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Cellular and genetic bases of cold nociception and nociceptive sensitization in Drosophila larvae

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CELLULAR AND GENETIC BASES OF COLD NOCICEPTION AND NOCICEPTIVE SENSITIZATION IN *DROSOPHILA* LARVAE

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CELLULAR AND GENETIC BASES OF COLD NOCICEPTION AND NOCICEPTIVE SENSITIZATION IN *DROSOPHILA* LARVAE

A

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DEDICATION

To the three most important men in my life:

Brenden who reminds me what is important,

William who always brings me joy,

and

Dad who taught me to always be curious and ask questions.

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CELLULAR AND GENETIC BASES OF COLD NOCICEPTION AND NOCICEPTIVE SENSITIZATION IN *DROSOPHILA* LARVAE

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Organisms from flies to mammals utilize thermoreceptors to detect and respond to noxious thermal stimuli. Although much is understood about noxious heat avoidance, our understanding of the basic biology of noxious cold perception is gravely minimal. Numerous clinical conditions disrupt the sensory machinery, such as in patients suffering from tissue damage (from wound or sunburn), or injury to the peripheral nerves, as in patients with diabetes or undergoing chemotherapy. Our goal is to determine the genetic basis for noxious cold perception and injury-induced nociceptive sensitization using the genetically tractable *Drosophila* model. Using a novel "cold probe" tool and assay we found larvae produce a mutually exclusive set of reactive behaviors to a defined noxious cold stimulus (3-12 ºC), including a full-body contraction and the bending of anterior and posterior segments to make a U-Shape. These behaviors are distinct from normal locomotion, responses to gentle touch, noxious heat or harsh mechanical stimuli. Through genetic manipulation, we found cold responses require specific classes of peripheral sensory neurons and receptors, which differ depending on the cold-evoked behavior. Our data indicates these cold-sensing neurons are multimodal, and the level of cellular activation determines the behavioral output to different stimuli.

To study cold nociceptive sensitization, we used a "sunburn assay" which exposes the dorsal side of the larva to UV-damage, and found larvae display a dramatic shift in cold responses after injury. This behavioral shift requires similar sets of peripheral sensory

neurons and receptors specific to each sensitized cold-evoked behavior. Lastly, we found the Tumor Necrosis Factor (TNF) and Tachykinin (Tk) pathways, both involved in sensitization to noxious heat, may also play a role UV-induced cold sensitization.

We have established the first system to study noxious cold and cold sensitization in *Drosophila*. Our unique tool and assay will allow us to further uncover the conserved molecular and genetic players involved in this process.

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ABBREVIATIONS

CT – Contraction

US – U-Shape

PR – Posterior Raise

HTR – Head and/or Tail Raise

AR – Anterior Raise

HW – Head Withdrawal

BR – Body Roll

NR – No Response (or non-responder)

RT – Room Temperature

S.E.M. – Standard Error of the Mean

TnTE – Tetanus Toxin Light-Chain

Hh - Hedgehog

TNF – Tumor Necrosis Factor

Tk - Tachykinin

TRP – Transient Receptor Potential

TRPVI – Transient Receptor Potential Vanilloid 1

TRPM8 – Transient Receptor Potential Melastatin

Pain – Painless (*Drosophila* TRPA channel)

Pyx – Pyrexia (*Drosophila* TRPA channel)

Iav – Inactive (*Drosophila* TRPV channel)

TRPP – Transient Receptor Potential Polycystic

Brv – Brivido (*Drosophila* TRPP-like channel)

Pkd2 – Polycystic Kidney Disease gene 2 (*Drosophila* TRPP channel)

Trpml – Transient Receptor Potential Mucolipin

Wtrw – Water witch

Nan – Nanchung

MD – Multiple Dendritic (neuron class)

Ppk – Pickpocket (neuron class)

Ch – Chordotonal (neuron class)

UAS – Upstream Activation Sequence

ChETA – Ultra fast Channelrhodopsin

ATR – all-trans-retinal

CaMPARI – Calcium Modulated Photoactivatable Ratiometric Integrator

UV – Ultraviolet

HIV – Human Immunodeficiency Virus

MS – Multiple Sclerosis

NSAID – Nonsteroidal Anti-Inflammatory Drug

CFA – Complete Freund's Adjuvant

VDRC – Vienna Drosophila RNAi Center

CHAPTER 1: Background and Significance

1.1. An Introduction to Nociceptive Pain

The perception of painful stimuli is an unpleasant yet a crucial part of everyday life that alerts us of potential or imminent tissue damage. Indeed, people that are unable to sense pain, such as in spinal cord injury (1), certain genetic disorders (2) or those with diabetes (3), often experience accidental self-harm that can lead to chronically open wounds that are at risk of getting infected (4, 5). There are many ways to label and define pain. Currently, pain is separated into a number of different subtypes, which describe various biological and clinical manifestations. The most broad definition of pain is the unpleasant sensory experience of potential or actual tissue damage (6) however, subcategories of pain include 'neuropathic pain': pain caused by damage to or disease of the sensory nerves, and 'nociceptive pain': pain that arises from actual or threatened damage that arises from the activation of pain-sensing sensory nerves and receptors (definitions outlined on the International Association for the Study of Pain website (6)). All three of these definitions include the unpleasant *experience* of pain, which is associated with emotional and psychological distress.

This study is primarily focused on 'nociception', which is the neural processes to detect and respond to potentially dangerous stimuli, since the emotional components of pain are exceedingly difficult to study in any organism other than humans (albeit some advances have been made in primates and rodents (7, 8), also see review (9)). Nociception can be found in diverse organisms across species and phyla (10), and many pain disorders share common critical alterations in nociception. Thankfully, this allows for a diverse set of models to study conserved aspects of nociception and identify novel drug targets to treat pain.

In both vertebrates and invertebrates, nociceptive stimuli are detected by specialized sensory cells, called nociceptors, that innervate the skin (11-13). Nociceptors express high threshold ion channels that translate noxious stimuli (capable of causing tissue damage) from the environment into electrical signals (12). These electrical signals, or action potentials, are transmitted along sensory nerves to the spinal cord and brain before resulting in descending motor outputs to avoid the stimulus and further tissue damage (12). Nociceptive stimuli are diverse, including harsh touch or pressure, chemical or extreme temperature.

1.2. Cold Nociception: An Investigation

The ability to sense temperature, including responses to painful temperatures, is highly conserved across species (14), including bacteria (15), plants (16), fish (17), worms (18), and flies (18, 19). In human skin, thermosensory cells are capable of sensing minute changes in temperature (11). For any type of stimulus, there is an intensity threshold that defines the boundary between innocuous and noxious. As a stimulus gets closer to a perceived "noxious" threshold, behavioral responses in humans (20) and animal models (21) become more frequent in a dose-response manner, often adapting into distinct nocifensive or protective behaviors (such as paw guarding or licking in rodents). In vertebrates, the nociceptive thresholds for temperature can vary slightly depending on the report, but rodents typically begin to exhibit nocifensive responses to cold below 15 ºC ((22)described as allodynia threshold), and to heat above 43 ºC (23). While the sensory mechanisms underlying detection of noxious heat have been fairly well characterized, those for noxious cold have not. Much work has been done to elucidate cold nociception mechanisms, but the precise cells and genes required are still debated (24, 25).

A wide array of nociceptive assays and tools have been used in vertebrates to investigate responses to cold stimuli, including: a cold plate (26), tail-flick (27), and exposure to acetone (28), or dry ice assays (29). Except for the tail-flick, these assays generally target the rodent paw and the behavioral responses measured include paw withdrawal, paw licking, and guarding of the paw. Response latencies are often measured and used to indicate the robustness of the response. These behavioral assays have identified a number of cells and channels important in sensing cold.

Vertebrates have four main peripheral sensory neuron types that are characterized by their size and conduction properties. Aα and Aß have large cell bodies and thickly myelinated axons resulting in fast conduction speeds; Aδ neurons are slightly smaller, with "medium-sized" cell bodies and thinly myelinated axons and therefore slightly slower conduction speeds; and C fibers have the smallest cell bodies and are unmyelinated, therefore they have the slowest conduction velocity (23). C and Aδ fibers are specifically activated by cold temperatures, resulting in changes in their firing frequencies to cool or noxious cold (23). Cool sensing neurons are activated between 20-37 ºC with increased firing upon cooling down to 20-17 ºC, while noxious cold sensing neurons are activated between 10-20 ºC with increased firing down to 0 ºC (23). Although the temperature thresholds between innocuous and painful cold seem fairly well defined in neurons, the sensory receptors required for cold sensing have been harder to pinpoint.

Thus far, there have been a number of cold-sensitive channels proposed as cool or noxious cold receptors. A large focus has been on Transient Receptor Potential (TRP) channels, which have known functions in nociception and thermosensation (14). TRP channels are variably selective cation channels containing multiple subunits and six

transmembrane domains that fall into several different gene families including: TRPC, TRPM, TRPV, TRPA, TRPP and TRPML. While TRPV channels have been characterized as warmth and heat activated (30), TRPM8 and TRPA1 are most notably referred to as cold receptors (31, 32). There is some debate however on whether either of these channels are required for behavioral responses to noxious cold in rodents (33). TRPA1 has been suggested to be primarily responsible for inflammatory or damage-induced cold hypersensitivity, while TRPM8 acts as a receptor for cooling rather than harsh cold (33). Indeed, some cold-sensing neurons do not express TRPM8 or TRPA1 (34), suggesting alternative noxious cold channels exist. To further complicate cold-sensing biology, sodium channels and potassium channels have also been implicated in sensing or modulating cellular responses to noxious cold (35-38).

In any case, it appears that noxious cold detection in vertebrates is a complex process, likely involving multiple receptors and/or modulators. Some work has been done to identify the cells and channels involved in cool-to-cold detection in invertebrates, including flies (18), worms (18, 39), and the leech (40). Thus far however, these studies have focused on fairly innocuous 'cool' ranges (> 12 ºC) and investigate on thermotaxis, or avoidance of temperatures just outside preferred ranges. Very little has been done to identify cells and genes required for detection of acute, noxious cold stimuli.

1.3. Maladaptive Pain: Sensitization gone awry

In the context of injury or damage (such as from a cut, burn or other wound), nociceptors will become temporarily sensitized. Pain sensitization under this context is extremely useful in alerting us to damaged tissue so that it can be protected while the healing

process is under way (41). Normally, once the wound has healed, nociceptive sensitization will resolve and nociceptors will return to normal response levels to innocuous and noxious stimuli. Many patients however, experience maladaptive chronic pain, or sensitization that persists after an injury has healed, concomitant with chronic disease, inflammation, central or peripheral nervous system damage, or as a result genetic mutations (41-44). Neuropathic pain, caused by damage to, or malfunction of, the peripheral nerves or central nervous system (45), is associated with chemotherapy (46, 47) and radiation treatment (48), diabetes (49), multiple sclerosis (50), stroke (45), spinal cord injury (51), and various infections such as leprosy and HIV (52, 53).

The symptoms accompanying neuropathic pain are debilitating and diverse, including sensitivity to innocuous or noxious stimuli (evoked pain from mechanical/gentle touch, temperature, pressure etc…), and spontaneous pain or discomfort (characterized as stabbing pains, sensation of pins and needles, tingling, deep aching, numbness, and burning). These symptoms can severely decrease the quality of life for a patient. In the case of chemotherapy-induced peripheral neuropathy, symptoms can arise after only one treatment dose and without relief can seriously impact the amount of chemotherapy a patient can withstand to adequately treat their cancer (47). While some patients eventually recover from neuropathic symptoms, others must deal with these symptoms for years or never find relief from chronic pain.

Treating neuropathic pain is especially difficult (54). While opiates are undoubtedly the most ubiquitous class of nociceptive pain drugs, they are rather ineffective at treating neuropathic pain (55). Even their use as nociceptive analgesics however is problematic due to their function in depressing the central nervous system, which is associated with unpleasant and detrimental side effects including: sedation, respiratory depression, dizziness, nausea,

and constipation (56). Opiates are also notorious for inducing tolerance, requiring higher doses to get the same pain relief, and physical dependence and addiction (56). In some cases, anticonvulsants (carbamaxepine or baclofen) and antidepressants have been used to treat neuropathic symptoms with varying success (55), but for many patients these drugs do little to treat their symptoms and are accompanied by their own undesired side-effects. It is undeniable that one of the major difficulties in developing better pain therapeutics is in understanding the complex mechanisms that underlie chronic and neuropathic pain.

While the mechanisms underlying neuropathic pain are heavily investigated in animal models, there seems to be a large gap between identified players in model nociception and effective therapeutic treatments. In part, this may be due to the higher degree of complexity of nociceptive and neuropathic mechanisms in human patients compared to animal models. For example, even as some specific neuropathic mechanisms are elucidated, patients with the same neuropathic etiology can have very different severity of symptoms, morphological phenotypes, and recovery outcomes, making it exceedingly difficult to find common mechanisms that are likely to help most patients (57). In addition, within a single patient, symptoms may depend on multiple distinct neuropathic mechanisms that are at play at different stages of their condition, requiring different treatment strategies (57). It doesn't help that the field still lacks reliable and effective methods of identifying neuropathy at early stages, making early adjustments to care and prevention of pain difficult (58). Indeed, with some chemotherapy treatment, neuropathic symptoms are often not detected until *after* the cessation of treatment, making it almost impossible to predict or prevent neuropathic outcomes (47). In this case, the chemotherapy drugs that most commonly cause delayed

neuropathic symptoms are platinum compounds such as oxaliplatin, which are notorious for causing severe sensitivity to cold stimuli (47).

1.4. Clinical conditions associated with cold hypersensitivity and potential mechanisms

A number of neuropathic conditions include hypersensitivity to temperature. For cool temperatures, this can be experienced as innocuous cool temperatures being perceived as painful (cold allodynia), or harsh cold temperatures perceived as *more* painful (cold hyperalgesia). Exposure to mild cooling has also been shown to exacerbate chemotherapy-induced spontaneous innocuous (paresthesias) and painful sensations (dysesthesias) (59). While cold allodynia is common in patients with multiple sclerosis (50), fibromyalgia (60), stroke (61, 62), and chemotherapy-induced neuropathy(47, 59), the mechanisms that underlie cold sensitization in these conditions are largely unknown. Generally, investigations into the mechanisms underlying nociceptive sensitization tend to focus on the observed increases in excitability of peripheral sensory neurons, increases in excitability of neurons in the dorsal horn of the spinal cord, altered gene expression in these neurons, and decreases in inhibition at the level of the spinal cord, leading to disinhibition of excitatory sensory neurons (63).

Sensitization mediators including neurotrophic factors, protons, bradykinin, prostaglandins, and ATP, play a role in these cellular and genetic changes. These mediators are released from damaged tissue or inflammatory cells, and can directly sensitize sensory neurons and receptors to induce sensitization (64). The inflammatory compounds, nerve growth factor (NGF) and the glial cell line-derived neurotrophic factor (GDNF) family ligand artemin, in particular have recently been specifically associated with cold hypersensitivity in rodents (65). For artemin, sensitization to cold in sensory neurons is dependent on the

TRPM8 channel(65), but TRPA1 has also been implicated in diabetic, chemotherapy-induced, and spinal cord injury-induced cold allodynia (66). Like other sensitization mediators, the chemotherapy drug oxaliplatin induces significant changes in ion channel expression in peripheral sensory neurons, making the neurons more sensitive to cold in rodents (67).

Whether these mechanisms are actually at play in patients experiencing cold allodynia however, is not known. This is a serious gap in knowledge, given that for patients taking oxaliplatin, for example, nearly *all* patients develop cold allodynia, severely impacting their quality of life (47). Undoubtedly, a more thorough investigation of the potential mediators of cold allodynia under different sensitization contexts will provide clinicians with a wider range of future drug targets to treat this condition.

1.5. Invertebrate Models: Usefulness in studying nociception and nociceptive sensitization

There are many advantages to working with invertebrate models. They provide genetically tractable systems where unbiased forward genetic identification of thermosensation and nociceptive genes is both feasible and cost-effective. A number of highly useful genetic tools are available in invertebrate models that are technically challenging or time and cost ineffective in rodents. Notwithstanding, invertebrate nociception is vastly simplified genetically and neuronally compared to their vertebrate counterparts, and it is virtually impossible to assess the *emotional* ramifications of pain in invertebrate models to the same extent as we can in humans. Despite this, unraveling the nociceptive processes in a simplified system can provide valuable insights into complex mechanisms and provide an

excellent starting point for forming and testing hypotheses in nociception and nociceptive sensitization.

Classic invertebrate models of nociception and nociceptive sensitization include the worm *Caenorhabditis elegans*, the mollusk *Aplysia californica* and the fly *Drosophila melanogaster. C. elegans* exhibit withdrawal reflexes to extreme temperatures through specific nociceptors (68) which can be blocked by administered opiates (69). Although *C. elegans* has only 302 neurons, this has allowed for precise manipulation and mapping of sensory circuits, including those involved in adaption, sensitization and associative learning (18, 70). In *Aplysia,* although nociceptors are unmyelinated, they are capable of sensing noxious mechanosensory stimuli and mediate robust withdrawal reflexes and behaviors (71). Interestingly, studies have paired noxious stimuli with non-salient stimuli to study conditioned fear in *Aplysia* (72). Similar experiments have also been done in *Drosophila,* where flies were conditioned to avoid an area of an enclosed chamber using high temperature (73). In fact, *Drosophila* have a number of nociceptive sensory neurons that mediate aversive behavioral responses (74, 75) that can be sensitized under the context of tissue damage (76, 77)(elaborated on further in next section).

These invertebrate studies start to scratch at the surface of the aforementioned barrier between *nociception* versus *pain,* the later, which has an implied emotional component (78, 79). That these models have associative learning and modifiable motivational states is particularly interesting given the vast differences in neural structures and presumably neural circuits between these models and vertebrates, suggesting that although the central nervous system may be drastically different between these models, invertebrates can offer valuable insights into conserved nociceptive and neurobiological functions.

1.6. *Drosophila*: A model for nociception and nociceptive sensitization

Drosophila has been used for over 100 years to help answer genetic and biological questions about vertebrate neuroscience (80). Their quick generation time and cost-effective maintenance make them a highly economical choice. In addition, *Drosophila* has a sophisticated genetic toolkit which makes them highly useful as a fast genetic screening tool to identify novel players in nociception (81). In addition, the *Drosophila* fly and larval stage have been used to study avoidance of temperatures outside their preferred range (82-84), and responses to potentially dangerous temperatures (19, 85). Larvae have been particularly useful for imaging due to their translucent epidermis, which allows both fixed and live imaging of their underlying peripheral sensory neurons and brain areas (86, 87).

Behavioral assays have already uncovered a number of conserved genetic players in nociception (81, 88-91) and damage-induced nociceptive sensitization in *Drosophila* (19, 77, 92). Although the cells and receptors important for noxious cold sensing are unknown, *Drosophila* does not survive prolonged exposure to cold temperatures (93), and will avoid cool, preferring warmer temperatures in behavioral preference assays (82, 83). For thermal preference, the neurons and sensory receptors required to avoid cool temperatures (> 12 ºC) have been identified, most of which are localized to the larval anterior segments and brain (18). In the periphery, *Drosophila* larvae utilize complex peripheral sensory neurons that sense gentle touch, heat and harsh mechanical stimuli (94) (**Figure 1.1**). These multiple dendritic (md) neurons possess naked nerve endings, similar to cold-responsive non-myelinated C fibers in vertebrates (86), but it is unclear if md neurons participate in noxious cold sensing.

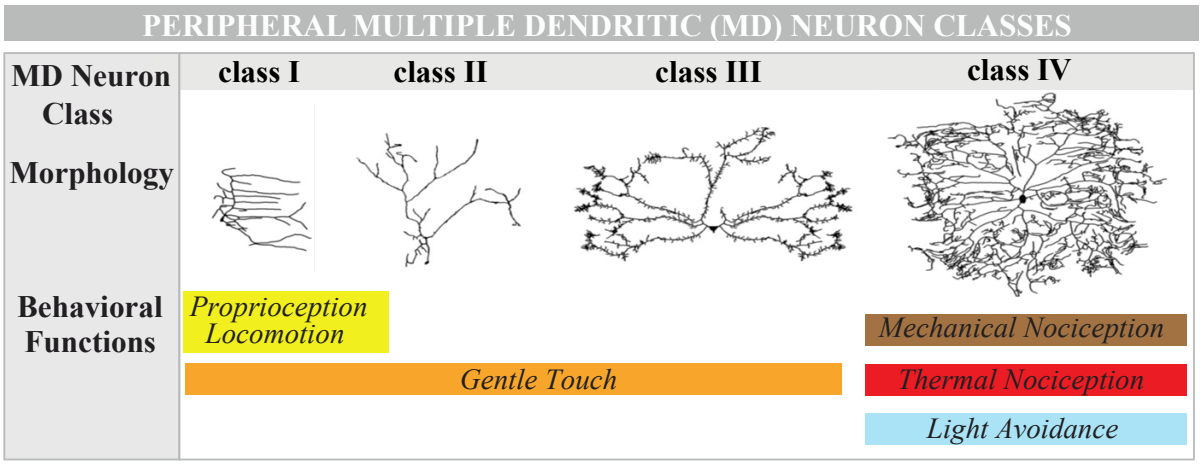

Figure 1.1. Md sensory neuron classes: morphology and function.
Modified version of figure originally published in (75).

TRP Family	TRP Channel Gene	Thermal Function
TRP C	*trp*	Cool Avoidance
	Trp γ	?
	trpl	Cool Avoidance
TRPM	*Trpm*	?
TRPN	*nompC*	?
TRPV	*nanchung*	?
	inactive	?
TRPA	*dTRPA1*	Warmth
	pyrexia	Noxious heat
	painless	Noxious heat
	waterwich	?
TRPP	*almost there, Pkd2*	?
	Brv1	Cool avoidance
	Brv2	Cool avoidance
	Brv3	Cool avoidance
TRPML	*Trpml*	?

Figure 1.2. TRP channel genes categorized by their family with known thermosensory functions.

Drosophila also expresses TRP channels that participate in thermosensation (**Figure 1.2**). TRPA channels are involved in noxious heat sensing in larvae, including the TRPA1genes *painless (pain)*(19)*, pyrexia* (*pyx*)(85)*,* and *TrpA1*(91)*,* while *inactive* (*iav,* a TRPV channel) (95), *brivido (brv,* a TRPP channel) (84), *trp,* and *trpl* (96) have been implicated in avoiding cool temperatures in temperature preference assays. It is unknown if any of these, or other TRP channels, are involved in sensing noxious cold temperatures (< 10-12 ºC).

It would be extremely useful for the field of pain biology to develop a genetically tractable model for studying acute cold nociception and nociceptive sensitization. With the understanding of basic cold nociception in *Drosophila*, researchers will be able to build on this knowledge to ask important questions about how innocuous cool and noxious cold stimuli become sensitized following tissue damage, or in other models of neuropathic pain syndromes where the mechanisms of sensitization are not clear. Based on the utility of *Drosophila* as a model for disease thus far, these organisms represent a very promising tool for future pain research.

CHAPTER 2: Baseline Cold Nociception in *Drosophila* Larvae

2.1. Introduction

Although the biology of cold nociception has been studied in vertebrates for some time, it has remained unknown whether *Drosophila* has behavioral responses to noxious cold temperatures. Much like vertebrates, *Drosophila melanogaster* has a fairly well defined preferred temperature range for optimal survival and reproductive success (93). This preferred range varies depending on their genetic background (93), but is generally between 24-27 ºC (97, 98). In the larval stage, *Drosophila* will avoid (crawl away from) temperatures below 22 ºC and above 28 ºC towards preferred temperatures (18, 82, 98). Behavioral responses to mild warmth or cooling include head casting (swinging anterior segments back and forth), turning, prolonged 'runs' (no turning), and reversal of locomotion to avoid the non-preferred temperature (18, 82, 99). At 39 ºC larvae begin to exhibit a robust 360º body roll that becomes more prevalent at shorter latencies as the temperature increases (77). At 45 ºC, 100 % of larvae exhibit this rolling response (77). Up to this point, there have been no studies on acute responses to noxious cold in *Drosophila* larvae, but larvae do not survive prolonged exposure to temperatures 10 ºC and below (93).

Cold-sensing neurons in *Drosophila*

Drosophila adult flies and larvae have a number of thermosensory neurons in the anterior segments (the head and antennae) that guide locomotion towards preferred temperatures (82-84, 98). In flies, specific cold-sensing neurons in the antenna detect very minute changes in temperature (~ 0.5 ºC) and exhibit a clear dose response in cellular activation with increasing or decreasing temperature (84). These neurons synapse onto corresponding distinct hot or cold glomeruli in the Proximal-Antennal-Protocerebrum of the

brain to ultimately guide thermotaxis (84). Larvae also have thermosensory neurons in the anterior segments that mediate thermal preference. Avoiding warm temperatures requires AC neurons in the brain (83), and dorsal organ neurons in the antenna are required for thermotaxis, or avoidance of warm or cool stimuli along a thermal gradient (82). Interestingly, the thermosensory neurons in the dorsal organ are activated by cooling (depolarization of the neuron) and deactivated by warming (hyperpolarization of the neuron), thereby communicating both sensory inputs to the brain and driving thermotaxis (82).

Drosophila also has a set of peripheral sensory neurons in the body wall (86) that respond to various innocuous (100-104) and noxious stimuli (74, 94, 105) (**Figure 1.1**). These neurons, particularly class III and class IV, have complex dendritic arbors that are embedded in the epidermis much like vertebrate nociceptors (87). The axons of md neurons project to the larval ventral nerve cord and brain (87). Class IV neurons are required for the body roll response to high temperature (74), harsh mechanical (74), and even aversive light (105), making them highly multimodal. The only neurons found to be involved in cool sensation in the periphery are the extrasensory chordotonal neurons(95). Chordotonal neurons are required for avoiding cool (abolished preference of 17.5 ºC over 14 ºC) but not cold (12 ºC) or warm (24 ºC) temperatures in larvae (95), and also sense stretch and gentle touch (102, 106).

Cold-sensing receptors in *Drosophila*

Thermosensation in the above cells are mediated primarily by thermosensitive TRP channels in *Drosophila* (**Figure 1.2**). While *trp, trpl* and *iav* appear to act in cool avoidance (10-20 °C)(95, 96) in larval cool avoidance, whether they are also involved in acute noxious

cold (≤ 10 °C) sensing is unknown. It also has yet to be determined if these channels are direct cold sensors. The TRP channel *iav* is expressed in chordotonal neurons where it is required for cool avoidance, however ectopic expression of *iav* in oocytes did not elicit cold-sensitive calcium currents (95). Therefore, the activation of this channel may be indirect or require other cellular components not found in oocytes.

Here we demonstrate a novel behavioral assay to study cold nociception in *Drosophila* larvae. This assay utilizes a Peltier-based probe, capable of applying a focal noxious cold to ambient (3-22 ºC) stimulus, resulting in quantifiable cold-specific behaviors. Our findings reveal that *Drosophila* uses a distinct set of cells, channels, and aversive behaviors to respond to extreme cold. This assay will allow further dissection of nociception at a cellular and molecular level.

2.2. Results

Cold exposure evokes distinct behavioral responses in *Drosophila* larvae

To determine if *Drosophila* larvae exhibit behavioral responses to acute noxious cold stimuli, we designed a custom-built "cold probe", which utilizes a closed loop Peltier device for cooling an aluminum shaft and conical tip through thermal conduction (**Figure 2.1A**). An embedded thermistor inside the conical tip of the probe reports the real-time temperature on the control unit. The probe is capable of gently applying a range of innocuous to noxious cold stimuli (23 º- 3 ºC) to 1-2 segments of the freely moving larva. For this study, we primarily targeted the dorsal midline, to segment(s) equidistant from anterior and posterior ends (roughly segment A4, see **Figure 2.1A**) of the larva. The smooth,

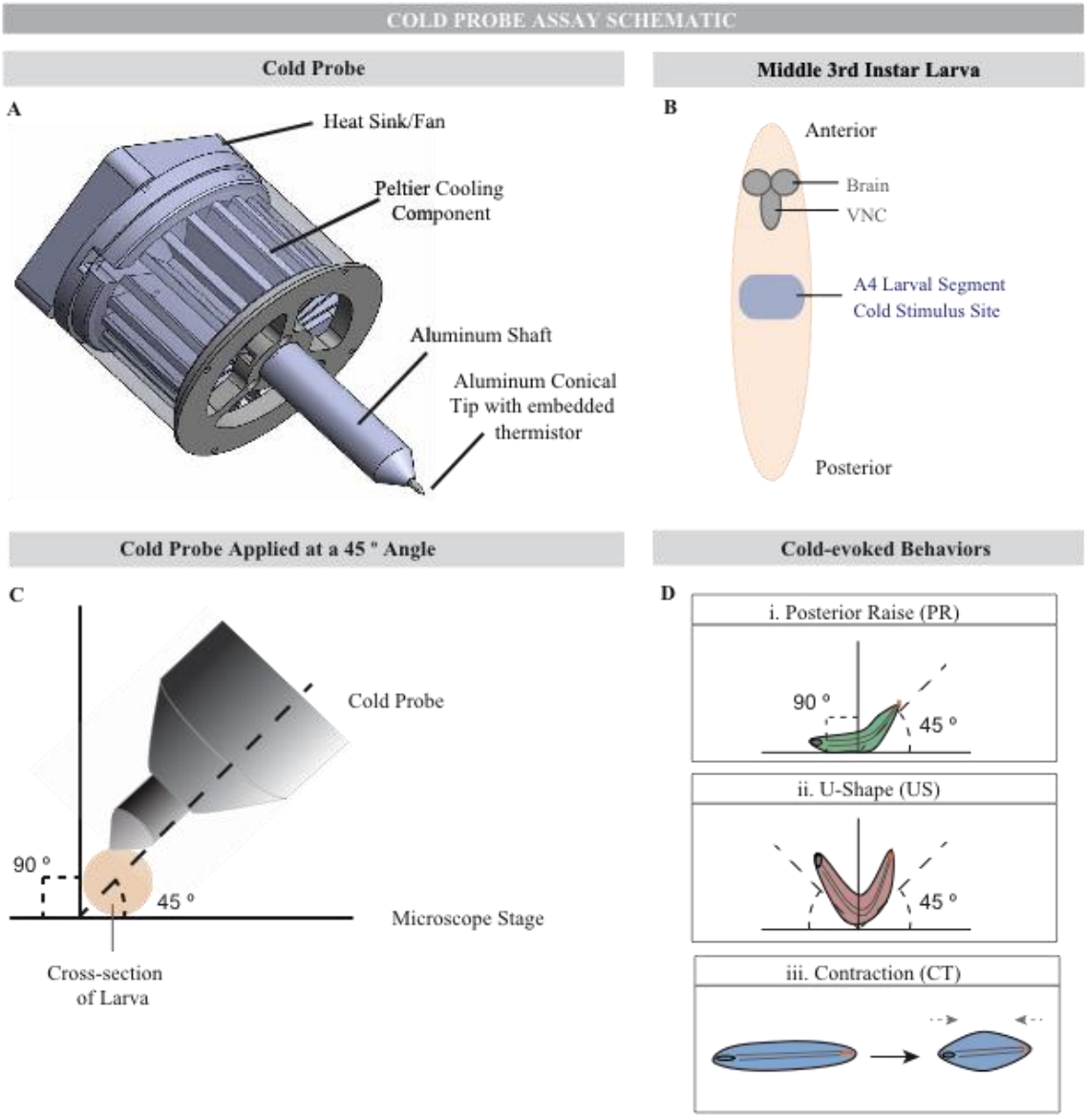

Figure 2.1. Cold probe tool and assay reveals unique cold-evoked behaviors in Drosophila larvae. (A) Diagram of cold probe and (B) application site on 3rd instar larva. (C). A cross-section of the larva indicating an approximate 45 ° angle of the cold probe to the microscope stage (dashed line), and perpendicular to the anteroposterior body axis of the larva during the stimulation. (D) Diagram of the three cold-evoked behaviors: (i) Posterior-raise (PR) – a 45-90º (dashed line) raise of the tail; (ii) U-Shape (US) – a 45-90º (dashed line) raise of the head and tail; and (iii) contraction (CT) of the head and tail towards the center of the larval body (grey arrows).

rounded tip of the probe is gently placed in this region at approximately a 90 º angle to the larva, and a 45 º angle to the microscope stage (**Figure 2.1B**) and held for up to 10 seconds. Upon cold stimulation larvae exhibited a mutually exclusive set of behaviors that preclude normal locomotion and differ significantly from behavioral responses to gentle touch (102, 107), light (105), noxious heat (19, 77), and harsh touch (19, 74, 89, 108). The cold-evoked behaviors were 1. A 45-90 ° raise of the posterior segments (posterior raise, PR); 2. A 45-90 ° simultaneous raise of the anterior and posterior segments into a U-Shape (US); 3. Or full body a contraction (CT) of the anterior and posterior segments towards the middle of the body (**Figure 2.1D**). This illustrates that *Drosophila* larvae do have behavioral responses to cold stimuli, and that they are distinct from those to noxious heat, harsh touch, light and gentle touch stimuli.

Characterization of cold-evoked responses in *Drosophila* larvae

To determine the range and specificity of these cold behaviors, larvae were tested over a range of noxious (3-12 ºC) to innocuous cool (13-23 ºC) temperatures. With decreasing temperature, the overall number of responders increased, but different behaviors peaked at distinct temperatures (**Figure 2.2**). US and PR responses peaked between 3-8 °C, while CT peaked between 9-14 °C (**Figure 2.2A-C**). CT was the only behavior occasionally observed in response to light touch, assayed by using a room temperature probe (**Figure 2.2C**). As opposed to a clear dose-response decrease in response latency seen with increasing hot temperatures(77), the latency of cold responses did not robustly decrease with temperature, however the majority of cold-evoked responses occurred within three seconds at temperatures below 14 ºC (**Figure 2.2D**).

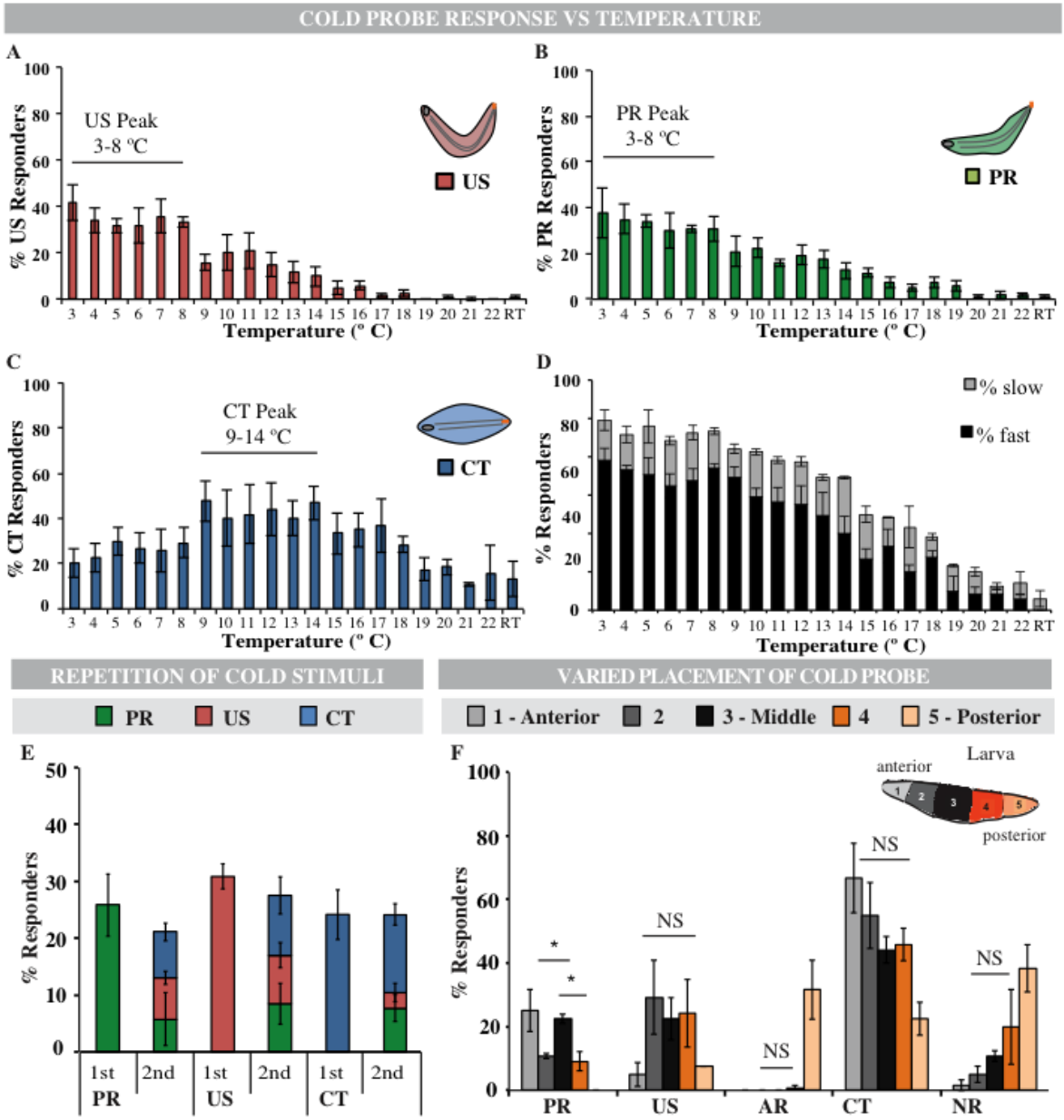

Figure 2.2. Characteristics of cold probe assay response. (A) Percentage of US, (B) PR and (C) CT responders to cold probe at various temperatures. (D) Latency of all responses to cold probe with respect to temperature. Fast = response within 3 s of onset of cold stimulus; slow = response 4-10 s after onset of cold stimulus. (E) Percent responders to cold probe (6 °C) during initial stimulus (1st) were separated based on response and tested with a second 'follow-up' stimulus (2nd). (F) Percent responses where the cold probe (6 °C) was applied to multiple regions of larvae (1-5). Colors indicate placement of the probe from anterior to posterior: 1 = on the most anterior segments; 2 = inbetween the most anterior and middle of larva, 3 = in the middle-most segment; 4 = inbetween the middle and posterior end; and 5 = on the posterior end. PR = posterior raise; US = U-shape; AR = anterior raise; CT = contraction; and NR = non-responder. (A-F) n = 3 sets of 40 larvae averaged ± s.e.m.. (F) NS = no significance between indicated bars by Fisher's exact test, $p > 0.05$. Figure reproduced from author's published work (75).

Given that three different cold-evoked behaviors were observed over a range of cold temperatures, we wanted to address a few variables that could potentially influence behavioral output. First, it could be that different cold-evoked responses represent distinct populations of responder types (US, PR and CT responders). To test this possibility, a population of larvae were tested for cold nociception, separated into groups based on their initial cold-evoked response, then tested again after an arbitrary 20-minute rest period. Upon the second cold stimulation however, most responses divided roughly equally into three different responder types (**Figure 2.2E**), suggesting that larvae do not fall into distinct responder groups.

Since variation in cold-evoked behaviors could be a result of user variability in the placement of the probe on the dorsal surface of the larva, we adjusted the cold stimulation site to five different "zones" along the larval body axis: 1. The most anterior segments (head/brain), 2. In between the anterior and middle (A4) region, 3. The middle most region, equidistant from anterior and posterior ends of the larva (roughly segment A4), 4. In between the middle and posterior end of the larva, and 5. The most posterior segments of the larva (**Figure 2.2F**). A new behavior arose when probing the posterior end of the larva: an anterior raise (AR), where the head comes 45-90 ºC into the air. This behavior may arise from the larva trying to complete a US but fails due to the probe placed on its tail end. Overall, cold-evoked responses increased significantly when probing zone 1 (the head), and the least number of responders was observed when stimulating zone 5 (most posterior end) (**Figure 2.2F**). However, the percent US, AR, CT, and percent non-responders (NR), did not significantly differ between zones 2-4, suggesting that subtle differences in probe placement

in these regions by the experimenter are unlikely to cause behavioral response variability (**Figure 2.2F**).

To further characterize the cold probe assay, cold-evoked behaviors were quantified up to 20 s (instead of 10 s), to a 3 ºC, 10 ºC, 20 ºC or a room temperature (RT) probe to determine the most effective and efficient assay cut-off. When extending the assay out to 20 s however, we found the majority of cold evoked responses were produced within a 10 s cut-off, and there were no significant differences between the percent of cumulative responders at 3 seconds, 11 seconds or 20 seconds, indicating a shorter cut-off is just as effective as a longer one (**Figure 2.3**). Interestingly, the latency curves differed depending on the behavior and temperature being tested however, with CT responders having significantly different latency curves (a more gradual slope and different peak/plateau level of response) at 10 ºC, 20 ºC and RT (**Figure 2.4**).

Lastly, since the relative surface area being stimulated could vary with larval size, we assayed early, middle and late 3rd instar larvae for cold nociception. Early, middle and late 3rd instar larvae were all collected 4-5 days after egg lay, and sorted based on size, early 3rd being the smallest and late 3rd larvae the largest. Although behavioral responses varied significantly with larval size at 3 ºC, there were no significant differences in cold-evoked responses at 10 ºC (**Figure 2.5**). These results indicate that cold-evoked behaviors differ in their peak temperatures and response latencies, but do not seem to arise from preferred behavioral output (**Figure 2.2E**), or be affected by subtle differences in probe placement (**Figure 2.2F**), longer assay cut-off times (**Figure 2.3**) or larval size (at least not at 10 ºC)(**Figure 2.5**).

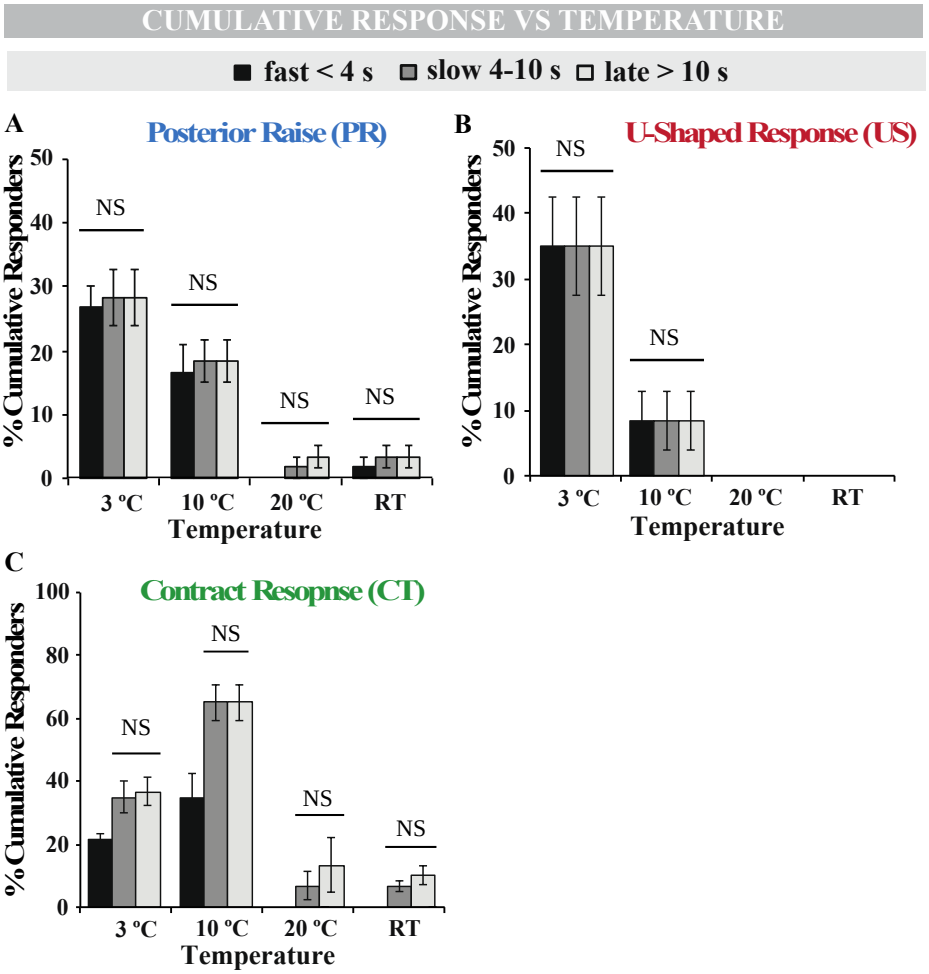

Figure 2.3. Cumulative cold-evoked response latency comparison.
(A) Percent of PR, (B) US or (C) CT responders to cold probe at vrious temperatures (3 ºC, 10 ºC, 20 ºC and RT). Responses are represented at different cut off points to compare cumulative fast (< 4 s), slow (4-10 s), or late (11-20 s) responders. Three sets of n = 40 were averaged at each temperature. PR = Posterior raise, US = U-Shape, CT = Contraction, RT = Room temperature. NS = no significant difference between indicated data sets by Fisher's exact test, $p > 0.05$. Error bars indicate ± s.e.m..

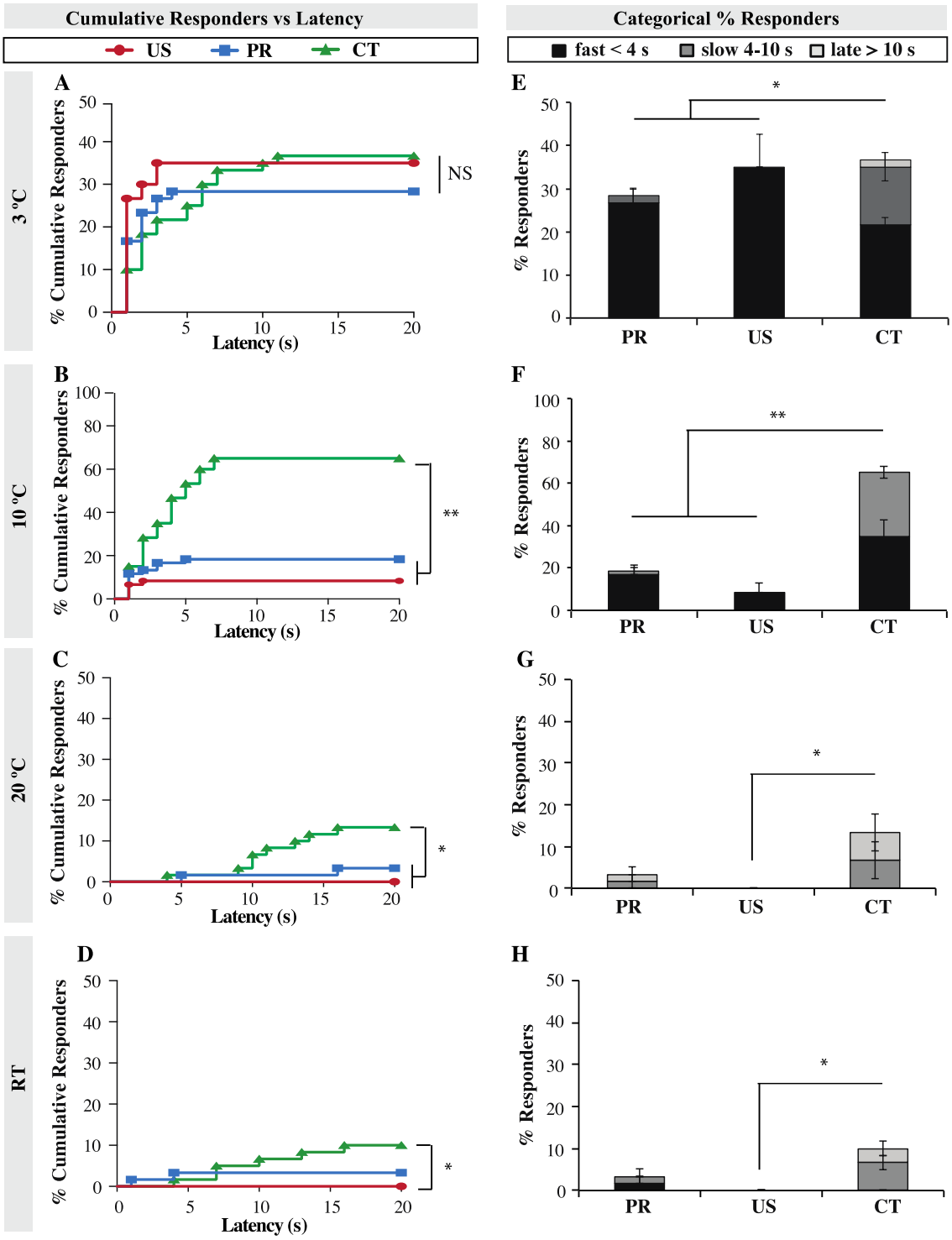
Cumulative Responders vs Latency
US
PR
CT
Categorical % Responders
fast < 4 s
slow 4-10 s
late > 10 s
3 °C
10 °C
20 °C
RT
A
B
C
D
E
F
G
H
% Cumulative Responders
Latency (s)
% Responders
NS
**
*
PR
US
CT

Figure 2.4. Cold-evoked response vs latency with categorical comparison.
(A-D) Percent of cumulative US (red), PR (blue), or CT (green) responders to cold probe at given temperatures: (A) 3º C, (B)10 ºC, (C) 20 ºC and (D) RT over time (1-20 s). (E-H) Average proportions of fast (<4 s), slow (4-10 s), and late (11-12 s) responding larvae to cold probe at given temperatures: (E) 3º C, (F)10 ºC, (G) 20 ºC and (H) RT. (A-H) For each temperature, n = 3 sets of 40 larvae were tested. PR = posterior raise, US = U-Shape, CT = Contraction, RT = Room temperature. (A-D) NS = no significant differences between data sets, * = p-value < 0.05, ** = p-value < 0.0001 by Long-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test. (E-H) Error bars indicate ± s.e.m.. * = p-value < 0.05, ** = p-value < 0.0001 by chi-square test.

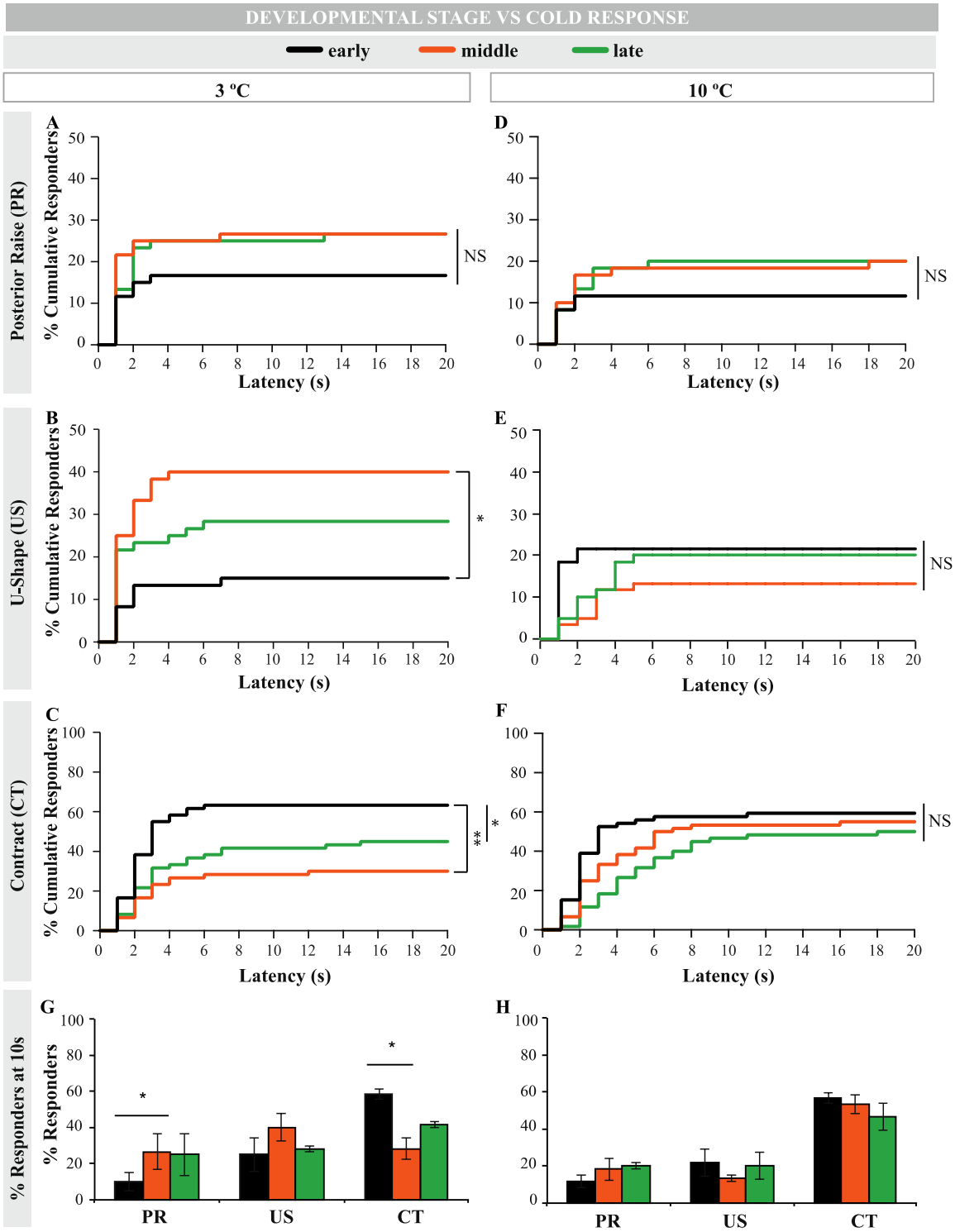
DEVELOPMENTAL STAGE VS COLD RESPONSE
early
middle
late
3 °C
10 °C
Posterior Raise (PR)
U-Shape (US)
Contract (CT)
% Responders at 10s
A
B
C
D
E
F
G
H
% Cumulative Responders
% Responders
Latency (s)
NS
*
**
PR
US
CT

Figure 2.5. Cold-evoked response latency in early, middle and late 3rd instar larvae.
Percent cumulative responders to a (A-C) 3 ºC or (D-F) 10 ºC cold probe over time (1-20 s) in early (black), middle (orange) or late (green) 3rd instar larvae. Percent responsders versus latency are separated by behavior: PR (A, D), US (B, E), CT (C, F). NS = no significant difference between indicated data sets, * = p-value < 0.05, ** = p-value < 0.001 by Long-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test. (G-H) Percent responders to a (G) 3 ºC or (H) 10 ºC cold probe within 10 s in early, middle or late 3rd instar larvae. Error bars indicate s.e.m..* = p-value < 0.05 by two-tailed Fisher's Exact test. (A-H) n = 3 sets of 20 larvae.

Identification of cold sensory neurons in *Drosophila* larvae

Drosophila larvae have four types of peripheral multiple dendritic (md) sensory neurons that are embedded in the larval epidermis (86) (**Figure 2.6A**). Each class has a distinct dendritic morphology, varying known sensory functions, and precise cell-specific GAL4 drivers available (**Figure 2.6A,** see also Section 5.1). To determine if cold evoked responses are mediated by a particular class or classes of md sensory neurons, larvae expressing a tetanus toxin transgene capable of preventing neurosynaptic transmission (109) was driven in a cell-specific manner, targeting and electrically silencing each class (**Figure 2.6B**), then larvae were tested with cold stimuli.

When all classes of md neurons were silenced using this method, a significant reduction in CT and US responses were observed to the cold probe, while PR responses remained unchanged (**Figure 2.7A**). Silencing class I or IV had no effect on cold-evoked CT responses, and although silencing class II resulted in a CT reduction, there was also significant reduction observed in the class II GAL4 control (**Figure 2.7B**). Silencing class III neurons using two different cell-specific drivers however, resulted in a significant reduction in cold-evoked CT responses compared to all genetic controls, while silencing class II and III together, resulted in equivalent reductions in CT versus silencing class III alone (**Figure 2.7B)**. In contrast, US responses were significantly attenuated when silencing class IV (or all md) sensory neurons when compared to all genetic controls (**Figure 2.7C**). These results suggest that distinct classes of md neurons mediate different cold-evoked behaviors, class III for CT and class IV for US, while the neurons mediating PR responses remain unknown but do not seem to be mediated by md neurons.

To determine if class III neurons are in fact responsive to cold, we live-imaged intact

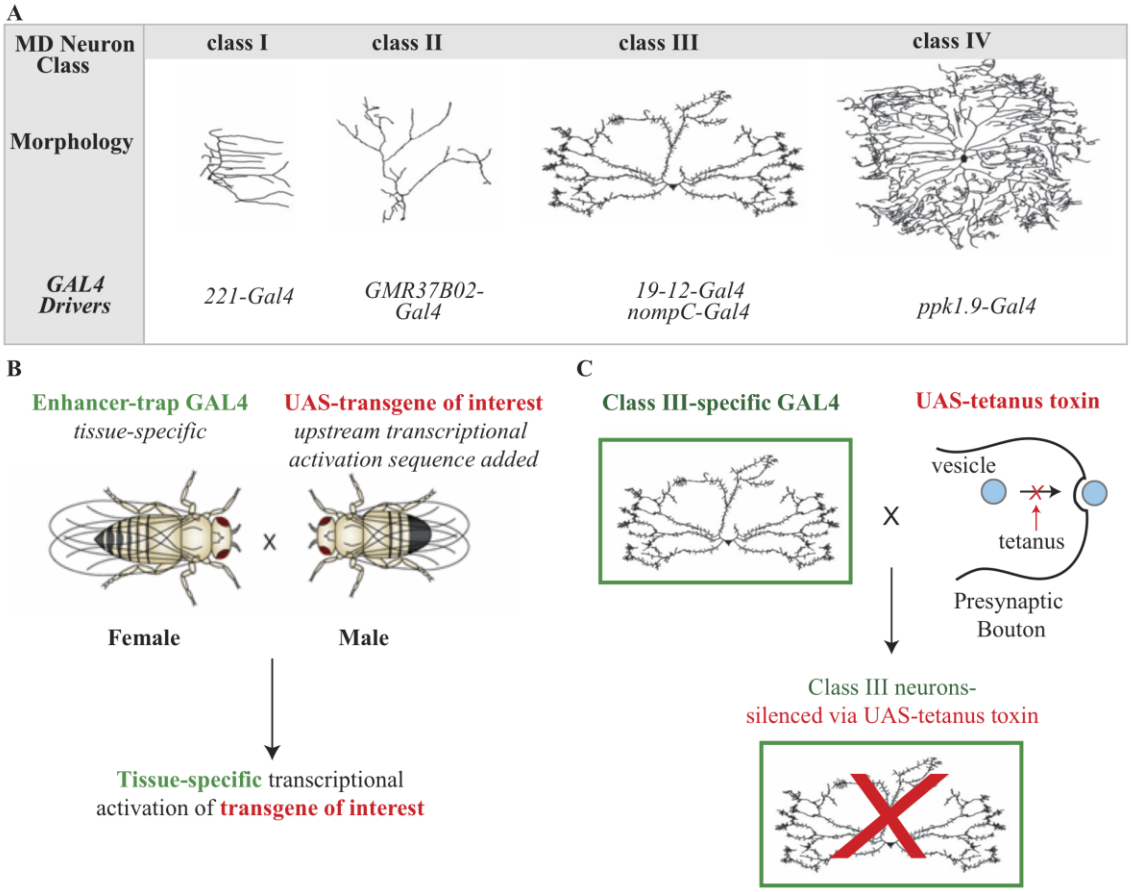

Figure 2.6. Md sensory neuron classes: morphology and tissue-specific drivers.
(A) Representative images of md neuron dendritic morphology shown with corresponding Gal4 drivers that mediate class-specific expression. (B) *Drosophila* UAS-GAL4 crossing scheme to create tissue-specific expression of a gene of interest. (C) Schematic of expression of a tetanus toxin transgene (UAS-tetanus toxin or UAS-TnTE) in class III neurons using the UAS-GAL4 system. Also shown is a diagram of how UAS-tentanus toxin acts to prevent neurosynaptic transmission (release of synaptic vesicles) at the presynaptic bouton to ultimately genetically induce silencing of class III neurons. (A) Modified from figure originally published in (75). (A) Images of neuronal morphology from the lab of Daniel Cox at Georgia State University.

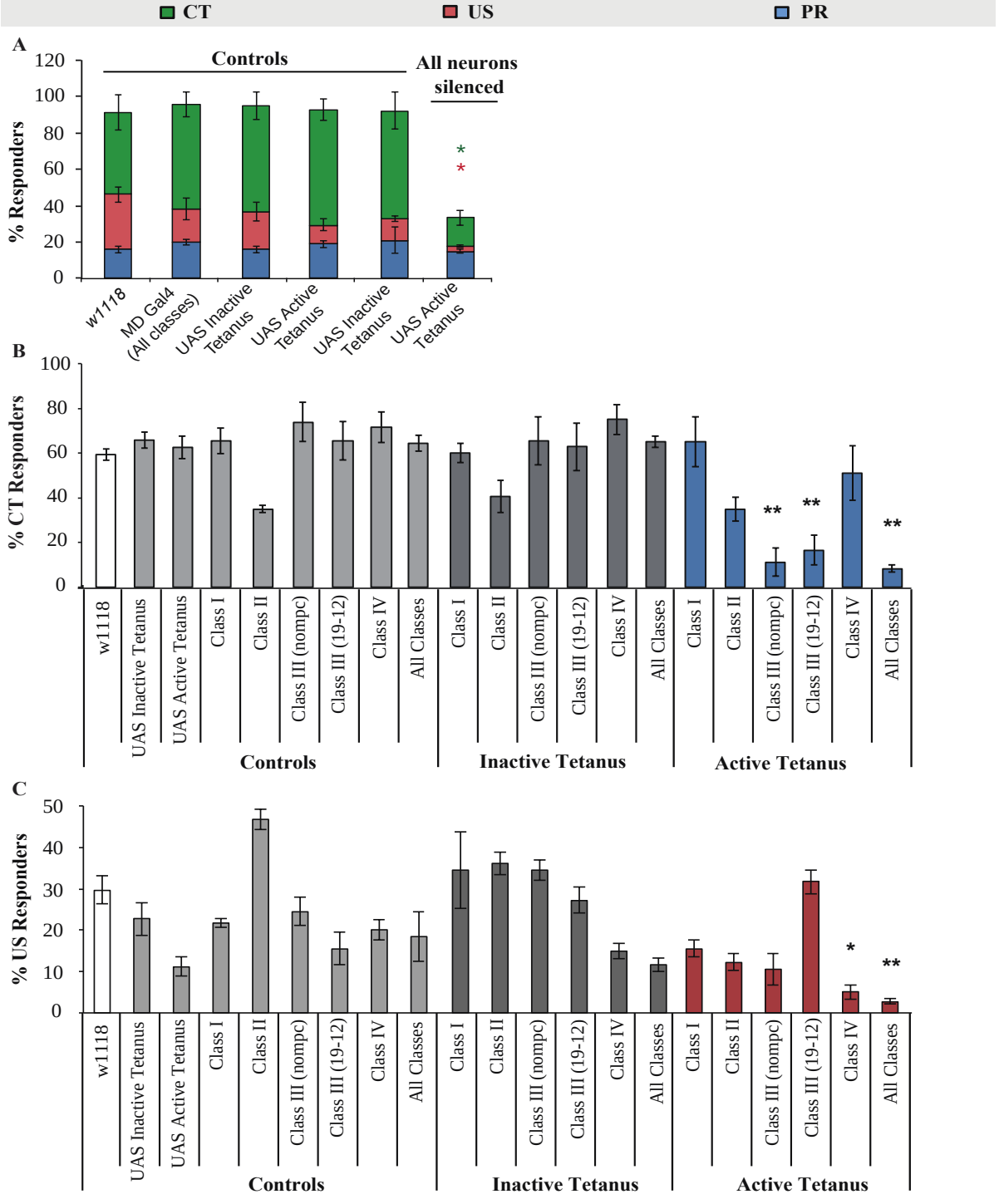
CT
US
PR
A
Controls
All neurons silenced
% Responders
w1118
MD Gal4 (All classes)
UAS Inactive Tetanus
UAS Active Tetanus
UAS Inactive Tetanus
UAS Active Tetanus
B
% CT Responders
w1118
UAS Inactive Tetanus
UAS Active Tetanus
Class I
Class II
Class III (nompc)
Class III (19-12)
Class IV
All Classes
Controls
Inactive Tetanus
Active Tetanus
C
% US Responders
Controls
Inactive Tetanus
Active Tetanus

Figure 2.7. Class III neurons are required for cold-evoked CT behavior.
(A) Percent responders observed upon silencing all md neurons (via MD-GAL4). (B-C) Percent of (B) CT or (C) US responders to cold probe (11 ºC) observed upon silencing different md neuron classes using class-specific drivers via an active tetanus tox in transgene. n = 3 sets of 40 larvae were averaged ± s.e.m.. (B-C) White and grey bars indicate controls, colored (blue or red) bars indicate class-specific silencing with tetanus toxin transgene expression. Stats: two-tailed Fisher's exact test with Bonferroni correction, * = $p < 0.0125$, ** = $p < 0.0001$. (B) Modfied figure from published work (75).

third instar larvae expressing GCaMP6 in md sensory neurons and quantified any changes in calcium-sensitive fluorescence (**Figure 2.8A**). Compared to their respective baselines, cold stimulation produced a slight increase in GCaMP6 signals in class I, a moderate increase in class II neurons, and no change in class IV neurons (**Figure 2.8B, C, E**). By contrast, class III neurons showed a robust increase in GCaMP6 fluorescence in response to cold temperatures (**Figure 2.8D**). Importantly, noxious heat stimulation does not inactivate GCaMP6 because heat followed by subsequent noxious cold still gave a robust class III GCaMP6 signal (**Figure 2.8F-G**). Together, these results demonstrate that class III, and to a lesser extent class II, neurons are directly activated by cold.

Lastly, to determine if activation of class III neurons in the absence of cold or touch is sufficient to provoke a CT response we expressed the ultrafast Channelrhodopsin-2 variant ChETA in different classes of md sensory neurons and directly activated these classes via high intensity blue light with or without all *trans*-retinal (ATR, a required cofactor for ChETA function). In controls, some larvae displayed a head and or tail raise (HTR) upon blue light stimulation (**Figure 2.9A-D**). While activating CI neurons resulted in no observable change in behavioral responses (**Figure 2.9A)**, activation of class II or class III neurons elicited a robust percent of CT responders (**Figure 2.9B-C**). Activation of CIV neurons elicited a aversive rolling response (**Figure 2.9D**) as previously reported(74). When directly comparing the robustness of CT responses upon class II or class III activation, we found class III activation elicited a greater percent change in larval body length and a longer duration CT, implying class III activation causes a more robust response than class II. Together these data reinforce and extend our GCaMP observations, demonstrating

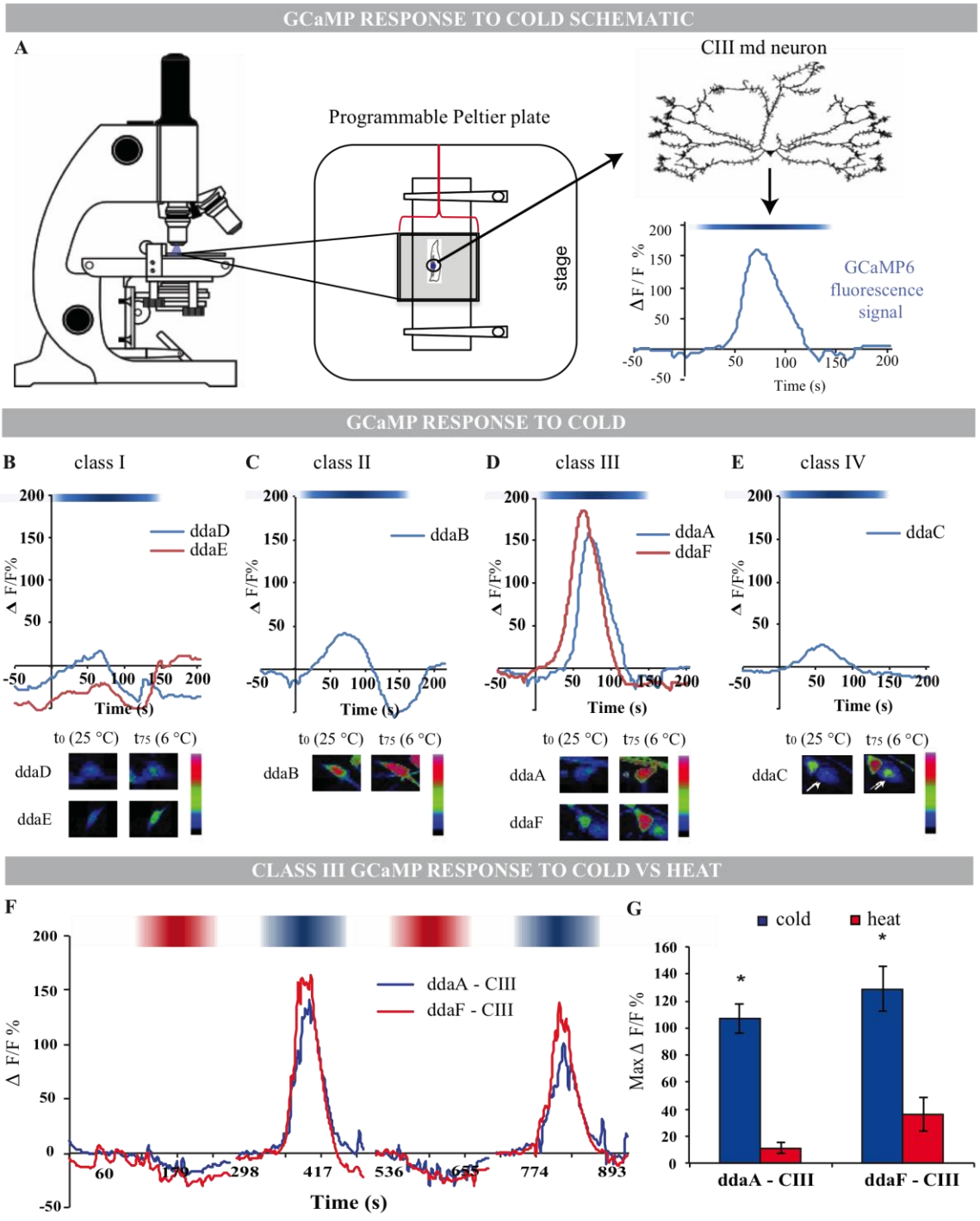
GCaMP RESPONSE TO COLD SCHEMATIC
A
Programmable Peltier plate
stage
CIII md neuron
GCaMP6 fluorescence signal
ΔF/F %
Time (s)
GCaMP RESPONSE TO COLD
B class I
C class II
D class III
E class IV
ddaD
ddaE
ddaB
ddaA
ddaF
ddaC
Δ F/F%
Time (s)
t0 (25 °C) t75 (6 °C)
CLASS III GCaMP RESPONSE TO COLD VS HEAT
F
G
ddaA - CIII
ddaF - CIII
Δ F/F %
Time (s)
cold
heat
Max Δ F/F %
ddaA - CIII
ddaF - CIII

Figure 2.8. Class III neurons are most specifically activated by cold via GCaMP signaling assay. (A) Diagram of time-lapse live imaging of GCaMP6 activation in response to cold stimulation. Intact third instar larvae are immobilized and placed on a programmable Peltier cold plate (boxed region in middle inset) under a confocal microscope. Shown here is a class III neuron identified under the microscope via expression of fluorescent transgenes, cell body shape and specific location in the larval segment. The Peltier plate is then cooled (6 °C) from a baseline control temperature (25 °C) while the change in GCaMP6 fluorescence signal in specific sensory neurons is recorded as a change in fluorescence over baseline over time. Blue bar indicates cooling of the plate over time, with coldest temperature shown in darkest blue. (B-E) Representative tracings of class-specific GCaMP6 responses (ΔF/F) with cold stimulation (6 °C) in (A) CI (ddaD, ddaE), (B) CII (ddaB), (C) CIII (ddaA, ddaF), and (D) CIV neurons (ddaC, marked by white arrow).White-blue spectrum bar signifies temperature range (25-6 ˚C). Bottom inserts: neuronal activation at a cold temperature (6 °C) and baseline temperature (25 °C). n = 20 per neuron type. (F) Representative tracing of GCaMP6 responses in class III neurons subjected to alternating noxious heat (44 ˚C) and noxious cold (6 ˚C) temperature cycles (indicated by red and blue bar across the top), n = 10. (G) Quantification of GCaMP activation in dorsal class III neuron subtypes (ddaA, ddaF) in response to cold (6 ˚C) vs hot (44 ˚C) stimuli.A two-tailed Welch's t-test was used to assess differences in average peak ΔF/F % ± s.e.m under noxious cold and heat exposure, * = $p < 0.001$, n = 10 per neuron subtype and condition. Error bars represent ± s.e.m.. Data collection and figure representation created in collaboration with Daniel Cox laboratory at Georgia State University, reproduced from (75).

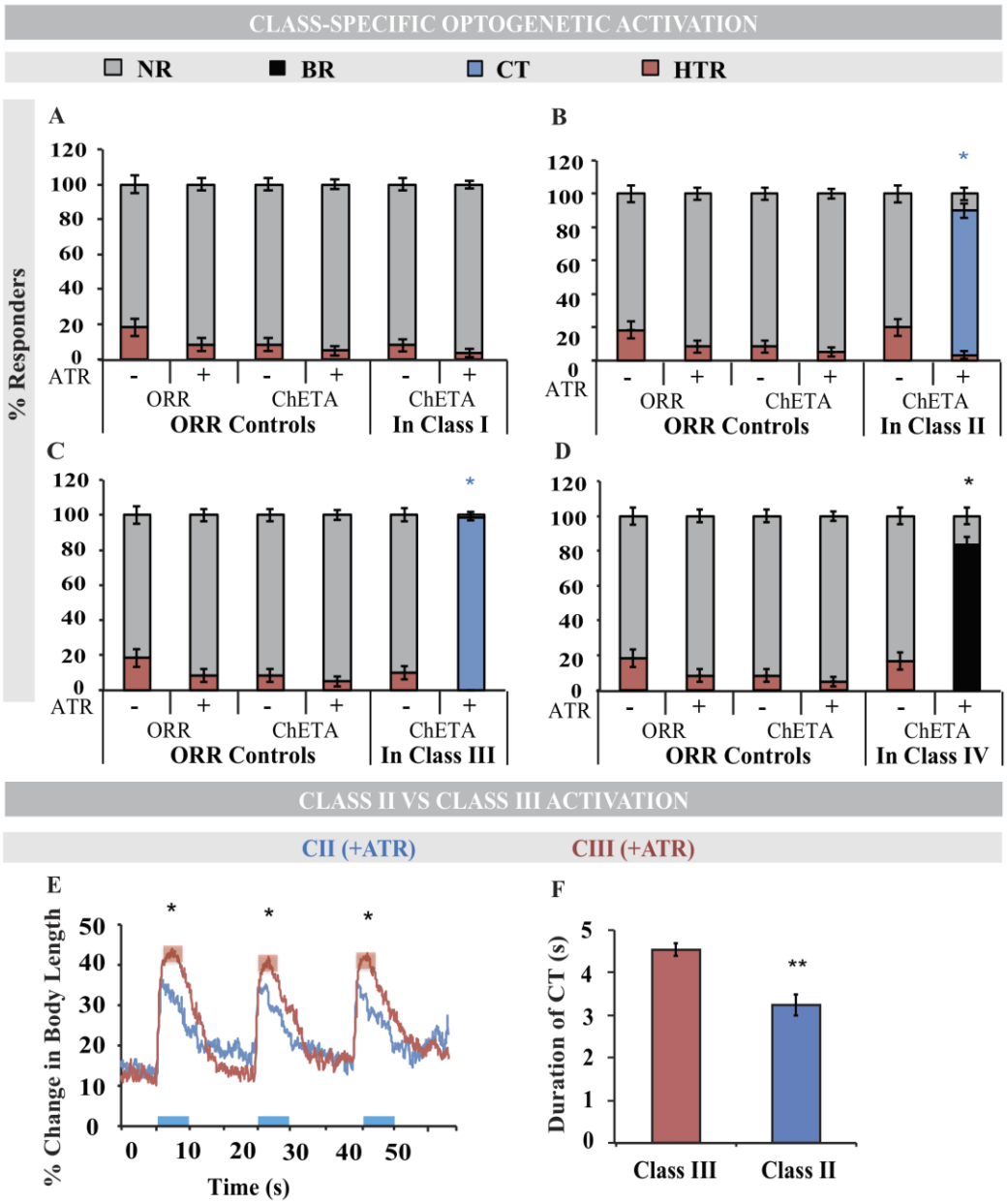

Figure 2.9. Optogenetic activation of class II and class III results in CT. (A) Behavioral responses observed upon optogenetic activation of class I, (B) class II, (C) class III and (D) class IV via ChETA expression in neurons. (A-D) The control strain (ORR), channelrhodopsin alone (ORR x CHETA), or larvae lacking the required ATR cofactor (-ATR) served as negative controls. HTR= head and/or tail raise; CT = contraction; BR = body roll; NR = non-responder. n = 15-20 per genotype/condition averaged ± s.e.p.. (E) Change in body length during optogenetic activation of class II or III neurons. Shaded red boxes indicate time period in which larvae exhibit a significantly increased change in body length in class III vs. class II. Light blue boxes indicate duration of 480 nm blue light stimulation. (F) Average duration of optogenetically-induced CT behavior (seconds) in class III vs. class II md neurons ± s.e.m.. Stats: (A-D) A two-tailed Fisher's exact test with Bonferonni correction, * = $p < 0.0001$, (E-F) A two-tailed Welch's t-test, * = $p < 0.05$, ** = $p < 0.001$, n = 20 per genotype. Error bars represent ± s.e.p.. Data collection and figure representation created in collaboration with Daniel Cox laboratory at Georgia State University, reproduced with permission from Current Biology (Turner et al. 2016).

that light-mediated activation of class III, or class II, neurons is sufficient to generate CT responses.

Role of TRP channels in *Drosophila* cold nociception

Transient receptor potential (TRP) channels play a diverse and significant role in sensory perception of vertebrates and invertebrates (14). The *Drosophila* genome contains 14 TRP channel genes, which have a number of known thermosensory functions (**Figure 1.2**). Peripheral expression of these genes in *Drosophila* larvae has begun to be characterized and a number of TRP channel genes are enriched in class III and class IV neurons (75, 100, 110). To determine if TRP channels play a role in *Drosophila* cold nociception, whole animal mutants for these genes transheterozygous for relevant deficiencies (large deletions spanning relevant gene of interest regions) were tested for changes in cold nociception. Interestingly, cold-evoked CT responses increased in some mutants and decreased in others compared to control larvae (**Figure 2.10A**). Those that were significantly decreased included *nompC, trp, Polycistic kidney disease gene 2 (Pkd2), Trpm, waterwich (wtrw), pyx, iav, Trpml,* and *nanchung (nan)* (**Figure 2.10A**). Within the same population of larvae, some mutants exhibited increases or decreases in cold evoked US responses compared to controls (**Figure 2.10B**). Mutants with significantly attenuated US responses to cold included *trpl, brv,* and *pain* (**Figure 2.10B**). It is interesting to note that TrpA1 mutant larvae exhibited no significant change in CT or US responses to the cold probe, indicating that while this channel is important in sensing warmth (83, 96) it is dispensable for sensing cold in *Drosophila*. Of mutants exhibiting CT response defects, *Pkd2*, *nompC* and *Trpm* genes were of particular interest given their enrichment in class III neurons (75, 110), and *pain* was especially

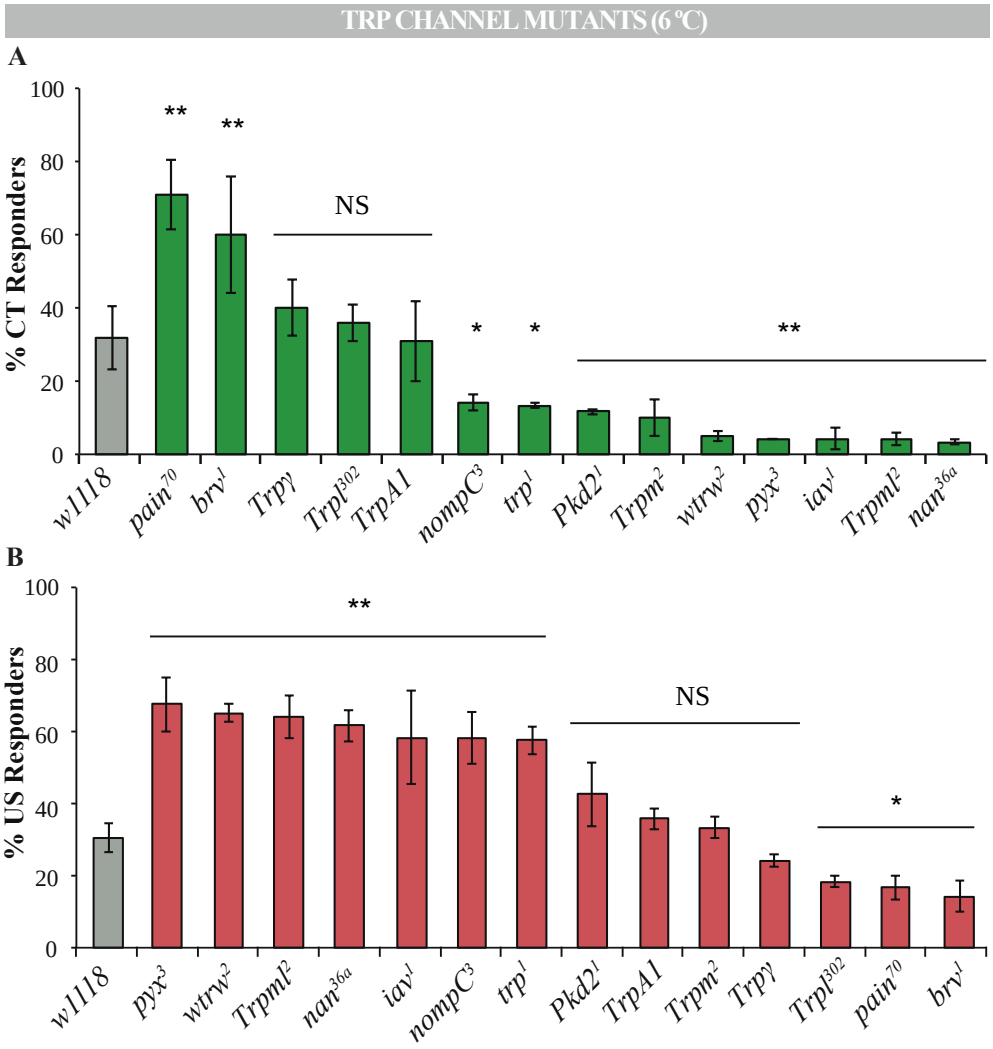

Figure 2.10. Cold Response in TRP Channel Mutants.
(A) Percent CT (green bars) or (B) US (red bars) responders to cold probe (6 °C) in TRP channel whole animal mutants compared to control *w1118* (grey bars) larvae. All mutants were transheterozygous over relevant deficiencies (lis ted in Section 5.1). n = 90-120, responders averaged ± s.e.m.. * = $p < 0.05$, ** = $p < 0.01$ in dicating significant compared to *w1118* by Fisher's exact test.

interesting given its known functions for mediating body roll responses to noxious heat and harsh touch (19).

Considering the supporting evidence for class III neuron's role in CT responses thus far, at this point we chose to focus on the possible functions of Pkd2, NompC, and Trpm in class III neurons to mediate cold-evoked CT. Larvae bearing RNAi transgenes *Pkd2*, *nompC*, or *Trpm* in class III neurons all showed marked defects in cold-evoked CT responses (**Figure 2.11A)**. Further, rescue to normal levels of CT responders was attained in mutants over-expressing *Pkd2* or *nompC* transgenes specifically in class III neurons (**Figure 2.11B**). These results suggest that different cells and TRP channels mediate different cold-evoked behavioral responses. In particular, US responses seem to be mediated by Trpl, Brv and Pain, while CT responses through NompC, Trpm and Pkd2 in class III neurons. It remains unknown whether expressing these RNAi transgenes or TRP overexpression (rescue) results in the same effects when expressed in class I, II or IV neurons.

2.3 Discussion

The results shown here illustrate and characterize a novel assay to study noxious cold detection in *Drosophila* larvae. This assay utilizes a custom-built Peltier probe, capable of applying a focal cold to ambient (3-22 ºC) stimulus, resulting in quantifiable cold-specific behaviors. These behaviors, US, PR and CT, are unlike larval responses to other types of nociceptive (19, 74, 77, 89, 108) or innocuous stimuli (102, 107). Several lines of evidence indicate that class III neurons are important for responding to cold temperatures. First, upon

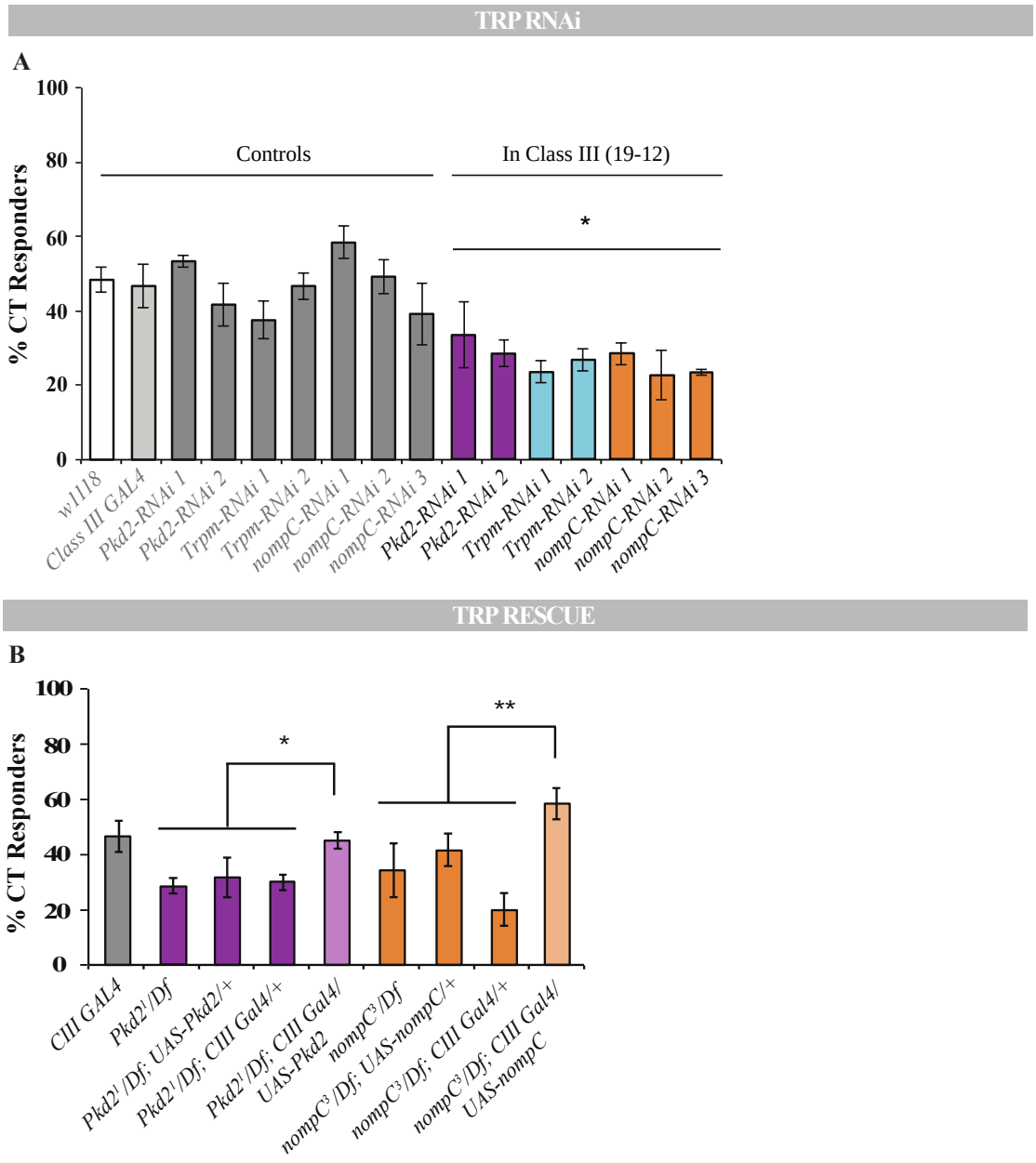

Figure 2.11. Function of TRP channels Pkd2, Trpm and nompC in class III sensory neu rons. (A) Pkd2, nompC and Trpm targeted expression of RNAi transgenes in class III neurons (CIII via 19-12 GAL4) responses in the cold probe (10 ºC) assa y. (B) Rescue of Pkd2 or nompC in class III neurons via TRP overexpression in mutant over deficiency background in cold probe assay (10 ºC). (A-B) n = 3 sets of n = 20 averaged ± s.e.m.. (B-F) White and grey bars: controls; colored bars: di fferent genes targeted. * = $p < 0.05$, ** = $p < 0.01$ via Fishe r's exact test (B) * indicates significant compared to relevant mutant over deficiency control. Modified figure from autho r's published work (75).

silencing class III neurons larvae exhibit significant defects in cold-evoked responses (CT and US responses respectively). Second, class III neurons are directly and specifically activated by cold temperatures as shown by GCaMP responses. Lastly, larvae produce a CT response upon direct optogenetic activation of class III neurons.

The data supporting class IV involvement in cold sensing are less definite. While silencing class IV neurons results in a defect in the cold-evoked US response, direct optogenetic activation of class IV neurons results in a body roll response, not a US (this study, (75) and (74)). It may be that, class IV neurons only lightly activated result in a US whereas strong activation results in a BR (see Chapter 3, Figure 3.2, for an example of this type of experiment). However, GCaMP imaging of class IV also resulted in no calcium fluorescence upon cold activation. This suggests that perhaps class IV neurons do not directly respond to cold, but instead play a more modulatory role in cold sensing (this hypothesis will be explored further in Chapter 4). Lastly, while class II neurons seem to be activated by cold (as indicated by their GCaMP responses), upon genetic silencing they do not appear to be required for cold-evoked responses. This may also imply a modulatory role for class II, however the precise roles of class II and class IV neurons in cold detection and their role in mediating cold behaviors need to be studied further.

At the molecular level, *Pkd2*, *nompC*, and *Trpm*, are enriched and appear to function in class III neurons to mediate cold-evoked CT. These are interesting hits given these channels' other known sensory functions, which indicate that these channels are multimodal. *nompC* is involved in gentle touch (100, 101), *Pkd2* in taste (111, 112) and mechanosensation in the primary cilia of the vertebrate kidney epithelium (113). Currently, it is unclear if patients suffering from polycystic kidney disease, or mice lacking the Pkd2 gene (114), have

cold nociception defects, but this will be an interesting area for future study. Lastly, although TRPM8 is a known cold sensor in vertebrates (115, 116), this is the first evidence that a *Trpm* family member acts in cold nociception in *Drosophila. Trpm* also plays a role in maintaining zinc and magnesium homeostasis in *Drosophila* (117, 118).

While there is much evidence for multimodality among TRPs and other sensory channels (see Painless (19, 92, 104); TrpA1 (90-92, 105, 119); Pickpocket1 (104, 120, 121) and Pickpocket 26 (88, 121)), it begs the question: how do these channels distinguish between innocuous and harsh stimuli? For *painless* and *TrpA1,* functionality may depend on different splice variations (122). In *nompC*, ankyrin repeats are required for its mechanosensory function (123, 124) but it is unknown if they are also required for responding to cold. Other studies suggest TRP channels may collaborate with different sets of partially overlapping channels for different functions. For gentle touch, this includes NompC, Ripped Pocket, Nmdar1 and Nmdar2 (100, 101). For noxious cold, vertebrate studies have revealed interactions between TRPM8 and potassium channels (Task-3, Kv1 and Kv7) (see review (125)) and voltage-gated sodium channels (Nav1.8 and 1.9)(35, 36). Such complex interactions, particularly in class III neurons, may help explain how one channel can alter responses to multiple types of stimuli. Microarray expression profiles of class III neurons seems to support this, as two particular calcium-activated potassium channels were found to be enriched in class III neurons (*SK,* 2.1 average fold change and *slowpoke,* 10.4 average fold change; see GEO accessions GSE69353 and GSE46154). It is unknown however, if either of these channels are required for responses to cold in our assay. Of the TRP channels identified here, Pkd2 seems the most likely to act as a direct cold sensor as calcium levels are decreased

upon cold exposure in *Pkd2* mutants and mis-expression of Pkd2 confers cold-responsiveness to other sensory neurons (75).

Taken together, our results identify the peripheral sensory neurons responsible for noxious cold detection in *Drosophila* larvae and conserved molecular players required for this process. The cold assay developed here offers a powerful model for the genetic dissection of cold nociception. Further exploitation of this model should yield exciting insight into cold nociceptive genes and circuitry.

CHAPTER 3: Behavioral Responses to Competing Sensory Stimuli: Circuits Underlying Responses to Noxious Heat, Cold and Gentle Touch

3.1 Introduction

Our results thus far indicate that class III neurons are capable of responding to cold stimuli, in addition to gentle touch (100, 101). Like cold, there are multiple gentle touch behaviors including: pause, head withdrawal/recoil, turn, reverse locomotion (single peristaltic wave backwards) and reverse retreat (multiple peristaltic waves backwards) (106). Unlike cold however, these behaviors are often scored and combined into an overall 'gentle touch score'. There are a number of channels that are required in class II and/or class III neurons to respond gentle touch, including NompC, Ripped Pocket, Nmdar1 and Nmdar2 (100, 101). The fact that class III neurons can mediate two different types of behavioral output (CT and gentle touch behaviors) presents an interesting biological question: How do class III neurons mediate responses to two very different stimuli?

Class III neurons are not the only neurons that can do this however. Class IV neurons are highly multimodal, and other than responding to noxious heat, they are known to mediate behavioral responses to harsh touch with a 360 º body roll (19, 88), mechanical stimuli for locomotion (102), and aversive light mainly through locomotor avoidance (turning)(105). This degree of multimodality is not limited to *Drosophila*. C-fibers are activated by heat, cold and mechanical stimuli (11). This biology leads to another interesting question: what happens if an animal is exposed to both hot and cold at the same time?

In adults and larvae, *Drosophila* avoids temperatures outside their comfortable range by utilizing a distinct set of thermosensitive antennal or dorsal organ neurons in the head (84, 95, 99). Interestingly, the cold sensitive neurons are inhibited by heat and vice versa to ultimately determine motor output to help the animal navigate along a temperature gradient (82, 84). Similar circuits have begun to be characterized for noxious stimuli (126, 127). The

full neuronal circuit for class III that mediates CT responses to cold however, is not known. Yet, we can start to get at this question by interrogating the behavioral responses to both stimuli to see which response is produced.

Here we show and discuss how *Drosophila* can be used to ask how neurons are activated by different sensory stimuli to ultimately mediate different behavioral outputs, and how they can be used to dissect competing nociceptive circuits.

3.2 Results

Cold versus Hot: Competing thermosensory stimuli result in primarily cold-evoked responses

Thus far, our data suggest that class III neurons mediate CT responses to cold, utilizing a set of sensory receptors that include Trpm, NompC and Pkd2. Previous studies have indicated that class IV neurons are required for the body roll (BR) response to high temperature, mediated primarily by the TRP channel Painless (19, 74). What would happen however, if class III and class IV neurons were activated simultaneously? Class III and class IV dendritic arbors both extensively cover the entire larval body surface and their axonal projections terminate near each other in the anterior ventral nerve cord (86, 87). By expressing *TrpA1,* which has been used to thermogenetically activate non-thermosensory neurons (83), in class III neurons, the application of a heat probe should activate both class III neurons (through TrpA1) and class IV neurons (through endogenous heat channels like Painless) simultaneously. Using this method, simultaneous class III and class IV activation resulted in predominantly CT responses, correlated with a dramatic reduction in the typical level of heat-evoked BR responses (**Figure 3.1A**). This result was supported by an

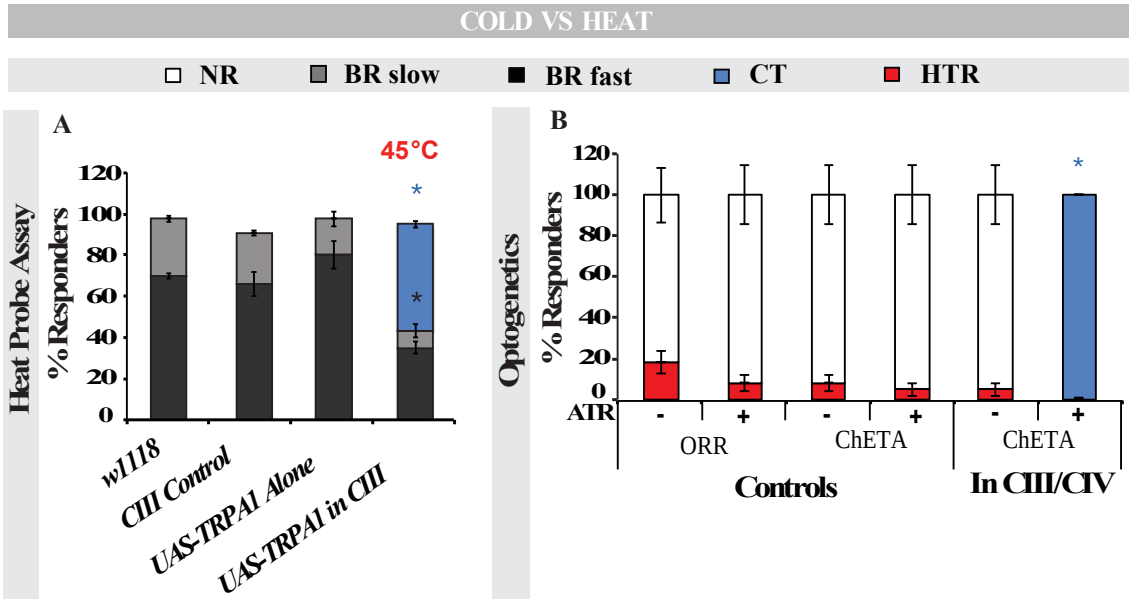

Figure 3.1. Coactivation experiments reveal preference for CT output response.
(A) Percent responders with class III (CIII) and class IV (CIV) coactivation via CIII TRPA1 expression and heat probe stimulation (45 ˚C). n = 3 sets of 40 averaged ± s.e.m..* p = < 0.001, (B) Percent responders upon CIII and CIV optogenetic coactivation. n = 45-60 averaged ± s.e.p.. (A-B) * = p < 0.001, colored * indicate significance between bars of same color. CT = contraction; HTR = head and/or tail raise; BR fast = body roll within 5 s; BR slow = body roll with 6-20 s; NR = non-responder. (B) Data collection and figure representation created in collaboration with Daniel Cox laboratory at Georgia State University. (A-B) Figure modified from published work (75).

alternative approach where class III and class IV neurons were simultaneously activated through channelrhodopsin expression and exposure to blue light (**Figure 3.1B**). In this case, the majority of responses were CT (**Figure 3.1B**). These results suggest that noxious cold signals, and the CT behavioral output, outcompetes signals from noxious heat and subsequent BR responses when class III and class IV neurons are activated simultaneously.

Class III behavioral output depends on level of activation

Given our results thus far implicating class III neurons as cold sensors, this makes them multimodal to both cold and gentle touch. One of the primary behavioral responses to gentle touch is a head withdrawal (HW)(106), which is like an asymmetric CT. To clarify how these cells might distinguish between cold and gentle touch we varied the dose of optogenetic light activating class III neurons, to see if a particular activation level evoked either HW or CT (**Figure 3.2A**). Optogenetic activation of class III neurons at the highest dose, resulted in CT almost exclusively (**Figure 3.2A**). The percentage of CT responders was reduced with decreasing light, however while HW responses increased (**Figure 3.2A**).

Our optogenetic dose-response suggests that cold may activate class III neurons more strongly than light touch or activate more class III neurons. To investigate this, we utilized the genetic tool CaMPARI, which upon exposure to photoconverting violet light shifts fluorescence from green to red as a function of intracellular calcium levels evoked by a specific stimulus(128). Class III neurons expressing CaMPARI exhibited a significant increase in photoconversion in response to cold versus gentle touch (**Figure 3.2B-D**).

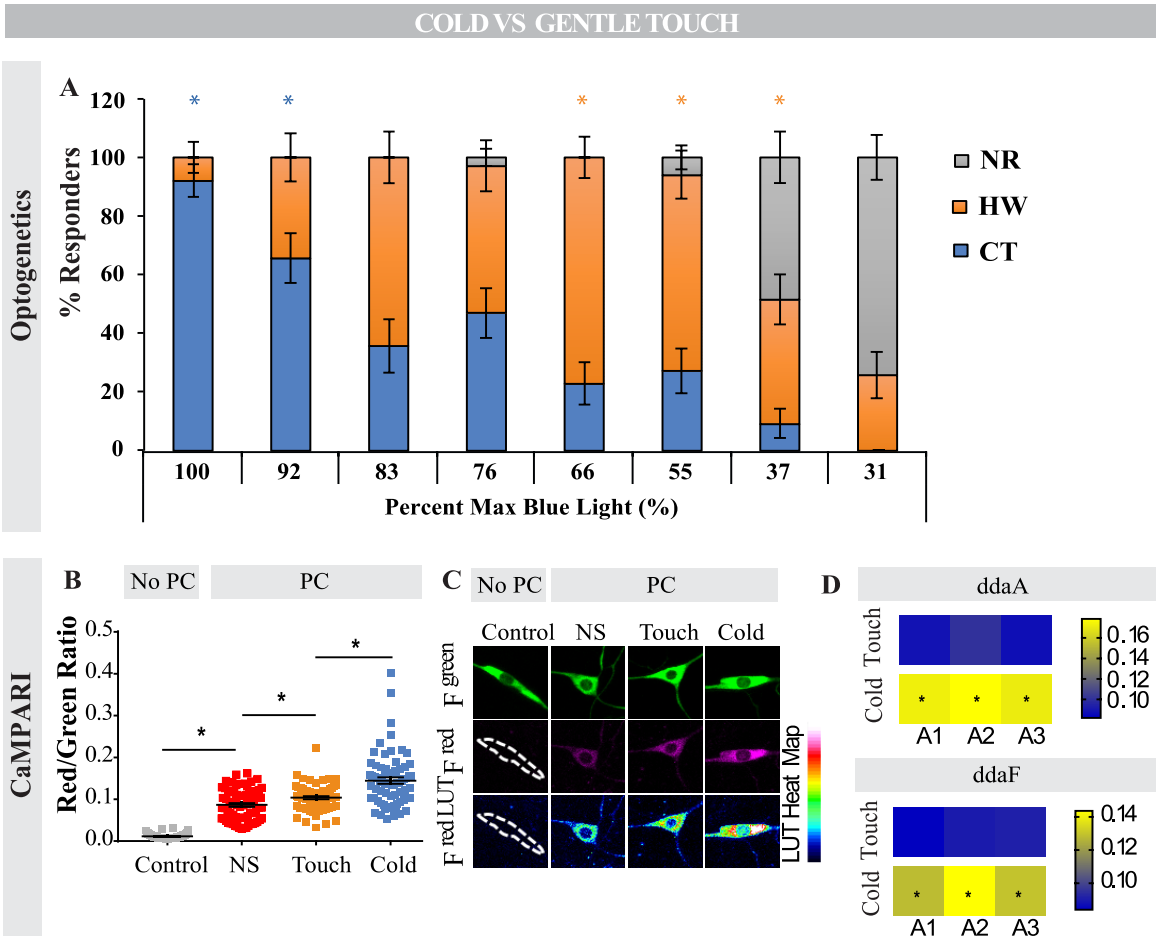

Figure 3.2. Optogenetic dose-response and CaMAPRI experiments reveal how different class III output behaviors are determined.

(A) Percent responders with varying light dose in larvae with CIII-ChETA expression. n = 25-35 averaged ± s.e.p.. Colored *: significance of indicated behavior compared to other behaviors at given light dose. Blue *p = ≤ 0.02; orange * p = ≤ 0.004. (B) Ratio of CIII green to red photoconversion (PC) in non-PC (grey) and PC non-stimulated (NS) (red) controls vs. PC touch-stimulated (orange) and cold-stimulated (blue) larvae bearing CaMPARI transgenes, n = 70-72 averaged ± s.e.m.. * = $p < 0.01$. (C) Representative images of CIII neuron PC. (D) PC ratio heat map analysis (n = 9-12 animals), yellow indicating stronger PC in abdominal segments A1-A3. * = $p < 0.05$. CT = contraction; HW = head withdrawal; HTR = head and/or tail raise; BR fast = body roll within 5 s; BR slow = body roll with 6-20 s; NR = non-responder. (A-D) Data collection and figure representation created in collaboration with Daniel Cox laboratory at Georgia State University. Modifed figure from published work (75).

While a similar number of activated class III neurons were observed between cold and gentle touch stimulated larvae, cold-evoked significantly larger CaMPARI responses in multiple larval segments (**Figure 3.2D**). These data suggest that noxious cold more strongly activates class III neurons compared to gentle touch.

Pkd2, Trpm and NompC also impact gentle touch

While class III and class IV neurons have different downstream behavioral outputs, cold (this study) and gentle touch responses are both mediated by class III neurons and result in different behavioral outputs. As stated above, this may be mediated by differences in the magnitude of cellular activation perhaps sensed through different suites of channels, but NompC has been implicated in both cold (here) and touch (100, 101). This raises the question: Are the other identified cold channels Pkd2 and Trpm also involved in responding to gentle touch?

To test whether Pkd2 and Trpm play a role in gentle touch, larvae bearing mutations for these genes transheterozygous for relevant deficiencies were tested in a gentle touch assay (**Figure 3.3A**). Gentle touch assays qualitatively identify a number of subtle behaviors that are typically weighted somewhat arbitrarily (**Figure 3.3B,** see Method 1). We scored larval responses using this scheme, but also with a modified scheme where each observed "touch" behavior is weighted equally (**Figure 3.3B,** see Method 2), and found that all three mutants displayed gentle touch defects (**Figure 3.3C-D**). Since one of the primary touch behaviors, "turning", also occurs frequently during normal locomotion, "locomotion scores" were calculated (based on same scoring criteria as "touch score") during a non-touch trial and compared to "touch scores". This comparison becomes important when comparing the two

GENTLE TOUCH ASSAY

Method

A

1. Measure Locomotion

a. Let larva acclimate for 60 s
b. Measure gentle touch behaviors for 10 s (no stimulus)
c. Wait 10 s
d. Repeat steps b & c, 4 times

2. Measure Gentle Touch

a. Stimulate larval T1-T3 segments with gentle touch stimulus
b. Measure gentle touch behaviors for 10 s
c. Wait 10 s
d. Repeat steps 2 & 3 four times

Scoring Scheme

B

	Method 1	Method 2
Observed Behavior	Point Value	Point Value
Pause	1	1
Head Withdrawal (HW) or Turn	2	1 for HW, 1 for Turn
Single Reverse (SR)	3	1
Reverse Retreat (RR)	4	1
Score Caluclation		
Single Trial	Highest scoring behavior	Sum of all behaviors observed
Total per Larva	Sum scores from all 4 trials	
Total Score	Average sum score, n= 20 larvae	

GENTLE TOUCH RESPONSES

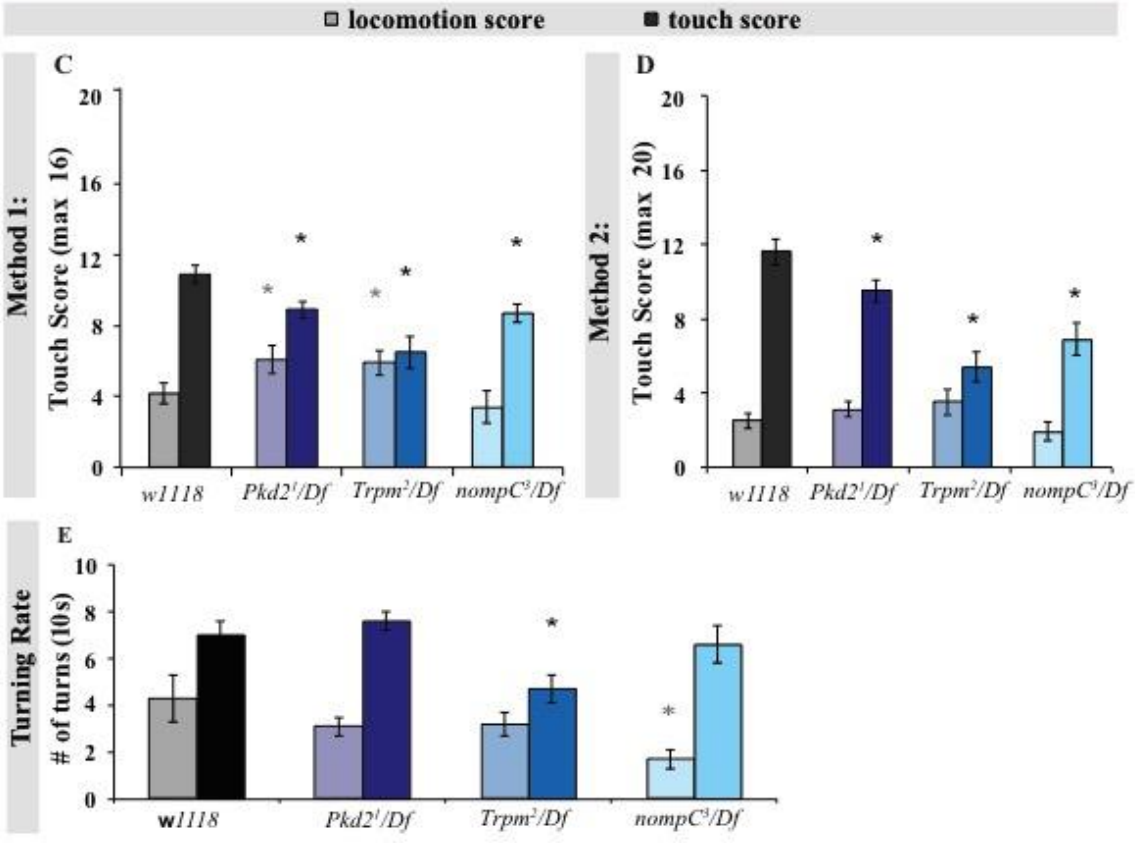

Figure 3.3. Pkd2, Trpm and NompC mutant larvae exhibit gentle touch defects.
(A) Diagram of gentle touch assay and (B) scoring schemes used to assess (C, D) locomotion and gentle touch responses in control (grey/black bars) larvae and mutants over relevant deficiencies (colored bars). (E) Comparison of the number of larval turns during normal locomotion or gentle touch trials. (C-E) Bars representing locomotion (light colors) or touch (darker colors) scores were averaged from 10 larvae. Each larvae was tested four times and scores were summed. Error bars indicate s.e.m.. Stats: two-tailed Welch's t-test * $p > 0.05$ compared to black control bar. * $p > 0.05$ compared to grey control bar. Figure reproduced from published work (75).

scoring schemes, as in method 1 TRP channel mutants have significantly altered "locomotion scores" compared to control larvae, where as in Method 2, they do not (**Figure 3.3C-D**). It is interesting to note, that the number of observed turns during a locomotion trial or touch trial, varied slightly only in *Trpm* mutants, with *Trpm* mutants having a significantly lower turning rate following a gentle touch stimulus (**Figure 3.3E**). In contrast, only *nompC* had significantly lower turning rates during normal locomotion, indicating a possible locomotion defect in these mutants (**Figure 3.3E**), which has been previously reported (103). These results indicate that Trpm, NompC and Pkd2 may not be sole noxious cold detectors, but may also be important for responding to gentle touch, and/or for amplifying sensory signals in the cell for necessary sensory transduction and ultimately behavioral responses to various sensory stimuli.

3.3 Discussion

Other studies have shown how single neuron types can be activated by or inhibited by contrasting stimuli such as high or low temperature (18, 84, 129). In this study however, we presented the animal with two different stimuli *simultaneously,* which gives us a different sort of information that is equally interesting. Coactivation of hot (class IV) and cold (class III) neurons, either thermogenetically or optogenetically, resulted in predominantly CT responders. This result is unlikely to arise solely from faster neuronal conduction since both neurons are of a similar size and terminate at similar locations in the anterior ventral nerve cord(87). These data suggest that class III neuronal activity is somehow able to interfere with class IV activity, or shift the behavioral output towards a CT at some point along the class III/cold circuit. Currently the class III/cold circuit has not been mapped out, but class IV neurons synapse on a set of basin interneurons in the ventral nerve cord of the fly (126). It

will be interesting to determine whether class III neurons also synapse on this interneuron and whether the basin interneuron is required for cold-evoked CT responses. However, it may be that CT responses require different interneurons, and that processing of competing hot and cold stimuli occurs in the brain, which could be similarly assessed through a combination of CaMPARI and other genetic techniques. For either of these outcomes, understanding the organization of this nociceptive circuit will be extremely useful in developing more specific pain models where additional environmental or genetic factors on pain processing could be explored. Obviously, the noxious hot and cold circuit in the periphery may be quite a bit more complex, given our data suggesting that cold responses may be mediated by multiple sensory neuron types (US by class IV and CT by class III), however, with further work using this assay and similar genetic and imaging approaches, elucidating the full thermosensory circuit should be achievable.

It has long been an open question in sensory biology how specific sensory cells and receptors can respond to different sensory stimuli, sometimes resulting in different behavioral outputs. Here we explore several possible explanations for how class III neurons may be activated by both noxious cold and gentle touch. First, class III neurons could be activated to different magnitudes by different stimuli, resulting in different firing frequencies and/or patterns which can be interpreted by the second order neuron or further downstream in the brain, which is ultimately translated into the proper avoidance behavior. An alternative hypothesis is that different sensory stimuli activate a different number of sensory neurons, similarly resulting in a lower or higher magnitude of input into the second order neuron and/or into downstream processing areas. Interestingly, with low doses of light larvae exhibited a gentle touch behavior (HW), while stronger doses of light resulted in CT,

indicating that the level of cellular activation plays a role in behavioral output. Further, measurements of calcium fluorescence via CaMPARI indicated a significant difference between the magnitudes of class III activation when larvae are stimulated with noxious cold versus gentle touch, but not an observable difference in the number of activated neurons. These data suggest that class III neurons may have different activation thresholds that ultimately determine the correct behavioral output to different stimuli.

How these levels of activation translate into action potential firing frequency, or firing patterns represents an interesting area of future study. It is still unknown how class IV neurons mediate behavioral responses to high temperature, harsh touch (BR), proprioceptive feedback (normal locomotion) and possibly contribute to US responses to cold (here). Although optogenetic activation of class IV neurons results in a rolling response, it may be that lower doses of activation result in US responses and/or increases in locomotor avoidance (turning rate and head casts). In any case, this dose-response optogenetic approach represents a useful starting point for dissecting multiple roles of sensory neurons and sensory circuits.

While we found *Pkd2*, *Trpm* and *nompC* mutants displayed gentle touch defects it remains unclear whether this is due to a specific loss of these channels in touch sensing neurons. It will be important to target these genes using RNAi and perform genetic rescue specifically in gentle touch neurons to confirm their role in these neurons and to determine if cell-specific rescue can restore gentle touch responses. Although for NompC ectopic expression conveys touch sensitivity (101) and its mechanism of mechano-activation, has begun to be elucidated (124), this will need to be further explored for Pkd2 and Trpm to determine their true roles in gentle touch. It may be that for many sensory neurons TRP channels act broadly to amplify signals to various stimuli, either through direct or indirect

gating. However, there have been a number of reports investigating how a single TRP channel can be activated in multiple ways by various stimuli (122, 123, 130), so a direct role for the TRPs identified here in both touch and cold is not inconceivable. Further studies are warranted however to confirm these roles, especially for gentle touch, and to uncover the mechanism(s) of channel activation. Alternatively, these TRP channels could be impacting behavior more broadly, such as on locomotion. Although one locomotion measure was quantified here (turning rate), there are much more sophisticated and sensitive techniques to measure locomotion defects in *Drosophila* larvae. It will be interesting to investigate whether these particular "gentle touch" defects we observe here, are actually an effect of an overall locomotion defect.

CHAPTER 4: Injury-Induced Cold Sensitization in *Drosophila* Larvae

4.1. Introduction

After tissue injury, the sensory system of cells and receptors that normally sense innocuous or painful stimuli can become hypersensitive (43). Sensitization is characterized by decrease in the perceived pain threshold, which can be categorized into two groups: 1. Allodynia: where previously perceived innocuous stimuli are now regarded as painful; or, 2. Hyperalgesia: where painful stimuli elicit a more robust response than before the injury. Injury-induced sensitization is thought to foster protective behavioral mechanisms to prevent further tissue damage and aid wound healing, but when sensitization extends beyond the time necessary for wound healing, chronic pain syndromes may be the culprit. Currently, given that chronic pain can arise under many different contexts (injury, inflammation, disease, etc…) finding the cellular and genetic mechanisms underlying these conditions has been difficult, but some important players have been found (12).

Vertebrate models of nociceptive sensitization

Using animals models, chronic and neuropathic pain research has been slowly but methodically pushed forward (9). One model for studying chronic neuropathic pain, is rodent spinal cord injury, which depending on the severity of the injury can be used to study hypersensitization or loss of sensory or pain processing (131). There are a multitude of genetic rodent models of disease associated with chronic pain (132), including those for diabetes (133), multiple sclerosis (50) and HIV (134). Another common area of pain hypersensitivity research in vertebrates is inflammation-induced hypersensitivity. These models usually involve injection of agents that directly activate nociceptive receptors such as formalin (TRPA1) or capsaicin (TRPV1), or inflammatory molecules such as TNFα,

carrageenan, substance P or Complete Freund's Adjuvant (CFA)(9). Thus far, rodent models have been useful in identifying cells and genes involved in clinical hypersensitivity and uncovering potential drug targets, however there is still much we do not know about the mechanisms underlying these models of chronic and neuropathic pain (9).

Drosophila models of nociceptive sensitization

Drosophila has been used as an efficient and cost-effective model for unbiased forward genetics to identify conserved players in nociceptive sensitization following tissue damage (77, 92, 135). To induce sensitization, larvae are exposed to UV irradiation mimicking a sunburn phenotype (77). UV causes a rapid apoptotic breakdown of the barrier epidermis between 16-24 hours after administration accompanied by behavioral sensitization to thermal stimuli in larvae (77). This behavioral sensitization to innocuous warm and noxious heat is mediated through conserved genetic pathways that include Hedgehog (Hh), Tumor Necrosis Factor (TNF), and Tachykinin (Substance P)(77, 92, 135). Further, using *Drosophila* genetics, these studies have identified the cells where the ligands and receptors are required, and novel genetic players in nociceptive sensitization to thermal stimuli. Specifically, class IV neuronal activity thresholds to heat are lowered after UV and the subsequent behavioral sensitization to heat requires Tachykinin (Tk) and Hh signaling components, and the TRP channel Painless (135).

Currently, it is unknown if *Drosophila* also sensitizes to other types of stimuli after UV damage, such as mechanical, chemical and cold. This study investigates whether *Drosophila* larvae sensitize to cold stimuli following tissue damage, and if so, whether they utilize identified nociceptors and genetic pathways to mediate cold sensitization.

4.2. Results

Drosophila larvae are sensitized to cold after tissue damage

Since *Drosophila* larvae sensitize to noxious heat after tissue damage (77, 92, 135), and respond to noxious cold without injury (here, and (75)), we wanted to determine if larvae also behaviorally sensitize to noxious cold after tissue damage. A UV-cross-linker was used to apply UV-induced damage to the dorsal epidermis (as done previously (77) with minor modifications, see methods in Chapter 5). Larvae were then allowed to recover for different amounts of time before being tested for changes in cold nociception. Interestingly, one of the cold behaviors, contraction (CT), was significantly decreased 16 and 24 hours after UV damage compared to mock-treated controls (**Figure 4.1A, E-F**). In contrast, the percent of U-Shape (US) responders to the cold probe was increased at 16 and 24 hours after UV (**Figure 4.1B, E-F)**. At 16 and 24 hours after UV, a significant number of larvae also respond to the cold probe with a body roll (BR), normally only seen in response to high temperature and harsh touch (**Figure 4.1B**). This result is surprising, given that BR responses are rarely, if ever, seen in response to a cold stimulus under baseline conditions (compare to mock-treated controls). Comparing the response versus latency at 24 hours after UV also resulted in significant differences between mock and UV treated larvae for all three behavioral responses (**Figure 4.1C-D**), however it does not appear that the percent of slow responders alters much between mock and UV-treated conditions (**Figure 4.1E-F**). There was no change in PR responses to cold following UV (data not shown).

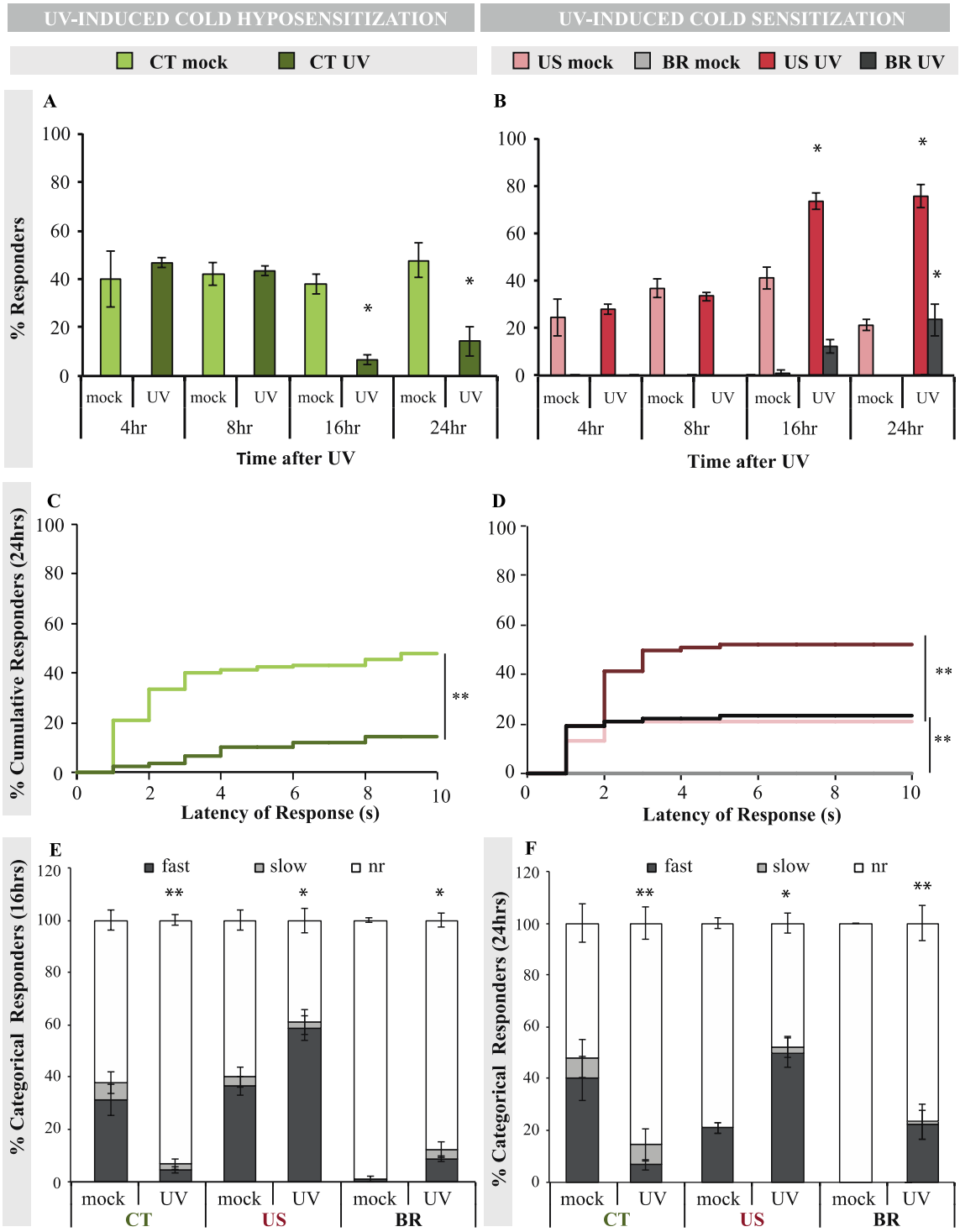
UV-INDUCED COLD HYPOSENSITIZATION
UV-INDUCED COLD SENSITIZATION
CT mock
CT UV
US mock
BR mock
US UV
BR UV
A
B
% Responders
mock
UV
4hr
8hr
16hr
24hr
Time after UV
C
D
% Cumulative Responders (24hrs)
Latency of Response (s)
E
F
fast
slow
nr
% Categorical Responders (16hrs)
% Categorical Responders (24hrs)
CT
US
BR

Figure 4.1. UV-induced cold sensitization peaks at 16 and 24hrs after UV.
(A) Percent of CT, (B) US or BR responders to cold probe (10 ºC) at different time points after UV. (C) Cumulative average latency of CT, (D) US or BR responders during a 10s cold stimulation (10 ºC) in mock or UV-treated larvae 24 hours after UV. (E) Percent average responders categorized as: fast (< 4s), slow (4-10 s) or no responders (no response within 10s) during a 10s cold stimulation (10 ºC) in mock or UV-treated larvae 16 or (F) 24 hours after UV. CT = Contraction; US=U-Shape; BR = Body roll, n = 90. (A-B, E-F) Data presented as mean ± s.e.m.. (C-F) as percent responders at given latency (accumulated over time). Stats: (A-B) two-tailed Fisher's Exact test, (C-D) Grehan-Breslow-Wilcoxon and Log-rank (Mantel-Cox) tests, (E-F) chi-square test. (C-F) * = $p < 0.05$, ** = $p < 0.0001$ comparisons were made between UV and mock control.

To determine if there is an optimal cold temperature for analyzing UV-induced cold sensitization, we tested larvae 24 hours after UV at different cold temperatures. Although we observed decreases in CT responses at 5, 10 and 15 ºC after UV compared to mock-treated controls, we only saw US sensitization at 10 ºC, and only saw BR responses with a 10 ºC or 15 ºC probe (**Figure 4.2**). Responses to a room temperature (RT) probe were also examined to determine if changes in gentle touch could be observed, however no change in cold-evoked responses after UV were seen with the RT probe (**Figure 4.2**). Lastly, since the precise UV dose can vary between 10-14 mJ/cm2 UV-C to the dorsal side, larvae grouped by received UV dose were tested in the cold assay 24 hours after UV and compared. The percent of CT responders at 13 mJ/cm2 UV-C was the only behavioral response statistically significant when compared to the other doses (**Figure 4.3A, B**), but when comparing response versus latency trends BR responses were also slightly different depending on UV dose (**Figure 4.3D**).

Peripheral sensory neurons are required for UV-induced cold sensitization

Given that baseline CT responses to cold require class III md sensory neurons (75) and BR responses to heat and mechanical stimuli are mediated by class IV neurons (74), we wanted to determine if either of these neuron classes are required for UV-induced cold sensitization. We were also interested in looking at the Type 1 sensory neurons, chordotonal neurons, (md neurons are Type 2) which are expressed in the lateral areas of the larval body (86). Chordotonal neurons have ciliated monopolar dendrites and were initially discovered as mechanoreceptors involved in gentle touch (102) and locomotion (103), but

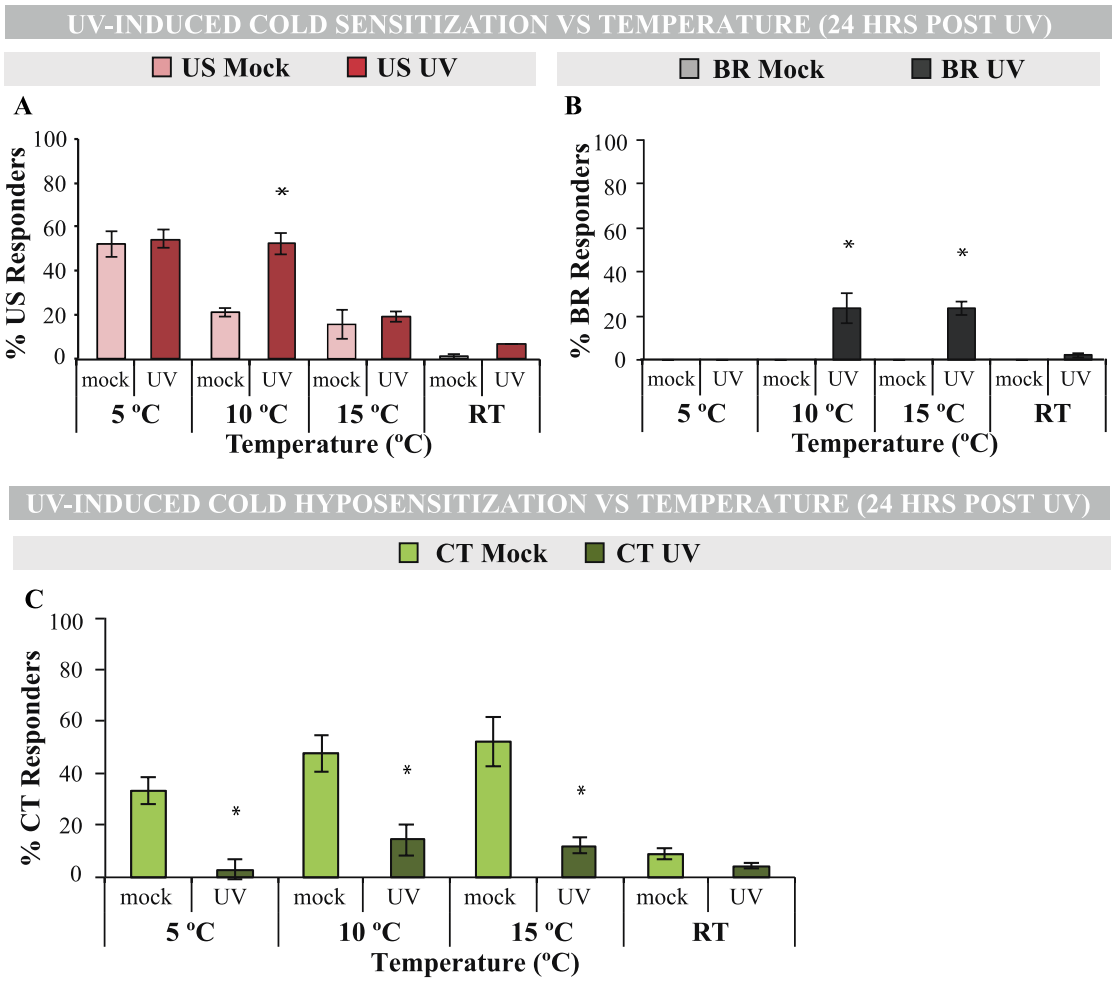

Figure 4.2. UV-Induced cold sensitization peaks at 10 ºC. Larvae were tested 24 hours after UV exposure or mock treatment with the cold probe either at 5 ºC, 10 ºC, 15 ºC or room temperature (RT) and the percent responders for US, (B) BR, (C) or CT were averaged. n = 90. Data are presented as the average ± s.e.m.. Stats: two-tailed Fisher's Exact test, * = $p < 0.05$, comparisons were made between UV and mock control at each temperature.

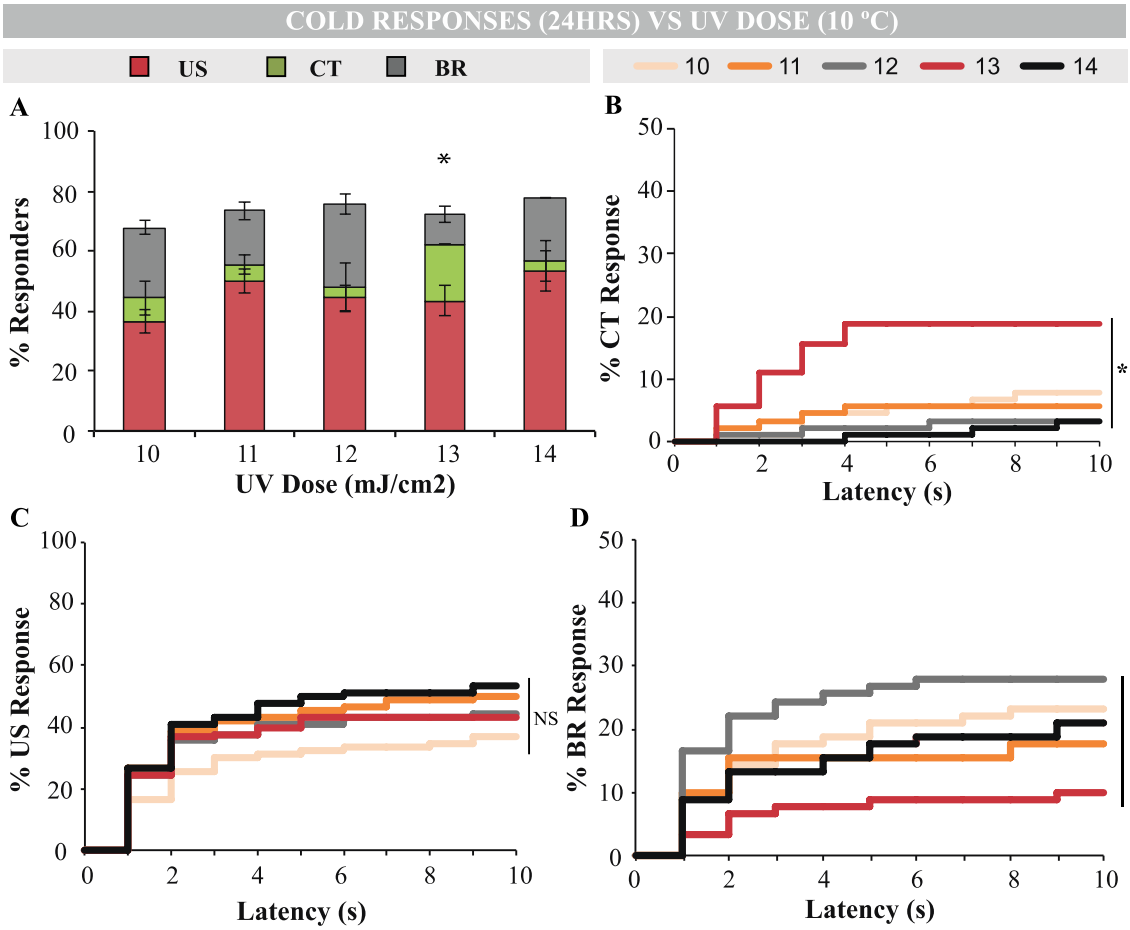

Figure 4.3. UV-dose varies cold responses only slightly.
(A) Larvae were tested 24 hours after UV exposure at varying doses or mock treatment with the cold probe (10 ºC) and the percent responders for US, BR, and CT were averaged ± s.e.m.. (B-D) Percent (B) CT, (C) US, or (D) BR responders to cold probe (10 ºC) 24hrs after UV at given dose. (A-D) Stats: (A) two-tailed Fisher's Exact test, * = $p < 0.05$, n = 90, (B-D) * = $p < 0.05$, ** = $p < 0.001$, ns (no significant differences) by Log-rank Mantel-Cox test, * by Log-rank test for trend. Comparisons were made between UV doses for each behavior.

have also been implicated avoiding cool temperatures in larvae (95) (See schematic of chordotonal morphology and function **Figure 4.4**). To determine if these neuron types are required for UV-induced sensitization to cold, the tetanus toxin transgene (UAS-TnTE) was expressed in class III, class IV, or chordotonal neurons to silence them and larval responses to cold after UV were compared. US sensitized responses 24 hours after UV were blocked when chordotonal neurons were silenced, but not when class III or class IV neurons were silenced compared to genetic and mock treated controls (**Figure 4.5A**). Interestingly, BR sensitized responses were blocked when class IV neurons were silenced, but also when chordotonal neurons were silenced (**Figure 4.5B**). Further, silencing class III neurons blocked CT responses in both mock (as shown previously (75)) and UV-treated larvae (**Figure 4.5C**). These data suggest a novel and important role for class IV and chordotonal neurons in UV-induced cold sensitization.

Role of thermal sensitization pathways in UV-induced cold sensitization

Since class IV neurons seem to play as an important role in cold sensitization as they do in heat sensitization, it follows that the genetic pathways required for heat sensitization may also be involved in cold sensitization. Mutants for *TNF* or *Tk* were tested for UV-induced cold sensitization 24 hours after UV. Although UV-induced changes in US or CT responses after UV were still present (a significant increase in US and decrease in CT respectively compared to mock controls)(**Figure 4.6A)**, *Tk* mutant larvae did not exhibit BR sensitization (**Figure 4.6B**). There was also a lack of BR sensitization in one of the TNF

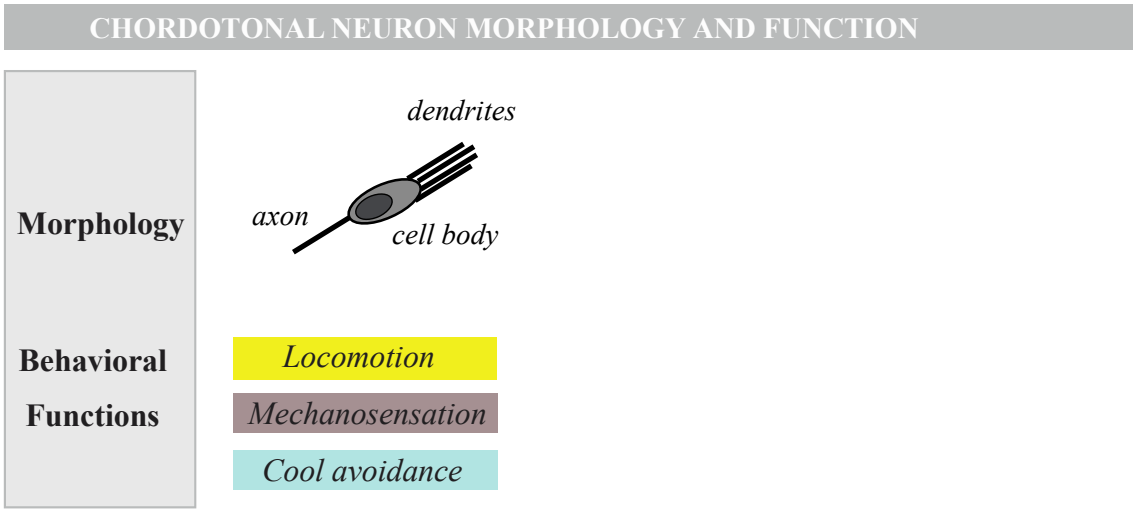

Figure 4.4. Chordotonal neuron morphology and function.

Schematic of peripheral chordotonal neuron morphology with known sensory functions.

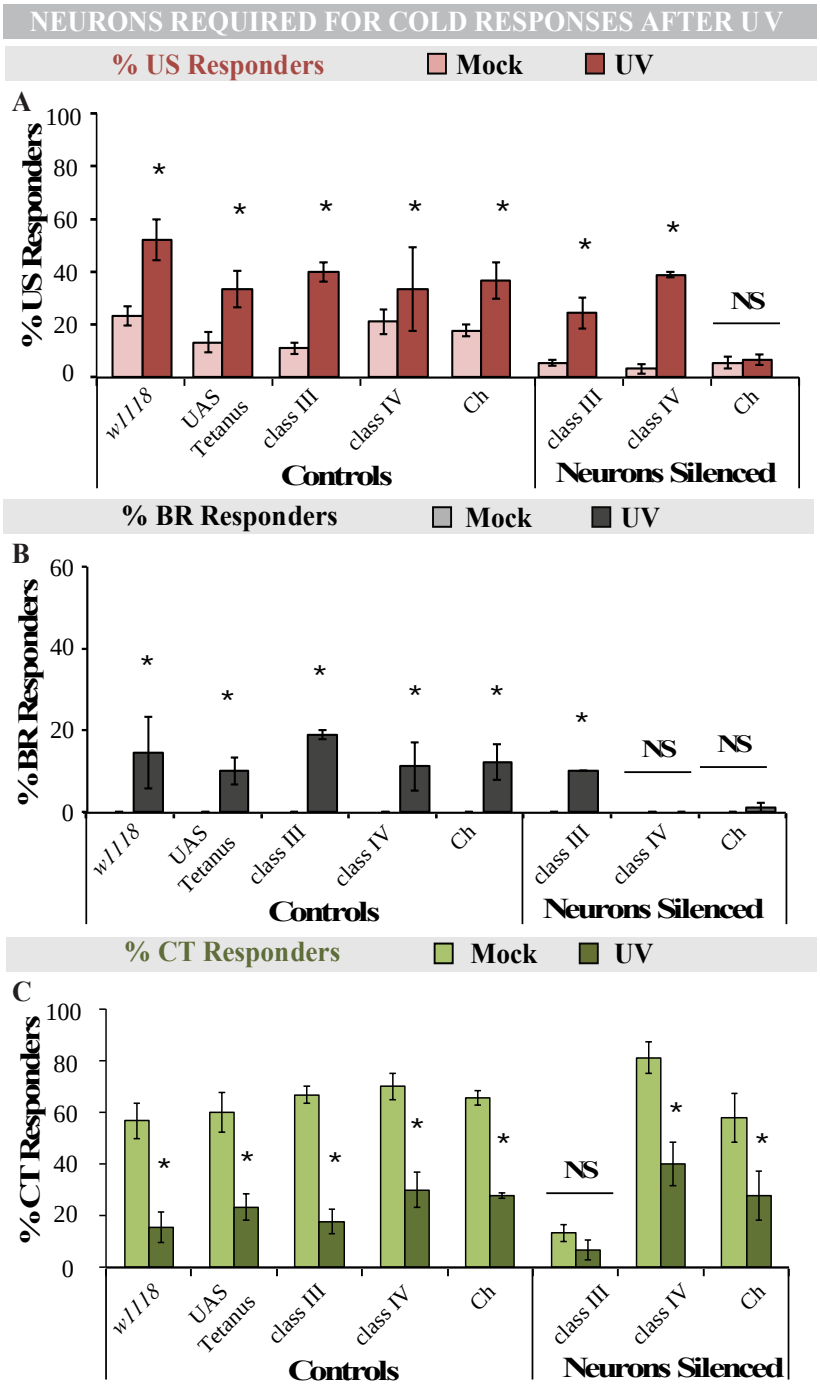

Figure 4.5. UV-induced cold sensitization requires class IV and Chordotonal sensory neurons. Larvae expressing active or inactive (control) form of the tetanus toxin transgene (see materials and methods) in class III, class IV or chordotonal neurons (Ch) were tested for cold responses 24 hours after UV exposure or mock treatment. (A) Percent US, (B) BR, or (C) CT responders were averaged and UV versus mock-treated responses were compared. n = 90. Data are presented as the average ± s.e.m. Stats: two-tailed Fisher 's Exact test * = $p < 0.05$.

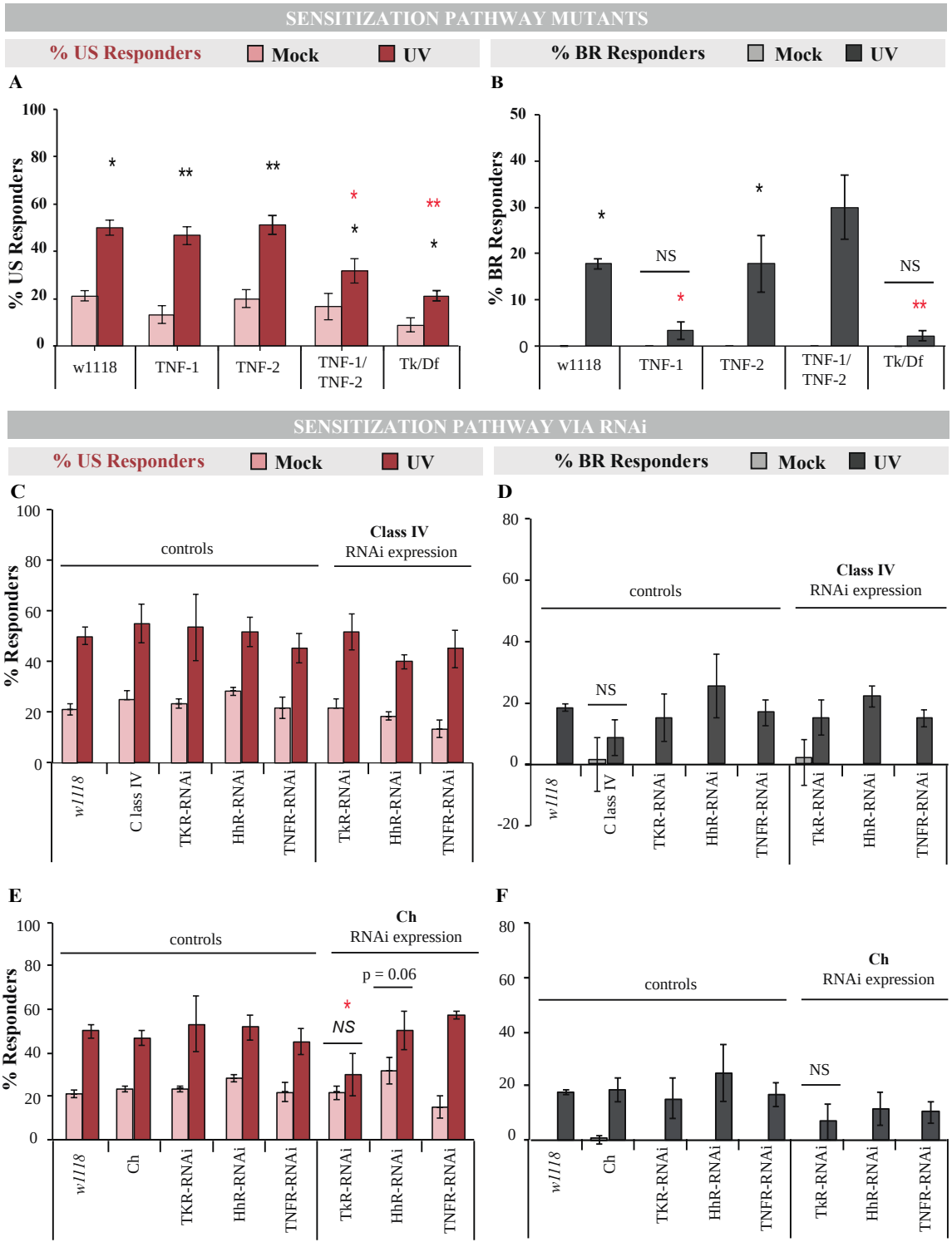
SENSITIZATION PATHWAY MUTANTS
% US Responders
Mock
UV
% BR Responders
Mock
UV
A
B
% US Responders
% BR Responders
w1118
TNF-1
TNF-2
TNF-1/ TNF-2
Tk/Df
NS
SENSITIZATION PATHWAY VIA RNAi
% US Responders
Mock
UV
% BR Responders
Mock
UV
C
D
% Responders
controls
Class IV RNAi expression
w1118
C lass IV
TKR-RNAi
HhR-RNAi
TNFR-RNAi
TkR-RNAi
HhR-RNAi
TNFR-RNAi
NS
E
F
Ch RNAi expression
p = 0.06
Ch
NS

Figure 4.6. The tachykinin pathway plays a role in UV-induced cold sensitization.
Larvae with mutant alleles for TNF or Tk ligands were tested for cold sensitization 24 hours after UV exposure (or mock-treatment) and the percent of (A) US or (B) BR responders recorded, n = 90. (C) Larvae expressing RNAi transgenes targeting the Tk, Hh or TNF receptors in class IV (CIV) neurons were tested for cold sensitization 24 hours after UV exposure (or mock treatment) and the percent of US or (D) BR responders recorded. (E) Larvae expressing RNAi transgenes targeting the Tk, Hh or TNF receptors in class IV (CIV) neurons were tested for cold sensitization 24 hours after UV exposure (or mock treatment) and the percent of US or (F) BR responders recorded. (C-F) n = 60. (A-F) Data are presented as the average ± s.e.m. Stats: two-tailed Fisher's Exact test * = $p < 0.05$, ** = $p < 0.001$. When UV-treated mutant/RNAi responses were significant compared to relevant genetic controls, annotated with red asterisk(s)*. (C-F) All comparisons between UV and mock for each genotype in were significant ($p < 0.05$) unless noted 'NS' (not significant).

mutants but this was not observed in larvae heterozygous for two different TNF mutant alleles (*eiger1/eiger3*, see Section 5.1) (**Figure 4.6B**).

To pinpoint where these genes, particularly *Tk*, are required for cold sensitization we targeted components of the TNF, Tk as well as the Hedgehog (Hh) pathway in class IV or chordotonal neurons using RNAi transgenes. Surprisingly, targeting receptors of these pathways in class IV neurons did not impact US or BR sensitization (**Figure 4.6C-D**). Interfering with the Tk receptor (TkR) in chordotonal neurons however resulted in a block of both US and BR sensitization (**Figure 4.6E-F**). Targeting the Hh pathway Smoothened receptor also slightly attenuated US sensitization responses to cold 24 hours after UV (**Figure 4.6E**). Targeting any of these pathways in class IV or chordotonal neurons had no affect on observed decreases in CT responses after UV (**Figure 4.7**). Together, these data suggest that UV-induced cold sensitization may be mediated by the Tk pathway in chordotonal neurons.

TRP channels are required for UV-induced cold sensitization in a class specific manner

Given that TRP channels mediate a multitude of thermosensory responses in *Drosophila* including those in nociceptive sensitization, we wanted to determine if they also play a role in cold sensitization following UV. Of particular interest were Pain, which acts downstream of Tk to facilitate UV-induced thermal sensitization in class IV neurons (135) and Pkd2, Trpm, NompC (shown here) and Brv (adult fly cold sensor (84)) which mediate behavioral responses to cold stimuli under non-injured conditions. For US responses, mutants of *nompC* and *Trpm* both attenuated responses after UV, blocking the sensitization that occurred in wild type (*w1118*) controls (**Figure 4.8A**). These were interesting hits, given that

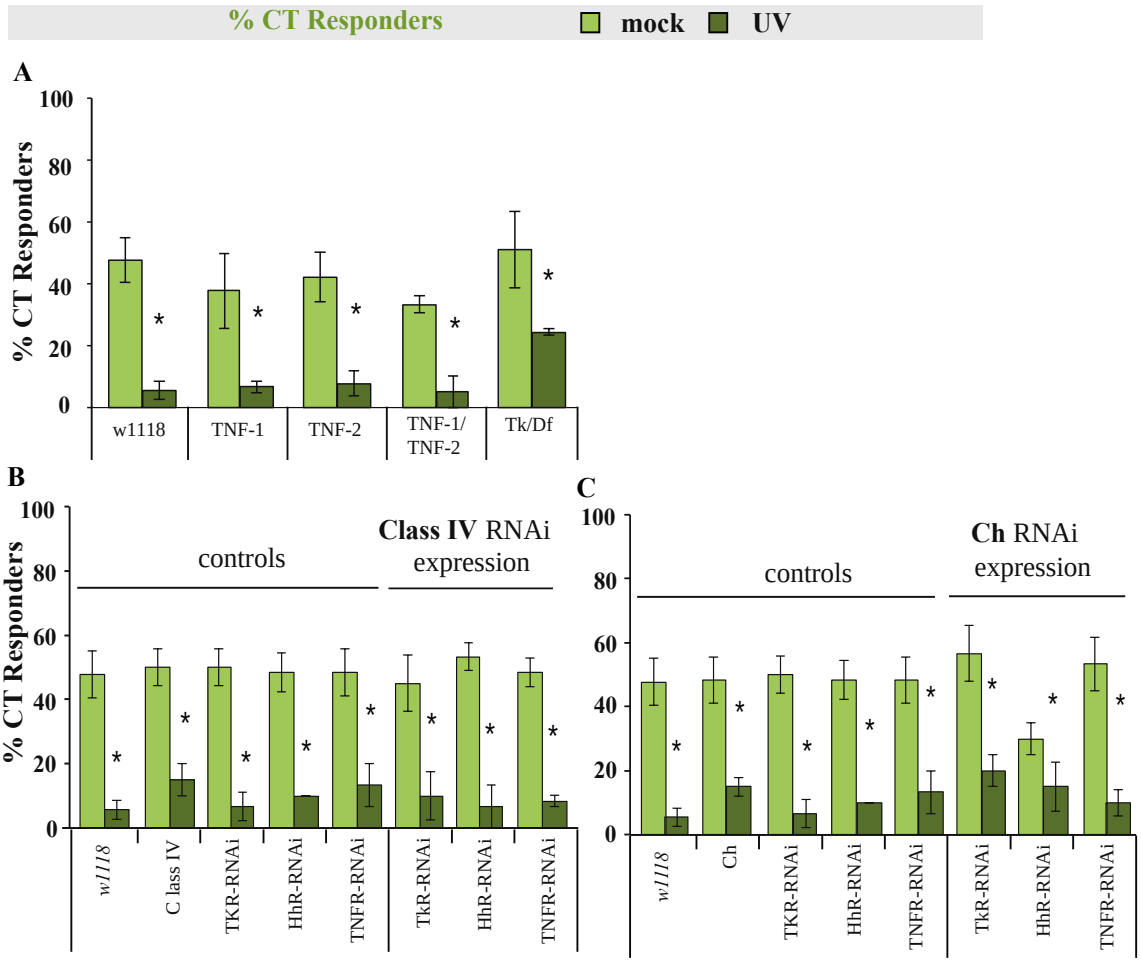

Figure 4.7. Known sensitization pathways are not required for decrease in CT after UV.
Larvae mutant for TNF or Tk ligands were tested for cold sensitization 24 hours after UV exposure (or mock-treatment) and the percent of (A) CT responders recorded. (B) Larvae expressing RNAi transgenes targeting the Tk, Hh or TNF receptors in class IV (CIV) or (C) chordotonal (Ch) neurons were tested for cold sensitization 24 hours after UV exposure (or mock treatment) and the percent of CT responders recorded. n = 60. Data are presented as the average ± s.e.m. Stats: two-tailed Fisher's Exact test * = $p < 0.05$, ** = $p < 0.001$.

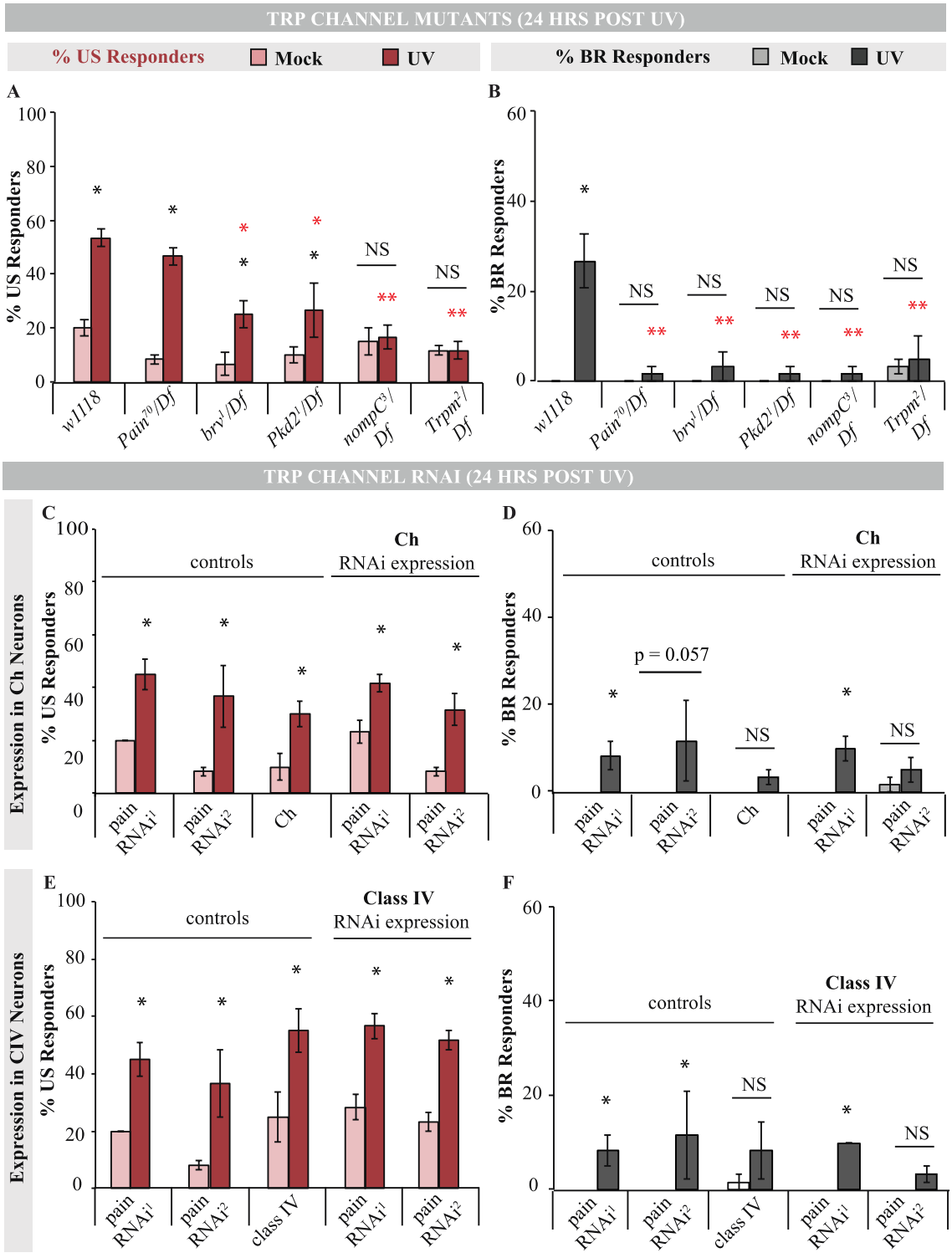
TRP CHANNEL MUTANTS (24 HRS POST UV)
% US Responders
Mock
UV
% BR Responders
Mock
UV
A
B
% US Responders
% BR Responders
w1118
Pain70/Df
brv1/Df
Pkd21/Df
nompC3/Df
Trpm2/Df
NS
TRP CHANNEL RNAI (24 HRS POST UV)
Expression in Ch Neurons
Expression in CIV Neurons
C
D
E
F
controls
Ch
RNAi expression
Class IV
p = 0.057
pain RNAi1
pain RNAi2
Ch
class IV

Figure 4.8. painless mediates UV-induced BR response to cold utilizing class IV and Ch neurons. (A) Percent of US or (B) BR responders to the cold probe (10 ºC) 24 hours after UV in TRP channel mutants for *painless, brivido, Pkd2,* or *nompC* over relevant deficiencies (Df). (C) Percent of US or (D) BR responder to the cold probe 24 hours after UV in larvae expressing painless RNAi transgenes in chordotonal neurons (Ch), or (E, F) class IV neurons. (A-F) n = 3 sets of 20 larvae averaged ± s.e.m.. Stats: two-tailed Fisher's exact test, * = $p < 0.05$, ** = $p < 0.001$. Black asterisks indicate comparison between UV-treated genotype and mock of same genotype, red askterisk indicate comparison between UV-treated w1118 and UV-treated of TRP mutants or RNAi genotypes. Black NS = no significance between UV and mock of same genotype, Red NS = no significance between UV-treated *w1118* and UV-treated of TRP mutants or RNAi genotypes.

baseline US responses in mock-treated *nompC* and *Trpm* mutants were normal compared to wild type controls, indicating a specific role for these genes in cold sensitization of US responses (**Figure 4.8A**). In contrast, US sensitization was still observed in *pain* and *brv* mutants, which also had baseline defects in US responses (mock condition) compared to controls, suggesting these channels are not required for US sensitization after UV (**Figure 4.8A**). Interestingly, for BR responses, sensitization after UV treatment was blocked in *painless, brv, Pkd2, nompC,* and *Trpm* mutants (**Figure 4.8B**).

While not all TRP channel RNAi were tested for cold sensitization, larvae expressing gene-specific *UAS-painless RNAi* in chordotonal or class IV neurons showed no changes in US responses, but they did show a block of BR responses (**Figure 4.8C-F**). Importantly, neither the TRP channel mutants nor *painless RNAi* tested blocked the UV-induced decrease in CT responses to cold (**Figure 4.9**). Collectively, these data suggest that Painless may act in class IV and/or chordotonal neurons to mediate UV-induced BR responses to cold. Additional work with TRP channel RNAi transgenes targeting other TRPs with mutant phenotypes should be tested for their function in class IV and chordotonal neurons as well, but even preliminarily this is an interesting result since it implies some overlap in the cells and channels required for heat and cold sensitization.

4.3. Discussion

We show that *Drosophila* larvae sensitize to cold after UV in a complex manner, namely a shift in behavioral output away from CT and towards US and BR responses. Both sensitized US and BR responses appear to require the TkR in chordotonal neurons instead of class IV neurons (See summary **Figure 4.10**). This data indicates that sensitized responses to

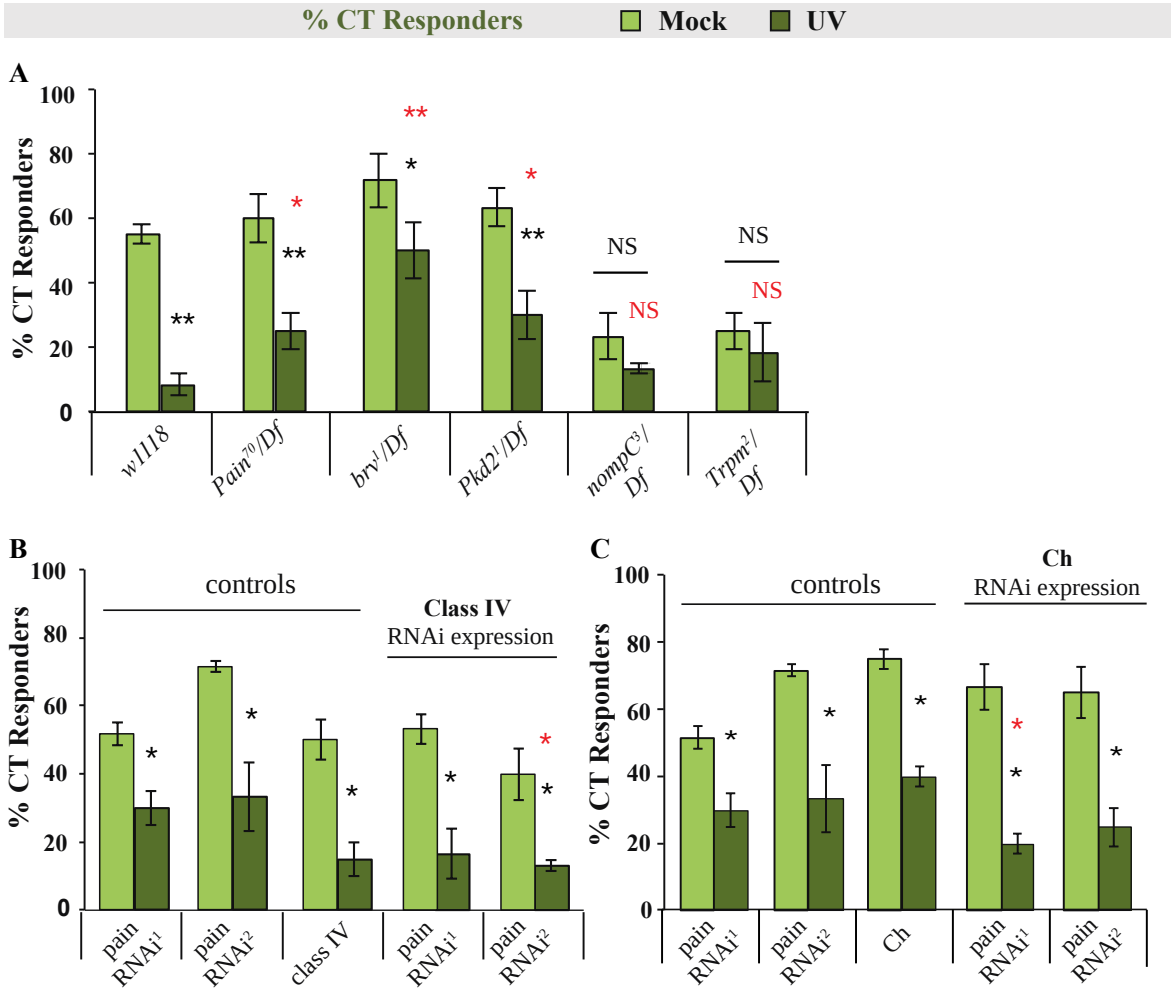

Figure 4.9. TRP channels don't seem to be required for decrease in CT after UV compared to mock controls.

(A) Percent of CT responders to the cold probe (10 ºC) 24 hours after UV in TRP channel mutants for *painless, brivido, Pkd2, nompC* or *Trpm* over relevant deficiencies (Df). (C) Percent of CT responder to the cold probe 24 hours after UV in larvae expressing painless RNAi transgenes in class IV or (C) chordotonal neurons. n = 3 sets of 30 larvae averaged ± s.e.m.. Stats: two-tailed Fisher's exact test, * = $p < 0.05$, ** = $p < 0.001$. Black asterisks indicate comparison between UV-treated genotype and mock of same genotype, red askterisk indicate comparison between UV-treated *w1118* and UV-treated of TRP mutants or RNAi genotypes. Black NS = no significance between UV and mock of same genotype, Red NS = no significance between UV-treated control (*w1118, UAS* and/or *Gal4*) and UV-treated of TRP mutant or RNAi genotype.

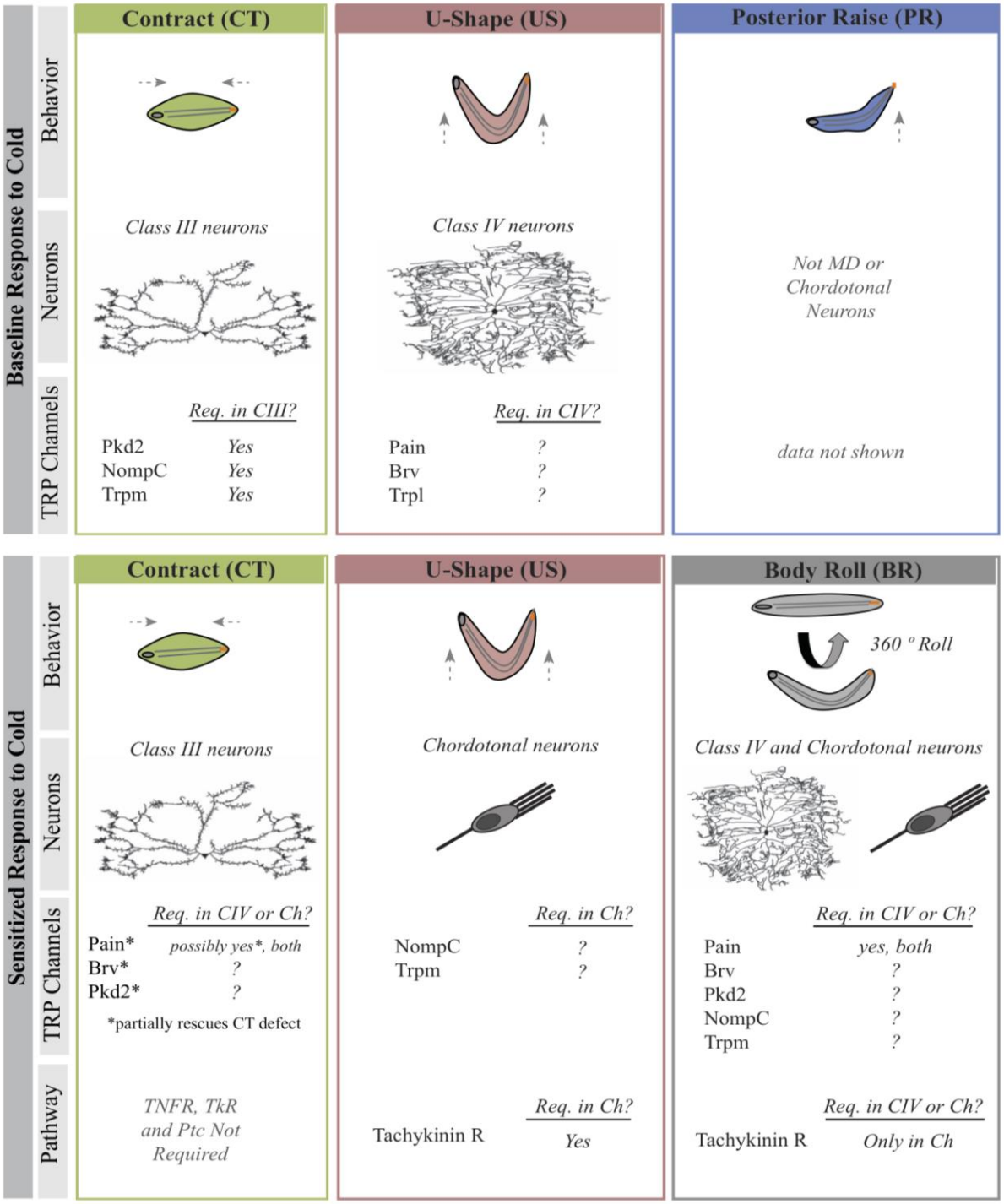

Figure 4.10. Summary figure of sensory neurons and genes required for cold nociception and cold sensitization in *Drosophila* larvae.

cold after UV-damage may be, at least partially, through distinct cells, receptors and genetic pathways.

Possible mechanisms for shift in cold responses after UV

First, let us examine why larvae may shift behaviorally away from CT responses to produce US and BR responses. At the level of perception, it could be that a full-body contraction is physically more painful than a US or BR to the larva. CT responses require a significant decrease in body length compared to US or BR (75). A CT response in particular, is accompanied by a considerable deformation of the epidermis to achieve this decrease in body length. Since the larval epidermis is undergoing cell death between 16 and 24 hours after UV (77), it could be much more painful for the larva at these time points to produce a CT compared to a US or BR, and hence a less desirable behavioral response. Even though class III and class IV co-activation results in a CT rather than a BR under normal conditions, we do not know if this is the case under UV conditions. This would be an interesting experiment to determine if the result is the same after UV damage, and if behavioral outputs shift to favor BR responses after injury. On a cellular level, it would be worth investigating whether class III neuronal firing is suppressed after UV damage to prevent CT responses, since this is a relatively simple way the observed behavioral shift could be mediated.

On a genetic level, the behavioral shift from CT to US/BR could be a result UV altering of the known sensitization pathways on class IV (and/or chordotonal) neurons to make them more sensitive to cold stimuli. This could give class IV a competitive advantage over class III neurons under damaged conditions. For chordotonal neurons this may be through Tk, but for class IV neurons it is unclear which genetic pathway is at play. Although

we focused on three genetic pathways that have been previously implicated in heat in this study, others have been identified in UV-induced heat sensitization (76) that may be worth pursuing, in addition to novel sensitization pathways. This will be especially important for identifying mediators of CT and US response changes after UV, which were both unaltered by impairment of the TNF, Tk or Hh pathways in class III and class IV neurons. This suggests there must be other unexplored pathways mediating behavioral changes to cold following UV damage in these neurons.

Possible role of Tk in cold-sensitization

Tk is produced by the brain, binds to its receptor on class IV neurons and requires Hh autocrine signaling within class IV neurons for UV-induced heat sensitization (135). In cold-sensitization, Tk was the only pathway that exhibited cold sensitization defects in the whole animal ligand mutant and in chordotonal driven Tk receptor RNAi, therefore it represents the best candidate genetic mediator of cold sensitization. It is curious that Tk seems to play a role in chordotonal neurons for cold sensitization, but not in class IV however. It also appears that Tk mediated cold sensitization in chordotonal neurons does not require Hh autocrine signaling (although more thorough validation of this is necessary), suggesting other downstream mediators may be involved. It will be interesting to determine if UV alters changes in TkR expression, localization, or activation threshold in chordotonal neurons to mediate cold-sensitization. Although ectopic over-expression of TkR in class IV neurons is sufficient to drive a genetic model of thermal allodynia (135), I did not observe US or BR sensitization to cold upon similar over-expression of TkR in class IV or chordotonal neurons (data not shown).

Role of class IV neurons in baseline cold and UV-induced cold sensitization

Class IV neurons play some role in baseline US responses to cold (**Figure 2.7C**), but they are not substantially activated by cold (via GCaMP **Figure 2.8E**) and are not sufficient to produce a US response upon robust optogenetic activation (**Figure 2.9D**). As discussed earlier, it will be valuable to determine if lower levels of class IV activation leads to US responses. It is also definitely worth exploring whether class IV neurons become activated by cold stimuli after UV damage, which could be assessed via live imaging with GCaMP transgenes or electrophysiologically as done previously ((75) and (135) respectively).

Role of TRP channel genes in UV-induced cold sensitization

It is plausible that UV could alter TRP channel sensitivity, localization, and/or expression on chordotonal and/or class IV neurons, but thus far this has not been addressed. Many TRPs, including those tested here, are expressed on class IV neurons (75, 110) but it is unknown whether they are also expressed on chordotonal neurons. Since NompC and Trpm seem to be required for cold sensitization, it will be worth examining whether they are required in specifically in class IV or chordotonal neurons for baseline US or sensitized responses (US or BR) to cold. Indeed, although all the mutants tested here blocked BR sensitization, it is not known for the majority of them (other than painless) whether these genes are required in class IV or chordotonal neurons for this sensitization, or if they are even expressed on chordotonal neurons.

Lastly, the majority of these channels have not been examined for their role in baseline responses to noxious heat. If it is found that these channels do function in BR

sensitization to cold following UV damage, it may be that they are also required for BR sensitization to heat, which would be a fascinating aspect of fly nociception to study further.

Together, we illustrate an intriguing shift in cold-evoked responses under the context of UV damage. While this shift seems to partially require similar cells and genes as thermal sensitization (class IV, *Tk* and *painless*), there is an interesting dichotomy in where these genes are required for cold-sensitization that are different from noxious heat. This project is fairly preliminary and therefore would benefit greatly from follow-up studies on additional genes that may be involved in cold-sensitization after UV. In all, this work establishes that *Drosophila* can be used to study nociceptive cold sensitization to identify key players in the process. This study should also encourage further work on cold sensitization following other types of injury or in models of chronic neuropathic pain.

CHAPTER 5: Materials and Methods

5.1. Drosophila stocks

Drosophila melanogaster larvae were raised on cornmeal food at 25°C. w^{1118} was used as a control strain. Mid-late 3rd instar larvae were used for all behavioral assays, selected based on age and size matching as well as documented developmental markers.

Mutants: $painless^{70}$(gift from Marc Freeman), brv^{1}(gift from Gallio), $Trp\gamma^{MB06664}$, $trpl^{302}$, $TrpA1^{ins}$, trp^{1}, $Pkd2^{1}(136)$, $Trpm^{2}$(117), $nompC^{3}$(137), $Trpm^{MI05302}$(gift of H. Bellen), $wtrw^{2}$, pyx^{3}, iav^{1}, $Trpml^{2}$, nan^{36a}, were from Kartik Venkatachalam unless otherwise noted, and are available on Bloomington, *eiger1 (TNF 1*) and *eiger3 (TNF 2*)(138), $Tk^{\Delta 1C}(135)$. Deficiencies: *Df(2L)BSC407* (*Pkd2*), *Df(2R)XTE-11* (*Trpm*), and *Df(2L)Exel6012* (*nompC*), *Df(3R)BSC747 (wtrw), Df(2L)BSC251 (trp), Df(3L)Exel6120 (nan), Df(1)BSC286 (iav), Df(2L)ED1109 (Trpγ), Df(3L)Exel6135 (Trpml), Df(3L)Exel6084 (pyx),brv Df gift from Marco Gallio, Df(2R)BSC602 (pain), Df(3L)ED4413 (TrpA1)*, and *Df(3R)Exel7312 (dTk)* were from Bloomington unless otherwise noted.

GAL4 Lines: *2-21-GAL4* (class I)(74), *GMR37B02-GAL4* (class II)(139), *19-12-GAL4(105)* and *nompC-GAL4*(140) (class III), *ppk1.9-GAL4* (class IV)(120), *1003.3-GAL4* (Class II/Class III)(141), *21-7-GAL4* (*md-GAL4,* classes I-IV) (142), *iav-GAL4* (Chordotonal)(140). *19-12-GAL4* was used for all behavioral experiments to drive expression in class III except where indicated in figure.

UAS transgenes: *UAS-TeTxLC* (active tetanus toxin)(109), *UAS-IMP TNT VI-A* (inactive tetanus toxin) (109), *UAS–GCaMP6m*(101), *UAS-channelrhodopsin-2* (ChETA-YFP)(140), *UAS-mCD8::*GFP(143), *UAS-CaMPARI*(128), *UAS-nompC-GFP*(103), *UAS-Pkd2* (gift of X. Lu), *UAS-TrpA1*(83), *UAS-RNAi* lines from Vienna *Drosophila* RNAi Center(144): 6940 (*UAS-Pkd2 RNAi 1),* 6941 (*UAS-Pkd2 RNAi 2),* 105579 (*UAS-nompC*

RNAi 2), 1372 (*UAS-TKR RNAi*), 9542 (targeting Smoothened, *UAS-HhR RNAi), wengen*^{IR} (145) , and 39477 (*UAS-pain RNAi 1*); from TRiP collection (146): 31291(*UAS-Trpm RNAi 1*), 31672 (*UAS-Trpm RNAi2*), 31689 (*UAS-nompC RNAi 1*), 31512 (*UAS-nompC RNAi 3*), and 31510 (*UAS-pain RNAi 2*) .

5.2. Behavioral assays

Cold Probe Assay

In all behavioral assays, freely moving mid 3rd instar larvae were used, age-matched and selected based on size. In the cold probe assay, larvae were placed under a bright field stereomicroscope (Zeiss Stemi 2000). The custom built probe (ProDev Engineering) consists of a temperature controlled Peltier device which cools the aluminum shaft, a thermistor (TE Technologies, Inc.) embedded inside the rounded conical tip, and a heat sink/fan to maintain the desired temperature (22 - 0 °C). The closed loop thermal management system measures and reports real time tip temperature and maintains the set point to within a half a degree. The tip tapers from 1.5 mm to a fine point, capable of contacting a single body segment. The tip of the probe was gently placed on the dorsal midline (segment A4) and held for either 10 s (up to 20 s in assay characterization assays) or until the first behavioral response. Larvae that did not respond within time limit were recorded as non-responders. For all assays cold-evoked behaviors precluded normal locomotion and each larva was only stimulated once (except Figure 2.2E). In all *GAL4/UAS* experiments, transgenes were heterozygous and no balancers or markers were present in the larvae tested.

Heat Probe Assay

The heat probe assay was conducted as previously described (77). Briefly, a metal tipped probe heated to 45 °C via electric thermocouple was applied gently to the mid dorsal surface of the larva and held for up to 20 s. A 360 ° body roll response within 5 s was recorded as a fast responder, 6-20 s, a slow responder, and normal locomotion for the full 20 seconds was recorded as no response.

Gentle Touch Assay

A single larva was first gently placed on an agarose plate under a bright field microscope and allowed to acclimate for 1 minute. The larva was then observed for four 10-second trials (20 seconds apart) without any stimulation to record normal locomotion behaviors (same as gentle touch behaviors outlined below) and the scores were summed. The larva was then gently stimulated with a light brushstroke of a feather in the thoracic segments (T1-T3) and gentle touch behaviors were recorded for 10 seconds. The same larva was tested for gentle touch responses during four 10-second trials 20 seconds apart and the scores were summed.

Gentle touch behaviors scored included the following: pause in locomotion (pause), retraction of the anterior segments (HW- head withdrawal), turn of the anterior segments between 45-180º (turn), single reverse wave of body segments (reverse), and multiple reverses in locomotion (retreat). Since "turning" is a behavior that is copiously observed during normal locomotion as well as after a gentle touch stimulus, gentle touch scores during a non-stimulated trial ("locomotion score") were compared for each genotype. Scoring of these behaviors was carried out in two ways: Method 1: Behaviors were weighted in ascending order such that pause = 1 point, turn or HW = 2 points, reverse = 3 points, retreat =

4 points. Each trial was given a single score depending on the "highest scoring" behavior observed (max total for each trial = 4, over four trials the summed max total score = 16). Method 2: Each behavior was weighted equally (1 point) and all behaviors for a trial were summed (max total for each trial = 5, summed max total score = 20). See schematic in Figure 3.3 for a summary outline of these scoring schemes. The number of turns was also recorded during normal locomotion trial and after gentle touch.

5.3. UV damage

To determine the affect of UV-induced tissue damage on larval cold nociception and sensitization, larvae were UV irradiated (as previously described (77)) and then allowed to recover on food in a 25 ºC incubator before being tested in the nociceptive assays 4, 8, 16 or 24 hours later. For this, early, middle, or late 3rd instar larvae were selected based on predicted final size when assayed. They were then placed on a cold slide, which immobilized them for a few minutes allowing for careful manipulation to prep them for UV. This required fine-tipped forceps to gently roll the larvae dorsal side up in a row along the length of the slide. A Spectrolinker XL-1000 UV crosslinker (Spectronics Corporation) was warmed up, and the UV dose was measured just prior to exposure to get an accurate reading of the predicted UV dose. Mounted larvae were exposed to 0 mJ/cm^2 ("mock treatment") or 10-14 mJ/cm^2 at a 254 nm wavelength over a duration of approximately 5 seconds which has been shown to induce epidermal cell death and behavioral sensitization to warm stimuli. Larvae were then rinsed into a clean petri dish and moved with a paintbrush into a small recovery vial of food and kept in a 25 ºC incubator until tested in behavioral assay.

For detailed methods on *in vivo* calcium imaging, optogenetics and CaMPARI techniques done by the Daniel Cox laboratory at Georgia State University, see (75).

CHAPTER 6: Conclusions and Future Directions

This study utilized a novel tool and behavioral assay to answer questions about noxious cold detection and sensitization in a highly useful, genetically tractable model. Like vertebrates, *Drosophila* can detect cold stimuli and respond behaviorally with quantifiable responses that are significantly altered under injury contexts. Much remains to be done however to get a complete picture of the nociceptive cold circuit and its role in nociceptive sensitization under various injury or disease contexts.

Remaining questions about the cold nociception assay

Even after a fairly thorough investigation and characterization of our acute noxious cold assay, there are still some remaining questions. We found that *Drosophila* larvae have three primary cold-evoked behaviors, which include: 1. A full-body contraction (CT), 2. A raise of the anterior and posterior segments so the larva forms a U-Shape (US), and 3. A raise of just the posterior segments (PR). These behaviors thus far, seem to be produced fairly randomly in response to cold temperatures within the cold (3-12 ºC) to cool range (roughly 13-18 ºC), with different peak response temperatures depending on the behavior observed. Varying the precise location of the cold probe in the mid-dorsal region does not appear to alter the behavioral output significantly, nor does altering larval size, even when observing cold responses out to 20 seconds. Why then, are there three different cold-evoked behavioral responses, and how does a larva choose which to produce in response to cold?

For larval responses to noxious heat, a robust rolling response should be effective in reducing the heat probe's contact and subsequent tissue damage caused to the epidermis (although whether the heat probe actually causes tissue damage to the larval epidermis is not known). All three cold-evoked behaviors may also function to reduce contact and perceived

temperature by underlying sensory neurons, because all three observably seem to cause a slight bunching up of the cuticle under the probe. The subsequent differences in behavioral output (from a body roll, or between US and CT for example) may then be due to different cold-specific channels being on distinct sensory neurons types (like class III and chordotonal), which mediate separate behavioral outputs through distinct sensory-motor circuits. A full screen of TRP channel genes in sensory neurons of interest would be useful here to see if this is the case, since only one candidate gene (*painless*) was tested in this study. Further, precise thermal imaging of *Drosophila* larvae during the application of the cold probe at different temperatures would be very interesting to see the extent of temperature change in the whole larva and if this correlates at all to behavioral output.

As proposed in section 4.3, class IV neurons may mediate baseline US responses to cold as well as the heat-induced body roll through different levels of class IV activation, as was observed in class III neurons. To dissect these possibilities, it will be important for future studies to investigate whether class IV neurons are sufficient to generate US at certain levels of activation (lower or possibly higher) (as done optogenetically in **Figure 3.2**). Since currently there are no reports of optogenetic activation of chordotonal neurons, it will be very interesting to activate these neurons at various levels as well to determine if they are sufficient to produce a US or BR response under any activation level**.**

Defining "cold sensing"

Even if these issues are addressed however, a rather confounding question remains: Why does *Drosophila* need multiple cell types and multiple receptors to respond to cold temperatures? This question is not easy to answer, but its answer will start to separate the

distinction between cold "detectors" versus "mediators" of cold. This study should illustrate that both thermosensitive TRP channels and thermosensory neurons are highly multimodal. They could mediate this modality through specific mechanisms for different stimuli or through broad modulatory mechanisms. Cold (and heat) is a particularly interesting sensory modality to investigate since it alters biological processes on a molecular level, creating many possibilities for cell membrane fluidity and protein structure to play interesting roles in thermosensation (147). It has also been proposed that especially for cold sensing, the dynamic levels of channel expression is a vital component of sensory processing (37, 38). These contributing factors are important to consider in furthering our understanding of thermosensation and nociception.

Beyond the peripheral nociceptor

We have focused solely on the peripheral sensory neurons in this study, but it is exceedingly likely that these neurons synapse on interneurons in the ventral nerve cord to form a thermosensory circuit that reaches the brain and descends to ultimately activate a set of motor neurons to evoke a behavioral response. There have been several recent genetic screens in *Drosophila* to identify interneurons and create useful interneuron GAL4 lines to allow specific targeting of these cell types (126). One such interneuron class, the basin interneuron, appears to synapse onto class IV sensory neurons (126). Whether class III or chordotonal neurons synapse on these interneurons, or whether they are required for responses to cold stimuli remains unknown.

In addition, although several cool and warm sensing circuits have been identified in the larval brain (82, 83), it is unclear whether these neurons are part of a common circuit with

peripheral hot and cold sensing neurons. Since the thermosensory neurons in the antennal segments of larvae are capable of directly sensing cold and their activation mediates different behavioral outputs, there may be no overlap in these circuits. It is worth noting however that the studies on antennal thermosensory cells were looking at calcium responses *in vitro*, and in behavioral assays more innocuous temperatures were tested. Further, when we directly probe the head with a 6 ºC cold probe we saw no significant decrease in CT responders upon md-neuron silencing (via tetanus toxin expression, data not shown), indicating other neuron classes in the head may be capable of eliciting a CT response to cold. This may help explain why CT responses were not completely abolished upon md neuron silencing when the cold probe was applied to the mid-body (**Figure 2.7**). It may be that application of the cold probe in the mid-dorsal region of the larvae is capable of rapidly chilling the underlying hemolymph, spreading a change in internal larval temperature anteriorly (and posteriorly) to activate the antennal thermosensory cells. It would be worth investigating whether silencing of the antennal cells prevents CT responses to acute noxious cold applied to the mid-body, or to the anterior segments, and whether optogenetic activation of the antennal cells (or others in this circuit) is sufficient to elicit a CT response.

Thermosensory circuits under the context of damage

Here we show that after UV damage, larvae exhibit a shift in behavioral output in response to cold temperatures. This includes a decrease in CT responders and an increase in US responders, accompanied by the appearance of a small but significant percentage of BR responders 16 and 24 hours after UV. It is difficult to determine the role of class III neurons in UV-induced decreases in CT responders in this study. Larvae have defects in CT responses

to cold when class III neurons are silenced under baseline (here, (75)) and UV-conditions (here). Presumably, if class III neurons were *not* required for the observed decrease in CT responses to cold after UV, then we should see CT responses at the same level as non-silenced controls, which is not the case. Further, silencing class IV or chordotonal neurons did not block UV-induced decreases in CT responses, suggesting they are not required for this shift in behavior. Likewise, silencing class III neurons does not alter US or BR sensitization to cold after UV. Lastly, while sensitization pathway RNAi and TRP RNAi transgenes were not expressed in class III neurons, in both of these experiments shifts in CT responses were not altered compared to controls. This suggests that the decrease in CT responses after UV must arise from other genetic mediators or mechanisms not tested here. Additional technical approaches will need to be applied to determine class III's specific role in behavioral responses to cold after UV.

To more thoroughly determine the roles of class III, class IV and chordotonal neurons in behavioral shifts to cold after UV, it will be important to investigate measures of neuronal activity after UV. One such method that the lab is currently pursuing is whether class III neurons' firing frequency is altered after UV damage via a cell-attached recording prep in live, dissected larvae (as described here (135)). We would predict, based on behavioral observations, that class III neurons exhibit a decrease in firing, while class IV and chordotonal neuronal firing rate increases in response to cold. Although, class IV and chordotonal electrophysiological responses to cold (with or without UV damage) has not been reported, these experiments would go a long way to help illuminate the cellular changes that occur following UV damage and how this might translate to behavioral changes to cold stimuli.

Further studies into damage-induced nociceptive sensitization

While we focused in cold sensitization induced by UV damage in this study, there are a multitude of ways one could cause tissue damage to *Drosophila.* Since other epidermal wounding assays have already been established in the field (148), it is worth investigating whether these types of epidermal wounds are capable of causing nociceptive sensitization. There are also multiple ways one could model peripheral neuropathy in *Drosophila* larvae, either through exposure to harmful chemicals (such as chemotherapy) or through genetically-induced neuronal damage (149) to investigate whether these types of injury cause cold hypersensitivity. Lastly, even if all of these types of damage cause cold hypersensitivity, they might not be through the same cell types or genetic pathways. Investigating the mechanisms underlying new models of nociceptive sensitization will greatly benefit our understanding of these processes in vertebrates and humans.

Ours is the first study that investigates cold nociception and attempts to model damage-induced cold hypersensitivity in *Drosophilae*. Given that the morphology and function of thermosensory neurons, structure and function of thermosensory channels, and many biological processes in *Drosophila* larvae resemble their vertebrate counterparts, flies represent a valuable tool for illuminating complexities within the pain field and uncovering therapeutic targets for a multitude of clinical pain conditions.

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