Direct neural pathways convey distinct visual information to Drosophila mushroom bodies

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Abstract Previously, we demonstrated that visual and olfactory associative memories of Drosophila share mushroom body (MB) circuits (Vogt et al., 2014). Unlike for odor representation, the MB circuit for visual information has not been characterized. Here, we show that a small subset of MB Kenyon cells (KCs) selectively responds to visual but not olfactory stimulation. The dendrites of these atypical KCs form a ventral accessory calyx (vAC), distinct from the main calyx that receives olfactory input. We identified two types of visual projection neurons (VPNs) directly connecting the optic lobes and the vAC. Strikingly, these VPNs are differentially required for visual memories of color and brightness. The segregation of visual and olfactory domains in the MB allows independent processing of distinct sensory memories and may be a conserved form of sensory representations among insects.

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Introduction Rewarding or punitive stimuli modulate behavioral responses to sensory stimuli. In insects, such associative modulation takes place in the mushroom body (MB) (Heisenberg, 2003). In the fruit fly, the MB receives distinct dopaminergic inputs that signal reward and punishment, and this valence circuit in the MB is shared for associative memories of different modalities: olfaction, gustation, and vision (Masek et al., 2015; Aso et al., 2012; Liu et al., 2012; Perisse et al., 2013). The role of MB output during memory acquisition and testing is similar in visual and olfactory memories (Vogt et al., 2014). However, the required subsets of MB Kenyon cells (KCs) are not the same (Aso et al., 2014a; Vogt et al., 2014), raising the possibility that memory-relevant visual and olfactory information may be represented by different KC subsets in the MB.

Studies of the neuronal circuits underlying olfactory learning have benefitted from well-characterized neuronal pathways conveying olfactory information to the MB (Turner et al., 2008; Butcher et al., 2012). Beyond visual associative memory, the MB was shown to be important in various vision-guided behavioral tasks (Liu et al., 1999; Brembs, 2009; Zhang et al., 2007). Yet, how visual information is conveyed and represented in the Drosophila MB is totally unknown. MBs of hymenoptera receive visual afferents in their calyces (Ehmer and Gronenberg, 2002; Gronenberg and Hölldobler, 1999; Pauk and Gronenberg, 2008), while such direct visual input from the optic lobes to the MBs has never been observed in dipteran insects (Mu et al., 2012; Otsuna and Ito, 2006). Therefore, indirect, multisynaptic pathways have been proposed to convey the visual input to the Drosophila MB (Farris and Van Dyke, 2015; Tanaka et al., 2008). In this
report, we demonstrate the existence of two visual projection neurons that directly connect the optic lobes to the MB.

**Results**

To understand the representation of visual memory in the MB, we blocked different subsets of KCs using split-GAL4 drivers and UAS-shi131 (Aso, 2014a) and behaviorally screened the flies for color discrimination memory using an aversive reinforcer (Figure 1C). Memory was consistently impaired when we used drivers labelling the γ-lobe neurons, confirming their importance for visual memory (Figure 1—figure supplement 1A) (Vogt et al., 2014). Strikingly, this screen further suggested a subset of the γ neurons (γd) to be specifically responsible (Figure 1, Figure 1—figure supplement 1A).

The γd neurons are embryonic born KCs consisting of ca. 75 cells in the adult brain (Butcher et al., 2012; Aso et al., 2009; Aso et al., 2014b). Two split-GAL4 drivers MB607B and MB419B showed strong expression in the γd neurons but had no detectable expression in other KCs (Figure 1A, Figure 1—figure supplement 1B). The blockade of the γd neurons using these lines severely impaired visual memory (Figure 1D,E, Figure 1—figure supplement 1C). In contrast, olfactory memory of these flies was not significantly affected (Figure 1F, Figure 1—figure supplement 1D). Given that both visual and olfactory memories were reinforced by the same aversive stimulus - electric shock punishment - the selective γd requirement for visual memory suggests that these KCs represent visual stimuli.

We characterized γd cell morphology using the MB607B-GAL4 and MB419B-GAL4 drivers to express axonal and dendritic markers. Their axons run in parallel to those of the other KCs in the peduncle, and project to the dorsomedial tip of the γd lobe (Figure 1B, 2A). The γd neurons are atypical in that their dendrites are highly enriched ventrolaterally outside the main calyx, where olfactory projection neurons (OPNs) terminate (Figure 1B, 2B), and form the ventral accessory calyx (vAC) (Butcher et al., 2012; Aso et al., 2014b). Nevertheless, the γd neurons are equipped with claw-like dendritic endings forming microglomeruli similar to those in the main calyx (Figure 2C,D).

To examine if the γd neurons are tuned to visual stimuli, we measured electrophysiological responses using whole-cell patch-clamp recordings. In line with the specific requirement of γd neurons for visual conditioning, we found spiking responses to blue and green light stimuli in some of these cells (Figure 2E,G). Stimulating flies with 5 different odors did not lead to excitatory responses of the γd neurons; olfactory stimulation rather evoked slow inhibitory responses, implying the existence of feedforward inhibition through other odor-responsive KC populations (Figure 2E,G). This response profile is in sharp contrast to what we observed with typical KCs (e.g. α/β neurons), many of which responded to odors but none to visual stimuli (Figure 2F,G, [Turner et al., 2008]). Thus, there is clear modality segregation between γd and α/β neurons.

The selective tuning of the γd neurons to visual stimuli prompted us to ask if Drosophila MBs receive direct visual input from the optic lobes in the γd dendrites. Performing an anatomical screen of a GAL4 driver collection (Jenett et al., 2012), we identified two types of neurons with arbors in the optic lobes and projections in the area of the vAC, making them candidates for visual projection neurons (VPN) to the MB (Figure 3—figure supplement 1). We named these two types of VPNs VPN-MB1 and VPN-MB2.

To precisely map the morphology of these VPNs, we generated split-GAL4 drivers, MB425B and MB334C. These drivers have strong expression in VPN-MB1 and VPN-MB2 with little other expression (Figure 3A,C). MB425B-GAL4 has predominant expression in VPN-MB1 (Figure 3B) projecting from the medulla to the vAC with many cell bodies on the anterior surface of the optic lobe. In contrast, MB334C-GAL4 strongly labels 1–3 cells of VPN-MB2 as well as the MB output neurons MBON-a1 (Figure 3C,D) (Aso et al., 2014a; 2014b). We found that the majority of VPN-MB1 and VPN-MB2 dendrites are localized to the optic lobes (Figure 3E,G). The VPN-MB1 neurons have dendritic arbors in the medulla enriched in layer M8 (Figure 3F). They cover a large field of the medulla with a conspicuous elaboration in the ventral half (Figure 3A,E,F). Single-cell labeling revealed that each VPN-MB1 cell samples input from approximately 20 optic cartridges in the medulla (Figure 3B). Dendrites of a single VPN-MB2 cover a large field of the ventral medulla, arborizing in layer M7 (Figure 3C,D,G,H). The output sites of both VPNs are restricted to the lateral protocerebrum,
including the vAC, forming pre-synaptic boutons in microglomeruli (Figure 3E,G; Figure 3—figure supplement 2).

To visualize possible connections between the VPNs and KCs in the vAC, we performed counter-staining of VPNs and the catalytic subunit of PKA that marks KCs (Wolff and Strausfeld, 2015) and found that the VPN terminals are enwrapped by the dendrites of the γd neurons (Figure 3—figure supplement 2). Differential labeling of KCs and the VPNs was consistent with their proposed connection (Figure 3—figure supplement 3). We also detected a GRASP signal between the VPNs and the ventrolateral projection of the γd dendrites (Figure 3I–L).

We examined the requirement of the VPNs as well as OPNs in visual memory by blocking their output using the driver lines MB425B-GAL4, MB334C-GAL4 and GH146-GAL4 (Figure 4A–C). Memory was not significantly altered upon the blockade of the OPNs (Figure 4A). In contrast, the blockade of VPN-MB1, but not VPN-MB2, significantly impaired color discrimination memory (Figure 4B, C). To further substantiate the results with MB425B-GAL4, we employed another driver line (VT008475) that labels similar VPNs to VPN-MB1. These VPNs in VT008475 project to the lateral protocerebrum but do not reach the vAC (Figure 4—figure supplement 1). The blockade of these VPNs left color discrimination memory intact (Figure 4—figure supplement 1). These results strongly suggest the existence of modality-specific input pathways to the MB and that VPN-MB1 is a key component of the visual learning pathway.
In our standard learning assay, flies discriminate the chromatic information of blue and green LED stimuli. In addition, flies can learn different light intensities of a single chromatic cue (Schnaitmann et al., 2013). We asked if the γd neurons and same VPNs are also required for brightness discrimination learning. We found that while the γd neurons were also necessary for this task, the blockade of VPN-MB1 did not significantly alter performance (Figure 4D,E). In contrast, learning was significantly impaired by blockade of VPN-MB2 (Figure 4F). Because MB334C-GAL4 has expression not just in VPN-MB2 but also in MBON-α1, we tested and ruled out the involvement of MBON-
by showing that its blockade did not significantly impair brightness discrimination learning (Figure 4—figure supplement 2). The differential requirements for VPN-MB1 and VPN-MB2 indicate that information about color and intensity of a visual cue is separately conveyed to the MB via distinct VPNs.

**Discussion**

Previous studies have shown the importance of MB circuits for visual memories and other visually guided behaviors in *Drosophila* (Vogt et al., 2014; Liu et al., 1999; Zhang et al., 2007); however, the MB had been thought to receive visual input from the optic lobe through an unknown indirect pathway, because direct connections had not been observed (Farris and Van Dyke, 2015; Tanaka et al., 2008). Our identification of direct pathways reveals similarity between dipteran and...
hymenopteran circuit design for visual processing, and provides experimental evidence for the behavioral roles of these circuits (Figure 4G).

VPN-MB1 and VPN-MB2 receive differential inputs in the medulla and convey distinct visual features - color and brightness - to the MB. The medulla layer M8 where VPN-MB1 arborizes (Figure 3F) contains presynaptic terminals of Tm5 neurons that are involved in color vision (Karuppudurai et al., 2014; Gao et al., 2008). VPN-MB2 is the first *Drosophila* interneuron shown to convey brightness information, and the position of its dendrites suggests that light intensity may be encoded in the medulla layer M7 (Figure 3H). Interestingly, the dendrites of VPN-MB1 and VPN-MB2 are preferentially distributed in the ventral half of the medulla, an arrangement consistent with the specialization of the ventral retina for color processing of landmarks during foraging (Giger and Srinivasan, 1997; Kinoshita et al., 2015; Wernet et al., 2015). The target region of the MB-projecting VPNs is separated from other described VPNs in the central brain, and largely segregated from other VPNs mediating innate spectral processing and motion vision (Mu et al., 2012; Otsuna and Ito, 2006; Zhang et al., 2013a). Parallel processing of different visual features with segregated...
projections may be a conserved circuit strategy for visual processing across phyla (Livingstone and Hubel, 1988).

Our results provide evidence that the Drosophila MB represents distinct sensory modalities in different KC subsets whose dendrites are segregated in subdomains of the calyx. Olfactory inputs project to the main calyx and visual stimuli to the vAC, while gustatory stimuli have been recently shown to project to yet another calyx domain (Kirkhart and Scott, 2015). In the fly MB, dopamine neurons including those encoding positive and negative valences divide the long axon terminals of KCs into distinct compartments (Aso et al., 2014a; Tanaka et al., 2008). As KCs send parallel axon fibers, a single dopamine neuron locally modulates the corresponding axonal compartment of multiple KCs (Hige et al., 2015; Cohn et al., 2016; Boto et al., 2014). Distinct KC subsets, yd and ym for example, can therefore share the same dopaminergic valence modulation, even if these KCs are devoted to different sensory modalities (Figure 4G) (Vogt et al., 2014). These local modulations in turn affect the information conveyed by shared MB output pathways (Figure 4G) (Aso et al., 2014; Vogt et al., 2014). Our results can thus explain the circuit mechanism by which the Drosophila MB processes memories of different modalities with shared modulatory and output pathways (Figure 4G).

There appears to be a close correlation between the ecological specialization of different insects and the organization of their MB calyces (Ehmer and Gronenberg, 2002; Lin and Strausfeld, 2012; Yilmaz et al., 2016; Groh et al., 2014), with the functional subdivision of the calyx reflecting the salient sensory environment. Olfactory processing, which is the dominant sensory modality in Drosophila subject to associative modulation, utilizes ~1800 of the 2000 KCs (Quinn et al., 1974). While fruit flies perform a wide range of behaviors driven by visual input, few could be modified by associative learning (Borst, 2009), given that fewer KCs subserve visual memory formation. The MB calyces of other insects also possess modality segregation, and different sets of KCs are presumably assigned to each sensory space (Gronenberg and Hölldobler, 1999; Kinoshita et al., 2015; Mobbs, 1982). Our results here are the first to show that individual KCs indeed have unimodal responses. The segregated sensory representation in the MB enables independent formation of different sensory memories, while allowing interaction among distinct KC populations that may underlie complex forms of learning involving multimodal integration (Zhang et al., 2013b).

Materials and methods

Flies and genetic crosses
Flies were reared at 25°C, at 60% relative humidity under a 12-12-hr light-dark cycle on a standard cornmeal-based diet. Flies were sorted by genotype at least two days prior to experiments. Each behavioral experiment used 30–40 flies of mixed gender under dim red light in a custom-made plastic box, containing a heating element on the bottom and a fan for air circulation.

For behavioral experiments, we used F1 progeny of crosses between females of wt:;pJFRC100-20x pJFRC100-20XUAS-TTS-Shibire-ts1-p10 in VK00005 (Pfeiffer et al., 2012) or WT-females and males of genotypes MB607B-GAL4 (Aso et al., 2014b), MB419B-GAL4 (Aso, 2014b), GH146-GAL4 (Stocker et al., 1997), MB425B-GAL4, MB334C-GAL4, MB310C-GAL4 (Aso et al., 2014b), VT008475-GAL4 (Vogt et al., 2014; Aso et al., 2014b), MB425B-GAL4, MB334C-GAL4, MB310C-GAL4 (Aso et al., 2014b), VT008475-GAL4 (Vogt et al., 2014; Aso et al., 2014b), MB425B-GAL4, MB334C-GAL4, MB310C-GAL4 (Aso et al., 2014b), and MB607B-GAL4 (Aso, 2014b). Split-GAL4 lines were generated using described vectors (Pfeiffer et al., 2010): MB425B carries 28F07-p65ADZp in attP40 and 10E05-ZpGdbd in attP2, MB334C carries 52G04-p65ADZp in attP40 and 49F03-ZpGdbd in VK00027. Split-GAL4 lines used in the KC screening (Figure 4—figure supplement 1) and for intensity conditioning (Figure 4—figure supplement 2) are as described in (Aso et al., 2014b). As all transgenes were inserted into the w+ mutant genome, the X chromosomes of the shi+ effector strain was replaced with that of wild-type Canton-S (w+).

Aversive visual conditioning
We used LEDs to present visual stimuli (green [520 nm] and blue light [465 nm]) from the bottom of the arena as previously described in (Schnaitmann et al., 2013). The intensities were controlled by current and calibrated using a luminance meter BM-9 (Topcon Technohouse Corporation) or a PR-655 SpectraScan Spectroradiometer: 19.4 mW/m2 (blue) and 8.58 mW/m2 (green) (Schnaitmann et al., 2013). To train flies with different light intensities, blue and green visual cues...
were replaced by different intensities of green light (1:10 ratio; 27.8 mW/m² (bright-green), and 2.77 mW/m² (dark-green)).

For aversive electric shock conditioning, we used an arena with a transparent shock grid as previously described (Vogt et al., 2014). During the test phase, the shock arena was video recorded from above with a CMOS camera (Firefly MV, PointGrey, Richmond, Canada) controlled by custom-made software (Schnaitmann et al., 2010). Four setups were run in parallel.

Differential conditioning was followed by binary choice without reinforcement (Vogt et al., 2014; Schnaitmann et al., 2010). Briefly, in a single experiment, approximately 40 flies were introduced into the arena using an aspirator. During a training trial, the entire arena was illuminated with alternating visual stimuli (60 s each) with one stimulus paired with aversive reinforcement. A 1-s electric shock (AC 60 V) was applied 12 times spaced over 60 s during presentation of the punished visual stimulus. Training trials were repeated four times per experiment. In the test, administered 60 s after the end of training, flies were allowed to choose between the two visual stimuli, which were each presented in two diagonally opposed quadrants of the arena. The distribution of the flies was video recorded for 90 s at 1 frame per second. No US was presented in the test period; however, a 1-s shock pulse (90 V) was applied 5 s before the beginning of the test to arouse the flies. Two groups were trained with reciprocal pairings and tested consecutively in the same setup, respectively. The difference in visual stimulus preference between the two groups was then used to calculate a learning index for each video frame. Reinforcement was paired with the first visual stimulus in half of the experiments, and with the second in the remaining experiments, to cancel any effect of order. The whole experimental setup was kept at 33°C for the temperature-induced effect of Shi¹s¹.

**Aversive olfactory conditioning**

Aversive olfactory conditioning was performed as described in (Aso et al., 2014a). A group of about 50 flies in a training tube alternately received octan-3-ol (OCT; Merck, Darmstadt, Germany) and 4-methylcyclohexanol (MCH; Sigma-Aldrich, MO) for 1 min in a constant air stream. OCT and MCH were diluted to 0.6% and 2%, respectively, in paraffin oil (Sigma-Aldrich) and presented in a cup with a diameter of 30 mm. For odor presentation, two of 3/2-way solenoid valves (MFH-3-3/4-S, FESTO, Germany) were used. Each valve was connected to two cups, one of which contains the diluted odor and the other of which contains pure paraffin oil. Four training tubes were connected to the valves. Twelve 1.5 s 90 V electric shocks (DC) were paired with one of the odor presentations. The delivery of electric shocks and the odors was controlled by a custom-made computer program. In the test, the trained flies were allowed to choose between MCH and OCT for 2 min in a T-maze. The odor cups used were identical to the ones for the conditioning. The distribution of the flies was imaged by cameras (FFMV-03M2M, Point Grey), and the preference index was calculated by taking the mean indices of the last 10 s in the 2 min choice. The learning index was then calculated by taking the mean preference of the two reciprocally trained groups. Half of the trained groups received reinforcement together with the first presented odor and the other half with the second odor to cancel the effect of the order of reinforcement. Temperature and humidity were 60% and 33°C, measured with a digital thermo-hydrometer (6011000, Venta Luftwäscher, Germany). Behavioral experiments were performed in dim red light for training and in complete darkness for test.

**Statistics**

Statistical analyses were performed with Prism5 software (GraphPad). Groups that did not violate the assumption of normal distribution (Shapiro-Wilk test) and homogeneity of variance (Bartlett’s test) were analyzed with parametric statistics: one-sample t-test or one-way analysis of variance followed by the planned pairwise multiple comparisons (Bonferroni). Experiments with data that were significantly different from the assumptions above were analyzed with non-parametric tests, such as Mann-Whitney test or Kruskal–Wallis test followed by Dunn’s multiple pair-wise comparison (Figure 1—figure supplement 1). The significance level of statistical tests was set to 0.05. Only the most conservative statistical result of multiple pairwise comparisons is indicated. Visual conditioning bar graphs show pooled data over total duration of test. Olfactory conditioning bar graphs show pooled data over last 10 s of test.
**Electrophysiology**

In vivo whole-cell recordings from KCs were performed as previously reported (Turner et al., 2008). Specific cell types were visually targeted using GFP signals, with a 60X water-immersion objective (LUMPlanFl/IR; Olympus) attached to an upright microscope (BX51WI; Olympus). \( \gamma d \) and \( \alpha/\beta \) KCs were specifically labeled with GFP by crossing flies bearing UAS-2eGFP (Bloomington) with GAL4 lines, MB607B (\( \gamma d \) KCs) or MB0088 (\( \alpha/\beta \) KCs). Adult F1 females were used at 2–3 days after eclosion. The patch pipettes were pulled for a resistance of 6–7 M\( \Omega \) lines, were specifically labeled with GFP by crossing flies bearing UAS-2eGFP (Bloomington) with GAL4 lines, MB607B (\( \gamma d \) KCs) or MB0088 (\( \alpha/\beta \) KCs). Adult F1 females were used at 2–3 days after eclosion. Odors were presented through a custom-built device as described previously (Honegger et al., 2011). Saturated vapors of pure odorants were diluted with air at a 1: 20 ratio. Final flow rate was 1 L/min. Odors were presented in a pseudo-random order so that no odor was presented twice in succession. The following five chemicals were used as stimuli: 2-heptanone (CAS# 110-43-0), 3-octanol (589-98-0), 4-methylcyclohexanol (589-91-3), isoamyl acetate (123-92-2) and apple cider vinegar (Richfood).

We observed distinct response profiles of \( \gamma d \) KCs and \( \alpha/\beta \) KCs with respect to both light and odor responses (Figure 2G). To see if excitatory drive from the two different modalities is segregated between the cell types, we performed similar statistical analysis (Fisher’s exact test) after combining the counts of ‘Spike’ with ‘Excitatory’, and ‘Inhibition Only’ with ‘No Response’ in Figure 2G. This analysis also showed highly significant differences both in light (\( p<10^{-5} \)) and odor responses (\( p<10^{-3} \)).

**Immunohistochemistry**

Adult fly brains were dissected, fixed, and stained using standard protocols (Aso et al., 2009). MB419B-GAL4, MB607B-GAL4, MB425B-GAL4, MB334C-GAL4, MB310C-GAL4, and VT008475-GAL4 were crossed to UAS-mCD8::GFP (Figure 1, Figure 2, Figure 3, Figure 1—figure supplement 1, Figure 3—figure supplement 1, Figure 3—figure supplement 2, Figure 4—figure supplement 1, Figure 4—figure supplement 2) and stained with anti-GFP antibody (AB) (rabbit anti-GFP polyclonal, Invitrogen, 1:1000) followed by Alexa Fluor 488 (goat anti-rabbit IgG highly cross absorbed, Invitrogen, 1:1000). For neuropil labeling, we used mouse anti-dskl AB (DSHB, 1:250) and double labeling of \( \gamma d \) and \( \alpha/\beta \) KCs. Adult F1 females were used at 2–3 days after eclosion. Odors were presented through a custom-built device as described previously (Honegger et al., 2011). Saturated vapors of pure odorants were diluted with air at a 1: 20 ratio. Final flow rate was 1 L/min. Odors were presented in a pseudo-random order so that no odor was presented twice in succession. The following five chemicals were used as stimuli: 2-heptanone (CAS# 110-43-0), 3-octanol (589-98-0), 4-methylcyclohexanol (589-91-3), isoamyl acetate (123-92-2) and apple cider vinegar (Richfood).
followed by Alexa Fluor 488 (anti-rat AB, Invitrogen, 1:250) and anti-dsRed AB (rabbit anti-dsRed, Clontech, 1:100) followed by Alexa Fluor 568 (anti-rabbit AB, Invitrogen, 1:200). DC0-positive neurons (PKA-C1, Figure 3—figure supplement 1) were visualized using anti-DC0 AB staining (anti-DC0 rabbit, 1:2000, Skoulakis et al., 1993) followed by Cy3 anti-rabbit (Jackson ImmunoResearch, 1:200). To visualize connections between VPNs and the vAC we crossed w-; mb247-DsRed; mb247-splitGFP11, UAS-splitGFP1-10 (Pech et al., 2013) to MB425B-GAL4 and MB334C-GAL4. For visualization of reconstituted GFP (Figure 3I–J), we used mouse anti-GFP AB (Clone N86/38, Neuro-Mab, Antibodies Inc., 1:100) followed by goat anti-mouse Alexa 488 (Invitrogen, 1:200). For MB labeling (Figure 3I–J), we used rabbit anti-dsRed AB (Clontech, 1:100) followed by Cy3 anti-rabbit (Jackson ImmunoResearch, 1:200). For neuropil labeling, we used rat anti-N-cadherin staining (anti-N-cad DNP Ex no.8, Developmental Studies Hybridoma Bank, 1:100) followed by Alexa 633 anti-rat (Invitrogen, 1:200). R7/R8 neurons (Figure 3F,H) were visualized using mouse anti-MAb24B10 AB (DSHB, Zipursky et al., 1984) followed by Cy3 anti-mouse (Jackson ImmunoResearch, 1:250). Optical sections of whole-mount brains were sampled with a confocal microscope (Olympus FV1000). Images of the confocal stacks were analyzed with the open-source software Fiji (Schindelin et al., 2012) and rendered using Fluorender (Schnaitmann et al., 2010). We applied the 3D mean filter (r = 2 pixels) followed by deconvolution to high magnification image stacks in Figure 3D–D’ using Fiji plugins Iterative Deconvolve 3D.

To obtain single-cell flp-out staining, males of the MB425B-GAL4 were crossed with females of y-w-, hsp70-flp [X); UAS>CD2 y+>mCD8::GFP/CyO; TM2/TM6b (Wong et al., 2002) to obtain F1 progeny carrying GAL4 insertion, hsp70-flp and UAS>rCD2,y+>mCD8::GFP. Crosses were raised at 25°C. One to six days before eclosion a mild heat shock was given by placing the vial into a 32°C incubator to remove the FLP-out cassette (rCD2, y+) in a subset of the neurons. The duration of the heat shock was 60–90 min. The eclosed flies were then transferred into a fresh vial and 2- to 5-day-old flies were used for dissection.

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