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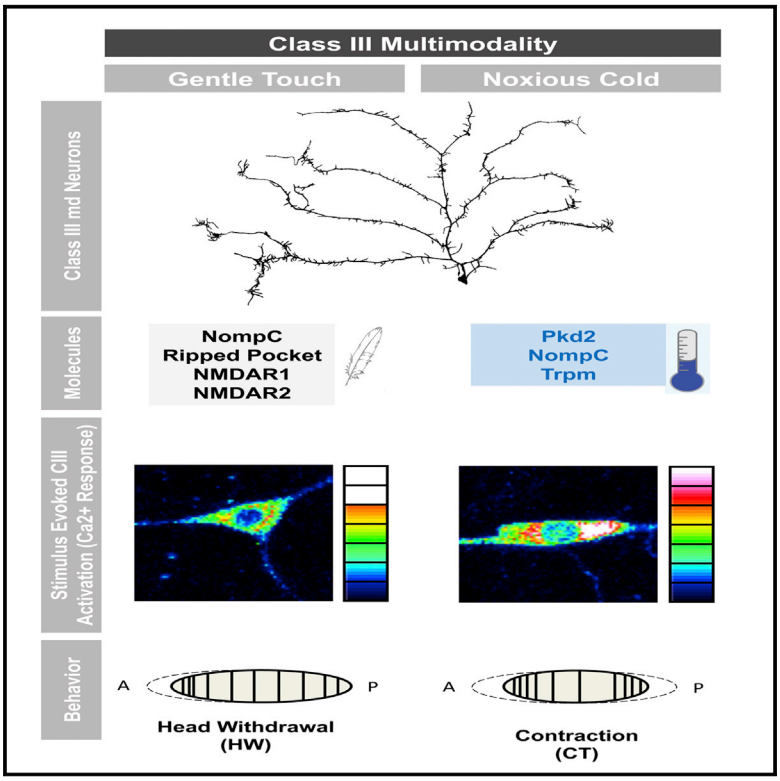
Graphical Abstract



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In Brief

Turner et al. define *Drosophila* responses to noxious cold. Optogenetic activation and genetic inactivation pinpoint class III neurons as cold nociceptors. Three TRP channels act in these neurons for cold nociception. Class-III-mediated multimodal behavioral output depends on activation levels, and circuit analysis reveals cold behavior is dominant.

Highlights

- *Drosophila* assays for cold nociception reveal unique cold-evoked behaviors
- Class III md neurons are cold responsive and mediate cold-evoked behavior
- Pkd2, NompC, and Trpm are required in class III md neurons for cold nociception
- Class-III-mediated multimodal behavioral output depends on activation levels

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The TRP Channels Pkd2, NompC, and Trpm Act in Cold-Sensing Neurons to Mediate Unique Aversive Behaviors to Noxious Cold in *Drosophila*

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SUMMARY

The basic mechanisms underlying noxious cold perception are not well understood. We developed *Drosophila* assays for noxious cold responses. Larvae respond to near-freezing temperatures via a mutually exclusive set of singular behaviors–in particular, a full-body contraction (CT). Class III (CIII) multidendritic sensory neurons are specifically activated by cold and optogenetic activation of these neurons elicits CT. Blocking synaptic transmission in CIII neurons inhibits CT. Genetically, the transient receptor potential (TRP) channels Trpm, NompC, and Polycystic kidney disease 2 (Pkd2) are expressed in CIII neurons, where each is required for CT. Misexpression of *Pkd2* is sufficient to confer cold responsiveness. The optogenetic activation level of multimodal CIII neurons determines behavioral output, and visualization of neuronal activity supports this conclusion. Coactivation of cold- and heat-responsive sensory neurons suggests that the cold-evoked response circuitry is dominant. Our *Drosophila* model will enable a sophisticated molecular genetic dissection of cold nociceptive genes and circuits.

INTRODUCTION

Diverse animals respond to noxious cold with stereotyped behaviors, which mostly reduce the tissue surface area exposed to low temperature. Such responses occur in endotherms [1, 2] and ectotherms [3], highlighting the importance of maintaining a healthy body temperature. The assays used to assess responses to noxious cold in laboratory animal models include the cold plate [4], tail flick [5], and exposure to acetone [6] or dry ice [7]. Although all are useful for analyzing genes implicated in cold nociception in rodents [8, 9], the field has lacked a genetically tractable system where unbiased forward genetic identification of cold nociception genes is both feasible and cost effective. *Drosophila* possess powerful genetic tools for analyzing genes and neural pathways [10] and have recently identified conserved players in thermal and mechanical nociception and nociceptive sensitization [11–14]. Although prolonged exposure to temperatures 10°C and below is fatal [15], how *Drosophila* larvae respond to acute noxious cold stimuli is currently unknown.

The *Drosophila* larval peripheral nervous system has four classes of type II multiple dendritic (md) sensory neurons that innervate the barrier epidermis. Each class has its own stereotypic location, dendritic arbor territory, and morphology [16]. Class IV (CIV) neurons respond to noxious heat [17] and mechanical [13, 17, 18] stimuli and mediate aversive rolling. Class II (CII) and class III (CIII) neurons mediate gentle touch responses [19, 20]. All md neurons possess naked nerve endings, similar to cold-responsive non-myelinated C fibers in vertebrates [16]. Myelinated peripheral Aδ fibers also contribute to cold responses in vertebrates [21]. It is not known which, if any, peripheral neuron class(es) are cold sensitive in *Drosophila*.

Transient receptor potential (TRP) channels are variably selective cation channels containing multiple subunits and six transmembrane domains. TRP channels function ubiquitously in sensory biology, including nociception [22]. Vertebrate TRPV1 responds to noxious heat in skin [23], while Painless (a TRPA channel) is required for aversive rolling to noxious touch and heat in flies [24]. However, the cells and channels required for responding to cold have been difficult to pinpoint. TRPM8 detects both innocuous and noxious cold in rat peripheral sensory neurons [25]. TRPA1 has also been implicated as a vertebrate

noxious cold receptor [26]; however, some cold-sensing neurons do not express TRPM8 or TRPA1 [27], suggesting that alternative noxious cold channels exist. Thermal preference assays in *C. elegans* have also identified genes responsible for cool sensing [28]. In *Drosophila* larvae, collective studies implicate *trp*, *trpl*, and *iav* in cool avoidance (10°C–20°C) [29, 30], and the TRPP-like *brivido* channel senses cool temperatures (12°C–15°C) in adult flies [31]. Although *Drosophila* TRP channels are clearly involved in cool avoidance, whether they are also involved in acute noxious cold (≤10°C) sensing is unknown.

Using global and local cold assays, we investigated the cellular and molecular bases of cold nociception in *Drosophila* larvae. Acute noxious cold provoked a set of unique behaviors with a full-body contraction (CT) as the primary response. The strongest and most specific calcium responses to cold were found in CIII md neurons. Additionally, direct optogenetic activation of CIII neurons in the absence of cold caused CT, while silencing CIII neurons attenuated responses to cold. The TRP channels NompC, Trpm, and Pkd2 play key roles in cold nociception in CIII neurons. Interestingly, the level of activation of CIII neurons determined whether the output response was a light-touch- or cold-evoked behavior. Consistent with this, light-touch- and cold-activated CIII neurons to different extents. Finally, coactivation of CIII and CIV (heat/mechanical) neurons evoked CT at the expense of aversive rolling, providing insight into nociceptive circuit organization. Our findings reveal that *Drosophila* uses a distinct set of cells, channels, and aversive behaviors to respond to extreme cold.

RESULTS

Drosophila Larvae Contract in Response to Noxious Cold

To determine how *Drosophila* larvae respond to noxious [15] cold, we developed two complementary behavioral assays. One involves global contact along the ventral body surface (Figure 1A), and the second involves a local noxious cold stimulus (Figure 1C). In the global assay, larvae were acclimated to a 25°C metal plate (Movie S1), then transferred to a cold or cool surface (4°C–16°C) (Figure 1A). Two unique behaviors were observed, distinct from normal peristaltic locomotion (Movie S1), gentle touch behaviors [32, 33], and aversive rolling to noxious heat [11, 24] or force [13, 17, 18, 24]. The cold-evoked behaviors were a 45°–90° head and/or tail raise (HTR) or a CT of the head and tail toward the middle of the body (Movie S2). At 14°C and below, CT was the predominant response, increasing with decreasing temperature (peak at 4°C), while HTR responses peaked at 10°C (Figure 1B).

Given that cold-induced muscular contractions [34] might induce CT in the global assay, we constructed a cold probe for locally stimulating larvae from ambient temperatures to 3°C (Figure 1C). The cold probe-evoked behaviors were as follows: (1) CT, identical to the global assay, (2) a 45°–90° simultaneous head and posterior raise (U shape [US]), and (3) a 45°–90° raise of the posterior body segments (posterior raise [PR]), with US and PR being similar to individual HTR behaviors observed in the global assay (Movie S3). These behaviors were not observed during normal locomotion, but their frequencies increased upon stimulation with decreasing temperatures (Figure 1D). Each behavior had a unique response-versus-temperature curve. US and PR exhibited broad peaks between 3°C and 8°C, while CT peaked between 9°C and 14°C (Figures S1A–S1C). CT was the only behavior occasionally observed in response to light touch (Figure 1D; Figure S1C). As with heat [11, 24], the average response latency decreased with decreasing temperature (Figure S1D). Since CT arises from the focal (one body segment) application of cold, CT is most likely not due to global cold-induced muscle contraction.

To determine whether there were three distinct classes of responders (CT, US, and PR), we retested larvae after an arbitrary 20-min period. Larvae did not consistently reproduce their initial behavior, although there was a slight preference for reproducing CT (Figure S1E). This ruled out three distinct responder classes. To test whether slight differences in the placement of the cold probe could affect behavioral output, we systematically varied the probe position along the anteroposterior axis. Positioning the probe one or two segments closer to the head (region 2) or posterior spiracles (region 4) had no significant effect on US or CT (Figure S1F). However, cold stimulation directly to the head (region 1) resulted in a dramatic increase in CT, while stimulation to the tail (region 5) resulted in fewer responders (Figure S1F).

The results from the global and local assays indicate that *Drosophila* larvae exhibit a unique set of behaviors in response to noxious cold that are distinct from normal locomotion, light-touch responses, and aversive rolling to noxious heat or mechanical stimuli.

Cold Stimulation Results in Specific Ca^{2+} Responses within Class III md Neurons

To investigate whether md neurons display Ca^{2+} responses to cold stimulation (6°C), we live-imaged intact third-instar larvae expressing GCaMP6 in md neurons (Figure S2A). Compared to their respective baselines, cold stimulation produced a slight increase in GCaMP6 signals in class I (CI) and a moderate increase in CII neurons (Figures 2A and 2B). By contrast, CIII neurons showed a robust increase in GCaMP6 fluorescence (Figure 2C; Movie S4). Heat-responsive CIV neurons were largely inert to cold (Figure 2D). Statistical comparisons of average peak GCaMP6 signals demonstrated significantly higher peak fluorescence levels in CIII neurons relative to the other subclasses (Figure S2B; Figure 2E).

To assess the specificity of GCaMP6 signals in md neuron subtypes as a function of temperature, we compared their average maximum changes in GCaMP fluorescence from ambient to cold temperatures and performed cross-correlation analyses (Figure 2F; Figure S2C). A strong negative cross-correlation coefficient was observed in CIII neurons at lower temperatures (Figure 2F; Movie S4), and they were not significantly activated by noxious heat stimulation (44°C) (Figure S2D), indicating cold specificity. Noxious heat stimulation does not inactivate GCaMP6 because heat followed by subsequent noxious cold still gave a robust CIII GCaMP6 signal (Figures S2D and S2E). Together these results demonstrate that CIII, and to a lesser extent CII, neurons are cold activated.

Optogenetic Activation of Class III md Neurons Causes CT Behavior

Cold-evoked Ca^{2+} responses observed in CIII neurons led us to ask whether direct optogenetic activation of these neurons, in the absence of cold, would elicit cold-specific behaviors. Larvae

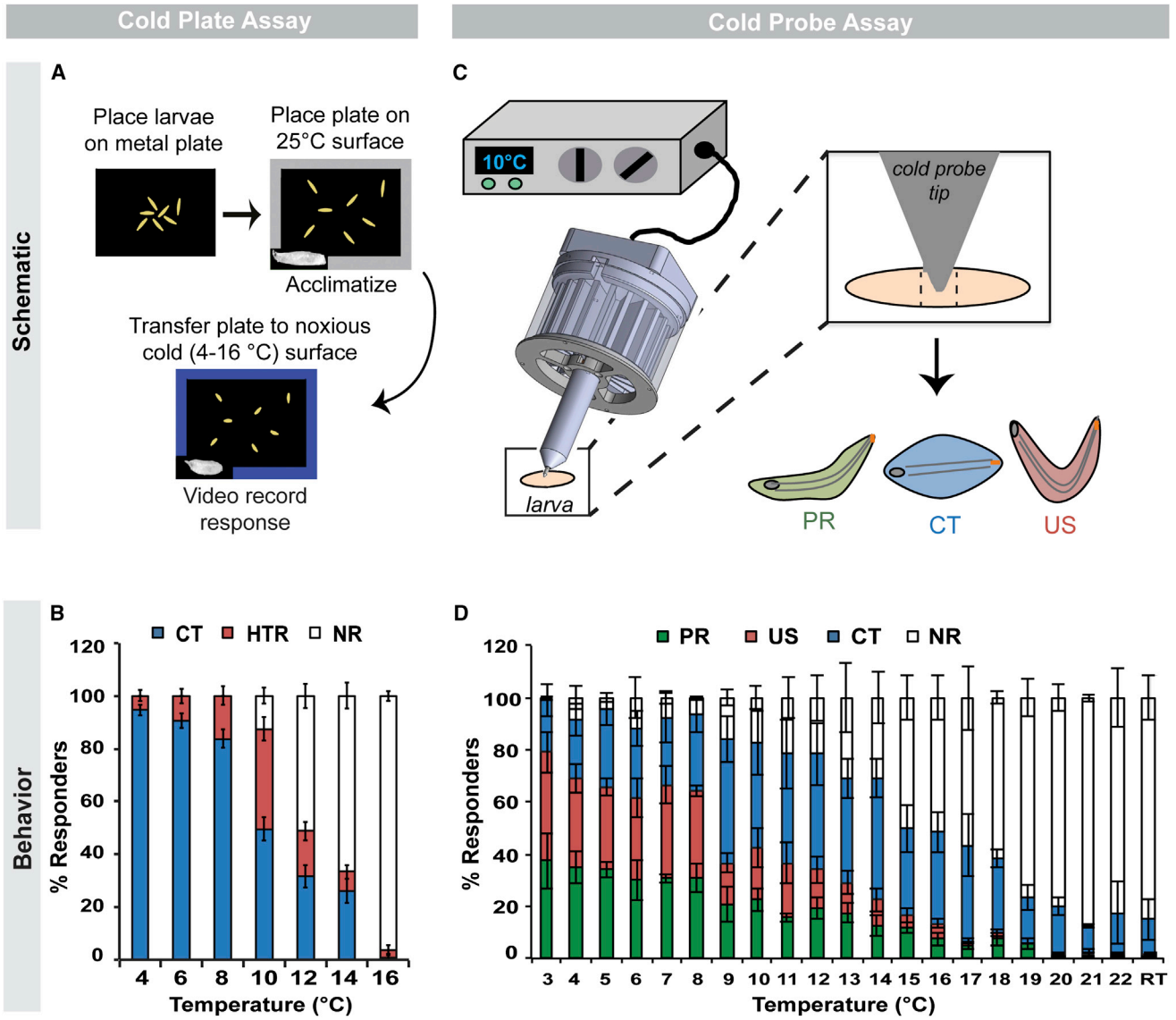


Figure 1. *Drosophila* Larvae Have Unique Behavioral Responses to Noxious Cold Stimuli

(A and B) Diagram (A) and averaged responses versus temperature in cold plate assay (B). Error bars, ± standard error of proportion (SEP); n = 96–119.

(C) Diagram of cold probe assay.

(D) Larval response versus temperature in cold probe assay averaged ± SEM (n = 120).

HTR, head and/or tail raise; US, U shape; CT, contraction; PR, posterior raise; NR, no response; RT, room temperature. See also Figure S1 and Movies S1, S2, and S3.

expressing the ultrafast Channelrhodopsin-2 variant ChETA in CIII neurons or in other md classes (Figure S3) were stimulated with high-intensity blue light with or without all *trans*-retinal (ATR, a required cofactor for ChETA function). In controls, most larvae continued normal locomotion and a few displayed HTR behavior upon light exposure (Figures S3F–S3I). Activating CI neurons resulted in an immediate ($\leq$1 s of stimulus) cessation of locomotion (Figure S3F; Movie S5) rather than CT (quantified as a change in body length) (Figure 3A). Optogenetic activation of CII neurons elicited CT, however, activation of CIII produced a more robust and longer CT in more larvae (Figures 3B and 3C; Figures S3G, S3H, S3J, and S3K; Movie S5). Activation of CIV neurons elicited aversive rolling (Figure 3D; Figure S3I; Movie S5) as previously reported [17]. Optogenetic activation of md neurons did not increase HTR responses (Figures S3F–S3I), further validating our focus on CT. Together, these data reinforce and extend our GCaMP observations, demonstrating that light-mediated activation of CIII or CII neurons is sufficient to generate CT responses.

Class III Sensory Neurons Mediate Cold-Evoked CT Behavior

We next examined whether CII, CIII, or other md neurons are required for cold-evoked CT. We electrically silenced md neurons

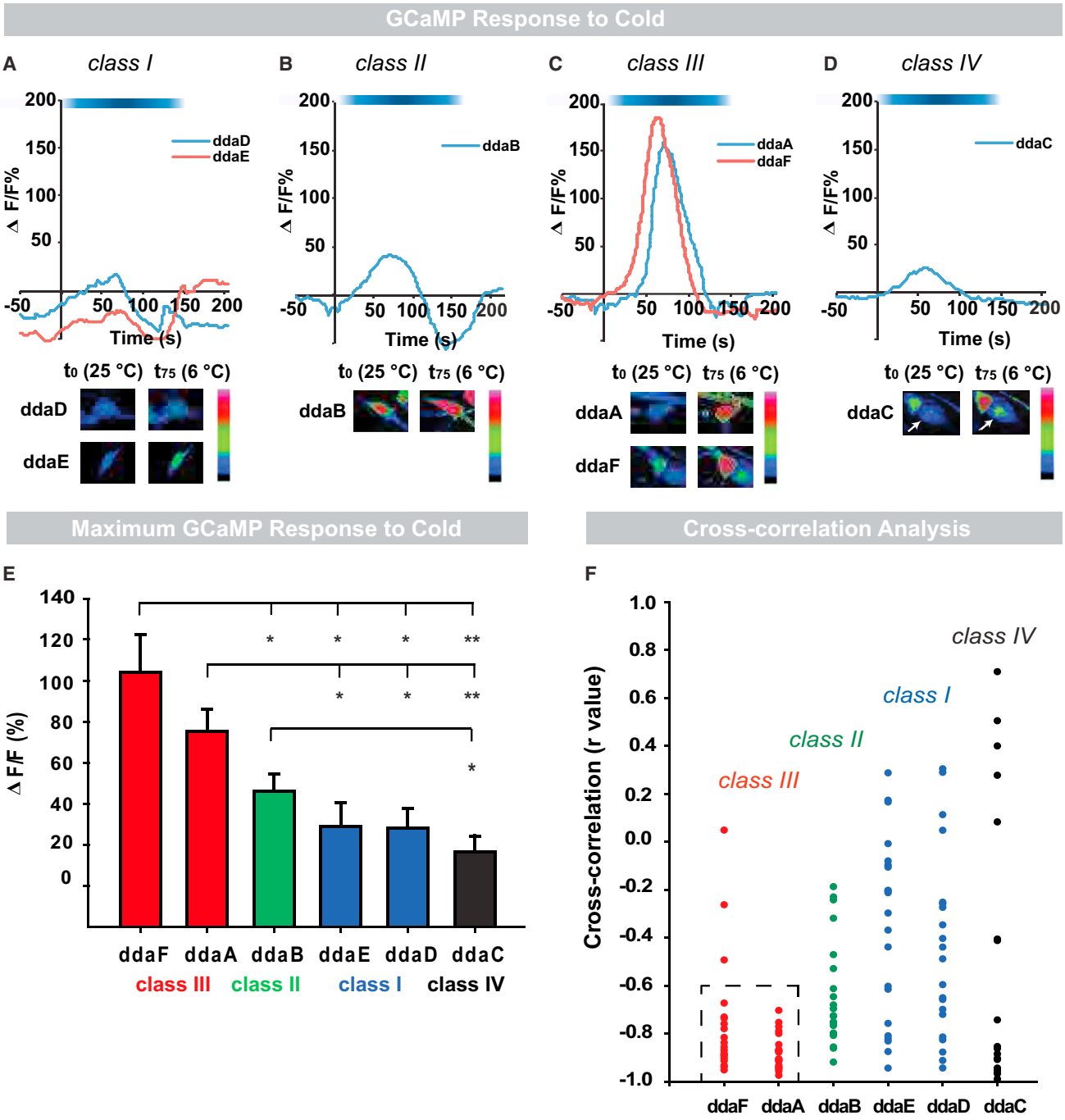


Figure 2. CII and CIII Sensory Neurons Are Activated by Cold

(A–D) Representative tracings of class-specific GCaMP6 responses (ΔF/F) with cold stimulation (6°C) in (A) CI (ddaD and ddaE), (B) CII (ddaB), (C) CIII (ddaA and ddaF), and (D) CIV neurons (ddaC, marked by white arrow). White-blue spectrum bar signifies temperature range (25°C–6°C). Bottom inserts show neuronal activation at a cold temperature (6°C) and baseline temperature (25°C).

(E) Average peak change in fluorescence (ΔF/F) ± SEM (n = 20 per neuron; *p < 0.01 and **p < 0.001).

(F) Cross-correlation analysis of neuronal activation with respect to temperature. Dashed box: clustered negative cross-correlation values are for CIII neurons (n = 20 per neuron).

See also Figure S2 and Movie S4.

via class-specific expression of tetanus toxin and examined their cold-evoked responses. In the cold plate assay, silencing CIII neurons significantly attenuated CT (Figure 4A). We also observed mild reductions in CT upon silencing CI neurons; however, silencing CII or CIV neurons had no significant effect on cold-evoked CT (Figure S4A). In the cold probe assay, silencing

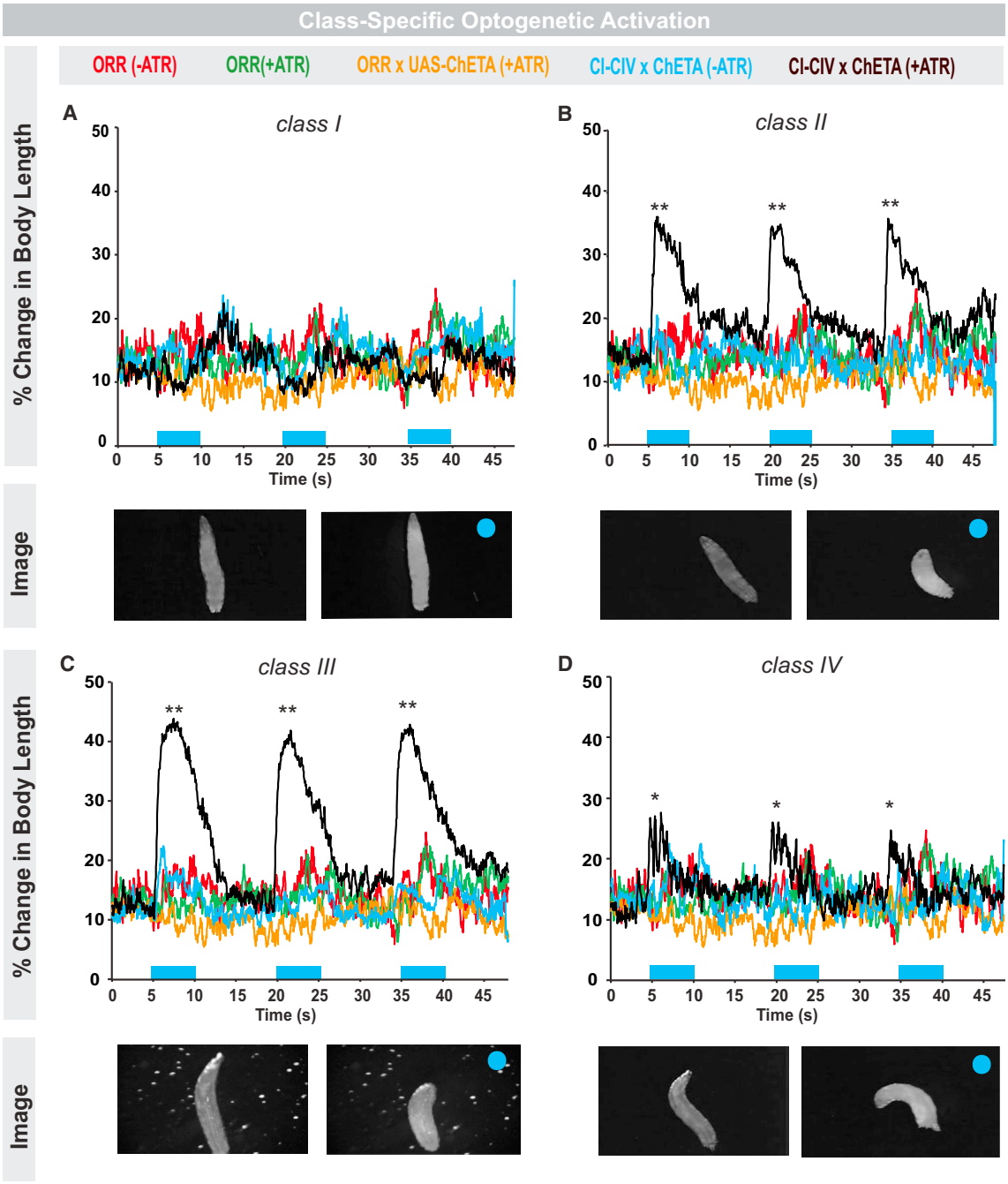


Figure 3. Optogenetic Activation of CII and CIII Neurons Results in CT Response

Average percentage change in body length over time upon light exposure (blue bars) and optogenetic activation (black) of (A) CI, (B) CII, (C) CIII, or (D) CIV neurons. Larvae not fed (red) or fed (green) ATR, larvae bearing the *UAS-channelrhodopsin* transgene (ChETA) alone (orange), or larvae expressing ChETA but not fed ATR (blue) acted as negative controls. Bottom inserts: larval images of optogenetic activation for each class pre- and post-light (blue circle) exposure (*$p < 0.05$ and **$p < 0.01$; n = 15–20). See also Figure S3 and Movie S5.

CIII neurons also resulted in a significant reduction in CT (Figure 4B), while silencing CI, CII, or CIV resulted in no change in cold-evoked CT versus genetic controls (Figure S4B). Silencing CII and CIII neurons together (Figure S3E) resulted in mild to equivalent reductions in cold responses in the plate or probe assays, respectively, versus silencing CIII alone (Figure S4).

As an alternative approach, we interfered with the *para* gene, which encodes an NaV-type sodium channel, to block action potential propagation in CIII neurons [35, 36]. This blocked cold-induced CT in both assays (Figures 4C and 4D). Together, these data support a prominent role for CIII neurons in mediating CT to noxious cold in *Drosophila* larvae. These results are consistent

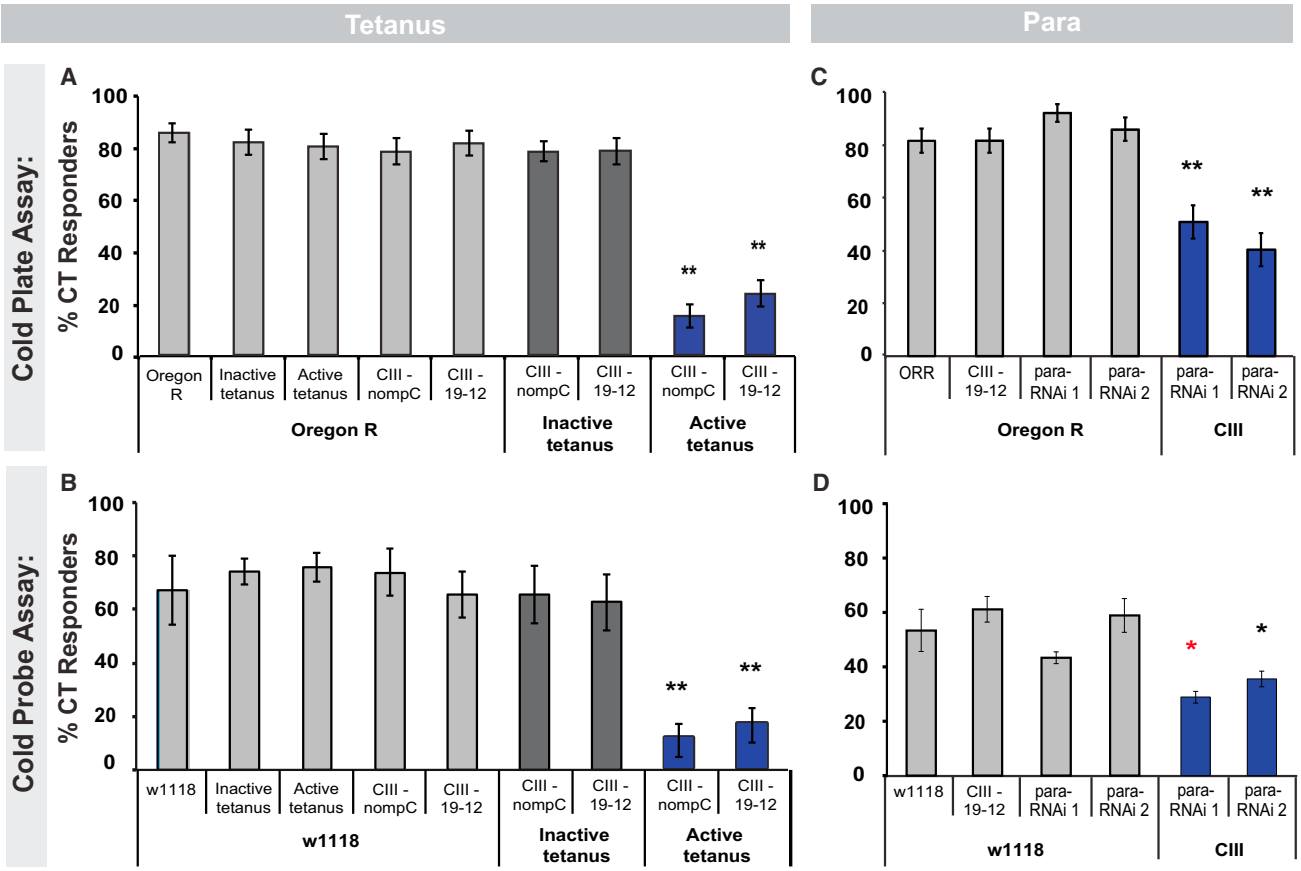


Figure 4. CIII Sensory Neurons Mediate Cold-Evoked CT Behavior

(A and B) Larvae with CIII neurons silenced via two independent drivers (*nompC-GAL4* or *19-12-GAL4*) were tested in the (A) cold plate (6°C; n = 60–100 averaged ± SEP) and (B) cold probe assays (11°C; n = 180 averaged ± SEM).

(C and D) Larvae with CIII neurons expressing *para-RNAi* transgenes in (C) cold plate (6°C; n = 62–71 averaged ± SEP) and (D) cold probe assays (10°C, n = 90 averaged ± SEM).

White and gray bars indicate controls and blue bars indicate experimental results (*p < 0.05, **p < 0.0125, and red *p < 0.05 compared to *w1118* and CIII Gal4 control). See also Figure S4.

with the neuron-specific GCaMP responses (Figure 2) and optogenetic activation data (Figure 3) and support that cold-evoked CT is sensory neuron mediated.

The TRP Channels Pkd2, Trpm, and NompC Are Expressed in Class III md Neurons

As TRP channels mediate diverse thermosensory responses in *Drosophila* and other animals [22], we hypothesized that cold-evoked CT might be mediated by one or more TRP channels expressed in CIII neurons. Whole-genome microarray expression comparisons between isolated CIII neurons and whole larval extracts identified the TRP channels *nompC* and *Trpm* RF isoform as significantly enriched in CIII neurons (Figure 5A). Comparisons between CIII and CIV neurons revealed that *Pkd2* and the *Trpm* RB isoform were specifically enriched in cold-responsive versus heat-responsive neurons (Figure 5A). Interestingly, while the *nompC* RD isoform was enriched in CIII neurons compared to whole larvae, isoform RA was enriched in both CIII and CIV compared to whole larvae (Figure 5A). Additionally, other TRP channel genes were found to be enriched in CIII versus CIV (Figure S5A).

Comparative qRT-PCR for *Pkd2*, *nompC*, and *Trpm* in isolated CIII or CIV neurons revealed significant enrichment of *nompC* and *Pkd2* in CIII neurons; however, *Trpm* expression showed only a mild increase that was not significant (Figure 5B). This latter case could be due to the amplicon used in the qRT-PCR analyses recognizing all *Trpm* isoforms, possibly masking class-specific enrichment (Figure 5B). A *nompC* promoter fragment driving Gal4 expression labeled CIII neurons (Figure S3D), and NompC protein was expressed in CIII neuron filopodia [19, 20]. Collectively, these data suggest that *nompC*, *Trpm*, and *Pkd2* are expressed in the proper cells to mediate cold-evoked behavior.

Pkd2, Trpm, and NompC Function in Class III Neurons to Respond to Noxious Cold

To test whether *nompC*, *Trpm*, or *Pkd2* is required for noxious cold detection, we assayed mutants. Larvae bearing *Pkd2*-, *Trpm*-, or *nompC*-null alleles over relevant deficiencies had significantly reduced cold-evoked CT in both assays (Figures 5C and 5D). Larvae bearing transheterozygous mutant alleles for these genes also showed CT defects (Figures S5B and

S5C). Larvae expressing *UAS-RNAi* transgenes targeting *Pkd2*, *Trpm*, or *nompC* in CIII neurons also showed significant decreases in CT (Figures 5C and 5D). Targeted overexpression of either *nompC* or *Pkd2* in CIII neurons in a mutant background rescued these defects (Figures 5E and 5F), with the exception of *nompC* in the cold plate assay. Importantly, disruption of *Pkd2*, *Trpm*, or *nompC* had no significant effect on CIII dendritic morphology (Figure S6). *nompC* has been implicated in both cold (here) and gentle touch [19, 20]. Thus, we tested whether *Pkd2* and *Trpm* also affect gentle touch responsiveness. All three mutants had slight defects in gentle touch sensation (see Figure S7 and the Experimental Procedures for assay details).

Roles of Pkd2, Trpm, and NompC in Sensing Cold

Do the identified TRP channels function in general neuronal excitability or do they have a specific thermosensory function in detecting noxious cold? Upon CIII neuron optogenetic activation with TRP-specific RNAi expression, larvae still exhibited robust CT (Figure 6A). In contrast, when co-expressing RNAi targeting *para*, CT was blocked upon optogenetic activation (Figure 6A).

To determine whether Pkd2, NompC, or Trpm is required for the observed calcium increases in CIII neurons stimulated with cold, we measured CIII GCaMP responses in TRP channel mutants. In all three mutants, cold-induced calcium responses were altered (Figure 6B). Cold-induced CIII calcium responses went up in *Trpm* mutants and down in *Pkd2* mutants, while *nompC* mutants exhibited a slight but non-significant increase (Figures 6B and 6C). These results suggest potentially complex roles for NompC and Trpm in regulating neuronal calcium homeostasis upon cold exposure and a more direct role for Pkd2 in cold sensing. Consistent with this, overexpressing *Pkd2* in non-cold-sensing CIV neurons resulted in a conferrence of GCaMP cold sensitivity (Figure 6D).

Class-III-Mediated Multimodal Behavior Depends on Activation Levels

Our results thus far implicate CIII neurons as cold sensors, making them multimodal given their gentle touch function [19, 20]. One of the primary reactions to gentle touch is a head withdrawal (HW) [32, 33], which is like an asymmetric CT. To clarify how these cells might distinguish between cold and gentle touch, we varied the dose of optogenetic light activating CIII neurons to see whether a particular activation level evoked either HW or CT (Figure 7A; Movie S6). Optogenetic activation of CIII neurons at the highest dose resulted in CT almost exclusively (Figure 7A). The percentage of CT responders was reduced with decreasing light, however, while HW responses increased (Figure 7A).

Our optogenetic dose response suggests that cold may activate CIII neurons more strongly than light touch or activate more CIII neurons. To investigate this, we utilized the genetic tool CaMPARI, which upon exposure to photoconverting violet light shifts fluorescence from green to red as a function of intracellular calcium levels evoked by a specific stimulus [37] (Figures 7B–7D). CIII neurons expressing CaMPARI exhibited a significant increase in photoconversion in response to cold versus gentle touch (Figures 7B–7D). While a similar number of activated CIII neurons was observed between cold- and gentle touch-stimulated larvae, cold evoked significantly larger CaMPARI responses in multiple larval segments (Figures 7B–7D). These data suggest that noxious cold more strongly activates CIII neurons compared to gentle touch.

Coactivation of Thermosensory Nociceptors Results in Predominantly Cold-Evoked Responses

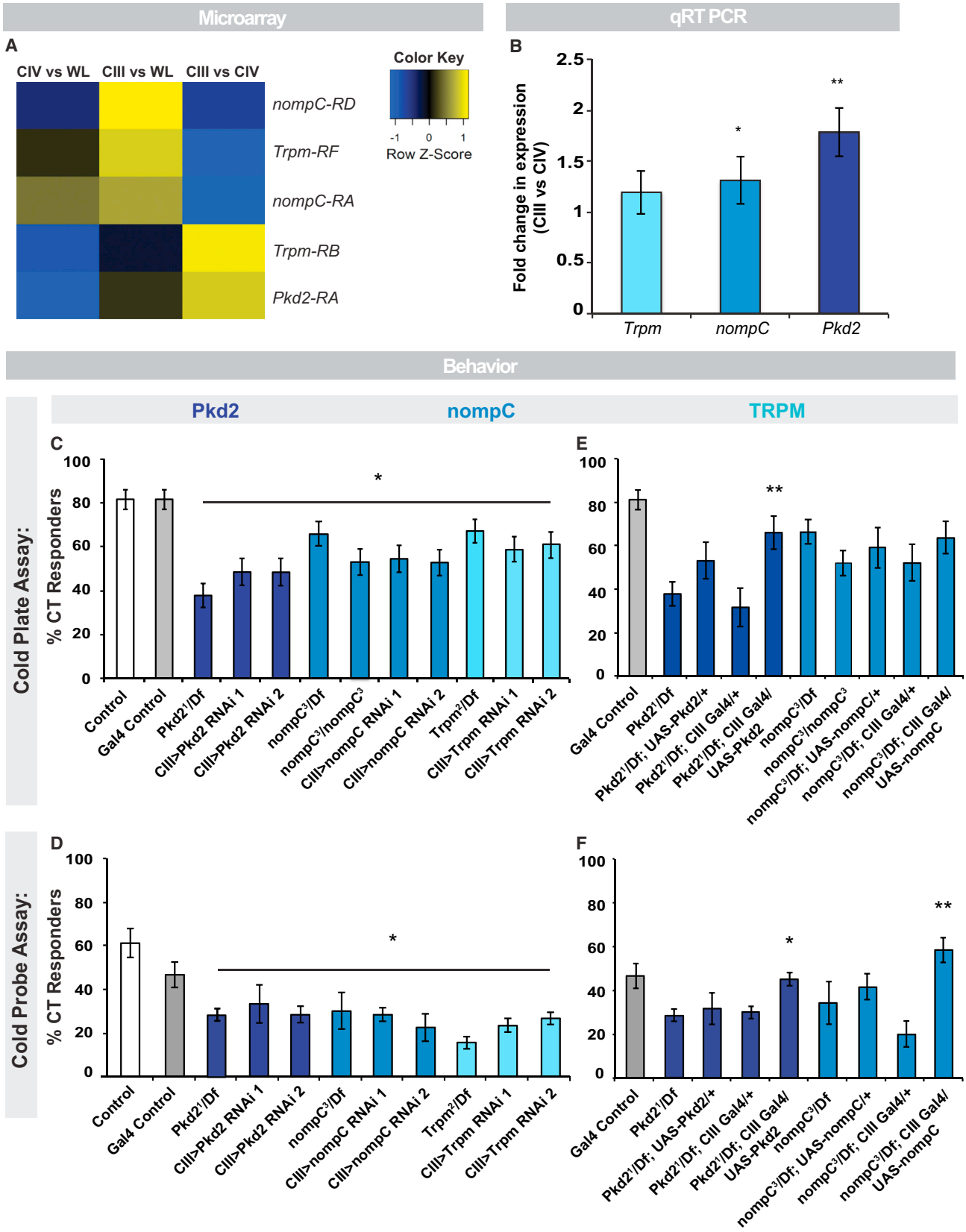
CIII and CIV dendritic arbors extensively tile the epidermis, and their axonal projections terminate near each other in the anterior ventral nerve cord [16, 38]. To understand how cold and hot stimuli are sensed, we asked what behavioral output would be observed upon directly stimulating CIII (CT) and CIV (body roll [BR]) neurons simultaneously. Optogentic coactivation of CIII and CIV neurons in live larvae produced exclusively CT (Figure 7E; Movie S7).

Additionally, we tried an alternative approach to address possible thermosensory competition between neuron types. TrpA1 expression has been used to thermogenetically activate non-thermosensory neurons [39]. Therefore, applying a heat probe to larvae overexpressing the warm-activated TRPA1 channel in CIII neurons should activate these neurons and endogenous CIV heat-sensitive channels. Similar to above, simultaneous CIII and CIV activation in this manner resulted in predominantly CT, correlated with a dramatic reduction in the typical level of heat-evoked BR responses (Figure 7F). These results suggest that noxious cold signals and subsequent CT override signals from noxious heat and BR responses when CIII and CIV neurons are simultaneously activated.

DISCUSSION

We developed assays to identify the cells and channels required to respond to noxious cold. The primary cold-evoked behavior, CT, is unlike larval responses to other types of nociceptive [11, 13, 17, 18, 24] and innocuous stimuli [19, 20, 32, 33]. Several lines of evidence indicate that CIII neurons are cold nociceptors. First, CIII neurons are directly and specifically activated by cold temperatures. Second, CIII neurons directly reproduce a robust CT in larvae upon strong optogenetic activation. Lastly, these neurons are required to produce a robust cold-evoked CT response, rather than the highly multimodal CIV neurons that mediate responses to heat [17, 24], harsh touch [13, 18], and UV light [40].

Multiple lines of evidence suggest how CIII neurons may be activated by both noxious cold (this study) and gentle touch [19, 20]. First, when CIII neurons are optogenetically stimulated with low doses of light, larvae exhibit a gentle touch behavior (HW), while stronger doses of light result in CT. Second, CaMPARI analysis indicates a significant difference between the magnitude of CIII activation when larvae are stimulated with noxious cold versus gentle touch. These data suggest that CIII neurons may have different activation thresholds that ultimately determine the correct behavioral output to different stimuli. This is an important finding since until now it has remained an open question of how multimodal neurons detect different types of stimuli to induce varying behavioral responses. For example, it is unknown how CIV neurons mediate behavioral responses to high temperature/harsh touch (BR) versus proprioceptive



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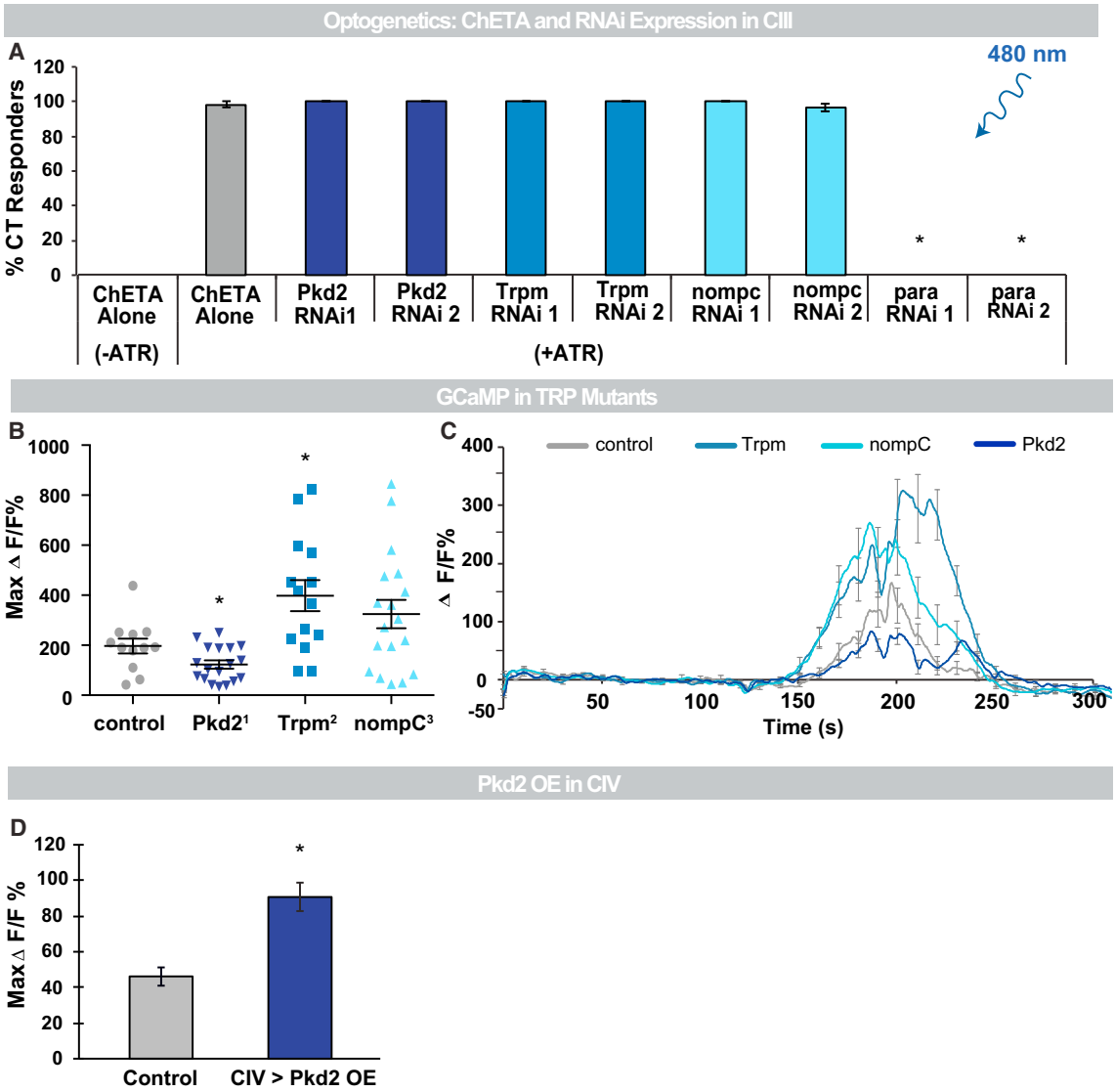

Figure 6. Role of Pkd2, Trpm, and NompC in Cold Detection

(A) Responses to optogenetic activation of CIII expressing *ChETA* and *RNAi* transgenes with (+) or without (–) ATR (n = 60 averaged ± SEP; *p < 0.0001).
(B) Maximum cold-induced change in GCaMP fluorescence in CIII neurons of TRP mutants (n = 12–18; error bars indicate SEM; *p < 0.05).
(C) Averaged cold-induced change in GCaMP fluorescence in CIII neurons of TRP mutants over time (n = 12–14; error bars indicate SEM).
(D) Cold-evoked (6°C) GCaMP response in CIV sensory neurons with targeted expression of *Pkd2* (n = 18 averaged ± SEM; *p < 0.0001).
Controls are labeled in gray, and specific genes targeted are labeled in blues.

feedback (normal locomotion) [41, 42]. The optogenetic dose-response strategy used here could be useful for any case where a single neuron gives two distinguishable behavioral outputs.

At the molecular level, *Pkd2*, *nompC*, and *Trpm* are enriched and appear to function in CIII neurons to mediate cold-evoked CT. This indicates that these channels are multimodal given their

Figure 5. Expression and Function of TRP channels *Pkd2*, *Trpm*, and *nompC* in CIII Sensory Neurons

(A) Microarray comparing *Pkd2*, *Trpm*, and *nompC* isoforms in CIII versus CIV neurons, CIII versus whole larva (WL), or CIV versus WL. For degree of enrichment, yellow indicates more enriched, and blue indicates not enriched by comparison (n = 3).
(B) qRT-PCR analysis of *Pkd2*, *Trpm*, and *nompC* expression in CIII neurons versus CIV (n = 4; values averaged ± SEP).
(C and D) *Pkd2*, *nompC*, and *Trpm* mutant and targeted expression of RNAi transgenes in CIII in the (C) cold plate (6°C) and (D) cold probe (10°C) assays (C, n = 60–81 responders averaged ± SEP; D, n = 90–120 responders averaged ± SEM).
(E and F) Rescue of *Pkd2* or *nompC* in CIII neurons via TRP overexpression in mutant over deficiency background in (E) cold plate and (F) cold probe assays (E, n = 22–74 responders averaged ± SEP; F, n = 3 sets of n = 20 averaged ± SEM).
In (B)–(F), white and gray bars indicate controls, and colored bars indicate different genes targeted; *p < 0.05 and **p < 0.01, indicating significant compared to relevant mutant over deficiency control (E and F). See also Figures S5 and S6.

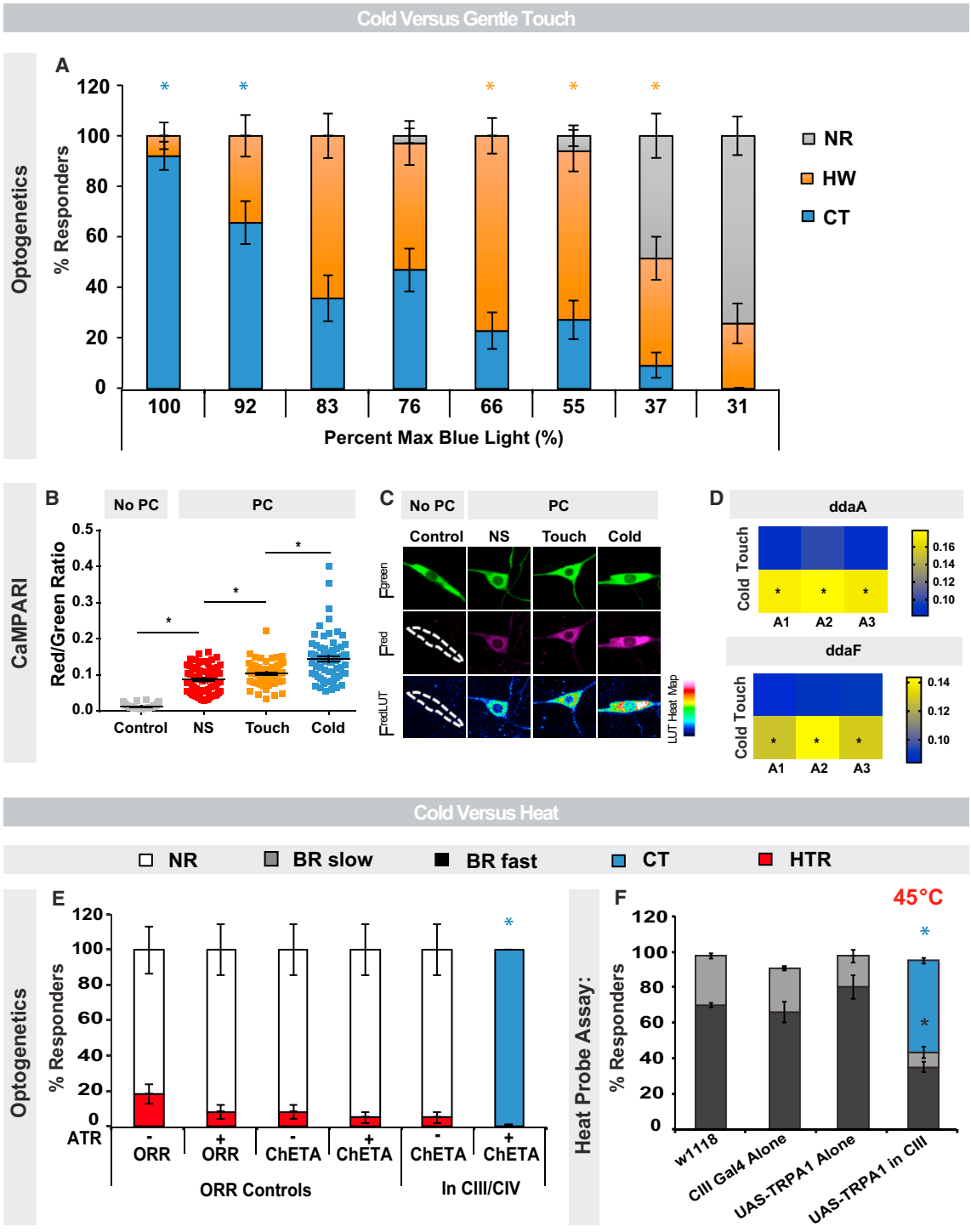

Figure 7. Optogenetic Dose-Response and Coactivation Experiments Reveal How Different Output Behaviors Are Determined

(A) Percentage of responders with varying light dose in larvae with CIII ChETA expression (n = 25–35 averaged ± SEP). Colored asterisks represent significance of the indicated behavior compared to other behaviors at the given light dose. Blue, *p ≤ 0.02; orange, *p ≤ 0.004.

(B) Ratio of CIII green to red photoconversion (PC) in non-PC (gray) and PC non-stimulated (NS) (red) controls versus PC touch-stimulated (orange) and cold-stimulated (blue) larvae bearing CaMPARI transgenes (n = 70–72 averaged ± SEM; *p < 0.01).

(C) Representative images of CIII neuron PC.

(D) PC ratio heatmap analysis (n = 9–12 animals), with yellow indicating stronger PC in abdominal segments A1–A3 (*p < 0.05).

(E) Percentage of responders upon CIII and CIV optogenetic coactivation (n = 45–60 averaged ± SEP; *p < 0.001).

(legend continued on next page)

other known sensory functions, such as *nompC* in gentle touch [19, 20] and *Pkd2* in taste [43, 44] and mechanosensation in the primary cilia of the vertebrate kidney epithelium [45]. This latter function may be related to the role of *Pkd2* in autosomal dominant polycystic kidney disease [46]. Currently, it is unclear whether patients suffering from this disease, or mice lacking the gene [47], have cold nociception defects. Lastly, although TRPM8 acts as a cold sensor in vertebrates [8, 48], it maintains zinc and magnesium homeostasis [49, 50] in *Drosophila*; ours is the first evidence that a *Trpm* family member acts in cold nociception in *Drosophila*.

While multimodality is common among TRPs and other channels (see painless [12, 24, 51], dTRPA1 [12, 40, 52–54], Pickpocket1 [41, 42, 51], and Pickpocket 26 [42, 55]), it begs the question, how do these channels distinguish between innocuous and harsh stimuli? For *painless* and *dTRPA1*, splice variants may function differently [56]. For *nompC*, ankyrin repeats are important for its mechanosensory function [57, 58], and it will be interesting to determine whether this is also true for cold sensation. Other studies suggest TRP channels may collaborate with different sets of partially overlapping channels for different functions. For gentle touch, this appears to include NompC, Ripped Pocket, Nmdar1, and Nmdar2 [19, 20]. For noxious cold, vertebrate studies revealed interactions between TRPM8 and potassium channels (Task-3, Kv1, and Kv7) (see review [59]). Such potential interactions may help explain how increased GCaMP responses to cold in *Trpm* mutants could lead to the inhibition of cold-evoked behavior. Altered cold-evoked GCaMP responses could suggest that these channels work in tandem with other channels, such as calcium-activated potassium channels, to regulate cellular and behavioral cold responses.

Excess calcium in *Trpm* mutants could lead to the activation of calcium-activated potassium channels and, thereby, promote hyperpolarization, which would contribute to the inhibition of CT behavior. In fact, when microarray expression profiles of CIII versus CIV neurons were examined, two particular calcium-activated potassium channels were found to be enriched in CIII neurons (SK, 2.1 average fold change, and slowpoke, 10.4 average fold change; see GEO: GSE69353 and GSE46154). Altered calcium responses in *Trpm* and *Pkd2* mutants also may indicate that cold-evoked behavior is sensitive to optimal calcium levels, such that, above or below these levels, the behavior is inhibited. Other models are also conceivable. Of the TRP channels identified here, Pkd2 seems the most likely to act as a direct cold sensor, as calcium levels are decreased upon cold exposure in *Pkd2* mutants, and misexpression of Pkd2 confers cold responsiveness to other sensory neurons.

In adults and larvae, *Drosophila* avoid temperatures outside their comfortable range by utilizing a distinct set of thermosensitive antennal or dorsal organ neurons in the head [31, 60]. Interestingly, the cold-sensitive neurons are inhibited by heat and vice versa; ultimately, these thermosensory circuits determine motor output to help the animal navigate along a temperature gradient [31, 60]. Similar circuits have begun to be characterized for noxious stimuli [61, 62]. Here, coactivation of hot (CIV) and cold (CIII) neurons provokes a dominant CT that is unlikely to arise solely from faster neuronal conduction, since both neurons terminate at similar locations in the anterior ventral nerve cord [38]. It is not known whether CIII neurons, like CIV, converge onto basin interneurons [61], but unraveling the architecture of this circuit will be an interesting avenue for future work. We suspect that optogenetic coactivation and CaMPARI techniques, as used here, will be valuable tools in this endeavor.

Taken together, our results identify the peripheral sensory neurons responsible for noxious cold detection in *Drosophila* larvae and conserved molecular players required for this process. The cold assays we have developed offer powerful models for the genetic dissection of cold nociception. Further exploitation of these models should yield exciting insight into cold nociceptive circuitry.

EXPERIMENTAL PROCEDURES

For detailed methods, see the Supplemental Experimental Procedures.

Behavioral Assays

In all behavioral assays, freely moving mid-third-instar larvae were used, age-matched and selected based on size. In the cold probe assay, larvae were placed under a bright-field stereomicroscope (Zeiss Stemi 2000). The tip of the custom-built probe (ProDev Engineering; Figure 1C) was gently placed on the dorsal midline (segment A4) and held for either 10 s or until the first behavioral response. Larvae that did not respond within 10 s were recorded as non-responders. In the cold plate assay, larvae were placed ventral side down on a 25°C thin metal plate (2 mm) coated with a fine mist of water. The metal plate was transferred to a Peltier-controlled plate pre-set to between 4°C and 16°C (Figure 1A), as verified with a Fluke 62 mini infrared thermometer, and responses were recorded (see the Supplemental Experimental Procedures). For both assays, cold-evoked behaviors precluded normal locomotion and each larva was only stimulated once (except Figure S1E). In all *GAL4/UAS* experiments, transgenes were heterozygous and no balancers or markers were present in the larvae tested. Unless otherwise stated, statistical analysis of behavior consisted of two-tailed Fisher's exact test with Bonferroni corrections.

ACCESSION NUMBERS

The accession number for the CIII microarray data, including metadata, raw data, and quantile normalized datasets reported in this paper, is GEO: GSE69353.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.09.038.

AUTHOR CONTRIBUTIONS

Writing – Original Draft, H.N.T. and M.J.G.; Writing – Review & Editing, H.N.T., M.J.G., K.A., A.A.P., and D.N.C.; Conceptualization, H.N.T., M.J.G., K.A., A.A.P., and D.N.C.; Investigation, H.N.T. (cold probe and gentle touch assays), K.A. (cold plate assays, GCaMP6 imaging, optogenetics, and morphology analyses), A.A.P. (cold plate analyses, GCaMP6 and CaMPARI imaging,

(F) Percentage of responders with CIII and CIV coactivation via CIII *TRPA1* expression and heat probe stimulation (45°C) (n = 120 averaged ± SEM; *p < 0.001; colored asterisks indicate significance between bars of the same color).

CT, contraction; HW, head withdrawal; HTR, head and/or tail raise; BR fast, body roll within 5 s; BR slow, body roll with 6–20 s; NR, non-responder. See also Figure S7 and Movies S6 and S7.

optogenetic dose response, qRT-PCR analyses, and contributed to morphology analyses), N.J.H. (cold plate analyses and optogenetic dose response), L.S. (contributed to cold plate assay development and data), S.C.I. (CIII neuron microarrays), S.B. (bioinformatic analyses of microarray data), E.P.R.I. (contributed to development of GCaMP6 imaging and identified the CII GAL4 driver used in this study), and C.L. (designed and engineered the cold probe). All authors critically read and commented on the final version of the manuscript.

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