Figure S1 (related to Figure 1)

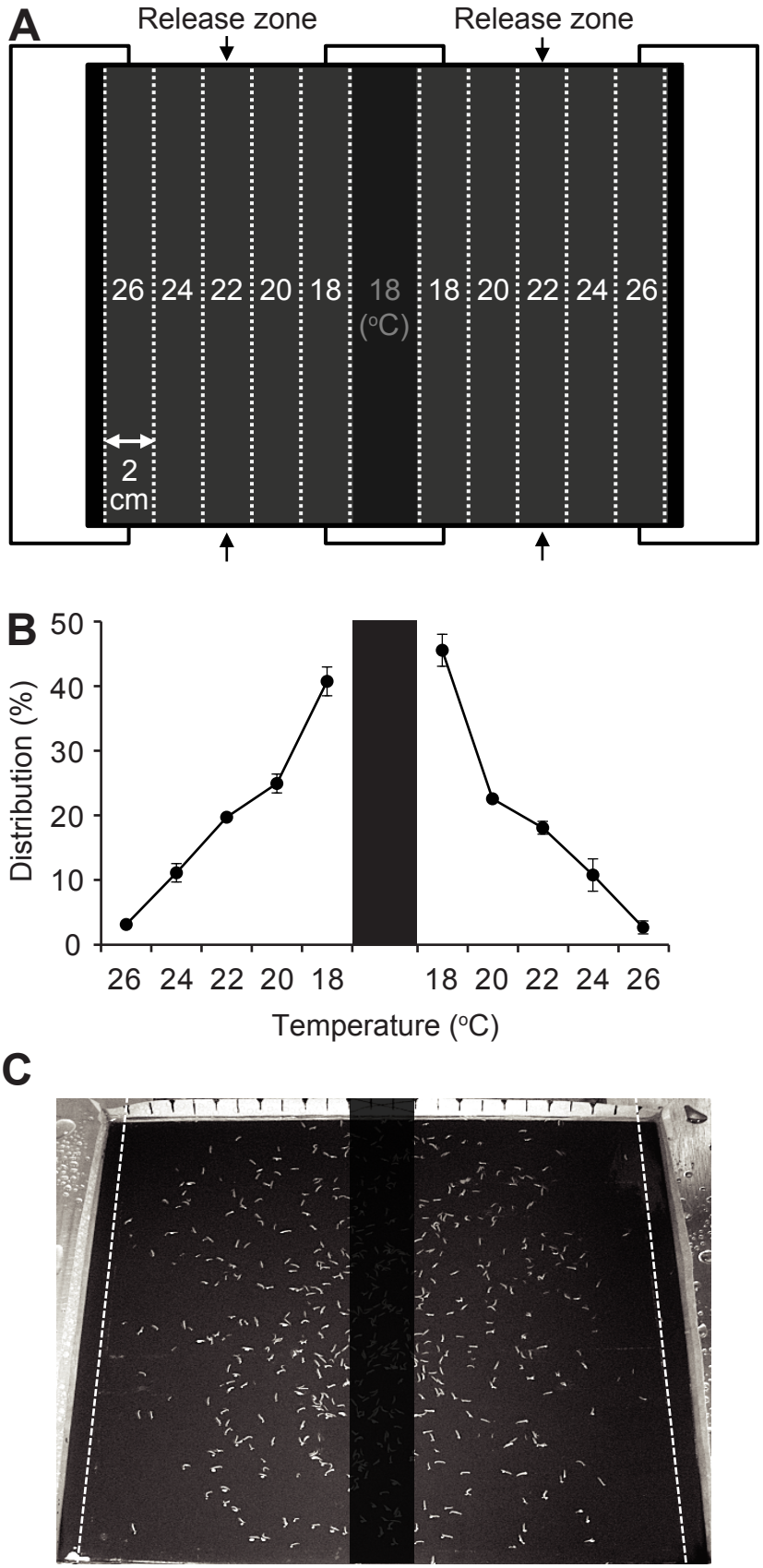


Figure S1. Thermal preference using a bidirectional-temperature gradient.
(A) Schematic of the dual-gradient temperature choice assay. The test plate is placed on three aluminum blocks, each of which is temperature controlled by a water bath. The blocks on the two sides are set at the high temperature (26°C) and the block in the center is set at the low temperature (18°C). Each side of the test plate had a linear temperature slope (18°—26°C) and is divided into five zones (2 cm each).
(B, C) Distribution of control late-3rd instar larvae (w^{1118}; 120 hours AEL) along the thermal gradient. The error bars in (B) represent the mean temperatures ±SEMs. n=3.
See also Figure 1.

Figure S2 (related to Figure 2)

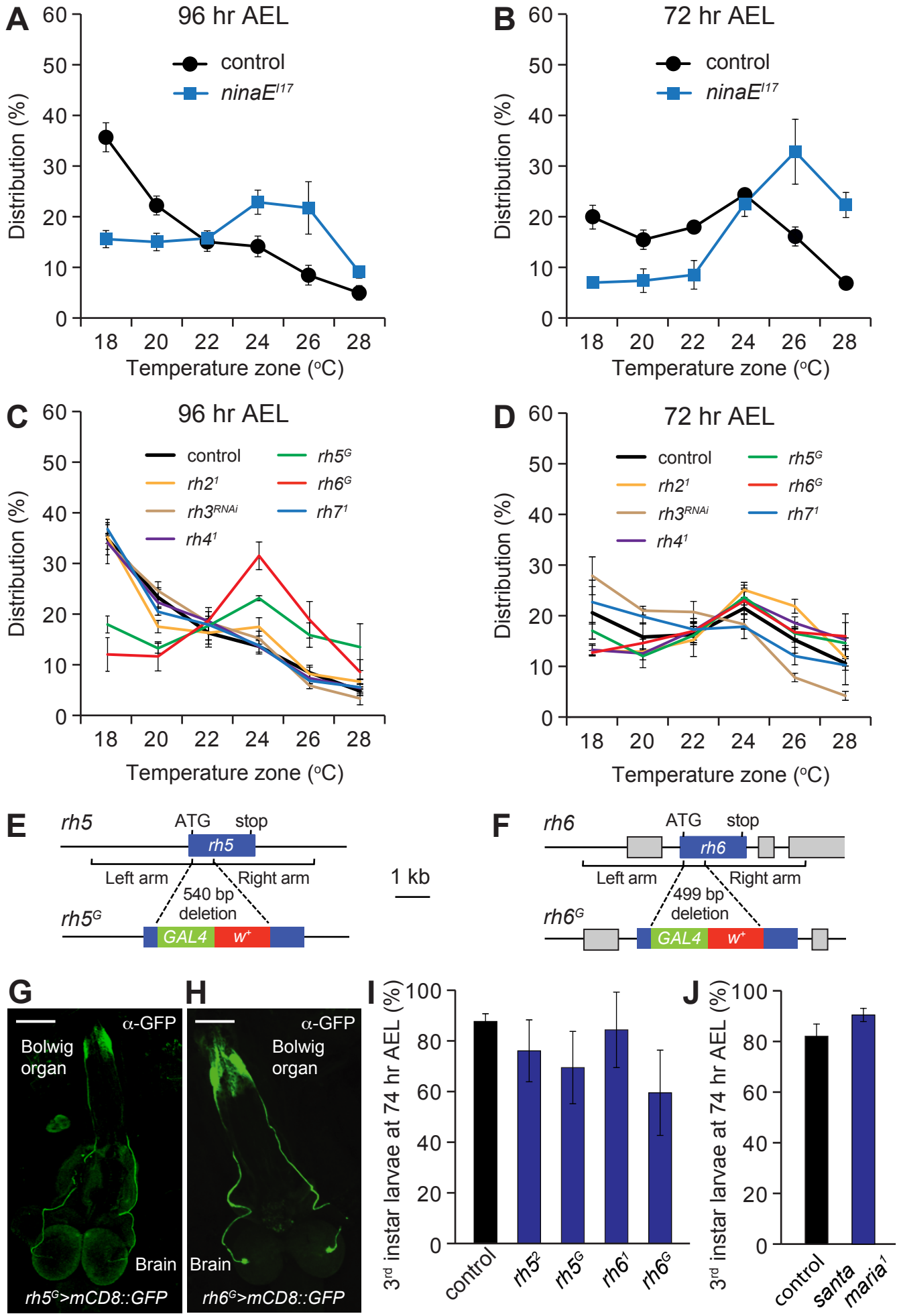


Figure S2. Thermal preferences of *rh1-7* mutant early- and mid-3rd instar larvae, generation of *rh5^{G}* and *rh6^{G}* alleles by homologous recombination, and 3rd instar entry assay for *rh5*, *rh6*, and *santa maria* mutants.

(A-D) Thermal preference of control (*w^{1118}*) and the indicated mutant mid- and early-3rd instar larvae (96 and 72 hours AEL, respectively) along 18°—28°C gradients. The *rh3^{RNAi}* in C and D were *dicer2*/+;;*elav-GAL4*/*UAS-rh3^{RNAi}*. The data represent the means ±SEMs. n=3-4.

(E, F) Cartoons showing deletions in *rh5* and *rh6* and the substitutions of the *GAL4* and *mini-white* genes at the positions of the original ATG start codons.

(G, H) Anti-GFP staining of late-3rd instar larvae expressing *UAS-mCD8::GFP* under control of the *GAL4* reporters knocked into *rh5^{G}* and *rh6^{G}*. The scale bars in G and H represent 100 μm.

(G) *rh5^{G}*/*UAS-mCD8::GFP*.

(H) *UAS-mCD8::GFP*/+; *rh6^{G}*/+.

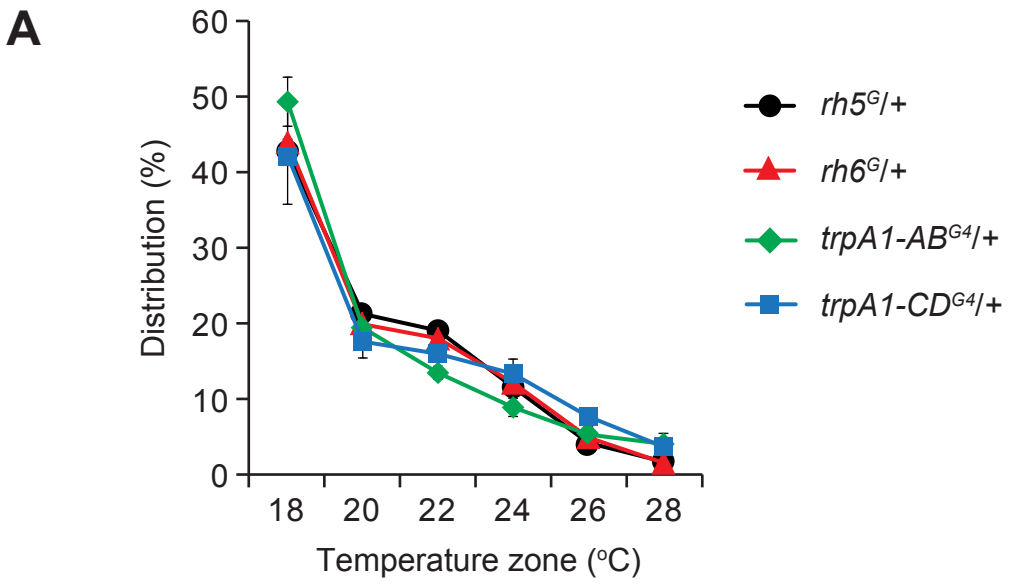
(I, J) The percentage of 3rd instar at 74 hours AEL based on the morphology of mouth hooks and spiracles. The controls were *w^{1118}*.

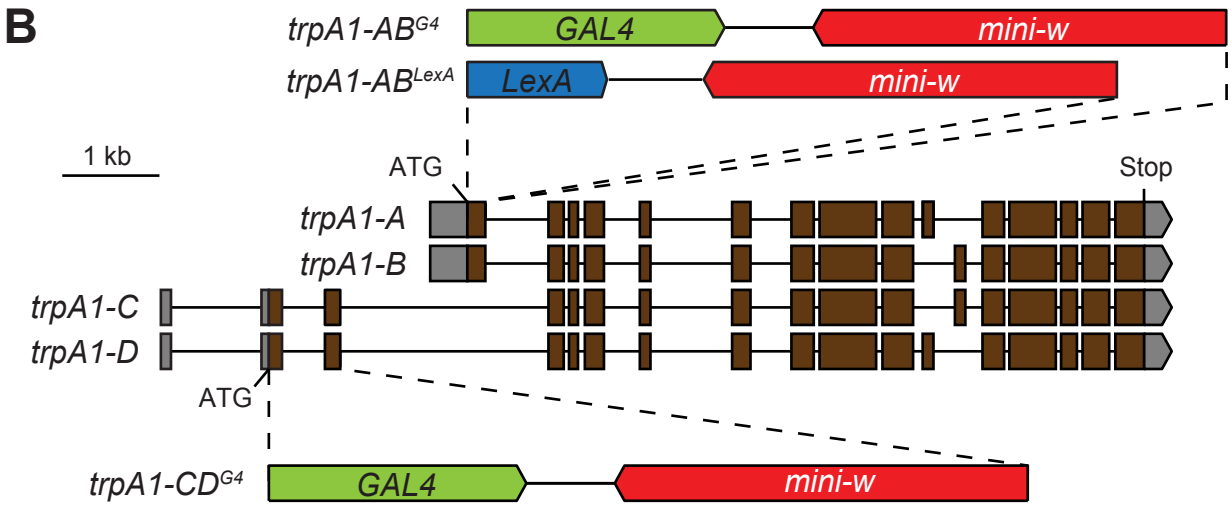
(I) Rhodopsin mutants.

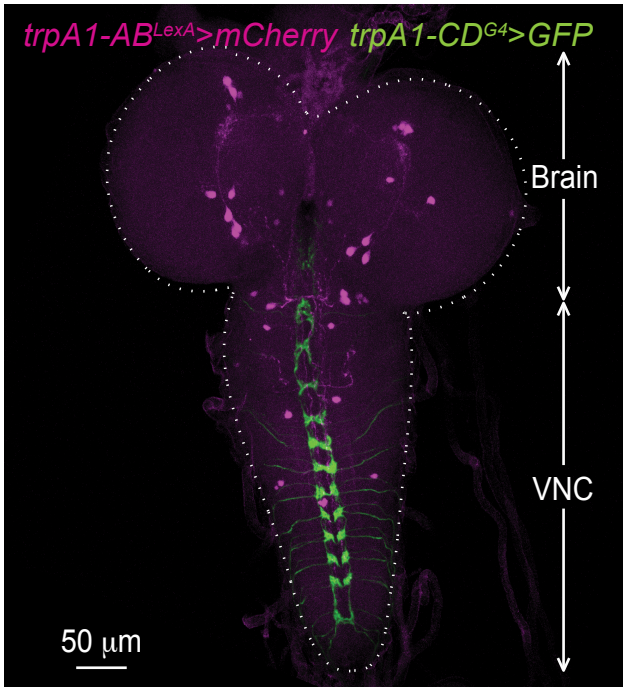
(J) *santa maria^{1}*. The data represent the means ±SEMs. n=6.

See also Figure 2.

Figure S3 (related to Figure 3)







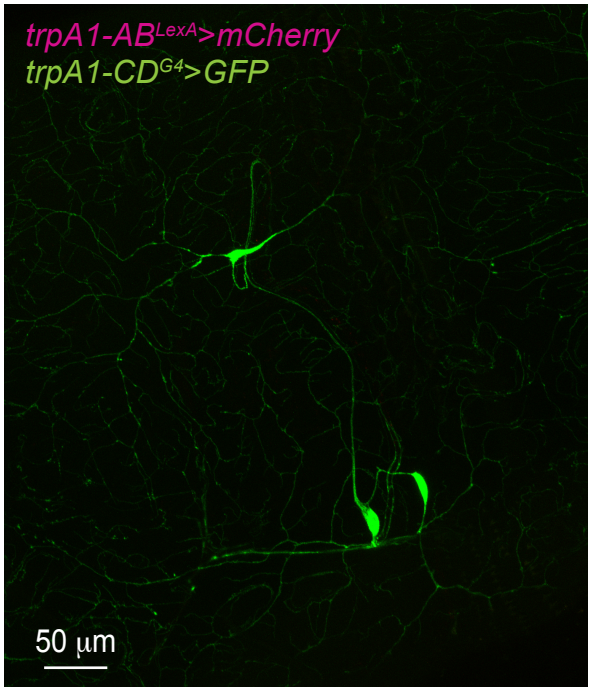


Figure S3. Thermal preferences of heterozygous *rh5, rh6* and *trpA1* larvae and design and expression patterns of reporters knocked into *trpA1-AB* and *trpA1-CD*.

(A) Shown are the percent distributions of the indicated heterozygous late-3rd instar larvae on 18°—28°C thermal gradients. The data represent the means ±SEMs. n=3-6.

(B) Cartoon showing the four *trpA1* isoforms and the insertion sites of the *GAL4*, *LexA* and *mini-white* genes knocked into *trpA1-AB* and *trpA1-CD*. The *trpA1-A* and *trpA1-B* isoforms share one promoter, and *trpA1-C* and *trpA1-D* share a second promoter. The coding and non-coding exons are indicated by the brown and gray rectangles, respectively. The *GAL4* and *LexA* reporters (and the *mini-white* marker) were knocked into the sites of the original ATG start codons. *trpA1-AB^{G4}* and *trpA1-AB^{LexA}* also contain a deletion of the first coding exon, and *trpA-CD^{G4}* includes a deletion of the first two coding exons (J. Luo, W.L. Shen and C. Montell, Nat. Neurosci. in press).

(C, D) Co-expression of the *trpA1-AB^{LexA}* (*trpA1-AB^{LexA}/LexAop-mCherry*) and *trpA1-CD^{G4}* (*UAS-mCD8::GFP*/+;*trpA1-CD^{G4}*/+) reporters.

(C) Reporter staining of the brain and the ventral nerve cord (VNC).

(D) Reporter staining of multidendritic type IV neurons and an external sensory organ neuron in the body wall.

See also Figure 3.

Figure S4 (related to Figure 4)

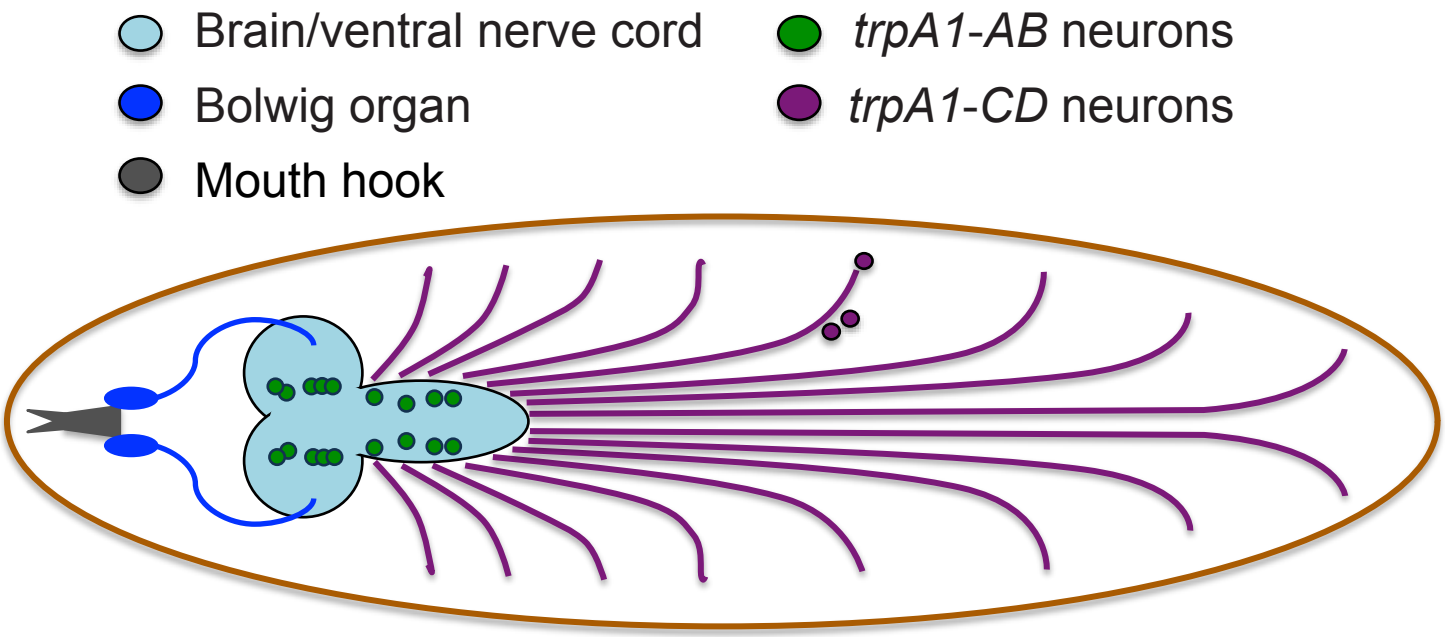
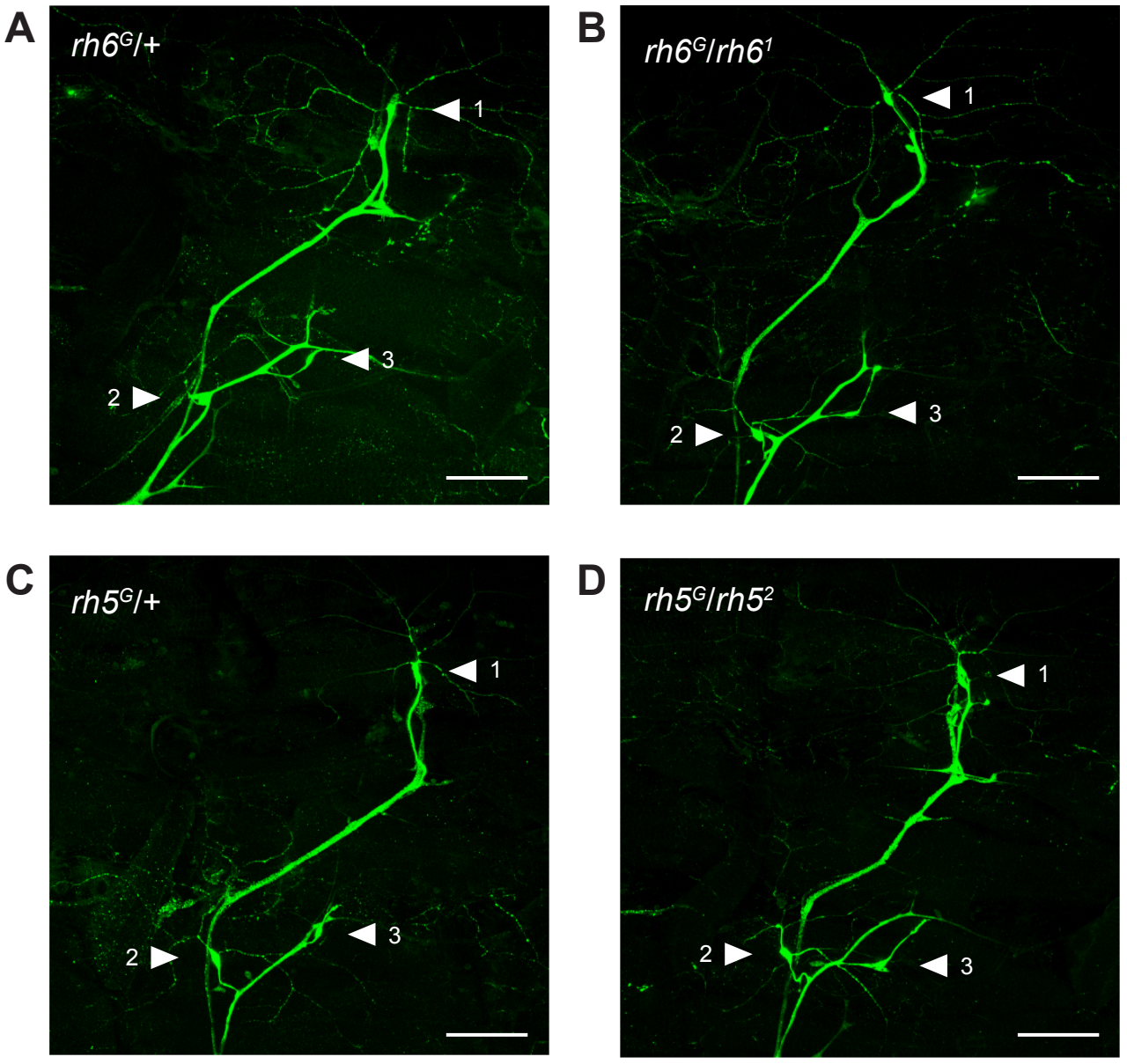


Figure S4. *rhodopsin* neurons in 3rd instar larvae.
Representative locations of *rhodopsin*-expressing neurons. The Bolwig organ (dark blue) on the anterior (left) side of the larvae is a light sensing organ and expresses *rh5* and *rh6*. However, the rhodopsins in the Bolwig organ do not function in thermotaxis behavior. We observed *rh5*- and *rh6*-reporter expression in a subset of *trpA1-CD* neurons in the body wall (purple circles indicate the cell bodies). 1 and 2 indicate multidendritic type IV neurons (1: ddaC, 2: v'ada) and 3 indicates an external sensory organ neuron (vp5). These three cells are repeated in every segment, but are shown here in one abdominal segment for simplicity. The cell bodies of *trpA1-AB* neurons (green) in the brain and the ventral nerve cord (light blue) require *rh5* and *rh6*. However, use of the TSA approach to detect *rh5* and *rh6* in these latter neurons was problematic due to background issues (see main text). Both *trpA1-AB* and *trpA1-CD* neurons are essential for temperature selection in 3rd instar larvae. See also Figure 4.

Figure S5 (related to Figure 5)



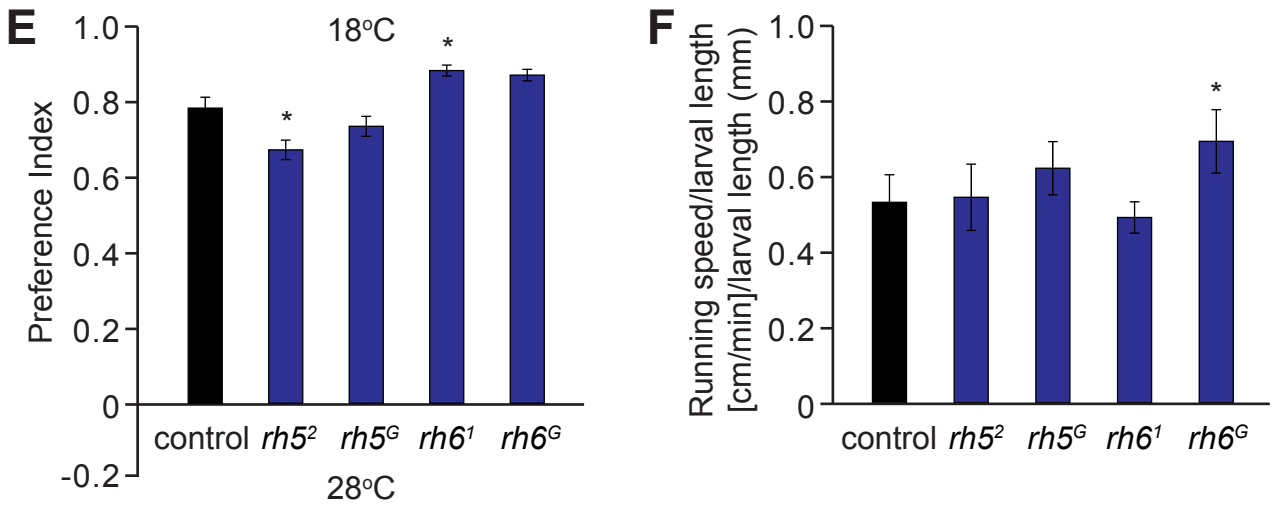


Figure S5. Rhodopsin mutants do not affect the gross morphology of *trpA1* neurons, 18° versus 28°C thermotaxis or running speed.

(A-D) Representative confocal images of *trpA1-CD* reporter expression from a late-3rd instar larval body segment. One copy of the *trpA1-CD* reporter drove expression of one copy of *20XUAS-6XGFP* (anti-GFP, green). The three arrowheads in each panel indicate the cell bodies of the *trpA1-CD* neurons. Arrowheads 1 and 2 correspond to the type IV neurons ddaC and v'ada, and arrowhead 3 corresponds to the external sensory organ neuron vp5. See Figure S4 for depictions of the locations of the ddaC, v'ada and vp5 neurons. In all images, anterior is the left and the dorsal side is at the top. The scale bars in all images represent 100 μm.

(A) $rh6^{G}$/+.

(B) $rh6^{G}/rh6^{1}$.

(C) $rh5^{G}$/+.

(D) $rh5^{G}/rh5^{2}$.

(E) Temperature preferences of control (w^{1118}) and rhodopsin mutant late-3rd instar larvae in a binary choice. Larvae were given a choice between 18° and 28°C. n=4.

(F) Running speed of late-3rd instar larvae at 23°C. The running speeds (cm/min) were calculated only during the times the larvae were moving and were normalized based on the body lengths (mm). n=6. The data represent the means ±SEMs.

See also Figure 5.

Supplemental Experimental Procedures

Sources of fly stocks, and rearing

We used w^{1118} as the wild-type control. The following flies were obtained from the Bloomington Stock Center (stock numbers are indicated): *Gα49B*^{1} (#42257), *ninaE*^{117} (#5701), *GMR-hid* (#5771), *UAS-dicer2* (#24646, #24650), *UAS-mCD8::GFP* (#5137), *20XUAS-6XGFP* (#52261, #52262), *LexAop-frt-mCherry-STOP-frt-ReaChR::Citrine* (#53745), *elav-GAL4* (#8760), *trp-GAL4* (#36359), *GMR-GAL4* (#8605), *inactive-GAL4* (#52273), *117Y-GAL4* (#30814), *trpA1-CD-QF* (#36348) and *10XQUAS-6XmCherry* (#52270). The following stocks were provided by the indicated investigators: *rh4*^{1}, *rh5*^{2}, *rh6*^{1}, *UAS-rh6* (C. Desplan), *norpA*^{P24} (W. Pak), *plc21c*^{P319} (G. Hasan) and *Gr66a-GAL4* (H. Amrein). The following stocks were created in our laboratory and some are available from the Bloomington Stock Center (stock numbers are indicated): *rh2*^{1}, *rh7*^{1} (J. Ni, L.S. Baik, T.C. Holmes and C. Montell, submitted), *trpA1*^{1} (#26504), *trpA1-AB*^{G4}, *trpA1-AB*^{LexA}, *trpA1-CD*^{G4}, *santa maria*^{1} (#24520), *Gr33a*^{GAL4} (#31425), *UAS-rh5*. The generation of *trpA1-AB*^{LexA} and *trpA1-CD*^{G4} is described in detail elsewhere (J. Luo, W.L. Shen and C. Montell, Nat. Neurosci. in press). The following RNAi lines were from the Harvard Transgenic RNAi Project (TRiP: http://www.flyrnai.org/TRiP-HOME.html): *rh3* (JF01584), *Gα49B* (JF02464) and *trpA1* (JF02461). The following RNAi lines were from the Vienna Drosophila RNAi Center (https://stockcenter.vdrc.at/control/main): *rh5* (#101930) and *rh6* (#102152).

Flies were reared on standard cornmeal-yeast medium: 22,800 ml distilled water, 190 g agar, 1,609.2 g cornmeal, 379.2 g yeast, 220.8 g soy flour, 240 ml Tegosept (30% in ethanol), 108 ml propionic acid, 8.5 ml phosphoric acid and 1,700 ml corn syrup. The mutant lines were outcrossed to the control line (w^{1118}) for five generations.

Generation of *rh5*^{G} *and rh6*^{G} flies

We generated 0.5 kb of deletions in *rh5* (*rh5*^{G}) and *rh6* (*rh6*^{G}) by ends-out homologous recombination (Gong and Golic, 2003). To create the DNA constructs to generate the mutations, we PCR amplified two genomic DNA fragments (~3 kb) flanking each side of the deletion region and subcloned the fragments into the pw35*GAL4* vector (Moon et al., 2009) using the Clontech In-Fusion Cloning System. We crossed the transgenic flies with *hs-FLP* transgenic flies to generate the targeted alleles. We confirmed the *rh5*^{G} and *rh6*^{G} mutations by PCR.

Temperature gradient assays

The apparatus for performing the gradient assays included an aluminum tray (outer: 14 x 10.1 x 0.9 cm, inner: 12.9 x 8.7 x 0.8 cm), which was filled with 25 ml of 2% agarose (Figure 1A). The upper and lower rims of each tray contained 12 demarcations, separated by 1 cm. The first and last demarcations were 0.45 cm from the inner edge of the trays. The gel trays were placed on top of two aluminum blocks (5.1 cm wide, 25.5 cm long and 1.4 cm thick) separated by 10 cm and individually temperature controlled using a circulating water bath (9106, Thomas Scientific). To establish an 18°—28°C temperature gradient, the water in the two baths were set at two different temperatures, which varied depending on the ambient temperature. We gently scratched the agarose surfaces and sprayed water to prevent the gels from drying. To verify the 18°—28°C gradients, we monitored the surface temperatures on the test plates in each of six zones (2 cm wide) using a probe (YC520-13K, Chino Corporation) connected to a thermometer (MC3000-000, Chino Corporation). A linear temperature gradient on the surface of the gels formed within 5 minutes.

To prepare synchronized larvae for the temperature gradient assays, we maintained flies at 25°C under 12-hour light/12-hour dark cycles. To boost egg production, we allowed the flies to recovery from CO_2 for ≥48 hours in vials containing standard food supplemented with yeast paste. Each vial contained 12—35 females and 8—15 males (1—20 days old) to produce comparable numbers of larvae per vial. To prepare 120 hours AEL samples, we tapped the flies over to new vials containing standard food with yeast granules, allowed the females to lay eggs for 3—6 hours on day 1, and aged the animals. In those cases in which we prepared 3rd instar larvae at 72 and 96 hours AEL, we transferred the same flies into vials supplemented with yeast paste without using CO_2 and incubated them overnight. On days 2 and 3, we again transferred the flies to fresh vials and allowed the females to lay eggs over a 3—6 hour period and aged the animals. All the egg laying was performed during a light period. Typically, 72 hours AEL coincided with the initiation of the 3rd instar larval period, while 120 hours AEL was immediately prior to the wandering stage.

Larvae were collected from the food into an 18% sucrose solution in 50 ml tubes (352070, BD Falcon) to allow the larvae to float and the debris to sink. The top layer containing larvae were transferred to another 50 ml tube, which was filled with fresh 18% sucrose solution. The top layer with larvae was again transferred to a fresh 50 ml

tube and the larvae were washed thoroughly with DI water three times. The larvae were kept in empty 35 mm dishes (353001, BD Falcon) at room temperature for 10—20 minutes to allow them to recover from the washing procedure, and subsequently used for the assays. If the number of larvae was less than ~150, the sample was discarded.

To initiate the gradient assays, larvae (~150) were released in a line at the border between the 22° and 24°C zones (Figure 1B). The whole surface of the gel was covered with a microplate lid to minimize escaping of larvae. The experiments were conducted under virtual darkness (<0.1 $\mu W/cm^2$) by covering the assay system with a cardboard box, unless indicated otherwise. We typically allowed the animals to make temperature selections for 16—20 min for 72 hours AEL samples, 14—17 min for 96 hours AEL samples and 11—14 min for 120 hours AEL samples, due to differences in their speed of movement.

The larvae in each of the six temperature zones (2 cm wide each) were counted and the distribution was calculated as follows: (number of larvae in a given 2-cm zone)/(total number of larvae in 6 zones) x 100%. We omitted from the tabulation larvae in the 0.45 cm borders between the gel and aluminum walls, larvae on the walls of the apparatus, larvae outside of the plates and immobile larvae in the release zone. Typically, the final tabulation included 150—300 larvae. The light intensities under dark condition and in the presence of light were measured at multiple locations using a photometer (Model 815, Newport), and the values were averaged.

To perform the bisymmetrical gradient assays (Figure S1), a 25 x 22 cm aluminum plate was filled with 120 ml of 2% agarose. Two aluminum blocks connected to one water bath were placed on the left and right edges of the plate and an aluminum plate connected to another water bath was placed in the center of the plate. The surface temperature gradient (18°—26°C) was formed bisymmetrically. A total of 250—400 late 3rd instar larvae were released and the number of larvae in each 2-cm zone was counted 15—20 minutes after the release. The numbers of larvae on each side were calculated separately.

Thermal two-way choice assays

The two-way choice assay was similar to that which we described previously (http://www.natureprotocols.com/2008/07/28/assaying_thermotaxis_behavior.php), but with modifications. We reared and collected late-3rd instar larvae (120 hours AEL) as described above for the gradient assays.

To prepare the assay plates, we used plexiglass plate covers (8.38 x 5.83 cm) from 6 x 12 mini trays (136528, Nunc) coated with 7.5 ml of 2% agarose. The three plates were placed on top of two adjacent aluminum blocks, which were separated using X-ray film as a spacer. The blocks were individually temperature controlled using a circulating water bath (9106, Thomas Scientific). We gently scratched the agarose surfaces and sprayed water to prevent the gels from drying. We monitored the surface temperatures on the center of each side of the test plate using thermometers (MC3000-000, Chino Corporation) with probe (YC520-13K, Chino Corporation).

To initiate the gradient assays, larvae (60—100) were released in a line at the border between the 18° and 28°C areas (release zone). The experiments were conducted under virtual darkness (<0.1 $\mu W/cm^2$) by covering the assay system with a cardboard box. The larvae on each side of the plates were counted after 10—12 minutes and the preference indexes (PIs) were calculated as follows: (number of larvae on the 18°C side) – (number of larvae on 28°C side)/(total number of larvae on both sides of the test plate). Larvae in the release zone (1 cm wide) and outside of the plates were not counted.

Locomotion assays

To perform the larval locomotion assays, we used a tracking assay described previously with modifications (Gershow et al., 2012). We reared and collected late 3rd instar larvae (120 hours AEL) in the same manner as described above for the gradient assays.

The arena to monitor the freely-moving larvae consisted of an aluminum plate (12 x 12 cm) coated with 40 ml of 2% agarose. We sprayed water on the surface of the agarose to prevent the gels from drying. The surface temperature of the arena was maintained uniformly at 23°C using a circulating water bath (9106, Thomas Scientific). We monitored the surface temperature throughout the experiment using thermocouples (TE Technology).

To initiate the assays, larvae (~30) were sparsely released in the arena to prevent physical contact. The experiments were conducted under red LED lights. The camera and illumination system was similar to that described previously (Ohyama et al., 2013). We acclimated the animals to the environment for 5 min and tracked their movements during the following 10 min. The running speeds and the body lengths were calculated using a program we prepared in MATLAB (MathWorks). The running speeds (cm/min) were measured only during the

periods they were moving and the values were normalized according to the body length (mm).

Evaluation of developmental rate

Flies were prepared for egg laying in the same manner as for the temperature gradient assay. To check the entry into the 3rd instar stage, 15 larvae were collected 74 hours AEL and transferred to a glass slide. Larvae were gently squashed between a glass slide and a cover glass, and 10 larvae were randomly picked to check the morphology of mouth hooks and spiracles under a Nikon TE300 microscope using a HMC 20x ELWD Plan Fluor/0.45 objective. The percentages of 3rd instar larvae were calculated based on the size of mouth hooks and the typical morphology of spiracles. To check pupation timing, 80 larvae were collected 74 hours AEL and transferred to a new vial containing standard food. The numbers of pupae on the vial walls were counted during the light periods. The percentages of pupae were calculated based on the maximum number at 227 hours AEL. T_{50} and T_{80} were the times at which 50% and 80% of the animals underwent pupation, respectively.

Immunostaining

The following primary antibodies were used at the indicated dilutions, and all secondary antibodies were used at a 1:1000 dilution except for the experiments employing the tyramide signal amplification (TSA) method (see below): 1) Rh6 staining: mouse anti-Rh6 (1:200, gift from S. Britt, University of Colorado, Denver) and Alexa Fluor 488 goat anti-mouse IgG (A11001, Invitrogen), 2) *rh5*^{G} and *rh6*^{G} reporter staining: rabbit anti-GFP (1:500; A11122, Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (A11008, Invitrogen), 3) *trpA1-CD*^{G4} reporter staining: chicken anti-GFP (1:500; A10262, Invitrogen) and Alexa Fluor 488 goat anti-chicken IgG (A11039, Invitrogen), and 4) *trpA1-AB*^{LexA} and *trpA1-CD*^{G4} reporter co-labeling: chicken anti-GFP (1:500; A10262, Invitrogen), rabbit anti-DsRed (1:500; 632496, Clontech), Alexa Fluor 488 goat anti-chicken IgG (A11039, Invitrogen) and Alexa Fluor 555 donkey anti-rabbit IgG (A31572, Invitrogen).

To perform immunostaining, 3rd instar larvae were placed on a cover glass, quickly killed on a 70°C heat block for 10 seconds and immersed in PBS (154 mM NaCl, 5.60 mM Na_2HPO_4 and 1.05 mM KH_2PO_4; pH 7.4). The larva was immobilized using insect pins (26002-10, Fine Scientific Tools, Inc.) and the cuticles were cut open under a dissection microscope. The guts, fat and trachea were removed while the mouth hooks, brains, ventral nerve cords and surrounding discs were kept intact. The body wall cuticle was flattened with insect pins and fixed with 4% paraformaldehyde/PBS for 15 min at room temperature. The samples were washed with PBS-0.3% Triton X100 (PBST) and blocked with 5% normal goat serum (NGS)/PBST for 30 min at room temperature, followed by incubation with the primary antibodies in 5% NGS/PBST for 1—3 days at 4°C. The samples were washed with PBST (10 min, 4 times) and incubated with the secondary antibodies in PBST overnight at 4°C in the dark. After the PBST wash, the samples were mounted between a slide and coverslip using Vectashield (H-1000, Vector Laboratory) and nail polish.

We incubated the samples as follows to apply the TSA method (Chao et al., 1996) for testing for co-labeling between the *rhodopsin-GAL4* reporters and *trpA1* reporters. We incubated the samples with the primary antibodies (mouse anti-GFP, 1:500; A11120, Invitrogen; rabbit anti-DsRed, 1:500; 632496, Clontech) in 5% NGS/PBST for 5—7 days at 10°C. The samples then were washed with PBST and incubated with the secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG, 1:100; TSA detection kit, T20912, Molecular Probes; Alexa Fluor 568 goat anti-rabbit IgG, 1:500; A11036, Invitrogen) in PBST overnight at 10°C in the dark. The samples were washed with PBST and incubated in TSA buffer containing Alexa Fluor 488-labeled tyramide (1:50) and H_2O_2 (0.0015%) for 10 min at room temperature to enhance the GFP signals. The samples were washed with PBST (30 min, 5 times) and mounted. We imaged the samples using a Zeiss LSM 700 confocal laser scanning microscope using a 20x/0.8 Plan-Apochromat DIC objective, and analyzed the images using Zen software.

To quantify the GFP signal intensities in an unbiased fashion, we prepared a computer script using MATLAB (MathWorks), to identify the regions of interest (ROIs) and the mean pixel intensities. The program measured the mean pixel intensities within ROIs using the GFP channel. The ROIs corresponded to the cell bodies of *trpA1-CD* neurons, which were detected using the RFP channel.

Statistics

Multiple comparisons between the wild-type control and test groups were performed using one-way ANOVA followed by the Dunnett's post-hoc test. To compare the entry of *santa maria*^{1} to the 3rd instar larval stage relative to controls, we used the Unpaired Student's t-test. Values are shown as mean ±SEM, unless indicated otherwise. A *p*

value <0.05 was considered significant.

Supplemental References

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