A gustatory receptor parologue controls rapid warmth avoidance in Drosophila

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Behavioural responses to temperature are critical for survival, and animals from insects to humans show strong preferences for specific temperatures1,2. Preferred temperature selection promotes avoidance of adverse thermal environments in the short term and maintenance of optimal body temperatures over the long term1,2, but its molecular and cellular basis is largely unknown. Recent studies have generated conflicting views of thermal preference in Drosophila, attributing importance to either internal3 or peripheral4 warmth sensors. Here we reconcile these views by showing that thermal preference is not a singular response, but involves multiple systems relevant in different contexts. We found previously that the transient receptor potential channel TRPA1 acts internally to control the slowly developing preference response of flies exposed to a shallow thermal gradient5. We now find that the rapid response of flies exposed to a steep warmth gradient does not require TRPA1; rather, the gustatory receptor GR28B(D) drives this behaviour through peripheral thermosensors. Gustatory receptors are a large gene family, widely studied in insect gustation and olfaction, and are implicated in host-seeking by insect disease vectors5–7, but have not previously been implicated in thermosensation. At the molecular level, GR28B(D) misexpression confers thermosensitivity upon diverse cell types, suggesting that it is a warmth sensor. These data reveal a new type of thermosensory molecule and uncover a functional distinction between peripheral and internal warmth sensors in this tiny ectotherm reminiscent of thermoregulatory systems in larger, endothermic animals5. The use of multiple, distinct molecules to respond to a given temperature, as observed here, may facilitate independent tuning of an animal’s distinct thermosensory responses.

Thermal preference is an important body temperature control mechanism from insects to humans1,2. In Drosophila two sets of warmth-sensing neurons (activated above ~25 °C) have been proposed to control thermal preference: the anterior cell (AC) neurons2, located inside the head, and the hot cell (HC) neurons3, located peripherally in the arista (Fig. 1a). However, different studies suggest conflicting cellular and molecular mechanisms for thermal preference control. At the cellular level, primary importance has been attributed to either internal2 or peripheral3 warmth sensors. At the molecular level, the internal AC neurons sense warmth via TrpA1 (ref. 3), which encodes a warmth-activated transient receptor potential (TRP) channel1,8, whereas the peripheral HC neurons seem to be TrpA1-independent9. To clarify the mechanisms of thermal preference, we sought to discover the molecular basis of HC neuron function.

The arista contains six neurons7: three warmth-responsive HC neurons (which can be labelled using cell-specific Gal4 expression in the HC-GAL4 strain7) and three cool-responsive (cold cell; CC) neurons (labelled in the CC-GAL4 strain4) (Fig. 1b–d). Three unidentified cells in the arista have been reported to express Gr28b.d-GAL4, a transgene in which promoter sequences upstream of the gustatory receptor GR28B(D) control Gal4 expression10. We found that these Gr28b.d-GAL4-expressing cells resembled thermoreceptors, with cell bodies near the arista base and thin processes in the shaft (Fig. 1e). To determine the thermoreceptor subset labelled, Gr28b.d-GAL4 was combined with each thermoreceptor-specific Gal4. Gr28b.d-GAL4 plus HG-CAL4 labelled three neurons (Fig. 1f, n = 5), whereas Gr28b.d-GAL4 plus CC-GAL4 labelled six neurons (Fig. 1g, n = 5), indicating that Gr28b.d-GAL4 is expressed in the HC neurons. Although in situ hybridization was unsuccessful (common for gustatory receptors), GR28B(D) transcripts were robustly detected in dissected antennae/arista from wild-type, but not Gr28b mutant, animals by reverse transcriptase PCR (RT–PCR) (Supplementary Fig. 1), demonstrating expression in this tissue.

Gustatory receptors are a large family of seven transmembrane proteins present in invertebrates2, with 68 members in Drosophila melanogaster11 (Supplementary Fig. 2). Insects also contain multiple gustatory receptor-related odorant receptors (62 in D. melanogaster12) and are involved in host-seeking by insect disease vectors5–7, but have not previously been implicated in thermosensation. We examined gustatory receptor involvement in thermosensation using a two-temperature choice assay5, exposing flies for 1 min to a steep thermal gradient (initially >5 °C per cm) created using tubes of ~25.5 °C (a preferred) and ~31.0 °C (an innocuous temperature) separated by 1 cm. Flies normally prefer the cooler tube, a behaviour termed ‘rapid negative thermotaxis’ (Fig. 1h, i). Consistent with a previous report5, inhibiting HC neurons by cell-specific expression of tetanus toxin light chain (TNT), a vesicle release inhibitor13, using HC-GAL4 strongly reduced such behaviour (Fig. 1h). In agreement with the importance of HC neurons, and in addition to previous studies14, third antennal segment/arista removal strongly reduced this behaviour, whereas ablating other tissues expressing HC-GAL4 and Gr28b.d-GAL4 did not (Supplementary Figs 3–5). By contrast, inhibiting AC neurons by TNT expression using TrpA1GAL4, a Gal4 knock-in at the TrpA1 locus15, had no effect (Fig. 1h). (This manipulation disrupted a previously reported AC-dependent thermosensory behaviour15.) These data indicate that rapid negative thermotaxis depends on the peripheral HC warmth sensors.

To probe the molecular basis of rapid negative thermotaxis, we first examined its dependence on TrpA1, which is required for AC neuron warmth-sensing15. Consistent with the TrpA1-independent HC neuron thermosensitivity4, a strong loss-of-function TrpA1 mutation did not affect this behaviour (Fig. 1i). By contrast, strong loss-of-function mutations in the gene encoding GR28B(D) eliminated the response; Gr28b mutants distributed nearly equally between ~25.5 °C and ~31.0 °C (Fig. 1k). The defect was specific: exciting the transposon in the Gr28b allele restored thermotaxis (Fig. 1k), and both a Gr28b-containing genomic transgene and GR28B(D) complementary DNA expression rescued the mutant (Fig. 1k, l). We also attempted rescue

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by expressing cDNAs for the other Drosophila Gr28 family members\textsuperscript{10,11} (four other Gr28b isoforms (Fig. I)) and Gr28\textsuperscript{a,12} under Gr28b.d-GAL4 control. Although a negative result could reflect a failure to be properly expressed, only Gr28b(E) yielded significant rescue (Supplementary Fig. 7). However, endogenous Gr28b(E) transcripts were not detected in the antenna/arista (Supplementary Fig. 1), consistent with a previous analysis indicating that GR28B(E) is not expressed there\textsuperscript{13}. Together, these data demonstrate that rapid negative thermotaxis depends not on TrpA1, but on Gr28b, consonant with the specific dependence of this behaviour on HC neuron function (Fig. 1h). Notably, cell-specific GR28B(D) expression using HC-GAL4 strongly rescued the Gr28b mutant (Fig. II), indicating that GR28B(D) function in the HC thermosensors is sufficient to restore rapid negative thermotaxis.

To test whether GR28B(D) might act as a thermosensor, we examined whether it conferred warmth-sensitivity when ectopically expressed. Unlike controls, flies broadly expressing GR28B(D) under Actin5C-GAL4 control were incapacitated when heated to 37°C for 3 min, recovering when returned to 23°C (Fig. 2a and Supplementary Video 1). This dramatic effect suggested that GR28B(D) might promote warmth-responsive neuronal activation. We showed previously that ectopic expression of the warmth-activated cation channel TRP1(A), a product of Drosophila TrpA1, renders flies thermosensory responsive\textsuperscript{16}. Like TRP1(A), chemosensor expression of GR28B(D) (using Gr28a-GAL4) conferred robust warmth-responsiveness (Fig. 2b). We examined the behavioural consequences of such GR28B(D) expression. When chemically activated, sweet-receptive chemosensors promote proboscis extension\textsuperscript{5,6}. When GR28B(D) was expressed in these cells, strong proboscis extension was elicited by warming to ~32°C (Fig. 2c). This ability to confer warmth-responsiveness is consistent with GR28B(D) acting as a warmth sensor.

Whether GR28B(D) requires sensory neuron-specific cofactors was examined in the neuromuscular system. Unlike controls, motor neurons expressing GR28B(D) (using OK371-GAL4) triggered warmth-responsive excitatory junction potentials at the neuromuscular junction (Fig. 3a). Thus, GR28B(D)-mediated warmth-responsiveness does not require sensory neuron-specific cofactors. The threshold for GR28B(D)-dependent muscle stimulation was 26.0 ± 0.3°C (± s.e.m., n = 12), just above TRP1(A)’s ~25°C threshold in this system\textsuperscript{17}, indicating that both molecules mediate responses to innocuous warming.

To quantify the thermosensitivity of GR28B(D)-dependent responses, currents were monitored using whole-cell patch-clamp electrophysiology. Unlike controls, voltage-clamped motor neurons expressing GR28B(D) exhibited warmth-responsive inward currents (Fig. 3b). The response’s temperature coefficient (Q10, fold change in current per 10°C change) was calculated by Arrhenius analysis\textsuperscript{18} (Fig. 3c). GR28B(D)-dependent currents were highly thermosensitive (Q10 of 25 ± 5 (s.e.m., n = 7), similar to mammalian neurons expressing thermosensitive TRP channels\textsuperscript{18}. Substituting N-methyl-D-glucamine (NMDG)\textsuperscript{+} for Na\textsuperscript{+} in the extracellular solution eliminated heat-responsiveness, consistent with cation channel activation (n = 3; Supplementary Fig. 8).

The potential dependence of GR28B(D) on neuron-specific cofactors was tested in muscle. Although control muscles voltage-clamped at ~60 mV exhibited modest warmth-responsive outward currents (Fig. 3d), muscles expressing GR28B(D) (using Mhc-GAL4) exhibited robust warmth-responsive inward currents (Fig. 3d). The ability of GR28B(D) to confer warmth sensitivity across diverse cell types supports the hypothesis that GR28B(D) acts as a molecular thermoreceptor. It further suggests GR28B(D) as a new class of tool for thermonergic neuronal activation, adding to the TRP-based toolbox currently used in Drosophila\textsuperscript{19}.

Although GR28B(D) resembles TRP1(A) in conferring warmth-sensitivity\textsuperscript{2,15,17}, these two proteins have distinct functions in the fly, with only Gr28b controlling rapid negative thermotaxis (Fig. 1). TrpA1 was found previously to control the slowly developing thermal preference response of flies on a shallow, broad thermal gradient (~0.5°C per cm, 18–32°C). We tested the contribution of Gr28b to this long-term body temperature selection behaviour. As reported previously\textsuperscript{19}, TrpA1 mutants selected unusually warm temperatures after 30 min on...
Figure 2 | GR28B(D) expression confers warmth-responsive neuronal activation and behaviour. a, Top, flies before and after warming. Bottom, knockdown quantification (n = 3 independent assays per genotype, >10 flies per assay). Ectopic GR28b(D) confers warmth-responsiveness upon diverse cell types. b, Gustatory bristle responses to warming. Top, extracellular recording traces. Bottom, average spike rate from gustatory bristles during warming, after subtracting electrolyte-only baseline. GrSa-GAL4 (n = 6) bristles; UAS-Gr28b(D) (n = 9), GrSa>Gr28b(D) (n = 17). c, Frequency of proboscis extension upon warming to ~32 °C (n = 32 flies per genotype). Data are mean ± s.e.m. **Significantly different from UAS and Gal4 alone controls (Tukey’s HSD, α = 0.01).

Figure 3 | GR28B(D) expression yields highly thermosensitive currents. a, Top, muscle response to warming in OK371>Gr28b(D) animals. Bottom, excitatory junction potentials (EJP) during temperature course. OK371-GAL4 (n = 12) muscles; UAS-Gr28b(D) (n = 13), OK371>Gr28b(D) (n = 9). b, Currents in voltage-clamped motor neurons upon warming. OK371-GAL4 (n = 5) motor neurons; UAS-Gr28b(D) (n = 5), OK371>Gr28b(D) (n = 7).

c, Arrhenius plot of warmth-responsive current of OK371>Gr28b(D) motor neuron in panel b. d, Currents in voltage-clamped muscles upon warming. Mhc-GAL4 (n = 3) muscles; UAS-Gr28b(D) (n = 3), Mhc>Gr28b(D) (n = 7). Data are mean ± s.e.m. **Significantly different from UAS and Gal4 alone controls (Tukey’s HSD, α = 0.01).

The fly’s reliance on distinct sensors for distinct aspects of thermal preference is reminiscent of complex thermosensory systems of larger, endothermic animals. In the fly, these warmth-responsive pathways potentially converge in the brain, where both sets of sensors innervate overlapping regions.

Finally, we tested whether Gr28b and TrpA1 were uniquely suited to their roles in the fly. Although TrpA1 was normally not required for rapid negative thermotaxis (Fig. 1I), when expressed in the arista using Gr28b.d-GAL4, TRP1(B) significantly rescued the Gr28b mutant defect (Fig. 4B). (As expected, a less thermosensitive TrpA1 isoform, TrpA1(A), did not rescue the defect (Fig. 4B.) Conversely, although Gr28b was not normally required for slowly developing thermal preference on the shallow gradient (Fig. 4A), GR28B(D) expression under TrpA1GAL4 control significantly rescued the TrpA1 mutant defect (Fig. 4C). Thus, when their expression is manipulated appropriately, GR28B(D) and TrpA1(B) can act in the same cells and support the same behaviours, indicating fundamental functional similarities.

Although studied extensively, the mechanisms of gustatory receptor action are not fully resolved. Gustatory receptors have been reported to act as cation channels22-24 and via G-proteins25. Whether GR28B(D) acts by either mechanism remains unknown. Although attempts to study GR28B(D) in heterologous cells (including Xenopus laevis oocytes and HEK cells; L.N., T. Lauer, P. Taneja, S. Nelson and P.A.G., unpublished observations) were unsuccessful, the ability of GR28B(D) to confer warmth-responsiveness upon diverse cell types argues against a requirement for cell-type-specific cofactors in the fly. Gr28b has been implicated in responses to strong illumination26. This seems to be unrelated to GR28B(D)-dependent thermosensation, as Gr28b-dependent photosensors are unresponsive to innocuous warming27 and appear to express other Gr28b isoforms28. GR28B(D)-expressing muscles were not light-responsive (n = 4, Supplementary Fig. 9).

Previous studies have demonstrated the importance of TRP channels in Drosophila thermosensation1; stimulating interest in their
potential involvement in warmth-host-seeking in insect disease vectors\(^\text{24}\). This work raises the possibility that gustatory receptors, including GR28 receptors in disease vectors such as tsetse flies and mosquitoes (Supplementary Fig. 2), regulate thermosensation more broadly. GR28(D) adds to a growing list of highly thermosensitive membrane proteins including not only TRPs, but the mammalian ANO1 chloride channel\(^\text{25}\) and calcium-channel regulator STIM1 (ref. 26). The presence of exceptional thermosensitivity in diverse proteins may facilitate temperature-responsive modulation of diverse physiological responses. Furthermore, using multiple molecules to mediate behavioural responses to similar temperatures may facilitate independent tuning of distinct thermosensory responses.

**METHODS SUMMARY**

**Fly strains.** Gr28b, TrpA1, HC-GALA and CC-GALA strains were previously described\(^\text{3,4,10,15,16,23,27}\). Df(Gr28b) is Df(2L)Exel7031 (Bloomington Stock Center). To control for transposon position effects, all UAS-Gr28 transgenes were inserted at the same landing site, attP2, by site-specific transgenesis\(^\text{22}\).

**Behaviour and physiology.** Two-temperature rapid negative tool toxicosis assay was as described\(^\text{5}\), except tube temperatures were 25.5 ± 0.3 °C and 31.0 ± 0.5 °C (± s.d.), ≥15 flies per trial. Thermal preference assay was as described\(^\text{12,11}\), with 20–60 flies (2–5 days old) per trial. For proboscis extension, female flies (1–5 days old) were starved overnight with water, then glued to glass slides and heated\(^\text{11}\). Flies were given three 5-s heat presentations at 5-s intervals. Physiology is detailed in methods.

**Molecular biology.** Transgenic fly creation and RT–PCR were performed as described\(^\text{22}\). RT–PCR primers straddled splice junctions to minimize genomic DNA amplification. Three independent tissue preparations gave similar results.

**Phylogeny.** As gustatory receptor sequence diversity creates the potential for alignment ambiguities, phylogeny was created using BAliPhy\(^\text{9}\), which performs simultaneous Bayesian inference of alignment and phylogeny. Further details provided in Methods.

**Full Methods** and any associated references are available in the online version of the paper.

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1. Garrity, P. A., Goodman, M. B., Samuel, A. D. & Sengupta, P. Running hot and cold: behavioral strategies, neural circuits, and the molecular machinery for thermotaxis in C. elegans and Drosophila. Genes Dev. 24, 2365–2382 (2010).

2. Flourens, A. D. Functional architecture of behavioural thermoregulation. Eur. J. Appl. Physiol. 111, 1–8 (2011).

3. Hamada, F. N. et al. An internal thermal sensor controlling temperature preference in Drosophila. Nature 454, 217–220 (2008).

4. Gallio, M., Ofstad, T. A., Macpherson, L. J., Wang, J. W. & Zuker, C. S. The coding of temperature in the Drosophila brain. Cell 144, 614–624 (2011).

5. Thorne, N., Chromey, C., Bray, S. & Amrein, H. Taste perception and coding in Drosophila. Annu. Rev. Neurosci. 30, 505–533 (2007).

6. Vosshall, L. B. & Stocker, R. F. Molecular architecture of smell and taste in Drosophila. Cell 123, 235–242 (2005).

7. Silbering, A. F. & Benton, R. Ionotropic and metabotropic mechanisms in chemoreception: ‘chance or design?’ EMBO Rep. 11, 173–179 (2010).

8. Vosshall, L. B. & Stocker, R. F. Molecular architecture of smell and taste in Drosophila. Nature Rev. Neurosci. 10, 505–533 (2009).

9. Foelix, R. F., Stocker, R. F. & Steinbrecht, R. A. Fine structure of a sensory organ in the arista of Drosophila melanogaster and some other dipterans. Cell Tissue Res. 258, 277–287 (1989).

10. Thorne, N. & Amrein, H. Apycical expression of Drosophila gustatory receptor genes in sensory and central neurons. J. Comp. Neurol. 506, 548–568 (2008).

11. Robertson, H. M., Warr, C. G. & Carlson, J. R. Molecular evolution of the insect chemoreceptor gene superfamily in Drosophila melanogaster. Proc. Natl Acad. Sci. USA 100, 14537–14542 (2003).

12. Sayeed, O. & Benzer, S. Behavioral genetics of thermosensation and hygroosensation in Drosophila. Proc. Natl Acad. Sci. USA 93, 6079–6084 (1996).

13. Sweeney, S. T., Broadie, K., Keane, J., Niemann, H. & O’Kane, C. J. Targeted expression of fetalbumin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14, 341–351 (1995).

14. Zars, T. Two thermosensors in Drosophila have different behavioral functions. J. Comp. Physiol. A 187, 235–242 (2001).

15. Kim, S. H. et al. Drosophila TRP1 channel mediates chemical avoidance in gustatory receptor neurons. Proc. Natl Acad. Sci. USA 107, 8440–8445 (2010).

16. Kang, K. et al. Modulation of TRP1 thermal sensitivity enables sensory discrimination in Drosophila. Nature 481, 76–80 (2012).

17. Pulver, S. R., Pashkovski, S. L., Horstein, N. J., Garrity, P. A. & Griffith, L. C. Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRP1 determine behavioral output in Drosophila larva. J. Neurophysiol. 101, 3075–3088 (2009).

18. Vyklicky, L. et al. Temperature coefficient of membrane currents induced by nosoxin heat sensitive neurons in the rat. J. Physiol. (Lond.) 517, 181–192 (1999).

19. Bernstein, J. G., Garrity, P. A. & Boyd, E. S. Optogenetics and thermogenetics: technologies for controlling the activity of targeted cells within intact neural circuits. Curr. Opin. Neurobiol. 22, 61–71 (2012).

20. Gingl, E., Hinterwirth, A. & Tichy, H. Sensory representation of temperature in mosquito warm and cold cells. J. Neurophysiol. 94, 176–185 (2005).

21. Sato, K., Tanaka, K. & Touchara, K. Sugar-regulated cation channel formed by an insect gustatory receptor. Proc. Natl Acad. Sci. USA 108, 11680–11685 (2011).

22. Liu, J. et al. A novel phototransduction pathway requires a 6 protein-dependent cGMP pathway and a taste receptor homolog. Nature Neurosci. 13, 715–722 (2010).

23. Xiang, Y. et al. Light-avoidance-mediating photoreceptors tile the Drosophila larval body wall. Nature 468, 921–926 (2010).

24. Wang, G. et al. Anopheles gambiae TRPA1 is a heat-activated channel expressed in thermosensitive sensilla of female antennae. Eur. J. Neurosci. 30, 967–974 (2009).
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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.A.G. (pgarrity@brandeis.edu).
METHODS

Fly strains. Gr28b, TrpAI, HC-GAL4 and CC-GAL4 strains were previously described. Df(Gr28b) is Df(2L)Eve7031 (Bloomington Stock Center). To control for transposon position effects, all UAS-Gr28 transgenes were inserted at the same landing site, atyp, by site-specific transgenesis. UAS-Gr28/B(B) was created from expressed sequence tag clone IP03356 (DGRC stock no. 1623277). Alternative amino termini of UAS-Gr28(A), UAS-Gr28(C) and UAS-Gr28(D) were amplified from CDNA with N-terminal primers (UAS-Gr28(A): 5′-CCG GATATGCACTCCTCAGGATTGGAC-3′; UAS-Gr28(C): 5′-CCGAATCTCAGAATGGTGACAGG-3′; and UAS-Gr28(D): 5′-CCGGAAT TCTCAGTATTTTTACTTGGCGA-3′). Amplified products were digested with EcoRI and SalI, and ligated into pUAST vector (Clontech) upstream of the UAS promoter.

Intron-deleted transgenes were created from genomic DNA with primers 5′-CCGAATTCATGTCATTTTACTTTTGCGAA-3′ and 5′-CCGCGAGATCTCTTTGTTACAAGTG-3′. The first intron of Gr28(E) was deleted by PCR (5′-GCACTACGGAGGTGTTGAAG-3′ and 5′-GGTCTTTACAACCTGGTTAAGTGC-3′). UAS-Gr28a transgene was amplified from genomic DNA with primers 5′-CCGAATTCATGGCCTTTAAGTTGACAGG-3′ and 5′-TCCGAGGATCTCTTTGTTACAAGTG-3′. The introns were deleted by PCR (first intron: 5′-TATCCGAGGATCTCTTTGTTACAAGTG-3′ and 5′-TATCCGAGGATCTCTTTGTTACAAGTG-3′; second intron: 5′-GGCGAGCCACGATGATCTCTTTGTTACAAGTG-3′ and 5′-GGCACTACGGAGGTGTTGAAG-3′). All clones were sequenced to confirm that no mutations were introduced. TRP1A (A) resembles dTRPA1-D (B), but contains 20 additional N-terminal amino acids. TRP1A (B) corresponds to dTRPA1-A (E).

Behaviour and electrophysiology. Two-temperature rapid negative thermotaxis assay was performed as described, except that tube temperatures were 25.5 ± 0.3 °C and 31.0 ± 0.5 °C (± s.d.), ≥15 flies per trial. Ablations were performed with Ultra Fine Clipper Scissors (Fine Science Tools) on ice-anaesthetized 1–2-day-old Canton-S flies. Recovery was 1 h to 2 days. For rapid (1 min) phototaxis, all flies were collected after thermotaxis assay and re-tested using same apparatus but clear-walled tubes (BD Falcon) in a dark-lined box exposed on one side to ultraviolet light (ULTRA-LUM, no. UVA-16). Thermal preference assay was performed as described, except that tube temperatures were 25.5 ± 0.3 °C. Flies were given three 5-s heat presentations (10–15 mW mm−2) across wavelengths measured: pulse 1: 4.3 at 400 nm, 0.75 at 577 nm, 0.02 at 700 nm; pulse 2: 2.4 at 430 nm, 1.02 at 488 nm, 0.75 at 577 nm, 0.1 at 700 nm. Intensities are minimum estimates; meter measured using PM100 light meter (Thor) with 400 nm wavelength correction. Intensity (in mW mm−2) across wavelengths measured: pulse 1: 1.4 at 400 nm, 0.25 at 488 nm, 0.17 at 577 nm, 0.02 at 700 nm; pulse 2: 4.3 at 400 nm, 1.02 at 488 nm, 0.75 at 577 nm, 0.1 at 700 nm. Intensities are minimum estimates; meter was ~2 mW further from source than preparation.

Motor neuron currents were recorded at ~60 mV by whole-cell patch clamp with Multiclamp700A amplifier (Molecular Devices) and patch pipettes (3.5–5 MΩ). External solution was a nominally Ca2+-free modified A solution (in mM: 118 NaCl, 2 KCl, 4 MgCl2, 5 TrisHCl, 45 sucrose, 5 HEPEs), 290 mOsM, pH 7.1–7.2, with 0.15 μM tetrodotoxin to limit spike frequency. The internal solution (in mM: 2 NaCl, 130 K-gluconate, 0.1 CaCl2, 2 MgCl2, 1 EGTA, 0.2 Na-GTP, 10 HEPEs) adjusted to 285 mOsM with glucose, and pH 7.1–7.2 with KOH. Dorsal motor neurons below nerve cord sheath were visualized with ×40 water immersion objective and exposed using 0.75% (w/v) protease (type XIV, Sigma) in modified A solution. For ion substitution, after initial heating, perfusion was changed to nominally Ca2+-free external Modified A solution of the same osmolarity with NaCl replaced by equimolar NMDG and HCl. After 5 min NMDG solution perfusion, preparation was re-heated. Perfusion was then reverted to nominally Ca2+-free modified A solution. After 5 min, a third heat ramp was recorded. Trace plotting and analysis performed in Matlab. All neuromuscular physiology used female third instar larvae.

The data presented reflect biological replicates as noted in each sample’s n. Sample sizes were chosen to reliably reveal robust distinctions among samples. No blinding or randomization was used. Nonparametric analysis (Kruskal–Wallis/Steel–Dwass All Pairs test (IMP10, SAS)) yielded results similar to Tukey’s HSD.

Molecular biology. Transgenic fly creation and RT–PCR were performed as described. RT–PCR primers straddled splice junctions to minimize genomic DNA amplification. Three independent tissue preparations gave similar results. Primers for RT–PCR reactions: Gr28a forward primer: 5′-CAGCAGCAGTA ATCTGAATAATTATC-3′; Gr28a reverse primer: 5′-TATGGTTAAAGAATCTGGTGATA-3′; sec- ond intron: 5′-GGCAGCCACGATGATCTCTTTGTTACAAGTG-3′ and 5′-CA CACTGATTTTTGATTACTCTGTTGCGA-3′. All clones were sequenced to confirm that no mutations were introduced. TRP1A (A) resembles dTRPA1-D (B), but contains 20 additional N-terminal amino acids. TRP1A (B) corresponds to dTRPA1-A (E).

Phylogeny. As gustatory receptor sequence diversity creates the potential for alignment ambiguities, phylogeny was created using BIaL-Phy, which performs simultaneous Bayesian inference of alignment and phylogeny. The analysis was performed using the RAxEL insertion/deletion model, LG substitution matrix, estimating equilibrium amino acid frequencies, with gamma distributed rate variation (four categories). Two independent chains were run until the average standard deviation of split frequencies (ASDSF) and potential scale reduction factor were <1.01 and 1.0, respectively.

29. Zhong, L. et al. Thermosensory and nonthermosensory isoforms of Drosophila melanogaster TRP1A reveal heat-sensor domains of a thermoTRP channel. Cell Rep. 1, 43–55 (2012).

30. Redelings, B. D. & Suchard, M. A. Incorporating indel information into phylogeny estimation for rapidly emerging pathogens. BMC Evol. Biol. 7, 40 (2007).

31. Le, S. Q. & Gascuel, O. An improved general amino acid replacement matrix. Mol. Biol. Evol. 25, 1307–1320 (2008).