

Neural substrates of cold nociception in *Drosophila* larva

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Abstract

Metazoans detect and differentiate between innocuous (non-painful) and/or noxious (harmful) environmental cues using primary sensory neurons, which serve as the first node in a neural network that computes stimulus specific behaviors to either navigate away from injury-causing conditions or to perform protective behaviors that mitigate extensive injury. The ability of an animal to detect and respond to various sensory stimuli depends upon molecular diversity in the primary sensors and the underlying neural circuitry responsible for the relevant behavioral action selection. Recent studies in *Drosophila* larvae have revealed that somatosensory class III multidendritic (CIII md) neurons function as multimodal sensors regulating distinct behavioral responses to innocuous mechanical and nociceptive thermal stimuli. Recent advances in circuit bases of behavior have identified and functionally validated *Drosophila* larval somatosensory circuitry involved in innocuous (mechanical) and noxious (heat and mechanical) cues. However, central processing of cold nociceptive cues remained unexplored. We implicate multisensory integrators (Basins), premotor (Down-and-Back) and projection (A09e and TePns) neurons as neural substrates required for cold-evoked behavioral and calcium responses. Neural silencing of cell types downstream of CIII md neurons led to significant reductions in cold-evoked behaviors and neural co-activation of CIII md neurons plus additional cell types facilitated larval contraction (CT) responses. We further demonstrate that optogenetic activation of CIII md neurons evokes calcium increases in these neurons. Collectively, we demonstrate how *Drosophila* larvae process cold stimuli through functionally diverse somatosensory circuitry responsible for generating stimulus specific behaviors.

eLife assessment

This is a **useful** study that investigates neural circuits mediating behavioral responses to cold in *Drosophila* larvae. Using a combination of behavioral analysis, neuronal manipulation, EM connectomics, and reporters of calcium activity, the authors **convincingly** show that cold-induced body contraction is mediated by specific central neurons. However, the strength of evidence is **incomplete** due to the concern that larval contraction is a result of chilling the nervous system and muscles, which causes spreading depolarization and mechanical contraction of the body, rather than an active sensorimotor response to cold. With these concerns addressed, this paper would be of interest to neuroscientists interested in temperature sensing.

Introduction

Metazoans detect innocuous and/or noxious environmental cues and appropriately generate relevant behavioral responses. There is a large diversity in types of nervous systems from relatively simple nerve nets to highly complex centralized organs dedicated for processing and executing behavioral commands. The ability of an organism to sense and respond to environmental cues is based on the underlying neural architecture and its connectivity to muscle groups for generating behavioral responses. Understanding the neural substrates underlying the execution of how behavioral commands are generated in the nervous system, spanning from the sensory neuron input to motor neuron output, constitutes one of the key areas of research in contemporary neuroscience.

Drosophila melanogaster is among the premier model organisms for studying molecular, cellular and circuit bases of behaviors in modern neuroscience driven by advances in electron microscopy (EM) connectomics, ability to perform high-throughput behavioral screens, characterization of stereotyped behavioral phenotypes, the ability to perform cell-type specific manipulations and genetic accessibility (for review see (Eschbach & Zlatić, 2020)). Another critical benefit of using *Drosophila* to study neural connectivity at the synaptic level is its relatively small, but complex CNS, where there are only ~10,000 neurons in the *Drosophila* larval CNS and ~100,000 neurons in adult *Drosophila melanogaster* brain compared to 86 billion neurons in the human brain (Herculano-Houzel, 2009; Scheffer et al., 2020). Highly detailed serial section transmission electron microscopy (ssTEM) whole brain volumes with synaptic level resolution have been obtained for both *Drosophila* larval (Ohshima et al., 2015) and adult (Zheng et al., 2018) brains, where neurons were reconstructed using a collaborative web-based software, CATMAID (Saalfeld et al., 2009; Schneider-Mizell et al., 2016). Concerted efforts from many laboratories have made tremendous advances in reconstructing the *Drosophila* larval connectome at synaptic level resolution and there has been great progress in mapping out select connectomes at the EM level (Clark et al., 2018; Eschbach & Zlatić, 2020; Kohsaka et al., 2017). Connectomes have been further validated using behavioral and functional imaging studies for olfaction in combination with learning and memory (Berck et al., 2016; Eichler et al., 2017; Eschbach et al., 2020; Saumweber et al., 2018), feeding (Miroshnikov et al., 2018; Schlegel et al., 2016), visual processing (Larderet et al., 2017), locomotion (Carreira-Rosario et al., 2018; Fushiki et al., 2016; Heckscher et al., 2015; Hiramoto et al., 2021; Kohsaka et al., 2019; Zarin et al., 2019; Zwart et al., 2016), chemotaxis (Tastekin et al., 2018), thermosensation (Hernandez-Nunez et al., 2021), mechanosensation (Jovanic et al., 2016; Jovanic et al., 2019; Masson et al., 2020), nociceptive modalities (Burgos et al., 2018; Gerhard et al., 2017; Hu et al., 2017; Imambocus et al., 2022; Kaneko et al., 2017; Ohshima et al., 2015; Takagi et al., 2017), among others (Andrade et al., 2019; Huckesfeld et al., 2021; Imura et al., 2020; Mark et al., 2021; Valdes-Aleman et al., 2021; Winding et al., 2022).

We are particularly interested in how larval somatosensation functions through peripheral sensory neurons located along the body wall just below the larval cuticle. Larval somatosensory neurons are comprised of type I, mono-ciliated dendrites, (external sensory (es) and chordotonal (Ch) neurons) and type II, bipolar dendritic (td and bd) and highly branched multidendritic (md) neurons (classes I-IV, referred to as CI-IV md). Functional and behavioral roles of these sensory neurons include proprioception (CI md & Ch) (Caldwell et al., 2003; He et al., 2018; Vaadia et al., 2018), heat thermoreception (CIV md) (Babcock et al., 2009; Im et al., 2015; Ohshima et al., 2015; Tracey et al., 2003), cold thermoreception (Ch, CII md, & CIII md) (Turner et al., 2016; Turner et al., 2018), chemoreception (CIV md) (Himmel et al., 2019; Lopez-Bellido et al., 2019) and mechanosensation (Ch, CII md, CIII md, & CIV md) (Hu et al., 2017; Hwang et al., 2007; Jovanic et al., 2019; Masson et al., 2020; Ohshima et al., 2015; Scholz et al., 2015). Recent studies have unraveled circuit bases underlying thermo-(heat), chemo-and

mechanosensation, however, circuit bases of noxious cold thermosensory evoked behaviors are yet to be described (Burgos et al., 2018; Hu et al., 2017; Hu et al., 2020; Imambocus et al., 2022; Jovanic et al., 2016; Jovanic et al., 2019; Hernandez-Nunez et al., 2021; Kaneko et al., 2017; Lopez-Bellido et al., 2019; Masson et al., 2020; Ohyama et al., 2015; Yoshino et al., 2017).

Neural circuitry downstream of select somatosensory neurons has been elucidated both physiologically and behaviorally in the context of nociceptive stimuli that evoke characteristic rolling behavior in *Drosophila* larvae. Specifically, Ch and CIV md neurons signal through multisensory integrator neurons (Basins) via ascending pathways to command neurons for mechanical and nociceptive stimulation that synergistically impact larval rolling escape responses (Ohyama et al., 2015). CIV md neuron mediated nociceptive escape behaviors also function through premotor neurons such as Down-and-Back (DnB) and medial clusters of CIV md interneurons (mCSI) (Burgos et al., 2018; Yoshino et al., 2017). Additionally, A08n projection neurons receive input from CIV md neuron and the peptidergic neuron DP-ilp7 to integrate multisensory inputs from CII, CIII and CIV md neurons (Imambocus et al., 2022). A08n and DP-ilp7 neurons are specifically required for nociceptive mechanical and chemical sensing but not for nociceptive thermosensation (Hu et al., 2017; Imambocus et al., 2022; Kaneko et al., 2017; Tenedini et al., 2019; Vogelstein et al., 2014). Lastly, Ch and CIII md neuronal connectivity, through ascending projection neurons A09e and TePns as well as premotor neuron Chair-1, are required for anemotaxis and innocuous mechanical sensing (Jovanic et al., 2019; Masson et al., 2020). While CIII md neuron connectivity to select second order neurons has been published (Masson et al., 2020), the functional and behavioral roles of these circuit components in the context of cold nociception remain unexplored. We hypothesized that the noxious cold sensitive somatosensory nociceptive circuit functions through shared circuitry amongst other somatosensory modalities.

Analyses of cold sensitive circuitry downstream of CIII md neurons elucidated dual pathways for signal transduction: (1) CIII md neurons directly signal through premotor neurons that modulate motor activity and (2) CIII md neurons also function via multisensory integrators and projections neurons to convey somatosensory information to higher order brain regions. Somatosensory neurons (Ch and CIII md) are required for cold-evoked responses but not CIV md neurons (Turner et al., 2016; Turner et al., 2018). Multisensory integration neurons, Basins 1-4, are required for cold nociception, facilitate CIII md evoked larval contraction (CT) responses, and select Basins exhibit cold evoked increases in calcium. Premotor neurons, DnB and mCSI, are required and facilitate cold-evoked behavioral responses, whereas Chair-1 premotor neurons are required for cold nociception but do not facilitate CIII md neuron mediated responses. Lastly, nociceptive projection neurons A09e and TePns also integrate cold stimuli but only A09e neurons enhance CIII md neuron mediated behavioral responses.

Results

Peripheral sensory neurons share synaptic partners

Recent work on competitive interactions and behavioral transitions in *Drosophila* larvae has reported CIII md and Ch connectomes in the context of larval mechanosensation (Masson et al., 2020). We performed a comparative circuit analysis of somatosensory (Ch, CIII md and CIV md) neurons using an online repository hosted by LMB Cambridge, which contains a ssTEM volume of *Drosophila melanogaster* first instar larval central nervous system and a repository of neural reconstructions (<https://neurophyla.mrc-lmb.cam.ac.uk/>). Here, we report and contextualize relevant CIII md neuron downstream neurons, whose functional roles in cold nociception were evaluated. The meta-analysis of previously published literature on *Drosophila* larval somatosensory connectomes revealed several common post-synaptic partners that are shared

amongst Ch, CIII md and CIV md neurons (**Figure 1A**). CIII md neurons are upstream of multisensory integration neurons, pre-motor neurons and projection neurons. Class III md neurons are synaptically connected to multisensory integration neurons (Basin-2, -3 & -4). The downstream pathway from Basins includes connectivity to A00c neurons and a polysynaptic pathway to the rolling command neuron (Goro) via A05q neurons. CIII md neurons are also connected to various pre-motor neurons, which are ventral nerve cord localized neurons providing synaptic input to motor neurons, including DnB, A02n and Chair-1, and projection neurons including A05q, A09e, TePn05 and A08n. The CIII md neuron connectome analyses reveal broad second order connectivity and complex interconnectivity amongst second order neurons (**Figure 1A**, **Figure 1-figure supplement 1**). Functional and behavioral roles of CIII md neuron second order interneurons in cold nociception remain unexplored and below we address this knowledge gap.

Functional analysis of somatosensory neurons in cold nociception

Due to shared post-synaptic neural connectivity of Ch, CIII md and CIV md neurons (**Figure 1A**, **Figure 1-figure supplement 1**), we first assessed functional roles of these sensory neurons in cold nociception by investigating: (1) stimulus-evoked calcium responses in the *Drosophila* larval ventral nerve cord; (2) cold-evoked calcium responses of these sensory neurons; (3) necessity and sufficiency of these sensory neurons in cold-evoked behaviors; and (4) examining whether co-activating multiple sensory neurons facilitates CIII-mediated behavioral response in *Drosophila* larvae.

1. CaMPARI analysis reveals distinct neuropil activation patterns by sensory stimuli

Drosophila larvae have distinct behavioral responses to various sensory stimuli including touch, heat or cold. Exposure to noxious cold temperatures ($\leq 10^{\circ}\text{C}$) evokes highly stereotyped head and tail withdrawal towards the center of the animal, termed here as contraction (CT) response (Patel et al., 2022; Turner et al., 2016). The cold-evoked CT response is defined as at least 10% reduction in larval surface area (**Figure 1-figure supplement 4**). Innocuous mechanical stimuli evokes a suite of behaviors including pausing, turning, head withdrawal, and/or reverse locomotion. Noxious heat exposure leads to a corkscrew body roll escape response (Babcock et al., 2011; Im et al., 2015; Ohyama et al., 2015; Tracey et al., 2003). Previous work on stimulus-evoked changes in neural ensembles in larval zebrafish (*Danio rerio*) used the genetically encoded calcium integrator CaMPARI to reveal distinct CNS neural activation patterns in response noxious heat or cold exposure (Fosque et al., 2015). CaMPARI affords great spatial resolution as stimulus-evoked neural responses are captured from a freely moving animal.

With CaMPARI and pan-neuronal imaging of ventral nerve cord neurons, we visualized central representations of somatosensory stimuli across sensory modalities including innocuous mechanical, noxious cold or noxious heat, which are primarily detected via Ch, CIII md, & CIV md neurons, respectively. We pan-neuronally expressed CaMPARI using *R57C10^{GAL4}* (Jenett et al., 2012; Pfeiffer et al., 2008) and *Drosophila* larvae were simultaneously exposed to diverse sensory stimuli and photoconverting light. Post-hoc imaging of intact *Drosophila* larvae ventral nerve cord revealed relatively little neural activity when larvae are not presented with any sensory stimulus (**Figure 1-figure supplement 2**). However, upon innocuous mechanical (gentle touch) stimulation, there is a marked increase in neural activation as reported by F_{redLUT} (**Figure 1-figure supplement 2**). *Drosophila* larvae exposed to noxious heat experience a large, spatially broad increase in neural activity. Incidentally, similar neuropil regions appear to be activated by both innocuous touch and noxious heat, albeit at different levels (**Figure 1-figure supplement 2**). Lastly, noxious cold exposure leads to robust neural activation medially, in the neuropil, however, cell bodies seem to have lower activation levels compared to touch or heat stimulations (**Figure 1-figure supplement 2**). These experiments measuring pan-neuronal

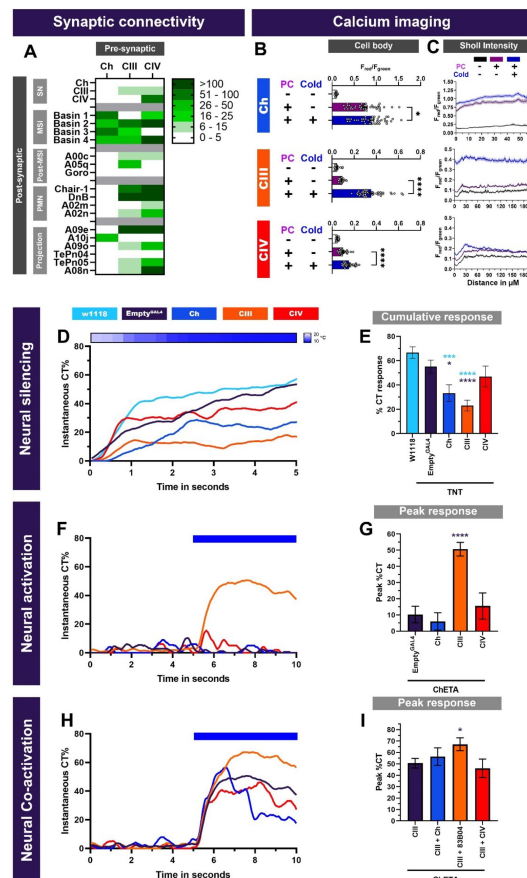


Figure 1

CIII md neurons are primary cold sensors and share common post-synaptic partners with mechanosensory and nociceptive Ch and CIV md neurons

(A) Sensory neuron second order connectome analyses. Heatmap plot of synaptic connections between sensory neuron subtypes including chordotonal (Ch), class III (CIII) md, class IV (CIV) md neurons and previously published centrally located neurons. Sensory neurons (SN) Ch, CIII md, and CIV md were analyzed. Multisensory integrator (MSI) neurons include Basin-1, -2, -3, and -4. Neurons downstream of MSI (Post-MSI) include A00c, A05q and Goro. Premotor neurons (PMNs) include Chair-1 (A10a), Down and Back (DnB), A02m and A02n. Lastly, sensory neurons are also connected to several projection neurons including A09e, A10j, A09o, TePn04 and TePn05. Synaptic connectivity data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-C) Calcium imaging of sensory neurons including chordotonal (*IAV^{GAL4}*), class III md (*19-12^{GAL4}*) and class IV md (*ppk^{GAL4}*) neurons using CaMPARI2. There were three conditions for sensory neurons: No photoconversion (PC) control (no stimulus and no photoconversion), photoconversion control (photoconversion and no stimulus) and stimulus (Stim) condition (photoconversion and 6°C stimulus). CaMPARI2 data are reported area normalized intensity ratios for F_{red}/F_{green} (mean $\pm$ SEM). Average N for each cell type and each condition is n=32. (B) CaMPARI2 response measured at the cell body for each neuron type. (C) Sholl intensity analysis performed using custom Fiji scripts. CaMPARI2 response is measured radially away from the center of the soma. (D-E) Cold-evoked responses of third instar *Drosophila* larva. Sensory neurons Ch, CIII md or CIV md neurons were silenced by inhibiting neurotransmitter release via cell type specific expression of tetanus toxin (TNT). (D) Instantaneous %CT over time. Heatmap on top represents change in temperature over time. (E) Cumulative %CT response for a duration of 5 seconds. Controls include *w1118* and *Empty^{GAL4}>TNT*. Significant stars: turquoise stars represent comparison to *w1118* and purple stars represent comparison to *Empty^{GAL4}>TNT*. Average n=72. (F-G) Neural activation of sensory neurons via cell type specific expression of ChETA relative to *Empty^{GAL4}>ChETA* control. *Empty^{GAL4}* n=35. Ch n=20, CIV n=20. & CIII n=143. (F) Instantaneous %CT over time. Blue bar represents optogenetic neural activation. (G) Peak %CT. (H-I) Neural co-activation of sensory neurons and CIII md neurons. Here each condition represents expression of *ChETA* in CIII md neurons and plus Ch (via *IAV-GAL4*), CIII (via *R83B04^{GAL4}*) or CIV md neurons (via *ppk^{GAL4}*). (H) Instantaneous %CT over time. Blue bar represents optogenetic neural activation. (I) Peak %CT response during optogenetic stimulation. CIII n=143 and average experimental n=50 Significant differences indicated via asterisks, where *p<0.05, ***p<0.001, and ****p<0.0001.

ventral nerve cord activity in response to sensory stimuli would be ideal for comparative neural activation analyses, were the spatial resolution of the optical microscope sufficient. Acknowledging the limitations of the approach, we heretofore focus instead on single-cell type specific genetic driver lines for identified neurons that can be assigned to known neurons in the connectome,

2. CIII md somatosensory neurons exhibit robust Ca^{2+} responses to cold

Somatosensory neurons function as primary sensors of external stimuli. Ch and CIII md neurons have previously been reported to present cold evoked increases in Ca^{2+} (Turner et al., 2016 [↗](#); Turner et al., 2018 [↗](#)). Previous reports indicate that CIV md neurons are weakly sensitive to cold stimuli (Turner et al., 2016 [↗](#)). Cold sensitivity of sensory neurons (Ch, CIII md, & CIV md) was assessed by selectively expressing the CaMPARI2 Ca^{2+} integrator in sensory neurons using cell-type specific driver lines (**Figure 1-figure supplement 3A** [↗](#)). CaMPARI2 signal, as assessed by $F_{\text{red}}/F_{\text{green}}$ ratios at the cell body, reveal all three neuron subtypes (Ch, CIII md and CIV md) have significantly higher response upon cold exposure compared to no stimulus controls (**Figure 1B** [↗](#), **Figure 1-figure supplement 3B** [↗](#)). Unsurprisingly, as high-threshold nociceptors, CIII md or CIV md neurons present relatively low responses upon photoconverting light exposure *sans* cold (**Figure 1B** [↗](#), **Figure 1-figure supplement 3B** [↗](#)). However, mechanosensitive Ch neurons exhibit relatively high responses upon exposure to only photoconverting light but no stimulus indicative of high baseline neuronal activity processing mechanosensory information (**Figure 1B** [↗](#), **Figure 1-figure supplement 3B** [↗](#)).

Similar to the analysis at cell bodies, in the dendrites of Ch neurons we observe marked increases in CaMPARI2 response to photoconverting light (control), however, upon cold exposure (experimental condition) there is a further increase in CaMPARI2 response (**Figure 1C** [↗](#)). Sholl intensity analysis (see methods) likewise reveals that CIII md neurons present relatively low CaMPARI2 response in control conditions, however, there is a robust cold evoked increase in CaMPARI2 response throughout the dendrites (**Figure 1C** [↗](#)). Interestingly, CIV md neurons also exhibited significant increases in CaMPARI2 response upon cold exposure in the cell body (**Figure 1B** [↗](#)). we measure a higher cold-evoked CIV md neuron CaMPARI2 response closer to the soma (**Figure 1C** [↗](#)). However, there is no change in distal dendritic CIV md neuron CaMPARI2 response between cold and no stimulus conditions (**Figure 1C** [↗](#)). CIII md have robust cold evoked Ca^{2+} responses compared to Ch and CIV md neurons, which have relatively low cold evoked Ca^{2+} increases (**Figure 1B,C** [↗](#)). Therefore, we identified CIII md neurons as the most sensitive to noxious cold temperatures, and thus we focused our study on the neural circuits postsynaptic to CIII md neurons.

3. CIII md somatosensory neurons are necessary and sufficient for the cold response

The analysis of neuronal activity responses to cold exposure revealed distinct patterns for both central and somatosensory neurons, with most robust responses to stimuli evoked in CIII md neurons.

We assessed the necessity of somatosensory neurons in noxious cold-evoked behavioral responses (**Figure 1-figure supplement 4** [↗](#)) by expressing tetanus toxin light chain, which inhibits neurotransmitter release (Sweeney et al., 1995 [↗](#)). Based on Ca^{2+} imaging, we expected both Ch and CIII md neurons may be necessary for cold evoked CT responses. Neural silencing of Ch and CIII md neurons led to significant reductions in cold evoked CT responses compared to either *w1118* (parental line) or *Empty^{GAL4}* controls (a GAL4 construct lacking the promotor sequence; **Figure 1D, E** [↗](#)). Instantaneous behavioral response curves also indicate lower cold sensitivity when either Ch or CIII md neurons are silenced. However, inhibiting neurotransmitter release in

CIV md neurons alone did not result in significant reductions in cold evoked CT responses (**Figure 1D, E**). Among the three somatosensory neuron types tested, neural silencing of CIII md neurons resulted in the strongest impairment in cold evoked behavioral response.

Next, we evaluated whether neural activation of sensory neurons via optogenetics would be sufficient to elicit the CT behavioral response (**Figure 1-figure supplement 5A**). We expressed the engineered channelrhodopsin ChETA in individual sensory neuron subtypes and assessed evoked behavioral responses (Gunaydin et al., 2010). When assessing evoked responses, we first analyzed CT responses as measured by changes in surface area (**Figure 1-figure supplement 5B**). Only neural activation of CIII md neurons led to CT responses in *Drosophila* larvae (**Figure 1F, G**) consistent with our previously published work (Turner et al., 2016). Additionally, we analyzed larval mobility, which refers to changes in larval postures as measured by changes in occupied space (**Figure 1-figure supplement 5C**). Upon neural activation of CIII md neurons, there is a large increase in *Drosophila* larval immobility compared to controls (**Figure 1-figure supplement 6A-C**). However, there was no difference in immobility when either Ch or CIV md neurons were optogenetically activated compared to control (**Figure 1-figure supplement 6A-C**). Of the somatosensory neurons tested, only CIII md neurons are sufficient to elicit the CT response.

4. Effect of co-activating CIII md somatosensory neurons plus additional somatosensory neuron classes

Both Ch and CIV md neurons share common first order post-synaptic partners with CIII md neurons and all three somatosensory present cold-evoked increases in calcium levels. However, neither Ch nor CIV are sufficient to elicit a CT response. To further clarify the roles of Ch and CIV in cold evoked behaviors, we simultaneously activated CIII md neurons plus either Ch or CIV md neurons. We expected that co-activation of multiple sensory neuron subtypes would facilitate optogenetically-evoked CT responses. Optogenetic activation of CIII md neurons using two cell type specific driver lines (*19-12^{GAL4}* and *R83B04^{GAL4}*) led to sustained increases in instantaneous CT responses compared to control, where only one driver line (*19-12^{GAL4}*) was used to activate CIII md neurons (**Figure 1H, I**). The activation of CIII md neurons using two driver lines (expressed in the same cell type, CIII md) led to a significant increase in immobility compared to single *GAL4* driver-mediated activation of these neurons (**Figure 1-figure supplement 6D-F**), suggesting a single *GAL4* line does not exhaust the dynamic range of the CT response. Co-activation of CIII md and Ch neurons, which are cold sensitive and required for cold evoked CT responses, led to a subtle initial increase in instantaneous CT response compared to CIII activation alone, however, the initial increase in evoked CT response was quickly reduced to well below control (**Figure 1H, I**). Therefore the co-activation of two somatosensory neuron types, Ch and CIII md, elicited a CT response that varied along the temporal axis relative to the activation of CIII md alone. *Drosophila* larval immobility was reduced during co-activation of Ch and CIII md neurons compared to CIII md neuron activation alone suggesting that Ch neurons do not facilitate CIII md neuron mediated CT responses (**Figure 1-figure supplement 6D-F**). CIV md and CIII md neurons share a large proportion of common second order interneuron connectivity including multisensory integration neurons, premotor and ascending neurons. We predicted that CIV md and CIII md neuron co-activation might potentiate CT responses. Interestingly, simultaneous activation of CIII and CIV md neurons led to small but insignificant reductions in instantaneous and peak CT responses compared to CIII md neuron activation alone (**Figure 1H, I**). Similarly, co-activation of CIII md and CIV md neurons did not alter larval immobility compared to only CIII md neuron activation (**Figure 1-figure supplement 6D-F**). Ch neurons are cold sensitive and required for cold nociceptive behaviors but do not facilitate CIII md neuron evoked behavioral responses. Meanwhile, CIV md neurons are modestly cold sensitive but are not required for cold nociception and do not facilitate CIII md neuron evoked CT responses. Collectively, CIII md neurons have the strongest cold-evoked calcium response, are required for cold-evoked behavioral response and sufficient for the CT response.

Multisensory integrators are required and facilitate cold nociception

Basin interneurons function as multisensory integrators receiving convergent inputs from mechano-, chemo- and thermo-sensitive peripheral sensory neurons (**Figure 2A**, **Figure 2-figure supplement 1A**). Previous behavioral and functional studies have revealed that Basin interneurons are required for nociceptive escape responses mediated by Ch and CIV md neurons (Ohyama et al., 2015). Both Ch and CIV md neurons play roles in CIII md mediated behavioral responses either in cold nociception and/or can detect cold stimuli. We hypothesized that Basin interneuron function is required downstream of cold nociceptive somatosensory neurons. We evaluated whether Basin interneurons are necessary and sufficient for cold nociception, exhibit cold evoked increases in calcium response and function downstream of CIII md neurons.

Silencing Basin interneurons reduces cold-evoked CT

Basin interneurons receive somatosensory cues from CIII md neurons, thus we predicted that inhibiting neurotransmitter release from Basin neurons will result in impaired cold evoked behaviors (**Figure 2A**). We assessed the requirement of all Basin neurons using “pan-” Basin driver lines and also assessed roles of individual Basin neurons using subtype specific driver lines. Neural silencing of all Basin (1-4) neurons, using two independent driver lines (*R72F11^{GAL4}* and *R57F07^{GAL4}*), led to significant reductions in cold-evoked CT responses compared to genetic background (*w1118*) and *Empty^{GAL4}* controls (**Figure 2B**). *Drosophila* larvae with impaired Basin (1-4) neuronal function had significant reductions in CT duration, magnitude and cumulative %CT response compared to controls (**Figure 2C-E**). CIII neurons have differential connectivity to individual Basin subtypes (**Figure 2A**). Therefore, we assessed requirement of Basin-1, -2 or -4 neurons, for which there are previously validated independent driver lines (Jovanic et al., 2016; Ohyama et al., 2015). Tetanus toxin mediated neural silencing of Basin-2 or -4 led to significant reductions in cold evoked CT response, as measured by cumulative %CT response, CT duration and CT magnitude, compared to controls (**Figure 2B-E**). CIII md neurons do not synapse onto Basin-1 neurons according to the EM-mapped connectome (**Figure 2A**) (Masson et al., 2020), however, Basin-1 & -2 neurons share synaptic connectivity to both feedback and feedforward GABAergic interneurons, both of which are downstream of sensory neurons (Jovanic et al., 2016). Furthermore, Basin-1 neural activation leads to depolarizations in Basin-2 through GABAergic disinhibitory pathway (Jovanic et al., 2016). Therefore, we expected that inhibiting neurotransmitter release in Basin-1 neurons would result in reduced cold-evoked responses. Neural silencing of Basin-1 neurons resulted in modest, but significant reductions in cold evoked responses (**Figure 2B-E**). Impaired Basin neuron signaling results in at least 25% reduction in cold evoked responses with the strongest reductions for all-Basin, Basin-2 or Basin-4 driver lines (**Figure 2J**).

Co-activation of Basin interneurons and CIII md somatosensory neurons enhances CT

Next, we evaluated whether neural activation of Basin neurons would impair or elicit a CT response. Optogenetic activation of Basin neurons, either using all Basin or individual Basin driver lines, did not elicit a CT response (**Figure 2-figure supplement 2**). But the simultaneous co-activation of CIII md and Basin neurons led to sustained increases in CT responses compared to controls, where only CIII md neurons were activated, across multiple behavioral metrics (**Figure 2F-I**). Coactivation did not result in a change in larval immobility (**Figure 2-figure supplement 1B-D**), whereas activation of all Basins led to significantly greater immobility (**Figure 2-figure supplement 1B-D**). These results indicate that Basin neurons are not sufficient for the CT response, but that the combined activation of CIII md and Basins not only suffices but also elicits an even stronger CT response than activating CIII md neurons alone.

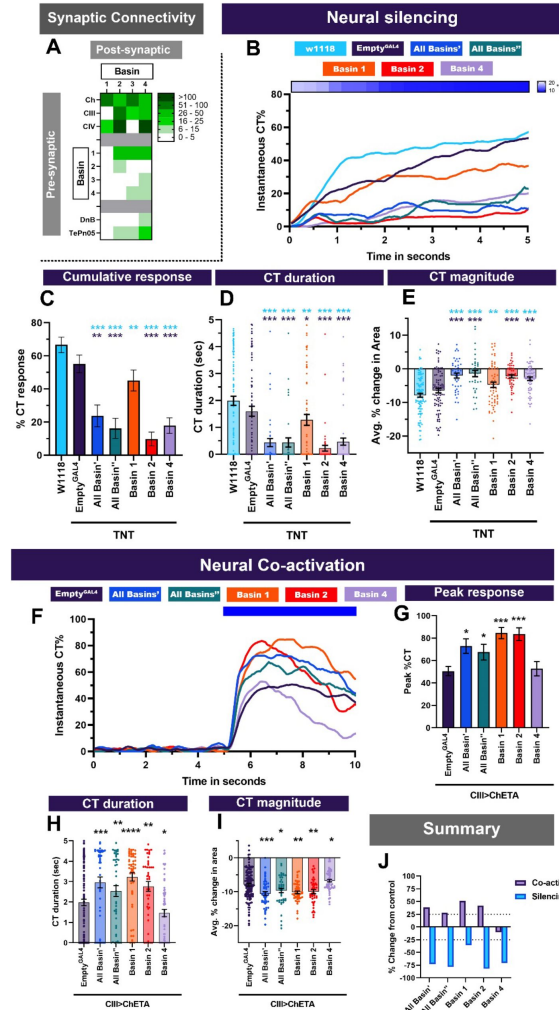


Figure 2

Multisensory integrator second order neurons are required for cold-evoked behaviors and facilitate CIII-evoked behaviors.

(A) Basins (1-4) receive inputs from sensory neurons (Ch, CIII md and CIV md), Basins, premotor neuron Down and Back (DnB) and projection neuron TePn05. Heatmap plot of pre-synaptic connections to Basins. Synaptic connectivity data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-E) Cold-evoked responses of third instar *Drosophila*. Basin (1-4) neurons were silenced by inhibiting neurotransmitter release via cell type specific expression of tetanus toxin (TNT), where All Basin' (*R72F11^{GAL4}*), All Basin'' (*R57F07^{GAL4}*), Basin-1 (*R20B01^{GAL4}*), Basin-2 (*SS00739^{splitGAL4}*) and Basin-4 (*SS00740^{splitGAL4}*). (B) Instantaneous %CT over time. Heatmap on top represents change in temperature over time. (C) Cumulative %CT response for a duration of 5 seconds. (D) CT duration in seconds. (E) CT magnitude as average percent change in area for the duration of stimulus. Controls include *w1118* and *Empty^{GAL4}>TNT*. Significant stars: turquoise stars represent comparison to *w1118* and purple stars represent comparison to *Empty^{GAL4}>TNT*. Average *n* = 64. (F-I) Neural co-activation of Basin neurons and CIII md neurons. Here each condition represents expression of ChETA in CIII md neurons and plus Basin (1-4) neurons. (F) Instantaneous %CT over time. Blue bar represents optogenetic neural activation. (G) Peak %CT response during optogenetic stimulation. (H) CT duration in seconds during optogenetic stimulation. (I) CT magnitude as average percent change in area for the duration of stimulus. Significant stars: purple stars represent comparison to CIII md + *Empty^{GAL4}>ChETA*. *Empty^{GAL4}* *n*=143 and experimental condition average *n*=49. (J) Overall percent change from control for either neural silencing or neural co-activation. The metrics for neural silencing include cumulative %CT, CT magnitude, and CT duration. The following metrics were used to calculate percent for neural co-activation: cumulative %CT, peak %CT, CT duration and magnitude. Significant differences indicated via asterisks, where **p*<0.05, ***p*<0.01, ****p*<0.001, and *****p*<0.0001.

To parse the contribution of individual Basin neuronal subtypes, we next assessed the role of Basin-1, -2 or -4, and all four Basin together, in either facilitating or suppressing CT responses using our co-activation paradigm (**Figure 2F-I**). Co-activation of Basin-1 or Basin-2 with CIII md neurons led to an enhanced CT response compared to controls across all measures of behavioral response including instantaneous %CT response, peak %CT response, CT duration and CT magnitude (**Figure 2F-I**), with a subsequent significant increase in larval immobility (**Figure 2-figure supplement 1B-D**). Basin-2 plus CIII md neuron co-activation led to strong facilitation of CT responses, but surprisingly resulted in significantly reduced immobility (**Figure 2-figure supplement 1B-D**). Unlike for all other Basin neurons tested, Basin-4 and CIII md neuron co-activation led to a suppression of CT response, where peak instantaneous %CT response was similar to controls, however, there was a rapid reduction in instantaneous %CT response compared to controls (**Figure 2F-I**). Both CT duration and CT magnitude were significantly impaired for Basin-4 plus CIII md neuron co-activation compared to controls (**Figure 2H, I**), which also showed significantly lower immobility (**Figure 2-figure supplement 1B-D**). Interestingly, dual activation of CIII md and all Basin neurons led to weaker CT enhancement compared to co-activation of either Basin-1 or -2 coupled with CIII md neurons (**Figure 2J**), consistent with the finding that co-activation with Basin-4 reduced the CT response. Collectively, second-order Basin neurons are required for cold evoked responses and specifically Basin-1 and Basin-2 are able to enhance CIII md neuron evoked behavioral responses.

CaMPARI reveals Basin-2- and Basin-4 are activated in CT responses

To further explore how Basin interneurons function in cold nociception, we sought to investigate cold-evoked Ca^{2+} responses of Basin neurons. Since somatosensory neurons are cholinergic (Salvaterra & Kitamoto, 2001), we expected that Basin neurons postsynaptic to CIII md neurons will exhibit cold-evoked increases in Ca^{2+} . Post-hoc imaging of evoked CaMPARI2 fluorescence revealed that Basin neurons have significantly higher $F_{\text{red}}/F_{\text{green}}$ ratios compared to their respective controls, as assessed by two independent all-Basin driver lines (**Figure 3A, B**). We further investigated Ca^{2+} responses in greater detail using individual driver lines for Basin-1, -2 or -4. Basin-1 neurons are weakly required for cold-evoked CT responses (**Figure 2J**), and coherently do not exhibit cold-evoked increases in Ca^{2+} response (**Figure 3C**). In contrast, Basin-2 and -4 neurons both exhibit significant increases in Ca^{2+} responses compared to their respective controls (**Figure 3D-E**). Collectively, Basin-2 and -4 neuron subtypes that are required for cold-evoked CT responses (**Figure 2J**), also exhibit cold-evoked increases in Ca^{2+} .

CIII md neurons and Basin-2 and -4 neurons are functionally connected

Basin-2 and -4 are postsynaptic to CIII md neurons, are required for cold nociception and have cold-evoked increases in Ca^{2+} . Next, we assessed whether CIII md neurons and Basin-2 or -4 neurons are functionally connected. From the EM-reconstructed connectome we predicted that the activation of CIII md neuron will result in increased Ca^{2+} levels of Basin-2 or -4. As expected, optogenetic activation of CIII md neurons led to significant increases in Ca^{2+} levels in Basin-2 neurons, and repeated stimulation of CIII md neurons did not lead to sensitization of Basin-2 Ca^{2+} responses (**Figure 3F, G**). Previously, it was shown that Ch and CIII md neurons could elicit Ca^{2+} response in Basin-4 neurons (Kaneko et al., 2017), however, in this previous study the authors were unable to determine which of the two sensory neuron cell types led to increases in the Basin-4 Ca^{2+} response (Kaneko et al., 2017). Here, we show that upon specifically activating CIII md neurons, Basin-4 neurons have large, rapid increases in cytosolic Ca^{2+} followed by quick return to baseline levels. In contrast to Basin-2, Basin-4 neurons showed reduced Ca^{2+} responses upon repetitive activations of CIII md neurons (**Figure 3H, I**). Therefore, CIII md neuron activation is differentially encoded by Basin-2 and -4 neurons, where Basin-4 neurons have much larger CIII md neuron evoked increase in Ca^{2+} levels but exhibit sensitization compared to Basin-2 neurons, which do not show any sensitization upon repetitive stimulations. Collectively, our data demonstrate that Basin neurons are required for cold-evoked behaviors and Basin-2 and -4 neurons functionally operate downstream of CIII md neurons (**Figure 3-figure supplement 1**).

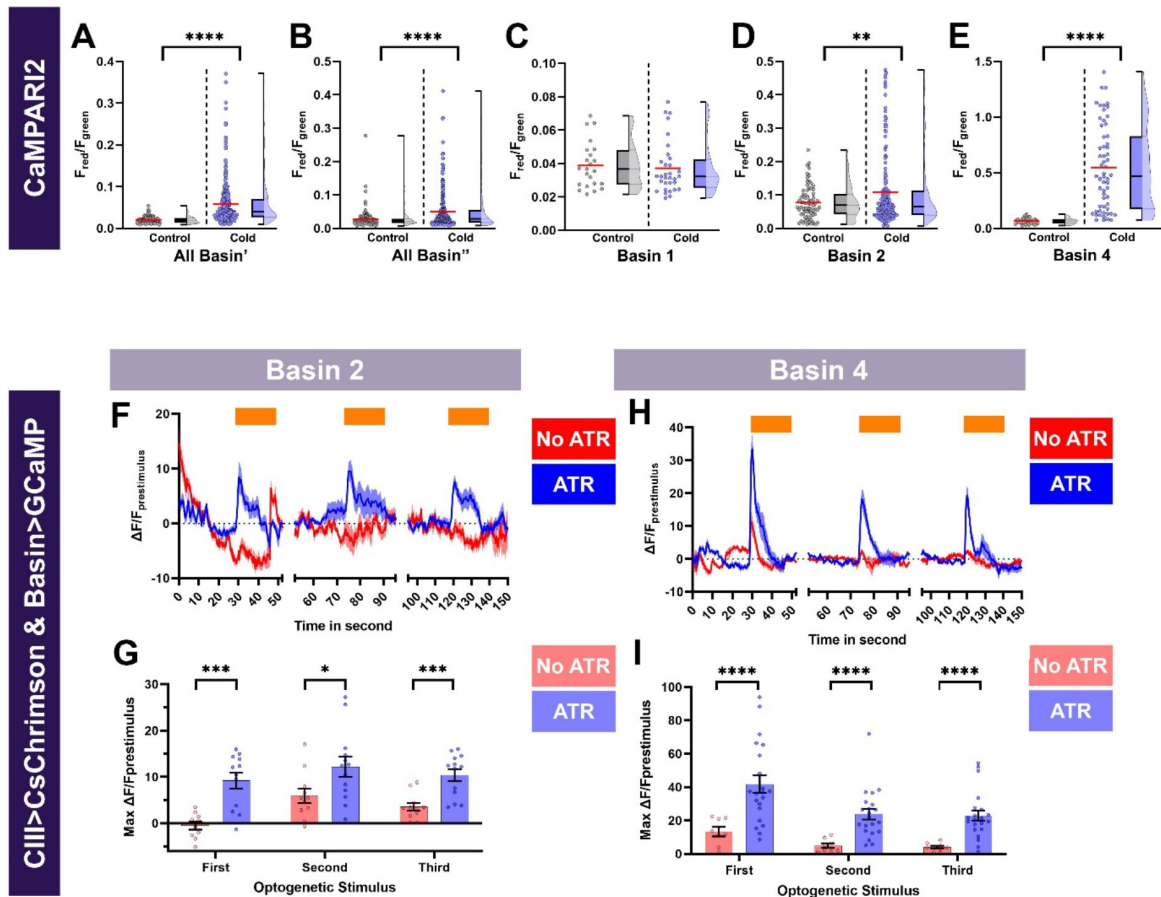


Figure 3

Cold- and CIII-evoked calcium responses of Basin neurons.

(A-E) Ca^{2+} responses of Basin neurons upon cold exposure vs. controls (room temperature). Neural responses (CaMPARI2) of Basin neuron cell bodies were analyzed using the following cell type driver lines (A) All Basin' ($R72F11^{GAL4}$) average $n=197$, (B) All Basin'' ($R57F07^{GAL4}$) average $n=182$, (C) Basin-1 ($R20B01^{GAL4}$) average $n=27$, (D) Basin-2 ($SS00739^{splitGAL4}$) average $n=119$ and (E) Basin-4 ($SS00740^{splitGAL4}$) average $n=46$. CaMPARI2 fluorescence ratio is reported as F_{red}/F_{green} . We report the data as individual datapoints, where the red line represents mean, and hybrid plots (boxplot & violin) for visualizing the distribution and quartiles of data. Significant stars represent $p<0.05$, where comparisons were made to their respective no stimulus controls. (F-I) To functionally assess CIII md neuron to Basin-2 or Basin-4 connectivity, we optogenetically activated CIII md neurons ($83B04^{lexA}>CsChrimson$) and visualized changes in evoked Ca^{2+} using $Basin-2^{splitGAL4}$ or $Basin-4^{splitGAL4}>GCaMP6m$. Control: No all *trans*-retinal (ATR) supplemented diet, which is required for optogenetic stimulation in *Drosophila*. Orange bars indicate optogenetic stimulation. (F,H) Basin-2 and Basin-4 changes in GCaMP reported as $\Delta F/F_{prestimulus}$, where prestimulus refers to 15 seconds prior to optogenetic stimulation. (G,I) Maximum Basin-2 and Basin-4 neuronal responses ($\Delta F/F_{prestimulus}$) upon optogenetic stimulation. Average n for each genotype was 13. Comparisons made to relevant controls and significant differences indicated via asterisks, where * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

Multisensory integrators function independently of Goro pathway for cold nociception

Basin neurons innervate a set of projection neurons (A05q and A00c), which are upstream of a command neuron (Goro) that is responsible for initiating CIV md neuron-mediated nociceptive escape behaviors (**Figure 4A**, **Figure 4-figure supplement 1A**) (Ohyama et al., 2015). We set out to test whether cold-evoked behavioral responses mediated by CIII md and Basin neurons function through A00c, A05q, and/or Goro neurons. We first assessed whether these neurons are required for cold-evoked behavioral responses (**Figure 4B-E**). Neural silencing of A05q neurons via tetanus toxin led to mild, yet significant reductions in cold evoked responses, when compared to *w1118* genetic control (**Figure 4B-E**). Neural silencing of A00c neurons resulted in significantly lower cold-evoked cumulative CT response compared to *w1118* (**Figure 4B, C**). There were also significant reductions in CT duration and CT magnitude when A00c neurons were silenced compared to both controls (**Figure 4D, E**). In contrast, the Goro command neuron for nociceptive rolling behavior is not required for cold-evoked CT responses (**Figure 4B-E**). Next, we assessed whether neural activation of these neurons led to evoked CT responses. Like Basin neurons, single cell-type activation of A00c, A05q, or Goro neurons did not lead to CT behavior (**Figure 2-figure supplement 2**). Similarly, optogenetic co-activation of CIII md neurons and A05q or Goro neurons did not lead to significant facilitation of the CT response (**Figure 4F-I**). However, simultaneously co-activating A00c and CIII md neurons led to significant increases in CT duration and CT magnitude (**Figure 4H, I**). There were no changes in *Drosophila* larval immobility upon co-activation of CIII md plus A00c, A05q or Goro neurons (**Figure 4-figure supplement 1B-D**). Only co-activation of A00c neurons leads to notable enhancement of CIII md mediated CT response, whereas neural silencing of A00c or A05q neurons led to greater than 25% impairment in cold evoked behavioral responses (**Figure 4J**). In conclusion, the Basin to Goro polysynaptic pathway does not significantly contribute to the cold-evoked CT response.

Premotor neurons function downstream of CIII md neurons to mediate cold nociceptive responses

Select *Drosophila* larval premotor neurons were previously implicated in CIV-mediated nociceptive escape responses. Specifically, DnB premotor neurons are involved in noxious thermal stimulus-evoked c-bending and rolling behavior (Burgos et al., 2018; Lopez-Bellido et al., 2019). *Drosophila* larvae also roll in response to activation of mCSI premotor neurons, which are synaptically connected to CIV md neurons and predicted to be A02m/n neurons from EM connectomes (Lopez-Bellido et al., 2019; Yoshino et al., 2017). Additionally, Chair-1 (A10a) premotor neurons have been implicated in anemotaxis (Jovanic et al., 2019). Collectively, these premotor neurons primarily receive inputs from both primary sensory neurons (CIII & CIV md) and multisensory integrators (Basin-2 & -4) (**Figure 5A**, **Figure 5-figure supplement 1A**). We predicted that premotor neurons are required for cold-evoked responses and function downstream of CIII md neurons in a stimulus-specific manner.

Inhibition of neural transmission via cell type specific expression of tetanus toxin in individual premotor neurons led to reduced cold evoked responses in *Drosophila* larvae (**Figure 5B-E**). Silencing Chair-1 neurons resulted in the strongest reduction of cold-evoked CT responses, where instantaneous %CT was the lowest of all premotor neuron subtypes tested (**Figure 5B**). CT duration, magnitude and cumulative percent response were all significantly reduced compared to controls (**Figure 5B-E**). Impairment in Chair-1 neuronal signaling leads to 75% reduction from controls in cold-evoked behaviors (**Figure 5J**). We silenced DnB neurons using two independent cell-type specific driver lines (DnB' (*IT4051^{GAL4}*) & DnB'' (*IT412^{GAL4}*)), where both resulted in reduced instantaneous %CT response, along with significant reductions in CT duration and magnitude compared to controls (**Figure 5B-E**). Silencing mCSI (*R94B10^{GAL4}*) neurons also resulted in significantly reduced cold-evoked CT responses compared to controls (**Figure 5B-E**).

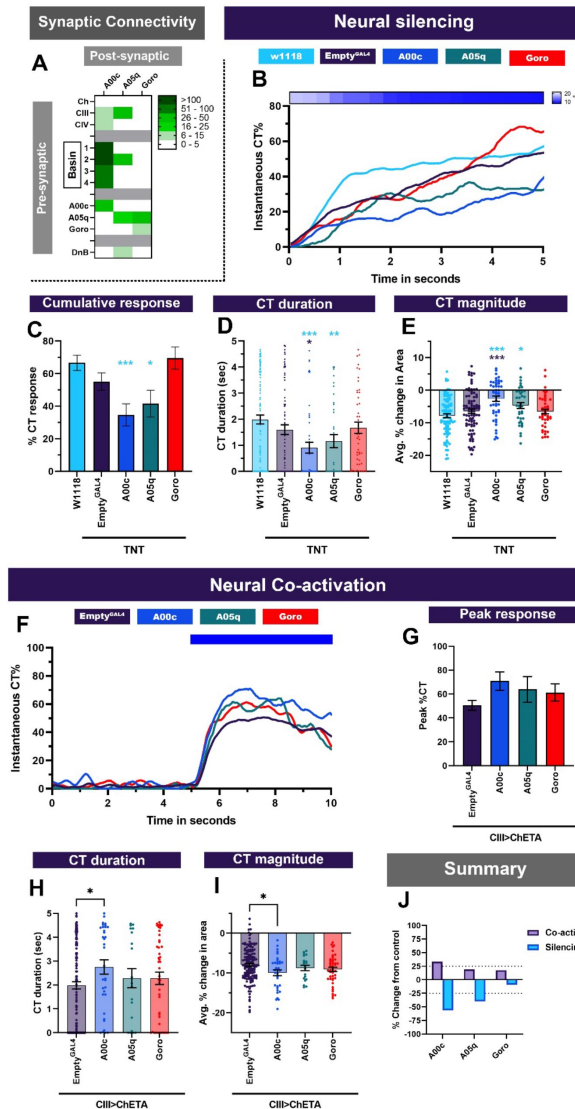


Figure 4

Projection neurons downstream of Basins and sensory neurons function in cold evoked responses.

(A) A00c and A05q primarily receive inputs from Basins and premotor neuron Down and Back (DnB). Goro neurons primarily receive inputs from A05q neurons. Heatmap plot of pre-synaptic connections to downstream neurons. Synaptic connectivity data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-E) Cold-evoked responses of third instar *Drosophila*. We used cell-type specific driver lines for downstream neurons to drive expression of tetanus toxin (TNT): A00c (*R71A10^{GAL4}*), A05q (*R47D0^{GAL4}*) and Goro (*R69F06^{GAL4}*). (B) Instantaneous %CT over time. Heatmap on top represents change in temperature over time. (C) Cumulative %CT response for a duration of 5 seconds. (D) CT duration in seconds. (E) CT magnitude as average percent change in area for the duration of stimulus. Controls include *w1118* and *Empty^{GAL4}>TNT*. For each genotype average n=64. Significant stars: turquoise stars represent comparison to *w1118* and purple stars represent comparison to *Empty^{GAL4}>TNT*. (F-I) Neural co-activation of downstream neurons and CIII md neurons. Here each condition represents expression of ChETA in CIII md neurons plus A00c, A05q or Goro neurons. (F) Instantaneous %CT over time. Blue bar represents optogenetic neural activation. (G) Peak %CT response during optogenetic stimulation. (H) CT duration in seconds during optogenetic stimulation. (I) CT magnitude as average percent change in area for the duration of stimulus. Significant purple stars represent comparison to *CIII md + Empty^{GAL4}>ChETA*. *Empty^{GAL4}* n=143 and experimental condition average n=33. (J) Overall percent change from control for either neural silencing or neural co-activation. The metrics for neural silencing include cumulative %CT, CT magnitude, and CT duration. The following metrics were used to calculate percent for neural co-activation: cumulative %CT, peak %CT, CT duration and magnitude. Significant differences indicated via asterisks, where *p<0.05, **p<0.01, and ***p<0.001.

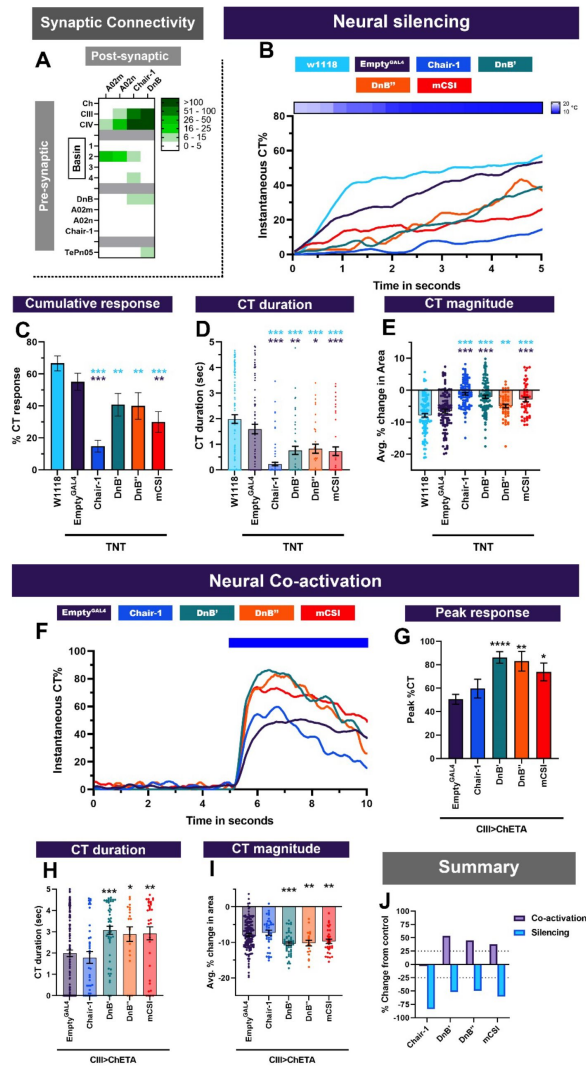


Figure 5

Premotor neurons downstream of sensory neurons and Basin neurons are required for cold-evoked responses.

(A) Chair-1 (A10a), A02m/n (predicted to be mCSI neurons) and Down and Back (DnB, A09l) primarily receive inputs from Basins, CIII md and CIV md neurons. Heatmap plot of pre-synaptic connections to premotor neurons. Synaptic connectivity data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-E) Cold-evoked responses of third instar *Drosophila*. Premotor neurons were silenced by inhibiting neurotransmitter release via cell type specific expression of tetanus toxin (TNT), where Chair-1 (*SS0091^{split}GAL4*), DnB' (*IT4015^{GAL4}*), DnB'' (*IT412^{GAL4}*) and mCSI (*R94B10^{GAL4}*). (B) Instantaneous %CT over time. Heatmap on top represents change in temperature over time. (C) Cumulative %CT response for a duration of 5 seconds. (D) CT duration in seconds. (E) CT magnitude as average percent change in area for the duration of stimulus. Controls include *w1118* and *Empty^{GAL4}>TNT*. Average *n* = 69. Significant stars: turquoise stars represent comparison to *w1118* and purple stars represent comparison to *Empty^{GAL4}>TNT*. (F-I) Neural co-activation of premotor neurons and CIII md neurons. Here each condition represents expression of ChETA in CIII md neurons plus premotor neurons. (F) Instantaneous %CT over time. Blue bar represents optogenetic neural activation. (G) Peak %CT response during optogenetic stimulation. (H) CT duration in seconds during optogenetic stimulation. (I) CT magnitude as average percent change in area for the duration of stimulus. *Empty^{GAL4}* *n*=143 and experimental condition average *n*=35. Significant stars represent *p*<0.05, where purple stars represent comparison to *CIII md + Empty^{GAL4}>ChETA*. (J) Overall percent change from control for either neural silencing or neural co-activation. The metrics for neural silencing include cumulative %CT, CT magnitude, and CT duration. The following metrics were used to calculate percent for neural co-activation: cumulative %CT, peak %CT, CT duration and magnitude. Significant differences indicated via asterisks, where **p*<0.05, ***p*<0.01, ****p*<0.001, and *****p*<0.0001.

DnB or mCSI inhibition of neurotransmitter release leads to approximately 50% reduction in cold-evoked CT responses (**Figure 5J**). Collectively, these premotor neurons are required for cold-evoked behavioral responses.

Based on EM connectivity and neural silencing experiments, we predicted that activation of these premotor neurons would be sufficient for *Drosophila* larval CT response. First, we found that activation of premotor neurons alone did not evoke CT response (**Figure 2-figure supplement 2**). Next, we performed optogenetic co-activation of CIII md and premotor neuron subtypes. Chair-1 neuron co-activation with CIII md neuron did not have an effect on CT responses (**Figure 5F-I**), however, there was a reduction in larval immobility, where average mobility was increased and duration of immobility was reduced compared to when only CIII md neurons are activated (**Figure 5-figure supplement 1B-D**). Simultaneous activation of CIII md neurons and DnB or mCSI both led to significant increases in CT responses, as measured by peak CT response, CT duration or magnitude, compared to CIII md neuron activation alone (**Figure 5F-I**). DnB and CIII md neuron co-activation led to short lived, subtle but insignificant increases in larval immobility (**Figure 5-figure supplement 1B-D**). Co-activation of mCSI and CIII md neurons resulted in mild reductions in larval immobility (**Figure 5-figure supplement 1B-D**). These data reveal DnB and mCSI neuronal activity enhances CIII md neuron mediated CT responses.

Since these premotor neurons are postsynaptic to CIII md neurons and their activity is required for proper cold evoked behaviors, we predicted that activation of CIII md neurons would elicit cold-evoked increases in Ca^{2+} . To test this prediction, we selectively expressed the Ca^{2+} integrator CaMPARI2 in premotor neurons. Unexpectedly, there was no change in cold evoked Ca^{2+} levels in mCSI or Chair-1 neurons (**Figure 6C-D**). Noxious cold exposure did however lead to significant Ca^{2+} increases in DnB neurons (**Figure 6A-B**). To further assess how CIII md neuronal activity affects DnB function, we optogenetically activated CIII md neurons and assessed evoked Ca^{2+} levels of DnB using GCaMP6. Interestingly, *Drosophila* larvae raised without all *trans*-retinal, a requisite light sensitive cofactor for optogenetic experiments, also had a mild light evoked increase in Ca^{2+} (**Figure 6E, F**). CIII md neuron optogenetic activation led to significant increases in DnB Ca^{2+} levels that slowly returned to baseline levels (**Figure 6E, F**). Upon repeated CIII md neuron activations, DnB neurons exhibit a blunted Ca^{2+} response relative to initial stimulation (**Figure 6E, F**). Taken together we find that premotor neuronal function is required for cold nociception and premotor neuron activity can facilitate CIII md neuron mediated CT responses (**Figure 6-figure supplement 1**).

Ascending interneurons are required for cold nociceptive responses

Sensory, second order multisensory integration neurons (Basins) and a premotor neuron (DnB) have further direct synaptic connectivity to a set of projection neurons including A09e, A08n, and *R61A01^{GAL4}* labeled neurons (labels: A10j, A09o, TePn04, TePn05) that have previously been implicated in anemotaxis, mechanosensory or chemosensory evoked behavioral responses (Hu et al., 2017; Jovanic et al., 2019; Kaneko et al., 2017; Masson et al., 2020; Vogelstein et al., 2014). Briefly, A09e receives synaptic inputs from CIII md, CIV md, A08n, DnB, and TePn05 (**Figure 7A**, **Figure 7-figure supplement 1A**). A09e and CIII md neurons are both required for *Drosophila* larval anemotaxis (Jovanic et al., 2019; Masson et al., 2020). A08n primarily receives inputs from CIV md neurons and very few inputs from CIII md neurons (**Figure 7A**, **Figure 7-figure supplement 1A**). However, *Drosophila* third instar larval synaptic connectivity visualization using GFP reconstituted across synaptic partners (GRASP) revealed that A08n are not synaptic partners of CIII md neurons (Kaneko et al., 2017). A08n neurons function downstream of CIV md neurons for noxious chemical and mechanical nociception (Hu et al., 2017; Kaneko et al., 2017) and neural activation of Ch and CIII md neurons does not lead to activation of A08n neurons (Tenedini et al., 2019). *R61A01^{GAL4}* labeled neurons receive inputs from Ch, CIII md,

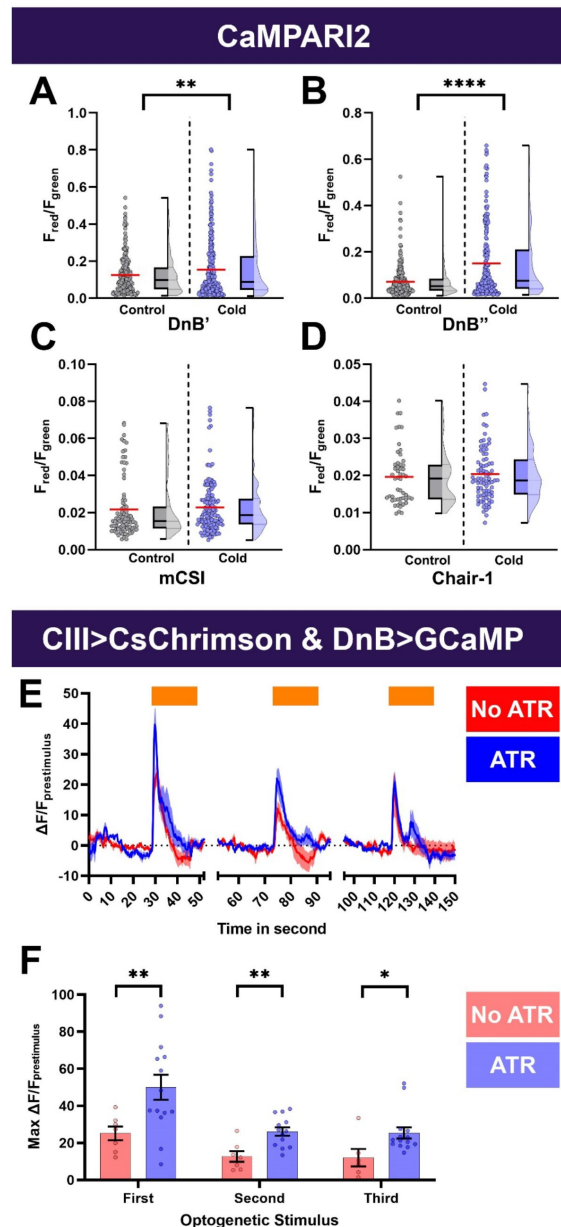


Figure 6

Cold- and CIII-evoked calcium responses of premotor neurons.

(A-D) Ca^{2+} responses of premotor neurons upon cold exposure vs. controls (room temperature). Neural responses (CaMPARI2) of premotor neuron cell bodies were analyzed using the following cell type driver lines DnB' (*IT4015^{GAL4}*) average n=250, (B) DnB'' (*IT412^{GAL4}*) average n=245, (C) mCSI (*R94B10^{GAL4}*) average n=116, and (D) Chair-1 (*SS00911^{GAL4}*) DnB' (*IT4015^{GAL4}*) average n=250, (B) DnB'' (*IT412^{GAL4}*) average n=245, (C) mCSI (*R94B10^{GAL4}*) average n=116, and (D) Chair-1 (*SS00911^{splitGAL4}*) average n=66. CaMPARI2 fluorescence ratio is reported as $F_{\text{red}}/F_{\text{green}}$. We report the data as individual datapoints, where the red line represents mean, and hybrid plots (boxplot & violin) for visualizing the distribution and quartiles of data. Significant stars represent $p < 0.05$, where comparisons were made to their respective no stimulus controls. (E-F) To functionally assess CIII md neuron to DnB connectivity, we optogenetically activated CIII md neurons (*83B04^{lexA}>CsChrimson*) and visualized changes in evoked Ca^{2+} using *DnB-GAL4>GCaMP6m*. Control: No ATR supplemented diet, which is required for optogenetic stimulation in *Drosophila*. Orange bars indicate optogenetic stimulation. (E) DnB changes in GCaMP reported as $\Delta F/F_{\text{prestimulus}}$, where prestimulus refers to 15 seconds prior to optogenetic stimulation. (F) Maximum DnB neuronal responses ($\Delta F/F_{\text{prestimulus}}$) upon optogenetic stimulation. Average n=10. Significant differences indicated via asterisks, where * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

CIV md, Basins, DnB, and A09e (**Figure 7A**, **Figure 7-figure supplement 1A**). Neurons labeled by *R61A01^{GAL4}* have been implicated in mechanosensation (Masson et al., 2020). We assessed whether the following projection neurons A09e, A08n, and neurons in the expression pattern of the GAL4 line *R61A01* (A10j, A09o, TePn04, TePn05) are required for cold evoked behaviors and function in conjunction with CIII md neurons for generating CT behavioral response.

Neural silencing of projection neurons using cell specific expression of tetanus toxin led to impairments in cold evoked behaviors. Neurotransmission inhibition in projection neurons (A09e and *R61A01*) that receive strong connectivity from CIII md neurons led to significant reductions in all the CT behavioral metrics that we analyzed (**Figure 7B-E**). As expected, A08n neurons, which primarily receive inputs from CIV md neurons, are not required for cold-evoked CT response (**Figure 7B-E**). Whereas neural silencing of A09e or *R61A01* neurons led to greater than 50% reduction in cold evoked responses from controls (**Figure 7J**).

Next, we assessed whether these projection neurons were able to elicit a CT response upon neural stimulation. Optogenetic stimulation of projection neurons alone did not lead to any CT response (**Figure 2-figure supplement 2**). Simultaneous co-activation of CIII md and *R61A01^{GAL4}* or A08n neurons led to mild but statistically insignificant increases in CT responses (**Figure 7F-I, J**). Co-activation of A09e and CIII md neurons led to facilitation of CT behavior, where compared to controls there were significant increases across all behavioral metrics that we analyzed (**Figure 7F, G-I**). There were mild, statistically insignificant, reductions in larval immobility when these projection neurons were co-activated with CIII md neurons, however, they were significant for A08n (**Figure 7-figure supplement 1B-D**). A09e, which receives substantial inputs from CIII md neuron, was sufficient to facilitate CIII md neuron evoked behavioral responses (**Figure 7J**).

Our neural silencing analyses suggest that A09e and *R61A01^{GAL4}* (A10j, A09o, TePn04, TePn05) neurons are required for cold evoked responses. We hypothesized that these neurons are cold sensitive and function downstream of CIII md neurons. We predicted that these neurons will have increases in Ca^{2+} upon cold stimulation or optogenetic activation of CIII md neurons. A09e neurons present indeed significant increases in Ca^{2+} levels upon cold stimulation, as measured by cell type-specific expression of CaMPARI2 (**Figure 8A**). Optogenetic activation of CIII md neurons led to significant increases in evoked Ca^{2+} levels of A09e neurons (**Figure 8B, C**). Multiple stimulations of CIII md neurons did lead to slightly lower levels of evoked Ca^{2+} but overall Ca^{2+} response was largely similar between stimulations (**Figure 8B, C**). For *R61A01^{GAL4}* Ca^{2+} imaging experiments, we restricted our analyses to TePn04 and TePn05 neurons, which were reliably identifiable in an intact *Drosophila* larval preparation. There were significant increases in CaMPARI2 response of TePn04/05 neurons upon cold stimulation (**Figure 8E**). Optogenetic stimulation of CIII md neurons led to strong increases in Ca^{2+} levels of TePn04/05 neurons, however, Ca^{2+} levels rapidly returned to baseline levels (**Figure 8F, G**). Repeated stimulations of CIII md neurons led to a blunted Ca^{2+} response in TePn04/05 neurons following the initial stimulation (**Figure 8F, G**). Combined, these data indicate A09e and *R61A01^{GAL4}* neurons function downstream of CIII md neurons for cold nociception (**Figure 8-figure supplement 1**), indicating that ascending neurons relay cold somatosensation to the brain.

Comparative analyses of cold sensitive neurons

Thus far, data were presented in logical groups based on their previously known cell-types and functions, however, these neurons function in an interconnected network and behavioral and functional imaging data must be assessed collectively to study circuit function. As discussed in previous sections, of all the cell types we tested only optogenetic activation of CIII md neurons alone is sufficient to elicit CT responses (**Figure 2-figure supplement 2**). Neural co-activation of CIII md neurons plus additional cell types resulted in marked increases in CT responses compared to only CIII md neuron activation (**Figure 9A**). Neural silencing of cell types downstream of CIII

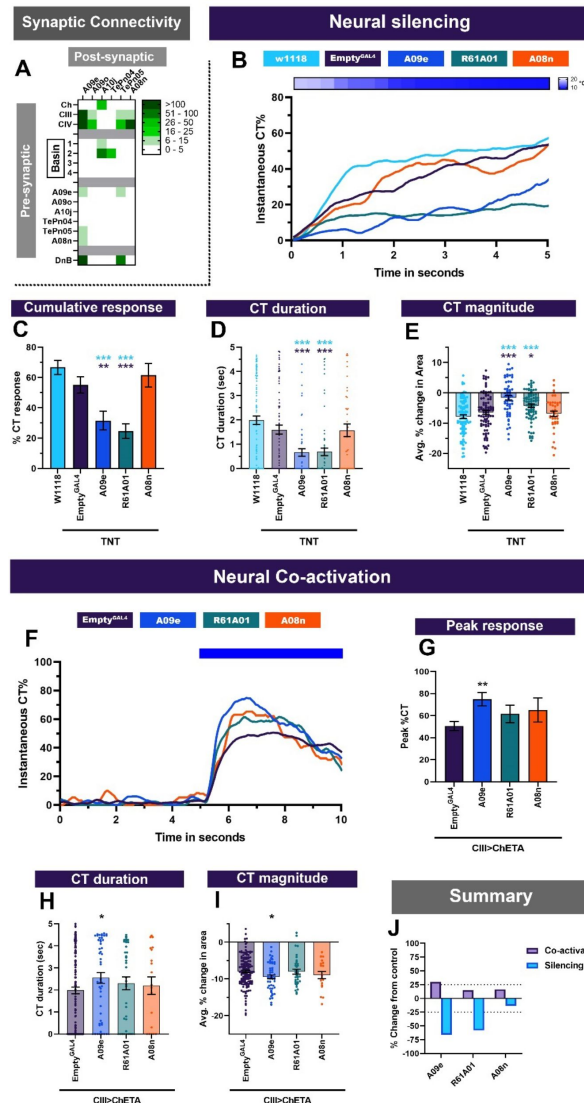


Figure 7

Projection neurons downstream of CIII md neurons are required for cold-evoked responses.

(A) A09e, A09o, A10j, TePn04, TePn05 and A08n primarily receive inputs from CIII md, CIV md, Basin-1, Basin-2 and DnB neurons. Heatmap plot of pre-synaptic connections to projection neurons. Synaptic connectivity data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-E) Cold evoked responses of third instar *Drosophila*. Projection neurons were silenced by inhibiting neurotransmitter release via cell type specific expression of tetanus toxin (TNT), where A09e (*SS00878^{splitGAL4}*), *R61A01^{GAL4}* (labels A09o, A10j, TePn04, and TePn05) and A08n (*R82E12^{GAL4}*). (B) Instantaneous %CT over time. Heatmap on top represents change in temperature over time. (C) Cumulative %CT response for a duration of 5 seconds. (D) CT duration in seconds. (E) CT magnitude as average percent change in area for the duration of stimulus. Controls include *w1118* and *Empty^{GAL4}>TNT*. Average *n*=73. Significant stars: turquoise stars represent comparison to *w1118* and purple stars represent comparison to *Empty^{GAL4}>TNT*. (F-I) Neural co-activation of projection neurons and CIII md neurons. Here each condition represents expression of ChETA in CIII md neurons plus projection neurons. (F) Instantaneous %CT over time. Blue bar represents optogenetic neural activation. (G) Peak %CT response during optogenetic stimulation. (H) CT duration in seconds during optogenetic stimulation. (I) CT magnitude as average percent change in area for the duration of stimulus. *Empty^{GAL4}* *n*=143 and experimental condition average *n*=35. Significant purple stars represent comparison to *CIII md + Empty^{GAL4}>ChETA*. (J) Overall percent change from control for either neural silencing or neural co-activation. The metrics for neural silencing include cumulative %CT, CT magnitude, and CT duration. The following metrics were used to calculate percent for neural co-activation: cumulative %CT, peak %CT, CT duration and magnitude. Significant differences indicated via asterisks, where **p*<0.05, ***p*<0.01, and ****p*<0.001.

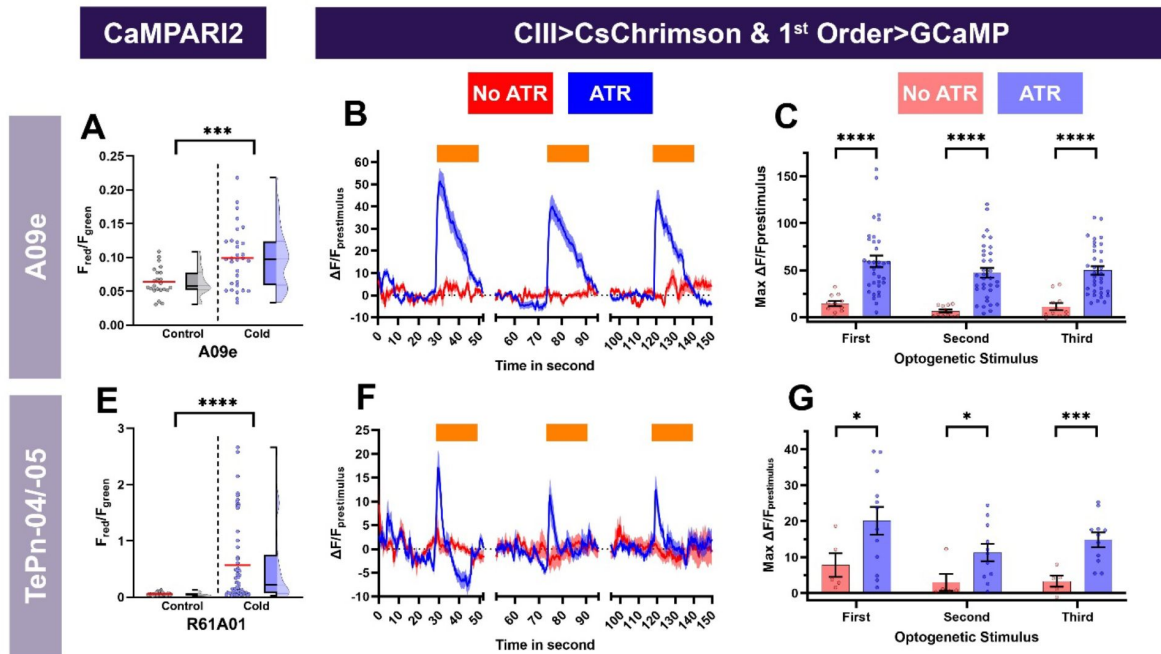


Figure 8

Cold- and CIII-evoked calcium responses of projection neurons.

Neural responses of A09e (*SS00878^{GAL4}*) (**A-C**) and terminally located TePns (–04, –05) were analyzed using *R61A01^{GAL4}* (**E-G**). (**A,E**) Ca^{2+} responses of projection neurons upon cold exposure vs. controls (room temperature). Cold evoked neural responses (CaMPARI2) of projection neuron cell bodies were analyzed for (**A**) A09e (n=27) and (**E**) TePns04, & TePns05 (n=42). CaMPARI2 fluorescence ratio is reported as F_{red}/F_{green} . We report the data as individual datapoints, where the red line represents mean, and hybrid plots (boxplot & violin) for visualizing the distribution and quartiles of data. Significant stars represent $p < 0.05$, where comparisons were made to their respective no stimulus controls. (**B-C, F-G**) To assess, if A09e or TePns functions downstream of CIII md neurons, we optogenetically activated CIII md neurons (*83B04^{lexA}>CsChrimson*) and visualized changes in evoked Ca^{2+} using projection neuron specific *GAL4>GCaMP6m*. Control: No ATR supplemented diet, which is required for optogenetic stimulation in *Drosophila*. Orange bars indicate optogenetic stimulation. (**B, F**) Changes in GCaMP reported as $\Delta F/F_{prestimulus}$, where prestimulus refers to 15 seconds prior to optogenetic stimulation. (**F**) Maximum neuronal responses ($\Delta F/F_{prestimulus}$) upon optogenetic stimulation. A09e average n=22. TePns average n=8. Significant differences indicated via asterisks, where * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$.

md neurons led to significant reductions in cold-evoked CT (**Figure 9B**). To understand how these behavioral phenotypes are interrelated, we performed t-distributed stochastic neighbor embedding (t-SNE) analysis on both neural silencing and co-activation behavioral datasets. We identified five different clusters that exhibit varying impacts on behavioral phenotypes upon either neural co-activation or silencing (**Figure 9C, D**). The first group clusters together with the control genotype and includes CIV, A05q, Goro, and A08n neurons. This group on average had less than 25% change in behavioral phenotypes in either neural co-activation or silencing experiments (**Figure 9C, D**). Basin-1 and DnB neurons clustered together, where on average they had 49% enhancement of CT response upon neural co-activation and 45% reduction in cold evoked CT response upon neural silencing (**Figure 9C, D**). *R61A01* and CIII md neurons formed a cluster, where neural silencing resulted in nearly 60% reduction cold evoked behavior and 20% enhancement of CIII evoked behaviors upon neural co-activation (**Figure 9C, D**). A group of neurons including Chair-1, Basin-4 and Ch were required for cold evoked behavioral responses, however, these neurons did not facilitate CIII evoked CT responses (**Figure 9C, D**). In the last group, neural silencing of all Basins, Basin-2, mCSI, A00c or A09e led to an overall 69% reduction in cold evoked behaviors and 34% enhancement in CIII evoked CT response (**Figure 9C, D**).

Analyses of *Drosophila* larval behavioral phenotypes revealed distinct roles for downstream neurons in cold nociceptive circuitry. Mapping synaptic connectivity of a particular circuit is only the first step in understanding how individual behaviors arise. Here, we draw attention to select CIII md neuron first-order neurons (Basins (-2 & -4), DnB, TePns and A09e) that showed robust requirements for cold nociceptive behaviors and had functional connectivity to CIII md neurons (**Figure 9E**). TePns synapse onto Basin-4 and form reciprocal connections to Basin-2, DnB, and A09e (**Figure 9E**). DnB further synapses onto Basin-4 and A09e. Within this circuit motif one might predict that A09e neurons function as the master integrators, where they might be computing synaptic information from various sources (**Figure 9E**). Consistent with EM connectivity, A09e neurons have the highest CIII md neuron evoked Ca^{2+} responses of the tested cell types (**Figure 9F, G**). Compared to Basin-4 neurons, Basin-2 neurons receive greater synaptic input from CIII md neurons (**Figure 9E**). However, CIII md neuron activation leads to nearly twice as much Ca^{2+} response in Basin-4 neurons than Basin-2 (**Figure 9F, G**). DnB neurons receive the second highest synaptic input from CIII md neuron and have the second highest CIII md neuron evoked Ca^{2+} response (**Figure 9F, G**). TePns receive significantly lower synaptic input compared to Basin-2 neurons, however, they both exhibit similar levels of CIII evoked Ca^{2+} response (**Figure 9F, G**). Comparative analyses reveal that synaptic connectivity is informative about functional neural activity, however, additional molecular and functional studies are required to fully understand roles of these neurons in cold nociception. Collectively, our findings on neural substrates of cold nociception indicate that second order multisensory integration by Basin neurons, select pre-motor neurons and projection neurons are preferentially activated in a stimulus specific manner to elicit appropriate behavioral responses.

Discussion

Environmental stimuli are detected by peripheral sensory neurons, which transduce relevant cues to downstream central circuitry responsible for processing multisensory input and generating stimulus-relevant behavioral responses. The study of the diverse molecular and cellular mechanisms involved in sensory discrimination have yielded key insights into how animals interact with their environment (Arnadóttir et al., 2011; Bandell & Patapoutian, 2009; Cho & Oh, 2013; Corfas & Vossell, 2015; Coste et al., 2010; Derby et al., 2016; Dietrich et al., 2022; Fowler & Montell, 2013; Freeman & Dahanukar, 2015; Himmel et al., 2017; Leung & Montell, 2017; Montell, 2021; Wu et al., 2018; Xiao & Xu, 2021). However, how animals distinguish between innocuous and noxious stimuli at molecular, cellular and circuit level

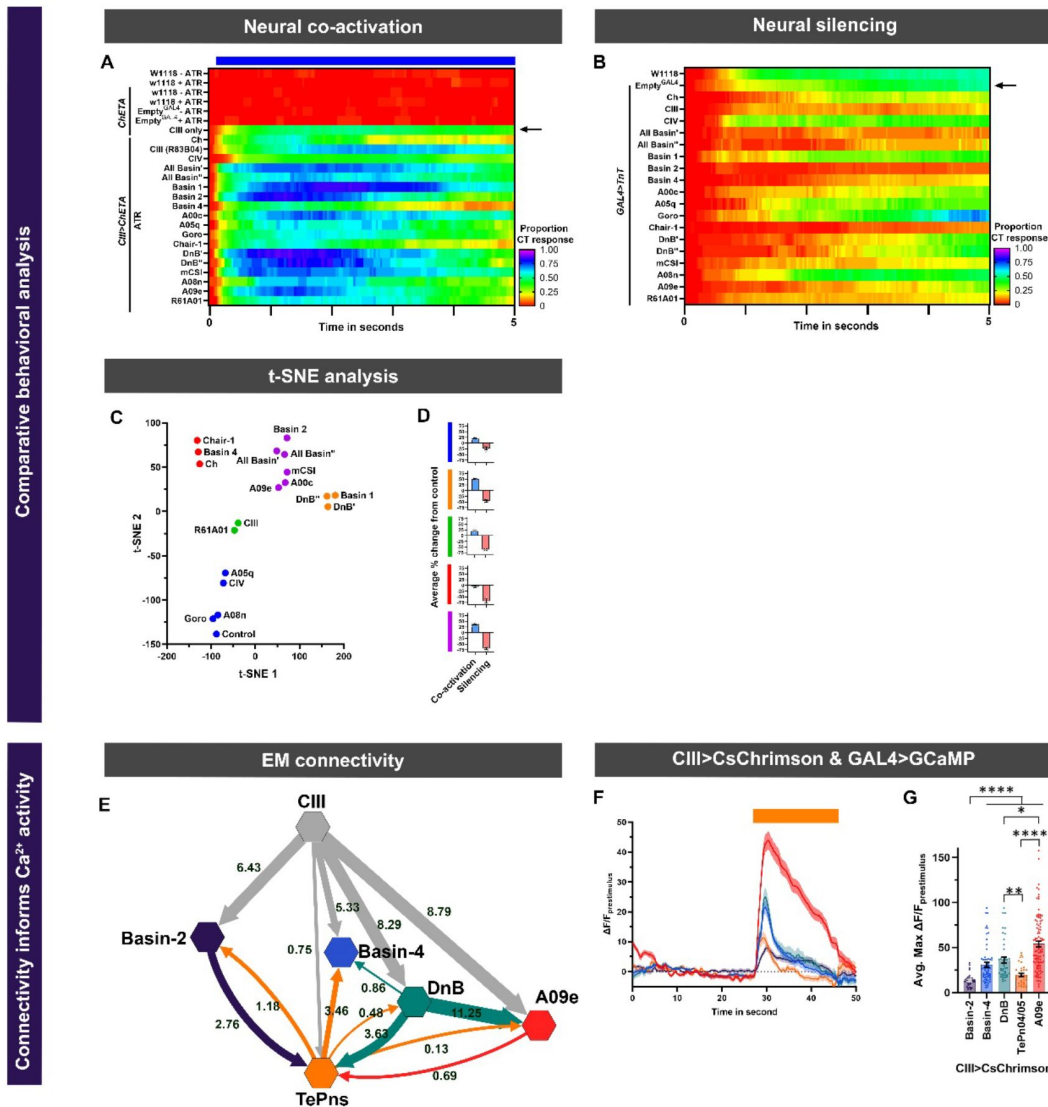


Figure 9

Dimensional reduction analysis of *Drosophila* larval behavioral responses and synaptic connectivity informs functional connectivity assessed via Ca^{2+} imaging

(A-B) Instantaneous CT proportions for all genotypes in this study. (A) Neural co-activation experiments, where CIII plus additional neuronal types were simultaneously optogenetically activated. Controls for optogenetic experiments were tested with or without ATR supplement and include the following conditions: background strain (*w1118*), background strain crossed to *UAS-ChETA*, and *Empty^{GAL4}* crossed to *UAS-ChETA*. Blue bar represents optogenetic stimulation. (B) Neurotransmitter release inhibition of individual neuronal types using cell-type specific expression of TnT. (C-D) t-distributed stochastic neighbor embedding (t-SNE) analysis of all neuronal subtypes role in both cold nociception (neural silencing data) and CIII md neuron evoked CT facilitation (co-activation data). (C) 2D plot of t-SNE analysis, where post-hoc clustering analysis based on “Euclidian complete” method revealed 5 unique groups. The following percent change from control (*Empty^{GAL4}*) data were included in the analysis: For neural co-activation (peak % CT response, cumulative % CT response, average % change in area, and CT duration) and for neural silencing (cumulative % CT response, average % change in area, and CT duration). (D) Average percent change from control for each cluster in ‘C’ across all neural co-activation or neural silencing metrics. (E-G) Analyses of connectivity upon select circuit components and comparative CIII md neuron evoked calcium responses in post-synaptic neurons. (E) Proportion of synaptic inputs amongst neurons are plotted. A09e neurons integrate responses from multiple pathways originating from CIII md neurons. Network map created using Cytoscape (Shannon et al., 2003) (F-G) CIII md neuron evoked calcium responses in post-synaptic neurons. (F) $\Delta F/F_{\text{baseline}}$ over time. (G) Average max $\Delta F/F_{\text{baseline}}$. Averages $\pm$ SEM of all trial are plotted. Significant differences indicated via asterisks, where * $p < 0.05$, ** $p < 0.001$, and *** $p < 0.0001$.

remains an important question in modern neuroscience (Bushnell et al., 1985 [DOI](#); Dannhauser et al., 2020 [DOI](#); Himmel et al., 2023 [DOI](#); Imambocus et al., 2022 [DOI](#); Moehring et al., 2018 [DOI](#); Patel et al., 2022 [DOI](#); Turner et al., 2016 [DOI](#); Ward et al., 1996 [DOI](#)).

Here, we assessed the downstream circuitry of a multimodal sensory (CIII md) neuron that detects both innocuous mechanical and noxious cold temperatures. In CIII md neurons, detection of innocuous cues occurs via low threshold activation and noxious cues are detected via high threshold activation leading to stimulus-relevant behaviors (Turner et al., 2016 [DOI](#)). Transduction of nociceptive cold stimulus is predominantly mediated by multimodal (innocuous and noxious) sensitive Ch and CIII md neurons. However, nociceptive peripheral sensory neurons, CIII md and CIV md neurons, function through shared downstream neural circuitry. We describe the behavioral and functional requirements of multisensory integration (Basins) neurons, premotor (DnB and mCSI) neurons and projection (A09e and TePns) neurons in noxious cold evoked behavioral responses. We identified circuit components that play a role in amplifying noxious cues and differentiating between opposing noxious (heat versus cold) stimuli-evoked behaviors at various levels of sensory processing. Our findings provide key insights into how environmental cues are processed by multiplexed networks for multisensory integration and decision making.

Basin-1 neurons function across sensory modalities as gain modulators for noxious stimuli-evoked escape responses

Accurately responding to potentially harmful stimuli by appropriately executing energetically expensive escape behaviors is critical for animal survival. Incorrectly performing escape responses in absence of dangerous stimuli can have long-term detrimental consequences. Therefore the need for neural mechanisms responsible for integrating various noxious and innocuous sensory stimuli for accurately executing stimulus-appropriate escape responses. Here, we focus on the role of Basin neurons, known to mediate various escape responses (Jovanic et al., 2016 [DOI](#); Masson et al., 2020 [DOI](#); Ohyama et al., 2015 [DOI](#)). EM connectome analyses revealed that somatosensory (Ch, CIII md and CIV md) neurons all synapse onto multisensory integrating Basin neurons, and that amongst the Basins subtypes, Basin-1 neurons are presynaptic to Basin-2,-3 and -4 neurons (Masson et al., 2020 [DOI](#); Ohyama et al., 2015 [DOI](#)). On the basis of the known connectome, we analyzed the function of Basin neurons in cold-evoked escape behaviors. Our findings indicate that optogenetic activation of Basin-1 is not sufficient to evoke noxious cold evoked behavioral response (Figure 2 [DOI](#) – Supplement 2) and it was known that thermogenetic activation of Basin-1 is also not sufficient for evoked rolling escape (Ohyama et al., 2015 [DOI](#)). Therefore, Basin-1 neuron activity alone is not sufficient to elicit a noxious stimuli-evoked behavioral response. However, due to intricate connectivity amongst the Basins, the total integrated output of multiple Basin neurons may lead to threshold activation of noxious stimulus evoked escape responses. And indeed, thermogenetic co-activation assays found that Basin-1 neural activity could facilitate a Basin-4-mediated rolling response (Ohyama et al., 2015 [DOI](#)), and also the activation of Basin-1 neurons leads to the activation of Basin-2 neurons through lateral disinhibition (Jovanic et al., 2016 [DOI](#)). Here, we found that co-activation of Basin-1 plus CIII md neurons led to strong evoked CT responses (Figure 2 [DOI](#)). Taken together, these studies concluded that Basin-1 facilitates the activation of other Basin neurons, and our results also provide additional support for the role of Basin-1 neurons in fine-tuning behavioral outcomes and enhancing cumulative output of multisensory integration Basin neurons leading stimulus specific escape responses.

Multisensory integration neurons drive behavioral selection downstream of sensory neurons

In order to evaluate the functional and behavioral roles of neurons within a circuit, a comprehensive analysis of both upstream and downstream synaptic connectivity is necessary. Basin-2 and -4 neurons integrate different amounts of synaptic input from somatosensory neurons, other Basin neurons, feedforward inhibitory local neurons, and projection neurons.

Basin-4 neurons receive greater excitatory synaptic input compared to Basin-2 neurons, which have greater connectivity with local inhibitory neurons (Jovanic et al., 2016; Masson et al., 2020; Ohyama et al., 2015). Larger inhibitory connectivity of Basin-2 neurons likely arises from much broader downstream connectivity including several projection neurons, premotor neurons and feedback inhibitory local neurons, whereas Basin-4 downstream connectivity is restricted to limited set of neurons. These synaptic level differences in multisensory integration neurons underlie noxious stimuli-specific evoked responses, and suggest that CIII md neuron mediated responses primarily function through Basin-2 neurons and CIV md neuron mediated environmental cues are processed via Basin-4 neurons.

Chemical nociception in *Drosophila* larvae primarily functions via CIV md. Among the md neurons, CIII md neurons are the least sensitive to noxious chemical stimulus (Lopez-Bellido et al., 2019). Analyses of Basin neuron requirement in chemical nociception revealed that all Basins are necessary for chemical-evoked rolling, where comparative analyses of Basin subtypes revealed that chemical nociception is primarily mediated through Basin-4 with weaker behavioral phenotypes of Basin-1 or -2 (Lopez-Bellido et al., 2019). Our work shows that inhibition of neurotransmission in Basin-2 or Basin-4 neurons leads to significant reductions in cold nociceptive responses, where across the analyzed behavioral metrics Basin-2 had stronger deficits in cold evoked responses (Figure 2). Furthermore, neural co-activation analyses revealed that Basin-2 plus CIII md led to enhanced CT response, whereas Basin-4 plus CIII md co-activation did not facilitate but rather suppressed the CT response (Figure 2). Basin-2 neural activity likely has cascading effects on functions of downstream neurons, which we also found to be required for cold nociception. Behavioral assessment of multisensory integration neurons supports the notion that CIII md neuron sensory input is processed primarily by Basin-2 and CIV md neuron sensory cues are processed via Basin-4 neurons.

Despite the accumulating evidence that cold-evoked responses are primarily mediated by Basin-2, our Ca^{2+} analyses revealed that compared to Basin-2, Basin-4 neurons have much greater cold-or CIII md neuron-evoked Ca^{2+} responses. There are a few possible explanations for greater Basin-4 activity levels. Firstly, greater Basin-4 Ca^{2+} activity could be due to greater synaptic input from projection neurons (TePn04/05) and premotor neurons (DnB), which are both required for cold nociception and exhibit cold-or CIII md neuron-evoked Ca^{2+} responses (Figure 7, Figure 8 – Supplement 1) (Masson et al., 2020). Secondly, reduced Basin-2 Ca^{2+} responses could be due to greater connectivity with feedback/feedforward inhibitory local neurons (Jovanic et al., 2016; Masson et al., 2020), therefore suggesting that Basin-2 could be mediating an onset response rather than a sustained response. Additional comparative Ca^{2+} imaging or electrophysiological analyses are required to further determine how Basin-2 and -4 neurons integrate sensory information across somatosensory modalities leading to distinct behavioral responses. However, to date, synaptic connectivity and behavioral analyses collectively indicate an initial sensory discrimination node amongst the Basin neurons, where downstream processing of CIII mediated sensory input is conveyed through Basin-2 and its downstream connectivity, meanwhile CIV somatosensory information is transduced through Basin-4 neuronal pathway.

Differential roles of pre-motor neurons lead to behavioral selection

Animal locomotive behavioral responses are implemented through neurosecretory systems and muscle contractions evoked by motor neuron activity. Motor neurons integrate a conglomerate of neural impulses from premotor neurons, leading to appropriate muscle activation patterns to implement specific behavioral responses (Bellardita & Kiehn, 2015; Berkowitz et al., 2010; Carreira-Rosario et al., 2018; Green & Soffe, 1996; Huang & Zarin, 2022; Liao & Fetcho, 2008; Talpalar et al., 2013; Zarin et al., 2019). Recent work in premotor and motor neuron connectomics has provided evidence for both labeled line and combinatorial connectivity between premotor and motor neurons that give rise to co-active motor neurons states leading to

selective muscle group activation (Huang & Zarin, 2022 [DOI](#); Zarin et al., 2019 [DOI](#)). Various *Drosophila* larval premotor neuron subtypes and/or motor pools have been implicated in locomotion, nociception, and innocuous mechanosensation (Burgos et al., 2018 [DOI](#); Huang & Zarin, 2022 [DOI](#); Jovanic et al., 2019 [DOI](#); Kohsaka et al., 2017 [DOI](#); Kohsaka et al., 2014 [DOI](#); Kohsaka et al., 2019 [DOI](#); Yoshino et al., 2017 [DOI](#); Zarin et al., 2019 [DOI](#)). Neural reconstruction efforts in *Drosophila* larvae have revealed that somatosensory (CIII md and CIV md) neurons are not directly connected to motor neurons, but rather feed into polysynaptic pathways leading to motor neurons via premotor neurons (such as the DnB, mCSI, & Chair-1 neurons studied here) (Jovanic et al., 2019 [DOI](#); Ohyama et al., 2015 [DOI](#); Winding et al., 2022). Premotor neurons (DnB, mCSI, and Chair-1) that receive synaptic input from CIII md neurons are required for cold-evoked responses. However, only premotor neurons previously implicated in nociceptive escape responses, DnB and mCSI, can facilitate CIII evoked CT responses (Burgos et al., 2018 [DOI](#); Lopez-Bellido et al., 2019 [DOI](#); Yoshino et al., 2017 [DOI](#)). Meanwhile, Chair-1 neurons, which are required for innocuous mechanosensation, do not facilitate CIII md neuron mediated CT responses (Jovanic et al., 2019 [DOI](#)). We showed, with Ca^{2+} imaging, that Chair-1 neurons are not activated upon cold exposure. It remains unclear whether Chair-1 neuronal function is sufficient to drive specific sensory evoked behaviors or requires a combinatorial pre-motor neuronal activity for generating evoked behavioral responses. Given the data, we speculate that Chair-1 neurons likely gate innocuous-to-noxious stimulus evoked behaviors; however, additional functional studies are required to tease out how Chair-1 neurons function in behavioral selection.

Heterogeneity in thermosensory processing reflects variety of thermal stimuli

Animals detect changes in temperature through a variety of sensors that occupy and function in different spatiotemporal timescales. Thermosensory systems function to detect changes at the epidermal, brain and visceral levels, and these broad thermosensory inputs must be integrated to generate appropriate behavioral responses (Nakamura, 2018 [DOI](#)). The underlying neural circuitry for thermosensation differs based on body plan and type of thermosensation. In mice, cool and warm sensory signals, which are detected by TRP channels, are transmitted to the spinal dorsal horn, where distinct cool/warm pathways relay epidermal changes in temperature to thalamocortical regions for perception and discrimination (Nakamura, 2018 [DOI](#)). In *Drosophila* larvae and adults, thermotaxis occurs through temperature detection via dorsal organ ganglion (DOG) and antennal thermoreceptors, respectively (Frank et al., 2015 [DOI](#); Gallio et al., 2011 [DOI](#); Klein et al., 2015 [DOI](#); Liu et al., 2003 [DOI](#)). Investigations of circuit bases of thermotaxis have revealed different strategies for larval and adult systems. Adult antennal thermoreceptors have distinct hot or cold sensors, which project to the proximal antennal lobe (PAL), where hot and cold information is segregated into discrete regions (Gallio et al., 2011 [DOI](#); Macpherson et al., 2015 [DOI](#)). Thermal coding properties of thermosensory projection neurons (tPNs), whose dendrites are located in PAL, include both slow/fast adapting tPNs and broadly/narrowly tuned tPNs (Frank et al., 2017 [DOI](#)). *Drosophila* larval thermosensory DOG contains both warm- and cool-sensing cells that are connected to individual cool or warm projection neurons (Hernandez-Nunez et al., 2021 [DOI](#)). DOG thermosensory information is further integrated by integration projection neurons (iPNs), which receive inputs from both warm and cool projection neurons (Hernandez-Nunez et al., 2021 [DOI](#)). The *Drosophila* larval noxious thermosensory system has distinct hot (CIV md) or cold (CIII md) sensing neurons (Babcock et al., 2009 [DOI](#); Tracey et al., 2003 [DOI](#); Turner et al., 2016 [DOI](#)). Larval somatosensory thermal inputs mediated by CIII md neurons are heavily integrated by second order neurons (Basins, DnB, A09e, etc.), where population coding leads to cold-evoked behavioral response. Noxious cold stimulus is encoded downstream of CIII md neurons by ascending neurons (A09e and TePns) that allow for systems-level integration of multiple thermal inputs including those from cool/warm sensitive larval DOG neural circuits and cold/heat information from CIII/CIV md neurons, respectively. Thermal information from the larval DOG and somatosensory neurons converges onto the mushroom body, where cool or warm projection neurons downstream of DOG and feed-forward projection neurons downstream of ascending

neurons (A09e) synapse onto the same mushroom body output neuron (Eichler et al., 2017 [↗](#); Hernandez-Nunez et al., 2021 [↗](#); Winding et al., 2023 [↗](#)). Thus far, ascending pathways into the brain have been characterized for larval cold nociception, however, descending output neurons responsible for cold-evoked behaviors remains unidentified. Diversity in processing thermosensory stimuli reflects heterogeneity at molecular, cellular, and circuit levels that collectively function to maintain proper thermal homeostasis and ultimately behavioral action selection. The *Drosophila* larval thermosensory system is well-suited to dissect mechanisms underlying central nervous system integration of varying spatial and functional thermosensory information by utilizing various molecules, sensory systems, and interconnected circuits for appropriate behavioral responses.

Methods

Fly Strains

All *Drosophila melanogaster* strains used in this study are listed in (Table 1 [↗](#)). All *Drosophila* reagents were maintained on standard cornmeal-molasses-agar diet in 12:12 hour light-dark cycle at ~22°C. All experimental crosses were raised in 12:12 hour light-dark cycle at 29°C, unless otherwise stated. The following strains were a gift from Marta Zlatić: Basin-2 (SS00739^{splitGAL4}), Basin-4 (SS00740^{splitGAL4}), A09e (SS00878^{splitGAL4}), and Chair-1 (SS00911^{splitGAL4}). We used two separate CIII md neuron driver lines in this study: 19-12^{GAL4} and R83B04^{lexA}.

Molecular cloning and transgenic generation of CIII^{lexA}

GMR83B04^{GAL4} was characterized as CIII md neuron driver using both optogenetics and visualization using membrane markers (Himmel et al., 2023 [↗](#); Patel et al., 2022 [↗](#)). The R83B04 enhancer containing entry vector was a gift from FlyLight team at Janelia Research Campus, Ashburn, VA. We performed Gateway cloning to insert R83B04 enhancer upstream of lexA. LR reaction was performed using R83B04 containing entry vector and lexA containing (pBPLexA::p65Uw) destination vector (Addgene: 26231). Transgenic fly generation was conducted by GenetiVision. R83B04^{lexA} was inserted in VK20 docking site using PhiC31 integrase mediated transformation.

EM connectomics

All data for synaptic connectivity, circuit diagrams and wire frame projections of neural cell types were extracted from Neurophyla LMB Cambridge (<https://neurophyla.mrc-lmb.cam.ac.uk/> [↗](#)). Relevant primary literature sources are cited within the main text. Sensory neurons from abdominal segments 1-4 were analyzed and for the remaining cell types, all reconstructed neurons were included (date accessed: March 04, 2022).

Cold Plate Assay

We assessed requirements of neurons downstream from peripheral sensory neurons in cold evoked responses using the cold plate assay (Patel & Cox, 2017 [↗](#); Patel et al., 2022 [↗](#); Turner et al., 2016 [↗](#)). *GAL4* drivers for select neuronal subtypes were used to express the light chain of tetanus toxin (*UAS-TNT*). We used two sets of controls: *w¹¹¹⁸*, genetic background control, and *Empty^{GAL4}*, which contains a *GAL4* construct but no regulatory promoter (*GAL4* only control). Genetic crosses were raised on standard cornmeal-molasses-agar diet and at 29°C. To assess cold evoked behavioral responses of age matched *Drosophila* third instar larvae, we exposed the ventral surface to noxious cold (10°C) temperatures. Briefly, using a brush we remove third instar larvae from food and place them on wet Kimwipe. Food debris is removed passively by allowing the larvae to freely locomote on wet Kimwipe. We place 6-8 larvae on a thin black metal plate that is

Designation	Nomenclature	Source or reference	Identifier
w1118		BDSC	
ChETA	<i>UAS-ChETA::YFP</i>	BDSC	36495
CaMPARI2	<i>UAS-CaMPARI2</i>	BDSC	78317
TnT	<i>UAS-TeTxLC.tnt-E2</i>	BDSC	28837
CsChrimson	<i>LexAop2-CsChrimson.tdTomato</i>	BDSC	82183
GCaMP6m	<i>UAS-GCaMP6m</i>	BDSC	42748
Pan-neural CaMPARI	<i>R57C10^{GAL4}, UAS-CaMPARI</i>	BDSC	58763
CHH>ChETA	<i>19-12^{GAL4}, UAS-ChETA::YFP</i>	BDSC	36495
CHH	<i>19-12 - GAL4</i>		
CHH	<i>R83B04 - lexA</i>	This Study	
Ch	<i>IAV - GAL4</i>	BDSC	52273
CIV	<i>ppk - GAL4</i>	BDSC	32079
Empty - GAL4	<i>pBDPGAL4Uw</i>	BDSC	68384
All Basin'	<i>R72F11 - GAL4</i>	BDSC	39786
All Basin''	<i>R57F07 - GAL4</i>	BDSC	46389
Basin-1	<i>R20B01 - GAL4</i>	BDSC	48877
Basin-2	<i>SS00739 - splitGAL4</i>	Zlatic Lab	
Basin-4	<i>SS00740 - splitGAL4</i>	Zlatic Lab	
A00c	<i>R71A10 - GAL4</i>	BDSC	39562
A05q	<i>R47D07 - GAL4</i>	BDSC	50304
Goro	<i>R69F06 - GAL4</i>	BDSC	39497
Chair-1	<i>SS00911 - splitGAL4</i>	Zlatic Lab	
DnB'	<i>IT4051 - GAL4</i>	Grueber Lab	
DnB''	<i>IT412 - GAL4</i>	BDSC	63300
mCSI	<i>R94B10 - GAL4</i>	BDSC	41325
A09e	<i>SS00878 - splitGAL4</i>	Zlatic Lab	
A08n	<i>R82E12 - GAL4</i>	BDSC	40153
R61A01/TePns	<i>R61A01 - GAL4</i>	BDSC	39269

Table 1

Drosophila melanogaster strains used in this study

subsequently placed on a pre-chilled (10°C) Peltier plate TE technologies Peltier plate (CP-031, TC-48-20, RS-100-12). Larval responses are recorded from above using Nikon DSLR (D5300). Changes in larval surface area were extracted using FIJI and Noldus Ethovision XT (<https://github.com/CoxLabGSU/CaMPARI-intensity-and-cold-plate-assay-analysis/branches>) (Patel et al., 2022). Next, we isolated individual larva from each video, removed background and used Ethovision to measure larval surface area. Using custom built r scripts, we compiled data from each larva and each genotype. Utilizing r, we calculated percent change in larval surface area ($\text{Area change} = (\text{Area}_N - \text{Average_Area}_{\text{baseline}}) / \text{Average_Area}_{\text{baseline}} * 100$). From the percent change in area dataset, we report three behavioral metrics: Average change in area, which is average percent change in area for the stimulus duration. We defined cold evoked CT response as change in area of -10% or less for at least 0.5 consecutive seconds. CT duration, time spent at or below -10% change in area. Lastly, percent CT response, which is cumulative percent of animals that CT for at least 0.5 consecutive seconds.

Statistical analysis: We preformed following statistical tests for all cold plate assay data analysis. %CT response: Fisher's exact with Benjamini-Hochberg for multiple comparison. We used r for performing comparisons of percent behavior response between genotypes, we used Benjamini-Hochberg multiple comparison correction. CT duration: Kruskal-Wallis with Benjamini, Krieger and Yekutieli for multiple comparisons. CT duration data are not normal. CT magnitude: One-way ANOVA with Holm-Šidák's for multiple comparisons.

Neural activation via optogenetics

We performed two types of neural activations to assess sufficiency for CT response: single downstream neural activation and co-activation, where we simultaneously activated CIII md neurons and individual downstream neurons. For optogenetic experiments in *Drosophila*, a light sensitive co-factor all *trans*-retinal (ATR) is required. For all conditions, all adult animals in the genetic cross were placed in ATR (1500μM) supplemented food and subsequently F1 progeny were also raised in food containing ATR and raised in dark. For control condition, we used an *Empty^{GAL4}* containing GAL4 construct but no regulatory promoter. Optogenetic experiments were conducted using a similar setup as previously described (Patel et al., 2022). Briefly, we used principles of dark field microscopy to enhance signal to noise ratio and capture high resolution larval videos. We created a custom dark field stage, where a Canon DSLR T3i camera captures video from above. Neural activation is performed by two blue led lights that are controlled remotely using the Noldus control box (Thorlabs: DC4100, DC4100-hub, and two M470L3-C4 led light. Noldus: mini-IO box). Larval behaviors are directly captured using Noldus Ethovision XT software, which also controls blue led activation. All optogenetic experiments were conducted in a dimly lit room. Third instar *Drosophila* larva were removed from food plug and placed onto wet Kimwipe, where larval locomotion allowed for passive removal of food debris. We lightly sprayed water onto a thin glass plate, then placed a single larva for optogenetic stimulation. The glass plate was manually moved on XY-axis to keep larva in the field of view. The following stimulus paradigm was used: 5 seconds of baseline (light off) and 5 seconds of neural activation (blue led lights on).

We performed video processing and behavioral analysis using FIJI and data compilation and analysis using r (https://github.com/CoxLabGSU/Drosophila_larval_optogenetic_analysis-area_and_mobility). In order to analyze *Drosophila* larval behavioral responses, we first automatically stabilized (XY axis) and then measured changes in larval surface area and mobility (described below).

Raw videos from Noldus Ethovision XT were uncompressed using video-to-video convertor (<https://www.videotovideo.org/>). The following steps were scripted in FIJI macro language for automatic video processing and data acquisition. For increasing processing speed, the uncompressed videos were automatically cropped to dimensions to contain all of the larva's movement. Next, using a pre-determined threshold, we created a mask followed by background removal using erode,

remove outlier and dilate functions. We then used the ‘Analyze Particles’ function to obtain XY coordinates of the larva in each frame. Larval movements were stabilized using XY coordinates and the ‘Translate’ function was used to create a highly stabilized video. Next, we measured larval surface area using automatic thresholding ‘Huang method’ and ‘Analyze Particles’ to obtain area. We define *Drosophila* larval mobility as changes in occupied pixels between two frames. We used our stabilized larval video to measure larval mobility, where larval peristaltic movements (XY displacement) are not captured. However, changes in occupied pixels resulting from turning and head sweeps are captured. Specifically, larval mobility was measured by subtracting thresholded larva in each frame from the previous frame (Raw mobility = Thresholded larva_{Frame N} - Thresholded larva_{Frame N-1}).

Data compilation and analysis was performed in R using custom scripts. Optogenetically evoked changes in behavior were analyzed independently for mobility and changes in area. For each larva, we calculated percent change in area (Area change = $(\text{Area}_N - \text{Average_Area}_{\text{baseline}}) / \text{Average_Area}_{\text{baseline}} * 100$). We measured CT duration as the amount of time the larva has -10% or lower change in area. We also measure CT magnitude by analyzing average change area for stimulus duration. We report percent instantaneous CT over time as percent of animals that are at or below -10% change in area. Additionally, we report peak %CT from percent instantaneous CT dataset for each genotype. For analysis of mobility, we calculated percent change in mobility (Mobility change = $(\text{Mobility}_N - \text{Average_Mobility}_{\text{baseline}}) / \text{Average_Mobility}_{\text{baseline}} * 100$). We report average percent change in mobility during stimulus for each genotype. We also plot percent immobility, which is calculated by the percent of animals with -25% or more reduction in mobility. Immobility duration is calculated based on amount of time individual animals spend at or below -25% mobility.

Statistical analysis: We performed following statistical tests for all behavioral optogenetic data analysis. %CT peak response: Fisher’s exact with Benjamini-Hochberg for multiple comparison. We used R for performing comparisons of percent behavior response between genotypes, we used Benjamini-Hochberg multiple comparison correction. CT/immobility duration: Kruskal-Wallis with Benjamini, Krieger and Yekutieli for multiple comparisons. CT duration data are not normal. CT/immobility magnitude: One-way ANOVA with Holm-Šidák’s for multiple comparisons.

CaMPARI Imaging

Post-synaptic neuron CaMPARI2 imaging: For assessing cold-evoked Ca^{2+} responses of sensory neurons and CIII md neuron downstream neurons, we utilized CaMPARI2, which upon photoconverting light and high intracellular Ca^{2+} stably photoconverts fluorescence from green to red. We performed the cold plate assay as described above. For stimulus condition, the Peltier plate was set to noxious cold (6°C) temperature and for control condition, the Peltier plate was turned off (room temperature). We placed individual third instar larvae onto the Peltier plate and simultaneously exposed the animal to photoconverting light for 20 seconds. CaMPARI2 fluorescence was imaged in live, intact larvae via confocal microscopy. Zeiss LSM 780 Axio examiner microscope, Plan-Apochromat 20x objective, and excitation wavelengths of 561nm and 488nm were used to image larval ventral nerve cord or peripheral sensory neurons. We mounted live intact larva onto microscope slide and immobilized the larva by placing a coverslip, as previously described (Im et al., 2018 [DOI](#); Patel & Cox, 2017 [DOI](#); Patel et al., 2022 [DOI](#); Turner et al., 2016 [DOI](#)). Three dimensional CaMPARI2 fluorescence in ventral nerve cord localized downstream neurons was imaged at 607.28μm x 607.28μm (XY resolution) and 2μm z-slices. Regions of interests were identified and area normalized fluorescence intensity for red & green signals were obtained via Imaris 9.5 software. CaMPARI responses are reported as ratio of $F_{\text{red}}/F_{\text{green}}$. Statistical comparisons: Parametric data – Welch’s t-test and non-parametric data – Mann-Whitney test.

PNS CaMPARI2 imaging: Sensory neuron CaMPARI2 responses were analyzed using custom FIJI macros that automatically detected cell bodies and shall intensity analyses were performed using semi-automated custom FIJI macros (<https://github.com/CoxLabGSU/CaMPARI-intensity-and-cold-plate->

Cell body analysis – We created a set of three sequential macros that draw ROIs around the cell body, user verification of the ROIs and lastly quantification of fluorescence intensities. The first custom FIJI script generates maximum intensity projections of z-stacks, image masks were created by thresholding (Moments method) GFP signal, and next, background and dendritic branches removed using erode and dilate functions. At the end of background clearing only cell bodies remain, where Analyze particle function is used to draw ROIs around soma. The second FIJI script is used for manual verification of each ROI and manually redrawing any incorrect ROIs. Lastly, upon ROI verification, area normalized F_{red} and F_{green} intensities are quantified. As previously described (Fosque et al., 2015; Im et al., 2018; Patel & Cox, 2017; Patel et al., 2022; Turner et al., 2016), we report evoked photoconverted CaMPARI signal as $F_{\text{red}}/F_{\text{green}}$ ratio. Statistical comparisons: Non-parametric data – Mann-Whitney test.

Sholl intensity analysis – Sholl intensity analysis was performed using a set of two custom FIJI scripts as previously described (Patel et al., 2022). Briefly, we first perform background clearing by manually thresholding the GFP signal and select all branches and soma of neuron of interest using the “Wand Tool” in FIJI and then a mask of neuron of interest is created. The second FIJI script draws five-pixel wide radial ROIs at a single pixel interval, here only the dendrites and soma from the neuron of interest are selected at each radial interval. After Sholl ROIs are drawn, area and area normalized F_{red} and F_{green} fluorescence intensities are extracted for each radial step away from the soma. Similar to CaMPARI cell body analysis, we CaMPARI2 signal as $F_{\text{red}}/F_{\text{green}}$ ratios away from the soma.

Ventral nerve cord CaMPARI imaging: We utilized Pan-neural ($R57C10^{GAL4}>CaMPARI$) driver to visualize ventral nerve cord Ca^{2+} responses to various stimuli including innocuous touch, noxious heat (45°C) and noxious cold (6°C). Stimulus and photoconverting light were delivered for 20 seconds. Whole ventral nerve cord was imaged at 607.28μm x 607.28μm (XY resolution) and 2μm z-slices. The rest of the stimulus delivery and imaging was similar to previously described CaMPARI experiments. No statistical analyses were performed.

CIII activation and second order neuron GCaMP imaging

CIII md neuron-evoked responses in downstream neurons were evaluated by using optogenetics and GCaMP. We expressed *lexAop-CsCrimson* in CIII md neurons using $R83B04^{lexA}$ and used downstream neuron specific *GAL4* to drive expression of *GCaMP6m*. For experimental condition, all adult animals in the genetic cross were reared in ATR (1500μM) supplemented food and subsequently F1 progeny were also raised in food containing ATR. For control condition, adult flies and F1 progeny were raised in standard cornmeal-molasses-agar diet. Both control and experimental crosses were reared in 24hr dark. We mounted live intact third instar larva in between microscope slide and a coverslip. Larval ventral nerve cord and cells of interest were located using epifluorescence on Zeiss LSM 780 confocal microscope. Larval GCaMP6 responses were allowed to return to baseline for at least 2 minutes. Time-lapse acquisition was imaged at 250.06μm x 250.06μm x 307.2ms using 488nm laser wavelength. CIII md neural activation was performed using two oblique 617nm leds (Thorlabs M617F2 and M79L01) that were manually operated using Thorlabs led controller (Thorlabs DC4100 and DC4100-hub). We performed three sequential neural activations using the following paradigm was used: Baseline light off (30 seconds)-> neural activation (617nm for 15 seconds)-> light off (30 seconds)-> neural activation (617nm for 15 seconds)-> light off (30 seconds)-> neural activation (617nm for 15 seconds)-> light off (30 seconds). Time-lapse videos were stabilized using Stack reg – Rigid transformation in FIJI (Linkert et al., 2010; Schindelin et al., 2012; Thevenaz et al., 1998). Regions of interest were manually drawn and area normalized GCaMP fluorescence over time was exported. We report changes in GCaMP6m fluorescence as $\Delta F/F = (F - F_{\text{prestimulus}}) / F_{\text{prestimulus}} * 100$ and max $\Delta F/F$ for each of the three neural activation epochs. Statistical comparisons: Parametric data – Welch’s t-test and non-parametric data – Mann-Whitney test.

Statistics and data visualization

Statistical analyses were performed using *r* (Fisher's exact test) and GraphPad Prism. All graphical visualization of the data were created using Prism GraphPad. Details on specific statistical tests are listed in the respective methods section.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization: AAP and DNC. Methodology: AAP, and DNC. Cold-plate assays: AAP; Optogenetics, AAP; Calcium imaging: AAP. Statistics and other formal analyses: AAP. EM Connectome analysis: AAP and AC. Writing – Original Draft: AAP and DNC; Writing – Review & Editing: AAP, AC and DNC. Visualization: AAP. Supervision: DNC. Funding acquisition: DNC.

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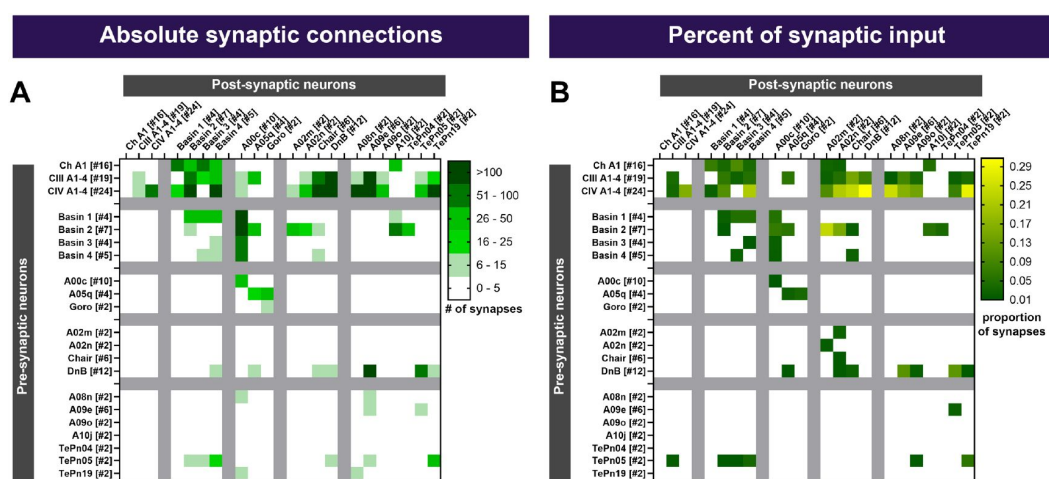


Figure 1-figure supplement 1

Sensory neuron connectivity matrix.

Synaptic connectivity matrix for neurons whose role in cold nociception was assessed. The number in brackets indicates the total number of neurons analyzed for each cell-type. **(A)** Absolute number of synaptic connections between pre- and post-synaptic neurons. **(B)** Connectivity represented as proportion of synaptic input to the post-synaptic neurons.

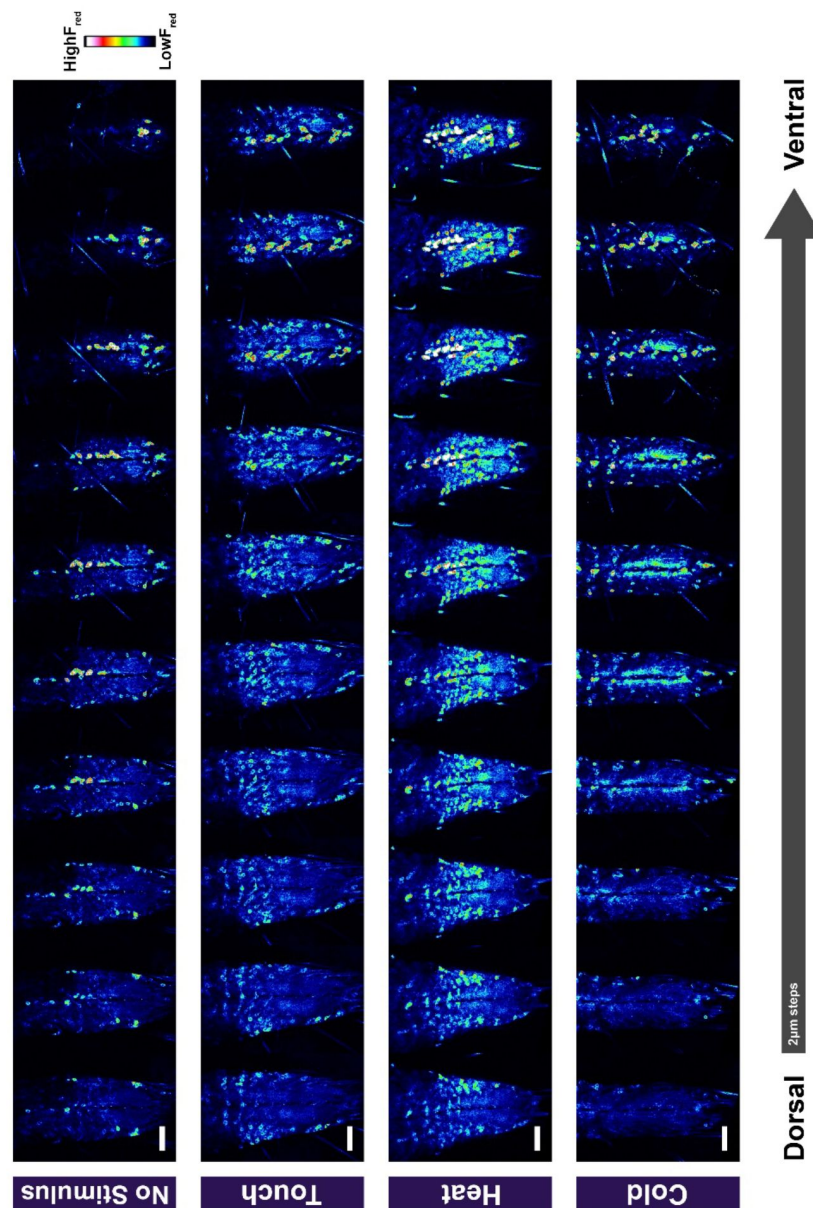


Figure 1-figure supplement 2

Stimulus evoked calcium responses of *Drosophila* larval ventral nerve cord.

Representative images of *Drosophila* larval *in vivo* intact animal ventral nerve cord calcium responses assessed via pan-neural CaMPARI expression. Freely moving larvae were exposed innocuous touch, noxious heat (45°C) or noxious cold (6°C) stimulus for 20 seconds and simultaneously exposed to 20 seconds of photo-converting light. Z-stacks of ventral nerve cord are shown with 2µm steps. Briefly, all animals were from the same batch, imaging was conducted using the same settings, and larvae were exposed to same levels of photo-converting light. For better visualization, only photoconverted red CaMPARI fluorescence is reported, and images were pseudo-colored for enhancing signal-to-noise ratio, where highest intensity represented as white and lowest intensity with dark blue. Scale bar represents 50µm.

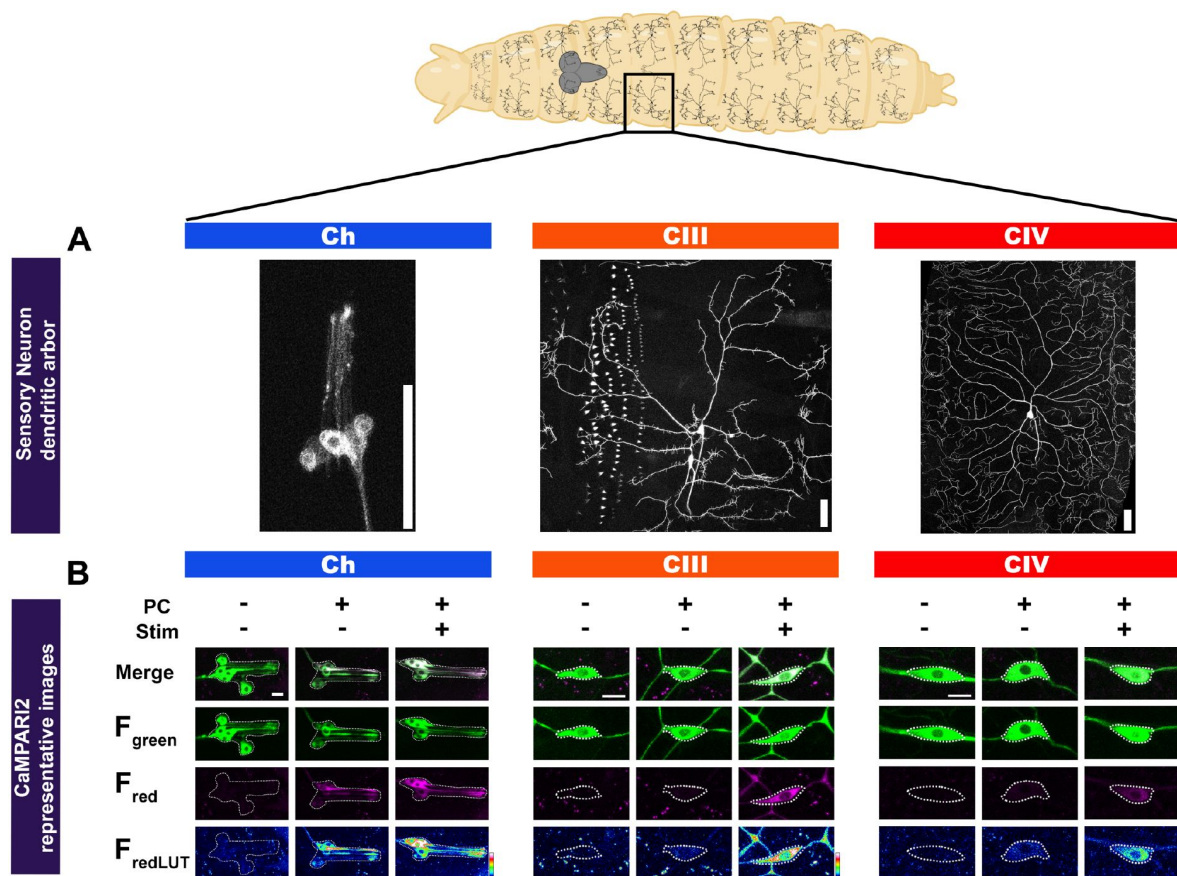


Figure 1-figure supplement 3

Somatosensory neural dendritic morphology and representative images of sensory neurons expressing CaMPARI2.

(A) Representative images of somatosensory neurons (Ch, CIII md and CIV md). Note, all images have separate magnification. Each image's scale bar represents 50µm. Drosophila larva and larval brain graphic was created with BioRender.com. (B) Confocal images of chordotonal (Ch: *IAV^{GAL4}*), class III md (CIII: *19-12^{GAL4}*) and class IV md (CIV: *ppk^{GAL4}*) neurons expressing CaMPARI2. There were three conditions for sensory neurons: No photoconversion control (no stimulus (Stim) and no photoconversion (PC)), photoconversion control (photoconversion and no stimulus) and stimulus condition (photoconversion and 6°C stimulus). Top row shows merge of F_{red} and F_{green}, second row is F_{green}, third row is F_{red} and last row contains F_{redLUT}, which is pseudo-colored with highest intensity being white and lowest intensity being dark blue (color scale bar on bottom right). Cell body and dendrites are outlined in white for Ch and cell body are outline in white for CIII and CIV neurons. Scale bar represents 10µm.

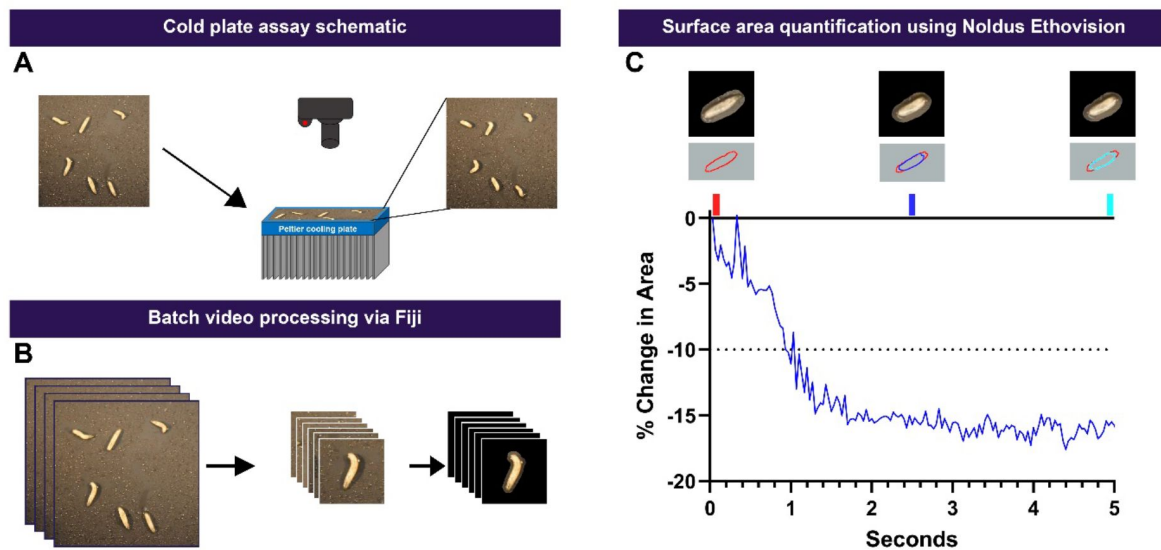


Figure 1-figure supplement 4

***Drosophila* larval cold plate assay and quantitative analysis.**

(**A**) A schematic of cold plate assay. Briefly, 3rd instar *Drosophila* larvae are plated on a thin metal plate, then we expose the larvae to noxious temperature by transferring the plate onto pre-chilled Peltier cold plate. (**B**) Behavior videos are automatically processed using custom macros in Fiji, where behavioral videos are cropped, and background is removed to improve quantitative analysis. (**C**) Larval surface area is measured using Noldus Ethovision. The following larval cold evoked contraction (CT) behavioral metrics are calculated using r: Instantaneous CT%, cumulative CT%, CT duration and CT magnitude. We define CT behavioral response as a reduction in surface area less than -10%.

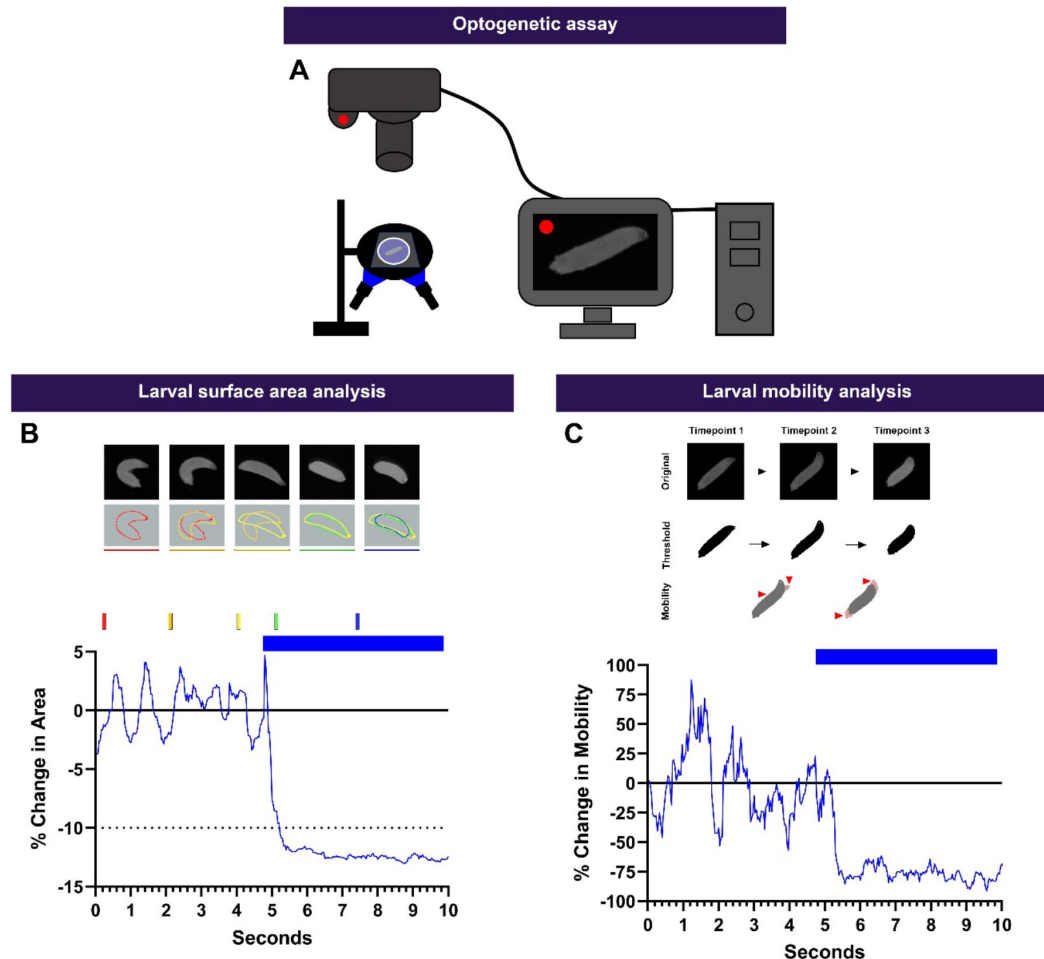


Figure 1-figure supplement 5

***Drosophila* larval neural activation assays using optogenetics.**

To perform neural activation experiments, we created a custom built optogenetic experimental setup, which has a very high spatial resolution and signal to noise ratio by using principles of dark field illumination. **(A)** Schematic of custom optogenetic rig, where stimulus and video recording are controlled via computer using Noldus Ehtovision. Individual *Drosophila* larva are plated on clear glass plate and illuminated from below with white light. Neuron activating blue light is also delivered from the below. Individual behavioral videos are automatically cropped and stabilized using custom Fiji macros. **(B-C)** We measured two variables *Drosophila* larval surface area and mobility. **(B)** Top, image stills from various timepoints before and during optogenetic stimulation of cold sensitive CIII md neurons resulting in contraction (CT) behavioral response. Bottom, percent change in area over time of an individual animal. During baseline, larval locomotion and turns results brief changes in surface area ($\pm 2-4\%$). Whereas upon neural stimulation, there is a distinct lasting reduction in surface area of less than -10% . **(C)** Larval mobility refers to changes in larval postures as measured by changes in occupied space. Since the amount of larval mobility is a function of various intrinsic and extrinsic factors, we normalized larval mobility to baseline period, where there was no neural activation. Top, conceptual framework of how larval mobility is measured. Original videos are motion stabilized in the XY axis and thresholded. Next, we perform image calculations ($\text{Mobility} = \text{Frame}_{\text{timepoint 2}} - \text{Frame}_{\text{timepoint 1}}$) to get just the red shaded portions denoting changes in postural locations (red arrowheads). *Drosophila* larval time series mobility data are reported as percent change in mobility, time spent being immobile and percent of immobile animals, or for a genotype percent of animals that are immobile over time, immobility is defined as -25% or more reduction in mobility.

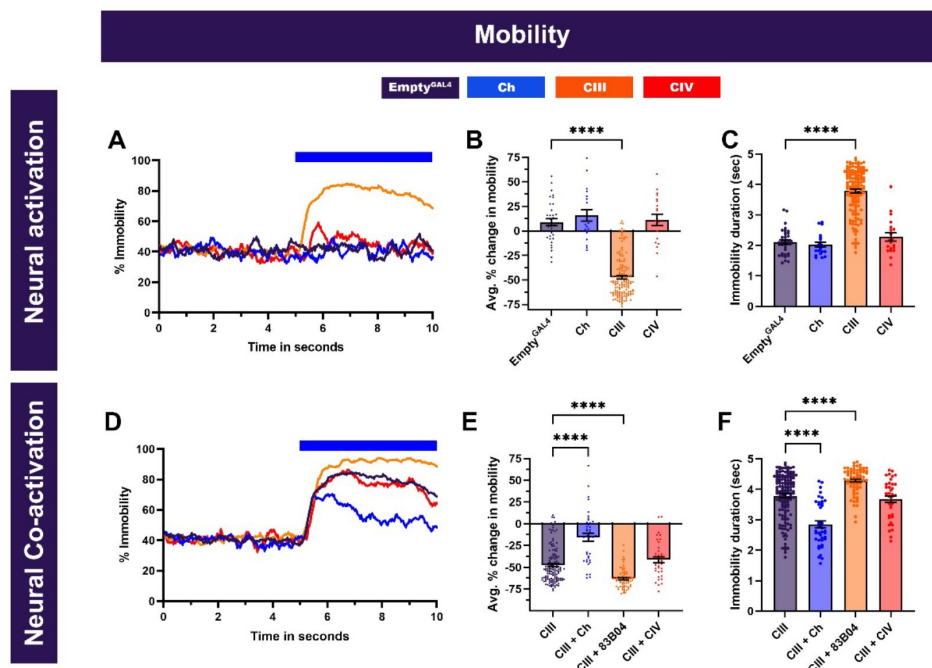


Figure 1-figure supplement 6

***Drosophila* larval mobility pipeline and effects of neural activation and co-activation of sensory neurons on larval mobility**

(A-F) *Drosophila* larval mobility following somatosensory neuron optogenetic activation (A-C) and CIII md neuron plus co-activation of Ch, additional CIII *GAL4* driver (*R83B04*^{GAL4}) or CIV md neurons (D-F). (A, D) Instantaneous percent immobility. Blue bar represents optogenetic neural activation. (B, E) Average percent change in mobility for each genotype, where greater percent immobility results in larger changes in average mobility. (C, F) Immobility duration in seconds during stimulation. Neural activation: Empty^{GAL4} n=35. Ch n=20, CIV n=20. & CIII n=143. Neural co-activation: CIII n=143 and average experimental n=50. Significant differences indicated via asterisks, where ****p<0.0001.

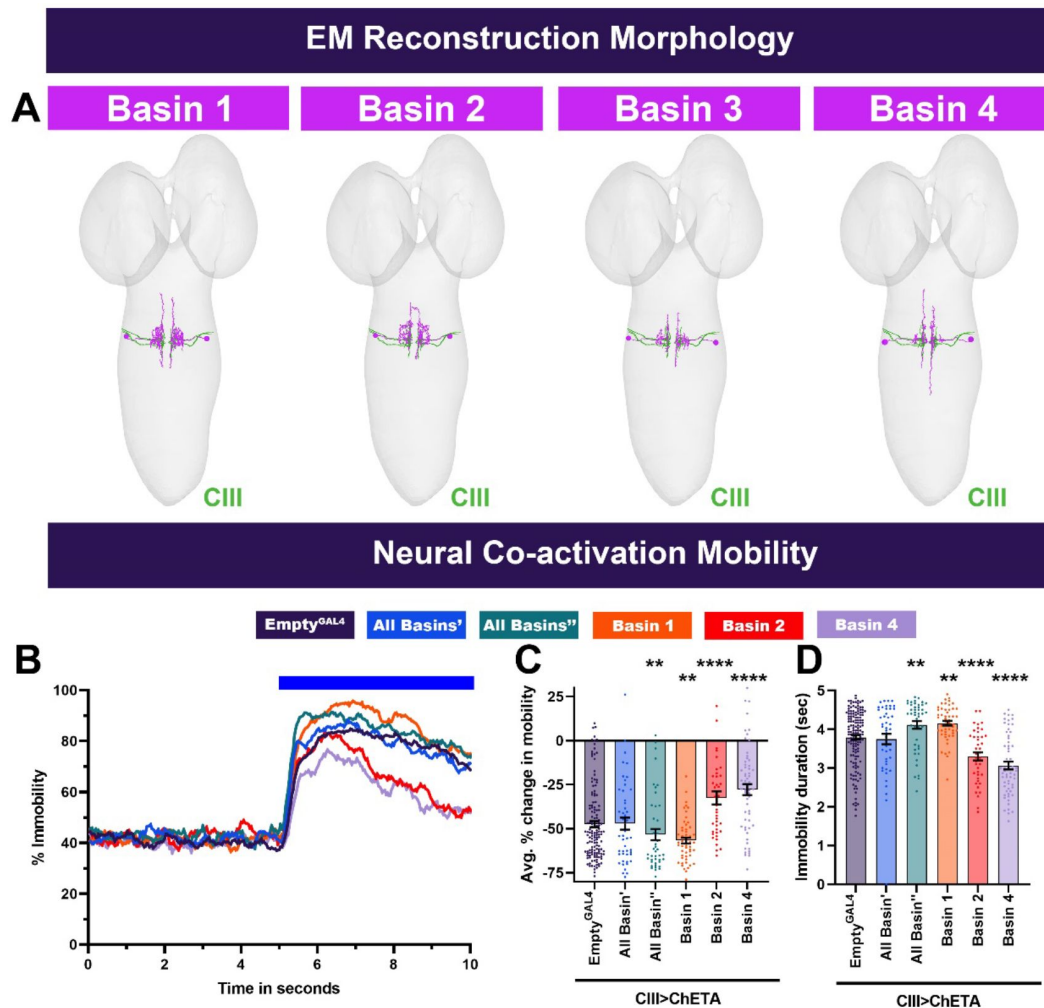


Figure 2-figure supplement 1

Neural reconstructions and larval mobility for multisensory integrator neurons.

(A) Individual Basin neuron subtypes (magenta) and CIII md neuron axons (blue). Neural reconstruction data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-D) *Drosophila* larval mobility for Basin plus CIII md neuron optogenetic co-activation. (B) Instantaneous percent immobility. Blue bar represents optogenetic neural activation. (C) Average percent change in mobility for each genotype, where greater percent immobility results in larger changes in average mobility. (D) Immobility duration in seconds during stimulation. *Empty^{GAL4}* *n*=143 and experimental condition *n*=49. Comparisons to *CIII md + Empty^{GAL4}>ChETA*. Significant differences indicated via asterisks, where ***p*<0.01, and *****p*<0.0001.

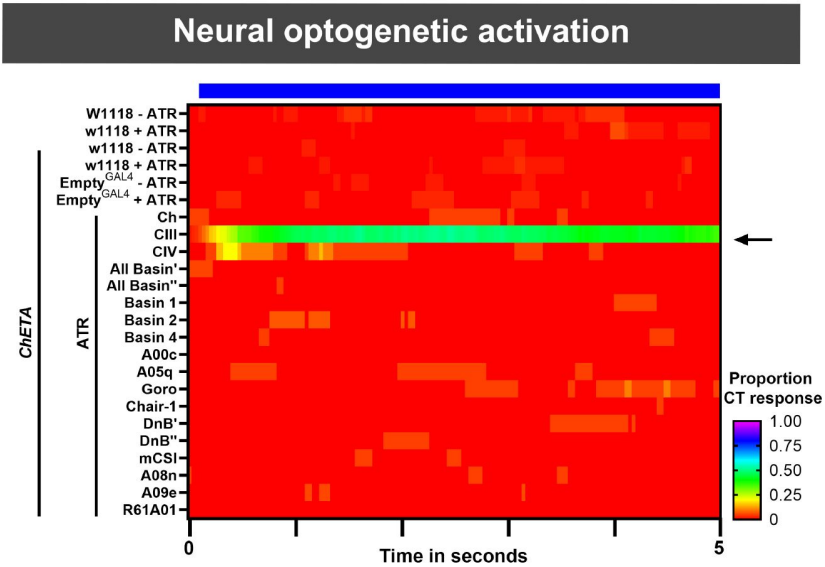


Figure 2-figure supplement 2

Optogenetic activation of individual neuronal cell-types.

Heatmap represents optogenetically evoked instantaneous contraction (CT) proportions of *Drosophila* larvae. Individual neuronal cell-types were optogenetically activated using cell-type specific expression of *ChETA*. Black arrow indicates optogenetic activation of primary cold somatosensory CIII md neurons, which are the only the genotype with high proportions CT. Average n= 31.

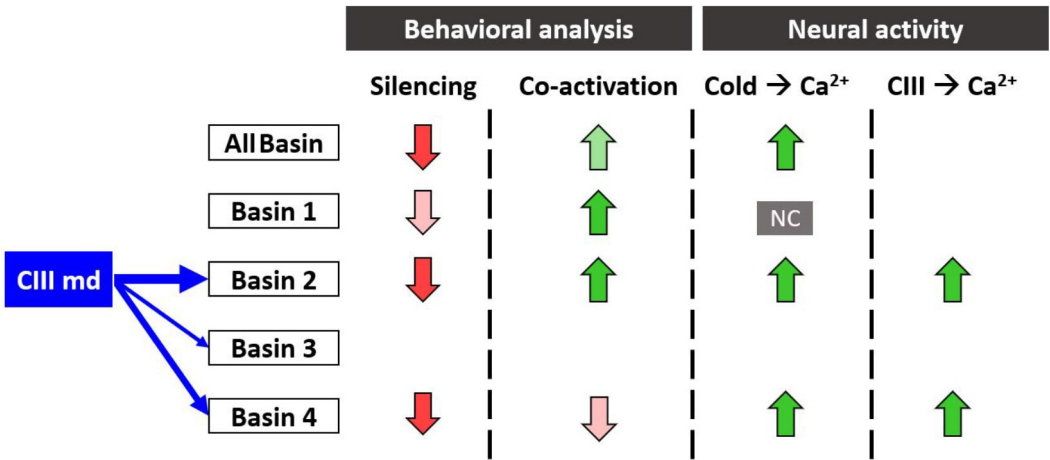


Figure 3-figure supplement 1

Summary of behavioral and functional roles of multisensory integrators in cold nociception.

Blue arrows indicate strength of synaptic connectivity between the CIII md neurons and second order neurons. In behavioral analysis column, the arrow direction indicates reduction or enhancement of CT response and shading indicates magnitude of change from control. In neural activity column: NC denotes no significant change in Ca²⁺ response, the arrow direction indicates reduction or enhancement in evoked Ca²⁺ response and shading indicates magnitude of change from control. Empty spaces indicate experimental analyses were not performed.

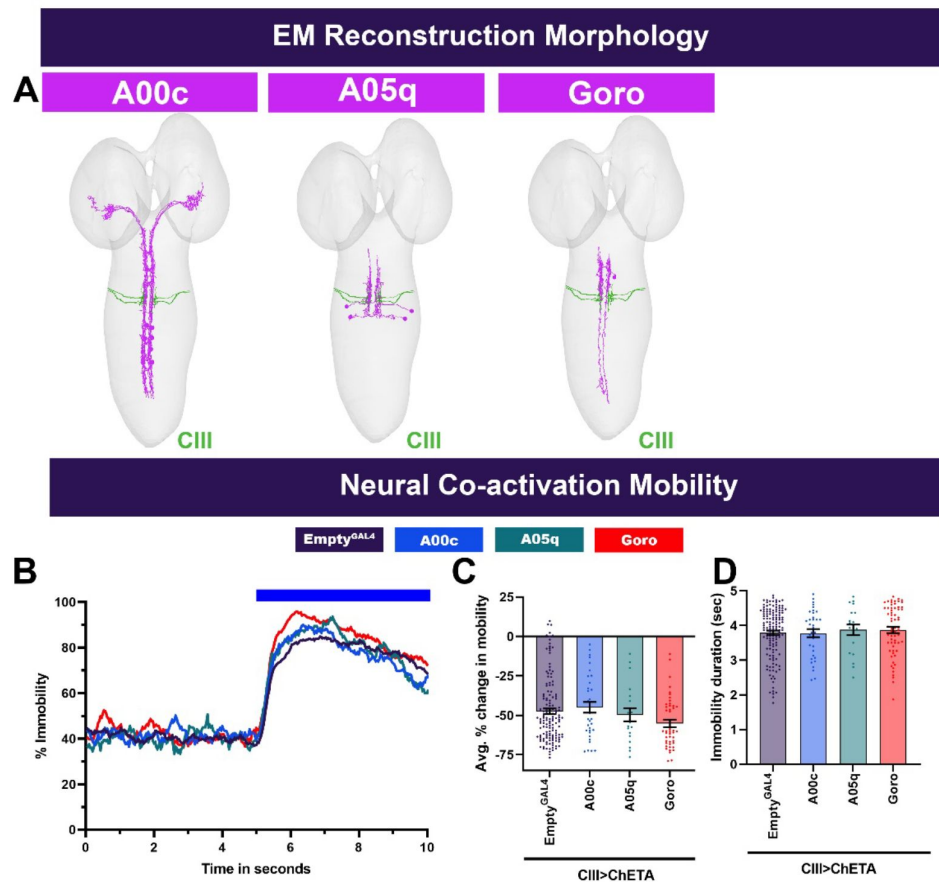


Figure 4-figure supplement 1

Neural reconstructions and larval mobility for A00c, A05q, and Goro neurons.

(A) Individual interneuron subtypes (magenta) and CIII md neuron axons (blue). Neural reconstruction data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-D) *Drosophila* larval mobility for A00c, A05q or Goro neurons plus CIII md neuron optogenetic coactivation. (B) Instantaneous percent immobility. Blue bar represents optogenetic neural activation. (C) Average percent change in mobility for each genotype, where greater percent immobility results in larger changes in average mobility. (D) Immobility duration in seconds during stimulation. Significant stars represent $p < 0.05$, where stars represent comparison to CIII md + Empty^{GAL4}>ChETA. Empty^{GAL4} $n = 143$ and experimental condition average $n = 33$.

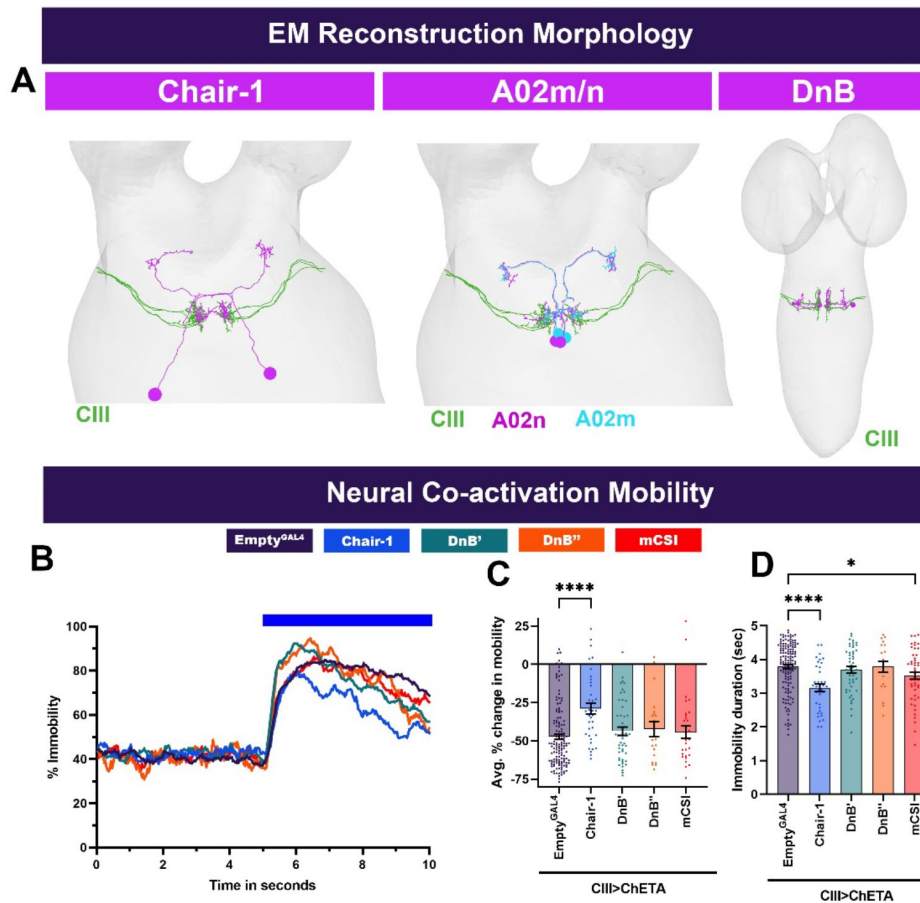


Figure 5-figure supplement 1

Neural reconstructions and larval mobility for premotor neurons.

(A) Individual premotor neuron subtypes (magenta/green) and CIII md neuronal axons (blue). Neural reconstruction data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-D) *Drosophila* larval mobility observed with premotor neurons plus CIII md neuron optogenetic coactivation. (B) Instantaneous percent immobility. Blue bar represents optogenetic neural activation. (C) Average percent change in mobility for each genotype, where greater percent immobility results in larger changes in average mobility. (D) Immobility duration in seconds during stimulation. *Empty^{GAL4}* n=143 and experimental condition average n=35. Comparison to *CIII md + Empty^{GAL4}>ChETA*. Significant differences indicated via asterisks, where *p<0.05, and ****p<0.0001.

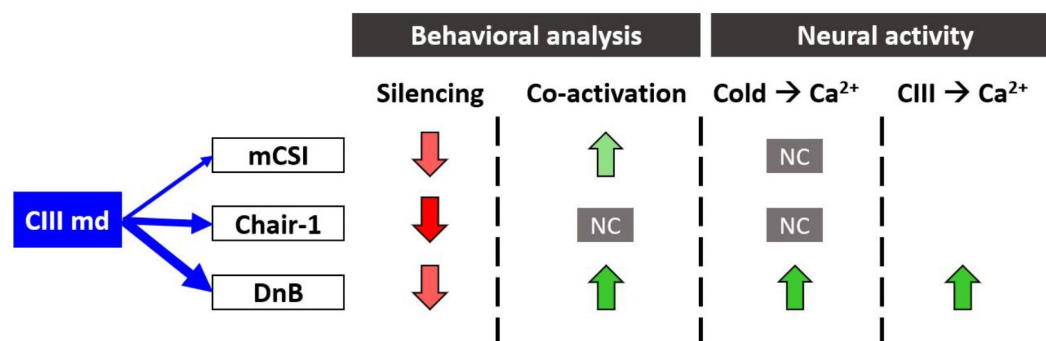


Figure 6-figure supplement 1

Summary of behavioral and functional roles of premotor neurons in cold nociception.

Blue arrows indicate strength of synaptic connectivity between the CIII md neurons and second order neurons. In behavioral analysis column, the arrow direction indicates reduction or enhancement of CT response and shading indicates magnitude of change from control. In neural activity column: NC denotes no significant change in Ca²⁺ response, the arrow direction indicates reduction or enhancement in evoked Ca²⁺ response and shading indicates magnitude of change from control. Empty spaces indicate experimental analyses were not performed.

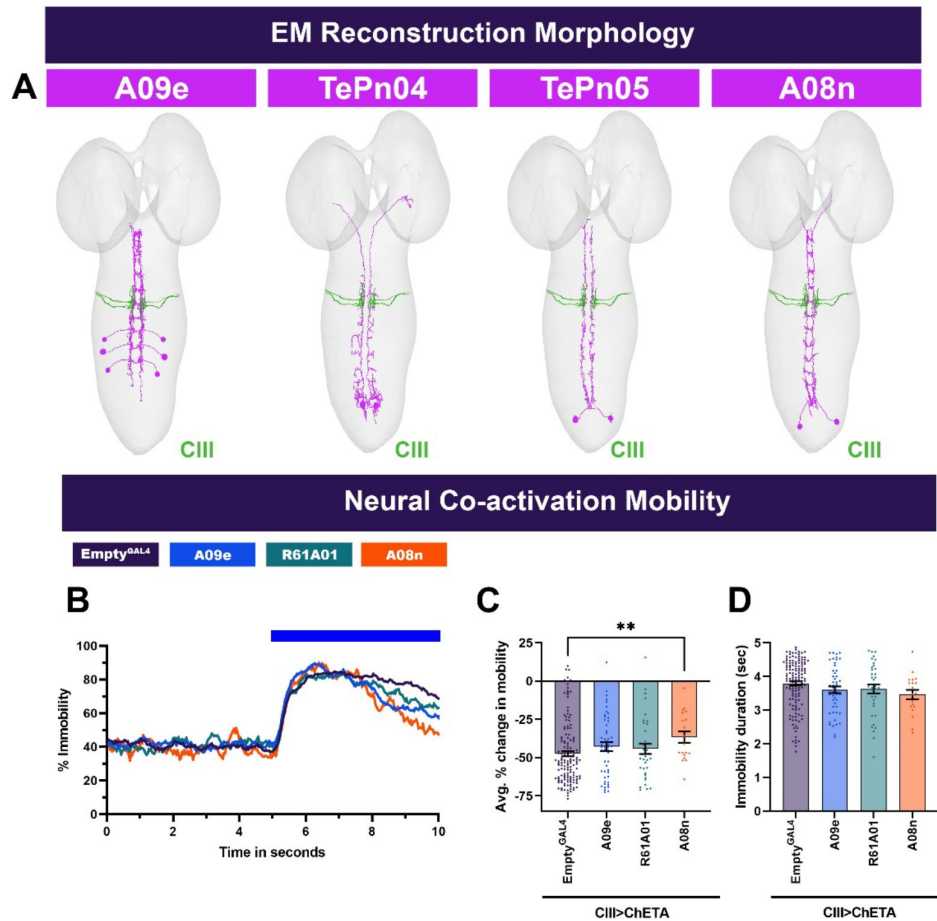


Figure 7-figure supplement 1

Neural reconstructions and larval mobility for projection neurons.

(A) Individual projection neuron subtypes (magenta) and CIII md neuron axons (blue). Neural reconstruction data was extracted from Neurophyla LMB Cambridge. (<https://neurophyla.mrc-lmb.cam.ac.uk/>). (B-D) *Drosophila* larval mobility observed with projection neurons plus CIII md neuron optogenetic coactivation. (B) Instantaneous percent immobility. Blue bar represents optogenetic neural activation. (C) Average percent change in mobility for each genotype, where greater percent immobility results in larger changes in average mobility. (D) Immobility duration in seconds during stimulation. Significant stars represent $p < 0.05$, where stars represent comparison to *CIII md + Empty^{GAL4} > ChETA*. Empty^{GAL4} $n = 143$ and experimental condition average $n = 35$. Significant differences indicated via asterisks, where $**p < 0.01$.

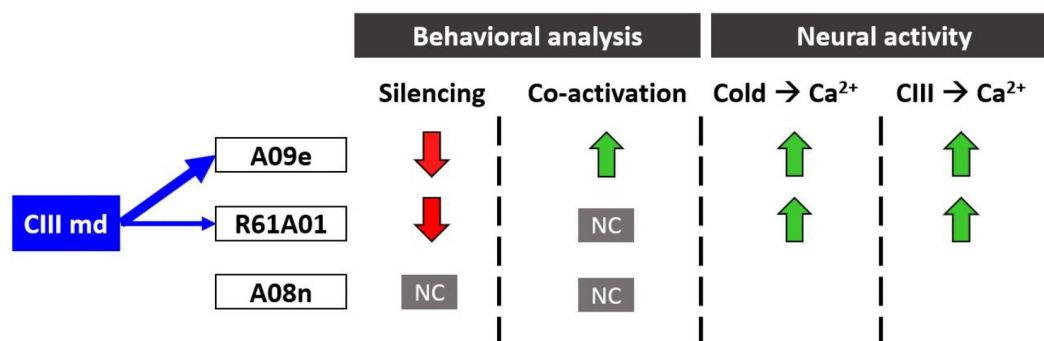


Figure 8-figure supplement 1

Summary of behavioral and functional roles of projection neurons in cold nociception.

Blue arrows indicate strength of synaptic connectivity between the CIII md neurons and second order neurons. In behavioral analysis column, the arrow direction indicates reduction or enhancement of CT response and shading indicates magnitude of change from control. In neural activity column: NC denotes no significant change in Ca²⁺ response, the arrow direction indicates reduction or enhancement in evoked Ca²⁺ response and shading indicates magnitude of change from control. Empty spaces indicate experimental analyses were not performed.

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Editors

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Reviewer #1 (Public Review):

Summary. The authors goal was to map the neural circuitry underlying cold sensitive contraction in *Drosophila*. The circuitry underlying most sensory modalities has been characterized but noxious cold sensory circuitry has not been well studied. The authors achieve their goal and map out sensory and post-sensory neurons involved in this behavior.

Strengths. The manuscript provides convincing evidence for sensory and post sensory neurons involved in noxious cold sensitive behavior. They use both connectivity data and functional data to identify these neurons. This work is a clear advance in our understanding of noxious cold behavior. The experiments are done with a high degree of experimental rigor.

Positive comments

-Campari is nicely done to map cold responsive neurons, although it doesn't give data on individual neurons.

-Chrimson and TNT experiments are nicely done.

-Cold temperature activates basin neurons, it's a solid and convincing result.

Weaknesses. Among the few weaknesses in this manuscript is the failure to trace the circuit from sensory neuron to motor neuron; and to ignore analysis of the muscles driving, cold induced contraction. Authors also need to elaborate more on the novel aspects of their work in the introduction or abstract.

Major comments.

-Class three sensory neuron connectivity is known, and role in cold response is known (turner 16, 18). Need to make it clearer what the novelty of the experiments are.

-Why focus on premotor neurons in mechano nociceptive pathways? Why not focus on PMNs innervating longitudinal muscles, likely involved in longitudinal larval contraction? Especially since chosen premotor neurons have only weak effects on cold induced contraction?

Reviewer #2 (Public Review):

Patel et al perform the analysis of neurons in a somatosensory network involved in responses to noxious cold in *Drosophila* larvae. Using a combination of behavioral experiments, Calcium imaging, optogenetics, and synaptic connectivity analysis in the *Drosophila* larval they assess the function of circuit elements in the somatosensory network downstream of multimodal somatosensory neurons involved in innocuous and noxious stimuli sensing and probe their function in noxious cold processing, Consistent with their previous findings they find the multidendritic class III neurons, to be the key cold sensing neurons that are both required and sufficient for the CT behaviors response (shown to evoked by noxious cold). They further investigate the downstream neurons identified based on literature and connectivity from EM at different stages of sensory processing characterize the different phenotypes upon activating/silencing those neurons and monitor their responses to noxious cold. The work reveals diverse phenotypes for the different neurons studied and provides the groundwork for understanding how information is processed in the nervous system from sensory input to motor output and how information from different modalities is processed by neuronal networks. However, at times the writing could be clearer and some results interpretations more rigorous.

Specific comments

1. In Figure 1 -supplement 6D-F (Cho co-activation)

The authors find that Ch neurons are cold sensitive and required for cold nociceptive behavior but do not facilitate behavioral responses induced but CIII neurons

The authors show that coactivating mdIII and cho inhibits the CT (a typically observed cold-induced behavioral response) in the second part of the stimulation period, while Cho was required for cold-induced CT. Different levels of activation of md III and Cho (different light intensities) could bring some insights into the observed phenotypes upon Cho manipulation as different levels activate different downstream networks that could correspond to different stimuli. Also, it would be interesting to activate chordotonal during exposure to cold to determine how a behavioral response to cold is affected by the activation of chordotonal sensory neurons.

1. Throughout the paper the co-activation experiments investigate whether co-activating the different candidate neurons and md III neurons facilitates the md III-induced CT response. However, the cold noxious stimuli will presumably activate different neurons downstream than optogenetic activation of MdIII and thus can reveal more accurately the role of the different candidate neurons in facilitating cold nociception.

2. Use of blue lights in behavioral and imaging experiments

Strong Blue and UV have been shown to activate MDIV neurons (Xiang, Y., Yuan, Q., Vogt, N. et al. Light-avoidance-mediating photoreceptors tile the *Drosophila* larval body wall. *Nature*

468, 921-926 (2010). <https://doi.org/10.1038/nature09576>) and some of the neurons tested receive input from MdIV. In their experiments, the authors used blue light to optogenetically activate CDIII neurons and then monitored Calcium responses in Basin neurons, premotor neurons, and ascending neurons and UV light is necessary for photoconversion in Campari Experiments. Therefore, some of the neurons monitored could be activated by blue light and not cdIII activation. Indeed, responses of Basin-4 neurons can be observed in the no ATR condition (Fig 3HI) and quite strong responses of DnB neurons. (Figure 6E) How do authors discern that the effects they see on the different neurons are indeed due to cold nociception and not the synergy of cold and blue light responses could especially be the case for DNB that could have in facilitating the response to cold in a multisensory context (where mdIV are activated by light). In addition, the silencing of DNB neurons during cold stimulation does not seem to give very robust phenotypes (no significant CT decrease compared to empty GAL4 control).

It would be important to for example show that even in the absence of blue light the DNB facilitates the mdIII activation or cold-induced CT by using red light and Chrimson for example or TrpA activation (for coactivation with md III)

Alternatively, in some other cases, the phenotype upon co-activation could be inhibited by blue light (e.g. chair-1 (Figure 5 H-I))

More generally, given the multimodal nature of stimuli activating mdIV , MdIII (and Cho) and their shared downstream circuitry it is important to either control for using the blue light in these stimuli or take into account the presence of the stimulus in interpreting the results as the coactivation of for example Cho and mdIII using blue lights also could activate mdIV (and downstream neurons, alter the state of the network that could inhibit the md III induced CT responses

Assessing the differences in behavioral phenotypes in the different conditions could give an idea of the influence of combining different modalities in these assays. For example, did the authors observe any other behaviors upon co-activation of MDIII and Cho (at the expense of CT in the second part of the stimulation) or did the larvae resume crawling? Blue light typically induces reorientation behavior. What about when co-activating mdIII and Basin-4?

Using Chrimson and red light or TrpA in some key experiments e.g. with Cho, Basin-4, and DNB would clarify the implication of these neurons in cold nociception

1. Basins

- Page 17 line 442-3 "Neural silencing of all Basin (1-4) neurons, using two independent driver lines (R72F11GAL4 and R57F07GAL4)

Did the authors check the expression profile of the R57F07 line that they use to probe "all basins"? The expression profile published previously (Ohyama et al, 2015, extended data) shows one basin neuron (identified as basin-4) and some neurons in the brain lobes. Also, the split GAL4 that labels Basin-4 (SS00740) is the intersection between R72F11 and R57F07 neurons. Thus the R57F07 likely labels Basin-4 and if that is the case the data in Figure 2 9 and supplement) and Figure 3 related to this driver line, should be annotated as Basin-4, and the results and their interpretation modified to take into account the different phenotypes for all basins and Basin-4 neurons

Page 19 l. 521-525 I am confused by these sentences as the authors claim that Basin-4 showed reduced Calcium responses upon repetitive activation of CDIII md neurons but then they say they exhibit sensitization. Looking at the plots in FIG 3 F-I the Basin-4 responses upon repeated activation seem indeed to decrease on the second repetition compared to the first. What is the sensitization the authors refer to?

On Page 47-In this section of the discussion, the authors emit an interesting hypothesis that the Basin-1 neuron could modulate the gain of behavioral responses. While this is an interesting idea, I wonder what would be the explanation for the finding that co-activation of Cho and MDIII does not facilitate cold nociceptive responses. Would activation of Basin-1 facilitate the cold response in different contexts (in addition to CHO-mediated stimuli)?

Page 48 Thus the implication of the inhibitory network in cold processing should be better contextualized

The authors explain the difference in the lower basin-2 Ca²⁺ response to Cold/ mdIII activation (compared to Basin-4) despite stronger connectivity, due a stronger inputs from inhibitory neurons to Basin-2 (compared to Basin-4). The previously described inhibitory neurons that synapse onto Basin-2 receive rather a small fraction of inputs from the class III sensory neurons. The differences in response to cold could be potentially assigned to the activation of the inhibitory neurons by the cold-sensing cho- neurons. However, that cannot explain the differences in responses induced by class III neurons. Do the authors refer to additional inhibitory neurons that would receive significant input from MdIII?

Alternative explanations could exist for this difference in activation: electrical synapses from mdII I onto Basin-4, and by stronger inputs from mdIV (compared to Basin-2 in the case of responses to Cold stimulus (Cold induces responses in md IV sensory neurons). Different subtypes of CD III may differentially respond to cold and the cold-sensing ones could synapse preferentially on basin-4 etc.

1. A00c

Page 26 Figure 4F-I line While Goro may not be involved in cold nociception the A00c (and A05q) seems to be.

A00c could convey information to other neurons other than Goro and thus be part of a pathway for cold-induced CT.

2. Page 31 766-768 the conclusion that "premotor function is required for and can facilitate cold nociception" seems odd to stress as one would assume that some premotor neurons would be involved in controlling the behavioral responses to a stimulus. It would be more pertinent in the summary to specify which premotor neurons are involved and what is their function

3. There are several Split GAL4 used in the study (with transgenes inserted in attP40 et attP2 site). A recent study points to a mutation related to attP40 that can have an effect on muscle function: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9750024/>. The controls used in behavioral experiments do not contain the attP40 site. It would be important to check a control genotype bearing an attP40 site and characterize the different parameters of the CT behavior to cold and take this into account in interpreting the results of the experiments using the Split-GAL4 lines

Reviewer #3 (Public Review):

Summary:

The authors follow up on prior studies where they have argued for the existence of cold nociception in *Drosophila* larvae. In the proposed pathway, mechanosensitive Class III multidendritic neurons are the noxious cold responding sensory cells. The current study attempts to explore the potential roles of second and third order neurons, based on information of the Class III neuron synaptic outputs that have been obtained from the larval connectome.

Strengths:

The major strength of the manuscript is the detailed discussion of the second and third order neurons that are downstream of the mechanosensory Class III multidendritic neurons. These will be useful in further studies of gentle touch mechanosensation and mechanonociception both of which rely on sensory input from these cells. Calcium imaging experiments on Class III activation with optogenetics support the wiring diagram.

Weaknesses:

The scientific premise is that a full body contraction in larvae that are exposed to noxious cold is a sensorimotor behavioral pathway. This premise is, to start with, questionable. A common definition of behavior is a set of "orderly movements with recognizable and repeatable patterns of activity produced by members of a species (Baker et al., 2001)." In the case of nociception behaviors, the patterns of movement are typically thought to play a protective role and to protect from potential tissue damage.

Does noxious cold elicit a set of orderly movements with a recognizable and repeatable pattern in larvae? Can the patterns of movement that are stimulated by noxious cold allow the larvae to escape harm? Based on the available evidence, the answer to both questions is seemingly no. In response to noxious cold stimulation many, if not all, of the muscles in the larva, simultaneously contract (Turner et al., 2016), and as a result the larva becomes stationary. In response to cold, the larva is literally "frozen" in place and it is incapable of moving away. This incapacitation by cold is the antithesis of what one might expect from a behavior that protects the animals from harm.

Extensive literature has investigated the physiological responses of insects to cold (reviewed in Overgaard and MacMillan, 2017). In numerous studies of insects across many genera (excluding cold adapted insects such as snow flies), exposure to very cold temperatures quickly incapacitates the animal and induces a state that is known as a chill coma. During a chill coma, the insect becomes immobilized by the cold exposure, but if the exposure to cold is very brief the insect can often be revived without apparent damage. Indeed, it is common practice for many laboratories that use adult *Drosophila* for studies of behavior to use a brief chilling on ice as a form of anesthesia because chilling is less disruptive to subsequent behaviors than the more commonly used carbon dioxide anesthesia. If flies were to perceive cold as a noxious nociceptive stimulus, then this "chill coma" procedure would likely be disruptive to behavioral studies but is not. Furthermore, there is no evidence to suggest that larval sensation of "noxious cold" is aversive.

The insect chill coma literature has investigated the effects of extreme cold on the physiology of nerves and muscles and the consensus view of the field is that the paralysis that results from cold is due to complex and combined action of direct effects of cold on muscle and on nerves (Overgaard and MacMillan, 2017). Electrophysiological measurements of muscles and neurons find that they are initially depolarized by cold, and after prolonged cold exposure they are unable to maintain potassium homeostasis and this eventually inhibits the firing of action potentials (Overgaard and MacMillan, 2017). The very small thermal capacitance of a *Drosophila* larva means that its entire neuromuscular system will be quickly exposed to the effect of cold in the behavioral assays under consideration here. It would seem impossible to disentangle the emergent properties of a complex combination of effects on physiology (including neuronal, glial, and muscle homeostasis) on any proposed sensorimotor transformation pathway.

Nevertheless, the manuscript before us makes a courageous attempt at attempting this. A number of GAL4 drivers tested in the paper are found to affect parameters of contraction behavior (CT) in cold exposed larvae in silencing experiments. However, notably absent from all of the silencing experiments are measurements of larval mobility following cold exposure. Thus, it is not known from the study if these manipulations are truly protecting the larvae

from paralysis following cold exposure, or if they are simply reducing the magnitude of the initial muscle contraction that occurs immediately following cold (ie reducing CT). The strongest effect of silencing occurs with the 19-12-GAL4 driver which targets Class III neurons (but is not completely specific to these cells).

Optogenetic experiments for Class III neurons relying on the 19-12-GAL4 driver combined with a very strong optogenetic actuator (ChETA) show the CT behavior that was reported in prior studies. It should be noted that this actuator drives very strong activation, and other studies with milder optogenetic stimulation of Class III neurons have shown that these cells produce behavioral responses that resemble gentle touch responses (Tsubouchi et al 2012 and Yan et al 2013). As well, these neurons express mechanoreceptor ion channels such as NompC and Rpk that are required for gentle touch responses. The latter makes the reported Calcium responses to cold difficult to interpret in light of the fact that the strong muscle contractions driven by cold may actually be driving mechanosensory responses in these cells (ie through deformation of the mechanosensitive dendrites). Are the cIII calcium signals still observed in a preparation where cold induced muscle contractions are prevented?

A major weakness of the study is that none of the second or third order neurons (that are downstream of CIII neurons) are found to trigger the CT behavioral responses even when strongly activated with the ChETA actuator (Figure 2 Supplement 2). These findings raise major concerns for this and prior studies and it does not support the hypothesis that the CIII neurons drive the CT behaviors.

Later experiments in the paper that investigate strong CIII activation (with ChETA) in combination with other second and third order neurons does support the idea activating those neurons can facilitate body-wide muscle contractions. But many of the co-activated cells in question are either repeated in each abdominal neuromere or they project to cells that are found all along the ventral nerve cord, so it is therefore unsurprising that their activation would contribute to what appears to be a non-specific body-wide activation of muscles along the AP axis. Also, if these neurons are already downstream of the CIII neurons the logic of this co-activation approach is not particularly clear. A more convincing experiment would be to silence the different classes of cells in the context of the optogenetic activation of CIII neurons to test for a block of the effects, a set of experiments that is notably absent from the study.

The authors argument that the co-activation studies support "a population code" for cold nociception is a very optimistic interpretation of a brute force optogenetics approach that ultimately results in an enhancement of a relatively non-specific body-wide muscle convulsion.

Author Response

We thank the reviewers for their suggestions in improving the manuscript. We are currently working on a formal revision and plan to submit a revised manuscript in the near future. However, we would be remiss, if we did not address concerns regarding the conceptual merits of the paper. Below we speak to major points of note that address select reviewer comments and the eLife assessment of our manuscript.

eLife assessment:

However, the strength of evidence is incomplete due to the concern that larval contraction is a result of chilling the nervous system and muscles, which causes spreading depolarization and mechanical contraction of the body, rather than an active sensorimotor response to cold.

Reviewer #3:

The scientific premise is that a full body contraction in larvae that are exposed to noxious cold is a sensorimotor behavioral pathway. This premise is, to start with, questionable. A common definition of behavior is a set of "orderly movements with recognizable and repeatable patterns of activity produced by members of a species (Baker et al., 2001)." In the case of nociception behaviors, the patterns of movement are typically thought to play a protective role and to protect from potential tissue damage.

Does noxious cold elicit a set of orderly movements with a recognizable and repeatable pattern in larvae? Can the patterns of movement that are stimulated by noxious cold allow the larvae to escape harm? Based on the available evidence, the answer to both questions is seemingly no.

We thank the reviewer for their questions and clarify, here. Exposure to cold temperatures does elicit a recognizable and repeatable pattern of behavior across multiple strains, including both wildtype and genetic control strains (w1118, Oregon R) and numerous control conditions that have been previously published (Himmel et al., 2021, Himmel et al., 2023, Patel et al., 2022, Turner et al., 2016, Turner et al., 2018, Tenedini et al., 2019). Our initial publication on *Drosophila* cold nociception demonstrated a variety of cold-evoked behavior responses including head and/or tail raising of the larva as well as contraction behavior. These behaviors were repeatedly observed in assays involving either local cold stimulation with a cold probe or global cold stimulation on a cold plate. Head and/or tail raise behaviors are consistent with behavior that displaces the larval body from the cold surface, however, exposure to increasingly colder temperatures leads to an increasing level of cold-evoked contraction (CT) responses which result in a reduction of larval area (Turner et al., 2016). Presumably, increasing the level of CIII md neuron activation leads to greater activation of downstream circuitry. We previously performed optogenetic dose response assays to further clarify the increased prevalence CT response to strong noxious cold stimuli and investigated how CIII md neurons discriminate between innocuous touch and noxious cold stimuli. Here, we found that lower-level activation of CIII md neurons lead to predominantly touch-evoked behaviors whereas high-level activation led predominantly to cold-evoked responses (Turner et al., 2016). These analyses were coupled with stimulus-evoked calcium imaging, which revealed that touch-evoked Ca^{2+} levels were significantly lower than cold-evoked Ca^{2+} levels (Turner et al., 2016).

In this manuscript, we confirm our previously published findings that neural silencing of CIII md neurons with either tetanus toxin expression or impairing action potential propagation results impaired cold-evoked CT responses (Turner et al., 2016, Turner et al., 2018). However, neural silencing of CIII md neurons did not eliminate cold-evoked CT responses. We interpret this finding as evidence that some component of cold-evoked CT response may be due to cold-induced muscle contraction. Furthermore, in this manuscript, we implicate the requirement of chordotonal (Ch) neurons in cold-evoked CT and demonstrate cold-evoked Ca^{2+} increases in Ch neurons. Furthermore, neural silencing of multiple sensory neuron types (CIII + Ch or CIII + CII) resulted in greater deficits in cold-evoked behaviors (Turner et al., 2016). Thus, the noxious cold stimulus is detected by multiple peripheral sensory neurons and inhibiting neural activity in CIII md neurons alone cannot eliminate cold-evoked CT responses.

In this manuscript and in several other publications, studies have shown that optogenetic activation of CIII md neurons, or CIII neurons plus CII neurons or Ch neurons elicits CT-like responses (Hwang et al., 2007, Shearin et al., 2013, Turner et al., 2016). Conversely, optogenetic stimulation of CIII md neurons knocked down for paralytic, the α -subunit of voltage-gated sodium channel, did not elicit blue light-evoked CT responses due to impaired action potential propagation. These analyses collectively indicate that CIII md neuron activation is sufficient for eliciting CT-like responses. Additionally, we have previously

published electrophysiological recordings of CIII md neurons under cold exposure. To address potential confounds of cold-induced muscle contraction on cold-induced electrical activity of CIII md neurons, we performed these analyses on de-muscle fillets revealing that CIII neural activity is not dependent upon muscles in response to cold. Exposure to noxious cold stimuli results in temperature-dependent increases in CIII neuron firing pattern consisting of both bursting and tonic firing (Himmel et al., 2021, Himmel et al., 2023, Maksymchuk et al., 2022, Patel et al., 2022, Himmel et al., 2022, Maksymchuk et al., 2023).

Reviewer #3:

Can the patterns of movement that are stimulated by noxious cold allow the larvae to escape harm?

We were similarly curious about the neuroethological and/or protective implications of cold-evoked behaviors. In *Drosophila* larvae, noxious mechanical stimuli-evoked body rolling allows for lateral escape from predatory wasp (Hwang et al., 2007). Reducing the overall surface area that is exposed to cold (e.g., huddling behavior) serves as a protective strategy in many species (Canals et al., 1997, Contreras, 1984, Gilbert et al., 2006, Vickery and Millar, 1984, Hayes et al., 1992). Low temperatures can be fatal to poikilotherms (e.g., insects), however, many species have evolved the ability to cold acclimate thereby increasing their cold tolerance. To explore the potential evolutionary benefit of CIII-mediated contraction response to cold, we previously published work revealing a neural basis for cold acclimation in *Drosophila* larvae implicating these neurons (Himmel et al., 2021). We demonstrated that cold-evoked CT behavior is evolutionarily conserved across 11 different drosophilid species and that other cold-induced behaviors (e.g., tail raise) were also observed. Furthermore, drosophilid species adapted to rapid temperature swings were more likely to retain the ability to locomote even at lower temperatures (Himmel et al., 2021). Next, we elucidated the role of CIII md neurons in cold acclimation. Silencing CIII md neurons resulted in the inability to cold acclimate. We additionally investigated roles of Ch or CII md neurons, which alone did not inhibit the ability of larvae to cold acclimate. However, combinatorial silencing of CIII with CII or Ch neurons resulted in an inability to cold acclimate but did not obviously increase baseline cold tolerance. We explored how developmental exposure to noxious cold temperature impacts CIII md neuron cold-evoked firing pattern. Electrophysiological analyses revealed that cold acclimation results in hypersensitization in CIII md neurons (Himmel et al., 2021). Lastly, developmental optogenetic activation of CIII md neurons led to increased cold tolerance. Therefore, CIII md neurons are necessary and sufficient for cold tolerance and our collective evidence demonstrate that CIII-mediated cold nociception constitutes a peripheral neural basis for *Drosophila* larval cold acclimation (Himmel et al., 2021).

Reviewer #3:

It should be noted that this actuator drives very strong activation, and other studies with milder optogenetic stimulation of Class III neurons have shown that these cells produce behavioral responses that resemble gentle touch responses (Tsubouchi et al 2012 and Yan et al 2013)...The latter makes the reported Calcium responses to cold difficult to interpret in light of the fact that the strong muscle contractions driven by cold may actually be driving mechanosensory responses in these cells (ie through deformation of the mechanosensitive dendrites).... Are the cIII calcium signals still observed in a preparation where cold induced muscle contractions are prevented?"

We agree with the reviewer that mild activation of CIII md neurons results in gentle touch-like responses. In this manuscript, and other previously published work, it has been shown that optogenetic activation of CIII neurons, or CIII neurons and other sensory neurons, using a variety of optogenetic actuators (ChR2, ChETA, and CsChrimson) promotes bilateral

contraction of the larval body along the anterior-posterior axis (Shearin et al., 2013, Hwang et al., 2007, Meloni et al., 2020, Turner et al., 2016, Patel and Cox, 2017, Patel et al., 2022, Himmel et al., 2023).

As described above, in our initial publication documenting larval cold nociception in *Drosophila*, we investigated how CIII md neurons discriminate multimodal stimuli to elicit stimulus relevant behavioral responses. We reported that increased activation of CIII md neurons results in cold-evoked behaviors, where lower activation results in touch-evoked behaviors. Subsequent, calcium analyses revealed greater stimulus-evoked calcium response to noxious cold and milder calcium response to gentle touch (Turner et al., 2016).

Though we have not performed cold-evoked Ca^{2+} imaging of CIII md neurons in larval preparations without muscles, we have recorded electrical responses of CIII md neurons in the absence of muscle contractions using de-muscled larvae fillets to analyze cold-evoked firing patterns of CIII md neurons (Himmel et al., 2021, Himmel et al., 2022, Himmel et al., 2023, Patel et al., 2022, Maksymchuk et al., 2022, Maksymchuk et al., 2023). These studies demonstrate the cold-evoked CIII neural activity is not dependent upon muscles.

Reviewer #3:

A major weakness of the study is that none of the second or third order neurons (that are downstream of CIII neurons) are found to trigger the CT behavioral responses even when strongly activated with the ChETA actuator (Figure 2 Supplement 2). These findings raise major concerns for this and prior studies and it does not support the hypothesis that the CIII neurons drive the CT behaviors."

We conducted extensive screening of interneuron populations post-synaptically connected to CIII neurons in an effort to identify post-synaptic partners that were sufficient to trigger CT response. Much to our surprise, we were unable to find any individual neuron type or driver line that was sufficient to elicit a CT response. However, we provide substantial supporting evidence for our co-activation experiments including neural silencing, EM connectivity and calcium imaging. We also report necessity for the reported second/third order neurons in cold-evoked behavioral responses, where inhibiting neural activity resulted in reduced cold-evoked behavior. Second/third order neurons also exhibit cold-evoked calcium responses. Lastly, we also report CIII-evoked (using optogenetics) increases in calcium response in downstream post-synaptic neurons.

Previously published literature investigating CIV md neuron circuitry has implicated downstream neurons that are not sufficient to elicit rolling behavior upon activation. In CIV md neuron circuit dissection, select neurons are reported as acting downstream of CIV md neurons that require additional circuit components in order to execute rolling behavior. For example, A00c neuron activation alone does not lead to rolling behavior, however, co-activation of A00c and Basin-4 neurons facilitates rolling response (Ohyama et al., 2015). Similarly, co-activation of Basin-1 and Basin-4 neurons significantly enhance rolling probability relative to Basin-4 alone (Ohyama et al., 2015). Further, DnB neurons require Goro command neuron activity to promote rolling behavior (Burgos et al., 2018). Thus, there is precedent for co-activation requirements to elicit robust behavioral output in sensorimotor circuits and we employed a similar strategy after we discovered that activation of second or third order neurons alone did not elicit CT response.

Reviewer #3:

Later experiments in the paper that investigate strong CIII activation (with ChETA) in combination with other second and third order neurons does support the idea activating those neurons can facilitate body-wide muscle contractions. But many of the co-activated cells in question are either repeated in each abdominal neuromere or they project to

cells that are found all along the ventral nerve cord, so it is therefore unsurprising that their activation would contribute to what appears to be a non-specific body-wide activation of muscles along the AP axis. Also, if these neurons are already downstream of the CIII neurons the logic of this co-activation approach is not particularly clear."

We agree with the reviewer's comment that various cell-types that were investigated are repeated in every abdominal neuromere, however, only select post-synaptic neurons (Basin 1-4, DnB, mCSI, and Chair neurons) are segmentally repeated in every abdominal segment. Conversely, other projection and ascending neurons we investigated (A09e, A00c, A05q, Goro, TePn04/05, and A08n) are not segmentally repeated in every section. We used connectome evidence to guide our experiments on populations of neurons to explore in cold-evoked behavior and as alluded to above our co-activation approach was driven by the observation that an individual subpopulation of connected interneurons was not found to be sufficient to elicit CT behavior. That said, it does not change the findings that inhibition of neural activity in these subpopulations impairs cold-evoked behavior, nor does it change the observation that connected interneurons exhibit cold-evoked Ca²⁺ responses that can also be observed with optogenetic activation of CIII neurons. Reviewer #3: "The authors argument that the co-activation studies support "a population code" for cold nociception is a very optimistic interpretation of a brute force optogenetics approach that ultimately results in an enhancement of a relatively non-specific body-wide muscle convulsion." Many studies exploring circuit bases of behavior have applied large-scale optogenetic, including co-activation strategies, or silencing screens to identify circuit components involved in specific behaviors under investigation. We employed similar methods in our circuit-based dissection and our conclusions are not solely based upon optogenetic analyses.

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