Short Communication

Anatomical Characterization of Thermosensory AC Neurons in the Adult Drosophila Brain

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Abstract: Temperature preference is vital for the survival of all animals. A small set of warm-activated anterior cell (AC) neurons acting as an internal thermosensor in the Drosophila brain is critical for optimal temperature selection (Hamada et al., 2008, Nature, 454, 217–220). Here, the authors analyze the circuit components of the AC neurons by characterization of its spatial distribution, dendrite-axon polarity, and the putative type of neurotransmitter released. The results show that the AC neurons are serotonergic, do not have any dendrites, and send axons bilaterally to the superior dorsofrontal protocerebrum (SDFP). Searching the FlyCircuit database for neurons with serotonin receptor and dendrites in the SDFP, the authors found a dorsal-anterior-lateral (DAL) neuron as a candidate postsynaptic partner of the AC neurons. In conclusion, by morphological analysis of the AC neurons, the authors show a general strategy for predicting brain circuits orchestrating thermosensory behaviors.

Keywords: AC neuron, DAL neuron, FlyCircuit, temperature, thermotaxis, TrpA1

INTRODUCTION

Animal behaviors are governed by activities in the interconnected brain circuits. Recent advances in transgenic and optogenetic tools to temporally perturb and monitor the activities of selective target neurons have allowed scientists to begin putting together neural circuits orchestrating complex behaviors (Luo et al., 2008). The ability to sense and respond to environmental and internal temperature changes is vital for the physiology and survival of all animals (Dillon et al., 2009). In Drosophila adults, warm temperature is sensed by a small set of four anterior cells (ACs) within the brain (Hamada et al., 2008). AC neurons’ activation occurs just above the fly’s optimal growth temperature and the disruption of their thermosensing via mutations or RNA interference (RNAi) knockdown of the dTrpA1 ion channels cause flies to move toward warmer temperature. To understand how AC neurons’ thermal information transforms to decision making and avoiding warm temperature, one must know the circuit components.

MATERIALS AND METHODS

Flies

Fly stocks were raised on standard cornmeal/agar/molasses medium at 25°C and 60–70% relative humidity under a 14/10-h light/dark cycle. The following transgenic fly lines were used: UAS-mCD8::GFP, UAS-Dscam::GFP, UAS-Syt::GFP, dTrpA1-GAL4, UAS-mKO, and hs-Flp;+;UAS > rCD2,y+ > mCD8::GFP.

Immunohistochemistry

Whole-mount immunolabeling of the adult brain was performed as previously described (Xia et al., 2005). Briefly, the brain samples were first dissected in phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde on ice with microwave irradiation (2450 MHz, 1100 Watts) for 90 s with continuous rotation, two times. Then the samples were transferred to 4% paraformaldehyde in PBS.
with 0.25% triton X-100 on ice in microwave for 90 s, two times. Subsequently the brains were penetrated and blocked in PBS containing 2% Triton X-100 and 10% normal goat serum and degassed in a vacuum chamber to expel tracheal air, four times (depressurize to ~70 mm Hg then hold for 10 min). After degassing, the samples were immersed in the same solution for 2 h on shaker at room temperature. Next, the brains were transferred to
the solution with primary antibodies and incubated at 4°C overnight.

The following primary antibodies were used: mouse 4F3 anti-DLG (*Drosophila* discs large) at 1:50 dilution (developmental studies Hybridoma Bank, University of Iowa); mouse anti-TH (tyrosine hydroxylase) at 1:100 dilution (ImmunoStar); rabbit anti-5-HT (5-hydroxytryptamine; serotonin) at 1:500 dilution (Sigma); rabbit anti-GABA (γ-aminobutyric acid) at 1:500 dilution (Sigma); and rabbit anti-VGlut (vesicular glutamate transporter) at 1:5000 dilution (Daniels et al., 2008). After washing in the PBS containing 1% Triton X-100 and 3% sodium chloride, the brains were transferred to the solution with secondary antibodies and incubated at 4°C overnight. The following secondary antibodies were used: biotin-conjugated goat anti-mouse (Invitrogen) and biotin-conjugated goat anti-rabbit immunoglobulin Gs (IgGs) (Invitrogen), all diluted to 1:200.

Next, the brain samples were washed and incubated in solution with 1:500 diluted Alexa fluor 635 streptavidin (Invitrogen) at 4°C overnight. Finally, after extensive washing, the immunostained brains were directly cleared and mounted in FocusClear (Celexplorer, Taiwan), an aqueous sugar-based solution rendering biological tissue transparent. Sample brains were imaged under a Zeiss LSM710 Confocal Microscope with a 40 × C-Apochromat water-immersion objective lens (N.A. value 1.2, working distance 220 μm).

**Circuit Prediction**

For genetic FLP-out labeling (Wong et al., 2002), flies carrying the *hs-Flp;dTrpA1-GAL4/+;UAS-rCD2,y+rCD2,y+;mCD8::GFP* transgene were heat shocked at the 4-day pupal stage. Individual single-neuron images were recorded, segmented, and uploaded to FlyCircuit as previously described (Chiang et al., 2011). In FlyCircuit, we used text-based search function with a query of “driver: 5-HT$_{1B}$-GAL4” and “innervation sites: innervations >1000 voxels in either SDFP or sdfp.” Among the five neurons satisfying

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**Figure 2.** The dendrite-axon polarity of AC neurons. (A–C) Visualization of postsynaptic terminals. UAS-mKO (red) indicates all dTrpA1-GAL4 neurons (A); UAS-Dscam::GFP (green) indicates putative dendrites (B); merged image (C). Genotype: UAS-Dscam::GFP/+;dTrpA1-GAL4/UAS-mKO,UAS-mKO. (D–F) Visualization of presynaptic terminals. UAS-mKO (red) indicates all dTrpA1-GAL4 neurons (D); UAS-Syt::GFP (green) indicates putative axon terminals (E); merged image shows the spatial relationship (F). Genotype: dTrpA1-GAL4/UAS-Syt::GFP,UAS-mKO.
the query, the dorsal-anterior-lateral (DAL) neuron is the only one with >5000 voxels in SDFP (9998 voxels) or sdfp (46416 voxels). To predict synaptic contacts between the DAL and AC neurons, we calculated their overlap regions by “Resample” and “Arithmetic” objects in Amira. “Resample” by enlarging voxel size with a twice-longer edge was used to compensate for individual variations and errors introduced during registration and warping. The overlapping voxels were generated by “Arithmetic” computation.

RESULTS AND DISCUSSIONS

We first reexamined the expression pattern of dTrpA1 promoter-driven GAL4 flies. dTrpA1-GAL4 neurons have been shown to be necessary and sufficient for normal thermal preference behavior. In the brain, the four AC neurons are the only dTrpA1-GAL4 neurons with anti-dTrpA1-immunopositive reaction (Hamada et al., 2008). We found that in addition to the four AC neurons and 10 other cells in the brain and subesophageal ganglion, dTrpA1-GAL4 also expresses in eight cells in the thoracic ganglion (Figure 1). Further functional analysis is required to see whether dTrpA1-GAL4 neurons outside the brain involved in thermal preference behavior.

Next, we labeled the polarity of the AC neurons to predict the direction of information flow. The somata of AC neurons are located near the brain surface where antenna nerve entering antennal lobe. We found that Dscam::GFP, a reporter of neuronal dendrites (Schmucker et al., 2000), labels only the cell bodies of the AC neurons (Figure 2A–C). Additional Dscam::GFP signals at the subesophageal ganglion and dorsolateral protocerebrum (DLP) (Figure 2B) are dendrites of other dTrpA1-GAL4 neurons (Figure 1, Figure 4B). This is consistent with the role of the AC neurons as internal thermosensors rather than inter-neurons receiving signals from other sensory neurons. The AC neurons’ axons, as reported by synaptotagmin::GFP labeling (Zhong et al., 1999), give extensive arborizations at the dorsal protocerebrum (Figure 2D–F).

How does thermal information transmit from the AC neurons? We addressed this question by immunolabeling to visualize the location of the following molecules: serotonin (5-HT), tyrosine hydroxylase (TH), γ-aminobutyric acid (GABA), and vesicular glutamate transporter (VGlut). Our results indicate that the AC neurons are immunopositive...
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Figure 4. Morphological characterization of AC neurons. (A) A representative AC neuron visualized by FLP-out labeling. (B, C) Two other dTrpA1-GAL4 neurons. (D) A representative AC neuron segmented into eight domains; each color indicates fibers innervating a distinct brain region. (E) Spatial distribution of the AC neuron in the brain. Each color corresponds to the fiber with the same color in (D). Spatial distribution represents the number of voxels in each brain region divided by the total number of voxels occupied by each neuron. The cell body is excluded. AL = antennal lobe; DLP = dorsolateral protocerebrum; DMP = dorsomedial protocerebrum; IDLP = inner dorsolateral protocerebrum; PAN = proximal antennal protocerebrum; SDFP = superior dorsofrontal protocerebrum; SOG = subsesophageal ganglion; VMP = ventromedial protocerebrum. Each datum represents mean ± SEM (N = 6). (F) A 5-HT$_{1B}$-GAL4 neuron with bilateral SDFP innervations from the FlyCircuit database (Chiang et al., 2011). (G) Putative synaptic contacts (red) between the AC neuron (dark gray) and the 5-HT$_{1B}$-GAL4 neuron (light gray).

to anti-5-HT (Figure 3A), but immunonegative to anti-TH (Figure 3B), anti-GABA (Figure 3C), and anti-VGlut (Figure 3D). This suggests that the Drosophila AC neurons are serotonergic, unlike the Caenorhabditis elegans, which use glutamate in their thermosensory neurons (Lee et al., 1999).

The Drosophila brain consists of 41 local processing units, six hubs, and 58 tracts (Chiang et al., 2011). How is an AC neuron connected to these information processing and relaying units in the brain? To address this question, we analyzed the morphology and spatial distribution of single AC neuron visualized with FLP-out labeling (Figure 4). The axon of an AC neuron branches near the antennal lobe projects obliquely toward ipsilateral posterior-dorsal protocerebrum, and then branches again before giving symmetric arborizations at both brain hemispheres (Figure 4A). Two other dTrpA1-GAL4 neurons also contribute to the arborizations in the dorsal protocerebrum (Figure 4B, C). The spatial distribution...
of single AC neuron was analyzed by uploading the segmented images to the FlyCircuit database (http://www.flycircuit.tw; Chiang et al., 2011).

After automated registration and 3D transformation using the DLG-counterstained brain as a reference, FlyCircuit divides an AC neuron into multiple domains; each intersects with one of the 58 brain regions (Chiang et al., 2011) (Figure 4D). Our analysis indicates that the AC neuron gives more than 50% of its fibers in the superior dorsofrontal protocerebrum (SDFP), also called superior lateral protocerebrum (Hamada et al., 2008), to communicate with the downstream neuron through serotonin (Figure 4E). In addition, it also innervates additional brain regions, including antennal lobe (AL) and subesophageal ganglion (SOG) as reported previously (Hamada et al., 2008). In Drosophila, there are four types of serotonin receptors, 5-HT$\text{1A}$, 5-HT$\text{1B}$, 5-HT$\text{2A}$, and 5-HT$\text{2C}$ (Tierney, 2001). Searching 5-HT$\text{1A}$ neurons in the FlyCircuit, we found a dorsal-anterior-lateral neuron (DAL) with bilateral innervations in the SDFP regions (Figure 4F). Putative contacts between the AC neuron and DAL neuron were calculated by assigning a new channel to voxels occupied by both neurons. We found that the two neurons are mainly intersected at the SDFP region, especially at the ipsilateral side (Figure 4G; Movie S1). After automated registration and 3D transformation using the DLG-counterstained brain as a reference, the AC neuron gives bilateral axonal terminals in the inferior dorsofrontal protocerebrum (IDFP) (Figure 4F). A neuron I.D. recording all of the above-mentioned morphological characterizations is available for each AC neuron submitted to the FlyCircuit (http://www.flycircuit.tw). This allows cross-experiment examination and further prediction of the AC neuron’s other candidate synaptic partners.

In conclusion, we have demonstrated an application of the FlyCircuit database to predict circuit connectivity between the AC neuron, an internal thermosensor, and the DAL neuron. The prediction is supported by five independent lines of evidence: (i) The AC neuron gives bilaterally projections at SDFP; (ii) the AC neuron’s arborizations at SDFP are presynaptic terminals; (iii) the AC neuron is serotonergic; (iv) the DAL neuron is a 5-HT$\text{1A}$ neuron; and (v) the two neurons overlap extensively at SDFP in both hemispheres. In the future, this prediction can be tested by manipulating the synthesis and release of serotonin in the AC neurons or regulating the expression level of serotonin receptors in the DAL neurons to see whether these components play a role in thermal preference behavior.

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REFERENCES


