TRPA1 mediates sensation of the rate of temperature change in Drosophila larvae

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Avoidance of noxious ambient heat is crucial for survival. A well-known phenomenon is that animals are sensitive to the rate of temperature change. However, the cellular and molecular underpinnings through which animals sense and respond much more vigorously to fast temperature changes are unknown. Using Drosophila larvae, we found that nociceptive rolling behavior was triggered at lower temperatures and at higher frequencies when the temperature increased rapidly. We identified neurons in the brain that were sensitive to the speed of the temperature increase rather than just to the absolute temperature. These cellular and behavioral responses depended on the TRPA1 channel, whose activity responded to the rate of temperature increase.

We propose that larvae use low-threshold sensors in the brain to monitor rapid temperature increases as a protective alert signal to trigger rolling behaviors, allowing fast escape before the temperature of the brain rises to dangerous levels.

RESULTS

Dependence of the nociceptive rolling response on the rate of temperature increase

To characterize the behavior of larvae in response to different rates of temperature change, we built an apparatus that allowed us to accurately control the heating speed while monitoring larval movement. The temperature control system was comprised of a Peltier pad and a programmable integrated circuit responsible for voltage regulation. We used this apparatus to heat and cool an agarose surface for larval navigation. A video camera recorded the larvae’s behavior, and a computer program, MAGAT Analyzer21, recognized the larvae in each frame.

To automatically and objectively analyze the large volume of data, we wrote an algorithm that employed several parameters to discern...
rolling from nonrolling larvae. These included the speed of the larvae, their direction of movement perpendicular to the body, acceleration and acceleration perpendicular to the body. We used a machine-learning approach to successively improve the ability of the computer to accurately identify rolling larvae with minimal noise.

We exposed wild-type second-instar larvae to temperature ramps with different slopes and determined how rolling was dependent on the rate of temperature change (\(dT/dt\)). In each experiment, we initially maintained the temperature at \(-23.5\) °C for 30 s and then increased the temperature to 40 °C. Larvae rolled when the temperature increased quickly (0.3 °C per s; Supplementary Video 1). As the temperature approached 40 °C, the larvae stopped moving and the rolling behavior ceased (Fig. 1a). However, the animals still responded to a mechanical stimulus (Supplementary Video 2).

We calculated the fraction of larvae displaying rolling behavior using two parameters: \(F_{peak}\) and \(T_{middle}\). \(F_{peak}\) was the maximum fraction of larvae that rolled during the temperature ramp. \(T_{middle}\) was the temperature at which the fraction of rolling was halfway between the baseline rolling behavior (at the start of the experiment) and \(F_{peak}\). We found that the relation between rolling and temperature (\(T\)) was dependent on \(dT/dt\). When the temperature rose at the fastest rate tested (0.5 °C per s), \(F_{peak}\) was 0.89 ± 0.09 and \(T_{middle}\) was 29.1 ± 0.3 °C (Fig. 1a,k). As \(dT/dt\) declined, \(F_{peak}\) decreased and \(T_{middle}\) increased (Fig. 1b–k). At the slowest rate examined (0.02 °C per s), \(F_{peak}\) fell to 0.47 ± 0.08 and \(T_{middle}\) increased to 34.5 ± 0.6 °C (Fig. 1j,k and Supplementary Video 3). Thus, when the rate of temperature increase was very gradual, only half as many larvae rolled at a temperature that was >5 °C hotter. These results demonstrate that when the temperature rose slowly, the tendency for the larvae to initiate an escape response was diminished greatly.

**Requirement for TRPA1-A for heat-induced rolling**

To identify a channel that might enable larvae to sense the fast temperature increases that stimulate rolling behavior, we screened for mutations in genes encoding channels known to detect temperatures in the noxious heat range (\(trpA1, pain\) and \(pyx\))\(^{12–15,24–26}\). We found that \(trpA1\) mutant flies exhibited severe defects in heat-induced rolling behavior (Fig. 2a,b and Supplementary Fig. 1), and this phenotype was suppressed by a duplication that included the wild-type \(trpA1\) gene (Fig. 2c). In contrast, \(pain\) and \(pyx\) mutations had no or minimal effects in this temperature change assay (Fig. 2d,e), although loss of \(pain\) has been reported to elevate the threshold for a thermal escape response (from 29 to 33 °C)\(^{27}\). We also found that \(Gr28b\) \(^{26}\), a mutation affecting a receptor protein required for sensing innocuously warm temperatures\(^{28}\), did not impact significantly on rolling behavior (Fig. 2f).

The \(trpA1\) gene encodes four mRNA isoforms: \(trpA1-A, B, C\) and \(D\) (Fig. 3a)\(^{14,16}\). The \(trpA1-A\) and \(trpA1-B\) isoforms (\(trpA1-AB\)) share one promoter and the \(trpA1-C\) and \(trpA1-D\) isoforms (\(trpA1-CD\)) share another promoter\(^{14}\). \(trpA1-A\) and \(trpA1-D\) have a common exon (Fig. 3a) that enables them to be activated by elevated temperatures. The different N termini in \(trpA1-AB\) and \(trpA1-CD\) influence their temperature thresholds (25 °C for \(trpA1-AB\) and 36 °C for \(trpA1-CD\))\(^{14,19}\).

To determine which \(trpA1\) isoform was required for heat-induced rolling, we first assessed the effects of knocking out each isoform pair (\(trpA1-AB\) and \(trpA1-CD\)). To address the requirements for the \(trpA1-AB\), we took advantage of the \(trpA1-AB\) \(^{Gal4}\) allele, which eliminates \(trpA1-A\) and \(trpA1-B\) (ref. 30). To disrupt \(trpA1-CD\), we used homologous recombination to create an allele containing a \(GAL4\) reporter in place of 732 nucleotides spanning the \(trpA1-CD\) translation initiation codon (Fig. 3a). We then exposed the mutant larvae to a temperature heat ramp (0.1 °C per s). We found that the \(trpA1-AB\) \(^{Gal4}\) larvae displayed substantially reduced rolling behavior, indicating that either the A or B isoform was required (Fig. 3b). In contrast, the \(trpA1-CD\) \(^{Gal4}\) larvae showed rolling behavior that was more reminiscent of the control larvae (Fig. 3c).

To address whether \(trpA1-A\) or \(trpA1-B\) was required for heat-induced nociception, we deleted two nucleotides in the exon that was present in \(trpA1-A\) but not in \(trpA1-B\) (Fig. 3a). We introduced this mutation (\(AD\)\(^*\)) in the \(trpA1-CD\) \(^{Gal4}\) background using the
In this mutant, only the B isoform of trpA1 remained (trpA1-ACDGAL4). We found that trpA1-ACDGAL4 larvae displayed a deficit in responding to the heat ramp, similarly to trpA1-ABGAL4 flies (Fig. 3b,d). These findings indicated that, in this assay, the key temperature sensor required for inducing rolling behavior was trpA1-A.

We attempted to rescue the trpA1-ABGAL4 mutant phenotype by expressing trpA1-A in trpA1-AB neurons. However, driving trpA1-A expression in trpA1-AB neurons decreased \( T_{\text{middle}} \) to 27.0 ± 0.2 °C (0.1 °C per s), which was ~5 °C lower than that for control animals (31.9 ± 0.5 °C; Supplementary Fig. 2a,b). This increased rolling behavior at lower temperatures suggested that the threshold for this avoidance behavior might be sensitive to expression levels, since the GAL4/UAS (upstream activation sequence) system potentially drove higher expression levels than the endogenous trpA1 promoter. Moreover, the rolling declined and then ceased at a lower temperature for mutant flies than for control flies due to an increasing proportion of heat-induced locomotor arrest. \( F_{\text{peak}} \): control, 0.90 ± 0.06; UAS-trpA1-A/+;trpA1-ABGAL4/trpA1\(^{C} \), 0.35 ± 0.05. We observed a similar effect of expressing trpA1-A in a heterozygous background (UAS-trpA1-A/+;trpA1-ABGAL4/trpA1\(^{C} \), 0.44 ± 0.03; \( T_{\text{middle}} = 25.9 \pm 0.2 °C \); Supplementary Fig. 2d). In contrast to the effects of driving expression of trpA1-A, we found that introducing trpA1-B in trpA1-AB neurons had no effect (Supplementary Fig. 2c).

To determine whether ectopic expression of trpA1-A could endow sensitivity to \( dT/dt \), we expressed trpA1-A in class IV multidenritic neurons in a trpA1\(^{B} \) mutant background (UAS-trpA1-A/ppk-GAL4;trpA1\(^{B} \); Supplementary Fig. 3). Both trpA1\(^B \) null mutant larvae and trpA1\(^{B} \) larvae harboring only the ppk-GAL4 or the UAS-trpA1-A transgene were virtually unresponsive to slow or fast temperature ramps (Supplementary Fig. 3a,i,j). However, when we applied a fast heat ramp (0.5 °C per s) to larvae ectopically expressing UAS-trpA1-A under the control of ppk-GAL4, \( F_{\text{peak}} \) was 0.75 ± 0.01 (Supplementary Fig. 3a,j). As \( dT/dt \) declined, \( F_{\text{peak}} \) decreased (Supplementary Fig. 3b-j). At the slowest rate examined (0.02 °C per s), \( F_{\text{peak}} \) fell to 0.35 ± 0.04 (Supplementary Fig. 3i,j). However, \( T_{\text{middle}} \) was not substantially different (Supplementary Fig. 3k).

### trpA1-AB neurons critical for sensing rapid temperature change

We performed homologous recombination using the CRISPR/Cas9 system \(^{31–34} \) to introduce the LexA gene into the trpA1-AB translation initiation codon, so that we could subsequently use this trpA1-AB\(^{LexA} \) reporter in combination with GAL4 reporters (Fig. 3a). The LexA gene and the \( w^+ \) marker replaced the same genomic region as in trpA1-ABGAL4 (ref. 30). The trpA1-AB LexA reporter (trpA1-ABLexA\(^+/+ \)) was expressed in a variety of neurons in the brain and ventral nerve cord (VNC; Fig. 4a,b and Supplementary Fig. 4). We observed an indistinguishable expression pattern in the trpA1-AB\(^{LexA} \) homozygous larvae (Supplementary Figs. 4a,b and 5), indicating that the trpA1-AB phenotype was not due to loss of trpA1-AB-expressing neurons.

To determine the specific trpA1-AB-expressing neurons required for rolling, we used RNAi-mediated gene silencing to assess the effects of knocking down trpA1 expression in different subsets of trpA1-AB neurons. To identify GAL4 lines for conducting the RNAi screen, we took advantage of the trpA1-AB\(^{LexA} \) reporter to test for overlap, using colabeling and intersectional flip-out strategies. The GAL4 lines we screened were lines from the Bloomington Drosophila Stock Center, in which the reporters labeled neurotransmitter- or neuropeptide-releasing neurons. We also reviewed the staining patterns of the Janelia Research Campus’s collection of ~7,000 GAL4 lines (https://www.janelia.org/open-science/gal4-fly-lines) by employing an image processing program we wrote to narrow down the candidates and then manually checked their expression patterns. Furthermore, we combined GAL80 lines from the Bloomington Drosophila Stock Center with a trpA1-ABGAL4 reporter to label a subset of trpA1-AB neurons.
We identified ~70 candidate GAL4 lines that might overlap with trpA1-ABGAL4. To clearly detect the neurons that expressed the GAL4 and trpA1-AB-lexA reporters, we used a ‘flipped out’ approach. We crossed the GAL4 lines into a genetic background such that trpA1-AB-lexA-positive neurons that did not express a given GAL4 were marked by mCherry. If the trpA1-AB-lexA-positive neurons also expressed GAL4, then the mCherry cassette was removed (genetically flipped out), thereby leading to expression of the Citrine marker. We found six GAL4 lines that overlapped with fewer than 10 pairs of trpA1-AB-lexA-positive neurons (Fig. 4c and Supplementary Figs. 6 and 7) and were therefore useful tools for manipulating small subsets of trpA1-AB-lexA-positive neurons.

We examined the requirements for different trpA1-AB neurons for rolling behavior in response to noxious heat by knocking down trpA1 expression by RNAi. We then performed temperature ramps at 0.1 °C per s and measured Fpeak. Knockdown of trpA1 using R60F07-GAL4 elicited severe rolling deficits (Fig. 4d and Supplementary Fig. 8a). R60F07-GAL4 was expressed in just three of the 12 classes of trpA1-positive neurons: brain lateral central neurons, brain lateral posterior (BLP) neurons and VNC anterior posterior neurons (Fig. 4b, c and Supplementary Fig. 7a–c). 386Y-GAL4, which overlapped with R60F07 in only the BLP class (Fig. 4b, c and Supplementary Fig. 7d–f), also caused strong impairment in rolling when we used it to knockdown trpA1 (Fig. 4d and Supplementary Fig. 8b). tsh-GAL80;trpA1-ABGAL4 only labeled trpA1-AB BLP neurons (Fig. 4b, c and Supplementary Fig. 6g–i). Using this latter GAL4 in combination with tsh-GAL80 to knock down trpA1 expression reduced Fpeak significantly comparing to control and trpA1-ABGAL4/+ (Fig. 4d and Supplementary Fig. 8c, d). We did not observe a significant reduction in rolling behavior after suppressing trpA1 using any of three GAL4 lines that were not expressed in BLP neurons (Fig. 4b–d, Supplementary Figs. 7 and 8e–g). Thus, we conclude that expression of trpA1 in BLP neurons influences rolling behavior.

To test whether BLP neurons were sufficient to trigger rolling behaviors, we performed optogenetic experiments. We found that expression of UAS-CsChrimson (ref. 35) under the control of trpA1-ABGAL4 (UAS-CsChrimson/CyO;trpA1-ABGAL4) triggered rolling behavior but not paralysis (Fig. 4e and Supplementary Video 4). Using tsh-GAL80, we suppressed trpA1-ABGAL4-dependent expression of UAS-CsChrimson but retained expression in two of the three BLP neurons (Supplementary Fig. 6g–i). We found that light also triggered rolling in these larvae (Fig. 4e and Supplementary Video 5) but not in control larvae harboring UAS-CsChrimson/+ alone (Fig. 4e and Supplementary Video 6).

trpA1-AB and trpA1-CD neurons function in a common circuit

We showed that trpA1-AB was required for the rolling behavior evoked by rapid heating of the entire larvae’s body. However, trpA1-CD neurons expressing trpA1-C and pain were necessary for rolling induced by touching a localized spot on the larvae with a hot (>39 °C) probe. Even though the temperature thresholds were different, trpA1-AB neurons and trpA1-CD neurons both triggered rolling behavior in response to noxious heat. This raised the possibility that the trpA1-AB and trpA1-CD neurons functioned in a common neuronal circuit.

To determine the relative expression patterns of trpA1-AB and trpA1-CD neurons, we performed double-labeling experiments. The trpA1-CDGAL4/+ reporter stained multidendritic neurons in the body wall (Fig. 5a), which extend axonal projections to the VNC (Fig. 5b). In contrast, the trpA1-AB-lexA/+ reporter stained neurons in the larval brain and VNC but not in body wall neurons (Figs. 4a and 5b).

Figure 4 Identifying trpA1-AB neurons in the larval brain required for heat-induced rolling. (a) Expression of the trpA1-AB reporter in the CNS of third-instar larvae. The fly line used for immunostaining with anti-DsRed was trpA1-AB-Gal4;LexAop-frt-mCherry;STO-frt-RedChr:Citrine+. trpA1-AB isoforms were expressed in the brain and VNC. Dashed line outlines brain and VNC; scale bar, 50 μm. (b) Map of trpA1-AB neuronal clusters in the brain and VNC. Colored boxes, regions that define the first two letters of the three letter nomenclature. First letter: brain (B) or VNC (V); second letter: general region within the brain or VNC that contained the neuronal cell bodies: A, anterior; C, central; L, lateral; P, posterior. Third letter: relative positions of the neuronal clusters within the general region: A, anterior; C, central; L, lateral; M, medial, P, posterior. (c) Summary of the expression patterns of indicated GAL4 reporters in trpA1-AB-positive neurons of third-instar larvae. +, expression in the indicated neurons. Expression patterns are shown in Supplementary Figures 6–7 and 8. fPeak, peak significantly comparing to control and +, expression in the indicated neurons. Expression patterns are shown in Supplementary Figures 6–7 and 8. (d) Effect on rolling behavior (fPeak) of second-instar larvae resulting from knockdown of trpA1. Temperature increased from 23.5 °C to 40 °C with dTId ≤ 0.1 °C per s. Center lines of boxes represent median values; left and right edges of the boxes represent the 25th (q25%) and 75th (q75%) percentiles of the sample data, respectively; +, symbols, outliers > q75% + 1.5 (q75% – q25%) or < q25% – 1.5 (q75% – q25%). Whiskers, minimum and maximum value of data points excluding outliers. One-way ANOVA of Fpeak values; f2,37 = 45.38; P = 8.1 × 10−12; n = 3, 4, 4, 8, 9, 3, 3 or 4 independent experiments. Tukey-Kramer test for statistically significant differences between control (wild-type) and experimental samples: trpA1-ABGAL4; q7,37 = 10.45; P = 3.9 × 10−3; UAS-Dicere;UAS-trpA1-RNAi-JF02461 × tsh-GAL80;trpA1-ABGAL4; q7,37 = 15.68; P = 9.0 × 10−8; UAS-Dicere;UAS-trpA1-RNAi-JF02461 × R60F07-GAL4; q7,37 = 13.12; P = 9.1 × 10−8; UAS-Dicere;UAS-trpA1-RNAi-JF02461 × 386Y-GAL4; q7,37 = 12.19; P = 9.7 × 10−8; ***P < 0.001. (e) Triggering rolling behavior of second-instar larvae by optogenetically activating trpA1-AB neurons. We manually recognized the rolling behaviors of each larva. We stimulated the larvae with light and scored the larvae as either 1.0 (rolled) or 0 (did not roll). Bar heights, fraction of larvae showed rolling behavior. Numbers above the bars are Nrolling/Total. Fisher’s exact test: UAS-CsChrimson+/UAS-CsChrimsonCyO;trpA1-ABGAL4, odds ratio = 32, P = 0.00116; UAS-CsChrimson+/UAS-CsChrimson; trpA1-ABGAL4, odds ratio = 22, P = 0.00228; UAS-CsChrimson/CyO;trpA1-ABGAL4 vs. UAS-CsChrimson/trpA1-ABGAL4, odds ratio = 0.69, P = 0.74, **P < 0.01, ***P < 0.001; n.s., not significant.

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Therefore, there was no apparent overlap between the expression patterns for trpA1-AB and trpA1-CD reporters.

Since trpA1-AB neurons and trpA1-CD neurons were located in close proximity to each other in the VNC, we wondered whether they formed synapses. GFP reconstitution across synaptic partners (GRASP) is a system for labeling membrane contacts between two neurons formed synapses, using LexAop-CD4:spGFP111+;UAS-CD4:spGFP1-10, trpA1-ABLexA trpA1-CDGAL4, (n = 3). Scale bars, 50 µm.

**BLP trpA1-AB neuronal activity increased by steep temperature ramps**

trpA1-AB neurons in the CNS might directly sense the rate of temperature change. Alternatively, these brain neurons may not be thermally activated but may instead receive signals from temperature-activated peripheral neurons. *Drosophila* TRPA1 is a Ca\(^{2+}\)-permeable channel\(^{24}\). To monitor Ca\(^{2+}\) increases in response to different dT/dt, we expressed UAS-GCaMP6f (ref. 37) under the control of trpA1-AB\(^{GAL4}\) (trpA1-AB\(^{GAL4/+}\)) and dissected out the CNS from the transgenic larvae. We varied the temperature ramps from 0.05 to 0.2 °C per s and monitored the changes in fluorescence (ΔF/F\(_0\)). We found that the rise of Ca\(^{2+}\) in BLP neurons was correlated with the steepness of the temperature ramps (0.2 °C per s, ΔF/F\(_0\) = 4.9 ± 1.0; 0.1 °C per s, ΔF/F\(_0\) = 3.2 ± 0.6; 0.05 °C per s, ΔF/F\(_0\) = 1.7 ± 0.2; P = 4.7 × 10\(^{-7}\) by one-way ANOVA; Fig. 6a,b,c,d,e,f,g,h,i). The Ca\(^{2+}\) increase was due to TRPA1, since it was eliminated in trpA1-AB\(^{GAL4}\) homozygous mutant flies (Fig. 6j,k). Brain lateral anterior (BLA) neurons also showed Ca\(^{2+}\) changes in response to the faster ramps (Fig. 6c,f,i,l). These changes were eliminated in trpA1-AB\(^{GAL4}\) mutants (Fig. 6l). However, the peak responses of BLA neurons were much smaller than those exhibited by BLP neurons (Fig. 6c,f,i). These findings indicated that trpA1-AB-expressing neurons were activated by rapid temperature changes and that BLP neurons responded most robustly, especially when dT/dt was highest. Consistent with the conclusion that BLP and BLA neurons were intrinsically responsive to temperature, these neurons also responded to temperature in the presence of tetrodotoxin (Supplementary Fig. 9), which depresses synaptic transmission by blocking voltage-gated Na\(^{+}\) channels and nerve conduction.

**TRPA1-A activity enhanced by rapid changes in temperature**

To address whether TRPA1 was sensitive to the rate of temperature change, we expressed TRPA1-A in *Xenopus* oocytes and performed two-electrode voltage-clamp experiments. We increased the temperature either slowly (0.05 ± 0.02 °C per s) or rapidly (0.2 ± 0.02 °C per s) and measured the currents (24 °C to 35 °C for TRPA1-A, 24 °C to 40 °C for TRPA1-D). When the temperature increased slowly, the peak current was −1.2 µA (Fig. 7a,b). However, when we employed the steeper temperature ramp, the peak current increased approximately threefold to −3.7 µA (Fig. 7a,b). Q\(_{10}\) is defined as the fold increase in activity caused by a 10 °C rise in temperature. In each pair of experiments, Q\(_{10}\) increased significantly with the faster temperature-change rate (P = 6.1 × 10\(^{-3}\); Fig. 7c). This suggested that the activity of the TRPA1-A channel was controlled both by the rate of temperature increase and by the absolute temperature. While the peak current was much larger during the fast heat ramp, there was more total current...
Figure 7 Effects of $dT/dt$ on the activities of the TRPA1-A and TRPA1-D channels. The indicated channels were expressed in *Xenopus* oocytes and currents were recorded at slow $dT/dt = -0.05 \, ^\circ\text{C}\, \text{s}^{-1}$ and fast $dT/dt = -0.2 \, ^\circ\text{C}\, \text{s}^{-1}$ in ND96 buffer containing either 1.8 mM Ca$^{2+}$ or 1 mM EGTA (0 mM Ca$^{2+}$ as indicated). (a) Oocytes were exposed to two temperature ramps as indicated by the trace. (b) Representative currents in an oocyte expressing TRPA1-A in the presence of 1.8 mM Ca$^{2+}$ upon exposure to a slow and fast $dT/dt$. (c-f) $Q_{10}$ values shown as a function of $dT/dt$. Black lines link data from the same oocyte. We changed the order of the slow and fast temperature changes so they were similar in number. Squares and triangles indicate the first and second temperature ramp in each experiment, respectively. We compared $Q_{10}$ values corresponding to the slow and fast temperature ramps using the Wilcoxon signed-rank test. The $P$ values are indicated. (c) TRPA1-A currents using a 24°–35° C ramp. Wilcoxon signed-rank test for $Q_{10}$ values: $n = 15$, $W = 120$, $P = 6.1 \times 10^{-5}$. (d) TRPA1-A currents using a 24°–35° C ramp. Wilcoxon signed-rank test for $Q_{10}$ values: $n = 18$, $W = 65$, $P = 0.16$. (e) TRPA1-D currents using a 24°–40° C ramp. Wilcoxon signed-rank test for $Q_{10}$ values: $n = 12$, $W = 54$, $P = 0.034$. (f) TRPV1 currents using a 24°–48° C ramp. Wilcoxon signed-rank test for $Q_{10}$ values: $n = 10$, $W = 47$, $P = 0.014$.

During the slow ramp (Supplementary Fig. 10). Nevertheless, we suggest that the peak current was the more relevant parameter since the lower peak during the slow ramp may have been insufficient to cross the threshold to trigger action potentials.

We tested whether Ca$^{2+}$ affected the differences in $Q_{10}$ during the slow and fast temperature ramps, as well as absolute $Q_{10}$ values under both conditions. When we eliminated Ca$^{2+}$ from the external bath, the differences in $Q_{10}$ at the slow and fast rates were not significant ($P = 0.16$; Fig. 7d). However, $Q_{10}$ values were much higher than in the presence of Ca$^{2+}$ (Fig. 7c,d). These findings suggest that inactivation of TRPA1-A is sensitive to Ca$^{2+}$, as is the case for mammalian TRPA1.38-41.

In addition to TRPA1-A, another TRPA1 isoform, TRPA1-D, is thermosensitive.14,29 We found that TRPA1-D also exhibited a higher $Q_{10}$ in response to the faster temperature ramp ($P = 0.034$; Fig. 7e). However, the differences between the slow and fast ramps were not as great as with TRPA1-A (Fig. 7c). Thus, the N-terminal exons that are distinct between TRPA1-A and -D (Fig. 3a) may have contributed to the degree of sensitivity to $dT/dt$. We also tested whether rat TRPV1 (rTRPV1) was sensitive to $dT/dt$. Like TRPA1, rTRPV1 also exhibited significant differences in $Q_{10}$ using slow and fast temperature ramps ($P = 0.014$, Fig. 7f; 24–48°C ramp).

**DISCUSSION**

We established *Drosophila* larvae as an animal model for dissecting the physiological basis through which an animal displays nociceptive behavior in proportion to the rate of heating. We found that if the temperature rose quickly, a much greater percentage of larvae initiated an escape response, which involved rolling perpendicular to its body axis. Moreover, they did so at much higher temperatures if the environmental temperature increased slowly. Thus, we conclude that *Drosophila* larvae respond to the rate of heating, rather than just the absolute temperature.

We demonstrated that the molecular sensor essential for detecting $dT/dt$ was a TRPA1 isoform, TRPA1-A. Peak TRPA1-A-dependent currents were larger when the heating was rapid, demonstrating that the activity of this thermoTRP was not strictly a function of the actual temperature but was also impacted by the heating rate. This finding was consistent with the larval behavioral response to different heating slopes. Rapid heating not only increased the percentage of larvae that rolled but also decreased the temperature at which the nociceptive behavior took place.

The trpA1-A-expressing neurons that were critical for sensing the heating speeds were in the brain. In support of this conclusion, the trpA1-AB reporter (trpA1-ABGAL4) was expressed in BLP neurons. Using a genetically encoded Ca$^{2+}$ sensor, we found that BLP neurons exhibited larger Ca$^{2+}$ responses when they were heated rapidly. The heat-induced Ca$^{2+}$ signals were eliminated in trpA1-AB mutant larvae. Nevertheless, we cannot exclude that voltage-gated Ca$^{2+}$ channels activated subsequent to TRPA1 contributed to the rise in Ca$^{2+}$. Because the tissue that we used for these experiments was devoid of the peripheral nervous system, our data indicated that the BLP neurons were directly sensing the rate of temperature change. Moreover, these neurons responded to temperature changes in the presence of tetrodotoxin, which suppresses voltage-gated Na$^+$ channels and synaptic transmission.

Neural accommodation is a potential mechanism through which BLP neurons could respond differentially to variations in $dT/dt$. It is a consequence of inactivation of voltage-gated Na$^+$ channels, due to slow depolarization. However, the slow depolarization that leads to neural accommodation typically occurs on a second timescale,42 and our fastest temperature ramps (0.5 °C per s) occurred over the course of many seconds. Thus, we suggest that the lower rolling propensity in response to the slow temperature ramps was not likely due neural accommodation, although our data do not formally rule this out. Nevertheless, a more likely mechanism is Ca$^{2+}$-dependent inactivation of the TRPA1 channels themselves. Consistent with this latter possibility, we found that the peak TRPA1-A currents were similar in response to slow ramps compared to fast heat ramps in the absence of external Ca$^{2+}$. However, during slow heat ramps in the presence
of external Ca\textsuperscript{2+} there is more time for Ca\textsuperscript{2+} to inactivate the TRPA1 channels, thereby reducing the peak currents. In addition to a role for trpA1-AB-expressing BLP neurons in thermal nociception, trpA1-CD-positive neurons in the periphery also sensed elevated temperatures\textsuperscript{14}. However, the activation threshold temperature for trpA1-AB neurons in the central brain was considerably lower than the temperature required for activation of trpA1-CD neurons in the periphery (> 40 °C)\textsuperscript{14}. Nevertheless, activation of either group of neurons elicited the same rolling behavior. Based on the GRASP analysis results, it appeared that the trpA1-AB and trpA1-CD neurons were in close proximity, suggesting that these neurons might have been functioning in a common neuronal circuit. Thus trpA1-AB neurons in the larval brain were not only primary thermosensors but may also have been transducers of peripheral signals.

We propose that having two groups of thermosensory neurons offers a dual defense. One class, the central brain trpA1-AB neurons, has a low threshold when heating is precipitous, thereby enabling the larvae to begin detecting rapid rates of temperature increase before the temperature rises to an acutely dangerous level. The faster the rate of temperature increase, the lower the threshold, thereby providing the animals ample time to initiate rolling and escape a noxious environment. However, if the rate of temperature increase is very gradual, the larvae have more time to respond and do not need to initiate an abrupt escape. Once the absolute temperature reaches an acutely noxious temperature, such as 39 °C, activation of the trpA1-CD neurons stimulates a rolling response, thereby providing a second line of defense via activation of the same neuronal circuit. Nevertheless, another TRP channel referred to as Painless also participates in thermal nociceptive responses\textsuperscript{12,17}. Thus, this TRP might provide a distinct backup mechanism that allows the animals to respond to noxious temperature changes. Finally, our findings that the robust behavioral response of larvae to rapid heating was mediated by a low-threshold TRP channel raises questions as to whether similar mechanisms occur in vertebrate animals from amphibians to mammals.

Data and code availability

The scripts and data that support the findings of this study are available from the corresponding author upon reasonable request.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank M. MacDonald (UC Santa Barbara) and H. Luo (Shanghai Jiao Tong University) for assistance in generating the knock-in fly lines; J. Liu and H. Chen (UC Santa Barbara) for assistance in performing blind optogenetic experiments; B. Afonso (Janelia Research Campus), M. Zlitic (Janelia Research Campus), M. Gershov (Harvard University) and A.D.T. Samuel (Harvard University) for help building the software and hardware for the larval tracking system; W.D. Tracey (Indiana University) for trpA1-ABAC (ref. 14); K. Scott (UC Berkeley) for GRASP flies\textsuperscript{26}; P.A. Garrity (Brandeis University) for the pOX-trpA1-A construct\textsuperscript{41}; and G.M. Rubin and J.W. Truman (Janelia Research Campus) for the expression data corresponding to the adult and larval Janelia GAL4 lines. W.L.S. was supported by National Science Foundation of China (X-0402-14-002). This work was supported by grants to C.M. from the National Eye Institute (EY010852) and the National Institute on Deafness and Other Communication Disorders (DC007864).

AUTHOR CONTRIBUTIONS

The study was designed by J.L., W.L.S. and C.M., and directed and coordinated by C.M. The behavioral experiments were performed by J.L. and W.L.S. J.L. generated the trpA1 alleles and performed the immunohistochemistry experiments and Ca\textsuperscript{2+} imaging experiments. W.L.S. and J.L. performed two-electrode recordings in Xenopus oocytes. The manuscript was prepared by J.L., W.L.S. and C.M.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Fly stocks. We used the following flies, all described previously: UAS-CD4::spGFP10, LexAop-CD4::spGFP11 (ref. 36), trpA1-ABGAL4 (ref. 30), UAS-trpA1-A (ref. 43), UAS-trpA1-B (ref. 44), pain2 (ref. 12), ppx2 (ref. 13), Gpa2GAL80 (ref. 45), trpA1-BAC (ref. 14), ppk-GAL4 (ref. 46), UAS-Kir2r1.2 (refs. 35,47) and tsh-GAL80 (ref. 48). We obtained the following flies from the Bloomington Drosophila Stock Center: UAS-MCD8::GFP (Bloomington Drosophila Stock Center) (BDSC #52261)19, UAS-FLP (BDSC #4539)50, LexAop-frt-mCherry-STOP-frt-RedChR::Citrine (BDSC #53745)51, UAS-GCaMP6f (BDSC #42477)52, 386Y-GALA (BDSC #25410)53, R06F07-GALA (BDSC #45358)53, R21E09-GALA (BDSC #48948)54, R21G01-GALA (BDSC #48951)53, R21F01-GALA (BDSC #49862)53 and UAS-CsChrimson (BDSC #5135)53.

Assaying temperature-induced rolling behavior in second-instar larvae. The assay and illumination system that we built was similar to one described previously55. Briefly, it consisted of a 12 cm × 12 cm temperature-control apparatus, which we assembled with nine 4 × 4 cm sections of a VT-127-1.4-1.15-71 Peltier device (TE Technology). The power controller for the Peltier device was a TC-36-25-RS232 temperature controller (TE Technology) (Supplementary Fig. 11). We also wrote a LabView program to drive the temperature controller and synchronized it to the recording camera. The scripts will be provided upon reasonable request.

To obtain second-instar larvae, we collected embryos for 4–6 h and incubated them for 72 h at 25 °C under 12 h light/12 h dark cycles. We tested their behaviors during the light cycle. We also tested wild-type third-instar larvae (96 h at 25 °C) and found that their rolling behaviors in response to heat ramps were similar to those of second-instar larvae (Supplementary Fig. 12). We focused our analyses on second-instar larvae in most of our behavioral experiments. Due to the small body size of second-instar larvae (< 1 mm in diameter), the temperatures of their brains were almost the same as the environment. The temperature differential between the center of the larva and the surrounding environment was < 0.05 °C when temperature was increased at a rate of 0.1 °C/s (ref. 56).

Before initiating the experiments, we separated the larvae from the food by washing them in 15% sucrose. The density of the 15% sucrose solution is higher than that of the larva but lower than the density of fly food. Thus, the larvae float above the sucrose, while the fly food sinks to the bottom. We stemmed the larvae from the top of the sucrose solution and washed them with water to remove the residual sucrose. Using a paint brush, we distributed ≤20 larvae evenly on the 12 cm × 12 cm Peltier pad coated with 1 mm of 2% agarose in water. The larvae were not selected by gender in the behavioral experiments. We kept the surface of the agarose moist by spreading enough water on the Peltier pad to lubricate the larvae during the experiments. For each experimental condition, we repeated the behavior experiments on 23 groups of larvae.

We used the MAGAT Analyzer21 to recognize the larvae in each video frame and calculated several parameters to discern rolling from nonrolling larvae. These included the speed of the larvae, the speed perpendicular to the body, acceleration and speed perpendicular to the body. We then created a machine-learning program and calculated several parameters to discern rolling from nonrolling larvae. These were not selected by gender in the behavioral experiments. We kept the surface of the agarose moist by spreading enough water on the Peltier pad to lubricate the larvae during the experiments. For each experimental condition, we repeated the behavior experiments on 23 groups of larvae.

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males with UAS-tsh-GAL80;trpA1-AβGal4 and tsh-GAL80/CyO;trpA1-AβGal4. We kept the individual larvae that we tested in the optogenetic experiments until they reached the adult stage to determine their genotypes.

We fed first-instar larvae food containing all-trans-retinal, which we prepared by adding 50 µl of 100 mM all-trans-retinal to 5 ml of fly food, and then allowed the animals to grow until they reached the second-instar larval stage. To conduct the experiments, we placed the larvae on a 2% agarose plate and stimulated the animals for 60 s with red light from an AmScope HC250DA 150W fiber-optic dual-gooseneck stereo microscope light illuminator. To obtain the red light, we used an AmScope CF-4 color filter for the fiber microscope illuminator. The light intensity was 5.2 mW/cm². We recorded the behavior of individual larvae using an AmScope 7×~45× trinocular stereo zoom microscope and a Basler acA2000-165uc camera and manually scored rolling behavior during light stimulation (roll score = 1; no-roll score = 0).

Blinding and randomization. To score rolling behavior in a blind fashion, we devised a computer algorithm to score the behavior without human intervention. To analyze control, mutant and transgenic flies, we selected animals in a random fashion. To perform the optogenetic experiments (Fig. 4c), we randomly permuted all of the videos and recognized the rolling behaviors manually without knowledge of the genotypes.

GCaMP imaging to assay changes in Ca²⁺ in response to different dT/dt. To assay the activities of BLP and BLA neurons in response to different dT/dt, we expressed UAS-GCaMP6f (ref. 37) under the control of trpA1-AβGal4 (ref. 30). We focused this analysis on third- rather than second-instar larvae since both they exhibit similar thermal nociceptive responses (Supplementary Fig. 12) and the small size of the second-instar larvae limits the resolution of the analysis. To control the temperature, we used a Q-1HC quick exchange heating/cooling platform with a CL-100 bipolar temperature controller (Warner Instruments, Hamden, CT, USA). To facilitate rapid temperature changes during the heating and cooling, we reduced the buffer volume using an apparatus consisting of a copper chamber. To accurately control the position of the brain in the center of the copper chamber. To determine the temperatures that the larvae were exposed to during the experiments, we placed a temperature probe directly adjacent to the larval brain in the center of the copper chamber. To accurately control the position of the temperature probe, we designed and 3D-printed the probe holder, which can be ordered from Shapeways (https://www.shapeways.com/product/SJWE7RXVX/temperature-probe-holder-for-ca2-imaging?key=8c8b76f4b919d0f5c9110304efab). We exposed each brain to only one temperature ramp.

We performed the Ca²⁺ imaging using an upright Zeiss LSM 700 confocal laser-scanning microscope, a 20×/0.1 Plan-Apochromat water immersion objective and Zen software. The wavelength of the laser used for exciting the GCaMP6f was 488 nm. The frame rate of the scanning was 2.2 frames per second. The increase in Ca²⁺ level in each neuron was indicated by the photobleaching stimuli according to the preceding equation, and then used factor k to compensate for photobleaching: (ΔF/Δt)corrected = ((ΔF/Δt)measured + 1)exp(-1). After making this correction, ΔF/Δt values before and after heat treatment were the same.

Two-electrode recordings in Xenopus oocytes. We recorded TRP1 currents in Xenopus laevis oocytes as we described previously54. We maintained the Xenopus laevis females (Xenopus 1 Inc.) at −19 °C under a 12-h light/dark cycle. The ovaries were surgically extracted from the female Xenopus and treated with 2 mg/ml collagenase A (Roche) in OR2 ringers (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5). We checked the status of the oocytes every 15 min to ensure that the oocytes were completely defolliculated. We cultured the oocytes at 18 °C for 12 h to let them recover in OR3 medium: 50% Leibovitz’s media L-15 (Sigma L1518), 13 mM HEPES, 90 µM gentamicin, 90 µg/ml fungizone (Amphotericin B), 90 µg/ml penicillin/streptomycin (pH 7.5).

We linearized the pOX-trpA1-A (ref. 43), pOX-trpA1-D (ref. 29) and pC5S- rTPR1 constructs overnight and generated trpA1-A mRNA using the Message Machine kit (Ambion). We injected the individual larvae with an AmScope 7×~45× trinocular stereo zoom microscope and a Basler acA2000-165uc camera and manually scored rolling behavior during light stimulation (roll score = 1; no-roll score = 0).

Statistical analyses. To obtain the Fpeak and Tmddle parameters from the assays of larval thermal nociception, we applied sigmoid functions (Frolloing = (Fpeak − Fbaseline))/(1 + e−(T − Tmddle)/k) + Fbaseline to fit the Frolloing–T curve. For each condition, we repeated the assays at least three times to obtain three sets of Fpeak and Tmddle data for the statistical tests. For the data shown in Figure 1j,k, we performed one-way ANOVA on Fpeak and Tmddle under different temperature-change rates (Fpeak = n = 3, 5, 7, 3, 6, 3, 7, 9; Tmddle = n = 3, 5, 7, 3, 6, 3, 7 and 9; Fpeak and Tmddle = 3, 5, 7, 3, 6, 3, 7 and 9; Fpeak and Tmddle = 10.56; P = 1.5 × 10⁻⁷). P-values for the ANOVA were one-sided. Variances between groups were verified using the Brown-Forsythe test (Fpeak; Fpeak = 5, 8, 24 = 1.79, P = 0.20; Tmddle: Fpeak = 0.49, P = 0.86). The P-value for the Brown-Forsythe test was two-sided. We used the Tukey-Kramer test to ascertain statistically significant differences relative to the highest temperature-change rate (0.5 °C/s). The conditions with significant differences are indicated by asterisks (*P < 0.05, **P < 0.01). The P-values for the Tukey-Kramer test were two-sided. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications41,43.

For the data shown in Supplementary Figure 3j,k, we performed one-way ANOVA on Fpeak and Tmddle, using different dT/dt, using the two-electrode voltage clamp experiments. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications41,43.

In Figure 4d, we performed one-way ANOVA on Fpeak values for the different genotypes (n = 3, 4, 8, 9, 8, 3, 4, 3, 5; Fpeak = 17.81; P = 2.4 × 10⁻⁷; Tmddle: n = 3, 3, 6, 3, 4, 3 and 5; Fpeak = 1.79; P = 0.13). P-values for the ANOVA were one-sided. Variances between groups were verified using the Brown-Forsythe test (Fpeak: Fpeak = 0.53, P = 0.82; Tmddle: Fpeak = 0.53, P = 0.82). The P-value for the Brown-Forsythe test was one-sided. We used the Tukey-Kramer test to ascertain statistically significant differences relative to the highest dT/dt (0.5 °C/s). The conditions with significant differences are indicated by asterisks (*P < 0.05, **P < 0.01). The P-values for the Tukey-Kramer test were two-sided. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications41,43.

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To compare the optogenetics data (Fig. 4e), we performed the Fisher’s exact test between each genotype (between UAS-CsChrimson/+ and UAS-CsChrimson/CyO;trpA1-ABGAL4, odds ratio = 32, P = 0.00016; between UAS-CsChrimson/+ and UAS-CsChrimson/tsh-GAL80.trpA1-ABGAL4, odds ratio = 22, P = 0.0028; between UAS-CsChrimson/CyO;trpA1-ABGAL4 and UAS-CsChrimson/tsh-GAL80.trpA1-ABGAL4, odds ratio = 0.69, P = 0.74). P-values for the Fisher’s exact test were two-sided: **P < 0.01, ***P < 0.001. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications35,31.

To analyze the in vivo Ca2+ imaging data (Fig. 6), we performed one-way ANOVA on the maximum ΔF/Δt values obtained from BLP neurons during the different temperature-change rates (Fig. 6b,c,h,k; n = 15, 13, 18, and 21; F3,63 = 13.86; P = 4.7 × 10−7). P-values for the ANOVA were one-sided. The variance similarities between groups were verified using the Brown-Forsythe test (F3,63 = 6.14, P = 9.9 × 10−4). No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications35,31.

We used the Tukey-Kramer test to ascertain statistically significant differences between two samples (between b and h: q0.05,4 = 6.07; P = 3.6 × 10−4; between b and k: q0.05,4 = 8.72; P = 3.3 × 10−7; between c and e: q0.05,4 = 5.10; P = 0.034). P-values for the Tukey-Kramer test were two-sided. We also performed one-way ANOVA on the maximum ΔF/Δt values obtained from BLA neurons during different dT/dt (Fig. 6c,f,k,l; n = 10, 11, 6, and 6; F3,29 = 8.01; P = 4.9 × 10−4). P-values for the ANOVA were one-sided. The variance similarities between groups were verified using the Brown-Forsythe test (F3,29 = 0.86, P = 0.47). The P-value for the Brown-Forsythe test was one-sided. We used the Tukey-Kramer test to ascertain statistically significant differences between two samples (between c and l: q0.05,4 = 3.80; P = 0.054; between c and e: q0.05,4 = 6.65; P = 3.2 × 10−4; between f and l: q0.05,4 = 4.85; P = 0.0093). P-values for the Tukey-Kramer test were two-sided. Data distribution was assumed to be normal but this was not formally tested. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications37,60,61.

To obtain Q10 values for each temperature ramp (0.05 °C/s and 0.2 °C/s; Fig. 7c–f), we fitted the current–temperature curve with the following equation: \( I = I_0 e^{(T_0 - T)/10} \) (ref. 59). We compared the Q10 values corresponding to the slow temperature ramps and fast temperature ramps using the Wilcoxon signed-rank test, which is a nonparametric test for two populations when the observations are paired. This nonparametric test is not based on the assumption that the means of the Q10 differences follow normal distributions. The results in Figure 7c–f indicated that Q10 was significantly larger when the rate of change was 0.2 °C/s relative to 0.05 °C/s (Fig. 7c: n = 15, W = 120, P = 6.1 × 10−5; Fig. 7e: n = 12, W = 54, P = 0.034; Fig. 7f: n = 10, W = 47, P = 0.014). The results in Fig. 7d indicated that Q10 was not significantly different when the rate of change was 0.2 °C/s versus 0.05 °C/s (n = 18, W = 65, P = 0.16). The P-value for the Wilcoxon signed-rank test was two-sided. We also used the Wilcoxon signed-rank test to compare the total current during the slow and fast temperature ramps (Supplementary Fig. 10; n = 15, W = 120, P = 6.1 × 10−5). No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications29,41,62.

A Supplementary Methods Checklist is available.

Data and code availability. The scripts and data that support the findings of this study are available from the corresponding author upon reasonable request.

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