (Fig. 6C).

We next calculated the direction (θ) and length (r) of the mean resultant vector for flies' control of each visual object. We defined frontal fixation as θ values in the front hemifield at r > 0.6 (Fig. 7A, *red region*). Antifixation is defined as θ in the rear hemifield at r > 0.6 (Fig. 7A, *purple region*). Spinning is defined by r <= 0.6 (Fig. 7A,

cyan region). Data from a single fly highlights instances of all three behavioral modes (Fig. 7A').

As with the residence probability distributions (Fig. 6C), increasing object size in clean air results in progressively stronger frontal fixation (higher probability values at the circumference near $\theta \sim 0°$), reduced antifixation (higher probability values at the circumference near $\theta \sim 180°$), and reduced spinning (lower probability values near the origin) (Fig. 7B). By visual inspection, for all three visual stimuli, switching from clean air to odor is accompanied by an increase in frontal fixation that is offset by a decrease in spinning (Fig. 7C). Accordingly the PI increased significantly with the transition from odor OFF to ON for all three visual objects ($p < 0.01$, Student's paired t-test, data are redundant with results of Fig. 6C and thus not shown).

We next computed the probability that flies engage in each behavioral mode under each experimental condition. In general, the frequency of antifixation or spinning decreases in the presence of odor for all three visual objects (Fig. 7D,D',D", purple & cyan). Conversely, odor increases frontal fixation behavior for all visual stimuli (Fig. 7D,D',D"; *$p<0.05$, **$p<0.01$, Student's paired t-test). Here, we show the effects for each fly, and for each experimental condition in which odor and clean air trials were interspersed. However, the effects of odor on visual behavioral modes were similar for the very first odor trial as well, suggesting that the influence of odor was immediate and not experience-dependent (data not shown).

DISCUSSION

Rigidly tethered flies tend to steer syn-directionally in response to an object moving across the visual midline. Thus, under virtual closed-loop feedback conditions, the object becomes fixated near the visual midline [58,168]. Smaller objects are frontally fixated less robustly (Fig. 6 C,C',C"). In the presence of odor, flies mores strongly fixate any sized visual object, while concomitantly decreasing antifixation and spinning (Fig. 6C, 7B, 7C, 7D). The effects of odor on both the distribution of behavioral modes and increased fixation would combine to bring a fly closer to a visual object, a behavioral response which has been observed in flies freely exploring a wind tunnel [151]. The modulation of visual salience by an appetitive odor can enhance foraging performance when meaningful sensory signals converge, and conserve neural processing resources when they do not.

Tethered flight experiments are crucial for exploring mechanistic interactions between sensory modalities, since stimuli can be precisely controlled. In open-loop conditions, in which the object is restricted to the visual periphery, flies tend to tonically steer in the opposite direction [168], or execute saccades oriented away from the object [186]. Attractive odors reverse aversion to approach [58]. But why do flies tend to approach (fixate) visual objects under closed-loop feedback conditions (Fig. 6C, 7B, 7C, 7D) but avoid them under open-loop conditions? This apparent paradox is resolved by the fact that the valence of a visual stimulus can vary across the visual azimuth. For example, a narrow grating or bar oscillating across midline elicits syn-directional steering responses [189]. Intuitively, this reaction would lead to frontal fixation under CL conditions. Indeed, a model of directionally selective motion detectors flanking the

visual midline is sufficient to explain frontal bar fixation [190]. However, positioning a bar or grating in the visual periphery generates a tonic steering effort and wing saccades oriented away from the grating [144,172,186,191]. Thus, the same visual cue triggers different behavioral outcomes depending on its location in the visual field [166]. Under tethered closed-loop control conditions, a visual object stimulates the entire visual azimuth, thereby driving motor responses with different azimuthal tuning.

We do not know whether fixation, antifixation or spinning behaviors are coordinated by different neural pathways. If so, then each subsystem may be individually and differentially modulated by odor. Alternatively, odor modulation may occur after signals from each subsystem have converged upon premotor descending neurons. Our behavioral results provide a conceptual framework for studying these interactions at the neuronal circuit level.

DATA REPOSITORY

Data for this chapter can be found at:

Cheng KY, Frye MA. 2021 Data from Odor boosts visual object approach in flies. Dryad Digital Repository. (https://doi.org/10.5068/D1GD5F)

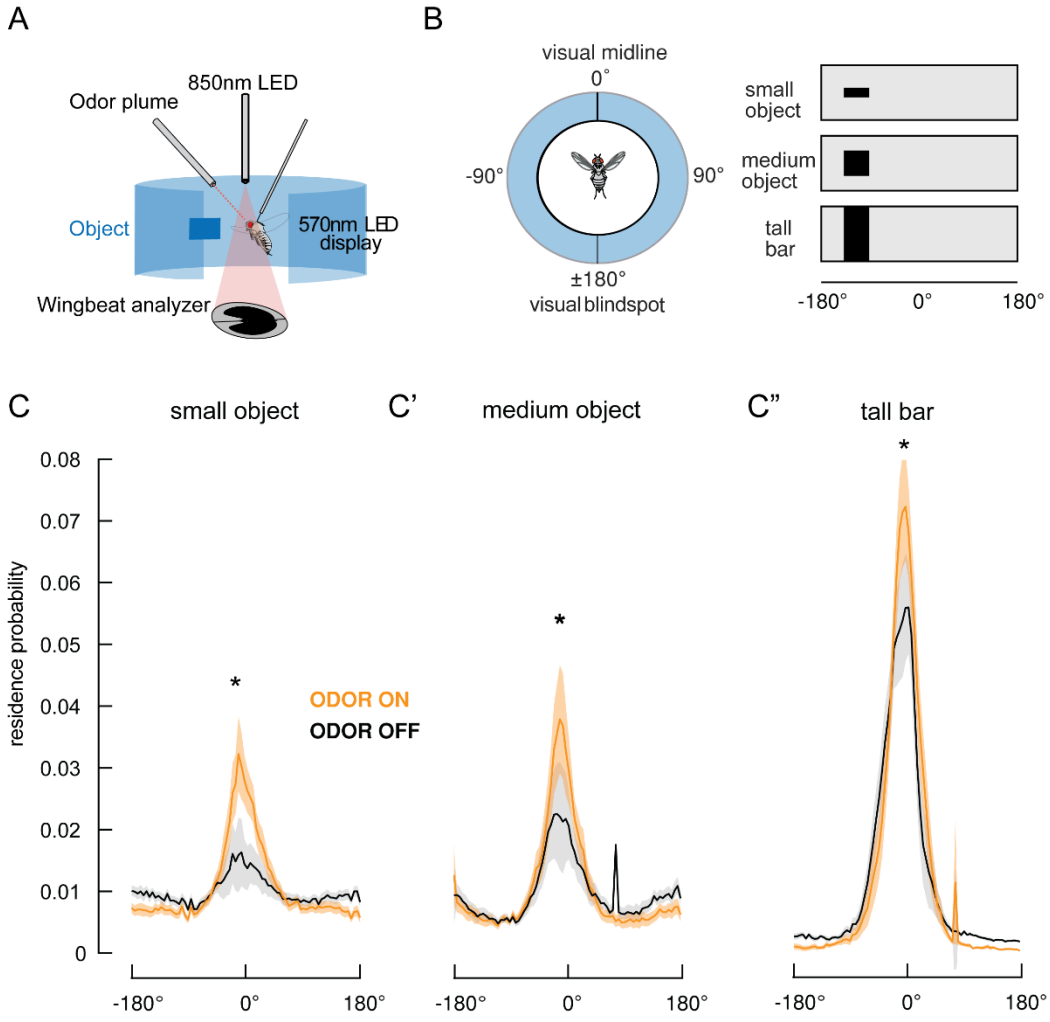

Figure 6

A) Schematic of the apparatus. A tethered fly is suspended within a cylinder of LED panels. Saturated odor vapor is delivered from a nozzle 1cm in front of the fly. An 850nm LED supplies a wingbeat analyzer so that steering effort controls the angular velocity of the visual stimulus.
B) (Left) The flight arena from above, 0° is visual midline. (Right) The three visual stimuli.
C-C") Average azimuthal residency histograms for air (black) and odor (orange). Solid lines represent the mean (n=17), shaded regions ±SEM. *p < 0.05, Student's paired t-test.

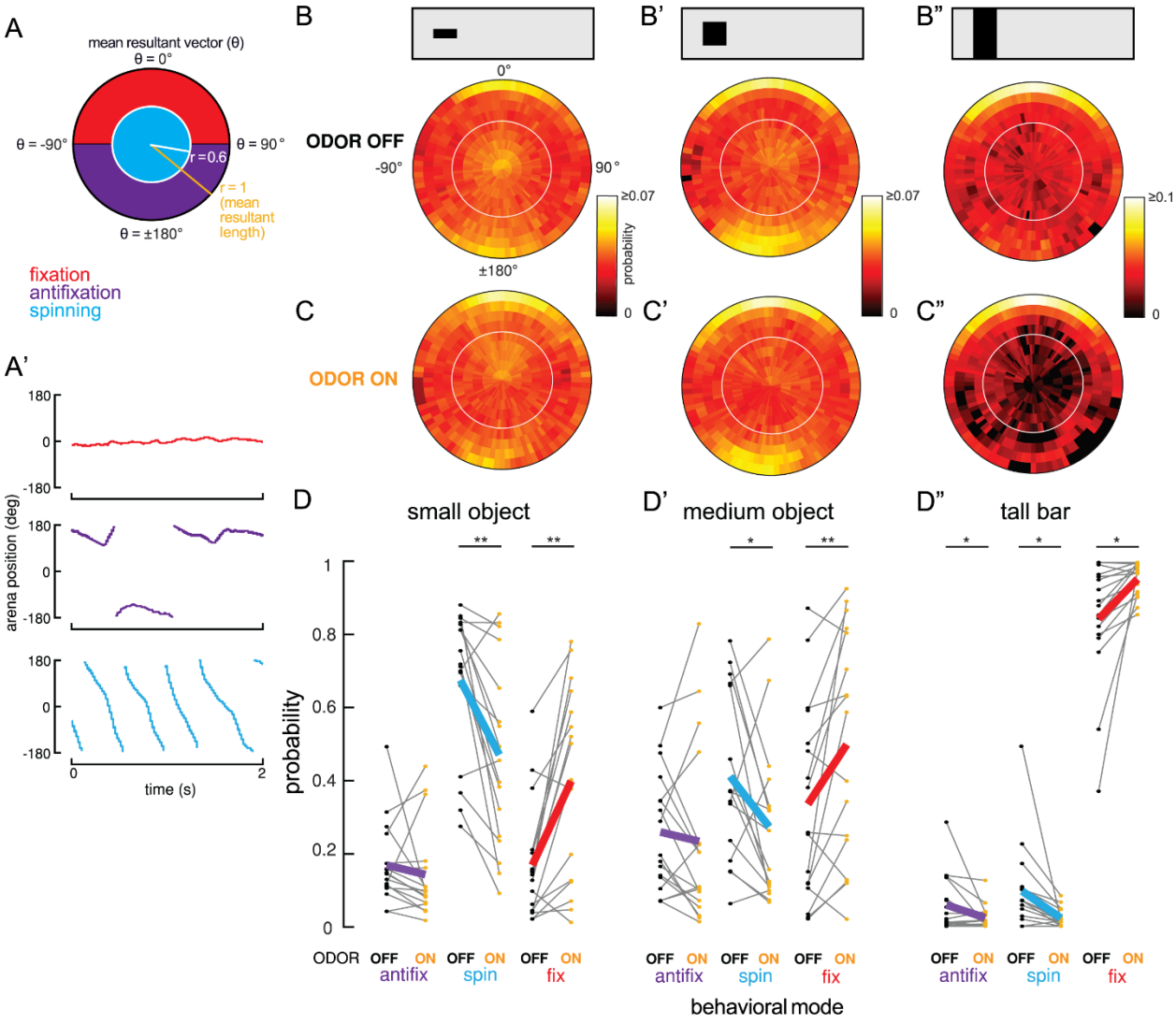

Figure 7

A) Notation of direction (θ, degrees), and length (r, unitless) of the mean resultant vector of object location. θ and r values define three behavioral modes. (A') Sample traces from a single fly with the 7.5°x 30° object in odorless air.
B&C) Mean density of θ and r in polar coordinates for n=17 flies.
D-D") Within-subjects comparison for each behavioral mode. Gray thin lines are individual flies; colored thick lines are means for all flies (n=17) (* $p < 0.05$, ** $p < 0.01$, Student's paired t-test).

4. OLFACTORY AND NEUROMODULATORY SIGNALS REVERSE VISUAL OBJECT AVOIDANCE TO APPROACH IN *DROSOPHILA*

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ABSTRACT

Behavioral reactions of animals to environmental sensory stimuli are sometimes reflexive and stereotyped, but can also vary depending on contextual conditions. Engaging in active foraging or flight provokes a reversal in the valence of carbon dioxide responses from aversion to approach in *Drosophila* [151,192], whereas mosquitoes encountering this same chemical cue show enhanced approach toward a small visual object [150]. Sensory plasticity in insects has been broadly attributed to the action of biogenic amines, which modulate behaviors such as olfactory learning, aggression, feeding and egg-laying [7–17]. Octopamine acts rapidly upon the onset of flight to modulate the response gain of directionally selective motion-detecting neurons in *Drosophila* [95]. How the action of biogenic amines might couple sensory modalities to each other or to locomotive states remains poorly understood. Here, we use a visual flight simulator [175] equipped for odor delivery [141] to confirm that flies avoid a small contrasting visual object in odorless air [168], but the same animals reverse their preference to approach in the presence of attractive food odor. An aversive odor does not reverse object aversion. Optogenetic activation of either octopaminergic neurons or directionally selective motion detecting neurons that express octopamine receptors elicits visual valence reversal in the absence of odor. Our results suggest a parsimonious model in which odor-activated octopamine release excites the motion detection pathway to increase the saliency of either a small object or a bar, eliciting tracking responses by both visual features.

RESULTS AND DISCUSSION

Visual object valence is reversed by appetitive odor, but not aversive odor

In the presence of odorless air, either flying freely or tethered within a wrap-around display of light emitting diodes (LEDs), a fly will steer toward an elongated vertical bar, which likely resembles a landscape feature such as a plant stalk. By contrast, reducing the vertical size of the bar into the shape of a small 'box' likely represents an approaching threat as it evokes a reflexive steering responses oriented away from the visual object [168]. However, a small visual object could represent food or another attractive resource. For mosquitoes flying freely in a wind tunnel, an attractive odor causes animals to approach and land near a small visual object with greater frequency than in clean air [150].

We tested the hypothesis that the odor of apple cider vinegar (ACV), which is highly attractive to *Drosophila* [137,147,187], modulates the innate behavioral aversion to a small visual object. We equipped the LED arena with an odor delivery nozzle [141] and measured wing steering kinematics in response to a small object oscillating in the visual periphery in the presence or absence of ACV (Figure 8A). Adopting an experimental approach similar to that of Maimon et al. 2008, we presented a 30-degree square object and a 30x94-degree vertical bar (Figure 8B). But, instead of using solid black objects set against a uniform white background [168], we used textured objects set against a textured background to reduce the confound between luminance and motion cues [193]. Steering responses are quantified as the difference between the left and right wing beat amplitude (ΔWBA) encoded by an optical analyzer. Positive values represent steering torque towards the fly's right side and negative values reflect steering towards

the left [144]. The bar and object were oscillated at 1Hz about a point centered 45-degrees to either side of the visual midline (Figure 8C). To facilitate visual inspection of fly steering direction, we plotted time along the vertical axis and ΔWBA on the horizontal axis. We observed similar approach and avoidance responses regardless of which side of the arena the visual stimuli were presented (Figure 9). Therefore, for simplicity, ΔWBA trajectories for stimuli presented on the right side of the arena were multiplied by -1, to reflect them about the visual midline, and pooled with the left-side data. The plot region to the left of visual midline therefore corresponds to responses toward the visual object (Figure 8, approach, blue shading), and *vice versa* for responses oriented opposite the visual object (avoid, gray shading).

Broadly consistent with prior work [168], in odorless air the steering responses of a single wild-type fly are variable - in some trials avoiding and in some trials approaching the small object (Figure 8D). By contrast, the same animal consistently approaches a long vertical bar oscillating at the same azimuthal position (Figure 8D'). Remarkably, upon switching the odor stream from air to ACV, the same fly strongly approaches the small visual object (Figure 8E), and more vigorously approaches the bar (Figure 8E'). A population of 18 animals showed significant reversal from avoidance to approach of the small object in the presence of ACV (Figure 8F,F' $p << 0.01$, Student's paired t-test of ΔWBA steady-state mean of the last two seconds).

This behavioral experiment consists of multiple trials in four different experimental conditions, lasting nearly 10 minutes for each individual. Thus, the visual valence response could have been impacted by classical conditioning, in which a fly might over time associate the small visual object (conditioned stimulus) with a strong,

attractive food odorant, ACV (unconditioned stimulus) [194]. To assess this possibility, we plotted mean steering responses over sequential trials and found that object responses were invariant over the duration of the experiment; flies switch to approach the small object within roughly two seconds after the first presentation of ACV (Figure 8G, trial 1). We found no statistical differences between the first and last trial ($p = 0.95$, Student's paired t-test of trial 1 vs. trial 6, Figure 8G). The effect of ACV on reversing the small object valence persists throughout the experiment, rather than building gradually over time.

To examine individual variation of odor-mediated valence reversal behavior, we calculated the endpoint ΔWBA steering responses and compared these measurements before and after ACV presentation for each individual fly (Figure 8H). Each black dot represents the average ΔWBA over the final two seconds in air, and each red dot represents the corresponding mean in ACV for the same animal. The two dots are connected by a blue line if the object valence was reversed by ACV, and by a black line for steering shifts in the same direction. 12 out of 18 flies tested exhibited visual object valence reversal (Figure 8H, blue lines). The inset shows the same plot (with an expanded time axis), but with the dots removed. Each blue line represents a shift from aversion to approach, and the projection on the x-axis indicates the strength of the shift for each individual fly. For 2 out of 18 flies, the steering responses to the small object were not influenced in either direction by ACV (Figure 8H, overlap of black and red dots). By comparison to reversing the valence of the object, ACV further increases the attractiveness of the bar by comparison to the odorless control air stream ($p < 0.01$,

Student's paired t-test, Figure 8F'). This was consistent across repeated trials (Figure 8G') and occurred in 10 out of 18 flies tested (Figure 8H').

We next examined whether visual valence reversal by odor persists across fly strain and odorant type. Similar to wild-caught population cage flies (PCF, Figure 8F, F'), OregonR wild-type flies in clean air steer to avoid the small object while robustly approaching the bar (Figure 8I,I'). In the presence of ACV, OregonR flies reverse their steering behavior to approach the small object ($p < 0.01$, Student's paired t-test over final 2-second epoch, Figure 8I). This reversal was observed in 11 out of 13 flies (Figure 8I, inset). Approach toward the bar was unchanged by ACV ($p=0.34$, Figure 8I').

We next tested a different odorant, ethanol (EtOH), which has been shown to be highly attractive in flight [151]. We found that like ACV, EtOH presented to WT-PCF flies reverses the valence of visual object avoidance to approach (Figure 8J, $p< 0.01$). Reversal was observed in 10 out of 13 flies tested (Figure 8J, inset), and the strength of the approach toward the elongated bar was unchanged by EtOH (Figure 8J', $p = 0.07$). By contrast to ACV and EtOH, when tested with benzaldehyde (BA), an odorant that flies actively avoid during flight [195], flies continue to avoid the small object and approach the vertical bar in a manner statistically indistinguishable from their responses in odorless air ($p=0.20$ object; $p=0.23$ bar, Figure 8K,K').

Our results suggest that visual valence reversal is elicited by two highly attractive odorants (ACV and EtOH, Figure 8F & 8J), but not by a canonically aversive odorant (BA, Figure 8K), each delivered at intensities known to evoke stable tracking or avoidance in flight [192,195]. To date, several lines of evidence suggest that attractive and aversive odorants are processed by anatomically segregated olfactory pathways

through the mushroom body and lateral horn [196–199]. The mushroom body is classically known for its role in olfactory learning in flies [200], but our analysis suggests that odor-induced visual valence reversal was learning-independent because it has a rapid onset and does not improve with repeated trials (Figure 8G). The lateral horn has been shown to mediate olfactory behaviors in a rapid, experience-independent manner, and also to segregate attractive and aversive odors into anatomical subdomains of the neuropil [196,197,199,201]. Our findings support the hypothesis that the attractive olfactory pathway is specifically engaged for visual object valence reversal [197,198,202].

Optogenetic activation of Tdc2 neurons induces visual valence reversal

To explore how olfactory signals are coupled with visual behaviors, we tested the hypothesis that aminergic neuromodulation is involved in odor-induced visual valence reversal. We expressed Chrimson, a red-shifted excitatory channelrhodopsin [203], in aminergic neurons and modified our experimental paradigm by replacing odor stimulation with 685nm Chrimson-exciting illumination (Figure 8A). The inducible nature of Chrimson allows us to compare each fly's flight steering response before (LED Off) and after (LED On) light-activated membrane depolarization. To account for the slow kinetics of some biogenic amines, we included a 2-minute priming excitation before presenting the visual stimuli. An enhancer-less Gal4 line 'Empty-Gal4' [204] served as a genetic control for transgene expression. Enhancerless controls show behavioral responses to the small object and bar that are similar to those of wild-type flies (Figure 10A,B), although the LED tends to increase approach toward the bar ($p=0.054$, Figure 10B'). Remarkably, in the absence of odor, optogenetic depolarization

of octopaminergic/tyraminergic neurons by the Tdc2-Gal4 driver [71] reverses the steering responses to the small object from aversion to approach, while also increasing the steering responses toward the bar (Figure 10C, Fig 11C, *** $p << 0.01$, Student's paired t-test). 15 out of the 16 flies tested showed valence reversal upon Tdc2>Chrimson activation (Figure 10C, inset). By contrast, optogenetic activation of dopaminergic neurons (TH>Chrimson, Figure 10D, $p=0.956$), mushroom-body specific dopaminergic neurons (PAM>Chrimson, Figure 10E, $p=0.045$), or serotonergic neurons (TRH>Chrimson, Figure 10F, $p=0.866$) failed to evoke visual valence reversal. The difference in steering amplitude of object responses to LED On and LED Off by PAM>Chrimson was statistically significant, but activating these neurons merely weakened the small object avoidance without reversing it (Figure 10E).

Depolarizing Tdc2-labeled neurons is sufficient to robustly induce visual valence reversal in the absence of appetitive odor in a flying fly (Figure 10C). Tdc2-Gal4 labels both octopaminergic (OA) and tyraminergic (TA) neurons. Indirect evidence implicates OA, as the two amines have antagonistic effects [156] and exert opposite effects on cAMP and Ca^{2+} concentrations downstream of the cognate G-protein coupled receptors [53]. OA has been implicated in gain modulation in every visual neuron studied [93,95,107,110,205]. Another important issue is the circuit mechanisms that stimulate Tdc2 neurons are unknown. Our lab previously reported calcium response increases in Tdc2 neurons increased upon the presentation of ACV in quiescent flies [148]. Other work has demonstrated that Tdc2 neurons in larvae are activated upon optogenetic excitation of Orco [152], a broadly expressed olfactory co-receptor [206]. Identifying the

specific circuitry and neuronal subdomains of Tdc2 neurons that are activated by attractive odorants, and the specificity of OA signaling, requires further investigation.

T4/T5 motion detectors are necessary for object aversion and sufficient to induce visual valence reversal

We assessed the synaptic organization of Tdc2 neurons in the optic lobe by co-labeling with DenMark [207] and synaptotagmin [208]. Consistent with previous findings [55], we found that Tdc2 neurons are broadly presynaptic in the optic lobe, showing strong and broadly distributed *syt* labeling throughout the medulla and lobula complex (Figure 12A). We found dense DenMark labeling within the central brain and subesophageal zone, but not within the optic lobe lamina (Figure 12A).

We next sought to identify Tdc2 targets in the optic lobe that could mediate odor-induced object valence reversal. Behavioral responses to visual objects are mediated by the superposition of directional motion signals and higher-order non-directional signals [174,189,209], and neither system alone is sufficient to drive the full complement of normal behaviors [172,190,191,210]. Identified neurons of the directionally selective motion detection system have been shown to be modulated by octopamine. In particular, several wide-field integration neurons of the third optic ganglion that control optomotor behavior [148] exhibit Tdc2-dependent increases in visual response gain upon flight initiation [95,152,206], and one has been shown to be modulated by odor [93]. Presynaptic inputs to the lobula plate, small-field T4 and T5 motion detectors, comprise the first stage of visual processing in which directional selectivity arises in individual cells, and express OA receptors albeit at a relatively low level by comparison

to other aminergic receptors [211]. Presynaptic inputs to T4/T5 neurons are also modulated by OA [110].

We tested the hypothesis that optogenetic activation of T4/T5 reverses the valence of object responses. We expressed Chrimson in T4/T5 neurons and subjected these flies to our visual optogenetics behavioral paradigm, but without the 2-minute priming excitation used in the Tdc2>Chrimson experiment because T4/T5 have rapid response kinetics (see Methods). Transgenic control animals showed qualitatively normal albeit slightly smaller amplitude object avoidance and bar tracking responses (Figure 12B,B'), neither of which were influenced by the LED stimulus (Figure 12B,B'). By contrast, optogenetically activating T4/T5 neurons mimicked the influence of both appetitive odor and Tdc2>Chrimson in all 18 flies we tested ($p << 0.01$, Figure 12C,C').

Since T4/T5 neurites innervate four layers of the lobula plate that each represent a separate cardinal direction of motion [30,212], one might expect that optogenetically depolarizing the full population of directionally tuned T4 and T5 small-field motion detecting neurons would render flies unable to perceive and respond to directional motion cues. Indeed, when we increased the LED intensity 4-fold from 0.010 mW/mm^2 to 0.040 mW/mm^2, we observed diminished approach behavior to both the small object and vertical bar (Figure 13A,A'), as well as diminished wide-field optomotor responses (Figure 13A''). These results show that mild T4/T5 depolarization is sufficient to induce visual valence reversal from avoidance to approach toward the small object (Figure 12C), while strengthening approach toward the bar (Figure 12C'), responses that are qualitatively similar to the effects of ACV or EtOH (Figure 8F,F', J,J'). These results

corroborate recent work showing that appropriately tuned Chrimson excitation can enhance the cellular responses to visual stimuli [94].

We next examined the effect on visual valence reversal when T4/T5 neurons were chronically hyperpolarized with Kir2.1. By contrast to the normal responses by genetic controls (Figure 12D,D'), flies with hyperpolarized T4/T5 neurons show essentially no steering responses to the moving object (Figure 12E), and diminished bar approach that was nevertheless significantly enhanced by odor ($p<0.01$, Figure 12E'). These results indicate that directional motion detectors play a crucial role in behavioral responses to moving objects and bars, yet odor modulation persists qualitatively when the performance of these cells is reduced.

T4/T5 activity is both necessary and sufficient for behavioral approach toward moving objects during flight (Figure 12). How might T4/T5 neurons participate in object aversion in clean air as well as object tracking in odor? It has previously been posited that the neural circuits involved in object classification may have overlapping cellular components [168]. T4/T5 neurons have been broadly implicated in bar tracking behaviors [190,191,193] as well as object-dependent male courtship behavior [209]. Thus, it stands to reason that T4/T5 may supply local motion information to many different visual circuits. Recent work has characterized several classes of columnar projection neurons (VPN) that encode visual features such as looming [213], movement of small contrasting targets [214], and optical disparities generated by the vertical edges of bar stimuli [88]. These cell types are postsynaptic in the lobula, but local trans-lobula plate neurons with dendrites in the lobula-plate and terminals in the lobula could convey directional motion signals to feature-based processes [25,215].

Octopaminergic neuromodulation of visual processing is hierarchical

Pharmacological delivery of OA (or an agonist), and induced excitation or chronic silencing of Tdc2 combine to demonstrate that Tdc2 release of OA modulates virtually every visual processing neuron so far tested. We therefore cannot determine whether the phenocopy of odor results (Figure 8) by optogenetic excitation of Tdc2 neurons (Figure 10) or T4/T5 neurons (Figure 12) is linked by causality or coincidence. Tdc2 activity might be increasing the response gain of upstream columnar inputs to T4/T5, which could be functionally equivalent to optogenetically increasing the response gain in T4/T5. We attempted to assess this issue by targeting RNAi against OA receptor genes specifically within T4/T5 neurons, but the genetic controls failed to show normal visual behavior (data not shown). Further analysis using more robust reagents will be required to discover specifically the visual neurons at the crux of odor or OA mediated visual valence reversal behavior.

An important aspect of our findings is that all behavioral manipulations were performed with animals in active flight. The transition from quiescence to active flight or walking behavior in *Drosophila* is associated with increased response gain of and shifted frequency tuning by wide-field neurons of the lobula plate [41,95,97,110,128]. The modulatory influence by locomotor state has been shown to depend upon the activity of Tdc2 neurons [41,95]. We posit that odor-evoked octopaminergic modulation of visual valence behavior implicates a hierarchy of OA neuromodulation, because this behavior is apparently superimposed upon the neuromodulation on motion vision driven by the onset of locomotion. In other words, OA modulation of motion vision circuitry triggered by the onset of active flight is by itself insufficient to trigger visual valence reversal (Figure 8). Rather, the presentation of either an appetitive odorant or

Tdc2 optogenetic activation in an already flying animal is required to induce visual valence reversal (Figure 8F & 10C). Such a hierarchy could explain why ACV failed to modulate visual responses by T4/T5 neurons in a quiescent imaging preparation [148].

Given the diverse neuronal morphologies contained within the ensemble of Tdc2-Gal4 positive neurons [55,216], it seems highly unlikely that all Tdc2 cells function as a single 'mega-interneuron'. Hierarchical OA neuromodulation by behavioral state and cross-modal sensory activation is more likely to be mediated by the recruitment of distinct subpopulations of Tdc2 neurons, or by differences in the distribution and molecular action of the various OA receptor types, or both [52]. Indeed, recent work has shown that, depending on experimental parameters, activation of Tdc2 neurons can decrease song behaviors [217] or promote male-to-male courtship [9], demonstrating the functional diversity of Tdc2 action.

CONCLUSIONS

In this study we characterized a novel behavior of *Drosophila melanogaster*, odor-induced visual valence reversal. Taken together, our results implicate a conceptual model for multisensory processing (Figure 14) in which an appetitive odor stimulates Tdc2 release that increases the response gain of the motion vision pathway. This excitatory modulation and the interaction between the motion and object vision pathways somehow induce object approach behavior. T4/T5 motion detectors might affect the object vision pathway via TLP neurons. What remains to be determined is how Tdc2 neurons of the optic lobe are driven by the olfactory system, how Tdc2 neurons interact with T4/T5 motion detectors or pre- and postsynaptic pathways, as well as the

underlying circuitry for avoidance of a small moving object, which in the presence of food odor is overridden by approach toward the object.

METHODS

Experimental Model and Subject Details

All Drosophila melanogaster were maintained in a humidity-controlled environment on a 12:12 hour circadian light:dark cycle. Crosses involving aminergic cell types were raised at 18ºC (Tritech Research) to reduce Gal4 toxicity. All other flies were raised in a 25ºC animal room. For behavior experiments, female flies 3-5 days post-eclosion were used, and experiments are conducted within 4 hours after lights-on or within 4 hours prior to lights-off.

Method Details

Rigid tether flight simulator and odor delivery

The rigid tether visual arena is previously described [168]. The arena comprises of computer-controlled 96x32 pixel array of 570nm LEDs arranged in a cylinder, each pixel subtending 3.75 degrees on the retina at the azimuth. Experimental flies were cold-anesthetized and tethered to 0.1mm-diameter tungsten pins. Flies were allowed to recover for one hour after tethering in a plastic box containing a dish of water and illuminated by a heat lamp to maintain humidity. In the flight arena, an infrared emitter and sensor are placed above and below the tethered fly to capture a shadow of the beating wings on the sensor (Figure 8A). The sensor and associated electronics measure the amplitude of each wing beat. The difference in amplitude of the left and right wing

signals (ΔWBA) is proportional to yaw torque [144] and indicates the fly's attempt to steer left (ΔWBA<0) or right (ΔWBA>0).

The following visual stimuli are used in all behavioral paradigms: a 30 degree, randomly textured square, and an elongated, textured bar subtending 30 degrees in width and 94 degrees in height at the eye (Figure 8B). Visual stimuli are presented in random order on the left or right 45-degrees from midline (Figure 8C). The stimuli oscillate via a 1Hz sine wave with an amplitude of 15 degrees. Each experimental condition (object/bar, arena left/right) is presented 4-6 times. Trials in clean air are presented before the odor-paired trials rather than being interspersed in order to limit potential effects of olfactory working memory [218]. A closed loop bar fixation trial is placed between open-loop test trials to keep the fly actively engaged in the experiment.

Odors used in this study were apple cider vinegar (Ralph's Grocery generic brand), ethanol (Decon Laboratories, Inc.) diluted to 70% and benzaldehyde (Sigma Aldrich, B1334) diluted to 40%. Because benzaldehyde precipitates easily, the odorant is placed on filter paper inside the odor delivery tube [195]. Odor delivery to the tethered fly has been described previously [141]. Briefly, saturated odor vapor was delivered through a pipet tip placed 1 cm in front of the fly's head and drawn away by vacuum in a tube positioned behind the fly. To confirm that each fly responded to the odor, we administered a 5-second odor response test without visual cues, and only included flies in the experiment that showed a significant increase in wingbeat frequency upon the onset of the odor pulse [142]. No flies were run more than once. At the beginning of each experiment day, a photoionization detector (miniPID Model 200B, Aurora Specific),

was used to confirm the ON/OFF switching of air/odor at the location of the tethered fly.

Rigid flight simulator and optogenetic activation

Optogenetics flight experiments are conducted in a similar setup to the odor experiments, except that blue LED panels (470nm, Adafruit) are used instead of green LED panels (570nm) to avoid Chrimson activation by the display [203]. To reduce the illumination intensity, three layers of neutral density filter (Rosco no. 59) were placed over the LED display. The odor delivery system is replaced by a red LED (685nm) that illuminates the entire fly. Similar to the odor experiment paradigm, all LED Off trials were conducted first, followed by the block of LED On trials. The same visual patterns from the odor experiments were used and presented with a random block experimental design.

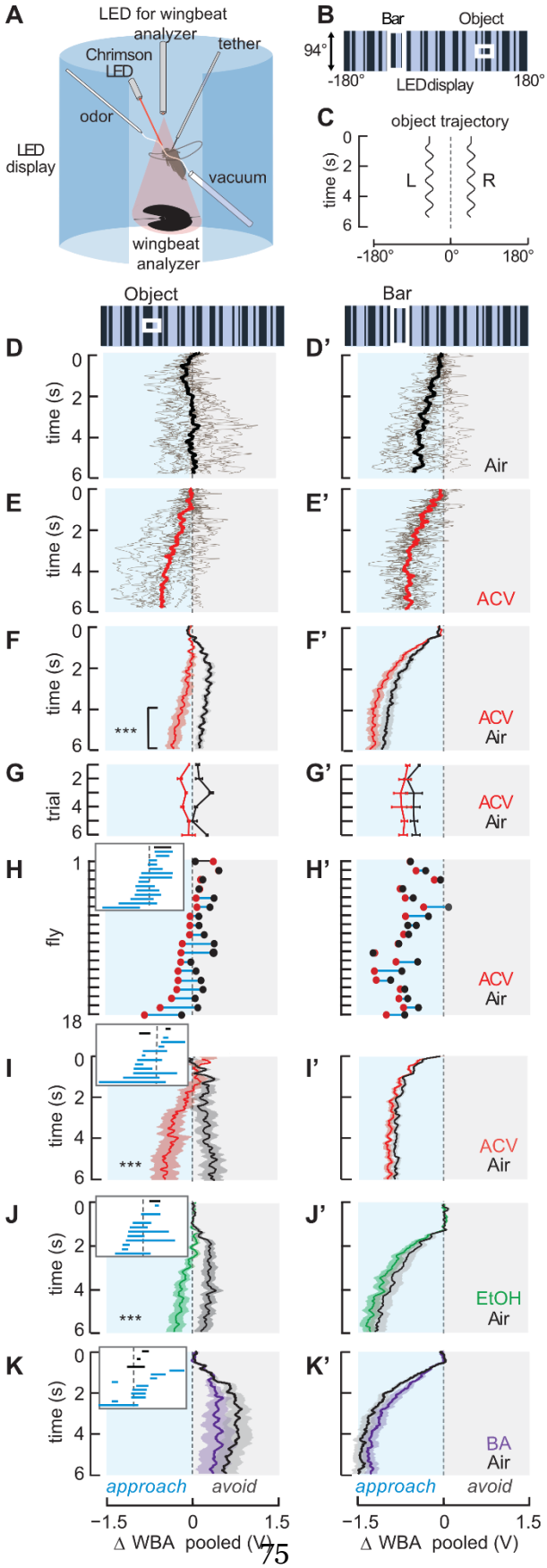
For flies expressing aminergic drivers, a 2-minute closed loop fixation period with the LED On is placed between the LED Off and LED On blocks. This was done to account for the slow kinetics of activation of biogenic amines reported in the literature, which predominantly mediate their effects via G-protein coupled receptors [219]. For flies expressing Chrimson under the control of Empty-splitGal4 and T4/T5-splitGal4, the 2-minute 'preincubation' LED On period is removed, and the LED is turned off in between trials during the LED On block. Except for the high-intensity experiment (Figure 13C-C"), all optogenetics experiments used a LED power intensity of 10μW/mm^2. Power intensity was increased to 40μW/mm^2 for the high-intensity experiment.

All-trans-retinal

For proper Chrimson protein conformation, all-trans-retinal (ATR, Sigma Aldrich, R2500) is required. Though flies endogenously produce retinal, additional ATR is added to the food to boost performance [220]. F1 Chrimson flies are raised in 0.5mM ATR food post-eclosion for at least 3 and no more than 5 days before being used for experiments.

Quantification and Statistical Analysis

Student's paired t-test of the last two seconds was performed in MATLAB 2017a (MathWorks, Inc.) to compare mean epoch ΔWBA.


A
LED for wingbeat analyzer
Chrimson LED
tether
odor
LED display
vacuum
wingbeat analyzer
B
Bar
Object
94°
-180°
LEDdisplay
180°
C
object trajectory
time (s)
L
R
-180°
0°
180°
Object
Bar
D
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E
E'
F
F'
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H
H'
I
I'
J
J'
K
K'
time (s)
trial
fly
***
Air
ACV
ACV
Air
EtOH
Air
BA
Air
approach
avoid
−1.5
0
1.5
Δ WBA pooled (V)

Figure 8. Odor-induced visual valence reversal is odorant specific and learning-independent

(A) Schematic of the rigid-tether visual flight arena equipped for odor delivery and Chrimson optogenetics. The arena is comprised of an array of LED panels controlled by MATLAB. For odor experiments, green panels (570nm) were used, and an odor port was placed in front of the fly. For optogenetics experiments, blue panels (470nm) were used to minimize unwanted Chrimson activation, and a 685nm activating LED was placed in front of the fly. By convention, ΔWBA is defined as left wing beat amplitude (WBA) - right WBA. ΔWBA<0 corresponds to turning to the left, ΔWBA = 0 corresponds to the fly flying straight or maneuvering in pitch, and ΔWBA>0 corresponds to turning to the right.
(B) Representation of the visual stimuli used in all flight behavior experiments. Random ON-OFF columns comprised both background and object stimuli. A 30x30° square object and 30x94° vertical bar were oscillated sinusoidally at 1Hz.
(C) Stimulus trajectory: ±15° peak-to-peak amplitude centered at ±45° from midline, left or right side selected at random for each trial.
(D,D') Individual repeated trials (gray) by a single fly when each visual stimulus was shown in air, superimposed with the mean ΔWBA response across trials (black). Data from right and left side presentations are inverted and pooled as if all visual stimuli were presented on the left side of the arena. Blue shaded rectangle (negative ΔWBA) indicates when flies are steering toward the visual stimuli ("approach"), and gray rectangle indicate flies steering to "avoid" the stimuli.
(E,E') Same fly as in panel D, with same visual stimuli, in in a plume of ACV. Note valence reversal for the small object (E).
(F,F') Mean ΔWBA (solid line) and SEM (shaded regions) for a population of wild-type PCF flies in response to an object (F) or bar (F') in air (black) or ACV (red). Bracket denotes the final two second epoch used to measure average responses for statistical analysis. Asterisks denote odor-induced visual valence reversal, n=18 $p < 0.01$, Student's paired t-test.
(G,G') Mean ΔWBA and SEM for each consecutive trial, averaged across flies, n=18.
(H,H') Mean ΔWBA of each fly in air (black) and ACV (red), sorted by ΔWBA values. Dots representing mean responses in clean air and ACV from the same fly are joined by a horizontal line. The dots are connected by a blue line for ACV-induced steering shifts toward the visual object and a black line for ACV shifts away from the object. This larger representation demonstrates how we made the inset, which is included in subsequent plots and figures. Inset: same as larger plot, but with dots removed. 12 out of 18 flies shifted their steering effort toward the visual object (blue line) when ACV was presented, and 1 out of 18 steered farther away (black line). Steering responses to the small object that were not influenced by ACV had no segment length value to plot and hence are not indicated, but were included in average points and statistical analyses.
(I,I') Same as row (F) for WT-OregonR flies. Asterisks indicate odor-induced visual valence switch, n=13 *** $p<0.01$.
(J,J') Same as row (F) for ethanol, n=13, *** $p<0.01$.
(K,K') Same as row (F) for an aversive odorant, benzaldehyde, n=14.

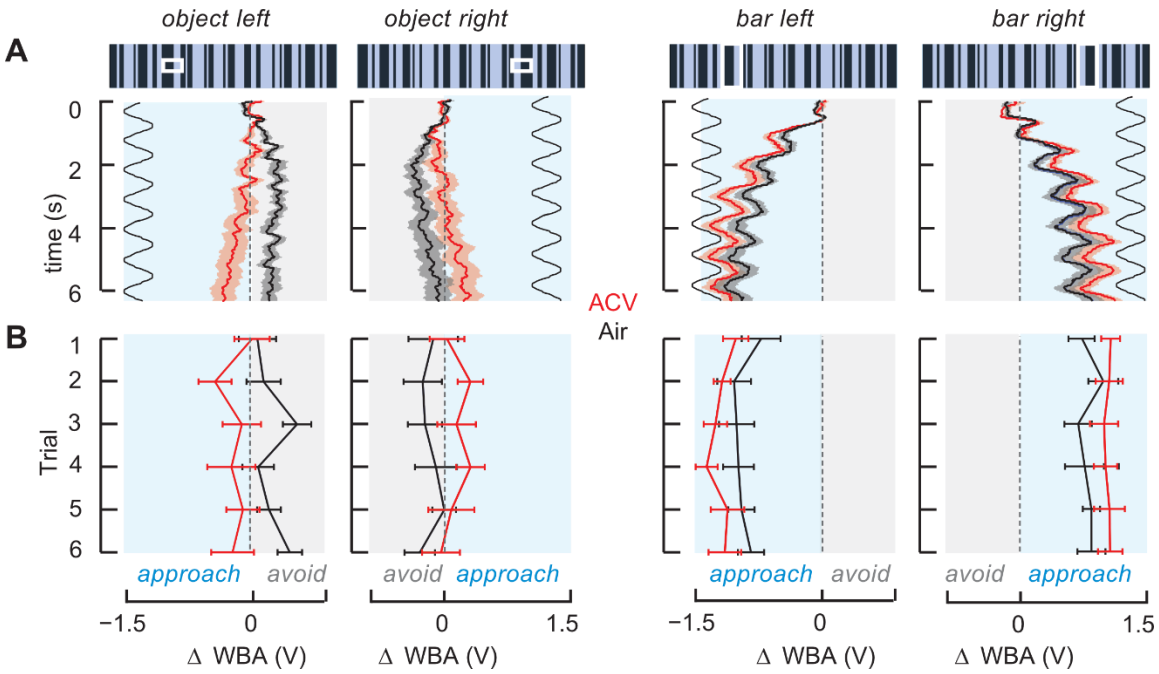

Figure 9. ΔWBA responses to bilateral stimuli for odor modulated object tracking behavior. (Related to Figure 8)

(A) Mean ΔWBA responses (solid lines, n=18) and SEM (shaded regions) of WT-PCF flies to object & bar presented on the left or right side of the visual arena. Red lines indicate responses when the stimulus is presented with ACV, and black lines indicate responses to the visual stimulus in air. Green rectangles denote region of the ΔWBA that correspond to "approach" behavior, which corresponds to the side of the arena that the visual stimulus is presented on. Gray rectangles represent region of the Δ WBA that correspond to "avoid" behavior. Black waveforms represent the 1Hz sine wave that was used to oscillate the visual stimulus ±15° in the left or right front quadrants of the arena. (B) Mean epoch (last 2 seconds) ΔWBA and SEM of single, consecutive trials, averaged across flies (n=18).

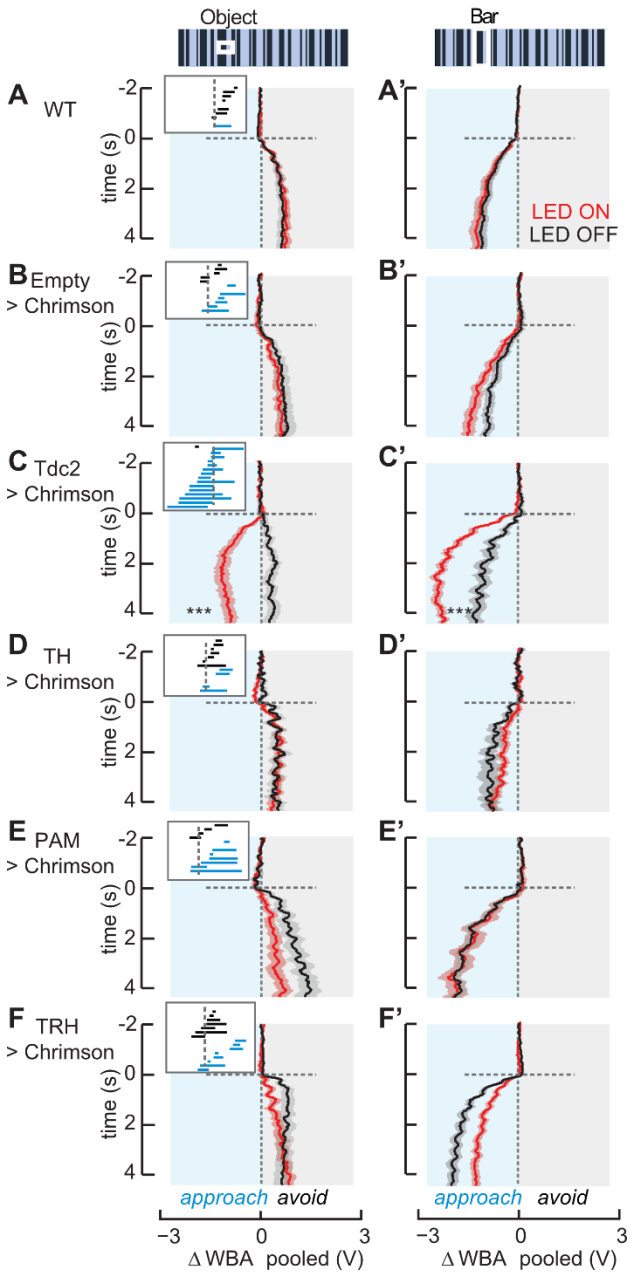

Figure 10. Optogenetic activation of aminergic neurons reveal that OA is sufficient for odor-induced visual valence reversal

All panels - mean ΔWBA (solid lines) and SEM (shaded regions) to an object (A-F) or bar (A'-F') in LED Off (black) or LED On (red) conditions. Each row represents flies of the genotype indicated. In LED On trials, the LED is switched on at time 0. Insets as in Figure 8 denote whether each fly steered more towards (blue) or away from (black) the stimulus upon Chrimson activation. Horizontal dashed line represents the onset of visual stimulus (time = 0). Vertical, dashed gray line represents visual midline (ΔWBA = 0).
(A, A') n=11; (B, B') n=12; (C, C') n=16; (D, D') n=13; (E, E') n=12; (F, F') n=16.
***p<<0.01, Student's paired t-test of the last 2 seconds

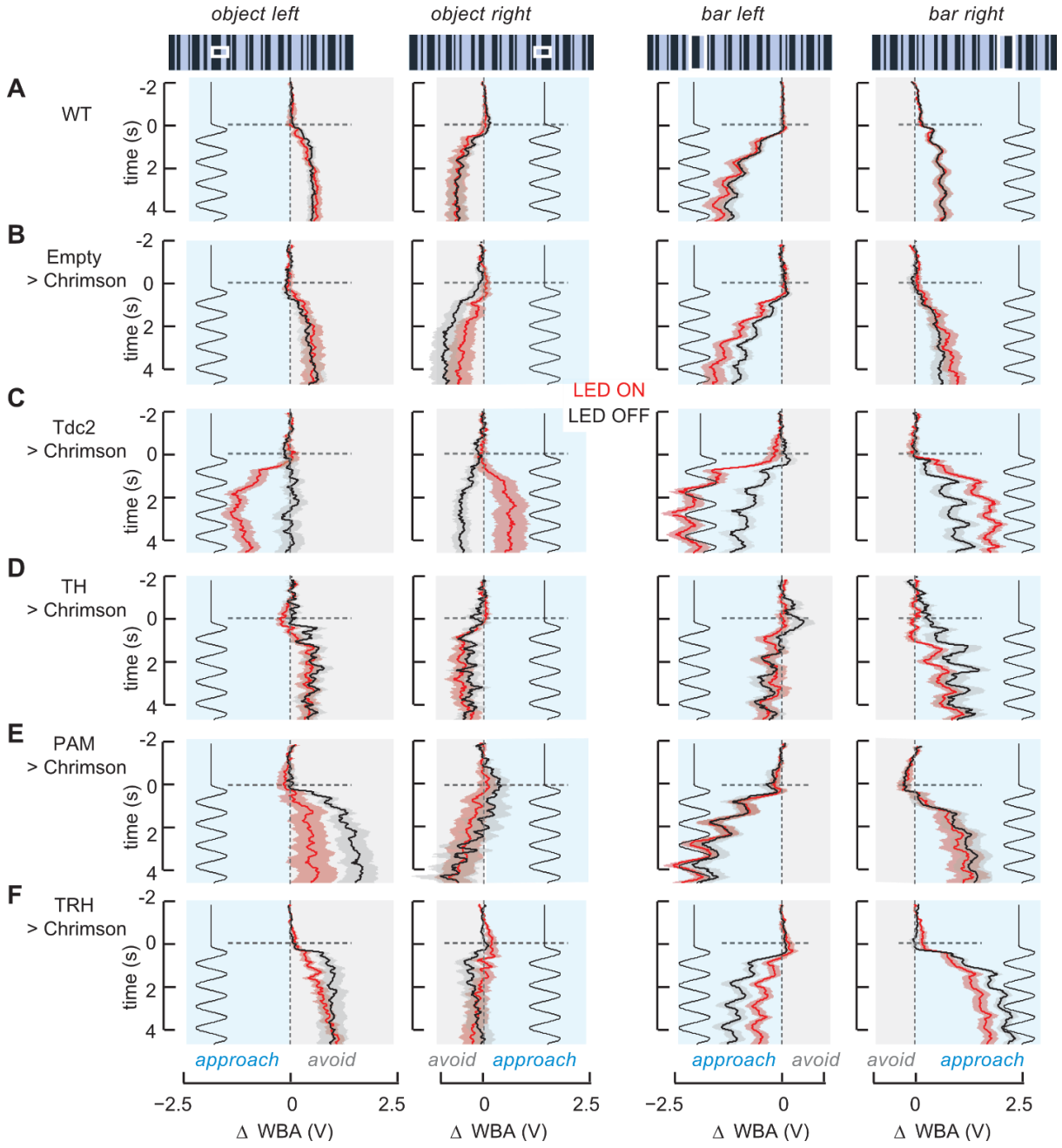

Figure 11. ΔWBA responses to bilateral stimuli for optogenetic manipulation of aminergic neurons. (Related to Figure 10)

Mean ΔWBA (solid line) and SEM (shaded regions) for each genotype to each of the four possible stimulus + arena side condition plotted in similar format as Figure 9. Horizontal dashed line represents the onset of visual stimulus motion. Black waveforms represent the 1Hz sine wave that was used to oscillate the visual stimulus ±15° in the left or right front quadrants of the arena. Each panel row shows the responses of one distinct genotype. (A) n=11; (B) n=12; (C) n=16; (D) n=13; (E) n=12; (F) n=16

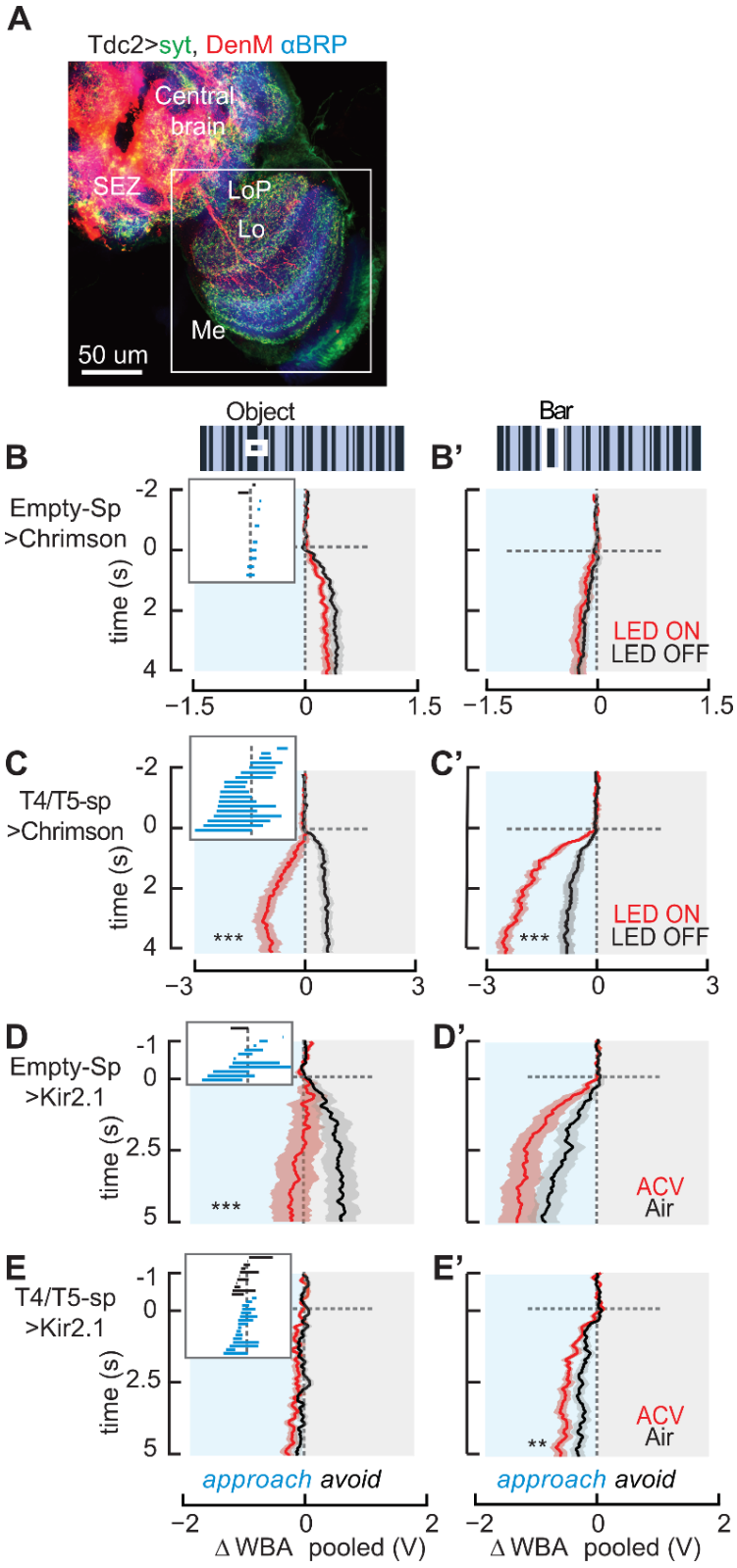
A
Tdc2>syt, DenM αBRP
Central brain
SEZ
LoP
Lo
Me
50 um
Object
Bar
B
B'
Empty-Sp >Chrimson
time (s)
LED ON
LED OFF
C
C'
T4/T5-sp >Chrimson
***
D
D'
Empty-Sp >Kir2.1
ACV
Air
E
E'
T4/T5-sp >Kir2.1
**
approach avoid
Δ WBA pooled (V)

Figure 12. Hyperpolarizing T4/T5 neurons eliminates object responses, depolarizing them induces visual valence reversal

(A) Distribution of presynaptic and dendritic neurites of Tdc2-Gal4 neurons. Red = DenMark labeling; Green = synaptotagmin labeling; Blue = anti-BRP labeling. Me: medulla; Lo: lobula; LoP: lobula plate; SEZ: subesophgeal zone.
(B,B') Genetic controls, enhancerlessless split-gal4 driving UAS-Chrimson, n=19. Mean ΔWBA (solid line) and SEM (shaded region) to an object (B) or bar (B') in LED Off (black) or On (red). Inset: as in Figure 8.
(C,C') Same as B for optogenetic depolarization of T4/T5 neurons, n=18 *** $p<<0.01$, Student's paired t-test of the last 2 seconds.
(D,D') Genetic controls, enhancerless split-Gal4 driving UAS-Kir2.1, for hyperpolarizing T4/T5 neurons. Mean ΔWBA (solid line) and SEM (shaded region) to an object (D) or bar (D') in clean air (black) or ACV (red) n=13 *** $p<<0.01$. Inset: as in Figure 8.
(E,E') Same as D, results of hyperpolarizing T4/T5 using Kir2.1, n=27 ** $p<0.01$, Student's paired t-test of the last 2 seconds.

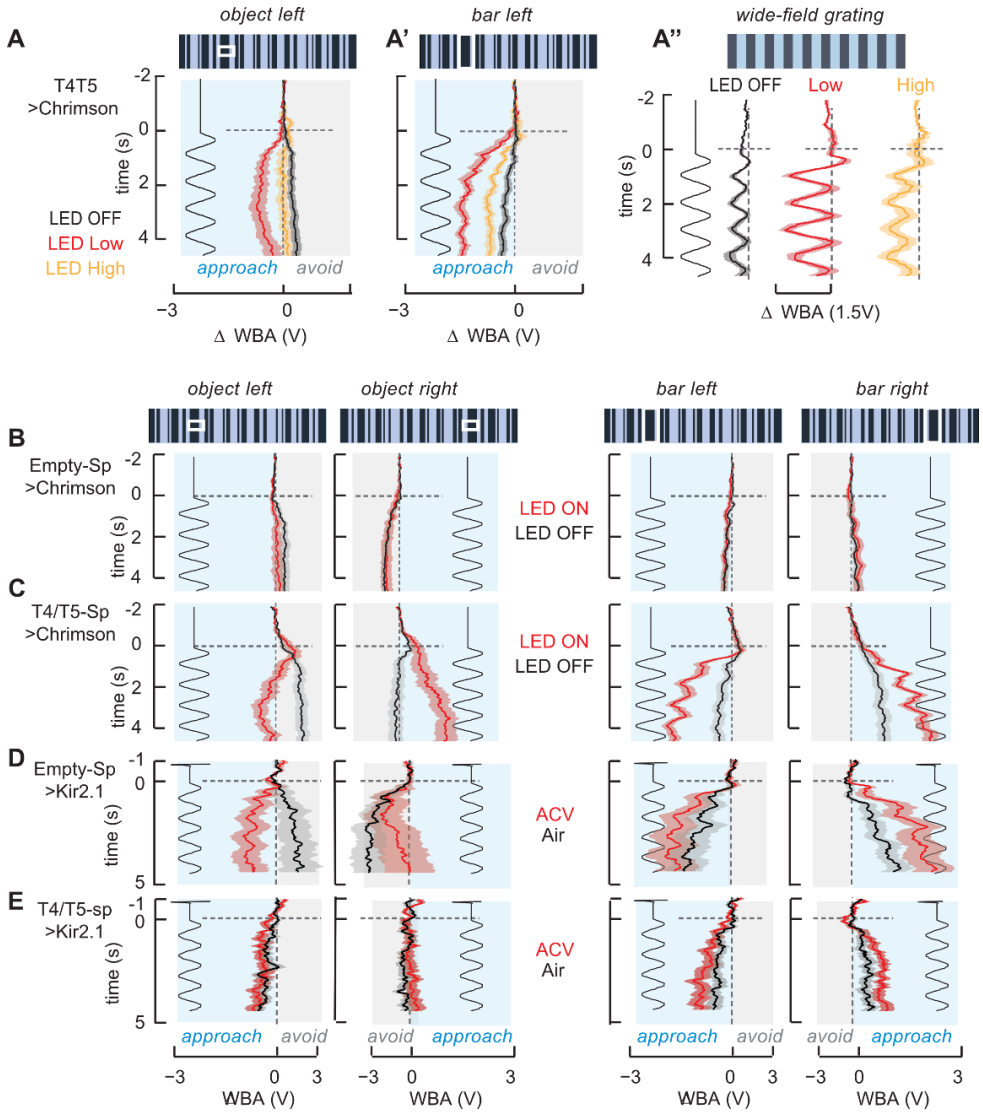

Figure 13. ΔWBA responses to bilateral stimuli for genetic and optogenetic manipulation of T4/T5 neurons. (Related to Figure 12)

(A) The effect of optogenetic activation of T4/T5 neurons (n=12) is dependent upon LED power intensity. High LED intensity (0.040 mW/mm²) abolishes odor-induced object tracking (yellow), while a lower LED intensity (0.010 mW/mm²), which is used in the aminergic optogenetics experiment also, phenocopies odor-induced object tracking (red), similar to Figure 3C. High LED intensity also diminishes bar tracking (A') and wide--eld grating response (A"). These are a separate set of experiments in addition to that presented in Figure 3. (B-C) Mean ΔWBA (solid line) and SEM (shaded regions) for optogenetic activation of enhancerless split-Gal4 (B, n=19) or T4/T5 (C, n=18), plotted in the same format as described in Figure S2. (D-E) Mean ΔWBA (solid line) and SEM (shaded regions) for Kir hyperpolarization of enhancerless split-Gal4 (D, n=13) or T4/T5 (E, n=27).

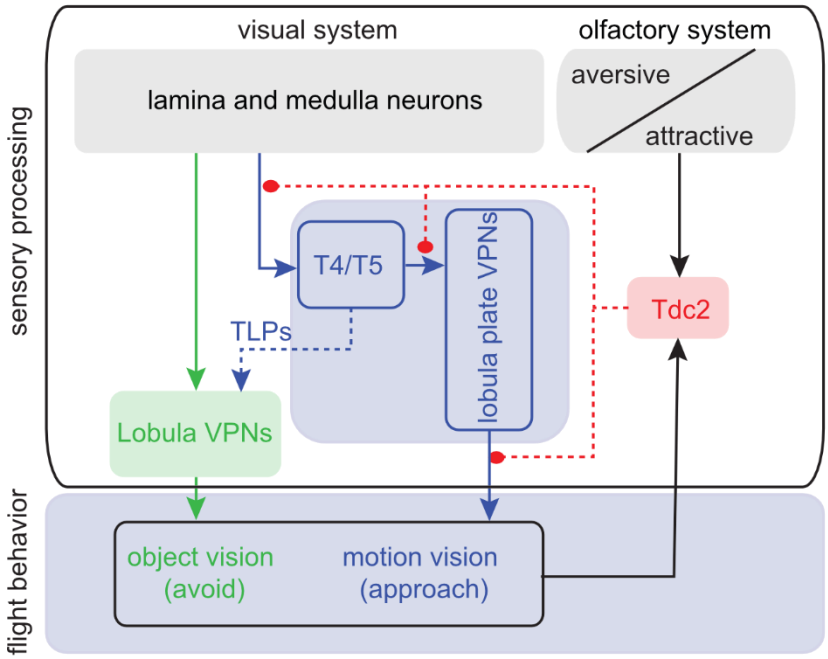

Figure 14. Hypothetical block diagram representing the circuit underlying odor-induced visual valence reversal

Our working model is that olfactory signals act through octopamine signaling to boost the response gain of motion detectors, which in turn elicits robust 'bar like' approach in response to an otherwise weakly aversive small object. Parallel feedforward columnar pathways from the medulla supply object detection via lobula VPNs (green), which combine object and motion signals to mediate weak aversion to small moving objects. Lobula plate VPNs (blue) are also supplied by directionally selective T4/T5 neurons, which are necessary for normal bar approach as well as small object avoidance behaviors - possibly (dashed line) via local translobula plate interneurons (TLPs). In flight, T4/T5 optogenetic activation is sufficient to elicit visual object valence reversal. The onset of flight behavior combines with attractive odor signals to activate Tdc2 neurons (red), which release octopamine. To date, evidence shows that octopamine modulates (filled circles) all components of the motion vision system, but whether octopaminergic excitation is received at one synaptic node of action or at all synaptic nodes within the motion vision circuit is unknown (dashed lines). In flight, optogenetic activation of Tdc2 is sufficient to elicit visual object valence reversal.

APPENDIX I

This appendix details the various loss-of-function experiment attempts to characterize the interactions between T4/T5 and Tdc2 neurons. Then, I propose future experiments using novel technology and reagents available in recent years.

INTRODUCTION

In Chapter 4, I showed that optogenetic activation of Tdc2 neurons and T4/T5 small field motion detectors independently induced object valence reversal, and the induced responses showed amplitudes much greater than those elicited by appetitive odors (ACV and ethanol). Previous studies have shown that pharmacological application of octopamine or chlordimeform (CDM), an octopamine agonist, modulates motion vision neurons downstream of T4/T5 small field detectors [41,95]. Furthermore, a set of RNA-sequencing data suggested that T4/T5 neurons express some levels of octopaminergic receptors [211]. Thus, we sought to determine whether T4/T5 and Tdc2 neurons could be mediating odor-induced valence reversal via the same neural circuit.

To address this, I selectively knocked down individual octopaminergic receptor types in T4/T5 neurons using RNA interference (RNAi) and tested these transgenic flies using the paradigm described in Chapter 4 [58]. Octopamine receptors in insects are classified into 3 main types: alpha receptors, beta receptors, and octopaminergic/tyraminergic (Oct/Tyr) receptors [60], all functioning as G-protein coupled receptors. Alpha and beta receptors, named for their structural similarities to vertebrate alpha- and beta-adrenergic receptors, respectively, differ in the downstream signaling cascade they elicit. Activation of Oct-alpha-receptors leads to an increase in downstream. Alpha-receptors lead to an increase in intracellular calcium levels, whereas beta-receptors lead to an increase in intracellular cyclic AMP [60].

RESULTS

RNAi of OAMB or OctB1 receptors in T4/T5 neurons (SS00324-split Gal4) independently abolished object tracking in the presence of appetitive odor (apple cider vinegar, ACV) (Figure 15 red). However, odor-induced object tracking was also abolished in all transgenic controls, (Figure 15,17). This "lack of control" behavior was observed across various transgenic control genotypes: in flies expressing receptor-RNAi under the control of an enhancerless-split Gal4, parental control flies (+>X-Receptor-RNAi & T4/T5-sp-Gal4 > +), or flies with an RNAi against an absent fluorescent protein (T4/T5sp-Gal4 > mCherry-RNAi). Similar observations were made in both experimental and control flies for RNAi of either OctB2 or OctB3 receptors (Figure 16).

Notably, additional loss-of-function approaches, including MiMIC gene traps, TRiP RNAi, Kir2.1, Kir2.1 under the control of temperature-sensitive Gal80 and amorphic mutants (see Table 1 for detailed genotypes) were also unsuccessful. These flies were too sickly to sustain the entire duration of the experiment (8-10 minutes). These flight defects were unsurprising, however, since octopamine is involved in both the initiation and maintenance of insect flight [122].

Given these challenges in the flight behavior paradigm, we performed *in vivo* calcium imaging experiments in quiescent flies expressing the genetic indicator, GCaMP6f, under the control of T4/T5 genetic driver (T4/T5-spGal4 > UAS-GCaMP6f). T4/T5 baseline responses were elevated after pharmacological application of 100μM octopamine (octopamine hydrochloride, Sigma Aldrich O0250) or 20μM CDM (Sigma Aldrich31099). However, modulatory effects on T4/T5 responses to visual object stimuli

varied greatly across animals (data not shown) and were therefore inconclusive. This observation persisted even with different concentrations or durations of application.

DISCUSSION

The failures of the control RNAi experiments highlight the importance of genetic backgrounds that may be especially emphasized in a physically taxing behavioral paradigm. One solution to mitigate this is to backcross each reagent to a w+ background before conducting the behavior experiments. This may be a more optimal solution before restricting RNAi expressing using Dicer. We hypothesize that defects from genetic backgrounds may also be partially responsible for the failures of other loss-of-function genotypes, in addition to the unavoidable motor defects from downregulating octopamine.

We hypothesize that one source of variability in the T4/T5 calcium responses to visual objects could be due to locomotor states. All of the relevant behavior experiments to date [58] have been conducted in flying flies, but the *in vivo* imaging preparations were done in quiescent animals. To address this, I have adapted a flying fly setup in our two-photon microscope modified from setups published by the Dickinson and Card labs. At the time of writing this appendix, I have tested only two T4/T5 GCaMP flies and saw that these neurons do not show a gain in response when the flies initiate and maintain flight. However, these results are only preliminary with an n=2.

Repeating the T4/T5 calcium imaging experiments with pharmacological applications of octopamine or CDM might appear to be a logical next step to test in the

flying fly set up. However, recent sequencing analyses from separate groups have led to contesting findings as to whether T4/T5 neurons actually express octopaminergic receptors [221,222] or whether efforts should be focused on the up- and downstream neurons of T4/T5 (Y. Kurmangaliyev, personal communication). Notably, octopamine modulation of both medulla presynaptic neurons and lobula-plate postsynaptic neurons have already been shown [95,110,111]. Additional object-encoding visual neurons have also since been identified and characterized, [45,214,223–225], some of them modulated by octopamine [224] or courtship [225].

Given these recent findings, in conjunction with the advent of new reagents, I propose alternative experimental approaches to assess aminergic modulation of motion and object vision. Specifically, to map receptor expression first in candidate neuron types using receptor-T2A-Gal4's [226,227] before optogenetic or pharmacological manipulations to assess physiological effects. Neuromodulator and neurotransmitter release can be monitored using G-protein-coupled receptor-activation-based (GRAB) sensors, after proper optimization, to identify candidate neuropils and neuron types [228]. Taken together, the approaches made possible by recent tool development, coupled with the hemibrain connectome [229], will help expand the understanding of neuromodulatory mechanisms and map a *melanogaster* functional connectome.

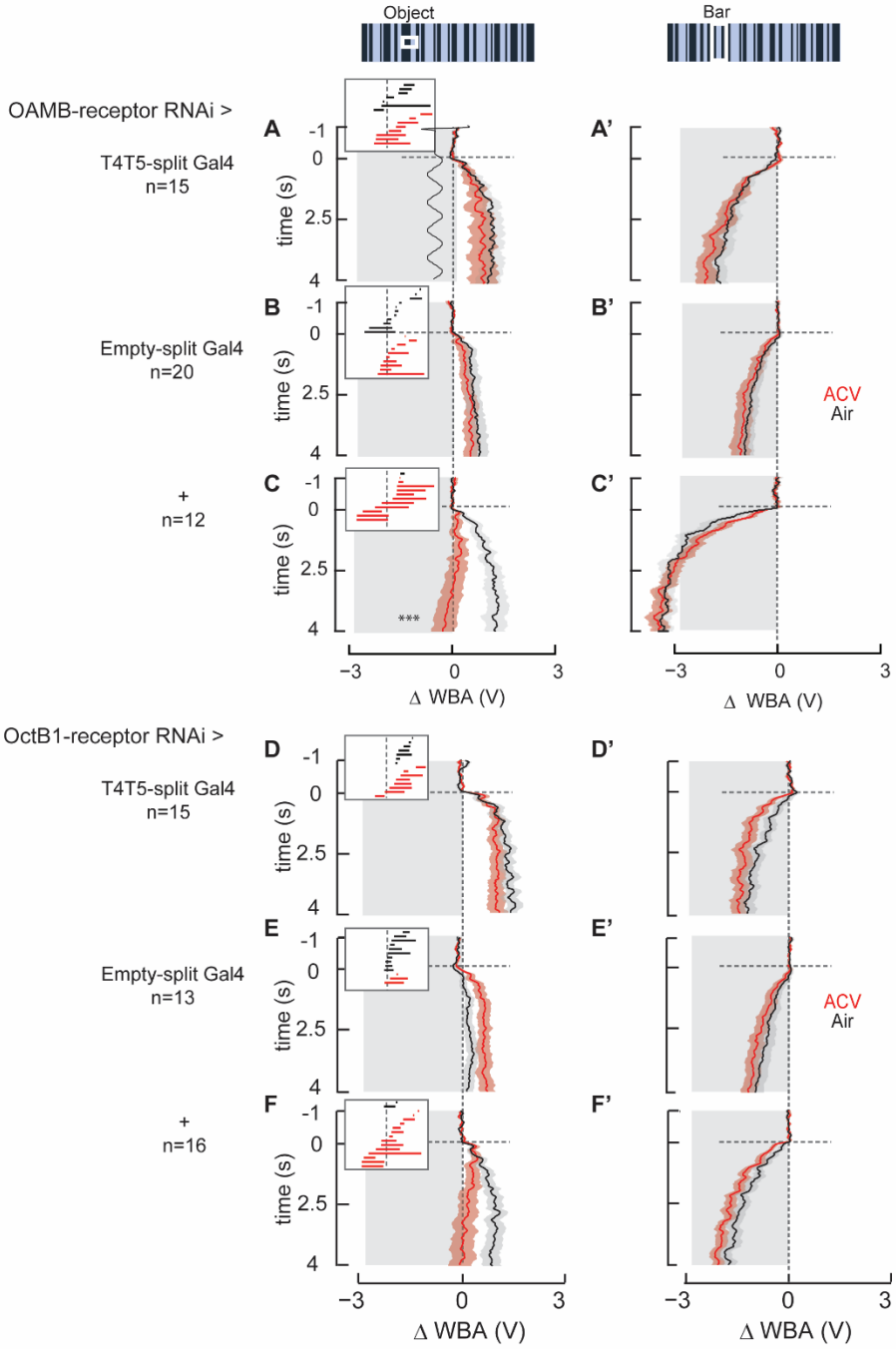

Figure 15. OAMB and Oct β1 receptor RNAi in T4/T5 neurons abolish object tracking, so do genetic controls

(A-C) OAMB-RNAi expressed in T4/T5 neurons (A,A'), under the control of enhancerless (empty) split-Gal4 (B,B'), and crossed with wild-type PCF flies. (***, $p<0.05$, Student's paired t-test of the last 2 seconds). Inset: as in Figure 8.
(D-F) Oct β1R-RNAi expressed in T4/T5 neurons (D,D'), under the control of Empty split-Gal4 (E,E'), and crossed with wild-type PCF (F,F').

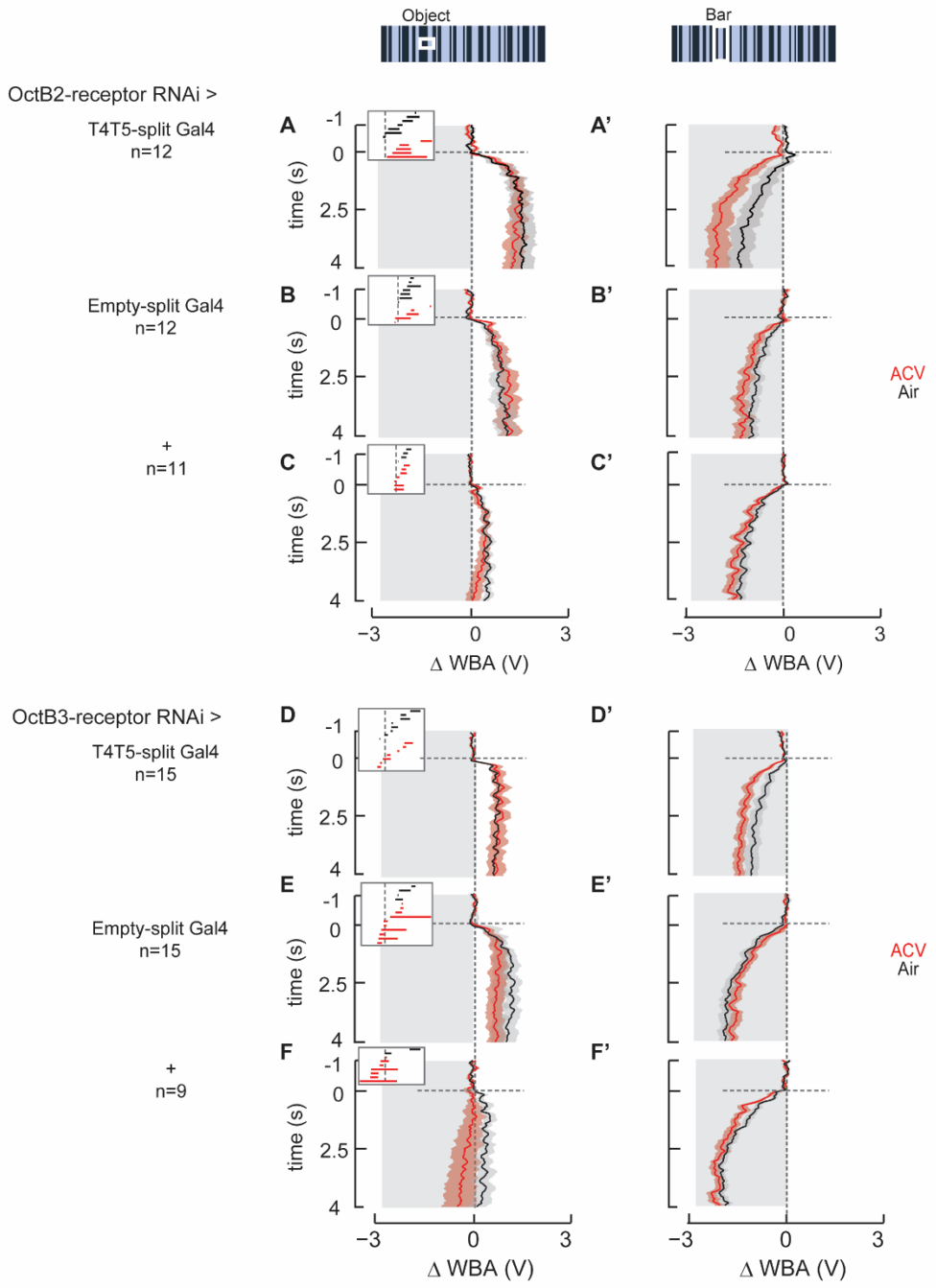

Figure 16. Octβ2 and Octβ3 receptor RNAi in T4/T5 neurons abolish object tracking, so do genetic controls

(A-C) Octβ2R-RNAi expressed in T4/T5 neurons (A,A'), under the control of enhancerless (empty) split-Gal4 (B,B'), and crossed with wild-type PCF flies. Inset: as in Figure 8.
(D-F) Oct β3R-RNAi expressed in T4/T5 neurons (D,D'), under the control of Empty split-Gal4 (E,E'), and crossed with wild-type PCF (F,F').

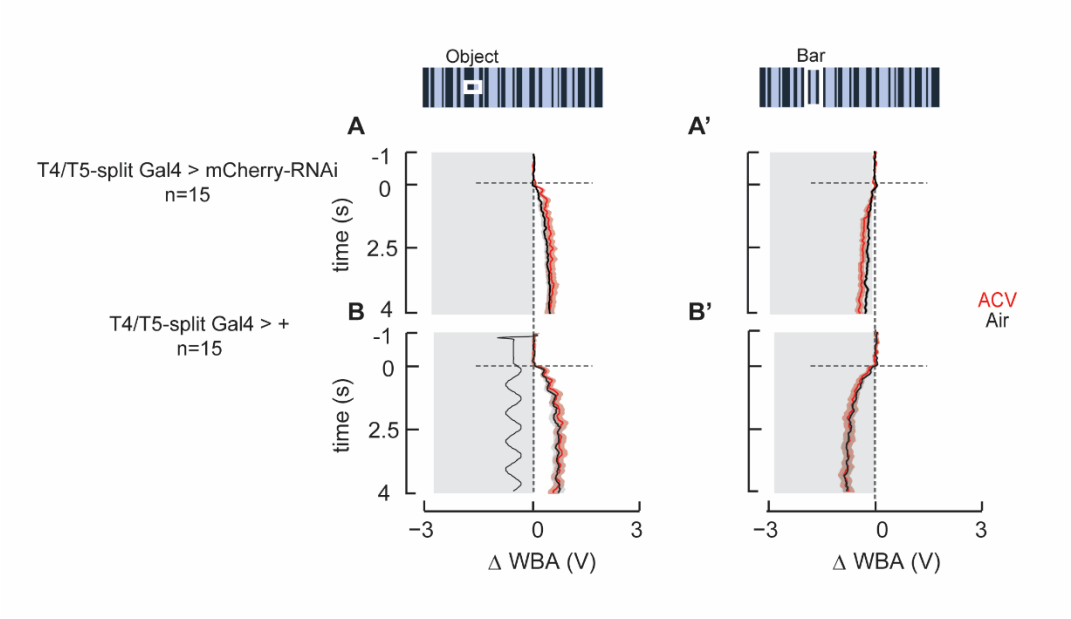

Figure 17. Odor-induced object tracking is abolished in T4/T5 genetic controls

(A,A') mCherry-RNAi expressed in T4/T5 neurons, supposedly targeting a non-existent protein in the transgenic flies.
(B,B') T4/T5 parental controls continue to avoid the small objects in the presence of apple cider vinegar (ACV, red).

Table 1. Loss-of-function genotypes tested

Driver	**Effector**
Tdc2-Gal4	w-; UAS-Gal80ts; UAS-Kir2.1
Empty-Gal4	w-; UAS-Gal80ts, UAS-Kir2.1
T4/T5-splitGal4	PCF (WT)
Tdc2-Gal4	w-;;UAS-Kir2.1
Empty-splitGal4	-
Empty-splitGal4	PCF (WT)
T4/T5-splitGal4	Tdc2-TRiP-RNAi
Empty-splitGal4	w+;; UAS-Kir2.1
T4/T5-splitGal4	w+;; UAS-Kir2.1
PCF(WT)	OAMB-receptor-RNAi
Empty-splitGal4	OAMB-receptor-RNAi
T4/T5-splitGal4	OAMB-receptor-RNAi
-	OAMB MiMIC (Bl#57940)
-	OAMB^286
PCF(WT)	Octβ1-receptor-RNAi
Empty-splitGal4	Octβ1-receptor-RNAi
T4/T5-splitGal4	Octβ1-receptor-RNAi
PCF(WT)	Octβ2-receptor-RNAi
Empty-splitGal4	Octβ2-receptor-RNAi
T4/T5-splitGal4	Octβ2-receptor-RNAi
PCF(WT)	Octβ3-receptor-RNAi
Empty-splitGal4	Octβ3-receptor-RNAi
T4/T5-splitGal4	Octβ3-receptor-RNAi

APPENDIX II

QUANTITATIVE ASSESSMENTS REVEAL IMPROVED NEUROSCIENCE ENGAGEMENT AND LEARNING THROUGH OUTREACH

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This appendix is a journal article about assessing the effectiveness of scientific outreach. I collaborated with Erin Sparck as Teaching Assistants for Project Brainstorm, the Neuroscience department's outreach program focusing on K-12 schools in the larger Los Angeles area. The program is subsequently passed on to other graduate students in the department. This appendix has its own references, a bibliography containing studies relevant to education and outreach.

INTRODUCTION

Opportunities for exposure to neuroscience are often limited in K-12 education due to a variety of factors including: 1) lack of curricular resources, 2) K-12 teachers having little formal training in neuroscience, 3) scarcity of the overall funding dedicated to develop K-12 neuroscience educational programs, and 4) limited textbook space devoted to the nervous system or other interdisciplinary intersections of neuroscience (National Center for Educational Statistics, 2011; Darling-Hammond, 2007). As a result, only a small number of K-12 students become aware of the exciting advances and wealth of information available about the nervous system. This lack of exposure to neuroscience, and to science in general, contributes to the relatively low number of K-12 students who pursue science in higher education or prepare to enter the science and technology workforce (National Science Board, 2010).

Meanwhile, the rapidly expanding field of neuroscience has encouraged an increasing number of higher education institutes to offer majors in neuroscience (Coskun & Carpenter, 2016). An undergraduate major in neuroscience is a worthwhile investment as it provides a strong foundation for graduate or professional education and it opens doors to multidisciplinary careers, including biomedicine, data analytics and health policy. The establishment of these majors has thus opened up the opportunity to integrate community outreach into college education. Having been recognized as a great complement to currently under-resourced public STEM education by federal agencies (Editorial, 2009; Stevens, 2011), outreach programs at universities engage faculty, graduate and undergraduate students, providing opportunities to impart much-needed

awareness and knowledge from their expertise to a broader audience. Given the lack of resources in K-12 education, anecdotal evidence indicates that some institutions of higher education have recognized these needs and are developing outreach activities/programs for neuroscience students both nationally (Brabb et al., 2008; Gittis, 2009; Butcher et al., 2010; McLaughlin et al., 2010; Stevens, 2011; Deal, 2014) and internationally (Yawson et al., 2016). Undergraduates additionally benefit from outreach activities as they get opportunities to develop communication skills, understand the public perception of neuroscience, and gain teaching experience while testing their own expertise. However, formal opportunities to engage in outreach as part of an undergraduate curriculum are still limited and even less effort has been devoted to developing assessment tools to evaluate the effectiveness of existing outreach programs. Thus, formalizing an assessment for effectiveness of these programs would serve as a useful step to integrating outreach efforts as part of neuroscience education.

To address this need, we sought to develop sustainable assessment tools for an existing outreach framework at the University of California, Los Angeles (UCLA). Project Brainstorm, a field experience and outreach course (Romero-Calderon et al., 2012) offered by the Interdepartmental Program in Neuroscience every year provides a well-defined opportunity for neuroscience graduate and undergraduate students at UCLA to interact with K-12 students in the local community. As originally conceived (Romero-Calderon et al., 2012), this ten-week course provides formal guidance to undergraduate students in developing lesson plans on a variety of timely neuroscience topics that are tailored to specific age groups (elementary, middle school, or high school), and requires that they design creative hands-on activities to complement their lesson plan (Figures 18A

& 23). In addition, undergraduate students present a series of interactive "stations" (Figure 18B) that demonstrate foundational concepts in neuroscience (e.g. human brain anatomy, comparative brain anatomy, brain injury, and brain plasticity) to K-12 students. Project Brainstorm students also participate in the annual Brain Awareness Week activities, a well-received global initiative to educate the public about the brain and diseases of the nervous system. Since the inception of Project Brainstorm in 2006, over 100 schools have been visited within the greater Los Angeles community. Locations and demographic distribution of schools visited between 2011 and 2017 is included in Figure 19B-C.

To improve and strengthen Project Brainstorm's outreach efforts, we developed a series of assessments to quantitatively measure the efficacy and effectiveness of this program. Herein, we have summarized these assessment tools and data collected from 298 K-12 students and 29 undergraduate students. We first studied the development of teaching and communication skills in undergraduate students, as well as their preference for teaching as a career, before and after participating in Project Brainstorm activities. We then examined K-12 students' neuroscience learning and interest in science before and after exposure to Project Brainstorm activities. Our results demonstrate that K-12 student participants and undergraduates alike show an improvement in neuroscience knowledge. Project Brainstorm's activities have a positive impact in motivating K-12 students towards pursuing higher education in science, as well as inspiring undergraduates to pursue teaching careers. The assessment tools and data presented here can be easily applied to facilitate the evaluation of other outreach programs in general and provide a data-driven pathway for optimizing outreach programs in the future.

METHODS

Participants

Twenty-nine UCLA undergraduates who enrolled in Project Brainstorm in 2016 and 2017 participated in this study along with 298 K-12 students. The latter were from 15 schools in the Los Angeles area, which we visited and managed to get complete survey back during these two years**.** Demographic information for representative schools visited are included in Figure 24. Prior to the day's activities, parents and/or legal guardians of the K-12 students provided signed a consent form to allow for the activities to be recorded and used for educational purposes.

Ethical Standards and Subject Consent

This study was reviewed by the Ethics Committee of the Medical Faculty of the University of California Los Angeles, and was found to be exempt under section 45 CFR 46.102(d) of the Federal Regulation for Protection of Human Subjects. Subject consent forms were collected and properly documented before all the surveys were performed.

Good Teaching Practices Training

The Project Brainstorm course began by providing undergraduate students with some basic teaching skills. Students received evidence-based training on effective teaching practices. Lectures introduced the 5E (Engage, Explore, Explain, Elaborate and Evaluate) Instructional Model (Bybee, 1997), and covered the importance of "desirable difficulties," or strategies that lead to better long-term retention and flexible representations of knowledge (e.g. retrieval practice, spacing of important points, etc.) in teaching and learning (Bjork &

Bjork, 2011). Students also played a “Tappers and Listeners” game that demonstrated the “curse of knowledge”, a cognitive bias that occurs when experts or individuals with more knowledge of a situation assume that novices understand and have access to the same knowledge (Froyd & Lane, 2008). The “curse of knowledge” is a roadblock to effective communication during teaching and learning, as teachers may have a difficult time placing themselves in the position of the learner (i.e., the presenters assume that K-12 students have the same scientific background knowledge and try to present their topic with materials and explanations geared towards undergraduate neuroscience majors). Students were required to implement these skills into their teaching preparation. Over the course of the class, student presentations were assessed through a series of practice presentations in class, before their school visits (Figures 18A & 23).

1.1 Undergraduate student teaching assessment

Teaching evaluation forms were created based on common good practices recommended to new teachers in general. Fifteen questions were chosen to form the assessment. Each question (Q) was carefully designed to measure different components of effective teaching: Q1-Q10 evaluated whether the 5E effective teaching approaches were properly applied; Q11-Q12 were content-related assessments to determine whether lesson plans were organized systematically with age appropriate information; Q13-Q15 tested improvement on general speaking skills, such as fewer verbal fillers, more eye contact or proper voice projection, to name a few. Teaching evaluation forms were scored on a Likert scale 7-point survey, where 7 indicated outstanding (needed no improvement) and 1 indicated poor (needed much improvement). Each lesson plan was evaluated twice, during

both the practice presentation and dress rehearsal presentation by instructors, coordinators and student peers involved in the outreach program. Additionally, presenters were given the opportunity for self-assessments through videotape recordings of practice presentations (Figure 18A & 23).

1.2 Undergraduate student survey on neuroscience and teaching interests

All undergraduate students completed an anonymous survey at the end of the Project Brainstorm course to assess: 1) their overall confidence/intention to pursue teaching as a potential career and to determine if there were any shifts after Project Brainstorm experience; 2) improvement in their ability to convey neuroscience topics to individuals with or without neuroscience background; and to determine; 3) whether Project Brainstorm helped to gain a deeper understanding of the particular neuroscience topic they chose, and 4) whether Project Brainstorm as a formal undergraduate course was an overall valuable experience. A Likert scale 7-point survey was used for these assessments, where 7 indicated "strongly agree" and 1 indicated "strongly disagree" (Figure 25).

2.1 Evaluation of K-12 student learning on neuroscience concepts

To measure K-12 students' comprehension of neuroscience topics, we developed assessments that evaluated their understanding and knowledge retention of the neuroscience topics presented in their classrooms. The "neuroscience topic questions" included 3-6 multiple-choice questions, which were designed based on key learning

objectives of their lesson plans and adjusted for age-appropriate difficulty levels (example shown in Figure 26).

An individual set of questions based on the specific chosen topic was created for each school visit by the presenting undergraduate students and vetted by the instructors of the course prior to being administered to K-12 classrooms before (pre-visit) and after (post-visit) Project Brainstorm's visits. Pre-visit surveys were administered to K-12 students either immediately before the presentations or a week before the school visit, while post-visit surveys were administered a week after the presentation, in order to assess long-term, but not immediate, knowledge retention (Soderstrom & Bjork, 2015).

2.2 Survey of K-12 student STEM interest

To gauge the interest of K-12 students in pursuing higher education in STEM, we designed another category of questions, i.e., "STEM interest questions" (Figure 27). This set included 6 questions constructed to assess K-12 students' overall interest in learning neuroscience and science in general, and their intention to pursue higher education. The same set of general questions was administered to all schools visited. Similar to the neuroscience-topic questions, each questionnaire was administered before (pre-visit), and after (post-visit) Project Brainstorm's K-12 classroom visits.

3. Statistical Analyses

For each evaluation question, summary statistics (mean, SEM and N) were calculated across all participants before and after participating Project Brainstorm. We performed

two sample t-test to assess the difference between two time points. Normality was checked by visually inspecting the histogram of each question. The analysis was performed in GraphPad Prism 7.0b (GraphPad Software; La Jolla, CA, United States; www.graphpad.com) or SigmaPlot (v. 13; Systat Software, San Jose, CA, United States). To assess K-12 students neuroscience learning with specific topic after attending lesson plan, we used the students' ID numbers on these surveys as identifiers, and applied a paired comparison test to detect differences between the pre and post-test per each student and assess individual progress. Cohen's d analysis was used to describe the standardized mean difference of an effect, measuring the practical significance of the work (http://staff.bath.ac.uk/pssiw/stats2/page2/page14/page14.html). Statistical significance was defined by $P < 0.05$ in all the analyses.

RESULTS

1.1 Undergraduate students showed improvement in teaching and presentation skills after attending Project Brainstorm

During the 1st quarter of 2017, shown as dotted and solid grey bars in the Figure 20A-B, undergraduate students showed significant levels of improvement between practice and dress rehearsal presentations (Figures 18 and 23) in all categories, except for one—general speaking skills (Q14: Spoke at the right volume to be heard). The biggest improvements were related to 5E teaching approach assessments, such as clearly stated learning objective (Q1), stated connection to prior student knowledge (Q4), defined new terms and principles (Q5), demonstrated clearly to explain abstract ideas (Q6), stated connections between presented

ideas (Q7), and repeated learning objectives throughout lesson(Q9). Approximately 75% of these students were part of a special program that required them to enroll in both Spring and Winter quarters to qualify for full course credit. Hence, we compared the 1st quarter dress-rehearsal presentations (solid grey bar) and the 2nd quarter practice presentations (purple dotted bar) to determine whether such improvements were maintained. No significant drop in scores were found for most of the questions, except Q5: defined new terms and principles (Figure 20A). When we compared performances between practice presentations versus dress rehearsal presentations from the 2nd quarter of 2017, students continued to show a significant improvement in 12 out of 15 categories (dotted versus solid purple bars in Figure 20A-B). The remaining three categories did not show significant improvement in the second quarter. This could be due to students having higher baseline scores to begin with, or students maintaining improvement through the course of the second quarter. The most significant improvements overall were still related to the 5E teaching approach assessment, such as stated connection to prior student knowledge (Q4), defined new terms and principles (Q5), and gave enough time to listeners to response (Q10). Moreover, we found significant improvement in all categories between 1st quarter practice presentation and 2nd quarter dress rehearsal presentation (dotted grey vs solid pink bars in Figure 20A-B).

1.2 Undergraduate students expressed increased confidence in communicating science and increased interest in pursuing teaching careers

Surveys (Figure 25) for gauging undergraduate interest in neuroscience and teaching revealed a significant increase in their interest in teaching (Q1) after participating in Project

Brainstorm (Figure 21). Importantly, they showed a significant boost of confidence in their overall teaching skills (Q2), as well as in communicating neuroscience to others, including a general audience unfamiliar with neuroscience topics (Q3-4; Figure. 21B). Moreover, majority of students strongly agreed that they had a better understanding of both the neuroscience topic that they picked for their presentations (Q5: Mean => 6.18/7) and of those their peers presented (Q6: 6.63/7). Most students (Q7: 6.9/7) strongly agreed that Project Brainstorm was overall a rewarding and worthwhile experience.

2.1 Project Brainstorm significantly enhanced K-12 students' neuroscience learning

We analyzed pre- and post-visit surveys pertaining to the neuroscience topic questions from 7 K-12 schools (3 elementary, 2 middle and 2 high schools) visited during the winter and spring quarters in 2016 (TABLE 2). In spite of the different topics and age-groups for each school, overall, all the subject groups showed gains of medium to very large effect size between the pre- and post-visits, suggesting that the presentations' main learning objectives had been met. Most importantly, long-term learning on various neuroscience topics was obtained as shown by the observed differences over the 7-day period (post-visit survey).

2.2 Project Brainstorm significantly enhanced K-12 students' STEM interest

Finally, pre- and post-visit surveys pertaining to the STEM interest survey (Figure 27) were collected from 298 K-12 students. A significant change was observed for all

questions between the pre- and post-visit surveys (Figure 22), indicating that Project Brainstorm effectively increased K-12 students' interest in learning science and understanding the brain and its functions (Q1, Q5-6, Figure 27). Notably, our analysis revealed that K-12 students showed a much stronger intention to attend college or pursue science as a future career (Q2-4; Figure 27) after our visit.

DISCUSSION

Here we described teaching and learning assessment tools developed to measure the effectiveness and efficacy of an existing outreach program, Project Brainstorm, at UCLA. Teaching evaluations were based on general common good practices recommended in training new teachers. Undergraduate students developed questions for the pre- and post-visit tests based on the main ideas K-12 students were taught. Through these newly developed tools, we found that Project Brainstorm is effective in improving undergraduate overall teaching/communication skills, developing their interest in pursuing teaching as a career, and increasing K-12 student science knowledge and interest in STEM.

Overall, not only the students who participated in Project Brainstorm retained the improved teaching/communication skills throughout the second quarter (Figure 20). It is worth noting that Q5 was the only skill that didn't retain the improvement in the beginning of the second quarter. Defining new terms and principles successfully requires that the presenter has a good "a priori" understanding of their audience's background, as well as their knowledge and comprehension of the topic. This observation suggests that the "curse of knowledge" is a continuous hurdle for students and initially can prevent effective communication. Remarkably, Q5 together with Q4 are also the two skill sets that got most

significant improvement ($P<0.0001$) across all three comparisons between practice presentations and dress rehearsal presentations (Figure 20A). The most effective teachers will connect students' previous knowledge to the novel unknown and guide them to explore and learn (Susan, 2010). Hence, the continuous positive effect that Project Brainstorm had on the students' ability to define new terms effectively and connect with the audience's previous knowledge strongly supports its usefulness in effectively improving teaching skills.

Furthermore, participation in Project Brainstorm clearly boosted the undergraduate students' confidence in communicating neuroscience and helped consolidate their neuroscience knowledge. A growing body of evidence suggests that teaching or even just preparing to teach others (Cohen, 1982; Nestojko, 2014; Peets, 2009; Rohrbeck, 2003; Roscoe, 2007) has learning benefits not only for the pupil, but also for the teacher. Effective teaching requires a strong grasp of knowledge, and above all that the knowledge be structured and communicated in a clear and logical fashion. From interactions during teaching, teachers are required to continuously update their knowledge, as well as refine the structure and methods of communication. University opportunities in which students teach others can thus serve as a valuable learning-through-teaching experience, consolidating student knowledge and developing communication skills that may help facilitate the transition to post-college positions. For instance, undergraduate students enrolled in Project Brainstorm have shown evidence of improved confidence in teaching and better understanding of the variety of neuroscience concepts. These are skills that would directly transfer to teaching or neuroscience research careers, but would also assist students in preparing for careers involving strong communication skills, such as journalism, public policy, and law.

Our observation also provides strong evidence that Project Brainstorm significantly benefited the K-12 school students who participated in the program. STEM interest survey questions administered in every school visit generated a large sample size of 298 K-12 students and their analysis suggested a significant improvement in every category, including both the general interest about neuroscience and basic neuroscience learning. For each school visit, a different topic specific questionnaire was designed to gauge learning specific to each lesson plan. Hence the sample size was limited to 30-50 students per class compared to sample size in the STEM interest survey. By using their student IDs as identifiers, we were able to detect improvements of each student before and after presentation. Additionally, based on our anecdotal observation, we noticed that when we sent pre-visit surveys before our school visit and asked K-12 classroom teachers to administer the survey, we usually obtained higher average scores in pre-visit surveys than what we obtained when we administered the survey ourselves right before presenting the lesson. One possible reason for this could be that teachers prime the students on the topic being evaluated. Thus, it is imperative to remind K-12 classroom teachers not to prime their students before testing, in order to generate an objective result. Future studies will also address the influence of gender of trainees and K-12 students on the outcome measures. This would be valuable in understanding the impact of outreach programs in motivating more women to pursue STEM careers.

This is a comprehensive study to quantitatively assess both neuroscience undergraduates' and K-12 students' knowledge gain through a neuroscience outreach program. In order to help outreach programs in other schools, adapt and generate classes and lesson plans about the brain, we have provided these assessment tools (surveys, pre-

and post- visit assessments, etc.) together with course description and curriculum (Figures 23, 25-27). Representative lesson plans / presentations and presentation videos can also be provided upon request.

In summary, UCLA's Project Brainstorm outreach program incorporates learning-through-teaching strategies in the undergraduate classroom and is truly making a significant impact on the community. It provides a valuable experience that can foster the undergraduates' interest and knowledge in neuroscience and a teaching career. Such efforts should not be and are not limited to neuroscience outreach, and can easily be adapted by and applied to other STEM fields. We make it our mission as a public university to bring our expertise from classrooms to communities, particularly those with modest resources (45.16% of K-12 schools we visited are Title 1 schools), and provide a dynamic and impactful learning experience. By bringing our enthusiasm and expertise to K-12 students, we strive to improve their understanding of neuroscience as well as to create an opportunity to promote and grow the interest in STEM. The quantitative assessment tools provided here, together with our outreach program framework and teaching resources, provide effective models for other educational outreach programs to adapt. Moreover, the assessment tools and data presented set up a data-driven pathway for optimizing outreach programs. We strongly believe these efforts into quantitative assessments to improve neuroscience learning and engagement through outreach will facilitate the making of a stronger STEM workforce.

ACKNOWLEDGMENTS

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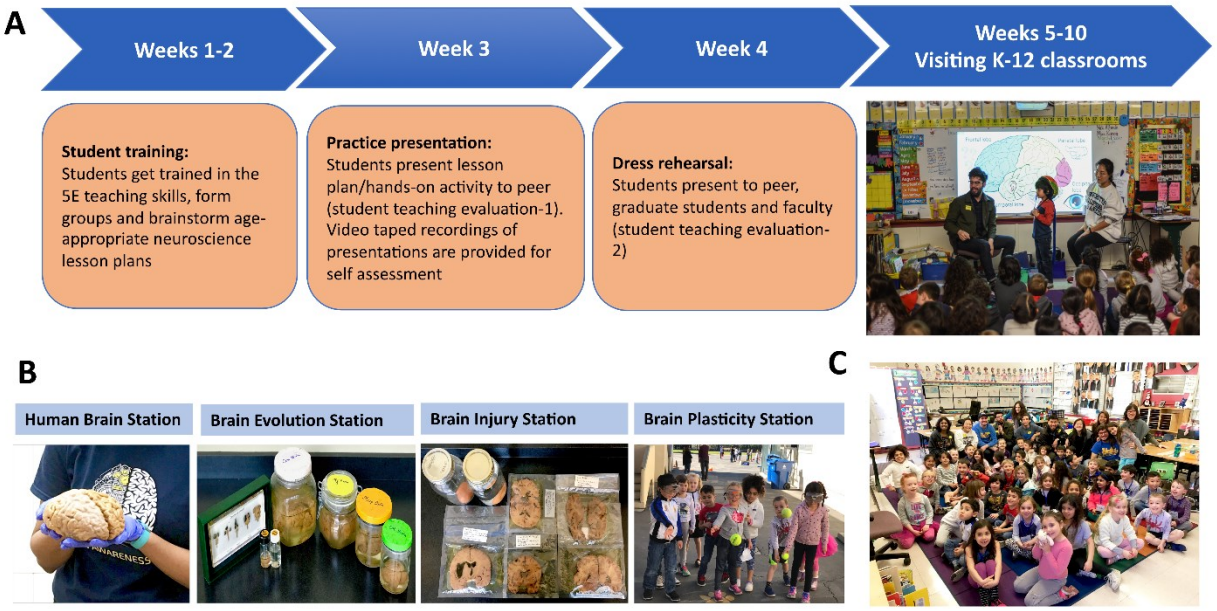


Figure 18. Project Brainstorm program outline

(A) Flow diagram of Project Brainstorm's 10-week course structure demonstrating how students collaborate, get trained in building interactive neuroscience lesson plans and take it to the community.
(B) Picture representations of the different activity stations that we present to K-12 students.
(C) Project Brainstorm group with a class of kindergarteners during a school visit in the winter quarter of 2017.

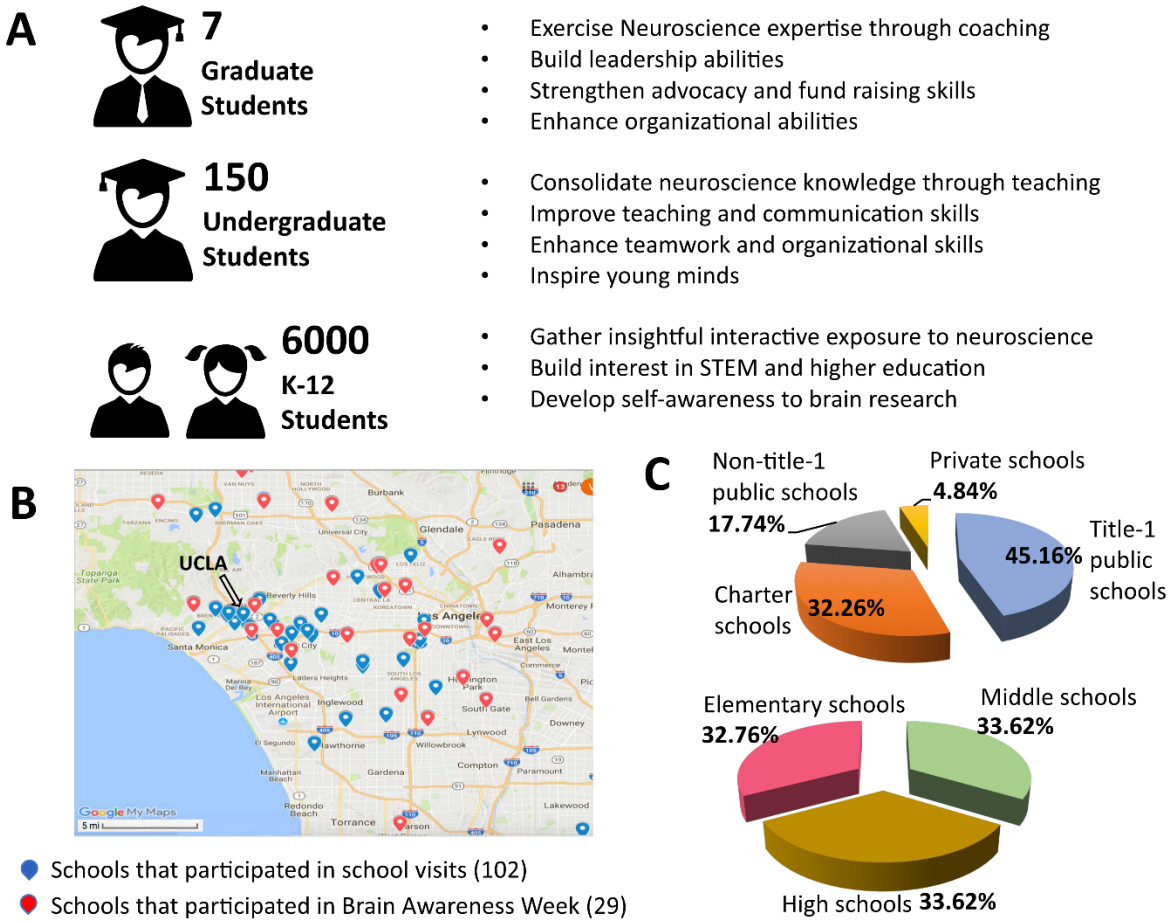


Figure 19. Impact of Project Brainstorm summary between the years 2011-2017

(A) Numbers and accomplishments of project brainstorm participants: graduate students, undergraduate students and K-12 students around the Los Angeles communities.
(B) Google map view of the K-12 LAUSD schools that have been part of both Project brainstorm and Brain Awareness Week between 2011-2017.
(C) Pie chart view of the distribution of education programs and grade-levels of all the participating LAUSD schools.

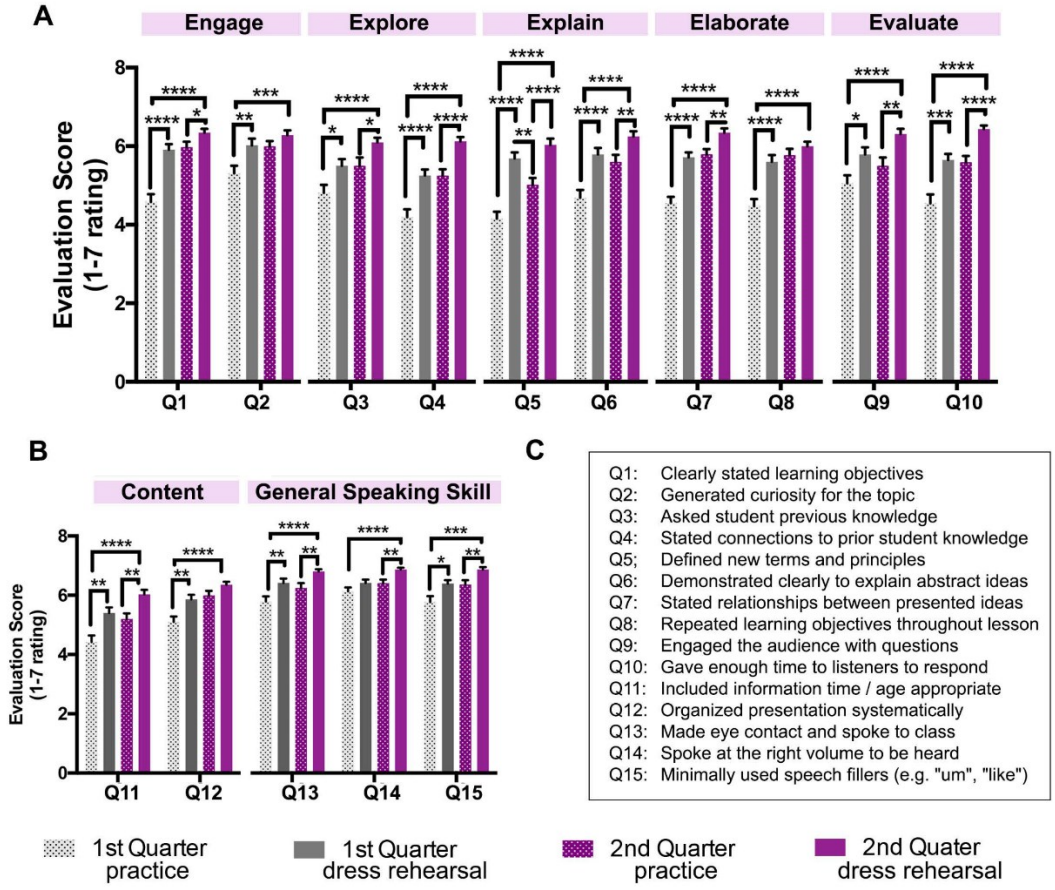


Figure 20. Undergraduate students showed continued improvement in their teaching and Communication abilities after having taken Project Brainstorm class

(A-B) Undergraduates showed Improvements in all measurements used to assess effective teaching skills, including the 5E approach of assessment, teaching content and general speaking skills. (C) A sample of the survey questions used to perform the assessment during undergraduate student presentations in class. (Mean ± SEM; n=11, ****P<0.0001, ***P<0.001, **P<0.01,*P<0.05, Unpaired t-test).

A

Q1: considering a career in teaching Science
Q2: Feel confident about my general teaching skills
Q3: Feel confident about teaching neuroscience concepts to others
Q4: Feel confident to communicate neuroscience effectively to individuals without a neuroscience background

B

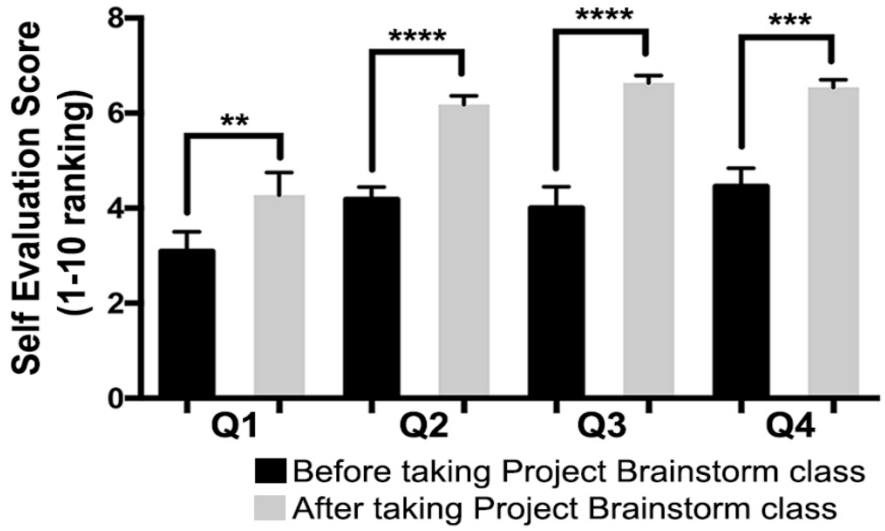


Figure 21. Project Brainstorm positively influenced undergraduate students' interest in pursuing a career in teaching, and boosted their ability to effectively teach and communicate their knowledge to a general audience

(A) A sample of the self-assessment survey questions used to assess undergraduate's improvement in their communication skills and interest in a teaching career.
(B) The students showed improvements in all levels of assessment. (Mean ± SEM; n=11, ****P<0.0001, ***P<0.001, **P<0.01, paired student t test)

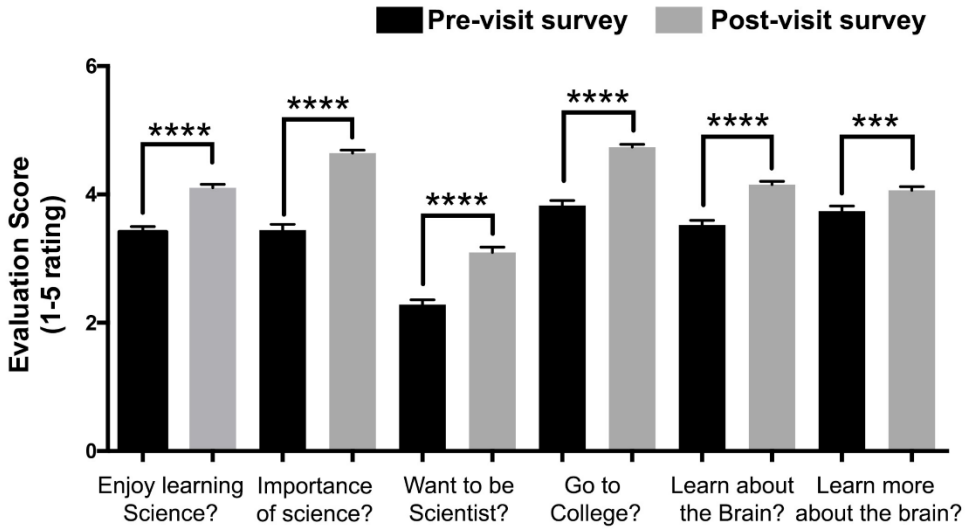


Figure 22

Project Brainstorm significantly enhanced K-12 students' interest in Brain research and motived them to pursue higher education (Mean ± SEM; n=287 for Pre-visit survey, n=298 for Post-visit survey,****P<0.0001, ***P<0.001, unpaired student t test)

Topic & Type of School	n	Number of Questions	Pre-TEST	Post-TEST	Inferential Statistics	Effect Size
Vision & Sleep Elementary School	24	3	1.750 ± 0.794	2.333 ± 0.761	t (23) = 2.933; P=0.007	0.599
Motor High School	33	4	1.364 ± 0.929	2.394 ± 1.029	t (32) = 4.36; P<0.001	0.759
Neuroplasticity Middle School	37	5	2.054 ±0.998	3.189 ± 0.938	t (36) = 6.524; P<0.001	1.073
Memory Elementary School	38	6	2.868 ± 1.398	4.895 ± 1.134	t (37) = 7.346; P<0.001	1.192
Senses Elementary School	40	3	1.216 ± 0.787	1.973 ± 0.726	t (39) = 4.976; P< 0.001	0.818
Motor Middle School	116	5	2.405 ± 1.165	3.578 ± 1.136	t (115) = 7.811; P<0.001	0.725
Stress High School	23	6	3.696 ± 1.396	4.783 ± 1.166	t (22) = 2.926; P=0.008	0.610

Table 2. Analysis of the pre- and post-visit surveys reveals significant retention of information on the topics students were taught

Note: An individual set of questions based on the specific chosen topic was created for each school visit. By using the students' school ID as identifiers, we could assess individual differences and thus, progress, between the pre- and post-test per each student. Data are shown as the mean ±SD; paired Student's t-test. To determine the practical significance of the work, effect size was assessed using the Cohen's d analysis and allowed us n=number of students per classroom. Degrees of freedom are reported in parentheses; alpha value < 0.05.

Neuroscience 192B Neuroscience Outreach
Project Brainstorm
(Offered in winter and spring quarter)
Syllabus

Course Instructors: name, office hours and contact info

TA / Coordinator: name, office hours and contact info

Classroom: classroom location

Contact information: brainstorm@ucla.edu

COURSE DESCRIPTION
Project Brainstorm is the K-12 science education outreach program of the Brain Research Institute (BRI) and the Neuroscience Interdepartmental Ph.D. and Undergraduate Programs. The goal of Project Brainstorm is to stimulate interest in science for children and adolescents in grades K-12 by providing hands-on learning experiences that emphasize the function and importance of the brain.

Project Brainstorm is coordinated by graduate students in the Neuroscience Interdepartmental Ph.D. Program and carried out by teams of undergraduate students who visit Los Angeles-area schools throughout the year. A typical visit involves group participation, interactive games, and hands-on exercises with teaching props, brain models and real animal and human brains.

The first half of the quarter is devoted to developing and practicing your own brain lessons that you will present at local K-12 schools during the last five weeks of the quarter. In small groups (two or three students), you will select a brain topic that you think is exciting and important and we will guide you in preparing your 45-minute presentation, which will be aimed at either an elementary, middle, or high school class. These lesson plans will include a general introduction to the nervous system, leading to a more specific example of the importance of the brain in everyday life accompanied by a fun and educational hands-on activity. Past topics have included neurodegenerative diseases, learning and memory, brain injury, development and aging, and sleep, just to name a few.

COURSE GOALS
Students will:

- Connect their academics and the "real world" using their community experiences.
- Gain teaching experience at the K-12 level.
- Improve writing and presentation skills.
- Explore options for using a science degree after graduation.

COURSE POLICIES
Both your TA and your faculty sponsor will evaluate you for your grade. Grading is Pass/No Pass for 4 units, which will count as *general* units. You must complete all course assignments and requirements in order to receive a passing grade, as well as attend ***ALL class meetings and school visits***. Units will ***not*** count toward satisfaction of the specific course requirements of the Neuroscience major in the Interdepartmental Undergraduate Program for Neuroscience. The close relationship between the student and the coordinator allows for strong evaluations of strengths and weaknesses.

ASSIGNMENTS AND REQUIREMENTS
Lesson Plan: Students will be paired into groups and assigned to either an elementary, middle, or high school class. Based on the age group of their class, they will prepare a relevant 20 minute lesson plan that will showcase the structure and function of the nervous system. The lesson plans must be completed by the 4th week.

The lesson plan will consist of the introduction, the specific topic, and a hands-on station:

1) An introduction to the nervous system (5 minutes). You should review general anatomy (the lobes of the brain, brain stem, and spinal cord along with their major functions) and the structure and function of neurons, so that your students will have a basic understanding of the brain and are prepared for the main topic of your lesson.

2) Your specific topic on the brain (10 minutes). Here are some ideas of topics that are appropriate for the following age groups. However, you are welcome to and encouraged to create your own ideas!

Elementary school (grades 1-5; 5-9 years):	***Middle school (grades 6-8; 10-13 years):***	***High school (grades 9-12; 14-18 years):***
→ senses, → memory and learning → motor systems and reflexes (spinal cord) → brain injury	All of the previous, plus: → sleep and dreaming → exercise → creativity	All of the previous, plus: → alcohol/smoking and the brain → stress and the brain → circadian rhythm

Presentation topics cannot be shared by more than one group: each group must have a different topic.

3) A hands-on practicum (5 minutes for one station, 5 total stations). In addition to the Presenter's station, there will 4 additional stations that will include teaching props, brain models and real animal and human brains. All students are needed to carry out this part of the lesson plan.
   a. Presenter's station: undergraduates will develop a station that is relevant to their specific topic
   b. Brain evolution, animal brains
   c. The human brain: lobes and neurons
   d. Brain damage: egg/jar + brain slices
   e. Neuroplasticity: prism goggles

Writing Assignments:
1) Students are required to write weekly journals (1 page) outlining the progress of their lesson plans and/or the teaching experience. They are due Monday at 5pm by email (brainstorm@ucla.edu). No late journals will be accepted. If a student has an incomplete journal without an adequate excuse, extra work will be added to the final assignment.

2) Students are also required to write a final written assignment, which will be due at the end of the quarter. The final assignment should be a 2-3 page instructive lesson plan on their project formatted so that others (teachers, students, and faculty) would be able to implement the lesson they developed over the course. The will also need to submit their final power point presentation.

❖ *Students enrolled in Project Brainstorm as a 199AB will be required to complete assignments in addition to the weekly journals. Additional details will be provided separately.*

Group Meetings: During the first four classes of the quarter, we will meet as a group to discuss and present lesson plans.

Classroom Teaching: In the second half of the quarter, each group will present their lesson plan at their assigned school. ***Every*** student is expected to attend ***all*** teaching sessions to help out with the hands-on practicum (there will be 5 teaching sessions). During Wednesday class time, we will meet on campus, drive to a school, set up, present, cleanup, and return to campus. Please be advised that we require you to have some flexibility during these weeks, so as to visit the schools during the times that they allow us.
During the final week, UCLA hosts Brain Awareness Week. Students are expected to volunteer for the equivalent amount of class time (at a minimum).

QUARTER SCHEDULE

Week	*Date*	*Assignment*	*Due*
1	Jan 11	**Class Meeting 1:** Introduction to the course. Group formation and assignment of grades to teach. Details on how to prepare the lesson plan (format, length, style). Resources to use to prepare the lesson plan. **Individual Journal #1:** Outline your lesson plan. What will be your topic and what are the 2-3 learning objectives you've set for your presentation? Is the information relevant to the age group of your students? What information is age-sensitive? Is this information fun and interesting? Is it going to engage the students' interest in the brain? How are you presenting the information? What sources will you use for your info (include 3). *You should discuss these with your group members, but write the journals separately.	
2	Jan 18	**Class Meeting 2:** 1) Two guest lectures will be provided to teach teaching and better communication skills. 2) Go over 4 general hands-on stations materials 3) Review of your proposed lesson plan. You should have a complete draft of your lesson plan by now. The course coordinators will go over the plan and advise you on changes that should be made. **Individual Journal #2:** Outline the 3rd part of your lesson plan (the hands on activity). What exactly will you do and say for your topic specific station? How does this relate to your presentation? What props will you need? Since you will rotate presenting each of the permanent stations, outline the information you will describe at each station. Give entertaining facts!	**Monday:** Individual Journal #1 Liability Waiver
3	Jan 25	**Class Meeting 3:** Presentation of your revised lesson plans to your peers and course coordinators only. In addition to peers' and course coordinators' assessment, presentation will be videotaped for self-assessment. After the presentations, briefly describe the hands on activity, information provided and the take home message to your peers. **Group Journal #3:** Design 5 topic specific pre- and post- test questions for K12 students, and submit for course coordinators to review. Assess the critiques you received on you lesson plan and describe the fine-tuning you intend to do before your final presentation. You only need to turn in one copy per group.	**Monday:** Individual Journal #2
4	Feb 1	**Class Meeting 4: Dress Rehearsal.** Presentation of your lesson plans to your peers, the course coordinators, previous TAs and a selected panel of faculty. The faculty will be there to offer helpful suggestions. Your topic specific station will be presented as well.	**Monday:** Group Journal #3
5	Feb 8	**1st School Visit:** Group 1 will present their lesson plan to the school grade assigned. All other groups will help out during the 'hands-on' practicum. **Presenter Group Journal:** Discuss the teaching session. How effective do you think it was? What went right/wrong? How could you improve your lesson plan? Did you get your take home message across? **Helper Group Journal #1:** What was the biggest challenge during your station? How did you deal with it, and what would you change? How was your experience different from what you expected?	*Nothing is due this week.*
6	Feb 15	**2nd School Visit:** Group 2 will present their lesson plan to the school grade assigned. All other groups will help out during the 'hands-on' practicum. **Presenter Group Journal:** Discuss the teaching session. How effective do you think it was? What went right/wrong? How could you improve your lesson plan? Did you get your take home message across? **Helper Group Journal #2:** What seemed to engage the students the most? Why do you think it was most engaging? What engaged the least? How can this be modified to make it exciting? Give specific examples and modifications.	**Monday:** Presenter OR Helper Journal
7	Feb 22	**3rd School Visit:** Group 3 will present their lesson plan to the school grade assigned. All other groups will help out during the 'hands-on' practicum. **Presenter Group Journal:** Discuss the teaching session. How effective do you think it was? What went right/wrong? How could you improve your lesson plan? Did you get your take home message across? **Helper Group Journal #3:** Reflect on what you have found exciting and interesting in the field of neuroscience, and design a short, fun activity for kids.	**Monday:** Presenter OR Helper Journal
8	Mar 1	**4th School Visit:** Group 4 will present their lesson plan to the school grade assigned. All other groups will help out during the 'hands-on' practicum. **Presenter Group Journal:** Discuss the teaching session. How effective do you think it was? What went right/wrong? How could you improve your lesson plan? Did you get your take home message across? **Helper Group Journal #4:** Discuss why you decided to major in science in general (and neuroscience in specific. Relate your experiences to what could be done to improve the way science is taught in schools.	**Monday:** Presenter OR Helper Journal
9	Mar 8	**5th School Visit:** Group 5 will present their lesson plan to the school grade assigned. All other groups will help out during the 'hands-on' practicum. **Presenter Group Journal:** Discuss the teaching session. How effective do you think it was? What went right/wrong? How could you improve your lesson plan? Did you get your take home message across? **Helper Individual Journal #5:** Discuss what you learned from this class about teaching and about yourself as a teacher. How can the class be improved? Strengths and weaknesses of the class (be honest).	**Monday:** Presenter OR Helper Journal
10	Mar 13-17	**Brain Awareness Week**	**Monday:** Presenter OR Helper Journal **Friday:** Final Assignment due (2-3 pages, plus power point; one per presentation group)

Class Schedule:

Week 1 (Jan 11):	9am-12pm	Classroom location
Week 2 (Jan 18):	9am-12pm	Classroom location
Week 3 (Jan 25):	9am-12pm	Classroom location
Week 4 (Feb 1):	9am-12pm	Classroom location (Dress rehearsal)

Classroom Visits:

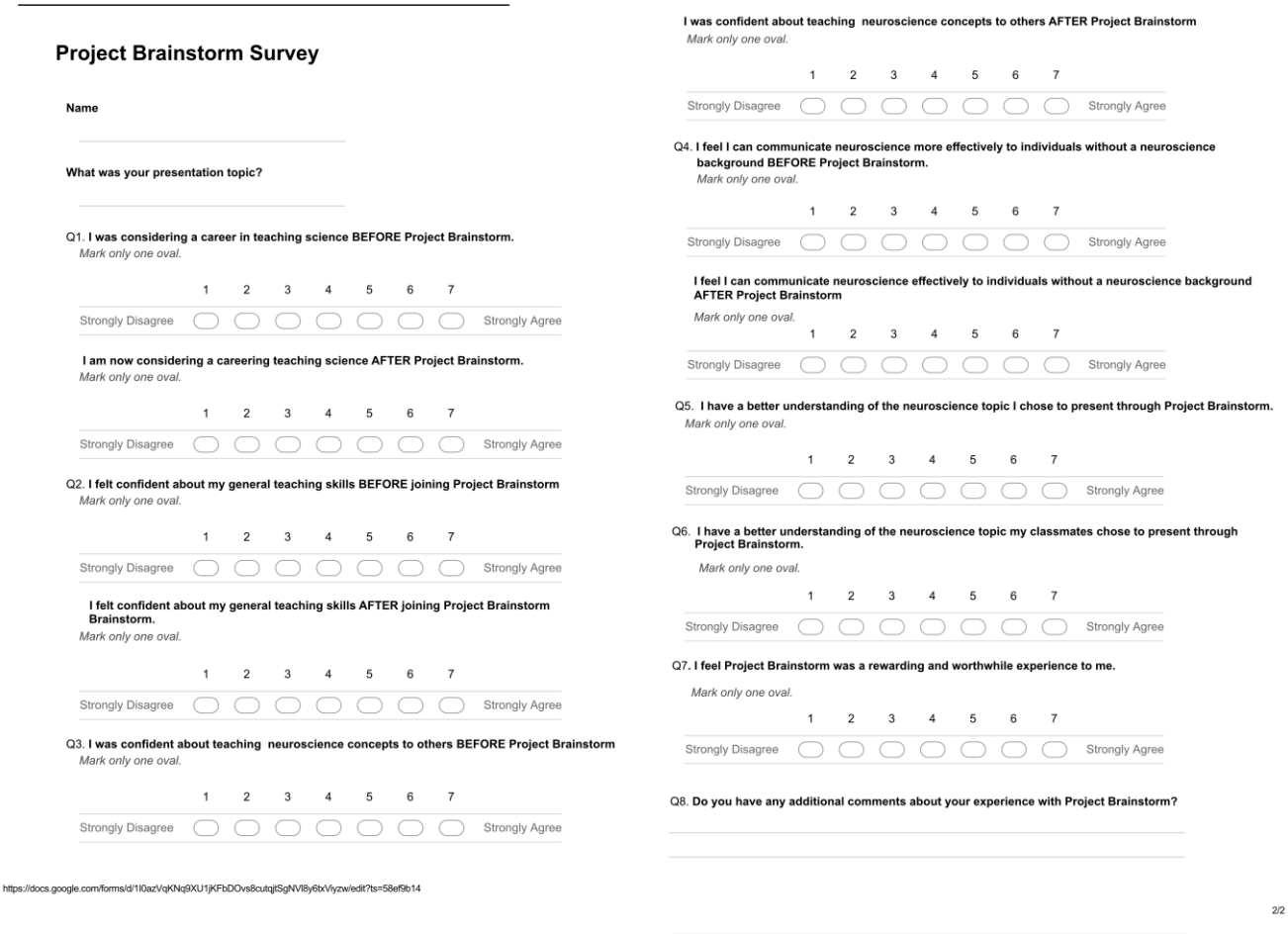
Week 5 (Feb 8):	K-12 School name 1
Week 6 (Feb 15):	K-12 School name 2
Week 7 (Feb 22):	K-12 School name 3
Week 8 (Mar 1):	K-12 School name 4
Week 9 (Mar 8):	K-12 School name 5
Week 10 (Mar 13-17):	*Brain Awareness Week*

Figure 23. Syllabus Copy

	Grades taught	Total number of students in school	% Male	% Female	% Hispanic	% Black	% White	% Asian	% Low Income
Elementary Schools									
Aspire Titan Elementary School*	4	328	53.00%	47.00%	100.00%	0.00%	0.00%	0.00%	88.00%
Citizens of the World Charter Elementary	4	206	52.00%	48.00%	24.00%	13.00%	58.00%	5.00%	27.00%
Da Vinci Innovation Academy Elementary	1 and 2	308	53.00%	47.00%	19.00%	13.00%	53.00%	6.00%	6.00%
Fairburn Elementary	5	459	52.00%	48.00%	8.00%	3.00%	66.00%	18.00%	26.73%
Nora Sterry Elementary School*	4 and 5	329	50.00%	50.00%	68.00%	12.00%	15.00%	3.00%	72.00%
Middle Schools									
Emerson Middle School*	7	550	48.00%	51.00%	49.00%	22.00%	22.00%	4.00%	58.40%
Virgil Middle School*	6,7 and 8	904	52.00%	48.00%	82.00%	3.00%	1.00%	6.00%	94.38%
Magnolia Science Middle School	6 and 7	160	54.00%	46.00%	73.00%	11.00%	8.00%	1.00%	79.00%
St. Brendan Middle School	6 and 7	305	N/A	N/A	10.00%	3.00%	60.00%	17.00%	N/A
High Schools									
Fairfax High School*	11 and 12	2101	52.00%	48.00%	54.00%	16.00%	8.00%	17.00%	81.11%
Hamilton High School*^	10, 11 and 12	2941	48.00%	52.00%	51.00%	27.00%	16.00%	3.00%	67.94%
New West High School	9 and 10	823	49.00%	51.00%	24.00%	19.00%	49.00%	6.00%	21.00%
University High School*	9 and 10	1763	51.00%	49.00%	56.00%	22.00%	11.00%	8.00%	74.72%

Table 3. Schools Visited

A list of representative schools visited and grades taught by Project Brainstorm during the 2016–2017 school years within the Greater Los Angeles Area. Elementary Schools represent kindergarten through fifth grade (5–10 y of age); Middle schools represent sixth through eighth grades (11–13 y); High schools represent ninth through 12th grades (14–18 y)
*Title I school (at least 40% of students come from families that qualify as low-income under the United States Census definitions).
^School visited multiple times. The gender and main four ethnic/racial groups are shown. N/A, data not available or missing. Not all percentage totals will equal 100 since other ethnicities are not shown.



Project Brainstorm Survey

Name

What was your presentation topic?

Q1. **I was considering a career in teaching science BEFORE Project Brainstorm.**
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

I am now considering a careering teaching science AFTER Project Brainstorm.
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

Q2. **I felt confident about my general teaching skills BEFORE joining Project Brainstorm**
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

I felt confident about my general teaching skills AFTER joining Project Brainstorm Brainstorm.
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

Q3. **I was confident about teaching neuroscience concepts to others BEFORE Project Brainstorm**
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

I was confident about teaching neuroscience concepts to others AFTER Project Brainstorm
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

Q4. **I feel I can communicate neuroscience more effectively to individuals without a neuroscience background BEFORE Project Brainstorm.**
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

I feel I can communicate neuroscience effectively to individuals without a neuroscience background AFTER Project Brainstorm
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

Q5. **I have a better understanding of the neuroscience topic I chose to present through Project Brainstorm.**
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

Q6. **I have a better understanding of the neuroscience topic my classmates chose to present through Project Brainstorm.**
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

Q7. **I feel Project Brainstorm was a rewarding and worthwhile experience to me.**
Mark only one oval.

	1	2	3	4	5	6	7	
Strongly Disagree	◯	◯	◯	◯	◯	◯	◯	Strongly Agree

Q8. **Do you have any additional comments about your experience with Project Brainstorm?**

https://docs.google.com/forms/d/1l0azVqKNq9XU1jKFbDOvs8cutqjlSgNVl8y6bxViyzw/edit?ts=58ef9b14

2/2

Figure 24. Survey of undergraduate interests in neuroscience and teaching

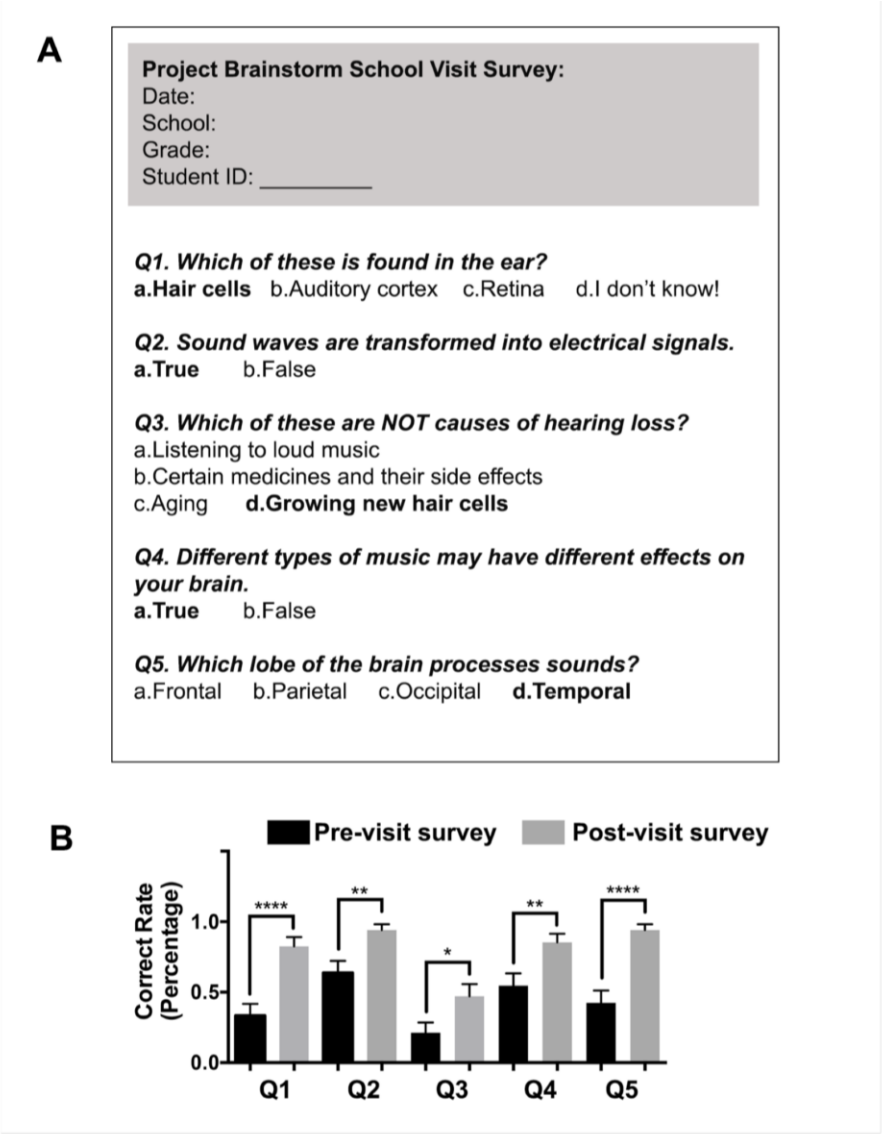


Figure 25. An example of topic specific questions to assess K-12 student learning about hearing

(A) Presentation topic-specific questions created by project brainstorm students to be presented to sixth
(B) graders at the Willows middle school, Los Angeles. Surveys were administered before and after
(C) school visit presentations in order to evaluate teaching effectiveness. (B) The correct rate of each
(D) question before and after school visit presentation (mean ± SEM; n =35, ****P<0.0001, **P<0.01
(E) *P<0.05, unpaired student t test)

Student ID: _____

Project Brainstorm School visit (Neuroscience 192B)

K-12 students' STEM interest

1. Do you enjoy learning about science?

1	2	3	4	5
no, not at all	rarely	sometimes	most of the time	yes, very much

2. Do you think science is important?

1	2	3	4	5
no, not at all	rarely	sometimes	most of the time	yes, very much

3. Do you want to be a scientist?

1	2	3	4	5
no, not at all	rarely	sometimes	most of the time	yes, very much

4. Do you want to go to college?

1	2	3	4	5
no, not at all	rarely	sometimes	most of the time	yes, very much

5. Do you enjoy learning about the brain?

1	2	3	4	5
no, not at all	rarely	sometimes	most of the time	yes, very much

6. Do you want to learn more about the brain?

1	2	3	4	5
no, not at all	rarely	sometimes	most of the time	yes, very much

Figure 26. Survey of K-12 student interest in STEM

APPENDIX II REFERENCES

1. Bjork, E. L., & Bjork, R. A. (2011). Making things hard on yourself, but in a good way: Creating desirable difficulties to enhance learning. In M. A. Gernsbacher, R. W. Pew, L. M. Hough, & J.
2. Ambrose, Susan A. (Eds.) (2010) *How learning works: seven research-based principles for smart teaching* San Francisco, CA : Jossey-Bass.
3. Brabb S, Lack J, Rector D (2008) Undergraduate neurophysiology students as role models for engaging elementary school students in science through a Kids Judge! Neuroscience Fair, Program No. 222.16. 2008 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience.
4. Butcher G, Do R, Wensler H, Shah G, Thorne S (2010) Brain Awareness Week as a vehicle for undergraduate service- learning, Program No. 22.14. 2010 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience.
5. Bybee, R. (1997). Achieving scientific literacy. Portsmouth, NH: Heinemann.
6. Cohen, P. A., Kulik, J. A., & Kulik, C. C. (1982). Educational outcomes of tutoring: A meta-analysis of findings. *American Educational Research Journal, 19*(2), 237–248.
7. Coskun, V., & Carpenter, E. M. (2016). Life science-based neuroscience education at large Western Public Universities. *J Neurosci Res, 94*(12), 1384-1392. doi:10.1002/jnr.23928
8. Darling-Hammond, L., Baratz-Snowden, J. (2007). A Good Teacher in Every Classroom: Preparing the Highly Qualified Teachers Our Children Deserve. *Educational Horizons, 85(2), 111-132.*

9. Deal, A. L., Erickson, K. J., Bilsky, E. J., Hillman, S. J., & Burman, M. A. (2014). K-12 Neuroscience Education Outreach Program: Interactive Activities for Educating Students about Neuroscience. *J Undergrad Neurosci Educ, 13*(1), A8-a20.
10. Editorial. (2009). encouraging science outreach. *Nature Neuroscience, volume 12, number 6, June 2009*.
11. Froyd, Jeff; Layne, Jean (2008). "Faculty development strategies for overcoming the "curse of knowledge"". 2008 38th Annual Frontiers in Education Conference. doi:10.1109/FIE.2008.4720529
12. Gittis A (2009) Brain Awareness as a laboratory project in an undergraduate behavioral neuroscience class, Program No. 24.3. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience.
13. McLaughlin M, Moeller M, Brandt S, Guns A, Kimbro A, Dever B, et al. (2010) Integrating course requirements with brain awareness objectives in the community, Program No. 22.16. 2010 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience.
14. National Center for Educational Statistics (2011).
15. National Science Board. (2010). Science and engineering indicators.
16. Nestojko, J. F., Bui, D. C., Kornell, N., & Bjork, E. L. (2014). Expecting to teach enhances learning and organization of knowledge in free recall of text passages. *Memory & Cognition, 42*, 1038-1048. DOI: 10.3758/s13421-014-0416-z
17. Peets, A. D., Coderre, S., Wright, B., Jenkins, D., Burak, K., Leskosky, S., & McLaughlin, K. (2009). Involvement in teaching improves learning in medical students: a randomized cross-over study. *BMC Medical Education*, 9(55).

18. Rohrbeck, C. A., Ginsburg-Block, M. D., Fantuzzo, J. W., & Miller, T. R. (2003). Peer-assisted learning interventions with elementary school students: A meta-analytic review. *Journal of Educational Psychology, 95*(2), 240–257.
19. Romero-Calderon, R., O'Hare, E. D., Suthana, N. A., Scott-Van Zeeland, A. A., Rizk-Jackson, A., Attar, A., . . . Watson, J. B. (2012). Project brainstorm: using neuroscience to connect college students with local schools. *PLoS Biol, 10*(4), e1001310. doi:10.1371/journal.pbio.1001310
20. Roscoe, R. D., & Chi, M. T. H. (2007). Tutor learning: The role of explaining and responding to questions. *Instructional Science, 36,* 321–350.
21. Soderstrom, N. C., & Bjork, R. A. (2013). Learning versus performance. In D. S. Dunn (Ed.), Oxford bibliographies online: Psychology. New York: Oxford University Press.
22. Stevens, C. (2011). Integrating community outreach into the undergraduate neuroscience classroom. *J Undergrad Neurosci Educ, 10*(1), A44-49.
23. Yawson, N. A., Amankwaa, A. O., Tali, B., Shang, V. O., Batu, E. N., Asiemoah, K., Jr., . . . Karikari, T. K. (2016). Evaluation of Changes in Ghanaian Students' Attitudes Towards Science Following Neuroscience Outreach Activities: A Means to Identify Effective Ways to Inspire Interest in Science Careers. *J Undergrad Neurosci Educ, 14*(2), A117-123.

REFERENCES

1. Chittka, L., and Niven, J. (2009). Are bigger brains better? Curr. Biol. *19*, R995–R1008.

2. Blenau, W., and Baumann, A. (2001). Molecular and pharmacological properties of insect biogenic amine receptors: lessons from Drosophila melanogaster and Apis mellifera. Arch. Insect Biochem. Physiol. *48*, 13–38.

3. Monastirioti, M. (1999). Biogenic amine systems in the fruit fly Drosophila melanogaster. Microsc. Res. Tech. *45*, 106–121.

4. Marder, E., and Calabrese, R.L. (1996). Principles of rhythmic motor pattern generation. Physiol. Rev. *76*, 687–717.

5. Burrows, M. (1996). The neurobiology of an insect brain (Oxford University Press on Demand).

6. Siegelbaum, S.A., and Tsien, R.W. (1983). Modulation of gated ion channels as a mode of transmitter action. Trends Neurosci. *6*, 307–313.

7. Alekseyenko, O.V., Chan, Y.-B., Li, R., and Kravitz, E.A. (2013). Single dopaminergic neurons that modulate aggression in Drosophila. Proc. Natl. Acad. Sci. U. S. A. *110*, 6151–6156.

8. Alekseyenko, O.V., Chan, Y.-B., Fernandez, M. de la P., Bülow, T., Pankratz, M.J., and Kravitz, E.A. (2014). Single serotonergic neurons that modulate aggression in Drosophila. Curr. Biol. *24*, 2700–2707.

9. Certel, S.J., Savella, M.G., Schlegel, D.C.F., and Kravitz, E.A. (2007). Modulation of Drosophila male behavioral choice. Proc. Natl. Acad. Sci. U. S. A. *104*, 4706–4711.

10. Dierick, H.A., and Greenspan, R.J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. Nat. Genet. *39*, 678–682.

11. Inagaki, H.K., Ben-Tabou de-Leon, S., Wong, A.M., Jagadish, S., Ishimoto, H., Barnea, G., Kitamoto, T., Axel, R., and Anderson, D.J. (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. Cell *148*, 583–595.

12. Inagaki, H.K., Jung, Y., Hoopfer, E.D., Wong, A.M., Mishra, N., Lin, J.Y., Tsien, R.Y., and Anderson, D.J. (2014). Optogenetic control of Drosophila using a red-shifted channelrhodopsin reveals experience-dependent influences on courtship. Nat. Methods *11*, 325–332.

13. Keene, A.C., and Waddell, S. (2007). Drosophila olfactory memory: single genes to complex neural circuits. Nat. Rev. Neurosci. *8*, 341–354.

14. Kim, S.M., Su, C.-Y., and Wang, J.W. (2017). Neuromodulation of Innate Behaviors in Drosophila. Annu. Rev. Neurosci. *40*, 327–348.

15. Rezával, C., Nojima, T., Neville, M.C., Lin, A.C., and Goodwin, S.F. (2014). Sexually dimorphic octopaminergic neurons modulate female postmating behaviors in Drosophila. Curr. Biol. *24*, 725–730.

16. Zhou, C., Rao, Y., and Rao, Y. (2008). A subset of octopaminergic neurons are important for Drosophila aggression. Nat. Neurosci. *11*, 1059–1067.

17. Zhou, C., Huang, H., Kim, S.M., Lin, H., Meng, X., Han, K.-A., Chiang, A.-S., Wang, J.W., Jiao, R., and Rao, Y. (2012). Molecular genetic analysis of sexual rejection: roles of octopamine and its receptor OAMB in Drosophila courtship conditioning. J. Neurosci. *32*, 14281–14287.

18. Hildreth, E.C., and Koch, C. (1987). The analysis of visual motion: from computational theory to neuronal mechanisms. Annu. Rev. Neurosci. *10*, 477–533.

19. Silies, M., Gohl, D.M., and Clandinin, T.R. (2014). Motion-detecting circuits in flies: coming into view. Annu. Rev. Neurosci. *37*, 307–327.

20. Borst, A., Haag, J., and Reiff, D.F. (2010). Fly motion vision. Annu. Rev. Neurosci. *33*, 49–70.

21. Mauss, A.S., and Borst, A. (2017). Motion Vision in Arthropods. In The Oxford Handbook of Invertebrate Neurobiology, J. H. Byrne, ed. (Oxford University Press).

22. Borst, A., Haag, J., and Mauss, A.S. (2019). How fly neurons compute the direction of visual motion. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. Available at: http://dx.doi.org/10.1007/s00359-019-01375-9.

23. Strausfeld, N.J. (2005). The evolution of crustacean and insect optic lobes and the origins of chiasmata. Arthropod Struct. Dev. *34*, 235–256.

24. Land, M.F., and Nilsson, D.-E. (2012). Animal Eyes (Oxford University Press).

25. Fischbach, K.-F., and Dittrich, A.P.M. (1989). The optic lobe of Drosophila melanogaster. I. A Golgi analysis of wild-type structure. Cell Tissue Res. *258*, 441–475.

26. Wardill, T.J., List, O., Li, X., Dongre, S., McCulloch, M., Ting, C.-Y., O'Kane, C.J., Tang, S., Lee, C.-H., Hardie, R.C., *et al.* (2012). Multiple spectral inputs improve motion discrimination in the Drosophila visual system. Science *336*, 925–931.

27. Osorio, D., and Bacon, J.P. (1994). A good eye for arthropod evolution. Bioessays *16*, 419–424.

28. Stuart, A.E. (1999). From Fruit Flies to Barnacles, Minireview Histamine Is the Neurotransmitter of Arthropod Photoreceptors. Neuron *22*, 431–433.

29. Frye, M. (2015). Elementary motion detectors. Curr. Biol. *25*, R215–R217.

30. Maisak, M.S., Haag, J., Ammer, G., Serbe, E., Meier, M., Leonhardt, A., Schilling, T., Bahl, A., Rubin, G.M., Nern, A., *et al.* (2013). A directional tuning map of Drosophila elementary motion detectors. Nature *500*, 212–216.

31. Takemura, S.-Y., Bharioke, A., Lu, Z., Nern, A., Vitaladevuni, S., Rivlin, P.K., Katz, W.T., Olbris, D.J., Plaza, S.M., Winston, P., *et al.* (2013). A visual motion detection circuit suggested by Drosophila connectomics. Nature *500*, 175–181.

32. Shinomiya, K., Karuppudurai, T., Lin, T.-Y., Lu, Z., Lee, C.-H., and Meinertzhagen, I.A. (2014). Candidate neural substrates for off-edge motion detection in Drosophila. Curr. Biol. *24*, 1062–1070.

33. Behnia, R., Clark, D.A., Carter, A.G., Clandinin, T.R., and Desplan, C. (2014). Processing properties of ON and OFF pathways for Drosophila motion detection. Nature *512*, 427–430.

34. Fisher, Y.E., Leong, J.C.S., Sporar, K., Ketkar, M.D., Gohl, D.M., Clandinin, T.R., and Silies, M. (2015). A Class of Visual Neurons with Wide-Field Properties Is Required for Local Motion Detection. Curr. Biol. *25*, 3178–3189.

35. Fisher, Y.E., Silies, M., and Clandinin, T.R. (2015). Orientation Selectivity Sharpens Motion Detection in Drosophila. Neuron *88*, 390–402.

36. Ammer, G., Leonhardt, A., Bahl, A., Dickson, B.J., and Borst, A. (2015). Functional Specialization of Neural Input Elements to the Drosophila ON Motion Detector. Curr. Biol. *25*, 2247–2253.

37. Serbe, E., Meier, M., Leonhardt, A., and Borst, A. (2016). Comprehensive Characterization of the Major Presynaptic Elements to the Drosophila OFF Motion Detector. Neuron *89*, 829–841.

38. Scott, E.K., Raabe, T., and Luo, L. (2002). Structure of the vertical and horizontal system neurons of the lobula plate in Drosophila. J. Comp. Neurol. *454*, 470–481.

39. Raghu, S.V., Joesch, M., and Borst, A. (2007). Synaptic organization of lobula plate tangential cells in Drosophila: γ-Aminobutyric acid receptors and chemical release sites. Journal of Comparative. Available at: https://onlinelibrary.wiley.com/doi/abs/10.1002/cne.21319.

40. Joesch, M., Plett, J., Borst, A., and Reiff, D.F. (2008). Response properties of motion-sensitive visual interneurons in the lobula plate of Drosophila melanogaster. Curr. Biol. *18*, 368–374.

41. Maimon, G., Straw, A.D., and Dickinson, M.H. (2010). Active flight increases the gain of visual motion processing in Drosophila. Nat. Neurosci. *13*, 393–399.

42. Schnell, B., Joesch, M., Forstner, F., Raghu, S.V., Otsuna, H., Ito, K., Borst, A., and Reiff, D.F. (2010). Processing of horizontal optic flow in three visual interneurons of the Drosophila brain. J. Neurophysiol. *103*, 1646–1657.

43. Otsuna, H., and Ito, K. (2006). Systematic analysis of the visual projection neurons of Drosophila melanogaster. I. Lobula-specific pathways. J. Comp. Neurol. *497*, 928–958.

44. Strausfeld, N.J., and Okamura, J.-Y. (2007). Visual system of calliphorid flies: organization of optic glomeruli and their lobula complex efferents. J. Comp. Neurol. *500*, 166–188.

45. Wu, M., Nern, A., Williamson, W.R., Morimoto, M.M., Reiser, M.B., Card, G.M., and Rubin, G.M. (2016). Visual projection neurons in the Drosophila lobula link feature detection to distinct behavioral programs. Elife *5*. Available at: http://dx.doi.org/10.7554/eLife.21022.

46. Ache, J.M., Polsky, J., Alghailani, S., Parekh, R., Breads, P., Peek, M.Y., Bock, D.D., von Reyn, C.R., and Card, G.M. (2019). Neural Basis for Looming Size and Velocity Encoding in the Drosophila Giant Fiber Escape Pathway. Curr. Biol. *29*, 1073-1081.e4.

47. Kloppenburg, P., and Erber, J. (1995). The modulatory effects of serotonin and octopamine in the visual system of the honey bee (Apis mellifera L.). Journal of Comparative Physiology A *176*, 119–129.

48. Nässel, D.R., and Klemm, N. (1983). Serotonin-like immunoreactivity in the optic lobes of three insect species. Cell Tissue Res. *232*, 129–140.

49. Nässel, D.R. (1991). Neurotransmitters and neuromodulators in the insect visual system. Prog. Neurobiol. *37*, 179–254.

50. Homberg, U. (1994). Distribution of neurotransmitters in the insect brain (Gustav Fischer Verlag).

51. Nässel, D.R., and Elekes, K. (1992). Aminergic neurons in the brain of blowflies and Drosophila: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. Cell Tissue Res. *267*, 147–167.

52. Farooqui, T. (2012). Review of octopamine in insect nervous systems. Open access insect physiol. *4*, 1–17.

53. Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. Annu. Rev. Entomol. *50*, 447–477.

54. Sinakevitch, I., and Strausfeld, N.J. (2006). Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. J. Comp. Neurol. *494*, 460–475.

55. Busch, S., Selcho, M., Ito, K., and Tanimoto, H. (2009). A map of octopaminergic neurons in the Drosophila brain. J. Comp. Neurol. *513*, 643–667.

56. Konings, P.N., Vullings, H.G., Geffard, M., Buijs, R.M., Diederen, J.H., and Jansen, W.F. (1988). Immunocytochemical demonstration of octopamine-immunoreactive cells in the nervous system of Locusta migratoria and Schistocerca gregaria. Cell Tissue Res. *251*, 371–379.

57. Stern, M., Thompson, K.S.J., Zhou, P., Watson, D.G., Midgley, J.M., Gewecke, M., and Bacon, J.P. (1995). Octopaminergic neurons in the locust brain: morphological, biochemical and electrophysiological characterisation of potential modulators of the visual system. J. Comp. Physiol. A *177*, 611–625.

58. Cheng, K.Y., Colbath, R.A., and Frye, M.A. (2019). Olfactory and Neuromodulatory Signals Reverse Visual Object Avoidance to Approach in Drosophila. Curr. Biol. *29*, 2058-2065.e2.

59. Roeder, T., and Nathanson, J.A. (1993). Characterization of insect neuronal octopamine receptors (OA3 receptors). Neurochem. Res. *18*, 921–925.

60. Evans, P.D., and Maqueira, B. (2005). Insect octopamine receptors: a new classification scheme based on studies of cloned Drosophila G-protein coupled receptors. Invert. Neurosci. *5*, 111–118.

61. Roeder, T. (1994). Biogenic amines and their receptors in insects. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. *107*, 1–12.

62. Farooqui, T., Vaessin, H., and Smith, B.H. (2004). Octopamine receptors in the honeybee (Apis mellifera) brain and their disruption by RNA-mediated interference. J. Insect Physiol. *50*, 701–713.

63. Beeman, R.W., and Matsumura, F. (1973). Chlordimeform: a pesticide acting upon amine regulatory mechanisms. Nature *242*, 273–274.

64. Evans, P.D., and Gee, J.D. (1980). Action of formamidine pesticides on octopamine receptors. Nature *287*, 60–62.

65. Hiripi, L., Nagy, L., and Hollingworth, R.M. (1999). In vitro and in vivo effects of formamidines in locust (Locusta migratoria migratorioides). Acta Biol. Hung. *50*, 81–87.

66. Nathanson, J.A., and Hunnicutt, E.J. (1981). N-demethylchlordimeform: a potent partial agonist of octopamine-sensitive adenylate cyclase. Mol. Pharmacol. *20*, 68–75.

67. Hollingworth, R.M., and Murdock, L.L. (1980). Formamidine Pesticides: Octopamine-Like Actions in a Firefly. Science *208*, 74–76. Available at: http://dx.doi.org/10.1126/science.208.4439.74.

68. Christensen, T.A., and Carlson, A.D. (1982). The neurophysiology of larval firefly luminescence: Direct activation through four bifurcating (DUM) neurons. J. Comp. Physiol. *148*, 503–514.

69. Kinnamon, S.C., Klaassen, L.W., Kammer, A.E., and Claassen, D. (1984). Octopamine and chlordimeform enhance sensory responsiveness and production of the flight motor pattern in developing and adult moths. J. Neurobiol. *15*, 283–293.

70. Benezet, H.J., Chang, K.-M., and Knowles, C.O. (1978). Formamidine Pesticides — Metabolic Aspects of Neurotoxicity. Pesticide and Venom Neurotoxicity, 189–206. Available at: http://dx.doi.org/10.1007/978-1-4615-8834-4_16.

71. Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J., and Hirsh, J. (2005). Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J. Biol. Chem. *280*, 14948–14955.

72. Williams, D.S. (1983). Changes of photoreceptor performance associated with the daily turnover of photoreceptor membrane in locusts. J. Comp. Physiol. *150*, 509–519.

73. Weckström, M. (1994). Voltage-activated outward currents in adult and nymphal locust photoreceptors. Journal of Comparative Physiology A *174*, 795–801.

74. Cuttle, M.F., Hevers, W., Laughlin, S.B., and Hardie, R.C. (1995). Diurnal modulation of photoreceptor potassium conductance in the locust. Journal of Comparative Physiology A *176*, 307–316.

75. Elias, M.S., and Evans, P.D. (1983). Histamine in the insect nervous system: distribution, synthesis and metabolism. J. Neurochem. *41*, 562–568.

76. Hardie, R.C. (1987). Is histamine a neurotransmitter in insect photoreceptors? J. Comp. Physiol. A *161*, 201–213.

77. Nässel, D.R., Holmqvist, M.H., Hardie, R.C., Håkanson, R., and Sundler, F. (1988). Histamine-like immunoreactivity in photoreceptors of the compound eyes and ocelli of the flies Calliphora erythrocephala and Musca domestica. Cell Tissue Res. *253*, 639–646.

78. Hevers, W., and Hardie, R.C. (1993). Serotonin modulates shaker potassium channels in Drosophila photoreceptors. Gene-brain-behaviour. Thieme, Stuttgart New York *631*.

79. Hevers, W., and Hardie, R.C. (1995). Serotonin modulates the voltage dependence of delayed rectifier and Shaker potassium channels in Drosophila photoreceptors. Neuron *14*, 845–856.

80. Eskin, A., and Maresh, R.D. (1982). Serotonin or electrical optic nerve stimulation increases the photosensitivity of the Aplysia eye. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. *73*, 27–31.

81. Crow, T., and Bridge, M.S. (1985). Serotonin modulates photoresponses in Hermissenda type-B photoreceptors. Neurosci. Lett. *60*, 83–88.

82. Chyb, S., Hevers, W., Forte, M., Wolfgang, W.J., Selinger, Z., and Hardie, R.C. (1999). Modulation of the light response by cAMP in Drosophila photoreceptors. J. Neurosci. *19*, 8799–8807.

83. Kaupp, U.B., Malbon, C.C., Battelle, B.A., and Brown, J.E. (1982). Octopamine stimulated rise of cAMP in Limulus ventral photoreceptors. Vision Res. *22*, 1503–1506.

84. Kass, L., and Barlow, R.B., Jr (1984). Efferent neurotransmission of circadian rhythms in Limulus lateral eye. I. Octopamine-induced increases in retinal sensitivity. J. Neurosci. *4*, 908–917.

85. Meinertzhagen, I.A., and Pyza, E. (1999). Neurotransmitter regulation of circadian structural changes in the fly's visual system. Microsc. Res. Tech. *45*, 96–105.

86. Chen, B., Meinertzhagen, I.A., and Shaw, S.R. (1999). Circadian rhythms in light-evoked responses of the fly's compound eye, and the effects of neuromodulators 5-HT and the peptide PDF. J. Comp. Physiol. A *185*, 393–404.

87. Alekseyenko, O.V., Chan, Y.-B., Okaty, B.W., Chang, Y., Dymecki, S.M., and Kravitz, E.A. (2019). Serotonergic Modulation of Aggression in Drosophila Involves GABAergic and Cholinergic Opposing Pathways. Curr. Biol. *29*, 2145-2156.e5.

88. Aptekar, J.W., Keleş, M.F., Lu, P.M., Zolotova, N.M., and Frye, M.A. (2015). Neurons Forming Optic Glomeruli Compute Figure–Ground Discriminations in Drosophila. J. Neurosci. *35*, 7587–7599.

89. Hausen, K. (1982). Motion sensitive interneurons in the optomotor system of the fly. Biol. Cybern. *45*, 143–156.

90. Hengstenberg, R., Hausen, K., and Hengstenberg, B. (1982). The number and structure of giant vertical cells (VS) in the lobula plate of the blowflyCalliphora erythrocephala. J. Comp. Physiol. *149*, 163–177.

91. Britten, K.H. (2008). Mechanisms of self-motion perception. Annu. Rev. Neurosci. *31*, 389–410.

92. Krapp, H.G., and Wicklein, M. (2008). Central Processing of Visual Information in Insects. The Senses: A Comprehensive Reference *1*, 131–203.

93. Longden, K.D., and Krapp, H.G. (2010). Octopaminergic modulation of temporal frequency coding in an identified optic flow-processing interneuron. Front. Syst. Neurosci. *4*, 153.

94. Busch, C., Borst, A., and Mauss, A.S. (2018). Bi-directional Control of Walking Behavior by Horizontal Optic Flow Sensors. Curr. Biol. *28*, 4037-4045.e5.

95. Suver, M.P., Mamiya, A., and Dickinson, M.H. (2012). Octopamine neurons mediate flight-induced modulation of visual processing in Drosophila. Curr. Biol. *22*, 2294–2302.

96. Longden, K.D., and Krapp, H.G. (2009). State-dependent performance of optic-flow processing interneurons. J. Neurophysiol. *102*, 3606–3618.

97. Jung, S.N., Borst, A., and Haag, J. (2011). Flight activity alters velocity tuning of fly motion-sensitive neurons. J. Neurosci. *31*, 9231–9237.

98. Lüders, J., and Kurtz, R. (2015). Octopaminergic modulation of temporal frequency tuning of a fly visual motion-sensitive neuron depends on adaptation level. Front. Integr. Neurosci. *9*, 36.

99. Nordstrom, K., de Miguel, I.M., and O'Carroll, D.C. (2011). Rapid contrast gain reduction following motion adaptation. Journal of Experimental Biology *214*, 4000–4009. Available at: http://dx.doi.org/10.1242/jeb.057539.

100. Maddess, T., and Laughlin, S.B. (1985). Adaptation of the motion-sensitive neuron H1 is generated locally and governed by contrast frequency. Proceedings of the Royal society of London. Series B. Biological sciences *225*, 251–275.

101. Harris, R.A., O'Carroll, D.C., and Laughlin, S.B. (2000). Contrast gain reduction in fly motion adaptation. Neuron *28*, 595–606.

102. Fairhall, A.L., Lewen, G.D., Bialek, W., and de Ruyter Van Steveninck, R.R. (2001). Efficiency and ambiguity in an adaptive neural code. Nature *412*, 787–792.

103. Neri, P., and Laughlin, S.B. (2005). Global versus local adaptation in fly motion-sensitive neurons. Proc. Biol. Sci. *272*, 2243–2249.

104. Kalb, J., Egelhaaf, M., and Kurtz, R. (2008). Adaptation of velocity encoding in synaptically coupled neurons in the fly visual system. J. Neurosci. *28*, 9183–9193.

105. Kurtz, R., Beckers, U., Hundsdörfer, B., and Egelhaaf, M. (2009). Mechanisms of after-hyperpolarization following activation of fly visual motion-sensitive neurons. European Journal of Neuroscience *30*, 567–577. Available at: http://dx.doi.org/10.1111/j.1460-9568.2009.06854.x.

106. Rien, D., Kern, R., and Kurtz, R. (2012). Octopaminergic modulation of contrast gain adaptation in fly visual motion-sensitive neurons. Eur. J. Neurosci. Available at: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1460-9568.2012.08216.x.

107. de Haan, R., Lee, Y.-J., and Nordström, K. (2012). Octopaminergic modulation of contrast sensitivity. Front. Integr. Neurosci. *6*, 55.

108. Bacon, J.P., Thompson, K.S., and Stern, M. (1995). Identified octopaminergic neurons provide an arousal mechanism in the locust brain. J. Neurophysiol. *74*, 2739–2743.

109. Rind, F.C., Santer, R.D., and Wright, G.A. (2008). Arousal facilitates collision avoidance mediated by a looming sensitive visual neuron in a flying locust. J. Neurophysiol. *100*, 670–680.

110. Arenz, A., Drews, M.S., Richter, F.G., Ammer, G., and Borst, A. (2017). The Temporal Tuning of the Drosophila Motion Detectors Is Determined by the Dynamics of Their Input Elements. Curr. Biol. *27*, 929–944.

111. Strother, J.A., Wu, S.-T., Rogers, E.M., Eliason, J.L.M., Wong, A.M., Nern, A., and Reiser, M.B. (2018). Behavioral state modulates the ON visual motion pathway of Drosophila. Proc. Natl. Acad. Sci. U. S. A. *115*, E102–E111.

112. Adamo, S.A., and Chase, R. (1991). "Central arousal" and sexual responsiveness in the snail, Helix aspersa. Behav. Neural Biol. *55*, 194–213.

113. Adamo, S.A., Linn, C.E., and Hoy, R.R. (1995). The role of neurohormonal octopamine during'fight or flight'behaviour in the field cricket Gryllus bimaculatus. J. Exp. Biol. *198*, 1691–1700.

114. Evans, P.D. (1980). Biogenic Amines in the Insect Nervous System. In Advances in Insect Physiology, M. J. Berridge, J. E. Treherne, and V. B. Wigglesworth, eds. (Academic Press), pp. 317–473.

115. Evans, P.D., and Siegler, M.V. (1982). Octopamine mediated relaxation of maintained and catch tension in locust skeletal muscle. J. Physiol. *324*, 93–112.

116. Heslop, J.P., and Ray, J.W. (1959). The reaction of the cockroach Periplaneta americana L. to bodily stress and DDT. J. Insect Physiol. *3*, 395–401.

117. Barron, A.B., Schulz, D.J., and Robinson, G.E. (2002). Octopamine modulates responsiveness to foraging-related stimuli in honey bees (Apis mellifera). J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *188*, 603–610.

118. Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J. Neurosci. *28*, 9377–9385.

119. Crocker, A., Shahidullah, M., Levitan, I.B., and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. Neuron *65*, 670–681.

120. Goosey, M.W., and Candy, D.J. (1980). The D-octopamine content of the haemolymph of the locust, Schistocerca americana gregaria and its elevation during flight. Insect Biochem. *10*, 393–397.

121. Orchard, I., Ramirez, J.M., and Lange, A.B. (1993). A Multifunctional Role for Octopamine in Locust Flight. Annu. Rev. Entomol. *38*, 227–249.

122. Brembs, B., Christiansen, F., Pflüger, H.J., and Duch, C. (2007). Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. J. Neurosci. *27*, 11122–11131.

123. Sombati, S., and Hoyle, G. (1984). Generation of specific behaviors in a locust by local release into neuropil of the natural neuromodulator octopamine. J. Neurobiol. *15*, 481–506.

124. van Breugel, F., Suver, M.P., and Dickinson, M.H. (2014). Octopaminergic modulation of the visual flight speed regulator of Drosophila. J. Exp. Biol. *217*, 1737–1744.

125. Ramirez, J.M., and Pearson, K.G. (1993). Alteration of bursting properties in interneurons during locust flight. J. Neurophysiol. *70*, 2148–2160.

126. Ramirez, J.M., and Pearson, K.G. (1991). Octopaminergic modulation of interneurons in the flight system of the locust. J. Neurophysiol. *66*, 1522–1537.

127. Ache, J.M., Namiki, S., Lee, A., Branson, K., and Card, G.M. (2019). State-dependent decoupling of sensory and motor circuits underlies behavioral flexibility in Drosophila. Nat. Neurosci. *22*, 1132–1139.

128. Chiappe, M.E., Seelig, J.D., Reiser, M.B., and Jayaraman, V. (2010). Walking modulates speed sensitivity in Drosophila motion vision. Curr. Biol. *20*, 1470–1475.

129. Fujiwara, T., and Chiappe, E. (2017). Motor-Driven Modulation in Visual Neural Circuits. In Decoding Neural Circuit Structure and Function: Cellular Dissection Using Genetic Model Organisms, A. Çelik and M. F. Wernet, eds. (Cham: Springer International Publishing), pp. 261–281.

130. Fujiwara, T., Cruz, T.L., Bohnslav, J.P., and Chiappe, M.E. (2017). A faithful internal representation of walking movements in the Drosophila visual system. Nat. Neurosci. *20*, 72–81.

131. Inagaki, H.K., Panse, K.M., and Anderson, D.J. (2014). Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in Drosophila. Neuron *84*, 806–820.

132. Stafford, J.W., Lynd, K.M., Jung, A.Y., and Gordon, M.D. (2012). Integration of taste and calorie sensing in Drosophila. J. Neurosci. *32*, 14767–14774.

133. Lee, G., and Park, J.H. (2004). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster. Genetics *167*, 311–323.

134. Yang, Z., Yu, Y., Zhang, V., Tian, Y., Qi, W., and Wang, L. (2015). Octopamine mediates starvation-induced hyperactivity in adult Drosophila. Proc. Natl. Acad. Sci. U. S. A. *112*, 5219–5224.

135. Yu, Y., Huang, R., Ye, J., Zhang, V., Wu, C., Cheng, G., Jia, J., and Wang, L. (2016). Regulation of starvation-induced hyperactivity by insulin and glucagon signaling in adult Drosophila. Elife *5*. Available at: http://dx.doi.org/10.7554/eLife.15693.

136. Longden, K.D., Muzzu, T., Cook, D.J., Schultz, S.R., and Krapp, H.G. (2014). Nutritional state modulates the neural processing of visual motion. Curr. Biol. *24*, 890–895.

137. Budick, S.A., and Dickinson, M.H. (2006). Free-flight responses of Drosophila melanogaster to attractive odors. J. Exp. Biol. *209*, 3001–3017.

138. Wong, J.-M.T., Malec, P.A., Mabrouk, O.S., Ro, J., Dus, M., and Kennedy, R.T. (2016). Benzoyl chloride derivatization with liquid chromatography-mass spectrometry for targeted metabolomics of neurochemicals in biological samples. J. Chromatogr. A *1446*, 78–90.

139. Wicher, D. (2007). Metabolic regulation and behavior: how hunger produces arousal - an insect study. Endocr. Metab. Immune Disord. Drug Targets *7*, 304–310.

140. Frye, M.A., Tarsitano, M., and Dickinson, M.H. (2003). Odor localization requires visual feedback during free flight in Drosophila melanogaster. J. Exp. Biol. *206*, 843–855.

141. Chow, D.M., and Frye, M.A. (2008). Context-dependent olfactory enhancement of optomotor flight control in Drosophila. J. Exp. Biol. *211*, 2478–2485.

142. Chow, D.M., Theobald, J.C., and Frye, M.A. (2011). An olfactory circuit increases the fidelity of visual behavior. J. Neurosci. *31*, 15035–15047.

143. Götz, K.G. (1968). Flight control in Drosophila by visual perception of motion. Kybernetik *4*, 199–208.

144. Tammero, L.F., Frye, M.A., and Dickinson, M.H. (2004). Spatial organization of visuomotor reflexes in Drosophila. J. Exp. Biol. *207*, 113–122.

145. Bhandawat, V., Maimon, G., Dickinson, M.H., and Wilson, R.I. (2010). Olfactory modulation of flight in Drosophila is sensitive, selective and rapid. J. Exp. Biol. *213*, 3625–3635.

146. Frye, M.A., and Dickinson, M.H. (2004). Motor output reflects the linear superposition of visual and olfactory inputs in Drosophila. J. Exp. Biol. *207*, 123–131.

147. Duistermars, B.J., Chow, D.M., and Frye, M.A. (2009). Flies require bilateral sensory input to track odor gradients in flight. Curr. Biol. *19*, 1301–1307.

148. Wasserman, S.M., Aptekar, J.W., Lu, P., Nguyen, J., Wang, A.L., Keles, M.F., Grygoruk, A., Krantz, D.E., Larsen, C., and Frye, M.A. (2015). Olfactory neuromodulation of motion vision circuitry in Drosophila. Curr. Biol. *25*, 467–472.

149. Stewart, F.J., Baker, D.A., and Webb, B. (2010). A model of visual–olfactory integration for odour localisation in free-flying fruit flies. J. Exp. Biol. *213*, 1886–1900.

150. van Breugel, F., Riffell, J., Fairhall, A., and Dickinson, M.H. (2015). Mosquitoes Use Vision to Associate Odor Plumes with Thermal Targets. Curr. Biol. *25*, 2123–2129.

151. van Breugel, F., Huda, A., and Dickinson, M.H. (2018). Distinct activity-gated pathways mediate attraction and aversion to CO2 in Drosophila. Nature *564*, 420–424.

152. Ma, Z., Stork, T., Bergles, D.E., and Freeman, M.R. (2016). Neuromodulators signal through astrocytes to alter neural circuit activity and behaviour. Nature *539*, 428–432.

153. Dolan, M.-J., Frechter, S., Bates, A.S., Dan, C., Huoviala, P., Roberts, R.J., Schlegel, P., Dhawan, S., Tabano, R., Dionne, H., *et al.* (2019). Neurogenetic dissection of the Drosophila lateral horn reveals major outputs, diverse behavioural functions, and interactions with the mushroom body. Elife *8*. Available at: http://dx.doi.org/10.7554/eLife.43079.

154. Vinck, M., Batista-Brito, R., Knoblich, U., and Cardin, J.A. (2015). Arousal and locomotion make distinct contributions to cortical activity patterns and visual encoding. Neuron *86*, 740–754.

155. Monastirioti, M., Linn, C.E., Jr, and White, K. (1996). Characterization of Drosophila tyramine β-hydroxylase gene and isolation of mutant flies lacking octopamine. Journal of Neuroscience *16*, 3900–3911.

156. Saraswati, S., Fox, L.E., Soll, D.R., and Wu, C.-F. (2004). Tyramine and octopamine have opposite effects on the locomotion of Drosophila larvae. J. Neurobiol. *58*, 425–441.

157. Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pflüger, H.-J., Blenau, W., and Broeck, J.V. (2010). The role of octopamine in locusts and other arthropods. J. Insect Physiol. *56*, 854–867.

158. Alkema, M.J., Hunter-Ensor, M., Ringstad, N., and Horvitz, H.R. (2005). Tyramine Functions independently of octopamine in the Caenorhabditis elegans nervous system. Neuron *46*, 247–260.

159. Roeder, T., Seifert, M., Kähler, C., and Gewecke, M. (2003). Tyramine and octopamine: antagonistic modulators of behavior and metabolism. Arch. Insect Biochem. Physiol. *54*, 1–13.

160. Maimon, G. (2011). Modulation of visual physiology by behavioral state in monkeys, mice, and flies. Curr. Opin. Neurobiol. *21*, 559–564.

161. Frye, M.A., and Dickinson, M.H. (2001). Fly flight: a model for the neural control of complex behavior. Neuron *32*, 385–388.

162. Muijres, F.T., Elzinga, M.J., Melis, J.M., and Dickinson, M.H. (2014). Flies evade looming targets by executing rapid visually directed banked turns. Science *344*, 172–177.

163. Mongeau, J.-M., Sponberg, S.N., Miller, J.P., and Full, R.J. (2015). Sensory processing within cockroach antenna enables rapid implementation of feedback control for high-speed running maneuvers. J. Exp. Biol. *218*, 2344–2354.

164. Sen, R., Wu, M., Branson, K., Robie, A., Rubin, G.M., and Dickson, B.J. (2017). Moonwalker descending neurons mediate visually evoked retreat in Drosophila. Curr. Biol. *27*, 766–771.

165. Card, G., and Dickinson, M.H. (2008). Visually mediated motor planning in the escape response of Drosophila. Curr. Biol. *18*, 1300–1307.

166. Tammero, L.F., and Dickinson, M.H. (2002). Collision-avoidance and landing responses are mediated by separate pathways in the fruit fly, Drosophila melanogaster. J. Exp. Biol. *205*, 2785–2798.

167. van Breugel, F., and Dickinson, M.H. (2012). The visual control of landing and obstacle avoidance in the fruit fly Drosophila melanogaster. J. Exp. Biol. *215*, 1783–1798.

168. Maimon, G., Straw, A.D., and Dickinson, M.H. (2008). A simple vision-based algorithm for decision making in flying Drosophila. Curr. Biol. *18*, 464–470.

169. Park, E.J., and Wasserman, S.M. (2018). Diversity of visuomotor reflexes in two Drosophila species. Curr. Biol. *28*, R865–R866.

170. Mongeau, J.-M., and Frye, M.A. (2017). Drosophila Spatiotemporally Integrates Visual Signals to Control Saccades. Curr. Biol. *27*, 2901-2914.e2.

171. Hassenstein, B., and Reichardt, W. (1956). Systemtheoretische Analyse der Zeit-, Reihenfolgen- und Vorzeichenauswertung bei der Bewegungsperzeption des Rüsselkäfers Chlorophanus. Z. Naturforsch. B J. Chem. Sci. *11*, 513–524.

172. Aptekar, J.W., Shoemaker, P.A., and Frye, M.A. (2012). Figure tracking by flies is supported by parallel visual streams. Curr. Biol. *22*, 482–487.

173. Fox, J.L., Aptekar, J.W., Zolotova, N.M., Shoemaker, P.A., and Frye, M.A. (2014). Figure-ground discrimination behavior in Drosophila. I. Spatial organization of wing-steering responses. J. Exp. Biol. *217*, 558–569.

174. Theobald, J.C., Duistermars, B.J., Ringach, D.L., and Frye, M.A. (2008). Flies see second-order motion. Curr. Biol. *18*, R464-5.

175. Reiser, M.B., and Dickinson, M.H. (2008). A modular display system for insect behavioral neuroscience. J. Neurosci. Methods *167*, 127–139.

176. Aptekar, J.W., Keles, M.F., Mongeau, J.-M., Lu, P.M., Frye, M.A., and Shoemaker, P.A. (2014). Method and software for using m-sequences to characterize parallel components of higher-order visual tracking behavior in Drosophila. Front. Neural Circuits *8*, 130.

177. Bender, J.A., and Dickinson, M.H. (2006). Visual stimulation of saccades in magnetically tethered Drosophila. J. Exp. Biol. *209*, 3170–3182.

178. Duistermars, B.J., and Frye, M. (2008). A Magnetic Tether System to Investigate Visual and Olfactory Mediated Flight Control in Drosophila. Journal of Visualized Experiments. Available at: http://dx.doi.org/10.3791/1063.

179. Heide, G., and Götz, K.G. (1996). Optomotor control of course and altitude in Drosophila melanogaster is correlated with distinct activities of at least three pairs of flight steering muscles. J. Exp. Biol. *199*, 1711–1726.

180. Heisenberg, M., and Wolf, R. (1979). On the fine structure of yaw torque in visual flight orientation ofDrosophila melanogaster. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *130*, 113–130.

181. Keleş, M.F., Mongeau, J.-M., and Frye, M.A. (2018). Object features and T4/T5 motion detectors modulate the dynamics of bar tracking by Drosophila. J. Exp. Biol. Available at: http://dx.doi.org/10.1242/jeb.190017.

182. Ferris, B.D., Green, J., and Maimon, G. (2018). Abolishment of Spontaneous Flight Turns in Visually Responsive Drosophila. Curr. Biol. *28*, 170-180.e5.

183. Schnell, B., Ros, I.G., and Dickinson, M.H. (2017). A descending neuron correlated with the rapid steering maneuvers of flying Drosophila. Curr. Biol. *27*, 1200–1205.

184. Muijres, F.T., Elzinga, M.J., Iwasaki, N.A., and Dickinson, M.H. (2015). Body saccades of Drosophila consist of stereotyped banked turns. J. Exp. Biol. *218*, 864–875.

185. Tanaka, R., and Clark, D.A. (2020). Object-displacement-sensitive visual neurons drive freezing in Drosophila. Curr. Biol. *30*, 2532-2550.e8.

186. Mongeau, J.-M., Cheng, K.Y., Aptekar, J., and Frye, M.A. (2019). Visuomotor strategies for object approach and aversion in Drosophila melanogaster. J. Exp. Biol. *222*. Available at: http://dx.doi.org/10.1242/jeb.193730.

187. Semmelhack, J.L., and Wang, J.W. (2009). Select Drosophila glomeruli mediate innate olfactory attraction and aversion. Nature *459*, 218–223.

188. Berens, P. (2009). CircStat: a MATLAB toolbox for circular statistics. J. Stat. Softw. *31*, 1–21.

189. Theobald, J.C., Shoemaker, P.A., Ringach, D.L., and Frye, M.A. (2010). Theta motion processing in fruit flies. Front. Behav. Neurosci. *4*. Available at: http://dx.doi.org/10.3389/fnbeh.2010.00035.

190. Fenk, L.M., Poehlmann, A., and Straw, A.D. (2014). Asymmetric processing of visual motion for simultaneous object and background responses. Curr. Biol. *24*, 2913–2919.

191. Bahl, A., Ammer, G., Schilling, T., and Borst, A. (2013). Object tracking in motion-blind flies. Nat. Neurosci. *16*, 730–738.

192. Wasserman, S., Salomon, A., and Frye, M.A. (2013). Drosophila tracks carbon dioxide in flight. Curr. Biol. *23*, 301–306.

193. Keleş, M.F., Mongeau, J.-M., and Frye, M.A. (2019). Object features and T4/T5 motion detectors modulate the dynamics of bar tracking by Drosophila. J. Exp. Biol. *222*. Available at: http://dx.doi.org/10.1242/jeb.190017.

194. Pavlov, P.I. (2010). Conditioned reflexes: An investigation of the physiological activity of the cerebral cortex. Ann Neurosci *17*, 136–141.

195. Wasserman, S., Lu, P., Aptekar, J.W., and Frye, M.A. (2012). Flies dynamically anti-track, rather than ballistically escape, aversive odor during flight. J. Exp. Biol. *215*, 2833–2840.

196. Grabe, V., and Sachse, S. (2018). Fundamental principles of the olfactory code. Biosystems. *164*, 94–101.

197. Sachse, S., and Beshel, J. (2016). The good, the bad, and the hungry: how the central brain codes odor valence to facilitate food approach in Drosophila. Curr. Opin. Neurobiol. *40*, 53–58.

198. Masse, N.Y., Turner, G.C., and Jefferis, G.S.X.E. (2009). Olfactory information processing in Drosophila. Curr. Biol. *19*, R700-13.

199. Schultzhaus, J.N., Saleem, S., Iftikhar, H., and Carney, G.E. (2017). The role of the Drosophila lateral horn in olfactory information processing and behavioral response. J. Insect Physiol. *98*, 29–37.

200. de Belle, J.S., and Heisenberg, M. (1994). Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. Science *263*, 692–695.

201. Fişek, M., and Wilson, R.I. (2014). Stereotyped connectivity and computations in higher-order olfactory neurons. Nat. Neurosci. *17*, 280–288.

202. Strutz, A., Soelter, J., Baschwitz, A., Farhan, A., Grabe, V., Rybak, J., Knaden, M., Schmuker, M., Hansson, B.S., and Sachse, S. (2014). Decoding odor quality and intensity in the Drosophila brain. Elife *3*, e04147.

203. Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z., *et al.* (2014). Independent optical excitation of distinct neural populations. Nat. Methods *11*, 338–346.

204. Hampel, S., McKellar, C.E., Simpson, J.H., and Seeds, A.M. (2017). Simultaneous activation of parallel sensory pathways promotes a grooming sequence in Drosophila. Elife *6*. Available at: http://dx.doi.org/10.7554/eLife.28804.

205. Creamer, M.S., Mano, O., and Clark, D.A. (2018). Visual Control of Walking Speed in Drosophila. Neuron *100*, 1460-1473.e6.

206. Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., and Vosshall, L.B. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron *43*, 703–714.

207. Nicolaï, L.J.J., Ramaekers, A., Raemaekers, T., Drozdzecki, A., Mauss, A.S., Yan, J., Landgraf, M., Annaert, W., and Hassan, B.A. (2010). Genetically encoded dendritic marker sheds light on neuronal connectivity in Drosophila. Proc. Natl. Acad. Sci. U. S. A. *107*, 20553–20558.

208. Zhang, Y.Q., Rodesch, C.K., and Broadie, K. (2002). Living synaptic vesicle marker: synaptotagmin-GFP. Genesis *34*, 142–145.

209. Ribeiro, I.M.A., Drews, M., Bahl, A., Machacek, C., Borst, A., and Dickson, B.J. (2018). Visual Projection Neurons Mediating Directed Courtship in Drosophila. Cell *174*, 607-621.e18.

210. Bahl, A., Serbe, E., Meier, M., Ammer, G., and Borst, A. (2015). Neural Mechanisms for Drosophila Contrast Vision. Neuron *88*, 1240–1252.

211. Pankova, K., and Borst, A. (2016). RNA-Seq Transcriptome Analysis of Direction-Selective T4/T5 Neurons in Drosophila. PLoS One *11*, e0163986.

212. Mauss, A.S., Meier, M., Serbe, E., and Borst, A. (2014). Optogenetic and pharmacologic dissection of feedforward inhibition in Drosophila motion vision. J. Neurosci. *34*, 2254–2263.

213. Namiki, S., Dickinson, M.H., Wong, A.M., Korff, W., and Card, G.M. (2018). The functional organization of descending sensory-motor pathways in Drosophila. Elife *7*. Available at: http://dx.doi.org/10.7554/eLife.34272.

214. Keleş, M.F., and Frye, M.A. (2017). Object-Detecting Neurons in Drosophila. Curr. Biol. *27*, 680–687.

215. Raghu, S.V., and Borst, A. (2011). Candidate glutamatergic neurons in the visual system of Drosophila. PLoS One *6*, e19472.

216. Claßen, G., and Scholz, H. (2018). Octopamine Shifts the Behavioral Response From Indecision to Approach or Aversion in Drosophila melanogaster. Front. Behav. Neurosci. *12*, 131.

217. O'Sullivan, A., Lindsay, T., Prudnikova, A., Erdi, B., Dickinson, M., and von Philipsborn, A.C. (2018). Multifunctional Wing Motor Control of Song and Flight. Curr. Biol. *28*, 2705-2717.e4.

218. Saxena, N., Natesan, D., and Sane, S.P. (2018). Odor source localization in complex visual environments by fruit flies. J. Exp. Biol. *221*. Available at: http://dx.doi.org/10.1242/jeb.172023.

219. Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., and Budnik, V. (1995). Octopamine immunoreactivity in the fruit fly Drosophila melanogaster. J. Comp. Neurol. *356*, 275–287.

220. Simpson, J.H., and Looger, L.L. (2018). Functional Imaging and Optogenetics in Drosophila. Genetics *208*, 1291–1309.

221. Davis, F.P., Nern, A., Picard, S., Reiser, M.B., Rubin, G.M., Eddy, S.R., and Henry, G.L. (2020). A genetic, genomic, and computational resource for exploring neural circuit function. Elife *9*. Available at: http://dx.doi.org/10.7554/eLife.50901.

222. Kurmangaliyev, Y.Z., Yoo, J., Valdes-Aleman, J., Sanfilippo, P., and Zipursky, S.L. (2020). Transcriptional Programs of Circuit Assembly in the Drosophila Visual System. Neuron. Available at: http://dx.doi.org/10.1016/j.neuron.2020.10.006.

223. Keleş, M.F., Hardcastle, B.J., Städele, C., Xiao, Q., and Frye, M.A. (2020). Inhibitory Interactions and Columnar Inputs to an Object Motion Detector in Drosophila. Cell Rep. *30*, 2115-2124.e5.

224. Städele, C., Keleş, M.F., Mongeau, J.-M., and Frye, M.A. (2020). Non-canonical Receptive Field Properties and Neuromodulation of Feature-Detecting Neurons in Flies. Curr. Biol. *30*, 2508-2519.e6.

225. Sten, T.H., Li, R., Otopalik, A., and Ruta, V. (2020). An arousal-gated visual circuit controls pursuit during Drosophila courtship. 2020.08.31.275883. Available at: https://www.biorxiv.org/content/10.1101/2020.08.31.275883v1?rss=1&utm_source=dlvr.it&utm_medium=twitter [Accessed September 7, 2020].

226. Diao, F., Ironfield, H., Luan, H., Diao, F., Shropshire, W.C., Ewer, J., Marr, E., Potter, C.J., Landgraf, M., and White, B.H. (2015). Plug-and-Play Genetic Access to Drosophila Cell Types using Exchangeable Exon Cassettes. Cell Rep. *10*, 1410–1421.

227. Sampson, M.M., Myers Gschweng, K.M., Hardcastle, B.J., Bonanno, S.L., Sizemore, T.R., Arnold, R.C., Gao, F., Dacks, A.M., Frye, M.A., and Krantz, D.E. (2020). Serotonergic modulation of visual neurons in Drosophila melanogaster. PLoS Genet. *16*, e1009003.

228. Sun, F., Zhou, J., Dai, B., Qian, T., Zeng, J., Li, X., Zhuo, Y., Zhang, Y., Wang, Y., Qian, C., *et al.* (2020). Next-generation GRAB sensors for monitoring dopaminergic activity in vivo. Nat. Methods *17*, 1156–1166.

229. Scheffer, L.K., Xu, C.S., Januszewski, M., Lu, Z., Takemura, S.-Y., Hayworth, K.J., Huang, G.B., Shinomiya, K., Maitlin-Shepard, J., Berg, S., *et al.* (2020). A connectome and analysis of the adult Drosophila central brain. Elife *9*. Available at: http://dx.doi.org/10.7554/eLife.57443.