**Letter to the Editor**

*Drosophila* Larval Light-Avoidance is Negatively Regulated by Temperature Through Two Pairs of Central Brain Neurons

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**Dear Editor,**

The innate preference behaviors of animals can be modified by external environmental conditions. In *Drosophila* for example, the preference for food and temperature are respectively influenced by the hardness of food and environmental light conditions [1, 2]. Comparatively, environmental modulation of *Drosophila* light preference has received less investigation. *Drosophila* avoids light and prefers darkness in the larval stage [3, 4]. *Drosophila* larval photoreceptors, Bolwig’s organs, and downstream neurons such as the 5th-lateral neurons [4, 5] and the posterior ventral lateral-09 neurons [6], are required for the light-avoidance response. Further downstream to these neurons is a neural pathway consisting of prothoracicotropic hormone neurons [7, 8], eclosion hormone neurons, and tyrosine decarboxylase-2 motor neurons that command the light-induced deceleration response [8], and another pathway includes LRIN⁸⁻¹⁻³⁻⁰⁻⁷ neurons (light repressed inhibitory neurons) and CLPN⁸⁻²⁻⁰⁻⁹ neurons (contralateral projecting neurons) that mediate light-induced reorientation [9].

As light is closely related to temperature in daily and seasonal cycles, it is probable that *Drosophila* larval light-avoidance is impacted by temperature. Thus, we investigated the effect of temperature on larval light-avoidance by testing 3rd instar larval light preference using a simple light/dark choice assay [7, 9] with a white light intensity of 550 lux for 10 min at temperatures ranging from 15 °C to 27 °C using a wildtype strain of WT-CS. In this range, larval light avoidance was increased as temperature decreased (Fig. 1A). Furthermore, we asked if the behavioral output was stable over longer times of testing. We tested the 3rd instar larval light-avoidance of another commonly-used control strain, *w¹¹¹八*, at 18 °C and 27 °C for 5 min, 10 min, 15 min and 20 min. In all cases, larvae showed a stronger preference for darkness at the cool temperature of 18 °C than at the warm temperature of 27 °C (Fig. 1B). Besides, we investigated whether the behavioral output was stable when the light intensity was changed, then we tested 3rd instar larval light-avoidance at 18 °C and 27 °C under 250 lux and 170 lux. Under these two light intensities, larvae showed a stronger preference for darkness at 18 °C than at 27 °C, similar to the behavioral output under 550 lux (Fig. S1). These results together showed that larval light avoidance is negatively regulated by temperature.

In order to explore the neural basis of the lower temperature-enhancement of larval light avoidance, we screened for candidate neurons by blocking neuronal activity and comparing larval light-avoidance performance at 18 °C and 27 °C. The difference between 18 °C and 27 °C in control lines disappeared when TNTG [9], a presynaptic inhibitor of neurotransmission, was expressed by *R60F09-GAL4* (Fig. 1C), and this phenomenon was...
**Fig. 1** ACLPR60F09 neurons participate in thermal regulation of larval light-avoidance. A WT-CS larvae show stronger light avoidance at a lower temperature in a 10-min light/dark choice assay (**P** < 0.001, one-way ANOVA followed by post hoc Tukey’s multiple comparison test, *n* = 16 for all temperatures except that *n* = 24 for 25 °C). B *w*1118 larvae always show stronger light avoidance at 18 °C than at 27 °C at different time points (n = 17, 15, 18, 16, 18, 16, and 16 from left to right). White light of 550 lux (23.3 μW/mm²) was used in both (A) and (B). C Blocking ACLPR60F09 neurons using TNTG abolishes the difference in light avoidance at cool and warm temperatures in a light/dark preference assay using white light at 170 lux (9.1 μW/mm²) (*n* = 26, 26, 29, 29, 38, and 38 from left to right). Note that performance index of larvae with ACLPR60F09 neurons blocked using TNTG is higher than in control larvae at 27 °C (+**P** < 0.05, **P** < 0.01, one-way ANOVA followed by post hoc Tukey’s multiple comparison test; *n* = 26, 29, and 38 from left to right). Error bars, SEM. D Expression of R60F09-GAL4 in the larval central nervous system. Arrowheads, the three neurons in one hemisphere; yellow ellipse, neurons in the SEZ; long thin arrows, the three neurons on one side of the SEZ; short thick arrow, neuron in the ventral nerve cord (VNC). E, F Morphology of single R60F09-GAL4 neurons in the hemispheres, including the ACLPR60F09 neuron (E) and the 3rd neuron lacking a contralateral projection (F). G Morphology of two ACLPR60F09 neurons located in the same hemisphere. Arrowheads, cell bodies; long thin arrow, dendrites; short thick arrow, axonal projections. Scale bars, 50 μm. H Ca²⁺ imaging of responses of ACLPR60F09 neurons and the 3rd neurons to white-light (170 lux) (*n* = 5 for ACLPR60F09 neurons; *n* = 6 for the 3rd neurons). I Mean peak Ca²⁺ responses in (H). J Ca²⁺ imaging of responses of ACLPR60F09 neurons to a temperature rise from 18 °C to 27 °C. Treating the neurons with 20 μmol/L TTX for 4 min abolishes the response (*n* = 4 for TTX-treated ACLPR60F09 neurons; *n* = 8 for control ACLPR60F09 neurons treated with AHD (adult hemolymph like saline)). K Statistics of peak responses within 40 s after temperature change in (J). L Ca²⁺ imaging of responses of ACLPR60F09 neurons to a temperature drop from 27 °C to 18 °C. Treating the neurons with 20 μmol/L TTX for 4 min abolishes the response (*n* = 7 for TTX-treated ACLPR60F09 neurons; *n* = 9 for control ACLPR60F09 neurons treated with AHD). M Statistics of peak responses within 40 s after the temperature change in (L). B, C, H–M n.s. *P* > 0.05, *P* < 0.05, **P** < 0.01, ***P** < 0.001, unpaired t-test. Error bars, mean ± SEM.

Independent of larval crawling speed (Fig. S2). In the larval central nervous system, R60F09-GAL4 marks three pairs of neurons in the brain hemispheres, three pairs of neurons in the subesophageal zone (SEZ), and one neuron in the ventral nerve cord (VNC) (Fig. 1D). We used flp-out technology [9] to reveal single neuronal morphology and found that the three pairs of neurons in the hemispheres (cells 1, 2, and 3) were divided into two categories: in the first category, each of the two pairs had dendrites and cell body ipsilaterally in anterior hemisphere and an axon projecting contraterally to the other hemisphere (Fig. 1E, G); in the second category, each of the third pair of neurons had the cell body and dendritic arborizations as well as axonal arborizations all in the same hemisphere (Fig. 1F). For convenience, we named the neurons in the first category ACLPR60F09 neurons (anterior contralateral projecting neurons) and neurons in the second category the 3rd neurons.

We proposed that thermal sensation might affect larval light-avoidance behavior by modulating neurons in the light-avoidance circuitry. If so, the neurons crucial for the cross-modality sensory integration should be responsive to both light and temperature change. We then tested this hypothesis using Ca²⁺ imaging. Upon light stimulation, we found that other neurons in the SEZ (cells 4, 5, and 6) and a neuron in the VNC (cell 7) did not show evident responses (Fig. S3). In addition, two of the three pairs of neurons in the hemisphere responded to light stimulation, and one did not (Fig. S4). To distinguish the functions of these two types of neuron, we used the SPARC technique (Sparse Predictive Activity through Recombinase Competition) [10] to separately express GCaMP in the ACLPR60F09 neurons and the 3rd neurons (Fig. S5). Ca²⁺ imaging showed that the ACLPR60F09 neurons clearly responded to light stimulation, while the 3rd neurons did not show a response (Fig. 1H, I). Therefore, we focused only on the two pairs of ACLPR60F09 neurons in subsequent experiments.

We next tested the responses of ACLPR60F09 neurons to temperature rises and drops. As shown in Fig. 1J–M, monotonic temperature rises from 18 °C to 27 °C within 30 s led to a rapid decrease in the Ca²⁺ signal by ~30%, while monotonic temperature drops from 27 °C to 18 °C within 30 s caused a notable response of an ~30% increase in fluorescence intensity. These responses were largely removed by application of 20 μmol/L tetrodotoxin (TTX) [11] which depresses synaptic transmission, suggesting that the thermal response originated from neurons other than the ACLPR60F09 neurons themselves. These data together showed that ACLPR60F09 neurons respond to both light and thermal stimulation. Taken together, integration of thermal and light signals in ACLPR60F09 neurons are crucial for the negative regulation of larval light avoidance by temperature.

As ACLPR60F09 neurons appeared to be morphologically close to CLPNR82B09 neurons [9] that were found to mediate the light-induced head-cast response in larval light avoidance (Fig. S6A, B), we asked if ACLPR60F09 neurons directly target on CLPNR82B09 neurons to affect light avoidance. We used the GRASP technique (GFP Reconstitution Across Synaptic Partners) [9] to test possible interaction between ACLPR60F09 neurons and CLPNR82B09 neurons. The three neurons in the hemisphere labeled by R60F09-GAL4 and CLPNR82B09 neurons showed robust GRASP signals (Fig. 2A, B) while no signal was seen in the controls (Fig. 2C, D), suggesting that the ACLPR60F09 neurons directly interact with CLPNR82B09 neurons. To further explore whether CLPNR82B09 neurons receive input from ACLPR60F09 neurons, we again combined

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*Note: The document contains technical and biological terminology and data presented in a research context.*
optogenetics and Ca$^{2+}$ imaging to see if directly exciting ACLPR60F09 neurons induced response in CLPN R82B09 neurons. In order to avoid the influence of the third neurons, we used the SPARC technique to express CsChrimson only in ACLPR60F09 neurons. When ACLPR60F09 neurons were excited by red light, CLPN R82B09 neurons showed a maximal decrease of $\sim 20\%$ in the Ca$^{2+}$ signal (Fig. 2E, F). This meant that CLPN R82B09 neurons received Ca$^{2+}$ responses in (E). G–R ACLPR60F09 neurons are GABAergic. The three neurons in one hemisphere (G–I) are separately shown in J–R; cell 1, 2, and 3 are separately shown in J–L, M–O, and P–R (magenta, anti-GABA signal; green, GCAMP signal driven by R60F09-Gal4). Arrowhead in (G) indicates the cell bodies of the three neurons. Note that R82B09-lexA and R82B10-Gal4 were used to label CLPN R82B09 neurons. Scale bars, 50 $\mu$m in G–I. S Ca$^{2+}$ imaging of responses of ACLPR60F09 neurons to white light (170 lux) at 18 $^\circ$C and 27 $^\circ$C ($n = 13$ for 18 $^\circ$C, $n = 12$ for 27 $^\circ$C). T Mean peak Ca$^{2+}$ responses in (S). E, F, S, T *$P < 0.05$, ***$P < 0.001$, unpaired $t$-test. Error bars, mean ± SEM.
inhibitory input from ACLP<sup>R60F09</sup> neurons. Indeed, when we co-stained <i>R60F09-Gal4</i>-labeled neurons with an antibody against GABA, an inhibitory neurotransmitter, co-localization was seen in cell bodies of all three <i>R60F09-Gal4</i>-labeled neurons in the hemispheres (Fig. 2G–R). This meant that ACLP<sup>R60F09</sup> neurons are GABAergic. As CLPN<sup>R82B09</sup> neurons had been identified to express the GABA<sub>A</sub> receptor RDL [9], it was highly likely that ACLP<sup>R60F09</sup> neurons inhibited CLPN<sup>R82B09</sup> neurons through GABA/RDL interaction. Therefore, ACLP<sup>R60F09</sup> neurons play inhibitory roles in larval light-avoidance behavior.

As ACLP<sup>R60F09</sup> neurons were activated by a temperature drop and inhibited by a temperature rise, how do the inhibitory ACLP<sup>R60F09</sup> neurons mediate the negative regulation of light-avoidance behavior, by temperature, that is, enhanced light-avoidance at lower temperature and repressed light-avoidance at higher temperature? To explain this apparent paradox, we investigated how light and thermal information are integrated in ACLP<sup>R60F09</sup> neurons. We then tested the response of ACLP<sup>R60F09</sup> neurons to light at different temperatures by recording their Ca<sup>2+</sup> response to white light pulses at 60-s intervals at 18°C and 27°C. To reduce the effect of spontaneous activity on the response, we used the average amplitude of the peak responses to the first two light pulses to measure the response amplitude. The response of ACLP<sup>R60F09</sup> neurons to light stimulation was significantly lower at 18°C than at 27°C (Fig. 2S, T). As the light response in ACLP<sup>R60F09</sup> neurons was weaker at a lower temperature, the light-induced inhibition of CLPN<sup>R82B09</sup> neurons by ACLP<sup>R60F09</sup> neurons was thus relieved to allow a stronger aversive reorientation response. Interestingly, CLPN<sup>R82B09</sup> neurons did not show a detectable response to a rapid temperature drop from 25°C to 15°C in our unpublished observations. Thus, a lower temperature did not impair larval light-avoidance by enhancing the ACLP<sup>R60F09</sup>–CLPN<sup>R82B09</sup> inhibition. Overall, our results well explain the paradox and are consistent with our previous finding that blocking ACLP<sup>R60F09</sup> neurons abolished the thermal regulation of larval light avoidance.

In summary, we found that <i>Drosophila</i> larval light-avoidance was negatively regulated by temperature. When temperature dropped, the response of ACLP<sup>R60F09</sup> neurons to light was decreased. As ACLP<sup>R60F09</sup> neurons inhibit the CLPN<sup>R82B09</sup> neurons that command the light-avoidance behavior, the reduction in light response of ACLP<sup>R60F09</sup> neurons at lower temperatures facilitated the light-avoidance behavior by relieving the inhibition of CLPN<sup>R82B09</sup> neurons.

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Conflict of interest All authors claim that there are no conflict of interest.

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