Octopaminergic neurons have multiple targets in *Drosophila* larval mushroom body calyx and regulate behavioral odor discrimination

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Abstract

Discrimination of sensory signals is essential for an organism to form and retrieve memories of relevance in a given behavioural context . These are modified dynamically by changes in behavioral state, facilitating context-dependent selection of behavior. These are mediated by brain signals carried by noradrenergic input in mammals, or octopamine (OA) in insects. To understand the circuit mechanisms of this signaling, we characterized the function of two OA neurons, in the input region to the memory center, the mushroom body calyx, in larval *Drosophila*. Here they target multiple neurons, including olfactory projection neurons (PNs), the inhibitory neuron APL, a pair of extrinsic output neurons, but relatively few mushroom body intrinsic neurons, Kenyon cells; PN terminals carried the OA receptor Oamb, a *Drosophila* α1-adrenergic receptor ortholog. We developed a behavioral assay to distinguish discrimination from learning ability, and showed that optogenetic activation of OA neurons compromised odor discrimination behavior but not learning ability. Our results suggest that OA neurons modify odor representations via multiple extrinsic inputs at the sensory input area to the olfactory learning circuit.

Introduction

Behavioral choices depend on discrimination among “sensory objects”, which are neural representations of multiple coincident sensory inputs, across a range of sensory modalities. For example, “odor objects” (Gottfried, 2009; Wilson and Sullivan, 2011; Gire et al., 2013) are represented in sparse ensembles of neurons, that are coincidence detectors of multiple parallel inputs from odor quality channels. This principle is used widely in animals, including in mushroom bodies (MBs), the insect center for associative memory (Masuda-Nakagawa et al. 2005; Honegger et al. 2011), and in the piriform cortex (PCx) of mammals (Stettler and Axel, 2009; Davison and Ehlers, 2011).

The selectivity of sensory representations can be modulated dynamically by changes in behavioral state, allowing an animal to learn and respond according to perceptual task. In mammals, the noradrenergic system originating in the locus coeruleus (LC) is implicated in signaling behavioral states such as attention, arousal and expectation (Sara and Bouret, 2012; Aston-Jones and Cohen, 2005). Noradrenergic modulation of the olfactory bulb enhances sensitivity of odor detection and discrimination, dependent on α-1-adrenergic receptors (Escanilla et al., 2010, 2012), and LC activation enhances the excitability of PCx pyramidal neurons (Bouret and Sara, 2002), and LC activation coincides with reward anticipation in appetitive odor discrimination (Bouret and Sara, 2004). However, the circuit and synaptic mechanisms of modulation by NA are not well understood.

In insects, octopamine (OA), chemically and functionally homologous to noradrenalin (NA) in mammals, can mediate changes in behavioral state that often promote activity, for example: sensitization of reflex actions in locust (Sombati and Hoyle, 1984); aggressive state in crickets (Stevenson et al., 2005); initiation and maintenance of flight state (Brembs et al., 2007; Suver et al. 2012); and enhanced excitability of *Drosophila* motion detection neurons during flight (Strother et al. 2018). Another potential role of OA is as a reward signal: although this role is at face value distinct from a role in promoting activity or preparedness, OA appears to encode unconditioned stimuli in honeybee (Hammer, 1993; Hammer and Menzel, 1998; Menzel 2012), cricket (Matsumoto et al. 2015), and *Drosophila* (Schwaertzel et al. 2003) appetitive learning paradigms. However, taken together, the OA system in insects is heavily involved in signaling behavioral states, comparable to NA in mammals.

Behavioral states facilitate the processing of visual sensory input associated to behavioral relevance (Gilbert and Li, 2013). At the circuit level, two models have been proposed, increase in response gain (Treue and Martinez Trujillo, 1999; Reynolds and Chelazzi, 2004), and changes in tuning properties of cortical visual neurons, reflecting sensory driven stimuli and behavioral context (Li et al. 2004). Although behavioral studies are extensive, the circuit and synaptic mechanisms of these signaling inputs are not well understood. We aimed to dissect the role of the OA circuitry in sensory processing. We used the simple sensory "cortex" of larval *Drosophila*, the calyx, which is the sensory input region of the mushroom bodies (MBs), the insect memory center. Here, each MB neuron (Kenyon cell, KC) typically arborizes in several glomeruli, most of which are organized around the terminus of an olfactory projection neuron (PN); KCs thus combinatorially integrate multiple sensory input channels (Masuda-Nakagawa et al., 2005; Eichler et al., 2017). Inhibitory feedback (Lin et al., 2014; Masuda-Nakagawa et al. 2014), helps to maintain KC sparse responses and odor selectivity (Honegger et al., 2011), analogous to inhibition in the mammalian PCx (Poo and Isaacson, 2009; Stettler and Axel, 2009; Gire et al. 2013).

The larval MB calyx is also innervated by two OA neurons, sVUMmd1 and sVUMmx1, ventral unpaired medial neurons with dendritic fields originating in the mandibular and maxillary neuromeres respectively of the subesophageal zone (SEZ) (Selcho et al., 2014). These sVUM1 neurons also innervate the olfactory neuropiles of the antennal lobe (AL) and lateral horn. This pattern of innervation is conserved in other insects, for example by dorsal unpaired median (DUM) neurons in locust (Bräunig, 1991), the VUMmx1 neuron in honeybee (Hammer 1993; Schröter et al. 2007), and OA-VUMa2 neurons in adult *Drosophila* (Busch et al. 2009). It also resembles the NA innervation of mammalian forebrain by LC neurons originating in the brainstem. Therefore, the network organization and coding principles in the larval MB calyx appear widely conserved, but with few neurons and genetically tractable tools, it provides many advantages to understand how individual neurons are integrated to generate a concerted neural activity pattern.

We therefore characterized the innervation pattern and synaptic partners of OA neurons in the calyx, the localization of the OA receptor Oamb in the calyx circuit, and the impact of OA calyx innervation on behavioral odor discrimination. We find that OA neurons innervate all the major classes of calyx neuron to some degree, although only a small fraction of KCs. The OAMB receptor is localized in the terminals of PNs, and activating OA neurons can affect odor discrimination during learning and memory, without affecting underlying olfactory learning and

memory ability. We suggest a broad modulatory effect of OA neurons in the calyx, including a potential role in modulating PN input at the second synapse in the olfactory pathway, that has an impact on odor discrimination in learning.

Results

sVUM1 neurons in the calyx and their polarity.

Two OA neurons innervate throughout the calyx without obvious regional preference. The larval calyx is innervated by two classes of OA neurons, sVUMmd1, and sVUMmx1, originating from the mandibular and maxillary neuromeres, respectively, in the subesophageal zone (SEZ), and labeled by the *Tdc2-GAL4* line (Selcho et al. 2014). To visualize the innervation pattern of both sVUM1 neurons in the calyx together, we used the Multicolor FlpOut technique (Nern et al. 2015). Flies of genotype *pBPhsFlp2::PEST(attP3); HA_V5_FLAG_OLLAS* were crossed to flies of *Tdc2-Gal4*, and single cell clones were generated by heat shock. Each sVUM1 neuron ramified throughout the calyx, and we only ever found a single sVUMmd1 or sVUMmx1 neuron labeled. When both sVUM1 neurons were labeled, they ramified through the calyx in a non-overlapping pattern, without obvious regional preference (Fig. 1).

OA neurons are presynaptic in the calyx. To visualize the polarity of the calyx-innervating sVUM1 neurons, we used *Tdc2-GAL4* to express either plasma membrane, presynaptic or dendritic markers (Fig. 2). The sVUM1 projections visualized by the plasma membrane marker CD4::tdTom showed dense ramification throughout the calyx, with bouton-like enlargements among glomeruli and in the core of the calyx (Fig. 2A). These enlargements contained OA, and were therefore presynaptic boutons; we observed 89 ± 7 (n= 12) OA-positive boutons per calyx. The presynaptic nature of the *Tdc2-GAL4*-expressing calyx boutons was further supported by the localization of the presynaptic markers nSyb::GFP (Fig. 2A) and Syt::GFP (Fig. 2B) in puncta throughout the calyx in these neurons, prominently between glomeruli or in the non-glomerular core of the calyx. The dendritic marker DenMark::mCherry is not detectable in the calyx (Fig. 2B), although it is strongly localized in the SEZ region (Fig. 2C), which may include other Tdc2-GAL4-expressing neurons as well as the sVUM1 neurons.

Identifying calyx OA neuron partners by GRASP

To visualize synaptic contacts between sVUM1 neurons and other calyx neurons, we used *Tdc2-LexA*, along with *GAL4* lines expressing in other calyx neurons, to drive the expression of the GRASP constructs *LexAop-CD4::spGFP11 and UAS-CD4::spGFP1-10* (Fig. 3). We labeled olfactory PNs using *NP225-GAL4* (Tanaka et al., 2004), and KCs using *MB247-GAL4* (Zars et al. 2000). We also tested for GRASP between sVUM1 neurons and two other classes of extrinsic calyx neurons. First, we labeled the larval APL using *NP2361-GAL4* (Masuda-Nakagawa et al., 2014). Second, the “Odd” class of neurons shows dendritic

arborizations throughout the calyx, with presynaptic terminals in the vicinity of the MB lobes (Slater et al., 2015), and appear to be the same as those designated as MBON-a1 and MBON-a2 by Eichler et al (2017); we identified a *GAL4* line, *OK263-GAL4*, which expresses in these neurons.

GRASP fluorescence. We detected GRASP using GFP fluorescence as widely distributed puncta in the calyx, suggestive of specific synaptic connections, between the sVUM1 neurons on the one hand, and PNs, KCs, the Odd, and APL neurons on the other (Fig. 3A). These findings suggest that the sVUM1 neurons may form synapses with all the neuronal classes that innervate throughout the calyx: PNs, KCs, the APL, and Odd neurons.

To test whether GRASP signals represented synaptic contacts of the sVUM1 neurons, we also immunolabeled brains with anti-OA. GRASP signals were identified, using a criterion that each GRASP signal was observed in at least two consecutive confocal sections, and discounting occasional GFP-positive axonal tracts that were negative for OA.

OA termini synapse with PNs. Using *Tdc2-LexA* and *NP225-GAL4* to express the GRASP constructs, we found GRASP signal at 49±3% of 80±4 (n=5) OA-positive boutons. OA-positive GRASP signals (Fig. 3B) were found in the core of the calyx away from glomeruli, in interglomerular spaces, and along the periphery of glomeruli. They are likely contacts between sVUM1 termini and PN axons. Around 13.5% (n=5) of total GRASP signals did not overlap with OA, and we cannot conclude that these are synaptic contacts. However, PNs are commonly postsynaptic to the termini of sVUM1 neurons, on their axonal or presynaptic processes, making axon-axon synapses.

OA termini synapse with KCs in the calyx. Using *Tdc2-LexA* and *MB247-GAL4* to express the GRASP constructs, we found GRASP signals at 51 ± 4 % of 185 ± 34 (n=3) OA boutons (Fig. 3C). GRASP puncta were found in the interglomerular space.

OA termini synapse with Odd neurons in the calyx. Using *Tdc2-LexA* and *OK263-GAL4* to express the GRASP constructs, we found GRASP signals at 51 ± 0.3 % of 123 ± 4.2 (n=3) OA labeled boutons. There were OA spots that were apposed to GFP (Fig. 3D).

OA termini synapse with the APL. Using *Tdc2-LexA* and *NP2361-GAL4* to express the GRASP constructs, we found GRASP signal at 77±4% of 74±12 (n=4) OA terminals, indicating that

sVUM1 neurons are presynaptic to the larval APL (Fig. 3E top panels). Most of these GRASP signals were found between glomeruli, and more abundant towards the ventral calyx.

Around 28.3 ± 1 GFP signals, 32.8 % ± 2.6 (n=3) GRASP signals, did not overlap with OA, and therefore do not represent synapses of the sVUM1 neurons onto the APL. Some of these appear to be tracts that lead to OA positive spots, rather than the characteristic round shape of synaptic GRASP signals, and might therefore be due to proximity of the sVUM1 axons with APL axons. Others could be due to synapses of the APL onto OA neuron axons. GABA labeling of brains of the same genotype, showed some GABA termini in close proximity to GRASP (Fig. 3E, bottom panels), suggesting that some contacts between APL and sVUM1 neurons could potentially be inputs from APL terminals onto the sVUM1 neurons. Labeling of Tdc2-GAL4>mCD8::GFP calyces with anti-GABA also showed some apposition of sVUM1 boutons to GABAergic termini of the APL (Fig. 3F).

Single cell GRASP. While the above GRASP experiments reveal the partners of the sVUM1 neurons in the calyx, they do not reveal whether the sVUMmd1 and sVUMmx1 have different partners. We therefore performed single-cell GRASP to label the contacts of each sVUM1 randomly, using Brp::mCherry as a presynaptic marker to verify whether GRASP signals have a synaptic localization. We distinguished the two sVUM1 neurons by the positions of their cell bodies in the SEZ, using local neuropil landmarks revealed by anti-Dlg labelling (Fig. 4A), and using single cell clones, we could identify sVUMmd1 and sVUMmx1 individually (Fig. 4B).

GRASP signals were detected between the *odd*-expressing neurons and both sVUMmd1 and sVUMmx1 (Fig. 4C), and similarly between the larval APL, and both sVUMmd1 and sVUMmx1 (Fig. 4D). Compared to standard GRASP, single cell GRASP signals were fewer, but clearly present and overlapping with the presynaptic marker Brp::mCherry. (Fig. 4D). Therefore, at least as judged by the APL and Odd neurons, both sVUM1 neurons appeared to have similar targets in the calyx.

Localization of a genomic Oamb::GFP fusion in PN terminals in calyx

To further understand how and where OA might act in the calyx, we investigated the localization of OA receptors in the calyx. *Drosophila* has a number of OA receptor classes defined both by sequence comparisons and pharmacology. Octopamine receptor in mushroom bodies (Oamb, also known as Dmoa1A or CG3856), an ortholog of human α1-adrenergic receptor (Roeder et al., 2003; Bauknecht and Jékely, 2017), is enriched in the

MBs (Han et al., 1998). *Drosophila* also has three OctβR receptors, which stimulate cAMP levels (Maqueira et al., 2005, Balfanz et al., 2005).

To detect the expression and subcellular localization of Oamb, we used recombinase-mediated cassette exchange (RMCE) with a MiMIC insertion (Venken et al., 2011), *MI12417*, in the third coding-region intron of *Oamb*, to tag endogenous *Oamb* with an exonic *EGFP-FlAsH-StrepII-TEV-3xFlag* fusion (Supp Figs. 1-4). Insertion of an EGFP-encoding exon here should tag all known splice variants of the Oamb protein in their third cytoplasmic loop, downstream of transmembrane (TM) domain 5 (Supp. Fig. 5,6); this includes the alternative TM6-TM7 regions encoded by two alternative groups of C-terminal exons (Supp Figs 4-6). Therefore, a protein trap generated from the *MI12417* insertion will not disrupt any transmembrane domains.

Six recombinant *Oamb::EGFP* stocks were recovered with the EGFP-encoding exon inserted in the same orientation as the *Oamb* transcript (Supp Fig. 7). One of these was designated as *Mi{PT-GFSTF.1}Oamb*$^{MI12417\text{-}GFSTF.1}$, or *Oamb(MI12417)::EGFP.1* or *Oamb::EGFP* for short. Both the original *MI12417 MiMIC* insertion, and *Oamb(MI12417)::EGFP* stocks were homozygous infertile, as expected from the egg-laying defects of *Oamb* mutants (Deady and Sun, 2015), suggesting that the Oamb::EGFP fusion might not be a functional Oamb protein. However, Oamb::EGFP was localized to glomeruli in the larval calyx (Fig. 5), implying that the protein folded normally and was not degraded by the ER unfolded protein response. Expression of *UAS-RFP* in the olfactory PN line *NP225-GAL4* showed localization of Oamb::EGFP in all PN termini labeled with the GAL4 line, as well as in some calyx glomeruli not labeled by *NP225-GAL4*, which may be either sites of non-olfactory sensory input, or olfactory glomeruli not labeled by *NP225-GAL4* (Fig. 5A). The restriction of Oamb::EGFP to specific glomeruli implies that it is unlikely to be expressed in KC dendrites in the calyx, which arborize through all glomeruli. We also found no overlap of Oamb::EGFP with GABAergic APL terminals in the calyx (Fig. 5B), implying that it was not expressed in the larval APL. *OK263-GAL4* calyx projections also showed little or no overlap with Oamb::EGFP (Fig. 5C), suggesting that Oamb is not expressed in the Odd neuron calyx dendrites.

We found no detectable localization of GFP-tagged DmOctβR receptors to the calyx (data not shown). Octβ1R::EGFP (*CG6919*) was expressed weakly in a few ventral and medial AL glomeruli, but was not detectable in the calyx. Octβ2R::EGFP (*CG6989*), was not detectable in either the calyx or AL, although it was expressed in a number of adjacent cell bodies that did

not colocalize with PNs as labeled by *NP225-GAL4* driving RFP expression. We could not detect any expression of Octβ3R::EGFP.

Activating an OA neuron subset including sVUM1 neurons impairs behavioral odor discrimination

Since the calyx is a site where MB neurons integrate sensory information that comprises conditioned stimuli (CS) in associative learning, we reasoned that modulating the processing of this information might affect the ability of the brain to discriminate among CSs while learning, but without affecting its underlying learning ability. Therefore, to test whether OA innervation of the calyx affected odor discrimination during learning, we developed an assay that could distinguish odor discrimination assay from learning ability (Fig. 6). The rationale of this assay was developed in the honeybee by Stopfer et al. (1997); molecularly similar odors are more difficult to discriminate than molecularly dissimilar odors, giving a measure of discriminability. In this case similar odor pairs are generated by varying the proportions of two dissimilar odors in two different odor mixes. By combining odor choice with an appetitive learning paradigm (Scherer et al. 2003), we tested the effect on behavioral odor discrimination of optogenetic activation of OA neurons in third instar larvae, using the long-wavelength absorbing channelrhodopsin, CsChrimson (Klapoetke et al., 2013).

Since we did not have *GAL4* or *LexA* drivers completely specific for sVUM1 neurons, we used an intersectional approach to restrict the expression of CsChrimson to a small subset of OA neurons including the sVUM1 neurons. We could use *LexAop-FLP* and a *GAL80* cassette flanked by two *FRT* sites to express *UAS-CsChrimson* only in neurons that expressed both *GMR34A11-GAL4* (which labels some VUM neurons in addition to non-OA neurons), and *Tdc2-LexA*. We thus expressed CsChrimson in only five OA neurons in the SEZ of the larval brain: two (1.8 ± 0.39 (n=12)) in the mandibular neuromere (including sVUMmd1), two (2 ± 0.43 (n=12)) in the maxillary neuromere (including sVUMmx1), and one (1 ± 0 (n=12)) in the labial neuromere (Fig. 8). The second neuron labeled in each neuromere could be sVUM2 or sVUM3, however, sVUM1 neurons are the only neurons of this subset to innervate the AL and calyces.

Activation of these OA neurons, during conditioning but not during testing, had no effect on the ability of larvae to discriminate odors in an appetitive learning assay using a dissimilar odor pair, but abolished their ability to discriminate a similar odor pair, implying that odor discrimination is affected by activation of these neurons, but not underlying learning ability (Fig. 7).

Discussion

OA neurons target extrinsic neurons within the calyx circuitry

Two OA neurons originating in the SEZ, sVUMmd1 and sVUM mx1, innervate the same brain neuropiles, with postsynaptic processes in the SEZ and presynaptic processes in the antennal lobe and MB calyces (Fig. 2). Using GRASP, we found contacts of sVUM1 presynaptic terminals with KCs, PNs, Odd and APL neurons in the calyx, suggesting that sVUM1 neurons can potentially regulate these neurons. However, most KCs cannot be directly affected by sVUM1 innervation: the tens of contacts between the sVUM1 neurons and KCs is less than the 300-plus KCs present in third instar larvae (Pauls et al., 2010), and Eichler et al. (2017) also find inputs of either sVUM1 neuron (which they call OAN-a1 and OAN-a2) into fewer than 10-15% of KCs in first instar larvae. Therefore, context-dependent signaling by OA in the calyx must principally affect MB activity via its other input neurons, not by direct action on KC dendrites.

Connectomic analysis of a six-hour first-instar larva (Eichler et al. 2017) shows that the sVUM1 neurons, with only 36 active zones in a left brain, and 48 in a right brain, have a qualitatively similar but less extensive calyx innervation pattern than we observe in third instar, with 89 ± 7 [n=12] OA-positive boutons per calyx, and even more active zones, assuming multiple active zones per bouton. Consistent with our findings, Eichler et al (2017) also report substantial presynaptic contacts of sVUM1 neurons with Odd neuron dendrites, and some synapses of sVUM1 neurons with KCs, but not with most KCs; they do not comment on synapses with PNs or the APL.

Roles of APL and Odd neurons in calyx activity

sVUM1 presynaptic termini make many contacts with both the APL and Odd neurons (Fig. 3, 4). Odd neurons ramify throughout the calyx and receive input from PNs generally, thus forming a channel for non-selective odor processing that is parallel to the main MB odor-specific processing through KCs. A similar division of processing is seen in the visual pathway downstream of area V1, the primary sensory area in the cortex, with the segregation of the “what” pathway, for object recognition, from the dorsal “where” pathway that is involved in motion detection, visually guided movements and attentional control (Livingston and Hubel, 1988, Gilbert, 2013). sVUM1 neurons have extensive contacts with Odd neurons and could potentially change their gain or tuning properties, to signal changes in behavioural state that

guide odor-driven choice behaviours. In fact Odd neurons have been implicated in chemotactic behaviour (Slater et al., 2015).

The APL mediates a negative feedback loop from KC outputs in the MB lobes to KC inputs in the calyx, thus potentially both limiting the duration of KC activity and improving their odor discrimination (Masuda-Nakagawa et al., 2014; Lin et al., 2014). sVUM1 synapses onto the APL in the calyx could therefore potentially regulate this feedback loop. This could increase signal-to-noise ratio, in a context-dependent manner, by sharpening odor representations in the calyx via APL inhibitory feedback, similar to the “gain control” mechanism with enhancement of behaviorally relevant responses and suppression of non-relevant ones, in monkey visual system, (Treue and Martinez Trujillo, 1999, Gilbert and Li, 2013). Similarly, songbirds possess a similar mechanism, with a gate open to the unique birdsong only during vocalization, in a behavioral state-dependent manner (Schmidt and Konishi, 1998). In addition, since we observed some GRASP signals adjacent to GABA termini (Fig. 3E), APL feedback could also inhibit OA release from sVUM1 termini, further increasing the complexity of interactions between OA innervation and the KC/APL negative feedback loop. NA regulation of inhibitory neurons is also a feature of the mammalian olfactory circuitry. In olfactory bulb, disinhibition of mitral cell (equivalent to PN) activity by NA regulation of inhibitory granule cells has been proposed (Nai et al. 2009). In mammalian PCx, feedforward and feedback inhibition are postulated to enhance cortical representation of strong inputs (Stokes and Isaacson, 2010), and the PCx receives extensive NA innervation from the LC, although its role in modulating inhibition has not been investigated.

PNs as potential targets of OA modulation

GRASP analyses suggest that PNs are postsynaptic to the sVUM1 neurons (Fig. 3). Indeed, an Oamb::eGFP exon-trap fusion is localized on PN presynaptic terminals, albeit more widely than GRASP puncta (Fig. 5). Similarly, the honeybee Oamb ortholog AmOA1 is found widely in the calyx (Sinakevitch et al., 2011), although these authors do not distinguish between PN terminals or KC dendrites. Much aminergic neurotransmission acts via extrasynaptic receptors (Bentley et al., 2016) and this may be the case for Oamb in PN terminals, Oamb is a GPCR that signals apparently through Gq, to release Ca^{2+} from intracellular stores (Balfanz et al., 2005; Morita et al, 2006); it may also elevate cAMP (Han et al., 1998), although this effect appears smaller (Balfanz et al., 2005). The mammalian counterpart, NA, is also found in the mammalian olfactory bulb: here, LC input improves the detection of peri-threshold stimuli (Jiang et al., 1996), and facilitates the detection of near threshold odors, when rewarded

(Escanilla et al., 2012), via an increase in mitral cell excitability mediated by NA action on α1-adrenergic receptor (Ciombor et al. 1999; Hayar et al. 2001). Therefore, we suggest that expression of OAMB receptors in terminals of PNs in the calyx, could potentially change the excitability of input PNs.

Odor discrimination learning

Here we observed that optogenetic activation of 5 neurons including the VUM1s, the discrimination of similar odors was compromised in an appetitive learning paradigm. One explanation is that VUM1 activation enhanced the gain of stimulus-driven PNs, increasing the magnitude and number of KCs responding, and therefore representations by KCs of CS+ and CS- increased in overlap, and lower discriminability of similar odors. On the other hand, another possibility is that the detection of odors was compromised, by a result of inhibition of stimulus induced activity, this could be an effect of VUM1s on the GABAergic APL.

A role for OA as a reinforcer in appetitive associative learning has been shown in honeybees and flies. The honeybee VUMmx1 neuron has properties of a reinforcer; its depolarization could replace sugar reinforcement in appetitive learning (Hammer, 1993), and injection of OA into the MB calyx induced memory consolidation (Hammer and Menzel, 1998). Furthermore, increased density of calyx microglomeruli is observed in long term memory (Hourcade et al. 2010) and KC claws showed increased responses after appetitive conditioning (Szyszka et al. 2008). It has been proposed that VUMmx1 learns about the value of the odor, and acts as a prediction error signal in appetitive learning (Menzel, 2012). However, associative plasticity in the MBs in *Drosophila*, is thought to reside mainly in the lobes rather than the calyx, for both appetitive (Schwaertzel et al. 2003; Schroll et al. 2006; Liu et al. 2012) and aversive learning (Aso et al., 2012, 2014). OA as a reinforcer in appetitive learning appears to act via Oamb expressed in PAM dopamine neurons that target the MB lobes, and a set of OA neurons that includes OA-VUMa2, the equivalent of larval VUMmx1, did not induce and is not required for appetitive learning in adult *Drosophila* (Burke et al. 2012). Furthermore, Oamb is required in KCs for adult appetitive learning (Kim et al., 2013), suggesting some direct input of an OA-encoded appetitive signal into KCs. Taken together, OA action as a reinforcer in associative learning might occur via unidentified inputs into dopaminergic neurons or KC lobes – a separate role from gating selected sensory information, which is favored by our behavioral data, and is also suggested as a role of NA (Berridge and Waterhouse, 2003).

Perspectives

Sensory representations are dynamically modified by higher brain signaling, according to behavioral state such as attention, expectation, and behavioral task; and LC (locus coeruleus) activation in mammals and OA activation in insects, correlate with changes in behavioral states. Mammalian olfactory neuropiles are densely innervated by noradrenergic input, similar to the dense octopaminergic sVUM1 neurons innervation of AL and calyx in insects. Although behavioral data is abundant, understanding of the circuit and synaptic mechanisms of NA/OA requires the identification of the synapses and receptors that they target. The innervation of sVUM1 neurons throughout the calyx, and their potential synaptic connections to PNs, KCs, APL, and odd-like neurons, mean that OA could induce a network level switch either by gating input afferent activity, and/or by interacting with the KC/APL feedback loop, and thus also affecting the output activity from the calyx – not only through KCs but also potentially via the Odd neurons. Behavioral demands would determine the balance between sensitivity and discrimination via OA; whether to escape from a predator at all cost, or the need to recognize closely similar smells associated to food or danger. NA/OA are teaching signals downstream higher order brain commands that compute cognitive processes, future work is necessary to understand the circuits upstream NA/OA and how they regulate the dynamics of bottom-up sensory circuits.

Materials and Methods:

Genetics and molecular biology.

Fly Stocks. Flies were raised on standard cornmeal medium at 25°C and subjected to a 12 hour day/night cycle. Stocks used are listed in Table 1.

MultiColor FlpOut. MultiColor FlpOut was performed according to Nern et al. (2015). Females of genotype *pBPhsFlp2::PEST(attP3); +; HA_V5_FLAG_OLLAS* ("MCFO-2") were crossed with male *Tdc2-Gal4* flies. Parents were left in vials to lay eggs for 24-hour intervals. After another 24 hours, larval progeny were heat shocked at 35-37°C by immersion in a heated circulated waterbath for 15-30 minutes, thus ensuring larvae were aged 24-48 hours after egg-laying (AEL) at the time of heat shock.

GRASP. Standard GRASP was according to Gordon and Scott (2009). Reconstituted GFP was detected using rat monoclonal anti-GFP. This did not detect either of the GRASP components GFP1-10 or GFP11, when *Gal4* or *LexA* drivers were used alone. GRASP signals had to meet a criterion of occurring in two consecutive 0.5-µm confocal sections. For single cell GRASP (Karuppudurai et al., 2014), we generated larvae carrying *P{hsFLP}*12, appropriate *GAL4* and *LexA* combinations, and a recombinant chromosome with insertions *LexAOp2-IVS>stop>spGFP11::CD4::HA-T2A-Brp::mCherry (attP2), UAS-spGFP1-10::CD4,* and *UAS- HRP::CD2.* To generate labelled single cells, parents were allowed to lay eggs initially for 24-hour intervals, then for 6-hour intervals in vials containing half the amount of food. At 0-24h, 24-48h, or later at 12-18 h, 18-24 h, or 24-30 h AEL, progeny were heat shocked as above for 10-50 minutes at 37 °C. Progeny were incubated from RT until dissection of non-tubby wandering third instar larvae.

Generation of an EGFP-tagged Oamb line. The *Mi{MIC}Oamb*^{MI12417} insertion in coding intron 3 of *Oamb* at 3R:20697059, (BDSC 57940; henceforth referred to as *MI12417*) was verified by PCR using primers *MI12417-5F/MiMIC-5R1* for the 5' end and *MI12417-3R/MiMIC-3F1* for the 3' end (Table 2; Supplementary Fig. 1). Sequencing of these PCR products and alignment with the *Drosophila* genome sequence using BLASTN (Altschul et al., 1990; https://blast.ncbi.nlm.nih.gov/) showed insertion of *MiMIC* at the recorded site of 3R 20697058-9 (Supplementary Figs. 2,3). The location of the *MI12417* insertion site relative to *Oamb* coding exons was determined by using Oamb-B sequences for BLASTN and TBLASTN queries of the *Drosophila* genome assembly (http://flybase.org/blast/;

Supplementary Fig. 4). TMHMM (Sonnhammer et al., 1998; http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the amino-acid coordinates of Oamb transmembrane (TM) domains (Supplementary Figs. 5,6).

To insert an EGFP-encoding exon into the *MI12417* insertion by RMCE, we chose the splice phase-1 version of *EGFP-FlAsH-StrepII-TEV-3xFlag* plasmid (DGRC 1306; Venken et al., 2011) as recommended by the Baylor Gene Disruption Project (http://flypush.imgen.bcm.tmc.edu/pscreen/rmce/rmce.php?entry=RM00888). This was co-injected with a helper phiC31-integrase plasmid (Venken et al., 2011) by the *Drosophila* microinjection facility (Department of Genetics, University of Cambridge). Injected embryos were left to hatch into adult flies and crossed to a *y w* double balancer stock. RMCE events were identified by loss of the *MiMIC yellow*$^{+}$ marker in F1 progeny. Four PCR reactions were carried out to determine the orientation of the EGFP cassette in each recombinant *Oamb::EGFP* stock (Table 2) as described in Venken et al. (2011).

***Octβ1R* EGFP fusions.** For *Octβ1R* and *Octβ3R*, we used lines *Octβ1R*$^{MI05807\text{-}GFSTF.2}$ and *Octβ3R*$^{MI06217\text{-}GFSTF.1}$ (Bloomington stocks 60236 and 60245), respectively, as *Octβ1R::EGFP* and *Octβ3::EGFP* exon traps. For *Octβ2R*, we used *Mi{MIC}Octβ2R*^{MI13416} to generate *Octβ2R*$^{MI13416\text{-}GFSTF.2}$ using a similar approach as that described above for Oamb::EGFP. We also generated a second *Octβ3::EGFP* exon trap, *Octβ3R*$^{MI06217\text{-}GFSTF.0}$, in a different reading frame from Bloomington stock 60245 , to allow for the possibility of the *EGFP* exon being spliced as in transcripts *RJ* or *RK* (frame 0, with only *RJ* able to encode all seven TM domains), rather than *RF* or *RG* (frame 1). Positions of each insertion were confirmed by PCR and sequencing similarly to Oamb, using primers as described in Table 2.

Molecular methods. Genomic DNA was extracted from 15-30 flies (1-7 days after eclosion) and homogenized in 100 mM Tris-HCl, 8.5; 80 mM NaCl, (Sigma 31434); 5% Sucrose (Sigma S0389); 0.5% SDS (Sigma L4509); 50 mM Na-EDTA (Sigma ED2SS), pH 8.0. The homogenate was incubated with RNase A (Roche 10109142001) for 1 hour at 37°C followed by Proteinase K (Roche 03115887001) for 1 hour at 50°C, and purified with phenol-chloroform (Sigma 77617) and chloroform (Sigma C2432). DNA was precipitated with 0.6 volumes of isopropanol (Sigma, 59304) and washed with 75% ethanol (Sigma E7023), dried overnight at room temperature and re-suspended in 10 mM Tris-HCl pH 8.0 (Sigma T6066).

PCR reactions (20 µl) contained 0.4 µl or 1 µl genomic DNA, 1 µl of each 10 µM primer (Sigma), 2µl of 10X PCR buffer (Qiagen 203203), 0.4 µl of 10 µM dNTP mix (Roche 11581295001), 0.08 µl of 5 U/µl HotStarTaq DNA polymerase (Qiagen 203203) and 15.1 µl or 14.5 µl milliQ water. PCR cycling in a G-Storm Thermal Cycler (GS4) was: 15 minutes at 95°C; 40 cycles of: denaturation at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 1 min; and a final elongation step at 72°C for 10 minutes. PCR products were loaded with 6X DNA gel-loading dye (ThermoFisher R0611) on a 1% Agarose Gel (LifeTech 16500500; 1X TBE buffer, LifeTech 16500500) with GelRed (Biotium 41003-T) for gel electrophoresis. 100 bp DNA ladder was used as a marker (LifeTech 15628019). PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, 28104), and sequenced at the Department of Biochemistry Sequencing Facility (University of Cambridge).

Immunohistochemistry and Confocal imaging.

Third instar wandering larval brains (144-176 hours AEL) were dissected in cold PBS (Sigma P4417), fixed in 4% Formaldehyde (Polysciences 18814) / PEM buffer (0.1 M PIPES, Sigma P1851; 2 mM EGTA, Sigma E3889; 1 mM $MgSO_4$; NaOH) for 2 hours at 4°C, washed for 3x10 minutes (or 4x15 minutes) in 0.3% Triton-X (Sigma T8787) in PBS (PBT) and incubated in 10% NGS (Normal goat serum; Vector Labs S-1000) in 0.3% PBT for 1 hour at room temperature. Brains were incubated in primary antibody in 10% NGS-0.3% PBT at 4°C for 2-3 days on a mini disk rotor (Biocraft, BC-710), washed for 3x15 minutes with 0.3% PBT and further incubated in secondary antibody in 10% NGS at 4°C for 2-3 days again on the mini disk rotor. Brains were finally washed 1x15 minutes with PBT, followed by 3x10 minutes with PBS, and left in 50% Glycerol/PBS at 4°C for at least one overnight prior to imaging.

Primary and secondary antibodies are listed in Table 3. Brains were incubated in primary antibody at 4°C for 2-3 nights, washed three times in PBT for 15-minutes, and incubated in secondary antibody for 2-3 more nights. To reduce background with the polyclonal chicken anti-GFP (Abcam, Ab13970), it was pre-incubated with *MI12417* larval brains which do not express GFP. Fifty *MI12417* larval brains were incubated in 1:20 chicken anti-GFP in 10% NGS in 0.3% PBT at 4°C for overnight. A further 50 *MI12417* larval brains were added and further incubated at 4°C over 2 nights. Mounting and orientation of brains for image acquisition was as described in supplemental information in Masuda-Nakagawa et al. (2009). Imaging was carried out using a Zeiss LSM710 Confocal Microscope with a 40X NA1.3 oil objective. Images were processed using ImageJ software (https://imagej.nih.gov/ij/download.html).

Behavioral assay.

Larval culture. Males of genotype *w; Tdc2-LexA; GMR34A11-GAL4/TM6B* were crossed to females of genotype *Tub84B(FRT-GAL80)1, w; LexAop-FLP; UAS-Chrimson::mVenus/TM6B* to generate F1 larvae in which *UAS-Chrimson::mVenus* could be expressed only in cells expressing both *Tdc2-LexA* and *GMR34A11-GAL4*, in which LexA-dependent *FLP* expression had removed the GAL4 inhibitor GAL80. Larvae were allowed to develop in food vials containing 100 µM all-trans-retinal (Sigma, R2500), in the dark at 21 degrees. For both the retinal and non-retinal food vials, transfer of adults into new vials happens both in the morning and in the evening, to then collect them after 108-120 hours in the case of non-retinal vials at 25°C, and after 132-144 hours for those kept in retinal vials at 23°C.

Behavioral arena. Agarose petri dishes were prepared the day before use, by using 100 ml of distilled water with 0.9% agarose (Sigma A9539). Fructose petri dishes were prepared similarly, but containing 35% fructose (Sigma-47740). Petri dishes had perforated lids, to facilitate creation of odorant gradients within the dish, by sucking air from a benchtop fume extractor (Sentry Air Systems (SAS), SS-200-WSL) at the back of the assay platform. Odorants were diluted in 10-µl volumes of paraffin oil (Sigma-Aldrich 76235), held in custom-built Teflon containers with pierced lids (7 holes), on the surface of agarose plates.

Light apparatus. The light apparatus contained a BK Precision pulse generator attached to a DC power pack, driving amber light LEDs (591 nm), constructed as described by deVries and Clandinin (2013), by the Psychology Department workshop of the University of Cambridge. One cycle of pulses consisted of 10-ms pulses at 10Hz for 30s, followed by 30s without pulses. This cycle was repeated 5 times, making a conditioning step of 5 minutes. This pulse frequency and width were chosen to replicate the activity of the only recorded sVUM1 neuron, the honeybee sVUMmx1, when activated by a sucrose reward (Hammer et al., 1993).

Behavior conditioning. Third-instar larvae were collected from vials using a metal sieve, and washed with tap water. Larvae were washed twice by transferring through a drop of water, and then placed on the conditioning agarose plate (35% fructose). A Teflon container with 10 µl of odor A to be reinforced was placed on the left side, and another containing paraffin oil (neutral) symmetrically on the right side, at positions marked on a black background, used to facilitate visualization of the larvae (Fig. 7). Larvae were conditioned on the fructose plate with odor A for 5 minutes under weak blue light. Larvae were then transferred to a water droplet using a brush, and again to a second water droplet to ensure no fructose traces remained, and then to

an agarose plate lacking fructose, on which a Teflon container with 10 µl of odor B (non-reinforced) was placed on the left side and a container with paraffin oil (neutral) on the right side. Larvae were conditioned for 5 minutes under weak blue light as above. This conditioning procedure on both plates was repeated for three cycles. For experiments using activation of OA neurons, the entire conditioning cycles were carried out under amber light (Fig. 7).

Odor dilutions. To avoid bias based on innate preferences of odors, odor concentrations were balanced to achieve a response index of around zero. Dilutions of ethyl acetate (EA) at 1:2000 and pentyl acetate (PA) at 1:500 were used. The dissimilar odor pair was ethyl acetate (EA) (Sigma-Aldrich, cat no. 319902) diluted 1:2000, and pentyl acetate (PA) (Sigma-Aldrich, cat no. 109584) diluted 1:500. The similar odor pair was mixtures of EA and PA at dilutions of 1:2000, and 1:500, respectively, at proportions of 1:4 EA:PA, and 4:1 of EA:PA.

Testing. Larvae were tested by placing them on an agarose plate carrying a container with odor A on one side, and a container with odor B on the other. Test was under blue light for 5 minutes. Larvae were counted on the side of odor A (conditioned), odor B (unconditioned) or in the neutral zone in the middle. In some experiments, larvae were transferred during the above procedures under white fluorescent ceiling lighting rather than weak blue light, but we detected no difference in learning scores between these conditions using ANOVA, as described in the Results section. Performance Index (PI) was calculated as:

$$PI = (N_{conditioned} - N_{unconditioned}) / N_{total}$$

$N_{conditioned}$: larvae on the side of the conditioned odor.

$N_{unconditioned}$: larvae on the side of the unconditioned odor.

N_{Total}: larvae on the side of conditioned odor, unconditioned odor, and middle zone.

Learning Index (LI) was calculated after both a conditioning with odor A and a reciprocal conditioning with odor B (with a different sample of larvae), using the formula:

$$LI = (PI_A - PI_B)/2$$

Statistical analysis. Planned comparisons were performed using t-tests. A four-factor ANOVA using SPSS software was used to test whether learning index was affected by food type (retinal vs. non-retinal), light (blue vs. amber), odor pairs (similar pair vs. dissimilar pair), or experimenter (either of two).

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Figure Legends

Figure 1. MutiColor FlpOut of *Tdc2-GAL4*-expressing neurons labels two calyx-innervating neurons. Single cell clones in larval progeny of *Tdc2-GAL4* crossed to *pBPhsFlp2::PEST(attP3); HA_V5_FLAG_OLLAS,* were generated by heat shock induction of FLP. **A.** 3D stereo projection of an sVUM1Md neuron (magenta, V5 tag) and an sVUM1Mx neuron (green, HA tag), both ramifying throughout the calyx. Arrowheads show the axons of the two neurons. Scale bar, 10 µm. **B**. Cell bodies in the SEZ of the same larva. In the mandibular (top) neuromere, two cell bodies labeled in magenta can be identified labeled by antibody to V5 (arrowheads). One of these is the sVUM1md neuron. In the maxillary (bottom) neuromere, a single cell body is only green, identified by anti-HA (arrowhead). Scale bar, 15 µm.

Figure 2. Polarity of calyx innervation by *Tdc2-GAL4*-expressing neurons. A. 3D stereo projection of a 3rd instar calyx expressing nSyb::GFP and CD4::Tom using *Tdc2-GAL4*. Two axonal tracts (arrowheads) enter the calyx and ramify throughout it (CD4::tdTom), while processes show nSyb::GFP in boutons (green). Note boutons at the borders of glomeruli (e.g. arrow) and core of the calyx (e.g. asterisk). **B.** Confocal section of a larval calyx expressing DenMark::mCherry and Syt::GFP using *Tdc2-GAL4*. **C.** Confocal section of a larval brain expressing DenMark::mCherry and Syt::GFP using *Tdc2-GAL4*. The primary processes of *Tdc2-GAL4*-expressing neurons in md (top arrowhead) and mx (bottom arrowhead) neuromeres are shown. **A**, **B**: Anterior is to the bottom, medial to the right. Right brain orientation is shown. Scale bars, 10 µm. **C**: Ventral view, anterior is to top. Scale bar, 50 µm.

Figure 3. GRASP shows calyx contacts of sVUM1 termini with PNs, larval APL, and *odd*-like neurons, and few KCs. A. GRASP signal between sVUM1 neurons (expressing *Tdc2-LexA*) and other calyx neurons expressing the *GAL4* lines shown, driving expression of GRASP constructs *UAS-CD4::spGFP1-10* and *LexAop-CD4::spGFP11*, is detected as native GFP fluorescence in confocal sections. Scale bar, 10 µm.

B-E. GRASP signal between sVUM1 neurons and other calyx neurons, from GRASP constructs driven by *Tdc2-LexA* and the *GAL4* lines shown, detected by anti-GFP. Examples of GRASP signal are highlighted with arrowheads; areas inside broken square are shown at higher zoom. sVUM1 termini are labeled using anti-OA, calyx glomeruli using anti-Dlg. Scale bars in main panels 10 µm, in insets 1 µm, here and throughout figure.

B. GRASP signal between sVUM1 termini and PNs, localized mainly around glomeruli. OA signals without GRASP are localized to the calyx core. Examples of GRASP signal are highlighted with arrowheads. GRASP signal overlaps partially with sVUM1 termini labelled with OA (inset). Areas inside broken lines, here and subsequently, are shown at higher zoom.

C-E. GRASP signals between sVUM1 termini and KCs (**C**), Odd neurons (**D**), or the APL neuron (**E**). **E.** GRASP signals between sVUM1 and APL neurons are localized around glomeruli, or in the core of the calyx. Upper panels show extent of GRASP overlap with OA-containing sVUM1 terminals; Lower panels show some GRASP signals overlapping partially with large GABA boutons.

F. Confocal sections of a 3rd instar larval calyx expressing *mCD8::GFP* using *Tdc2-Gal4* and labeled with anti-GABA and anti-Dlg. Top is dorsal section of calyx, bottom is a ventral section of the same calyx. Arrowheads indicate GABA boutons apposed to or overlapping with *Tdc2* neuron terminals. Arrows indicate the two axonal tracts of *Tdc2* neurons innervating the calyx.

Figure 4. Single cell GRASP of sVUM1 neurons in the calyx. A. Identification of sVUM1 neurons based on SEZ anatomical landmarks. Larval brains carrying *Tdc2-LexA*, *GMR34A11-GAL4*, *LexAop-FLP*, *tub>GAL80>* and *UAS-Chrimson::Venus*, showing Venus expression in a subset of the cells expressing both *Tdc2-LexA* and *GMR34A11-GAL4*. sVUM1md neurons (Md) are in a small anterior medial Dlg-negative gap, with characteristic bilaterally symmetrical gaps (asterisks) nearby. sVUM1mx neurons (Mx) are localized in a large medial gap that lies just anterior to a commissural DLG pathway (arrowheads). sVUMlb neurons (Lb) are localized posteriorly to this commissural pathway. Scale bar 24 µm.

B-D. Single-cell GRASP was induced between sVUM1 neurons expressing *Tdc2-LexA*, and potential calyx partner neurons that expressed different *GAL4* lines. sVUM1 neurons were individually labeled by heat-shock-induced FlipOut of larvae carrying *P{hsFLP}12, Tdc2-LexA, GAL4*, *LexAOp2-IVS>stop>spGFP11::CD4::HA-T2A-Brp::mCherry (attP2), UAS-spGFP1-10::CD4, UAS- HRP::CD2*.

B. Single cell clone generation showing expression of Brp::mCherry in subsets of *Tdc2-LexA*-expressing neurons; each row shows a separate clone. Left image pairs are stereo views of reconstructions showing the entire trajectory of labelled neurons; MB calyx (Ca), pedunculus (Pe), and medial and vertical lobes (ML, VL) are labeled in the top row. Right images are single confocal sections through the SEZ of the same larvae, showing the primary process of each labelled sVUM1 (arrows) and the anatomical landmarks shown in **A**. First row, sVUMmd1 and sVUMmx1. Second row, sVUMmd1. Third row, sVUMmx1. Scale bar is 45 µm in 3D images (Left), 30 µm in SEZ sections (Right).

C-D. Single-cell GRASP between sVUM1 neurons expressing *Tdc2-LexA*, and (**C**) Odd neurons expressing *OK263-GAL4*, or (**D**) larval APL expressing *NP2631-GAL4*. Left panels: larval calyx labelled with anti-DsRed to visualize Brp::mCherry (enriched at presynaptic sites but not confined to them), anti-Dlg to label neuropile, and anti-GFP to visualize GRASP signals. Arrowheads indicate GRASP signals. Right panel: region of same brain labelled with anti-Dlg to visualize neuropile, and anti-DsRed to visualize the VUM neurons. Top rows show sVUMmd1 (Md), bottom rows show sVUMmx1 (Mx). Arrows indicate the primary process of each sVUM1 neuron. Scale bars: 10 µm.

Figure 5. Oamb-GFP localization in PN presynaptic termini. **A.** Calyces of a larva carrying *Oamb::EFGP*, *NP225-GAL4* and *UAS-RFP* labelled with antibodies to GFP, DsRed and Dlg. Oamb::EGFP localizes to PN terminals in all glomeruli labeled by *NP225-GAL4*, and to a few additional glomeruli innervated by PNs, not labeled by *NP225-GAL4* (arrowheads). A few glomeruli show neither *NP225-GAL4* nor Oamb::EFGP expression (asterisks). **B.** Oamb::EGFP does not overlap with APL termini labeled by anti-GABA (top row, example at arrowhead) or with APL projections labeled using *NP0732-GAL4* (bottom row). **C.** Oamb::EGFP does not overlap substantially with Odd neuron dendrites labeled by *OK263-GAL4*. Scale bar, 10 µm.

Figure 6. Behavioral discrimination assay and optogenetics. Larvae are conditioned for three cycles of: a container with one odor (in this case ethyl acetate, EA) and a container with mineral oil (C) on an agarose sucrose plate (red), followed by a container with a different odor (in this case pentyl acetate, PA) and a container with mineral oil, on an agarose plate (light gray). Larvae are then tested by being placed on an oblong area in the middle of an agarose plate, with a choice of EA or PA, and a black background to provide contrast for counting larvae. A different group of larvae are conditioned and tested with reciprocal odor pairing. Control experiments (top row) are carried out in dim blue light. Optogenetic activation of CsChrimson is accomplished by amber light during the conditioning phases.

Figure 7. Activation of a small subset of OA neurons including the sVUM1 neurons disrupts odor discrimination but not learning. A. A pair of stereo images showing CsChrimson::mVenus expressed in a small subset of OA neurons, using the genotype shown in Fig. 4A. The mandibular, maxillary and labial (Md, Mx, Lb) clusters are shown, which include the sVUMmd1 and sVUMmx1 neurons, and the calyx innervation (Ca). Scale bar, 40 µm. **B.** Activation of the neurons labelled in A by CsChrimson occurs in the presence of retinal and

amber light, and abolishes odor choice learning using similar odor pairs, compared to controls exposed to blue light, lacking retinal, or tested with dissimilar odor pairs. No effect of CsChrimson activation is seen on learning using dissimilar odor pairs. Planned statistical comparisons shown are t-tests (***, $P < 0.0001$, NS, $P > 0.6$). An ANOVA test across all data from two experimenters showed no significant effect of experimenter either alone, or as interactions with other factors ($P>0.2$). ANOVA tests also showed no significant effect of odor alone ($P>0.03$, not significant with multiple testing), retinal alone ($P>0.4$), or light alone ($P>0.2$), when comparisons that would have included the retinal, amber, similar combination with the strong effect were excluded.

Table 1. *Drosophila* stocks used.

Genotype	Source	Reference	RRID	Use	Figure
NP2631-GAL4 (II)	T Awasaki, K Ito	Masuda-Nakagawa et al., 2014	DGGR_104266	APL neuron	3A,E, 4D
NP0732-GAL4 (I)	T Awasaki, K Ito	Masuda-Nakagawa et al., 2014	DGGR_112307	APL neuron	5B
NP225-GAL4 (II)	T Awasaki, K Ito	Masuda-Nakagawa et al. 2005	DGGR_112095	PN	3A,B, 5A
OK263-GAL4 (II)	COK, LMN	This work		*Odd* neurons	3A,D, 4C, 5C
MB247-GAL4 (III)	BDSC 50742	Zars et al. 2000	BDSC_50742	KCs	3A,C
GMR34A11-GAL4 (III)	BDSC 49767	Jenett et al., 2012	BDSC_49767	sVUM1 neuron	4A, 7
Tdc2-GAL4 (II)	BDSC 9313	Cole et al., 2005	BDSC_9313	OA neuron	1, 2, 3F
Tdc2-LexA (II)	S Certel, S Waddell	Burke et al., 2012		OA neuron	3, 4
Tdc2-LexA (III)	S Certel, S Waddell	Burke et al., 2012		OA neuron	3
MB247-LexA (III)	T Lee	Pitman et al., 2011		KC	
GMR34A11-LexA (II)	BDSC 52755	Jenett et al, 2012	BDSC_52755	sVUM1	
UAS-mCD8::GFP (III)	BDSC 5130	Lee and Luo, 1999	BDSC_5130	mCD8::GFP reporter	3F, 5B,C
10XUAS-IVS-mCD8::RFP (attP40) (II)	BDSC 32219	Pfeiffer et al., 2010	BDSC_32219	mCD8::RFP reporter	5
13XLexAOp-mCD8::GFP (III)	BDSC 32203	Pfeiffer et al., 2010	BDSC_32203	mCD8::GFP reporter	
UAS-CD4::tdTomato (II)	BDSC 35841	Han et al., 2011	BDSC_35841	Neuronal polarity	2A
UAS-nSyb::GFP	M Ramaswami	Estes et al., 2000		Neuronal polarity	2A
UAS-Syt::GFP UAS-DenMark::mCherry (III)	BDSC 33065	Nicolaï et al., 2010	BDSC_33065	Neuronal polarity	2B,C
10XUAS-IVS-mCD8::RFP, 13XlexAOp2-mCD8::GFP (X)	BDSC 32229	Pfeiffer et al., 2010	BDSC_32229	Double Reporter	
UAS-CD4::spGFP1-10	K Scott	Gordon and Scott, 2009		GRASP	3
LexAop-CD4::spGFP11	K Scott	Gordon and Scott, 2009		GRASP	3
LexAOp2-IVS>stop>spGFP11::CD4 ::HA-T2A-Brp::mCherry (attP2), UAS-spGFP1-10::CD4, UAS-HRP::CD2	C-H Lee	Karuppudurai et al., 2014		Single cell GRASP	4B-D
P{hsFLP}12 y w; CyO/Sco	BDSC 1929		BDSC_1929	Single cell GRASP	4B-D
pBPhsFlp2::PEST(attP3); +; HA_V5_FLAG_OLLAS (III)	M Landgraf	Nern et al., 2015	BDSC_64086	Multicolor Flpout (MCFO-2)	1
Tub84B(FRT-GAL80)1, w; Bl/CyO; TM2/TM6B	BDSC 38879		BDSC_38879	sVUM1 instersection	4A, 7
P{8xLexAop2-FLPL}attP40 (II)	B Pfeiffer, Janelia		BDSC_55820	sVUM1 instersection	4A, 7
UAS-Chrimson::mVenus (III)	BDSC 55136	Klapoetke et al., 2014	BDSC_55136	Effector/reporter for intersectional lines	4A, 7
y^1 w^*; +; *Mi{MIC}Oamb*^{MI12417}	BDSC 57940	Venken et al., 2011	BDSC_57940	Progenitor of *Oamb::EGFP*	
y^1 w^*; +; *Mi{PT-GFSTF.1}Oamb*$^{MI12417\text{-}GFSTF.1}$/ *TM6C*	Fly Facility, Dept. of Genetics	This work		*Oamb::EGFP*	5
Mi{MIC}Octβ1R^{MI05807}	BDSC 42119	Venken et al., 2011	BDSC_42119	Progenitor of *Octβ1R::EGFP*	
Mi{MIC}Octβ2R^{MI13416}	BDSC 59133	Venken et al., 2011	BDSC_59133	Progenitor of *Octβ2R::EGFP*	

Mi{MIC}Octβ2R^{MI06217}	BDSC 43050	Venken et al., 2011	BDSC_59133	Progenitor of *Octβ3R::EGFP*	
Mi{PT-GFSTF.2}Octβ1R$^{MI05807\text{-}GFSTF.2}$	BDSC 60236	Venken et al., 2011	BDSC_60236	*Octβ1R::EGFP*	
Mi{PT-GFSTF.2}Octβ2R$^{MI13416\text{-}GFSTF.2}$	Fly Facility, Dept. of Genetics	This work		*Octβ2R::EGFP*	
Mi{PT-GFSTF.0}Octβ3R$^{MI06217\text{-}GFSTF.0}$	Fly Facility, Dept. of Genetics	This work		*Octβ3R::EGFP line, GFP intron phase 0*	
Mi{PT-GFSTF.1}Octβ3R$^{MI06217\text{-}GFSTF.1}$	BDSC 60245	Nagarkar-Jaiswal et al., 2015	BDSC_60245	*Octβ3R::EGFP line, GFP intron phase 1*	

Table 2. Primers

Primer	Sequence	Purpose
MiMIC-5R	CTTGAGATTAAGGTAGCTTACGC	Verifying Mi{MIC} insertion
MiMIC-3F	TGCAGGTCGACGAATTCAAC	Verifying Mi{MIC} insertion
MI12417-5F	CCACAATCAACGTCCTGCTC	Verifying Mi{MIC} insertion
MI12417-3R	GATTATCGCCACCACAGAGTC	Verifying Mi{MIC} insertion
MI05807-5F	TCCTTTCATTCCCGAGCACC	Verifying Mi{MIC} insertion
MI05807-3R	CTCGTTAACAATCGCTCGCC	Verifying Mi{MIC} insertion
MI13416-5F1	CGGAGTCACTGAGTAATGGCG	Verifying Mi{MIC} insertion
MI13416-5F2	ATGGCGAGTGGTATGAGCAG	Verifying Mi{MIC} insertion
MI13416-5F3	GTGCTCTAGATGGCGAGTGG	Verifying Mi{MIC} insertion
MI13416-5F4	ACCGAGGCTCATTAACACAG	Verifying Mi{MIC} insertion
MI13416-5F5	GAGGCTCATTAACACAGCGC	Verifying Mi{MIC} insertion
MI13416-3R	GCTGCCTCATTGAACTCCAG	Verifying Mi{MIC} insertion
MI06217-5F	GCAGGAGAACAGCGACAGTC	Verifying Mi{MIC} insertion
MI06217-3R	CCTGTCTCTGGAAGTAGGTCG	Verifying Mi{MIC} insertion
Orientation-MiL-F (OriF)	GCGTAAGCTACCTTAATCTCAAGAAGAG	Verifying GFP swap orientation
Orientation-MiL-R (OriR)	CGCGGCGTAATGTGATTTACTATCATAC	Verifying GFP swap orientation
EGFPdo-Seq-F (EGFP-F)	GGATGACGGCACCTACAAGAC	Verifying GFP swap orientation
EGFPdo-Seq-R (EGFP-R)	GTGGCTGTTGAAGTTGTACTC	Verifying GFP swap orientation

Table 3. Antibodies

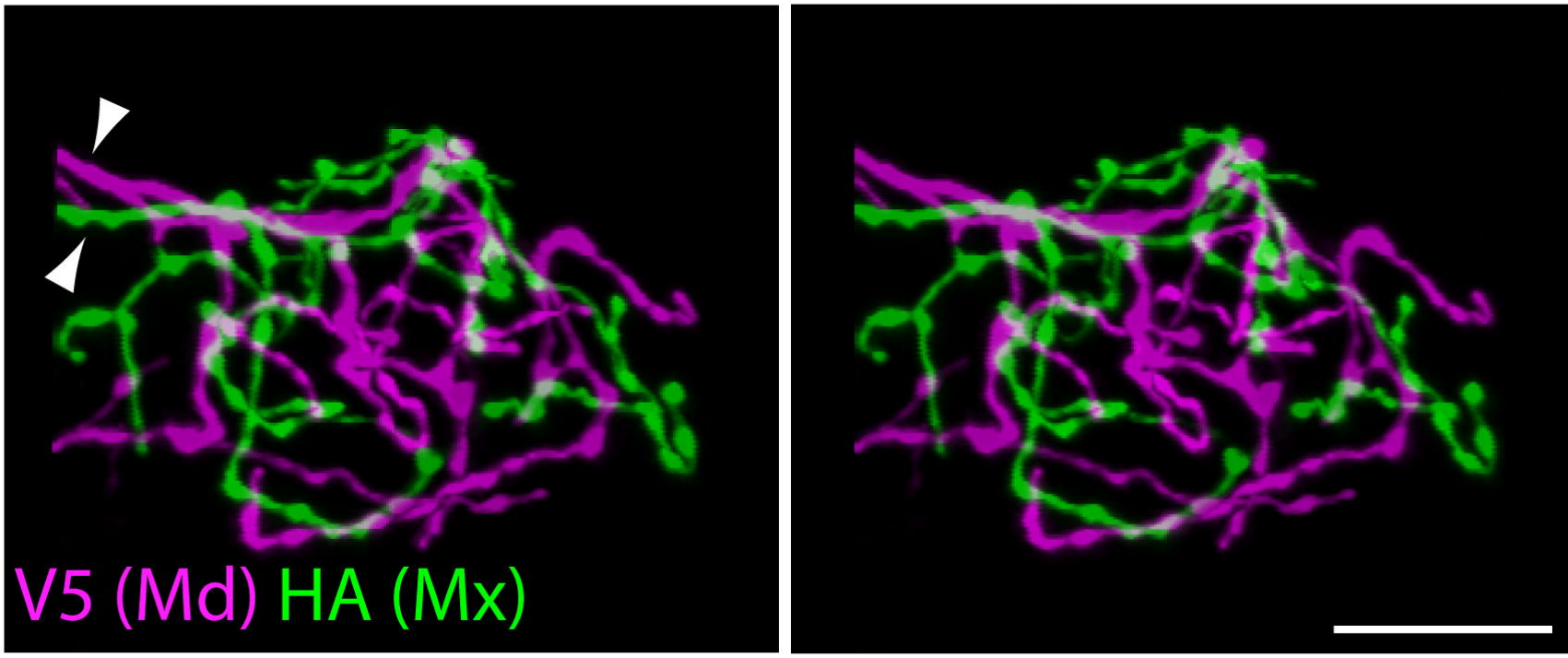
Antibody	Host	Source	RRID	Dilution	Experiment or Figures
Anti-GFP	Rat, monoclonal	Nacalai 440426 (Clone GF090R)	AB_2314545	1:1000	GRASP (3B,C,D,E) UAS-CD8::GFP
Anti-GFP	Chicken, polyclonal	Abcam, Ab13970	AB_300798	1:1000/ 1:2000	Oamb::EGFP (5)
Anti-GFP	Rabbit, polyclonal	Invitrogen, A11122	AB_221569	1:1000/ 1:2000	
Anti-GFP	Rabbit, polyclonal	Abcam, Ab290	AB_303395	1:800	
Anti-DsRed	Rabbit, polyclonal	Clontech, 632496	AB_10013483	1:1000	UAS-tdTom (2A), UAS-DenMark (2B,C) Brp::mCherry (4B,C,D) UAS-RFP (5),
Anti-GABA	Rabbit, polyclonal	Sigma, A2052	AB_477652	1:1000	Figs. 3E,F, 5B
Anti-OA	Rabbit, polyclonal	MoBiTec, 1003GE	AB_2314999	1:1000	Figs 3B-E
Anti-Dlg	Mouse, monoclonal	DSHB, 4F3	AB_528203	1:200	Figs. 2-5
Anti-FLAG	Mouse, monoclonal	Sigma, F1804	AB_262044	1:4000	MCFO
Anti-HA	Rat, monoclonal	Roche, 3F10	AB_2314622)	1:1000	MCFO (1A,B)
Anti-V5	Chicken, polyclonal	Abcam Ab1993	AB_302743	1:1000	MCFO (1A,B)
Secondary Antibody	**Host**	**Source**		**Dilution**	
Anti-Rat Alexa 488	Goat, polyclonal	Invitrogen, A11006	AB_2534074	1:200	
Anti-Rat Alexa 594	Goat, polyclonal	Invitrogen, A11007	AB_10561522	1:200	
Anti-Chicken Alexa 488	Goat, polyclonal	Invitrogen, A11039	AB_142924	1:200	
Anti-Rabbit Alexa 488	Goat, polyclonal	Invitrogen, A11034	AB_2576217	1:200	
Anti-Rabbit Alexa 568	Goat, polyclonal	Invitrogen, A11036	AB_10563566	1:200	
Anti-Mouse Alexa 647	Goat, polyclonal	Invitrogen, A21236	AB_141725	1:200	
Anti-Rabbit Alexa 647	Goat, polyclonal	Invitrogen, A21245	AB_2535813	1:200	
Anti-Chicken Alexa 568	Goat, polyclonal	Invitrogen, A11041	AB_2534098	1:200	

Table 4. Other reagents

Reagent	Source	Experiment or Figures
Paraffin oil	Sigma-Aldrich, 76235	Behavior
Pentyl Acetate	Sigma-Aldrich, 109584	Behavior
Ethyl Acetate	Sigma-Aldrich, 319902	Behavior
Ethanol	Sigma-Aldrich, 32221	Behavior
all-trans-retinal (ATR)	Sigma-Aldrich, R2500	Behavior
D-(-)-Fructose	Sigma-Aldrich, 47740	Behavior
Agarose	Sigma-Aldrich, A9539	Behavior

Fig 1

A



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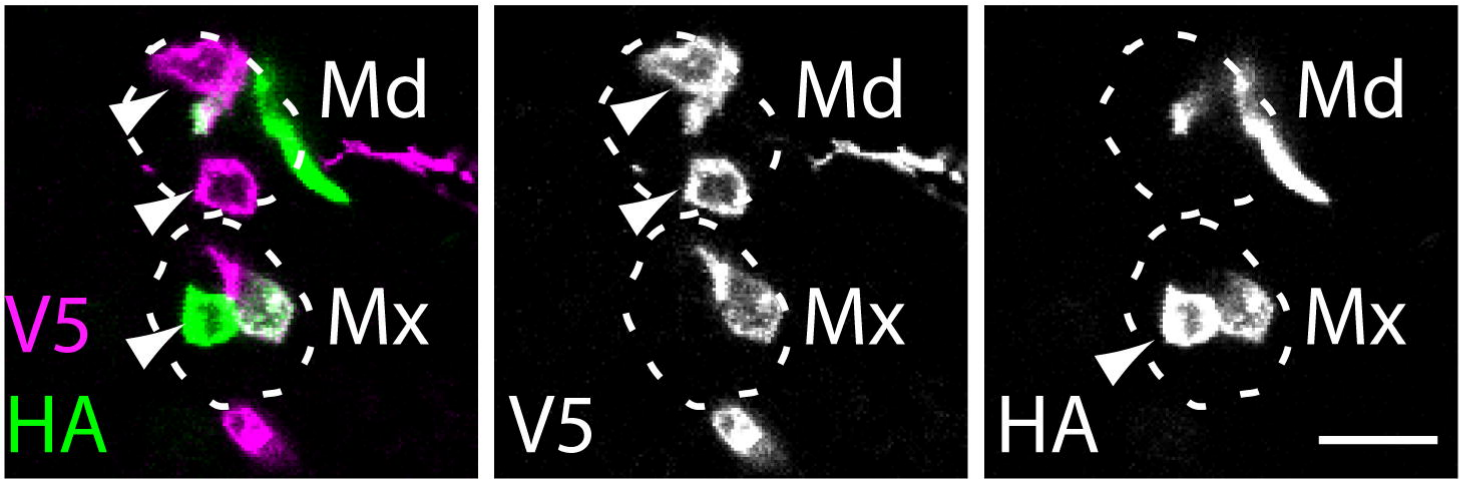
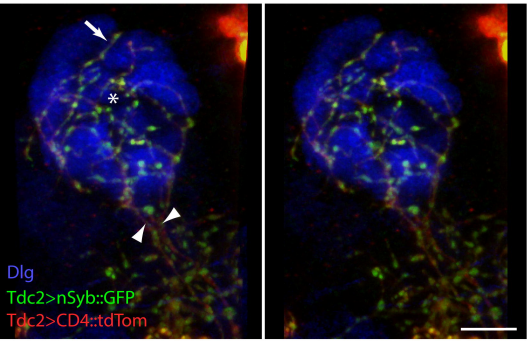


Fig 2

A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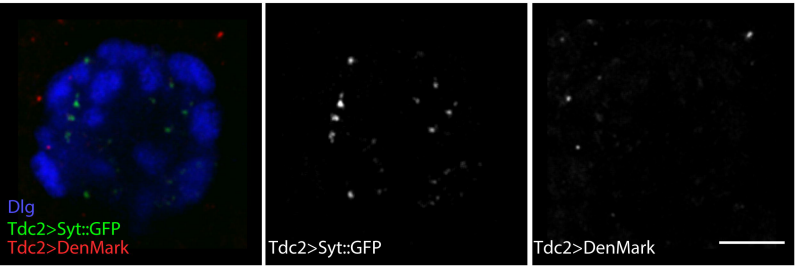
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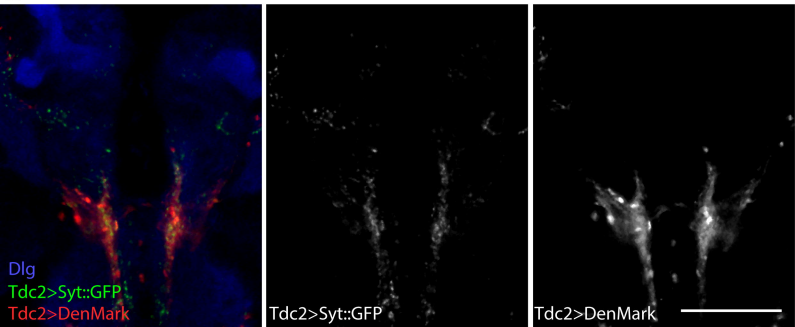
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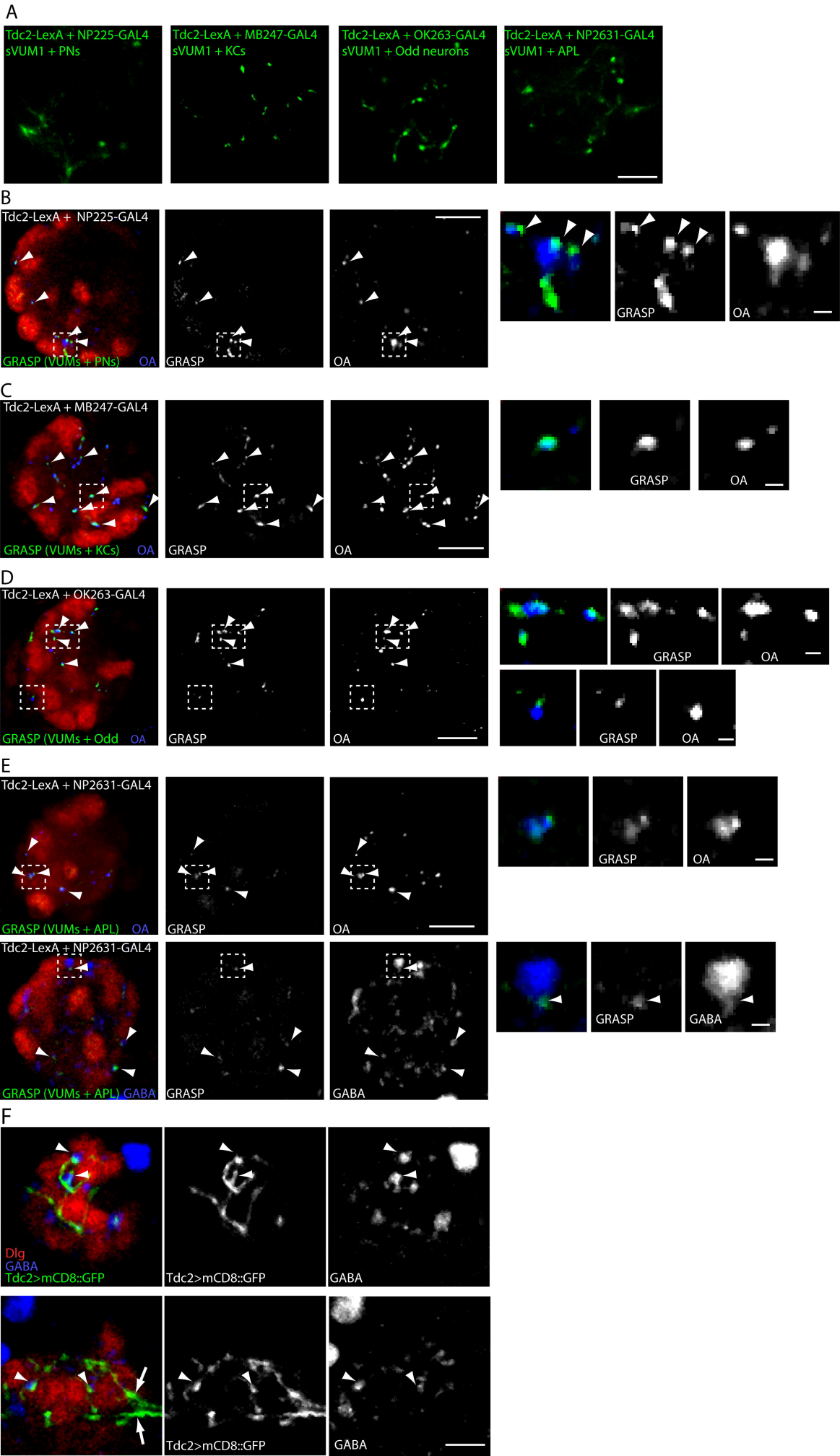
Figure 3


A
Tdc2-LexA + NP225-GAL4
sVUM1 + PNs
Tdc2-LexA + MB247-GAL4
sVUM1 + KCs
Tdc2-LexA + OK263-GAL4
sVUM1 + Odd neurons
Tdc2-LexA + NP2631-GAL4
sVUM1 + APL
B
Tdc2-LexA + NP225-GAL4
GRASP (VUMs + PNs)
OA
GRASP
OA
GRASP
OA
C
Tdc2-LexA + MB247-GAL4
GRASP (VUMs + KCs)
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GRASP
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Tdc2-LexA + NP2631-GAL4
GRASP (VUMs + APL)
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GRASP
OA
Tdc2-LexA + NP2631-GAL4
GRASP (VUMs + APL)
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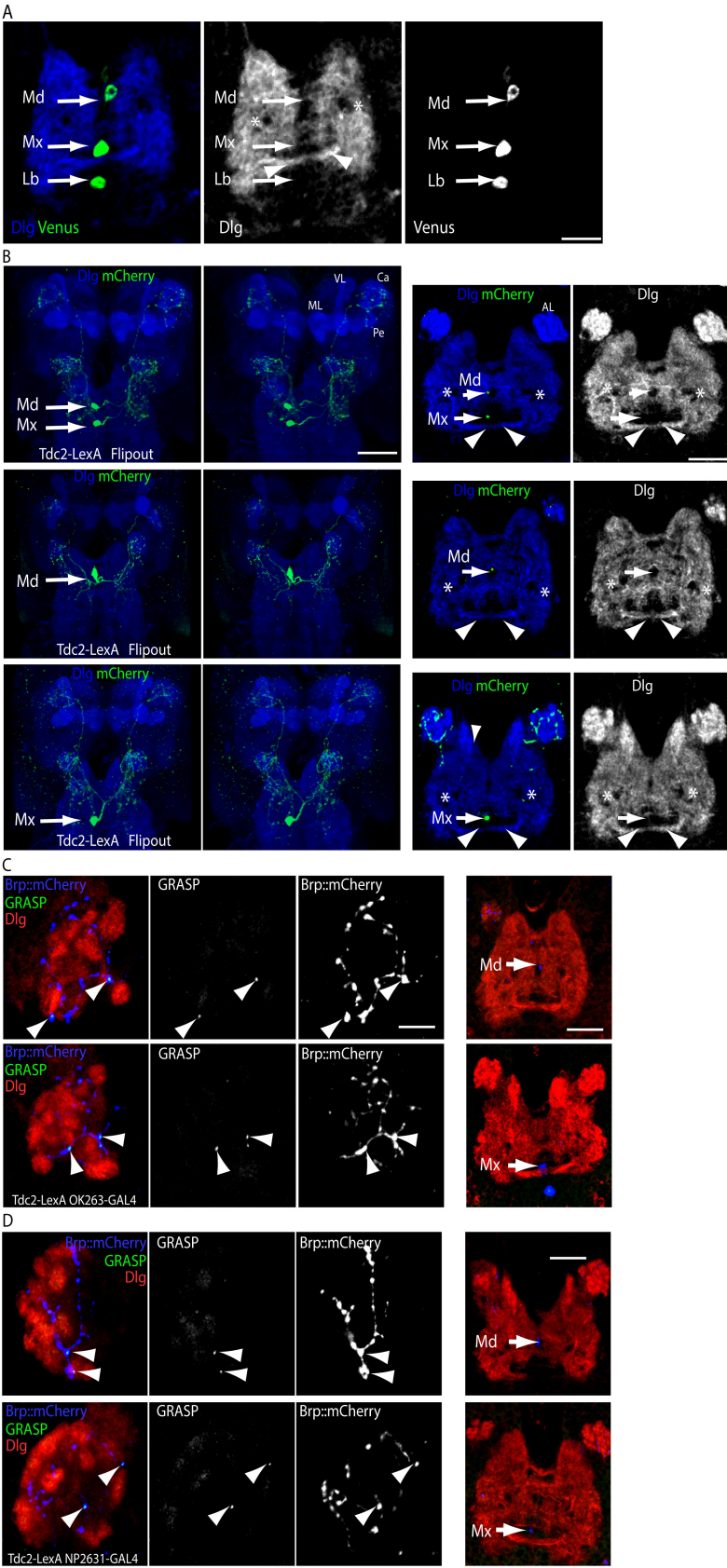
Tdc2>mCD8::GFP
GABA

Figure 4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Venus
B
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Tdc2-LexA Flipout
VL
Ca
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Pe
Dlg mCherry
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Mx
Dlg
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Brp::mCherry
GRASP
Dlg
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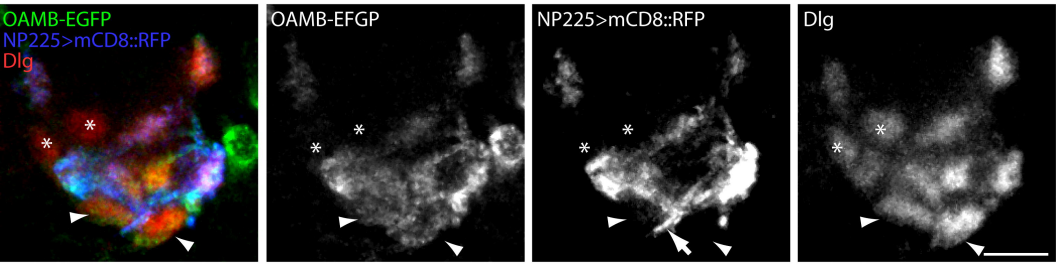
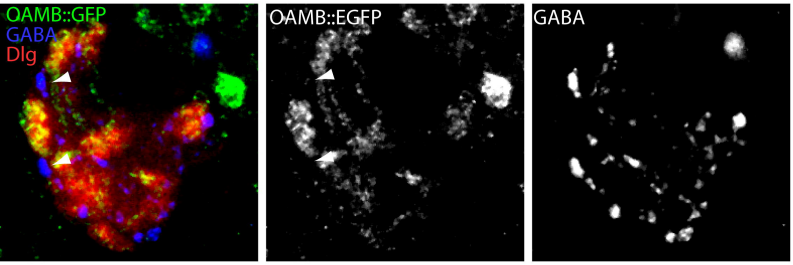
Tdc2-LexA NP2631-GAL4
GRASP
Brp::mCherry
Mx

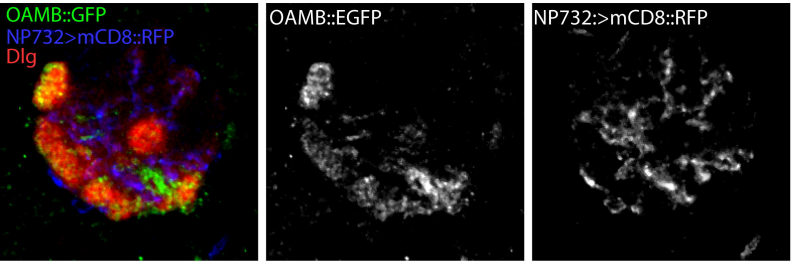
Figure 5

A


OAMB-EGFP
NP225>mCD8::RFP
Dlg
OAMB-EFGP
NP225>mCD8::RFP
Dlg

B


OAMB::GFP
GABA
Dlg
OAMB::EGFP
GABA


OAMB::GFP
NP732>mCD8::RFP
Dlg
OAMB::EGFP
NP732:>mCD8::RFP

C

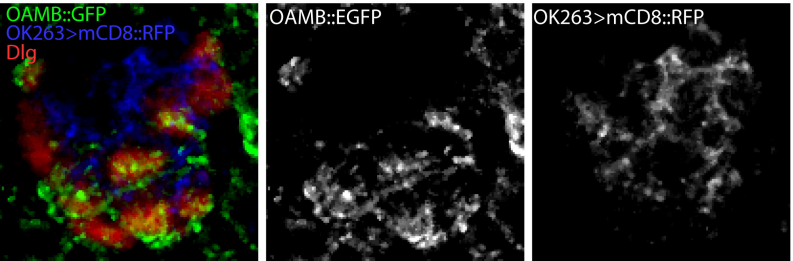

OAMB::GFP
OK263>mCD8::RFP
Dlg
OAMB::EGFP
OK263>mCD8::RFP

Fig ure 6

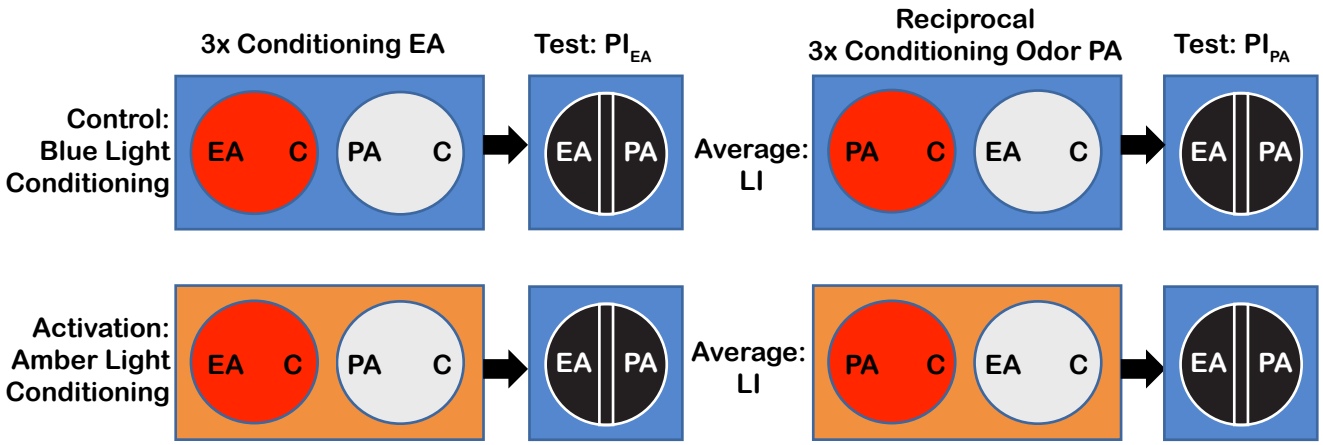
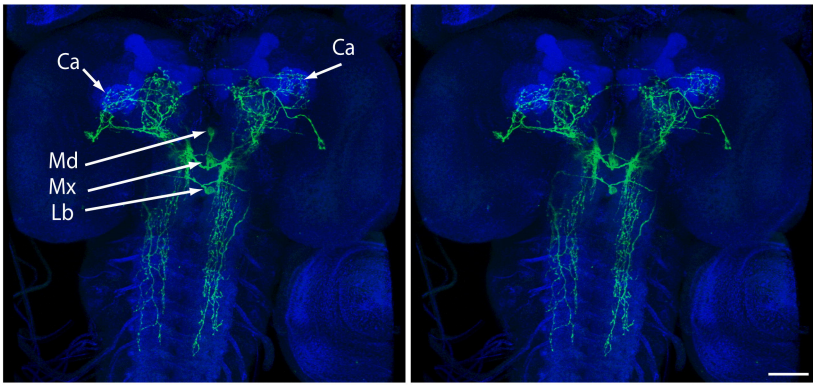
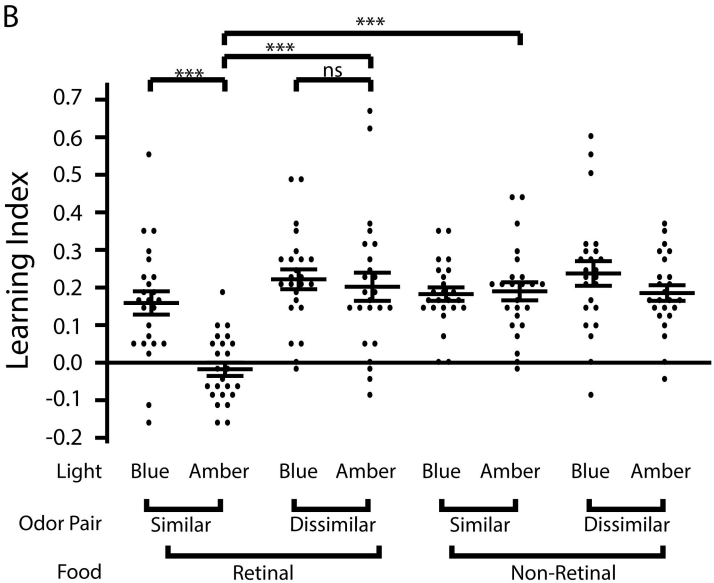

3x Conditioning EA
Test: PI_{EA}
Reciprocal
3x Conditioning Odor PA
Test: PI_{PA}
Control:
Blue Light
Conditioning
EA
C
PA
C
EA
PA
Average:
LI
PA
C
EA
C
EA
PA
Activation:
Amber Light
Conditioning
EA
C
PA
C
EA
PA
Average:
LI
PA
C
EA
C
EA
PA

Figure 7

A


Ca
Ca
Md
Mx
Lb

B


***
***
***
ns
Learning Index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
Light
Blue
Amber
Blue
Amber
Blue
Amber
Blue
Amber
Odor Pair
Similar
Dissimilar
Similar
Dissimilar
Food
Retinal
Non-Retinal