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A gustatory second-order neuron that connects sucrose-sensitive primary neurons and a distinct region of the gnathal ganglion in the *Drosophila* brain

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Abstract

Although the gustatory system provides animals with sensory cues important for food choice and other critical behaviors, little is known about neural circuitry immediately following gustatory sensory neurons (GSNs). Here, we identify and characterize a bilateral pair of gustatory second-order neurons in *Drosophila*. Previous studies identified GSNs that relay taste information to distinct subregions of the primary gustatory center (PGC) in the gnathal ganglia (GNG). To identify candidate gustatory second-order neurons (G2Ns) we screened ~5,000 GAL4 driver strains for lines that label neural fibers innervating the PGC. We then combined GRASP (GFP reconstitution across synaptic partners) with presynaptic labeling to visualize potential synaptic contacts between the dendrites of the candidate G2Ns and the axonal terminals of *Gr5a*-expressing GSNs, which are known to respond to sucrose. Results of the GRASP analysis, followed by a single cell analysis by FLPout recombination, revealed a pair of neurons that contact *Gr5a* axon terminals in both brain hemispheres, and send axonal arborizations to a distinct region outside the PGC but within the GNG. To characterize the input and output branches, respectively, we expressed fluorescence-tagged acetylcholine receptor subunit (Dα7) and active-zone marker (Brp) in the G2Ns. We found that G2N input sites overlaid GRASP-labeled synaptic contacts to *Gr5a* neurons, while presynaptic sites were broadly distributed throughout the neurons' arborizations. GRASP analysis and further tests with the Syb-GRASP method suggested that the identified G2Ns receive synaptic inputs from *Gr5a*-expressing GSNs, but not *Gr66a*-expressing GSNs, which respond to caffeine. The identified G2Ns relay information from *Gr5a*-expressing GSNs to distinct regions in the GNG, and are distinct from other, recently identified gustatory projection neurons, which relay information about sugars to a brain region called the antennal mechanosensory and motor center (AMMC). Our findings suggest unexpected complexity for taste information processing in the first relay of the gustatory system.

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Keywords

Taste; interneuron; subesophageal zone; higher-order circuits

INTRODUCTION

The sense of taste is critically important for several behaviors in animals including the ability to choose appropriate food sources. In adult *Drosophila*, which offer experimenters genetic control over neuronal labeling and function, gustatory sensory neurons (GSNs) are found in the tarsal segments of the legs, the ovipositor, the wing margins and the internal mouthparts, but the largest population of the GSNs is housed in the distal lobes of the mouthparts called labellum (Vosshall and Stocker, 2007). About 130 GSNs in the labellum send their axons to distinct subregions of the primary gustatory center (PGC) in the gnathal ganglia (GNG, formerly called the subesophageal ganglion, SEG, but renamed for greater precision, cf. Ito et al., 2014) (Singh, 1997). We previously classified the PGC into three branches that form along anterior-posterior axis: the anterior maxillary sensory center (AMS), the posterior maxillary sensory center (PMS) and the labellar sensory center (Miyazaki and Ito, 2010).

Genetic dissection of GSNs has revealed distinct subpopulations that respond to different groups of taste compounds. GSNs that express a set of gustatory receptor (Gr) genes including *Gr5a* are activated by sucrose, trehalose and some other sugars (Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006). Another set of Grs including *Gr66a* is expressed in a different GSN population, which responds to caffeine and other compounds that, in high concentrations, can sometimes be toxic (Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006). Use of a genetic method led to the identification of a GSN population that participates in responses to low osmolality (Inoshita and Tanimura, 2006), and later *ppk28* was identified as the gene responsible for taste detection in these GSNs (Cameron et al., 2010). *Ir76b* is expressed in a different set of GSNs and responds to low concentrations (~100 mM) of sodium chloride (Zhang et al., 2013). Another population of GSNs is activated by carbonated water (Fischler et al., 2007).

These subpopulations of GSNs distribute their axon terminals in distinct subregions in the PGC. *Gr5a*⁺ GSNs send their axons to the ventral and lateral portion of the PMS (PMS4), while *Gr66a*⁺ GSNs project to the dorsal and medial subregions of the PMS (PMS1-3) (Wang et al., 2004). *Ir76b*⁺ GSNs distributed axon terminals in the AMS and the PMS. In the PMS, axon terminals of *Ir76b*⁺ GSNs overlap with those of *Gr5a*⁺ and *Gr66a*⁺ GSNs (Zhang et al., 2013). *ppk28*⁺ GSNs also send their axons to the PMS4 (Cameron et al., 2010), while GSNs recognizing carbonated water project to the AMS (Fischler et al., 2007). These observations suggest that the locations of GSN terminals may be compartmentalized by taste quality. However, it is necessary to know how second-order neurons integrate their inputs to understand how taste information is processed in this first gustatory center.

Taste inputs mediated by GSNs exert a variety of effects on animals' behavior, but many questions remain about the neural pathways linking the GSNs to downstream circuits. Activation of GSNs expressing *Gr5a* evokes a series of feeding behaviors such as proboscis

extension and cibarial pumping, while activation of GSNs expressing *Gr66a* antagonizes the activation of GSNs with *Gr5a* (Marella et al., 2006). The neural circuits involved in these behaviors have been investigated. Proboscis extension is driven by a bilateral pair of motor neurons that elicits contraction of the muscles in the basal segment of the proboscis (Gordon and Scott, 2009), and several additional neurons innervate the muscles of the cibarial pump (Manzo et al., 2012). A pair of cells identified as feeding command neurons induces a series of related behaviors: foreleg bending, proboscis extension, labellum opening and cibarial pumping (Flood et al., 2013). However, these motor circuits do not receive direct synaptic input from the axon terminals of GSNs. In addition, presentation of sugar or water to the proboscis functions as an unconditioned cue to reinforce appetitive learning about olfactory stimuli, suggesting that taste information may be transmitted to the mushroom body, a site of olfactory learning (Schwaerzel et al., 2003; Liu et al., 2012; Lin et al., 2014). Yet, the neural pathways linking these regions have not been identified.

Recently, a group of projection neurons has been reported to be gustatory second-order neurons (Kain and Dahanukar, 2015). These neurons, identified through a behavioral screen, and found to participate in proboscis extension, make synaptic connections onto *Gr5a*-expressing GSNs and innervate the antennal mechanosensory and motor center (AMMC), a brain region adjacent to the GNG. However, other interneurons that immediately follow GSNs remain to be identified and characterized. Understanding the roles of such neurons is essential for understanding how the brain processes gustatory information. Here, we used an anatomical screen to identify a novel type of gustatory second-order neuron. We started by examining >5,000 strains of GAL4 driver collections (Yoshihara and Ito, 2000; Hayashi et al., 2002; Jenett et al., 2012) for lines that sparsely label neural fibers projecting to the PGC. Then, we investigated the neural connectivity between these candidate neurons and *Gr5a*-expressing GSNs using the GRASP technique, which we combined with a presynaptic label to discriminate synapses from incidental contacts between neural fiber membranes. Among the resulting strains, we identified a line that labels a bilateral pair of short projection interneurons different from previously reported neurons (Kain and Dahanukar, 2015). Further, using FLP-out recombination, we genetically isolated and characterized the morphologies of single candidate gustatory second-order neurons. Finally, using fluorescence-labeled markers, we identified input and output sites of the neurons. Our findings suggest that information about taste is processed by circuitry within the GNG before it is sent further into the brain.

MATERIALS AND METHODS

Experimental animals

Fly strains were maintained on standard *Drosophila* medium at 19-25°C. The following transgenic lines were used in this study: 1) *Gr5a-LexA::VP16* (Gordon and Scott, 2009), 2) *NP763-GAL4* (Yoshihara and Ito, 2000; Hayashi et al., 2002), 3) *R12C04-GAL4* (Jenett et al., 2012), 4) *R20G06-GAL4* (Jenett et al., 2012), 5) *R77C10-GAL4* (Jenett et al., 2012), 6) *R73G10-GAL4* (Jenett et al., 2012), 7) *UAS-DsRed S197Y* (Verkhusha et al., 2001), 8) *lexAop-spGFP11::CD4* (Gordon and Scott, 2009), 9) *UAS-spGFP1-10::CD4* (Gordon and Scott, 2009), 10) *lexAop-Brp::mCherry*, 11) *UAS-CD8::GFP* (Lee and Luo, 1999), 12)

hsFLP¹²² (also known as *hsp70-FLP*) (Struhl and Basler, 1993), 13) *UAS-FRT-CD2, y⁺-FRT-CD8::GFP* (Wong et al., 2002), 14) *20xUAS-IVS-FRT-stop-FRT-spGFP1-10::CD4::HA* (see below), 15) *lexAop-CD2::RFP* (Awasaki et al., 2011), 16) *UAS-CD8::mCherry*, 17) *UAS-Da7-GFP*, 18) *UAS-Brp::mCherry*, 19) *UAS-HRP::CD2*, 20) *R12C04-LexA::p65* (see below), 21) *lexAop-HRP::CD2* (Karuppururai et al., 2014), 22) *Gr5a-GAL4* (Wang et al., 2004), 23) *Gr66a-GAL4* (Scott et al., 2001), 24) *Ir76b-GAL4* (Zhang et al., 2013), 25) *ppk28-GAL4* (Cameron et al., 2010), 26) *Ir76b-QF* (Zhang et al., 2013), 27) *QUAS-mtdTomato::3xHA* (Potter et al., 2010), 28) *lexAop-CD2::GFP* (Lai and Lee, 2006), 29) *13xLexAop2-IVS-FRT-stop-FRT-spGFP11::CD4::HA-T2A-Brp^{D3}::mCherry (attP2)* (Karuppururai et al., 2014), 30) *UAS-Syb::spGFP1-10* (Karuppururai et al., 2014). To label single cells by the FLP-out recombination method, wandering larvae or early pupae were treated with 39°C heat shock for 20 min (for *UAS-FRT-CD2, y⁺-FRT-CD8::GFP*), 5 min (for *20xUAS-IVS-FRT-stop-FRT-spGFP1-10::CD4::HA*) or 0–2 min (for *13xLexAop2-IVS-FRT-stop-FRT-spGFP11::CD4::HA-T2A-Brp^{D3}::mCherry (attP2)*) and then kept at 25°C until dissection.

Generation of transgenic flies

To generate the *20xUAS-IVS-FRT-stop-FRT-spGFP1-10::CD4::HA* fly strain, first, the 1469-base DNA fragment of the *spGFP1-10::CD4::HA* accompanied by *C. elegans pat-3* signal peptide at its N-terminal was synthesized by a commercial service (Genscript, Piscataway, NJ): the sequence of the transgene was optimized for the codon usage of *Drosophila*, *NotI-KpnI* and *XbaI* sites were added at the 5'- and 3'-terminals, respectively, and the synthesized DNA was subcloned in pUC57. Second, the *NotI-XbaI*-digested *spGFP1-10::CD4::HA* fragment was subcloned into the pJFRC7-derived vector (Pfeiffer et al., 2010), which were also digested by the same set of enzymes. Third, the resulting vector was digested by *KpnI*, and then the stop cassette derived from the *KpnI*-digested *13xLexAop2-IVS-STOP-spGFP11CD4-HA-T2A-Brp^{D3}-mCherry* (Karuppururai et al., 2014) was inserted. The transgene was integrated into the *VK00033* site on 3L.

To generate the *R12C04-LexA::p65* fly strain, the 10.4 kb DNA enhancer fragment of *R12C04* (Jenett et al., 2012) was amplified from the Canton-S genome by PCR (primer sequences 5'-CGGTATCTCAAGAATCGTCGCCATA-3' and 5'-GCATGACCAATTCGTGTGGGTAAAC-3') using Phusion High-Fidelity DNA polymerase (Thermo Scientific). The fragment was cloned using a pCR8/GW/TOPO TA Cloning Kit (Invitrogen) and was then transferred into a transformation vector *pBPLexA::p65Uw* (Pfeiffer et al., 2010) using Gateway LR Clonase II Enzyme mix (Invitrogen). The transgene was integrated into the *attP2* site on 3L.

The transgenes were integrated using the standard PhiC31-mediated transformation protocol by Rainbow Transgenic Flies Inc. (Camarillo, CA). The sequences of constructs were confirmed by sequencing.

Anatomical screening of GAL4 strains

Two GAL4 driver collections, NP (Yoshihara and Ito, 2000; Hayashi et al., 2002) and FlyLight (Jenett et al., 2012), were examined for lines that label specific and sparse subsets

of neurons in the brain. From the NP collection, we examined 1314 GAL4 enhancer-trap strains and identified 41 lines that label neural fibers innervating the GNG. Next, to refine the selection, these 41 lines were crossed with the *UAS-DsRed S197Y* reporter strain, yielding 8 candidates with specific labeling in the PGC. From the FlyLight collection, lines that label neural fibers in the GNG sparsely were examined using descriptors in the collection's online search program, yielding 963 strains. Next, we examined high-resolution confocal stacks of these strains to identify lines that show specific labeling in the PGC, yielding 24 candidate strains. Altogether, our anatomical screening yielded 32 candidates.

Immunohistochemistry

Female flies aged at least 5 days after eclosion at 25° were examined. Fixative was prepared as follows: 4% paraformaldehyde (methanol free, RNase free, EM grade, #15713-S; Electron Microscopy Sciences, Hatfield, PA) in 0.1 M Sorenson's buffer (61 mM Na₂HPO₄, 39 mM NaH₂PO₄). The brain was dissected from the head capsule in the fixative and fixed for 2-6 hours on ice. Samples were rinsed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 6.1 mM Na₂HPO₄, 3.9 mM NaH₂PO₄), twice with PBS containing 0.5% (v/v) Triton X-100 (PBT), were subsequently incubated in blocking solution (BS) containing 10% (v/v) normal goat serum and 0.02% (v/v) ProClin 150 in PBT for 1 hour, then in a mixture of primary antibodies in BS overnight. Samples were then rinsed three times with PBT and incubated with a mixture of secondary antibodies in BS overnight. After three more rinses with PBT and two rinses with PBS, samples were cleared with a 2-hour incubation in 50% (v/v) glycerol in PBS and subsequent overnight incubation in 80% (v/v) glycerol in PBS. The samples were mounted on slides with 0.0085 inch-thick vinyl electrical tape (Super 88; 3M Electrical Markets Division, Austin, TX) as spacers. For experiments in which only raw fluorescence of GFP and mCherry/RFP was detected (Figure 1 and Figure 2D), treatments with PBT, BS and antibody mixture were omitted from the protocol; that is, fixed samples were just rinsed with PBS and then cleared in glycerol-PBS.

GFP fluorescence in all experiments and mCherry/RFP fluorescence shown in figures other than Figure 3D and Supplementary Figures S1A-C were detected directly without enhancement by immunolabeling to avoid increased background noise. Primary antibodies used were nc82 (mouse anti-Brp (Wagh et al., 2006); 1:20 dilution), mouse anti-HRP (2H11, Abcam), rat anti-HA (3F10, Roche), rat anti-RFP (5F8, Allele Biotechnology), rabbit anti-human CD4 (Sigma HPA004252) and chicken anti-GFP (Abcam #13970; 1:10,000 dilution). Secondary antibodies used were goat anti-mouse IgG conjugated with Alexa Fluor 647 (Life technologies, A-21236), goat anti-rat IgG conjugated with Alexa Fluor 488 (Life technologies, A-11006) and Alexa Fluor 568 (Life technologies, A-11077), goat anti-rabbit IgG conjugated with Alexa Fluor 568 (Life technologies, A-11036), goat anti-chicken IgG conjugated with Alexa Fluor 647 (Life technologies, A-21449). Antibodies were used at 1:500 dilution unless otherwise specified.

Confocal serial optical sections at 0.20–1.24-μm intervals were taken by LSM 510 or LSM780 confocal microscopes (Carl Zeiss) with water-immersion 40× C-Apochromat (NA 1.2), oil-immersion 63× Plan-Apochromat (NA 1.4) and glycerol-immersion 63× Plan-Neofluar (NA 1.3) objective lenses. Confocal datasets were processed by Image J software.

When a region of interest was larger than the viewing field of the microscope, confocal sections of adjacent viewing fields were stitched using an Image J plug-in (Preibisch et al., 2009). To enhance some magnified images (Figures 2C4, 2C5, 3E, 3F), confocal datasets were deconvolved by Huygens Pro software (Scientific Volume Imaging, Hilversum, The Netherlands). For morphological comparisons of labeled neurons across animals, confocal datasets were registered to the nc82 channel using the Computational Morphometry Toolkit (Neuroimaging Informatics Tools and Resources Clearinghouse). For 3D reconstruction, confocal datasets were processed with FluoRender software (ver. 2.13, University of Utah, UT) (Wan et al., 2012). The size, resolution, and contrast of the final images were adjusted uniformly with Photoshop software (Adobe Systems, San Jose, CA).

RESULTS

Identification of candidate gustatory second-order neurons

By definition, the dendrites of gustatory second-order neurons (G2Ns) receive synaptic inputs from the axonal terminals of gustatory sensory neurons (GSNs). To find candidate neurons with dendrites in the PGC, we screened anatomically the neurite patterns of more than 5,000 strains from two GAL4 driver collections (see Materials and Methods). We identified 32 candidate strains, each of which labels a distinct subset of neurons that extends neural fibers into the PGC.

To determine whether these neurons form synaptic contacts with GSN terminals, we used the GRASP (GFP reconstituted across synaptic partners) method (Gordon and Scott, 2009) combined with presynaptic labeling (Karuppururai et al., 2014). We used the *Gr5a-LexA* driver to express one half of the GFP moiety (CD4::spGFP11) in the *Gr5a*-expressing GSNs, and various candidate GAL4 drivers to express the other half of the GFP moiety (CD4::spGFP1-10) in the candidate G2N neurons. Functional GFP was reconstituted at the membrane contacts of these two neuronal populations. To differentiate true synapses from mere membrane contacts, we additionally expressed a presynaptic marker, Brp-mCherry, in the *Gr5a*-expressing GSNs to label their presynaptic sites, and used co-localization of reconstituted GFP (GRASP) and Brp-mCherry signals (Figure 1) to indicate potential synaptic contacts.

Among the 32 candidate GAL4 driver strains, 18 strains showed overlaps between the GRASP signals and the presynaptic label (examples are shown in Figures 1A-C), suggesting that neurons labeled with these lines are directly postsynaptic to *Gr5a*-expressing GSNs. Notably, nine strains showed significant GRASP signals that did not overlap with presynaptic signals (Figure 1D). This observation showed that, in principle, false positive GRASP signals, those not indicating synapses, could be identified and excluded from our sample. Among the 18 positive candidates, *R12C04* exhibited the sparsest labeling pattern (Figures 2C, E, F). Because the sparse labeling allowed us to delineate the structures and connectivity of individual neurons, we focused on neurons labeled with this line.

Anatomical characterization of a candidate gustatory second-order neuron

To characterize the location and structure of candidate G2Ns labeled with the *R12C04* driver line, we visualized their entire structures and specific cellular components by expressing in them a series of reporter proteins (Figures 2A-C, D, Figure 3). *R12C04-GAL4* indeed sparsely labeled neural fibers projecting to the GNG. This driver also labeled neurons in other parts of the brain that do not overlap the GNG: the mushroom body (MB) lobes; the ellipsoid body (EB); and olfactory local interneurons in the antennal lobes (ALs) (Figures 2A, B). But in each hemisphere, in the region around the GNG, only two neurons were specifically labeled, with their neural fibers confined entirely within the GNG (Figure 2C). One bilateral pair has cell bodies in the most anterior and dorsal part of the GNG (black arrowheads in Figure 2C) and extends neural fibers to the anterior dorsal GNG (Figures 2C2-2C5), while the other bilateral pair has cell bodies in the ventrolateral GNG (white arrowheads in Figures 2C) and distributes neural fibers in ventral and more posterior regions of the GNG (Figures 2C2-2C5). The neural fibers from these two pairs of neurons are distributed to adjacent subregions within the GNG, but the fibers are isolated from each other throughout their lengths, allowing us to clearly distinguish them from one another (see Figures 2C2-2C5). Dual labeling using *Gr5a-LexA* and *R12C04-GAL4* drivers revealed that only the labeled neurons with ventrolateral cell bodies innervate the axon terminals of *Gr5a*⁺ GSNs; labeled neurons with anterior and dorsal cell bodies do not (Figure 2D). Therefore, the *R12C04*-labeled neurons whose cell bodies are located in the ventrolateral GNG are G2Ns. We named these neurons G2N type 1 (G2N-1).

The neural process extending from the G2N-1 cell body runs along the ventral edge of the GNG, and contacts sucrose-responsive GSNs where they terminate in a region called PMS4 (Miyazaki and Ito, 2010). G2N-1's processes first enter the ventral PMS4 (vPMS4) and widely bifurcate (Figure 2D1). Three main branches extend from the vPMS4, extending in ventral-medial, dorsal-medial, and dorsal-lateral directions. While the ventral-medial branch bifurcates only a few times, the other two branches form many arborizations. The ventral-medial branch reaches beyond the midline, the dorsal-medial reaches the dorsal portion of the PMS4 (dPMS4), and the dorsal-lateral branch distributes its arborizations into a region close to PMS4 in the anterior-posterior direction (Figures 2D1, D2). Because the location of the target region of the third branch implied that it belongs to the posterior part of the maxillary neuromere of the GNG, we named this region the “lateral posterior maxillary zone” (IPMZ).

The branch projecting to the dPMS4 reaches the midline, and the ventral-medial branch extends beyond the midline, so that neural processes from G2N-1 cell bodies on both sides of the brain intermingle. To differentiate these processes, we used the FLPout technique (Wong et al., 2002) to label a single G2N-1. The single-cell labeling revealed that G2N-1 extends processes to the ipsilateral vPMS4, dPMS4 and IPMZ, and the contralateral vPMS4 and dPMS4; no neural processes reach the IPMZ on the contralateral side (Figure 3A). Although the processes of G2N-1 reliably targeted stereotyped locations, the positions of their cell bodies varied substantially from animal to animal (Figure 3A3).

Next, to visualize the localizations of output sites of a single G2N-1, we used a transgenic construct for bicistronic expression of cellular-membrane and presynaptic markers in a single cell selected by FLPout recombination (*lexAop-FRT-stop-FRT-spGFP11::CD4::HA-T2A-Brp^{D3}::mCherry*) (Karupudurai et al., 2014). To use this system for single-cell labeling of the R12C04 neuron, we first generated a LexA version of the driver strain (*R12C04-LexA::p65*), which visualized two pairs of cell bodies including those of G2N-1s, like the *R12C04-GAL4* line (Supplementary Figures S1A-C). Subsequent FLPout recombination carried out with this driver-reporter combination revealed a projection pattern similar to those obtained by the conventional single-cell labeling (Figure 3B). This approach also revealed that Brp^{D3}::mCherry signals were distributed in every projection target of the labeled cell, indicating that G2N-1 has output sites in all of its projection targets: the ipsilateral vPMS4, dPMS4 and IPMZ, and the contralateral vPMS4 and dPMS4.

When we expressed CD8::GFP in G2N-1s as a cell-membrane marker, we were able to observe fine structures of the neural fibers (see Figure 3E). To characterize in detail the distribution of output areas of G2N-1s, we co-expressed fluorescence-tagged Brp protein pre-synaptic markers (Figures 3C, 3E). Brp::mCherry presynaptic signals are localized predominantly in the IPMZ, though limited presynaptic label was also visible in the vPMS4 and the dPMS4 (Figures 3C1, 3C2). Detailed observation of the IPMZ region (Figures 3E1, 3E2) revealed labeled branches with many bleb-like structures co-localized with the presynaptic marker. This feature generally corresponds to characteristics of axons in insect neurons (Fischbach and Dittrich, 1989) and suggests that these branches comprise the axonal portion of G2N-1.

To characterize the input areas of the G2N-1s, we expressed in them acetylcholine receptor subunits (D α 7) fused with GFP (Figures 3D, 3F). The D α 7::GFP fusion protein appeared only in the vPMS4 and the dPMS4 (Figures 3D1, 3D2), where the neural processes of G2N-1 were dotted with many spines (Figures 3F1, 3F2). Intriguingly, most of the post-synaptic signals came from the tips and kinks of the spines. These features generally correspond to characteristics of dendrites in insect neurons (Fischbach and Dittrich, 1989), suggesting that the vPMS4 and the dPMS4 arborizations of G2N-1 are dendritic.

In sum, the bilateral G2N-1s appear to receive synaptic inputs exclusively in the vPMS4 and the dPMS4, and provide synaptic output predominantly in the ipsilateral IPMZ. The signals of the presynaptic marker in vPMS4 and dPMS4 suggest that the dendrites of G2N-1s are not exclusively postsynaptic but rather both pre- and post-synaptic, probably forming local collateral output as also seen in optic lobe neurons (Gao et al., 2008; Takemura et al., 2013).

G2N-1s receive synaptic inputs from sucrose-responsive GSNs, but not caffeine-responsive GSNs

Thus, G2N-1s form post-synaptic terminals in the vPMS4 and the dPMS4, regions that also receive axonal projections from *Gr5a*-labeled GSNs. We also observed GRASP signals between neurons labeled with *Gr5a* and R12C04 in vPMS4 and dPMS4, consistent with synaptic connectivity between the receptor neuron responsive to sugars and G2N-1 (Figures 4A, B). Next, we tested whether another type of GSN makes synaptic connections onto the G2N-1s.

Gr66a, a receptor responsive to caffeine (Moon et al., 2006), is expressed in GSNs other than those expressing *Gr5a*. *G66a*⁺ GSNs, which are activated by a variety of compounds aversive to flies (Marella et al., 2006), send axons to the more dorsal and medial region of the posterior maxillary sensory center (PMS1, 2, 3) in the PGC, a region different from the projection site of *Gr5a*⁺ GSNs, the ventral part (PMS4) (Thorne et al., 2004, Wang et al., 2004, Miyazaki and Ito, 2010). Because *Gr66a*⁺ axons pass through the region where G2N-1's dendrites bifurcate, there was a possibility that G2N-1s might form synaptic contacts also with the terminals of *Gr66a*⁺ GSNs. To test this we used GRASP combined with the presynaptic marker Brp::mCherry to check for synaptic contacts between these neurons. No colocalized GRASP- Brp::mCherry signals were observed between G2N-1s and GSNs expressing *Gr66a* (Figure 4C), suggesting that the *Gr66a*⁺ axons do not provide synaptic outputs onto the G2N-1s.

These anatomical results suggest that G2N-1 receives synaptic input selectively from GSNs expressing *Gr5a* in the vPMS4 and the dPMS4, but not from GSNs expressing *Gr66a*. However, because GFP fragments are expressed throughout the entire surface of the neurons with the conventional GRASP method, it remained unclear whether all the colocalized GRASP and presynaptic signals we observed represented *bona fide* synaptic contacts. We therefore employed a new version of GRASP method called Syb-GRASP (Karuppururai et al., 2014). With this method, N-terminal fragments of split GFP expressed in the presynaptic neurons are tethered to the synaptic vesicle protein synaptobrevin (Syb::spGFP1-10), which are exposed to the cellular surface only at presynaptic sites. Thus, although postsynaptic neurons present complementary fragments of split GFP throughout their entire surface, contact between split GFP components occur only at synapses.

We first expressed Syb::spGFP1-10 in the *Gr5a*⁺ GSNs and confirmed that the split GFP components were selectively localized in their presynaptic terminals, as expected (Figure 5A2). We then expressed Syb::spGFP1-10 in the *Gr5a*⁺ or *Gr66a*⁺ GSNs, and expressed spGFP11::CD4 in G2N-1s. We detected clear GRASP signals between *Gr5a*⁺ GSNs and the G2N-1s, but not between *Gr66a*⁺ GSNs and G2N-1s (Figure 5A3, B3). These more specific observations are consistent with the rest of our results, and together suggest G2N-1s receive synaptic inputs from sucrose-responsive GSNs activated but not caffeine-responsive GSNs.

DISCUSSION

To identify G2Ns, we first conducted a large-scale screening of GAL4 driver strains that sparsely label neurons projecting into the brain region receiving outputs from GSNs. Narrowing our selection by using GRASP to identify G2N candidates that form synapses with *Gr5a* GSNs, we identified the R12C04 driver line, which labels a pair of neurons (G2N-1s) in the GNG. Single-cell labeling showed that each G2N-1 innervates dPMS4, vPMS4 and lPMZ ipsilaterally, and sends neural fibers to dPMS4 and vPMS4 contralaterally. Labeling with marker proteins showed all of these projection targets contain presynaptic output sites, but postsynaptic input sites are distributed only within dPMS4 and vPMS4. A further analysis using conventional and Syb-GRASP showed that G2N-1s do not receive synaptic inputs from *Gr66a* GSNs, which respond to caffeine.

Screening strategy to identify gustatory second-order neurons

Genetic manipulations have been used to reveal the wiring of primary sensory neurons in the *Drosophila* gustatory system. For example, genetic labeling with promoters of gustatory receptor genes showed that *Gr5a*- and *Gr66a*-expressing GSNs project their axons to separate subregions in the PGC (PMS4,5 and PMS1-3, respectively) (Wang et al., 2004). Similar targeted strategies showed that a different population of *Ir76b*-expressing GSNs innervates distinct regions that overlap with the projection targets of *Gr5a* GSNs (Zhang et al., 2013). However, such a targeted genetic strategy is unsuitable for the identification of second-order neurons in the gustatory system because little is known about the gene expression patterns in these neurons.

Another approach to identify specific neuronal populations is to conduct screens based on transgenic expression driven by promoter constructs or trapped endogenous enhancers. For example, GAL4 enhancer-trap strains were used to identify GSNs that respond to water (low osmolality) (Inoshita and Tanimura, 2006) and carbonated water (Cameron et al., 2010). Other GSNs and taste-related mechanosensory neurons have also been identified through large-scale screenings of GAL4 enhancer-trap strains (Miyazaki and Ito, 2010). Behaviors, combined with genetic manipulations, can also serve as read-outs. For example, motor neurons that control components of feeding behavior, such as proboscis extension and cibarial pumping (Gordon and Scott, 2009; Manzo et al., 2012) were identified through tests that screened for behavioral changes following cell-type selective silencing of neurons directed by the enhancer-trap system. Similarly, motor command neurons that evoke a series of feeding behaviors were identified by observing the behavioral effects of cell-type selective manipulations of neurons by the enhancer-trap method (Flood et al., 2013).

Recently it was shown that enhancer-trap strain NP1562 labels secondary gustatory neurons that receive synaptic inputs from *Gr5a* GSNs that participate in feeding behaviors (Kain and Dahanukar, 2015). Although these neurons were shown to innervate the AMMC, details of their morphology and connectivity remain to be elucidated because, as is common with driver lines that induce strong behavioral phenotypes, the enhancer-trap strain used in this study labeled many neurons. Additional genetic manipulations of these strains, such as the restriction of labeled cells by chromosomal recombination, can be used to achieve single-cell resolution (Gordon and Scott, 2009; Flood et al., 2013).

To identify G2Ns, we followed an anatomical approach. We first searched through GAL4 driver lines for sparse labeling of neural fibers in the PGC. Candidate lines such as NP1562 that label many neurons were excluded from further testing. After this primary screen, we conducted a secondary anatomical assay, using GRASP to test whether the candidate labeled neurons make synaptic contacts with GSNs that respond to sucrose. Among the resulting candidate strains, we focused on the line that exhibited the sparsest expression pattern, R12C04. This line visualized a bilateral pair of neurons near the PGC, which we named G2N-1 (Figure 2). Further analysis using the FLP-out technique restricted label expression to single neurons, and allowed us to trace the innervation patterns of neural fibers from single G2Ns (Figure 3). This anatomical approach led us to directly and efficiently identify neural circuitry that immediately follows GSNs.

Our screening process yielded 17 candidate strains in addition to R12C04 that exhibited positive GRASP results. The labeling patterns of these lines included neurons other than G2N-1s. The line R20G06, for example, labels 4-6 cell bodies in the ventrolateral GNG that distribute neural fibers mainly in the most ventral part of the PGC (Figure 2F), while G2N-1s project neural fibers to more dorsal parts (dPMS4 and IPMZ) (Figures 2C, D). The R20G06-labeled neurons are also different from another recently identified population of gustatory second-order neurons whose cell bodies are located in the dorsal GNG (Kain and Dahanukar, 2015). These observations show that additional types of G2Ns remain to be identified.

To use GRASP for screening candidate second-order neurons, we expressed presynaptic split GFP fragments in *Gr5a*⁺ GSNs, allowing us to identify candidate gustatory neurons that follow sugar-responsive GSNs. However, other types of GSNs also project to the PGC. Thus, identifying the full set of G2Ns will require additional screening for neural connections between other types of GSNs and candidate followers. It will be interesting to determine whether some G2Ns receive input from multiple types of GSNs.

Neural circuits of G2N-1

The functions of G2N-1 are unknown, but our analysis of its morphology and connectivity provides some interesting clues. Our FLPout analyses showed that a single G2N-1 neuron extends neural fibers to the dPMS4, vPMS4, IPMZ on the ipsilateral side of the brain, and to the dPMS4 and vPMS4 on the contralateral side (Figure 3A, 3B). Interestingly, although G2N-1s receive postsynaptic signals in the dPMS4, vPMS4s, the output targets were observed in the dPMS4 and vPMS4 (Figure 3C-3F). Thus, a pair of G2N-1s interconnects both hemispheres.

Notably, The *Gr5a*⁺ neurons, the presynaptic partners of the G2N-1s, make only unilateral projections (Wang et al., 2004). Unilateral projection patterns are also found in some other neurons involved in feeding behavior evoked by sugar stimuli, such as motor command neurons, which innervate neural fibers only on the ipsilateral side (Flood et al., 2013), and motor neurons that control muscles for proboscis extension, which form dendritic arborizations only on a single side (Gordon and Scott, 2009). However, other classes of motor neurons required for cibarial pumping extend neural processes contralaterally (Manzo et al., 2012). One can speculate that G2N-1's cross-midline branches may help synchronize movements of both sides of the mouthparts.

The secondary gustatory projection neurons that innervate the AMMC have been shown to be necessary and sufficient to evoke proboscis extension behavior (Kain and Dahanukar, 2015). Therefore, the G2N-1s may contribute to behaviors other than proboscis extension, such as cibarial pumping. G2N-1s may also serve a modulatory role in feeding behavior. Using the driver line (*R12C04-GAL4/LexA::p65*), calcium flux or voltage changes in G2N-1s can be specifically recorded by fluorescence imaging *in vivo* and used to assess neural activity. The neural functions of G2N-1s can also be manipulated by selective expression of neural toxins or photo/thermosensitive cation channels. Combined imaging and behavioral experiments will no doubt shed light on the functions of G2N-1s.

Taste stimuli detected by *Gr5a*⁺ GSNs have been shown to serve multiple functions in *Drosophila*. First, activation of *Gr5a*⁺ GSNs evokes feeding behavior (Marella et al., 2006). Second, stimuli activating *Gr5a*⁺ GSNs provide effective cues for associative learning (Schwaerzel et al., 2003; Liu et al., 2012). It will be interesting to determine whether G2N-1s, which receive synaptic inputs from *Gr5a*⁺ GSNs, are involved in either or both of these processes.

A recently identified population of second-order gustatory neurons receiving synaptic input from *Gr5a* GSNs was found to project neural fibers to, and form output synapses in, the AMMC, a brain region adjacent to the GNG (Kain and Dahanukar, 2015). Thus, these neurons can be classified as secondary gustatory projection neurons. The G2N-1s we identified collect inputs from GSNs in subregions of the PGC –dPMS4, vPMS4 – and send their outputs to lPMZ, a nearby region that is adjacent to but not within the PGC. Because they link the inside and outside of the PGC and have polarized distributions of postsynaptic input, these cells can be regarded as short projection interneurons. The presence of widely-branching gustatory short projection interneurons within the GNG suggests that information about taste is processed by circuitry within the GNG before it is sent further into the brain, suggesting unexpected complexity for taste information processing in the first relay of the gustatory system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

GRASP analyses revealed potential synaptic contacts between *Gr5a*-expressing GSN and candidate second-order neurons. (A-E) Candidate Gal4 lines, *NP763-GAL4* (A), *R12C04-GAL4* (B), *R20G06-GAL4* (C), *R77C10-GAL4* (D), and *R73G1-GAL4* (E) were used to drive the expression of a membrane-tethered split-GFP moiety (spGFP1-10::CD4) while *Gr5a-LexA::VP16* drove the expression of the other split-GFP moiety (spGFP11::CD4). Membrane contacts between these two cell populations were examined by the native fluorescence of reconstituted GFP (A2-E2). The presynaptic terminals of the *Gr5a*⁺ GSNs, were labeled by the active zone marker, *Brp::mCherry* (A1-E1). A3-D3 show colocalization of GSN presynaptic terminals and GRASP signals. The right panels (A4-D4) show overlays of the GSN presynaptic areas shown in A1-A4 (magenta) and the colocalization shown in A3-D3 (green). (A-C) Three GAL4 driver strains (*NP763*, *R12C04* and *R20G06*) showed positive colocalization between the GSN synaptic areas and the GRASP signals. (D) *R77C10-GAL4* showed GRASP signals (D2), but there is no colocalization between the presynaptic areas and the GRASP signals (D3). *R73G10-GAL4* driver does not show GRASP signals (E2). (F) A schematic diagram showing the GNG region and the axonal terminals of *Gr5a*-expressing GSN. Scale bar: 10 μ m in A1 for A1-E2.

Figure 2.

Expression patterns of three candidate lines. (A) A schematic diagram of the fly's central brain (frontal view). The GNG (cyan) is located in the ventral region of the brain. The cell bodies and neural fibers of the G2Ns are shown in green. Other prominent brain regions, including the antennal lobes (ALs), the mushroom bodies (MBs) and the ellipsoid body (EB), are also shown. (B-F) Candidate GAL4 lines R12C04 (B-D), NP0763 (E) and R20G06 (F) were used to drive expression of a membrane-tethered GFP reporter (CD8::GFP, white in B, C, E, F; green in D). Neuropils were visualized with nc82 antibody staining (dark orange, in B, C1-C3). The axon terminals of *Gr5a* GSNs are labeled with the CD2::RFP reporter driven by *Gr5a-LexA* driver (magenta, D1-D2). (B-D) Labeling pattern of the R12C04 driver strain in the central brain. Frontal views, (B, C1, D1); side views, (C2, C4, D2); ventral view (C3, C5). (B) In addition to neural fibers in the GNG, the R12C04 driver strain labels the ALs, the MB lobes and the EB. (C1-C3) Magnified views of the GNG (orange dotted rectangle of [B]). The R12C04 driver strain labels two pairs of neurons, a ventrolateral (white arrowheads) and a dorsal (black arrowheads) pair, in the GNG. With respect to panel C1, only the signals within the observer's right hemisphere (C2) and the GNG (C3) are visualized. (C4, C5) Magnified views of the region where neural fibers extending from the different neurons are in close proximity (orange dotted rectangles in [C2] and [C3], respectively). The neural fibers extending from the ventrolateral cell body (white arrows) and the dorsal cell body (black arrows) are separate from each other. (D) The ventrolateral, but not the dorsal, neurons innervate the terminal areas of the *Gr5a* GSNs and are thus considered candidate G2Ns. The area corresponding to the blue dot-and-dash rectangles in [C] is shown. For clarity, signals of the R12C04-labeled fibers were separated (see Supplementary Movie S1), and only signals from neural fibers extending from the

ventrolateral cell body are visualized. The neural fibers extending from the ventrolateral neuron innervate the *Gr5a* terminal areas (vPMS4, dPMS4) and another zone (IPMZ). (E, F) The labeling patterns of NP763 (E) and R20G06 (F) driver strains in the GNG. These strains label many neurons, making it difficult to distinguish and trace single neural fibers. D: dorsal; V: ventral; M: medial; L: lateral; A: anterior; P: posterior. Scale bars: 50 μ m in B; 50 μ m in C1 for C1-C3; 10 μ m in C4 for C4, C5; 10 μ m in D1 for D1, D2; 50 μ m in E for E, F.

Figure 3.

Single-cell morphology and pre- and postsynaptic sites of G2N-1s. (A1-A3) Single R12C04 neuron clones were generated using the FLPout method to express the CD8::GFP marker (see Methods for details). The morphologies of the neurons were visualized with the native fluorescence of GFP or anti-HA immunolabeling against spGFP1-10::CD4::HA (green). Neuropil landmarks were visualized by nc82 immunolabeling (gray). (A1, A2) Two examples of the ventrolateral neurons. Neural fibers extending from the cell body (white arrowheads) innervate vPMS4, dPMS4 and IPMZ on the ipsilateral side of the cell body, but they innervate only vPMS4 and dPMS4 on the contralateral side. In A1, the branch innervating the ipsilateral dPMS4 extends into the contralateral side, while the dorsal branch ends at the midline in A2. (A3) Overlay of four single neurons from different flies registered to neuropil landmarks (grey). While the distributions of the neurites are stereotypical, the locations of cell bodies vary substantially. (B1-B2) The distribution of presynaptic sites in a single neuron clone. Single R12C04 neuron clones were generated by the FLPout method to express the membrane marker spGFP11::CD4::HA, and the active zone marker Brp^{D3}::mCherry. Neural fibers and presynaptic sites of the labeled neuron were visualized by anti-HA immunolabeling (green) and native mCherry fluorescence (magenta), respectively. The presynaptic sites of R12C04 neurons are visible in neurites projecting to all major regions, ipsilateral dPMS4, IPMZ and vPMS4, and contralateral sPMS4 and vPMS4. Neuropils labeled by nc82 immunostaining (blue) were used as landmarks. (B1) frontal view; (B2) side view. Because anti-HA labeling yielded dot-like background staining (see Supplementary Figure S1D), the signals from the labeled neuron have been extracted and 3D-reconstructed. (C1, C2, E1, E2) Detailed distribution of the presynaptic areas of the G2N-1s. The R12C04 driver was used to express the active zone marker, Brp^{D3}::mCherry

(magenta in C1, E1; single-channel gray-scale in C2, E2) and a membrane GFP marker, CD8::GFP (green in C1, E1) to label presynaptic sites and neurites, respectively. The presynaptic sites are mainly localized in IPMZ. Sparse presynaptic markers were also observed in vPMS4 and dPMS4. (D1, D2, F1, F2) Postsynaptic sites of the G2N-1s. The postsynaptic areas and the entire neural fibers are visualized with *UAS-Da7-GFP* (pseudocolored magenta in D1, F1; single-channel gray-scale in D2, F2) and *UAS-CD8::mCherry* (pseudocolored green in D1, F1) reporters, respectively. Postsynaptic areas are distributed in the vPMS4 and the dPMS4, but not in the IPMZ. (E) A magnified view of the dotted rectangle area in [C1]. The branches in the IPMZ showed bleb-like structures that labeled with presynaptic markers. (F) A magnified view of the dotted rectangle area in [D1]. In vPMS4 and dPMS4, neural fibers have many spine-like structures, labeled with postsynaptic markers. D: dorsal; V: ventral; M: medial; L: lateral; A: anterior; P: posterior. Scale bars: 50 μm in A1 for A1-A3; 50 μm in B1 for B1, B2; 10 μm in C1, for C1, C2, D1, D2; 1 μm in E1 for E1, E2, F1, F2.

Figure 4.

The conventional GRASP method revealed that candidate G2Ns were postsynaptic to *Gr5a*-expressing GSNs, but not *Gr66a*-expressing GSNs. Conventional GRASP was used to examine potential synaptic contacts between candidate G2Ns and two different GSNs, *Gr5a*-expressing (A, B) and *Gr66a*-expressing (see Materials and Methods for details). *R12C04-Gal4* (A) or *-LexA* (B, C) was used to express a membrane marker, HRP::CD2, to visualize the morphologies of the candidate G2Ns (A1-C1). *Gr5a*- (A,B) or *Gr66a*- (C) expressing GSN also expressed the active zone marker Brp^{D3}::mCherry to visualize their presynaptic sites (A2-C2). Colocalization of the GRASP signal and presynaptic marker is shown in A3-C3. Overlay of three channels is shown in A4-C4. Prominent colocalization of the GRASP signal and the presynaptic marker was observed for *Gr5a*-expressing GSNs and candidate G2Ns (A, B). However, little colocalization was observed for *Gr66a*-expressing GSNs and candidate G2Ns, suggesting that they form few or no synaptic contacts (C3). Scale bar: 10 μ m in A1 for A1-C4.

Figure 5.

The Syb-GRASP method reveals functional synapses between G2Ns and *Gr5a*-expressing GSNs. Flies expressing Syb::spGFP1-10 in *Gr5a* (A) or *Gr66a* (B) GSNs, and CD4::spGFP11 in candidate G2Ns were examined for native fluorescence of reconstituted GFP. The morphologies of G2Ns were visualized by anti-CD4 immunolabeling (A1, B1) while the terminals of GSNs were visualized by anti-GFP staining (A2, B2). In the Syb-GRASP method, half of the GFP moiety is selectively localized in presynaptic terminals, and not in other parts, of the *Gr5a*-expressing GSNs (A2). Strong GRASP signal was observed along with *Gr5a*-expressing GSN axons and their terminals (A3). On the contrary, no GRASP signal was observed in *Gr66a*-expressing GSNs (B3). (A4, B4) Overlay of three channels. D: dorsal; V: ventral; M: medial; L: lateral. Scale bar: 10 μ m in A1 for A1-B4.

Table 1

Summary of Experimental Genotypes

Figures	Genotypes
1A	<i>w NP0763/w Gr5a-LexA::VP16; +/-lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 lexAop-Brp::mCherry; +/-[+ or TM2]</i>
1B	<i>w/w Gr5a-LexA::VP16; +/-lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 lexAop-Brp::mCherry; R12C04-GAL4/lexAop-HRP-CD2</i>
1C	<i>w/w Gr5a-LexA::VP16; +/-lexAop-HRP-CD2; R20G06-GAL4/lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 lexAop-Brp::mCherry</i>
1D	<i>w/w Gr5a-LexA::VP16; +/-lexAop-HRP-CD2; R77C10-GAL4/lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 lexAop-Brp::mCherry</i>
1E	<i>w Gr5a-LexA::VP16/w; lexAop-HRP-CD2/+; lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 lexAop-Brp::mCherry/R73G10-GAL4</i>
2B, C	<i>w/y w hsFLP¹²²; +/-UAS-CD8::GFP; R12C04-GAL4/UAS-Brp::mCherry</i> (mCherry fluorescence is not shown)
2D	<i>w/w lexAop-CD2::RFP Gr5a-LexA::VP16; +/-UAS-CD8::GFP; R12C04-GAL4/[+ or TM6, Sb Tb]</i>
2E	<i>w NP0763/w lexAop-CD2::RFP Gr5a-LexA::VP16; +/-UAS-CD8::GFP</i>
2F	<i>w lexAop-CD2::RFP Gr5a-LexA::VP16/w; UAS-CD8::GFP/+; +/-R20G06-GAL4</i>
3A	<i>y w hsFLP¹²²/w; UAS-FRT-CD2, y⁺-FRT-CD8::GFP /+; [TM2 or 7M6B]/R12C04-GAL4</i> (animals #1, #2) <i>y w hsFLP¹²²/w; [+ or CyO]/+; R12C04-GAL4/20xUAS-IVS-FRT-stop-FRT-spGFP1-10::CD4::HA</i> (animals #3, #4)
3B	<i>w/y w hsFLP¹²²; [CyO, SM1 or Sp]/+; lexAop-FRT-stop-FRT-spGFP11::CD4::HA-T2A-Brp^{D3}::mCherry UAS-spGFP1-10::CD4/R12C04-LexA::p65</i>
3C, E	<i>w/y w hsFLP¹²²; +/-UAS-CD8::GFP; R12C04-GAL4/UAS-Brp::mCherry</i>
3D, F	<i>w; +/-UAS-CD8::mCherry; R12C04-GAL4/UAS-Da7::GFP</i>
4A	<i>w Gr5a-LexA::VP16/w; UAS-HRP-CD2/+; lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 lexAop-Brp::mCherry/R12C04-GAL4</i>
4B	<i>w; Gr5a-GAL4/lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 UAS-Brp::mCherry; lexAop-HRP-CD2/R12C04-LexA::p65</i>
4C	<i>w; Gr66a-GAL4/lexAop-spGFP11::CD4 UAS-spGFP1-10::CD4 UAS-Brp::mCherry; lexAop-HRP-CD2/R12C04-LexA::p65</i>
5A	<i>w; UAS-Syb::spGFP1-10/Gr5a-GAL4; lexAop-spGFP11::CD4/R12C04-LexA::p65</i>
5B	<i>w; Gr66a-GAL4/UAS-Syb::spGFP1-10; R12C04-LexA::p65/lexAop-spGFP11::CD4</i>
S1A-C	<i>w lexAop-CD2::RFP/w; UAS-CD8::GFP/+; R12C04-GAL4/R12C04-LexA::p65</i>
S1D	<i>w/y w hsFLP¹²²; [CyO, SM1 or Sp]/+; lexAop-FRT-stop-FRT-spGFP11::CD4::HA-T2A-Brp^{D3}::mCherry UAS-spGFP1-10::CD4/R12C04-LexA::p65</i>
Movies	Genotypes
S1	<i>w/w lexAop-CD2::RFP Gr5a-LexA::VP16; +/-UAS-CD8::GFP; R12C04-GAL4/[+ or TM6, Sb Tb]</i>