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Modulation of TRPA1 thermal sensitivity enables sensory discrimination in *Drosophila*

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Abstract

Discriminating among sensory stimuli is critical for animal survival. This discrimination is particularly essential when evaluating whether a stimulus is noxious or innocuous. From insects to humans, TRP channels are key transducers of thermal, chemical and other sensory cues[1, 2]. Many TRPs are multi-modal receptors that respond to diverse stimuli[1–3], but how animals distinguish sensory inputs activating the same TRP is largely unknown. Here we determine how stimuli activating *Drosophila* TRPA1 are discriminated. While *Drosophila* TRPA1 responds to both noxious chemicals[4] and innocuous warming[5], we find that TRPA1-expressing chemosensory neurons respond to chemicals but not warmth, a specificity conferred by a chemosensory-specific TRPA1 isoform with reduced thermosensitivity compared to the previously described isoform. At the molecular level, this reduction results from a unique region that robustly reduces the channel's thermosensitivity. Cell-type segregation of TRPA1 activity is critical: when the thermosensory isoform is expressed in chemosensors, flies respond to innocuous warming with regurgitation, a nocifensive response. TRPA1 isoform diversity is conserved in malaria mosquitoes, suggesting similar mechanisms may allow discrimination of host-derived warmth, an attractant, from chemical repellents. These findings indicate that reducing thermosensitivity can be critical for TRP channel functional diversification, facilitating their use in contexts where thermal sensitivity can be maladaptive.

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Author contributions: K.K., V.C.P., and P.A.G. designed experiments. K.K performed molecular biology, genetics, and oocyte physiology. V.C.P. performed genetics and sensory neuron electrophysiology. E.C.C. performed genetics and behavior. A.M.D. performed behavior. L.N. performed immunohistochemistry. A.M.J., K.R. and M.A.T.M. grew and harvested mosquitoes. P.A.G. performed bioinformatics. K.K., V.C.P., and P.A.G. wrote the paper.

Keywords

TRP; polymodal; pain; nociception; thermosensation; chemosensation

Highly temperature-responsive Transient Receptor Potential (TRP) cation channels, thermoTRPs, mediate thermosensation from insects to mammals[1, 2] and are important for human pain and inflammation[6]. Like mammalian thermoTRPs, *Drosophila melanogaster* TRPA1 is both a thermal and chemical sensor, responding to innocuous warmth (above ~25–27°C)[5, 7] and noxious chemicals[4]. TRPA1 acts in thermosensors within the brain to modulate thermal preference over 18–32°C[5], innocuous temperatures compatible with fly survival[8], and in gustatory chemosensors to inhibit ingestion of electrophiles[4], reactive chemicals like allyl isothiocyanate (AITC, found in wasabi) and N-Methyl Maleimide (NMM) that rapidly incapacitate flies (Supp. Fig. 1). TRPA1's responsiveness to both innocuous and noxious stimuli raises the question of how these stimuli are distinguished to elicit distinct behavioral responses. Mammals face similar issues; for example, TRPM8 transduces both innocuous and noxious cold[1–3].

We previously reported TRPA1-expressing chemosensors in the labral sense organ[4]; using improved immunostaining conditions, we now also detect specific TRPA1 protein expression in labellar chemosensors (Fig. 1a, b). Extracellular tip-recording[9] indicated these neurons were TRPA1-dependent chemosensors; they responded to the electrophile NMM with robust spiking in wild type but not *TrpA1* mutants (Fig. 1c, Supp. Fig. 2). The mutant defect was electrophile-specific, as *TrpA1* mutants responded like wild type to berberine chloride (Fig. 1c), a bitter compound that also activates these neurons[10]. In contrast, warming to ~39°C, from innocuously warm to the noxious range, elicited no spiking in these cells (Fig. 1d, e). This is striking as TRPA1's effectiveness in conferring warmth sensitivity has led to its use as a thermogenetic tool[5, 11]. Thus, despite TRPA1's known sensitivity to both temperature and chemicals, these chemosensors are warmth-insensitive.

In addition to the previously characterized transcript, TrpA1(B), a transcript with an alternative 5' end, TRPA1(A), has been annotated[12] (Fig. 2a). These transcripts encode protein isoforms with distinct amino termini, but the same ankyrin and transmembrane domains (Fig. 2b). RT-PCR demonstrated differential expression: TrpA1(A) was expressed in the proboscis, which houses the TRPA1-expressing chemosensors, while TrpA1(B) predominated elsewhere in the head, where TRPA1-expressing thermosensors are located (Fig. 2c).

Examined in *Xenopus* oocytes, TRPA1(A) was much less thermosensitive than TRPA1(B), as reflected in its temperature coefficient (Q10), the fold change in current per 10°C change[1, 2]. Arrhenius plot analysis[13] yielded a Q10 of ~9 for TRPA1(A) versus ~116 for TRPA1(B) (Fig. 2d–f). In addition, while TRPA1(B) was essentially inactive at low temperatures, TRPA1(A)-dependent currents were observed ≤ 15°C, further reducing TRPA1(A)'s temperature-dependent activity differential (Supp. Fig. 3). TRPA1(A)'s maximum heat-activated current was also significantly lower (Supp. Fig. 4). Finally, the transition (or threshold) temperature for increased temperature responsiveness was 29.7 +/− 0.3 °C for TRPA1(A) versus 27.8 +/− 0.4 for TRPA1(B) ($P<0.01$, t-test). As the fly's

innocuous warm temperature range is of particular behavioral relevance, the Q10 from 27°C to 37°C (below the fly's ~38°C nociceptive threshold[14]) was also calculated, yielding 6.2 +/− 0.5 for TRPA1(A) and 90 +/− 8 for TRPA1(B) (Fig. 2f). Other properties were largely unaffected; both channels responded robustly to electrophiles and had similar voltage sensitivities (Fig. 2g, h). TRPA1(A) and TRPA1(B) had similar maximum current amplitudes at 300 μM NMM, with EC50s of 176 +/− 12 and 128 +/− 9 μM, respectively (Fig. 2i).

TRPA1(A)'s reduced thermosensitivity could account for the chemosensors' warmth-insensitivity. But while less temperature-sensitive than TRPA1(B), TRPA1(A)'s Q10 resembles several TRPs suggested to mediate warmth sensitivity[15–17]. To assess whether TRPA1(A) could confer warmth sensitivity upon fly chemosensors, each isoform was used to rescue a *TrpA1* mutant. We previously demonstrated that expressing TRPA1(B) in TRPA1-dependent chemosensors using Gr66a-Gal4 rescues the *TrpA1* mutant behavioral defect[4]. Using electrophysiology, we found both isoforms restored NMM responsiveness (Fig. 3a, Supp. Fig 5a,b), but only TRPA1(B) conferred warmth-sensitivity (Fig. 3b, 3c). These differences did not require properties unique to TRPA1-dependent chemosensors. Each isoform was expressed ectopically in sweet-responsive chemosensors using Gr5a-Gal4[18]. Both isoforms conferred electrophile sensitivity upon these normally electrophile-insensitive neurons, but only TRPA1(B) conferred thermosensitivity (Fig. 3d–f, Supp. Fig. 4c, d). TRPA1(A)'s inability to confer warmth sensitivity on fly chemosensors emphasizes that while a Q10 above 5 makes TRPA1(A) more thermally sensitive than most ion channels, *in vivo* testing is important in evaluating whether a channel is sufficiently thermosensitive to make a specific neuron warmth-responsive.

These data support a model in which the specificity of TRPA1-expressing gustatory neurons for chemicals is established by their selective expression of TRPA1(A), an isoform unable to confer warmth sensitivity. In contrast, TRPA1(B)'s chemical sensitivity should render TRPA1-dependent thermosensors sensitive to reactive chemicals. However, the location of TRPA1-dependent Anterior Cell (AC) thermosensors inside the head[5] should minimize exposure to environmental irritants. Interestingly, multiple TRPV1 and TRPM1 isoforms are present in humans and other mammals[2, 17, 19, 20], suggesting the potential generality of isoform diversity in modulating TRP functions.

The behavioral significance of discriminating noxious from innocuous TRPA1 activators was examined by testing gustatory responses of *TrpA1* mutants rescued by chemosensor expression of each isoform. *TrpA1* mutants exhibit decreased avoidance of reactive electrophile-containing food[4]. Each isoform rescued this behavior (Fig. 3g). However, TRPA1(B) also triggered a nocifensive response to innocuous warming. When allowed to ingest water to satiation and warmed to ~32°C, neither wild-type nor TRPA1(A) rescue animals showed detectable gustatory responses (Fig. 3h, 3i). However, warming TRPA1(B) rescue flies caused ~75% to regurgitate (Fig. 3h, 3i; Supp. Movie 1). Thus, substituting TRPA1(B) for TRPA1(A) in chemosensors disrupts discrimination of noxious from innocuous stimuli and demonstrates the negative behavioral consequence of misregulated thermosensitivity.

To probe how TRPA1's alternative N-termini confer distinct properties, conserved residues within these regions were mutated (Fig. 4a). Mutating either a cysteine (C105) or two basic residues (R113, R116) in TRPA1(A) dramatically increased temperature-responsiveness (Fig. 4a–c, Supp. Figs. 6 and 7). While wild-type TRPA1(A)'s Q10 was <10, the TRPA1(A) mutants exhibited Q10s >50 (Fig. 4b, c), greater than the reported Q10s of canonical thermoTRPs like TRPM8 (~24)[21] and TRPV1 (~40)[22]. In addition, the TRPA1(A) mutants conducted little current below the threshold, increasing the temperature-dependent activity differential (Fig. 4c, Supp. Fig. 8). The mutants' enhanced sensitivities appeared temperature-specific, as NMM sensitivity was not increased (Supp. Fig. 8). These data indicate that TRPA1(A) retains all the requirements for robust thermosensation, but contains a modulatory region preventing those elements from exerting their full effect.

For TRPA1(B), mutating either a conserved tryptophan or two basic residues in the N-terminus yielded channels retaining robust thermosensitivity (Q10 >50; Fig. 4b, Supp. Fig. 6). The thresholds of the TRPA1(A) and TRPA1(B) mutants were all ~30–34°C, within the innocuous warm range but above wild-type TRPA1(B)'s ~28°C (Fig. 4b, c). Thus, while TRPA1(B)-specific sequences are unnecessary for robust responsiveness to innocuous warming, they may tune channel threshold within this range.

In insect disease vectors, TRPA1 orthologs have been implicated in detecting both warmth and chemical repellents[4, 23, 24], cues with opposing effects on host-seeking. We found the malaria mosquito *Anopheles gambiae* also contains TRPA1(A) and TRPA1(B) isoforms of differing thermosensitivity (Fig. 4d–h). In oocytes, AgTRPA1(A) had a Q10 of ~4 versus AgTRPA1(B)'s ~200; from 27 to 37°C, AgTRPA1(A)'s Q10 was ~2 versus AgTRPA1(B)'s ~60 (Fig. 4h). AgTRPA1(A) yielded lower maximum heat-induced current than AgTRPA1(B) (Supp. Fig. 4) and had a higher threshold (34.2 +/− 1.8°C vs. 25.2 +/− 0.9°C, $P<0.01$). AgTRPA1(A) also exhibited significant conductance below threshold (Fig. 4f, g). Both channels responded to electrophiles (Supp. Fig. 9). TRPA1(A) and TRPA1(B) are conserved in other hematophagous insects including *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes and *Pediculus humanus corporis* lice (Fig. 4a, Supp. Fig. 10), which transmit dengue, West Nile fever and typhus, respectively. TRPA1's functional diversity provides a potential explanation for how insect vectors discriminate noxious chemicals from host-derived warmth, suggesting TRPA1 presents two distinct molecular targets for disrupting pest behavior.

TRPA1-based electrophile detection likely emerged ≥500 million years ago in a common vertebrate/invertebrate ancestor[4]. However, the larger TRPA family extends to choanoflagellates, separated from animals ≥600 million years[4]. As divergent TRPA clades contain highly temperature-sensitive channels[1], thermosensitivity may be ancestral. In this scenario, TRPA1's specialization for noxious chemical detection would necessitate reducing thermosensitivity, consistent with the N-terminus' effect in TRPA1(A). The ability of N-terminal variation to sculpt channel properties is intriguing as the N-terminus is the most divergent region of TRPA1 within insects and from flies to humans[5].

TRPs are a large family of channels, with 27 human and 13 fly members, that vary greatly in thermosensitivity and function[2]. Significant diversity is evident even among closely related

TRPs. In mammals, for example, TRPM8 (Q10 ~24[21]) mediates thermosensation[3], while the less thermosensitive TRPM4 and TRPM5 (Q10 ~8.5 to 10[15]) mediate insulin secretion[25] and TRPM7 (with no reported thermal sensitivity) is implicated in ion homeostasis[2]. The mechanisms underlying such diversification are unclear. While studies of thermal sensing by TRPs have focused on identifying regions promoting thermosensitivity[1, 26–29], our work indicates that regions reducing thermosensitivity are also critical. Here we find that selectively reducing insect TRPA1's thermosensitivity facilitates its use in a context where thermosensitivity is undesirable. Similar mechanisms could mediate functional diversification not only among isoforms of a single TRP, but also contribute to the remarkable functional diversification observed between different TRP family channels.

METHODS SUMMARY

Fly strains and immunohistochemistry

UAS-TrpA1(B) and *Gr66a-Gal4* transgenic strains and the *TrpA1^{ins}* mutant have been described[4]. The *UAS-TrpA1(A)* transgene was amplified from fly cDNA with an isoform specific primer (5'-TATAAAGCTTAAGCCACCATGATTACAGCTCCGGCCACGGCCA-3') and a reverse primer (5'-GAGACTCGAGCTACATGCTCTTATTGAAGCTCAGGGCG-3'). As detailed in methods, the *UAS-TrpA1(A)* transgene was inserted in the same genomic location used for the *UAS-TrpA1(B)* transgene to control for transgene position effects. Anti-TRPA1 immunohistochemistry was as described [4], except secondary antibody was incubated three days.

Behavior

Proboscis extension assay was as described [4], with seven flies per experiment, three experiments per genotype. For heat-sensitive regurgitation, >20 flies per genotype (2 to 3 days old) were starved overnight with water, then glued to glass slides. After 2–3 hour recovery, flies were satiated with water. Only flies drinking longer than 5 sec were tested. Drinking times did not significantly differ between wild type and rescue flies (E.C. and P.G., unpub. data), consistent with similar ingestion behaviors. Flies were heated with a radiant heater at 800 W (H-4438, Optimus, USA) and temperature monitored by adjacent thermocouple microprobe (IT-23, Physitemp Instruments Inc., USA) wrapped in fly cuticle.

Physiology

Oocyte physiology was performed as described [4], with additional details provided in Methods. Extracellular recordings of gustatory neurons were obtained by tip-recording[9], as detailed in Methods.

Molecular Biology

Complementary DNA was prepared from dissected *Drosophila melanogaster* tissue (RETROscript, Ambion, Austin, TX, USA) and subjected to PCR with three primers, two forward isoform-specific primers and a reverse common primer detailed in Methods. Each primer was designed to straddle a splice junction, minimizing amplification of

contaminating genomic DNA. In all cases, similar results were obtained from four independent tissue preparations.

METHODS

Fly strains and immunohistochemistry

The *UAS-TrpA1(A)* transgene was inserted into the genome by site-specific transgenesis [30] at same landing site as *UAS-TrpA1(B)*, attp16 [31]. TRPA1(A) Genbank accession number is JQ015263.

Behavioral analysis

Chemicals used in incapacitation assays were Sucrose (Calbiochem LC8510 Gibbstown NJ), Sorbitol (Sigma S-1876, St. Louis, MO), Ficoll (Sigma F-4375), Agarose (Invitrogen 15510-027, Carlsbad, CA), Caffeine (Sigma C0750), NMM (Sigma 389412), Isopropanol (100%, J.T.Baker 9083-03 Phillipsburg NJ), Ethanol (100%, Decon Lab 2716,†King of Prussia, PA) and Allyl isothiocyanate (95%, Sigma 377430).

Characterization of TRPA1 isoforms in *Xenopus* oocytes

TRPA1 currents were recorded as described[4, 5]. To evaluate temperature sensitivities, oocytes were perfused in the recording buffer (96 mM NaCl, 1 mM MgCl2, 4 mM KCl, and 5 mM HEPES, pH 7.6), the temperature of which was increased ~0.5°C/sec from 10 to 45°C by SC-20 in-line heater/cooler (Warner Instruments, Hamden, CT, USA) with a CL100 bipolar temperature controller (Warner Instruments, Hamden, CT, USA). Temperature-evoked current was recorded at −60 mV. From the recorded current, Q10 was calculated as described[13, 16]. Arrhenius Q10=exp[10×(−S_{arrhe})/(T_1×T_2)], where S_{arrhe} is the slope of linear phase of an Arrhenius plot between absolute temperatures, T_1 and T_2. Transition temperature was assessed as the temperature at which the least-squares fit lines from the two linear phases intersect[13, 16]. Q10 from 27–37°C was calculated from currents at temperatures of interest using the equation, $Q10=(I_2/I_1)^{10/(T_2-T_1)}$, where $I_1$and I_2 are currents observed at temperatures of T_1 and T_2, respectively. Q10 determinations were validated by using *Crotalus atrox* TRPA1[16] as a control with known Q10 (K.K. and P.G. unpub.). To assess sensitivity to NMM, voltage across the membrane was initially held at −80 mV, and a 300-ms voltage ramp (−80 mV to 80 mV) per second was applied. The oocytes were perfused for 1 min with the recording buffer containing indicated concentrations of NMM with 30-second washes between NMM applications. Current amplitudes at −80 mV after application of each NMM concentration were fitted to the Hill equation through Sigmaplot 10. The first coding exon of AgTrpA1(B) was chemically synthesized (Genscript, Piscataway, NJ, USA).

Gustatory Neuron Electrophysiology

Extracellular recordings of gustatory neurons were obtained using the tip-recording method[9]. Adult female flies, aged 1–4 days, were prepared by inserting a glass reference electrode containing *Drosophila* Ringer's solution into the thorax and advancing the electrode through the head to the labellum. A glass recording electrode with an ~15 μm opening was used to apply tastants to individual sensilla. Raw signals were amplified using a

TasteProbe preamplifier (Syntech, The Netherlands) and were digitized and analyzed using a PowerLab data-acquisition system with LabChart software (ADInstruments Inc., Australia). Amplified signals were digitized at a rate of 20 kb/s and filtered using a 100 Hz-3000 Hz band-pass filter prior to analysis. Individual action potentials were sorted using a visually-adjusted threshold and average spike rate was calculated beginning 200 ms after electrode contact. Recording times varied by experiment: berberine chloride and sucrose positive controls, 5 s; electrolyte-only, 20 s; NMM on i-type bristles, 60 s; NMM on L-type bristles, 120 s; heat-ramps, >60 s. For heat-ramp experiments, recordings were performed using electrolyte-only as tastant. After ~30 s of recording to determine baseline activity, heat was applied manually to the fly using a radiant heater (PRESTO HeatDish, National Presto Industries, Inc., USA). Application of heat was maintained for ~10–30 s and the distance between the heat source and the preparation was reduced to obtain a temperature of ≥39°C. Bristle temperature was estimated using thermocouple microprobe (IT-23, Physitemp Instruments Inc., USA) wrapped in fly cuticle. All tastants were dissolved in 30 mM tricholine citrate as the electrolyte to inhibit the activity of the water cell in L-type bristles[32]. Tastants were stored at −20°C and aliquots maintained at 4°C for up to one week. For all experiments, a positive control was used to confirm the viability of the target bristle. For i-type bristles, 1 mM berberine chloride was used as control. For L-type bristles, 30 mM sucrose was used. Individual tastant presentations were separated by a minimum delay of 60 s. At least two animals and six bristles were examined for each condition.

Molecular biology

Primers for RT-PCR reactions:

Drosophila melanogaster TrpA1(A)-F: 5'GCC GGA ACA GCA AGT ATT3'

Drosophila melanogaster TrpA1(B)-F: 5'GTG GAC TAT CTG GAG GCG3'

Drosophila melanogaster TrpA1 common-R: 5'TAT CCT TCG CAT TAA AGT CGC3'

Mutagenesis of *Drosophila* TRPA1 was performed as described [4]. Briefly, for a desired mutation, each of two mutually complementary mutant primers was paired for PCR with a primer (outer primer) that anneals outside of either *SalI* or *HpaI* restriction recognition site. The two resulting PCR fragments that overlap only in the region of the two mutant primers were combined and served as template for the next PCR reaction that contained only outer primers. The second PCR product was digested by *SalI* and *HpaI*, and subsequently replaced the corresponding wild type region of TRPA1 cDNA. The fragment between the two restriction sites was sequenced. Sequences were aligned using MUSCLE 3.7[33].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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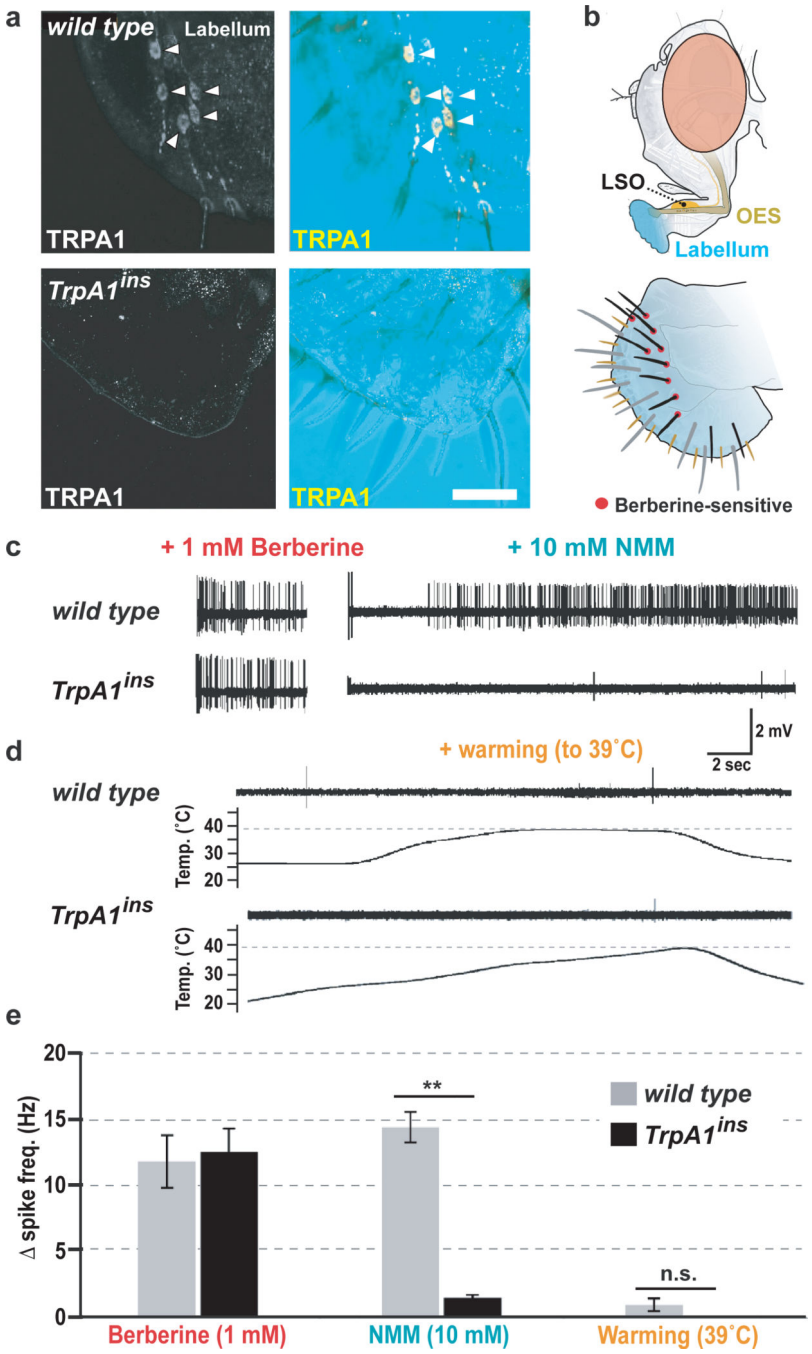

Figure 1. TRPA1-dependent gustatory neurons do not respond to heat

a, TRPA1 immunostaining of wild-type (top) and *TrpA1*^{ins} (bottom) labella. Right, DIC reveals labellar structures. Arrowheads, chemosensor cell bodies. **b,** *Drosophila* gustatory organs (top). LSO, Labral Sense Organ; OES, oesophagus. Labellar bristles (bottom). Brown, s-type; grey, L-type; black, i-type; berberine-sensitive bristles were targeted for electrophysiology. **c–d,** Bristle responses to: berberine (1 mM) and N-Methyl Maleimide (NMM, 10 mM) (**c**); warming (**d**). **e,** Average spike rate after subtracting electrolyte-only

baseline. **P<0.01; ns, not significant (P>0.05), t-test. All data are mean ± s.e.m. Warming reached average maximum temperature of 39.0 ±0.6 SD.

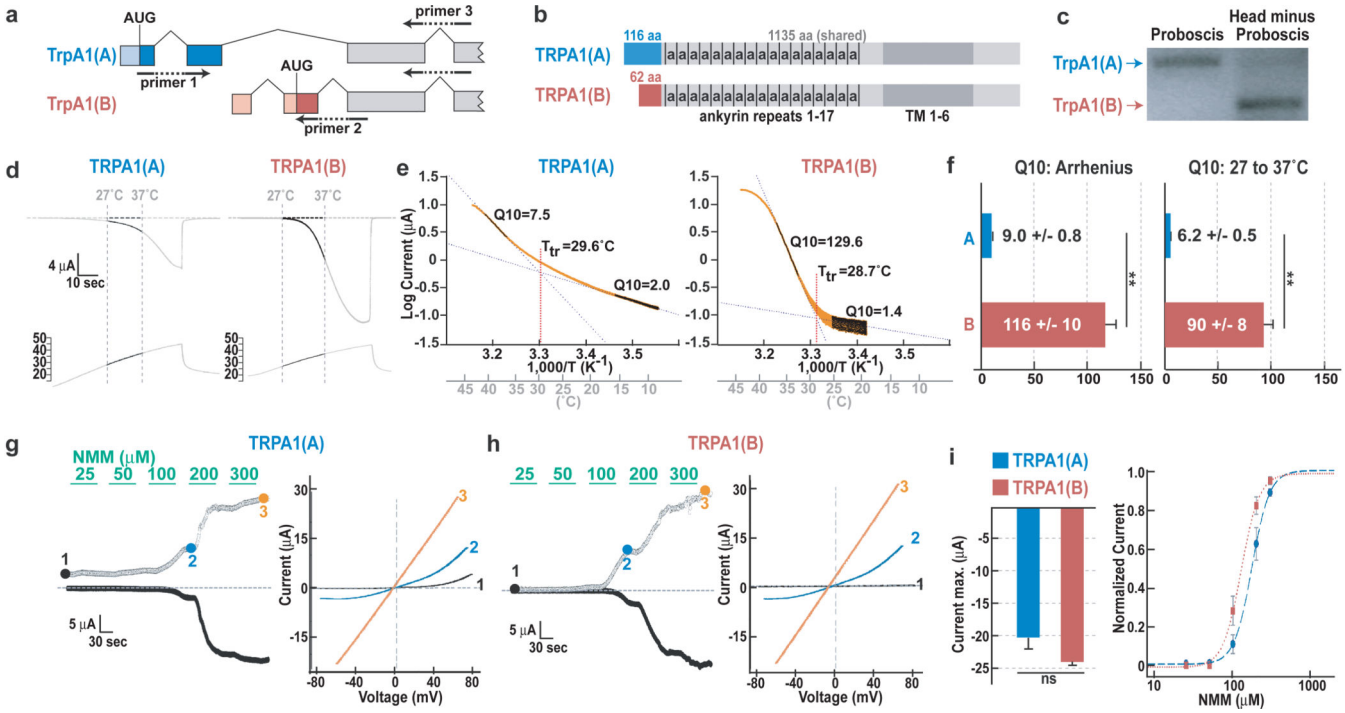

Figure 2. TRPA1 isoform diversity yields tissue-specific channels with different thermal sensitivities

a, *TrpA1* gene structure and primer locations. **b,** Red and blue boxes denote isoform-specific sequences. a, ankyrin repeat. Dark grey, transmembrane region. **c,** RT-PCR. **d–e**, TRPA1(A)- and TRPA1(B)-dependent currents (**d**) and Arrhenius plots (**e**) in oocytes. **f,** Q10s from Arrhenius plot (left) or 27–37°C (right). **g, h,** *Left panels*, NMM responsiveness of TRPA1(A) (**g**) and TRPA1(B) (**h**). *Right panels*, I–V relationships at points marked at left. **i,** Mean amplitudes at 300 μM NMM (left) and NMM dose-response (right). All data, mean +/− s.e.m. **P<0.01; n.s., not significant, t-test.

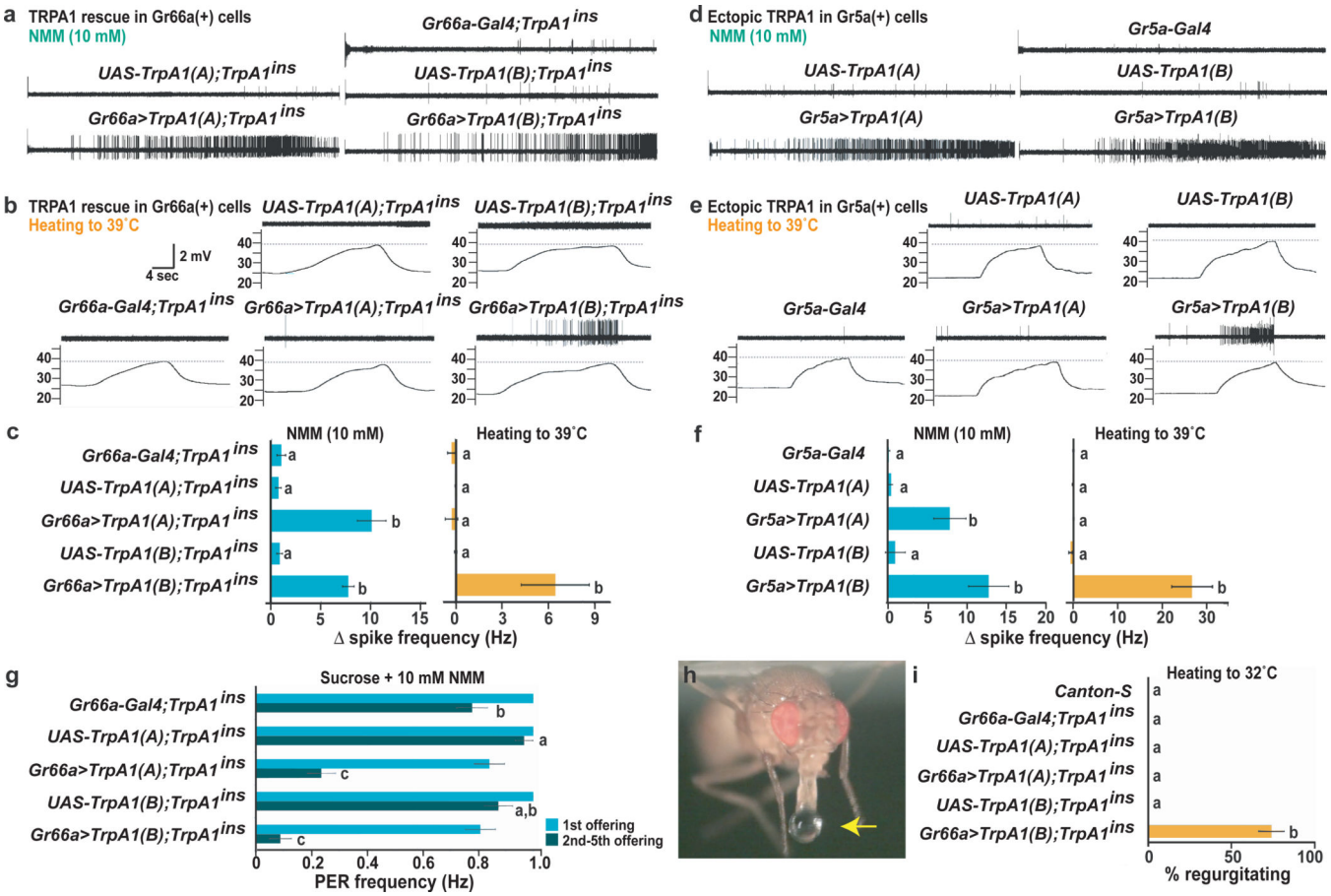

Figure 3. TRPA1 isoform diversity determines sensory specificity of gustatory neurons

a–c *TrpA1* mutant, berberine-sensitive i-type bristles expressing different TRPA1 isoforms. Responses to NMM (**a**) and warming **(b)**. **c,** Quantitation. **d–f,** L-type bristles expressing TRPA1 isoforms. Responses to NMM (**d**) and warming **(e)**. **f,** Quantitation. **g,** Rescue of *TrpA1* mutant behavioral response to NMM-containing food. PER, proboscis extension response. **h,** Warmth-induced regurgitation in *TrpA1* mutant rescued with TRPA1(B). **i,** Regurgitation upon warming from room temperature to 32°C. In **c, f, g** and **i**, statistically distinct groups marked by different letters (Tukey HSD, $\alpha = 0.01$). Data are mean ± s.e.m.

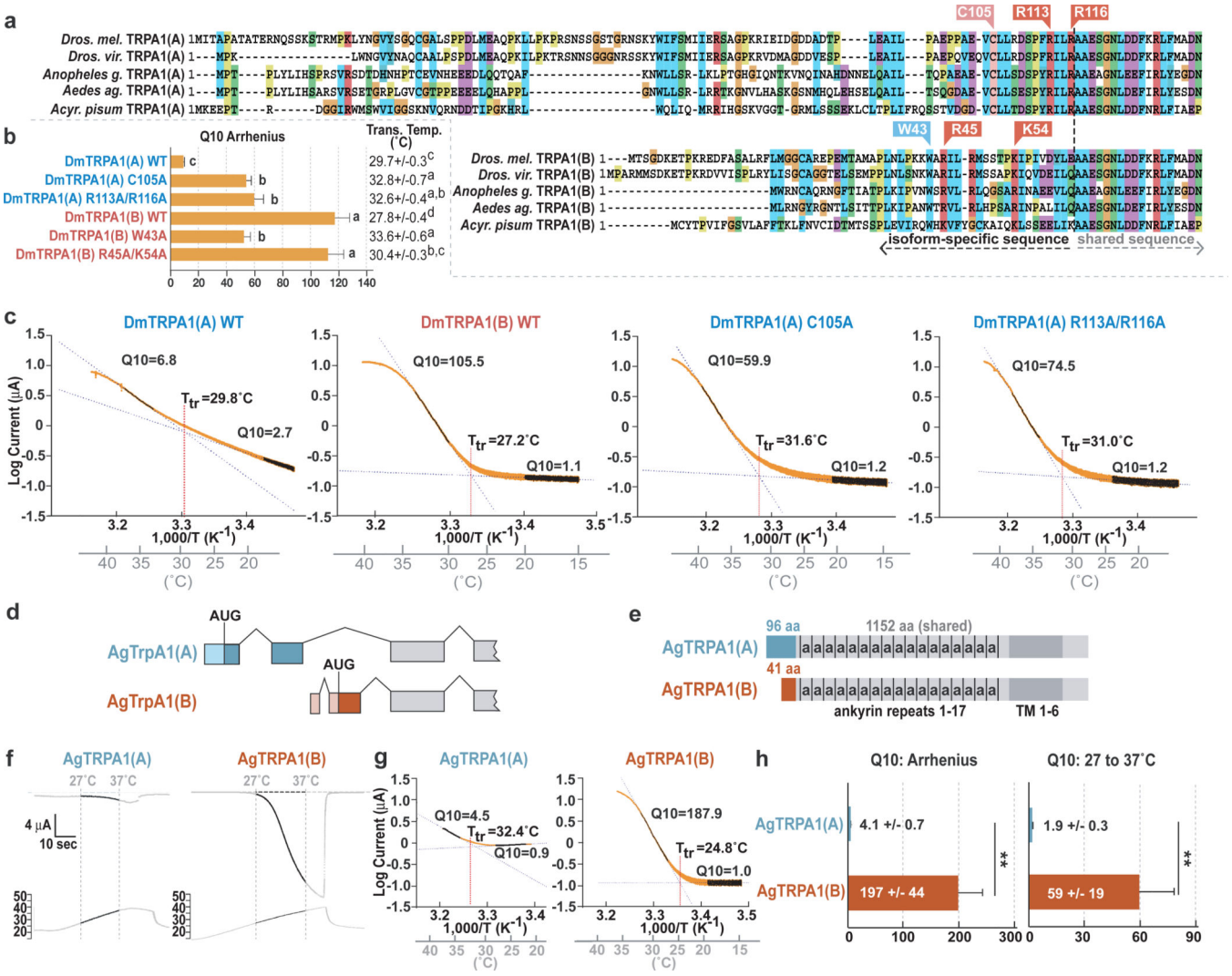

Figure 4. Regulation of insect TRPA1 thermosensitivity by alternative N-termini

a, TRPA1 sequence alignments. **b,** Q10 and transition temperatures of wild type and mutant TRPA1s. Letters denote statistically distinct groups (Tukey HSD, $\alpha = 0.02$). **c,** Arrhenius plots of indicated channels. **d,** *AgTrpA1* gene structure. **e,** AgTRPA1 isoforms. 'a': an ankyrin repeat. *Light blue* and *maroon*: isoform-specific amino acids. *Dark grey*: transmembrane region. **f–h**, Temperature sensitivity of AgTRPA1(A) and AgTRPA1(B). Traces (**f**) and Arrhenius plots (**g**) of temperature dependent current recordings at −60 mV in *Xenopus* oocytes. **h**, Q10s from Arrhenius plot (left) or 27–37°C (right) (**P<0.01, t-test).