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A gustatory receptor paralog controls rapid warmth avoidance in *Drosophila*

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Abstract

Behavioral responses to temperature are critical for survival, and animals from insects to humans show strong preferences for specific temperatures[1, 2]. Preferred temperature selection promotes avoidance of adverse thermal environments in the short-term and maintenance of optimal body temperatures over the long-term[1, 2], but its molecular and cellular basis is largely unknown. Recent studies have yielded conflicting views of thermal preference in *Drosophila*, attributing importance to either internal[3] or peripheral[4] warmth sensors. Here we reconcile these views by demonstrating that thermal preference is not a singular response, but involves multiple systems relevant in different contexts. We previously found that the Transient Receptor Potential (TRP) channel TRPA1 acts internally to control the slowly developing preference response of flies exposed to a shallow thermal gradient[3]. Here we find that the rapid response of flies exposed to a steep warmth gradient does not require TRPA1; rather, the Gustatory receptor (Gr) Gr28b(D) drives this behavior via peripheral thermosensors. Grs are a large gene family widely studied in insect gustation and olfaction and implicated in host-seeking by insect disease vectors[5–7], but not previously implicated in thermosensation. At the molecular level, Gr28b(D) misexpression confers thermosensitivity upon diverse cell types, suggesting 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 animals[2]. The use of multiple, distinct molecules to respond to a given temperature,

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as observed here, may facilitate independent tuning of an animal's distinct thermosensory responses.

Keywords

Gr28b; thermosensation; TRPA1; TRP; thermosensor; thermoreceptor

Thermal preference is an important body temperature control mechanism from insects to humans[1, 2]. In *Drosophila* two sets of warmth-sensing neurons (activated above ~25˚C) have been proposed to control thermal preference: the Anterior Cell (AC) neurons[3], located inside the head, and the Hot Cell (HC) neurons[4], 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 internal[3] or peripheral[4] warmth sensors. At the molecular level, while the internal AC neurons sense warmth via *TrpA1*[3], which encodes a warmth-activated Transient Receptor Potential (TRP) channel[3, 8], the peripheral HC neurons appear *TrpA1*-independent[4]. To clarify the mechanisms of thermal preference, we sought the molecular basis of HC neuron function.

The arista contains six neurons[9]: three warmth-responsive HC neurons (which can be labeled using cell-specific Gal4 expression in the *HC-Gal4* strain[4]) and three cool-responsive CC neurons (labeled in the *CC-Gal4* strain[4]) (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 expression[10]. We found 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 labeled, *Gr28b.d-Gal4* was combined with each thermoreceptor-specific Gal4. *Gr28b.d-Gal4* plus *HC-Gal4* labeled three neurons (Fig. 1f, n=5), while *Gr28b.d-Gal4* plus *CC-Gal4* labeled six neurons (Fig. 1g, n=5), indicating *Gr28b.d-Gal4* is expressed in the HC neurons. Although *in situ* hybridization was unsuccessful (common for Grs[5]), Gr28b(D) transcripts were robustly detected in dissected antennae/aristae from wild type, but not *Gr28b* mutant animals by RT-PCR (Supp. Fig. 1), demonstrating expression in this tissue.

Grs are a large family of seven transmembrane proteins present in invertebrates[7], with 68 members in *Drosophila melanogaster*[11](Supp. Fig. 2). Insects also contain multiple Gr-related Odorant Receptors (Ors; 62 in *D. melanogaster*[11]). Grs and Ors form a gene family distinct from and apparently unrelated to the GPCR superfamily[7]. Gr/Or's have been extensively studied as chemoreceptors for sweet and bitter tastants, food odors, carbon dioxide and other chemicals[5–7], but not previously implicated in thermosensation. We examined Gr involvement in thermosensation using a two-temperature choice assay[12], exposing flies for 1 min to a steep thermal gradient (initially >5˚C/cm) created using tubes of ~25.5 and ~31.0˚C air (a preferred and an elevated but innocuous temperature, respectively) separated by 1 cm. Flies normally prefer the cooler tube, a behavior termed "rapid negative thermotaxis" (Fig. 1h, i). Consistent with prior report[4], inhibiting HC neurons by cell-specific expression of tetanus toxin light chain (TNT, a vesicle release

inhibitor[13]) using *HC-Gal4* strongly reduced such behavior (Fig. 1h). In agreement with the HC neurons' importance, and previous studies[14], third antennal segment/arista removal strongly reduced this behavior, while ablating other tissues expressing *HC-Gal4* and *Gr28b.d-Gal4* did not (Supp. Figs. 3–5). In contrast, inhibiting AC neurons by TNT expression using $TrpA1^{Gal4}$, a Gal4 knock-in at the *TrpA1* locus[15], had no effect (Fig. 1h). (This manipulation disrupted a previously reported AC-dependent thermosensory behavior[3] (Supp. Fig. 6).) These data indicate rapid negative thermotaxis depends on the peripheral HC warmth sensors.

To probe rapid negative thermotaxis' molecular basis, we first examined its dependence on *TrpA1*, which is required for AC neuron warmth-sensing[3]. Consistent with the *TrpA1*-independence of HC neuron thermosensitivity[4], a strong loss-of-function *TrpA1* mutation did not affect this behavior (Fig. 1i). In 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: excising the transposon in the $Gr28b^{Mi}$ allele restored thermotaxis (Fig. 1k), and both a *Gr28b*–containing genomic transgene and Gr28b(D) cDNA expression rescued the mutant (Fig. 1k, l). We also attempted rescue by expressing cDNAs for the other *Drosophila* Gr28 family members[10, 11] (four other *Gr28b* isoforms (Fig. 1j) and *Gr28a*[11, 12]) under *Gr28b.d-Gal4* control. While a negative result could reflect a failure to be properly expressed, only Gr28b(E) yielded significant rescue (Supp. Fig. 7). However, endogenous Gr28b(E) transcripts were not detected in the antenna/arista (Supp. Fig. 1), consistent with prior analysis indicating Gr28b(E) is not expressed there[10]. Together, these data demonstrate rapid negative thermotaxis depends not on *TrpA1*, but on *Gr28b*, consonant with the behavior's specific dependence on HC neuron function (Fig. 1h). Importantly, cell-specific Gr28b(D) expression using *HC-Gal4* strongly rescued the *Gr28b* mutant (Fig. 1l), indicating 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 *Actin-Gal4* control were incapacitated when heated to 37˚C for 3 min, recovering when returned to 23˚C (Fig. 2a; Supp. Video 1). This dramatic effect suggested Gr28b(D) might promote warmth-responsive neuronal activation. We previously showed that ectopic expression of the warmth-activated cation channel TRPA1(B), a product of *Drosophila TrpA1*[16], renders fly chemosensors warmth-responsive. Like TRPA1(B), chemosensor expression of Gr28b(D) (using *Gr5a–Gal4*) conferred robust warmth-responsiveness (Fig. 2b). We examined the behavioral consequences of such Gr28b(D) expression. When chemically activated, sweet-responsive chemosensors promote proboscis extension[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 co-factors 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 (NMJ) (Fig. 3a). Thus, Gr28b(D)-mediated warmth-responsiveness does not require

sensory neuron-specific co-factors. The threshold for Gr28b(D)-dependent muscle stimulation was 26.0 +/− 0.3˚C (+/− SEM, n=12), just above TRPA1(B)'s ~25˚C threshold in this system[17], indicating 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[18] (Fig. 3c). Gr28b(D)-dependent currents were highly thermosensitive (Q10 of 25 +/− 5 (SEM, n=7)), similar to mammalian neurons expressing thermosensitive TRP channels[18]. Substituting $NMDG^+$ for Na^+ in the extracellular solution eliminated heat-responsiveness, consistent with cation channel activation (n=3, Supp. Fig. 8).

Gr28b(D)'s potential dependence on neuron-specific co-factors was tested in muscle. While 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). Gr28b(D)'s ability to confer warmth sensitivity across diverse cell types supports Gr28b(D) acting as a molecular thermoreceptor. It further suggests Gr28b(D) as a new class of tool for thermogenetic neuronal activation, adding to the TRP-based toolbox currently used in *Drosophila*[19].

While Gr28b(D) resembles TRPA1(B) in conferring warmth-sensitivity[3, 16, 17], they have distinct functions in the fly, with only *Gr28b* controlling rapid negative thermotaxis (Fig. 1). *TrpA1* was previously found to control the slowly developing thermal preference response of flies on a shallow, broad thermal gradient (~0.5˚C/cm, 18–32˚C)[3]. We tested *Gr28b*'s contribution to this long-term body temperature selection behavior. As reported[3], *TrpA1* mutants selected unusually warm temperatures after 30 min on the gradient, with many accumulating at ≥28˚C (Fig. 4a). In contrast, strong loss-of-function *Gr28b* mutants behaved indistinguishably from wild type (Fig. 4a). This cleanly distinguishes *Gr28b* and *TrpA1*, with the former controlling rapid negative thermotaxis and the latter long-term body temperature selection.

These findings reconcile previously disparate views of *Drosophila* thermosensation[3, 4] by demonstrating that thermal preference is not a singular behavior, but involves multiple systems relevant in different contexts. It suggests a model in which *Gr28b*, acting peripherally, controls rapid responses to ambient temperature jumps, while *TrpA1*, acting internally, controls responses to sustained temperature elevations reaching the core. In the arista, *Gr28b* could experience ambient temperature fluctuations in advance of core changes, eliciting rapid avoidance. Such behavior could be critical for a tiny animal where ambient and core temperatures equalize rapidly[1]. *Gr28b's* dispensability for responses on the shallow gradient (Fig. 4a) could relate to observations in other insects where peripheral thermoreceptors respond more to temperature fluctuations than absolute values[20]. The fly's reliance on distinct sensors for distinct aspects of thermal preference is reminiscent of complex thermosensory systems of larger, endothermic animals[2]. In the fly, these warmth-

responsive pathways potentially converge in the brain, where both sets of sensors innervate overlapping regions[4].

Finally, we tested whether *Gr28b* and *TrpA1* were uniquely suited to their roles in the fly. While *TrpA1* was normally not required for rapid negative thermotaxis (Fig. 1i), when expressed in the arista using *Gr28b.d-Gal4*, TRPA1(B) significantly rescued the *Gr28b* mutant defect (Fig. 4b). (As expected, a less thermosensitive *TrpA1* isoform, TRPA1(A)[16], did not rescue (Fig. 4b).) Conversely, while *Gr28b* was not normally required for slowly developing thermal preference on the shallow gradient (Fig. 4a), Gr28b(D) expression under $TrpA1^{Gal4}$ 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 behaviors, indicating fundamental functional similarities.

Although extensively studied, the mechanisms of Gr action are not fully resolved[7]. Grs have been reported to act as cation channels[7, 21] and via G proteins[22]. Whether Gr28b(D) acts by either mechanism remains unknown. While attempts to study Gr28b(D) in heterologous cells (including *X. laevis* oocytes and HEK cells, L.N., T. Lauer, P. Taneja, S. Nelson and P.G., unpub.) were unsuccessful, Gr28b(D)'s ability to confer warmth-responsiveness upon diverse cell types argues against a requirement for cell-type-specific co-factors in the fly. *Gr28b* has been implicated in responses to strong illumination[23]. This appears unrelated to Gr28b(D)-dependent thermosensation, as *Gr28b*–dependent photosensors are unresponsive to innocuous warming[23] and appear to express other *Gr28b* isoforms[10]. Gr28b(D)-expressing muscles were not light-responsive (n=4, Supp. Fig. 9).

Prior studies demonstrated the importance of TRP channels in *Drosophila* thermosensation[1], stimulating interest in their potential involvement in warmth-dependent host-seeking by insect disease vectors[24]. The present work raises the possibility that Grs, including Gr28's in disease vectors like tsetse flies and mosquitoes (Supp. Fig. 2), regulate thermosensation more broadly. Gr28b(D) adds to a growing list of highly thermosensitive membrane proteins including not only TRPs, but the ANO1 chloride channel[25] and the calcium-channel regulator STIM1[26]. The presence of exceptional thermosensitivity in diverse proteins may facilitate temperature-responsive modulation of diverse physiological responses. Furthermore, using multiple molecules to mediate behavioral responses to similar temperatures may facilitate independent tuning of distinct thermosensory responses.

METHODS SUMMARY

Fly strains

Gr28b, *TrpA1*, *HC-Gal4* and *CC-Gal4* strains were previously described[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[16].

Behavior and Physiology

Two-temperature rapid negative thermotaxis assay was as described[12], except tube temperatures were 25.5+/−0.3˚C and 31.0 +/− 0.5˚C (+/− s.d.), ≥15 flies/trial. Thermal

preference assay was as described[3, 12], with 20–60 flies (2–5 days old)/trial. For proboscis extension, female flies (1–5 days old) were starved overnight with water, then glued to glass slides and heated[16]. Flies were given three 5 sec heat presentations at 5 sec intervals. Physiology is detailed in methods.

Molecular Biology

Transgenic flies creation and RT-PCR performed as described[16]. RT-PCR primers straddled splice junctions to minimize genomic DNA amplification. Three independent tissue preparations gave similar results.

Phylogeny

As Gr sequence diversity creates the potential for alignment ambiguities, phylogeny was created using BAli-Phy[28], which performs simultaneous Bayesian inference of alignment and phylogeny. Further details provided in Methods.

METHODS

Fly strains

Gr28b, *TrpA1*, *HC-Gal4* and *CC-Gal4* strains were previously described[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[16]. *UAS-Gr28b.b* was created from EST clone IP03356 (DGRC stock No. 1623277). Alternative N termini of *UAS-Gr28b.a*, *UAS-Gr28b.c* and *UAS-Gr28b.d* were amplified from cDNA with N-terminal primers (*UAS-Gr28b.a*: 5'-CCGGAATTCATGATCCGCTGCGGATTG GAC-3'; *UAS-Gr28b.c*: 5'-CCGGAATTCATGGACATTGAAATGGCCAAGG-3' and *UAS-Gr28b.d*: 5'-CCGGAATTCATGTCATTTTACTTTTGCGAA-3') and common primer (5'-TCCGCAGGATCCTTGGTTACAATGG-3'). *UAS-Gr28b.e* was amplified from genomic DNA with primers 5'-CCGGAATTCATGTGGCTCCTTAGGCGATCGG-3' and 5'-TCCGCAGGATCCTTGGTTACAATGG-3'. *Gr28b.e*'s first intron was deleted by PCR (5'-GCACTTAACGAGGTGTTGAAGAACC-3' and 5'-GGTTCTTCAACACCTCGTTAAGTGC-3'). *UAS-Gr28a* transgene was amplified from genomic DNA with primers 5'-CCGGAATTCATGGCCTTTAAGTTGTGGGAG-3' and 5'- TCCCCTCGAGGTATATATAATTTTAATCAATCG-3'. The introns were deleted by PCR (first intron: 5'-TATCCTGCAGGATTTCGTTTAACATACTAA-3' and 5'-TTAGTATGTTAAACGAAATCCTGCAGGATA-3'; second intron: 5'-GGCAGCACCAGTAATCGTAAAAATCAGTGTG-3' and 5'-CACACTGATTTTTACGATTACTGGTGCTGCC-3'). All clones were sequenced to confirm no mutations were introduced. TRPA1(A) resembles dTRPA1-D[29], but contains 20 additional N-terminal amino acids. TRPA1(B) corresponds to dTRPA1-A[29].

Behavior and Electrophysiology

Two-temperature rapid negative thermotaxis assay was as described[12], except tube temperatures were 25.5+/−0.3˚C and 31.0 +/− 0.5˚C (+/− s.d.), ≥15 flies/trial. Ablations were performed with Ultra Fine Clipper Scissors (Fine Science Tools) on ice-anesthetized

1–4 day old *white;Canton-S* flies. Recovery was 1 hour 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, Cat. #352051) in dark-lined box exposed on one side to UV light (ULTRA-LUM #UVA-16). Thermal preference assay was as described[3, 12], with 20–60 flies (2–5 days old)/trial. For proboscis extension, female flies (1–5 days old) were starved overnight with water, then glued to glass slides and heated[16]. Flies were given three 5 sec heat presentations at 5 sec intervals.

Extracellular recordings of gustatory neurons were as described[16]. At least three animals and six bristles examined per genotype.

NMJ potentials were recorded by current clamp from muscle 6 with 3 M KCl-filled intracellular electrodes (20–30 MΩ) in 0.4 mM Ca^{2+} HL3.1, using an Axoclamp2B (Molecular Devices) and a Digidata1322A (Molecular Devices), recording at 5 kHz with pClamp8 (Molecular Devices). Muscles had −45 mV or lower resting potentials. Perfusate was heated with SC-20/CL100 cooler/controller (Warner Instruments) and temperature monitored with bath thermistor (Warner Instruments) or IT-23 thermocouple (Physitemp) connected to 80TK Thermocouple (Fluke).

Muscle currents were recorded by two electrode voltage clamp at −60 mV from muscle 6 as above, but using 0 Ca^{2+} HL3.1 solution with 0.5 mM EGTA, 12 mM $MgCl_2$, 100 microM quinidine and 1 mM 4-AP, and 3 M KCl-filled voltage-sensing (10–15 MΩ) and 3 M CH_3COOK-filled current-passing (5–10 MΩ) electrodes. For light responsiveness, experiments were as above with 0 Ca^{2+} HL3.1 with 0.5 mM EGTA, 4 mM $MgCl_2$, 100 microM quinidine and 1 mM 4-AP. 30–50 sec dark baseline (<0.1 μW/mm^2 at 400 nm) was recorded, followed by two ~30 sec pulses from halogen source at indicated intensities, followed by heat ramp. Intensity was 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 ~2mm farther 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 (3.5–4 MΩ). External solution was a nominally Ca^{2+}-free modified A solution (in mM: 118 NaCl, 2 KCl, 4 $MgCl_2$, 5 Trehalose, 45.5 sucrose, 5 HEPES) 290 mOsm, pH 7.1–7.2, with 0.15 micromolar TTX to limit spiking. The internal solution (in mM: 2 NaCl, 130 K-Gluconate, 0.1 $CaCl_2$, 2 $MgCl_2$, 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 40x 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 Ca^{2+}-free external Modified A solution of same osmolarity with NaCl replaced by equimolar NMDG and HCl. After 5 min NMDG solution perfusion, preparation was reheated. Perfusion was than reverted to nominally Ca^{2+}-free modified A solution. After 5 minutes, 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. Non-parametric analysis (Kruskal-Wallis/Steel-Dwass All Pairs test (JMP10, SAS)) yielded results similar to Tukey HSD.

Molecular biology

Transgenic flies creation and RT-PCR performed as described[16]. 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′- CAGCACCAGTAATCGTAAAAATC −3′

Gr28a reverse primer: 5′-TATGTTAAACGAAATCCTGCAGG −3′;

Gr28b.a forward primer: 5′-AACGTTTGCGAAGTCCTGTC-3′;

Gr28b.b forward primer: 5′-GCTGTGATTTATACGTCGGC-3′;

Gr28b.c forward primer: 5′-CTGTCATCTACCTGACTGCC-3′;

Gr28b.d forward primer: 5′-TTCCTCTGCAGCAGCATTCG-3′;

Gr28b.a, *Gr28b.b*, *Gr28b.c* and *Gr28b.d* common reverse primer: 5′-TCCTGTATAATCTCCGCAGG-3′;

Gr28b.d reverse primer (used in Supp. Fig. 1a): 5′- CTTGACCTCAGCACTTTTGG −3′;

Gr28b.e forward primer: 5′-GGCCCCGCTGATCGTGAAA-3′;

Gr28b.e reverse primer: 5′-GCACTTAACGAGGTGTTGAAG-3′.

Phylogeny

As Gr sequence diversity creates the potential for alignment ambiguities, phylogeny was created using BAli-Phy[28], which performs simultaneous Bayesian inference of alignment and phylogeny. The analysis was performed using the RS07 insertion/deletion model[30], LG substitution matrix[31], estimating equilibrium amino acid frequencies, with gamma distributed rate variation (four categories). Two independent chains were run until the ASDSF and PSRF-80%CI criteria fell below 0.01 and 1.01 respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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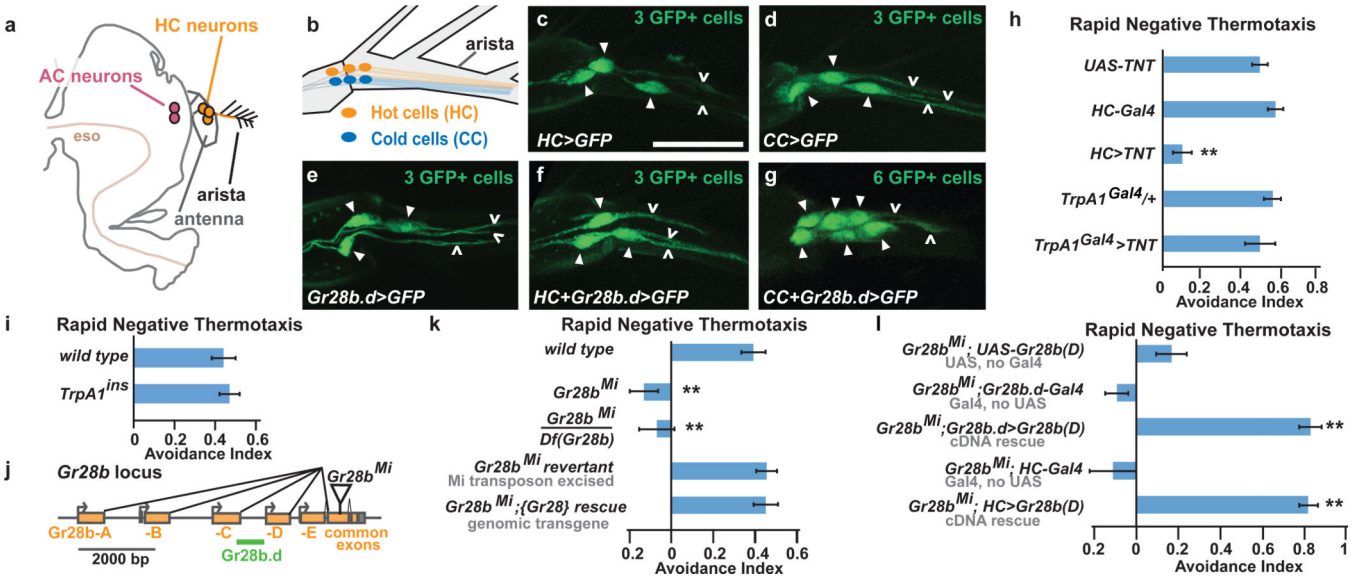

Figure 1. Gr28b is required for rapid negative thermotaxis

a, Head. Eso, esophagus. **b**, Arista. **c***HC-Gal4;UAS-nls:GFP* **d***CC-Gal4;UAS-nls:GFP* **e***Gr28b.d-Gal4;UAS-nls:GFP* **f***HC-Gal4;Gr28b.d-Gal4;UAS-nls:GFP* **g***CC-Gal4;Gr28b.d-Gal4;UAS-nls:GFP*. Arrowheads, cell bodies. Carats, processes. Scale, 15 μm. **h***UAS-TNT*, n=22; *HC-Gal4*, n=15; *HC>TNT*, n=29; $TrpA1^{Gal4}$, n=9; $TRPA1^{Gal4}$ *>TNT*, n=9. **i***wild type*, n=12; $TrpA1^{ins}$, n=29. **j***Gr28b*. Arrows denote isoform-specific start sites. Green line, *Gr28b.d-Gal4* promoter. **k***wild type*, n=12; $Gr28b^{Mi}$, n=13; $Gr28b^{Mi}$/ *Df(Gr28b)*, n=6; revertant n=10; rescue n=8. Data, mean ± s.e.m; n, independent assays. ** significantly different from wild type (**k**) or UAS and Gal4 controls (**h, l**) (Tukey HSD, α = 0.01).

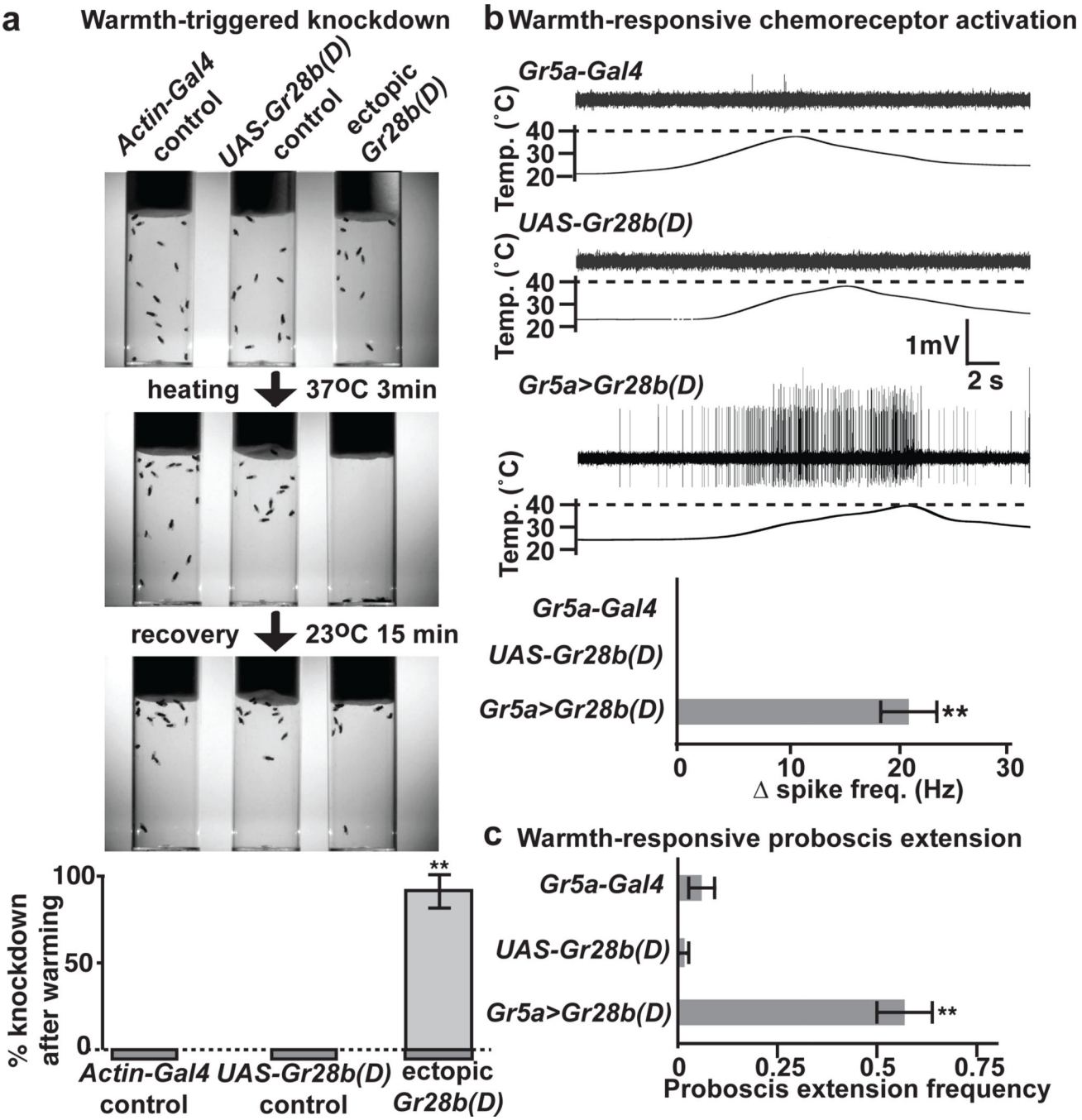

Figure 2. Gr28b(D) expression confers warmth-responsive neuronal activation and behavior

a, Top panels, flies before and after warming. Bottom panel, knockdown quantitation (n=3 independent assays/genotype, >10 flies/assay). Ectopic Gr28b(D) denotes *Actin-Gal4;UAS-Gr28b(D)***b**, Gustatory bristle responses to warming. Top panels, extracellular recording traces. Bottom panel, average spike rate from gustatory bristles during warming, after subtracting electrolyte-only baseline. *Gr5a–Gal4*, n=6 bristles; *UAS-Gr28b(D)*, n=9; *Gr5a>Gr28b(D)*, n=17. **c**, Frequency of proboscis extension upon warming to ~32˚C (n=32

flies/genotype). Data are mean ± s.e.m. ** denotes significantly different from UAS and Gal4 alone controls (Tukey HSD, $\alpha = 0.01$).

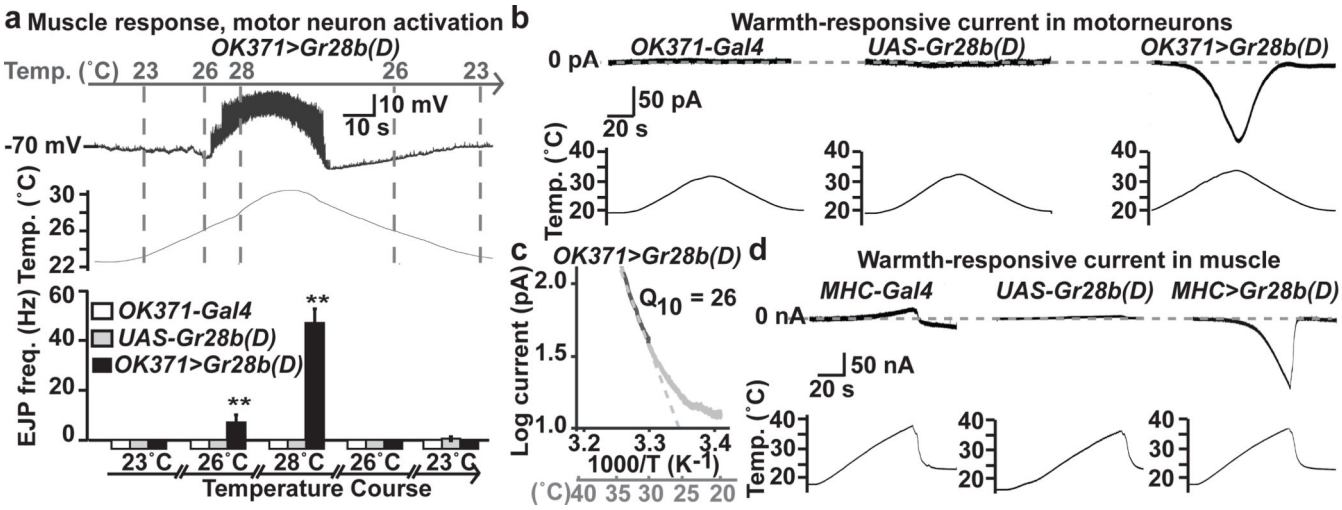

Figure 3. Gr28b(D) expression yields highly thermosensitive currents

a, Muscle response to warming in *OK371>Gr28(D)* animals (top panel). Excitatory junction potentials during temperature course (bottom panel). *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>Gr28(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 differ from UAS and Gal4 alone controls (Tukey HSD, $\alpha = 0.01$).

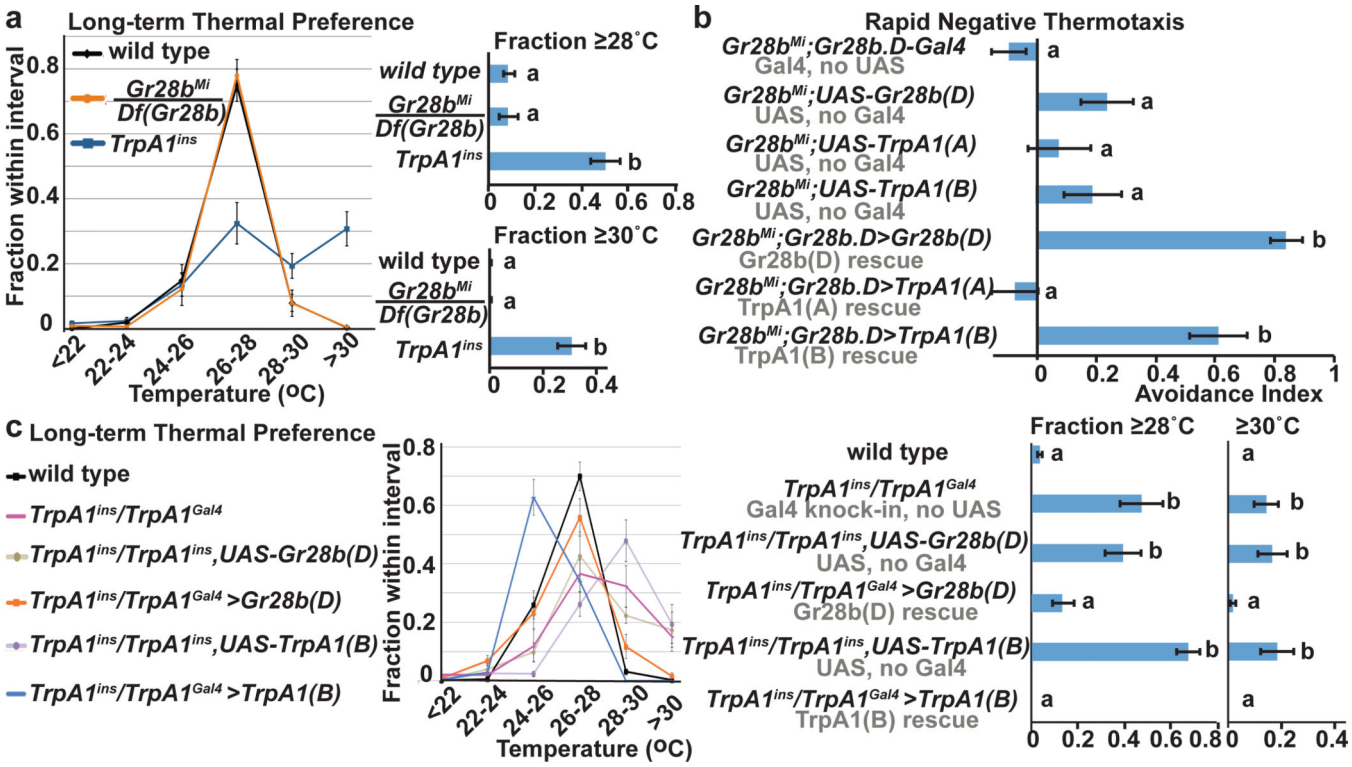

Figure 4. Gr28b(D) and TRPA1(B) functionally substitute for one another

a, Left panel, fly distribution across gradient (30 min). Right panel, flies in ≥28˚C and ≥30˚C regions. *wild type*, n=11, *Gr28b^{Mi}/Df(Gr28b)*, n=8, *TrpA1^{ins}*, n=17. **b**, Rapid negative thermotaxis. *Gr28b^{Mi};Gr28b.d-Gal4*, n=14; *Gr28b^{Mi};UAS-Gr28b(D)*, n=10; *Gr28b^{Mi};UAS-TRPA1(A)*, n=8; *Gr28b^{Mi};UAS-TRPA1(B)*, n=9; *Gr28b^{Mi};Gr28b.d>Gr28b(D)*, n=7; *Gr28b^{Mi};Gr28b.d>TRPA1(A)*, n=9*; Gr28b^{Mi};Gr28b.d>TRPA1(B)*, n=9. (*Gal4* alone, *UAS-Gr28b(D)* alone and *Gr28b(D)* rescue data from Figure 1l.) **c**, Data presented as in **a***wild type*, n=11; *TrpA1^{ins}/TrpA1^{Gal4}*, n=12; *TRPA1^{ins},UAS-Gr28bD*, n=11; *TrpA1^{ins}/TrpA1^{Gal4}>Gr28b(D)* n=14; *TRPA1^{ins},UAS-TrpA1(B)*, n=6; *TrpA1^{ins}/TrpA1^{Gal4}>TrpA1(B)* n=5. Data are mean ± s.e.m; n, independent assays. Letters denote statistically distinct groups (Tukey HSD, $\alpha = 0.01$).