Neuronal and Molecular Basis of Nociception and Thermosensation in *Drosophila melanogaster*

By

Lixian Zhong

Department of Pharmacology and Molecular Cancer Biology
Duke University

Date:_____
Approved:

_____
W. Daniel Tracey, Supervisor

_____
Hiroaki Matsunami

_____
Nina Tang Sherwood

_____
Dennis Thiele

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Xiao-Fan Wang

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Pharmacology and Molecular Cancer Biology in the Graduate School
of Duke University

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ABSTRACT

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Abstract

From insects to mammals, the ability to constantly sense environmental stimuli is essential for the survival of most living organisms. Most animals have nocifensive behaviors towards extreme temperatures, mechanical stimuli or irritant chemicals that are considered to be noxious. Nociception is defined as the neural encoding and processing of noxious stimuli. This process starts from the activation of pain detecting peripheral sensory neurons (nociceptors) that can detect noxious mechanical, thermal or chemical stimuli. On the other hand, animals also have the ability to discriminate innocuous temperatures and to direct their locomotions to their favorable environmental temperatures and this behavior is called thermotaxis.

In this study, I used *Drosophila melanogaster* as a genetic model organism to study the molecular and cellular basis of nociception and thermotaxis. *Drosophila* larvae exhibit a stereotyped defensive behavior in response to nociceptive stimuli (termed nocifensive escape locomotion behavior, NEL). Using this behavior as a readout, we manipulated the neuronal activities of periphery sensory Type II multidendritic neurons and have identified a specific class of neurons, class IV multidendritic neurons, to function as nociceptors in *Drosophila* larvae.

After identifying the nociceptors, I next investigated several ion channels that are critical molecular components for larval nociception. The Degenerin Epithelial Sodium

Channel (DEG/ENaC) protein called *pickpocket (ppk)* is required specifically for larval mechanical nociception but not for thermal nociception. Being specifically expressed in class IV multidendritic neurons (the nociceptors), *pickpocket* is likely to function as a first detector of mechanical stimuli and upstream of general neuronal action potential propagation. In addition, I have found that the *Drosophila* orthologue of mammalian *TRPA1* gene, *dTrpA1*, is required for both mechanical and thermal nociception in *Drosophila* larvae. I have cloned a new isoform of dTRPA1 and have found it to be specifically expressed in class IV md neurons. Unlike the known dTRPA1 isoform that is warmth activated, this new isoform is not directly activated by temperatures between 15-42 °C. Instead, it may function downstream of sensory transduction step in the nociceptors.

Interestingly, *dTrpA1* mutants are also defective in their thermotaxis behavior within innocuous temperature ranges. In addition to the previously reported defects in avoiding warm temperatures, I have found these flies also failed to avoid cool temperatures between 16-19.5 °C. This defect is likely to be mediated by temperature sensing neurons in the antennae. I have detected antennal expression using a GAL4 reporter of *dTrpA1*. Significantly, these neurons exhibit elevated calcium levels in response to cooling. *dTrpA1* mutants have a premature decay of the cooling response at temperatures below 22 °C during a cooling process. I have also identified another population of cells in the antennae that can respond to temperature changes. These

neurons express the olfactory co-receptor Or83b and are known to be olfactory neurons. Calcium oscillations triggered by cooling were detected in these neurons and they were terminated by warming. Severe behavioral defects in avoiding cool temperatures were found in animals lacking *Or83b*. Our results suggest that there are multiple pathways regulating cooling sensation in the fly antennae.

Taken together, I have shown that *Drosophila* serves as a great model system to study nociception and thermosensation at molecular, cellular and behavioral levels.

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Chapter 1 Introduction

1.1 Nociception

1.1.1 Pain and nociception

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" according to the International Association for the Study of Pain . Pain is a major symptom associated with many medical conditions. It serves as a detection mechanism to protect living organisms from harmful stimuli as well as a warning signal of pathological conditions. However, misregulation of the protective pain pathway can lead to hypersensitivity and cause chronic or persistent pain. Pain incorporates both sensory and emotional components and is mediated by complex neuronal pathways. The sensory component, which includes the neural processes of encoding and processing noxious stimuli, is called nociception. The primary sensory neurons that transduce noxious sensory signals into neuronal signals are called nociceptors. Behavioral avoidance responses to noxious stimuli (nocifensive response) are widely present throughout the animal kingdom.

Understanding the mechanisms of peripheral pain signaling is of clinical as well as academic interest due to the unmet medical need for the treatment of pain. Previous studies on pain sensing and nociception have revealed several important cellular and

molecular components involved in the pain pathways. In this study, I focused on understanding the cellular and molecular basis of nociception in *Drosophila melanogaster* and use it as a model system to study molecular signaling in pain sensation.

1.1.2 Pain pathways in mammals

The perception of pain depends on specific receptors and pathways (Hensel 1981; Scott 1992). In mammals, noxious stimuli are detected by neurons with naked unspecialized nerve endings (named nociceptors) that innervate the surface of the body as well as many internal organs (Sherrington 1906). The axons associated with nociceptors are either lightly-myelinated, medium-diameter (Aδ fiber) or unmyelinated, small-diameter (C fiber) with the cell bodies located in the trigeminal or dorsal root ganglia (Basbaum and Jessell 2000). These afferents functionally differ from the larger diameter and rapidly conducting Aβ fibers that respond to innocuous mechanical stimuli or light touch (Purves, Augustine et al. 2008). The central axons of nociceptive nerve cells enter the spinal cord via the dorsal roots and make synaptic connections with neurons in the dorsal horn. Some of these dorsal horn neurons further project axons to higher brain centers, mainly in the thalamus and the cortex. These are the ascending pathways that carry information about noxious stimuli to the brain. In addition, descending pathways modulate the transmission of ascending pain signals. They originate in the cortex, and modulate the ascending pathways at many different sites.

For example, endogenous opioids are released from these descending neurons to regulate activities of the afferent neurons. The central pain pathways and circuitry are incredibly complex. As a result, the interpretation of an identical pain stimulus can vary according to different circumstances (Purves, Augustine et al. 2001).

1.1.3 Nociceptors in mammals

By far, most of our knowledge on nociception comes from research conducted in mammals. Nociceptors are the highly specialized sensory neurons that detect noxious stimuli. They provide information to the CNS not only about the environment but also about the state of the living organism itself. Based on their peripheral targets, primary afferents of nociceptors can be classified into cutaneous, articular and visceral afferents, of which the skin associated cutaneous nociceptors are mostly extensively studied. Nociceptors have highly branched naked nerve endings. The axons associated with nociceptors are either lightly myelinated (Aδ fiber) or unmyelinated (C fiber) with the cell bodies located in the trigeminal or dorsal root ganglia (Basbaum and Jessell 2000). Both C and Aδ fibers are polymodal and can respond to multiple stimulus modalities, including noxious mechanical, noxious heat/cold as well as irritant chemicals (Wall and Melzack 1999). Nociceptive C fibers can be further categorized into two groups based on their neurochemical properties: peptidergic C fibers, which are mainly associated with skin and non-peptidergic C fibers, which innervate various other tissues as well as

deeper regions of the skin. C fibers have a narrow activation threshold between 39-41 °C, which is also the thermal nociception threshold (Wall and Melzack 1999). They can also be categorized based on their functional properties. A subset of C fibers are both heat and mechanically sensitive (CMHs); another subset are heat-responsive but mechanically insensitive (silent nociceptors).

Aδ nociceptors can also be categorized into two main classes based on their electrophysiological properties. Type I (HTM: high-threshold mechanical nociceptors) respond to mechanical and chemical stimuli and have relatively high threshold for thermal nociception (>50 °C). These afferents get sensitized in case of tissue damage and inflammation and respond to lower threshold thermal and mechanical stimuli. Type II Aδ nociceptors have very high mechanical activation threshold but much lower temperature threshold (~43 °C) (Basbaum, Bautista et al. 2009).

Aδ and C fibers innervate lamina I and much of lamina II in the dorsal horn of DRG.

Both the Aδ and C fibers, like all other primary afferents, use glutamate as their principal fast transmitter and thus are excitatory to their post synaptic targets (Wall and Melzack 1999).

Unmyelinated C fiber has a conduction velocity of 0.5-2 m/s. Lightly myelinated Aδ fiber has a conduction velocity of 20 m/s (Purves, Augustine et al. 2008). Selectively

activation and blocking C fibers and Aδ fiber demonstrated that Aδ fibers are responsible for first, sharp pain, while C fibers function for duller, longer-lasting second pain (Purves, Augustine et al. 2008). It's worth noting that not all C fibers are nociceptors. Small subsets of them are involved in cool sensation (Basbaum, Bautista et al. 2009).

1.1.4 Molecular signaling in nociceptors

Two classes of molecules have been implicated as receptors for transducing sensory signals into biochemical or electro- signals. The first class contains G protein-coupled receptors (GPCR) that are widely used in vision, taste and the olfaction system. The sensory stimuli, such as photon or small molecules activate GPCRs and lead to action potential via activation or inhibition of ion channels. The second class of molecules includes ion channels that can be directly gated by sensory stimuli such as temperature or mechanical stimuli. The identification of heat activated TRPV1 ion channel represents the first example of ion channel gated by a physical stimuli in eukaryotes (Caterina, Rosen et al. 1999).

Various receptors expressed in primary nociceptors have been found to be activated by painful stimuli. An important group of players are transient receptor potential (TRP) channels (Caterina, Schumacher et al. 1997; Tracey, Wilson et al. 2003; Bautista, Jordt et al. 2006). TRP channels are 6 transmembrane cation channels that can

be activated by different temperatures, chemical ligands or downstream of other signaling pathways such as the activation of phospholipase C (PLC) (Bandell, Story et al. 2004; Wang and Woolf 2005). For example, TRPV1 can be activated by capsaicin (the spicy ingredient of chili pepper) as well as by temperature above 43°C (Caterina, Schumacher et al. 1997; Tominaga, Caterina et al. 1998).

A *Drosophila* TRP channel Painless was discovered in a genetic screen for mutants defective in nociception (Tracey, Wilson et al. 2003). *Drosophila* larvae bearing mutation in *painless* show increased sensory thresholds to elicit nociception behavior in response to noxious heat of 46°C as well as to mechanical stimulus. Painless is expressed in multidendritic (md) neurons that structurally resemble nociceptors in mammals. The mammalian homologue of Painless is TRPA1 (Tracey, Wilson et al. 2003). TRPA1 channel is activated by allyl isothiocyanate (AITC), the pungent compound in wasabi and mustard oil (Bandell, Story et al. 2004; Jordt, Bautista et al. 2004) and allicin, the pungent compound in raw garlic (Bautista, Movahed et al. 2005). Similarly, *painless* mutant flies are defective in avoidance of AITC (Al-Anzi, Tracey et al. 2006). In addition to TRP channels, there are other channels, such as some members of the degenerin (DEG/ENaC) family that can be activated by mechanical force, acid-sensing ion channels (ASIC) that can be activated by protons and P2X, P2Y receptors that are gated by ATP, the release of which is a result of tissue damage (Julius and Basbaum 2001). Nociceptors

are sensitized in response to inflammatory mediators released at the site of tissue injury (Julius and Basbaum 2001).

1.1.5 *Drosophila* nociception

Drosophila larvae produce a stereotyped escape behavior in response to noxious stimuli, which is characterized by rotation around the long body axis in a corkscrew like motion. This behavior requires highly coordinated muscle contractions and is distinct from their normal locomotion behavior which is composed of peristaltic waves. This behavior is specifically triggered by noxious stimuli (mechanical, thermal or chemical). Interestingly, the thermal nociception threshold in *Drosophila* larvae is 39 °C, which is similar to the thermal nociception threshold in human. After characterized this nocifensive behavior in *Drosophila* larvae, Tracey et al. developed a simple yet elegant forward genetic screen to identify genes that are required for nociception. Through this screen, a TRP gene mutant, *painless* was isolated, which showed delayed response to a 46 °C stimulus. This was the first evidence that the molecular mechanisms mediating temperature sensation in mammals and insects are similar as the molecular basis for temperature sensation was just being discovered. *painless* mutants also showed defects in responding to a noxious mechanical stimulus of a 45 mN von Frey filament.

This also made *Drosophila* an appealing new system to study molecular signaling in nociception given all the powerful tools available for genetic manipulation and the

simplicity of the behavioral assay. *painless* encodes a TRPA1 subfamily member and is expressed in multidendritic neurons of *Drosophila* larvae. The expression pattern of Painless suggested that the multidendritic neurons, or a subset of them, could function as nociceptors in *Drosophila* larvae. The *Drosophila* larval sensory neurons fall into two groups: type I that terminates in a single ciliated dendrite and Type II that lacks sensory cilia and extends highly branched dendrites (multidendritic neurons) (as reviewed in (Tobin and Bargmann 2004) and is also talked in more details in Chapter 1.2.2.1). Indeed, the naked, highly branched nerve endings of multidendritic neuron morphologically resemble those of mammalian nociceptors (Tracey Jr, Wilson et al. 2003). Also expression of the tetanus toxin light chain (TeTxLC) neuronal silencer in these neurons prevented the nocifensive rolling response that is elicited by thermal stimuli of 46 °C in wild type larvae, indicating that these neurons or a subset of them are required for nociception (Tracey Jr, Wilson et al. 2003).

Using electrophysiological recordings from abdominal nerves, two types of neurons responding to temperature were observed. One type of neuron increased spiking at ~28 °C and the other increase firing at a threshold of 38 °C, suggesting the existence of at least two functionally distinct thermosensors in the larval abdominal wall. By contrast, the increase of firing above 38 °C was absent when recording from the nerves of *painless* mutant (Tracey Jr, Wilson et al. 2003).

The Painless channel itself was shown later to be activated by noxious heat (~42 °C) in heterologous expression system, which is consistent with the idea that Painless functions as the transduction channel in those neurons (Sokabe, Tsujiuchi et al. 2008).

Very little research on nociception has been done outside the mammalian system. The identification of the *Drosophila* nocifensive response allows us to use *Drosophila*, a genetically tractable and easily manipulated system to identify new genes involved in nociception and their mechanisms. Recently, a genome-wide RNAi screen for *Drosophila* thermal nociception mutants have been conducted and one of the hits, a $\alpha 2\delta$ family calcium channel was shown to be involved in pain sensation in flies, mouse as well as in humans (Neely, Hess et al. 2010). This is a good example of showing how studying nociception in *Drosophila* can be translated into human pain research.

1.2 Thermosensation

The abilities to detect internal and external temperature and changes in temperature and respond accordingly are fundamental to all living organisms. Temperature can greatly affect cellular and metabolic homeostasis, cause tissue damages and can be critical to survival. Sensory afferents that transduce temperature signals into neuronal signals can be categorized into two general populations, those that detect innocuous temperatures and those that detect extreme noxious temperatures. As I discussed earlier, the sensory neurons mediating the latter responses are called

nociceptors. Nociception is essential for eliciting avoidance behavior to potentially harmful stimuli, including extreme temperatures, intense mechanical stimuli and irritant chemicals. In this section, I will focus on temperature sensation within the innocuous temperature ranges. This process is essential for organisms to locate to favorable living temperatures, to find food and/or to maintain body temperature. In this dissertation, unless otherwise specified, I will use the term thermosensation to refer to innocuous temperature sensation. Various species use different mechanisms for thermosensation and the cellular and molecular mechanisms are gradually being discovered.

1.2.1 Thermosensation in *C. elegans*

Despite the simple composition of its nervous system with only 302 neurons, *C. elegans* is able to respond to complex environmental cues such as odors, tastes, touch, temperature and nociceptive stimuli (Ward 1973; Hedgecock and Russell 1975; Chalfie and Sulston 1981; Bargmann, Hartwieg et al. 1993; Sengupta, Colbert et al. 1993).

C. elegans exhibit a strong preference for the temperature at which they are cultivated and well fed between 16 °C and 25 °C. When placed on a thermal gradient, they would migrate to that particular growth temperature and move isothermally. Conversely, when cultivated at a certain temperature at which food is deprived, they subsequently exhibit aversion to that temperature (Hedgecock and Russell 1975; Mohri, Kodama et al. 2005).

Components of the neuronal circuit underlying thermotactic behaviors in *C. elegans* have been identified (Mori and Ohshima 1995). The bilateral AFD thermosensory neurons are major thermosensory neurons in the circuit and mediate most aspects of temperature sensation and thermotaxis behavior. AFD neurons are located near the tip of the nose. When it's ablated with laser microbeams, the nematode is no longer able to isothermal track to their cultivation temperature and instead preferred for cold temperatures (Mori and Ohshima 1995) . The AIY interneuron is the primary postsynaptic partner of AFD and further synapse on another interneuron AIZ. Neuronal ablation assay of either one of AIY or AIZ caused the nematode incapable of isothermal tracking but the effect is opposite. AIY-ablated animals are cryophilic (prefer cooler temperatures) while AIZ-ablated animals are thermophilic (prefer warmer temperatures). Activation of these neurons produce consistent phenotypes: AIY activation cause these nematodes to migrate to warmer temperatures while activating AIZ made them cryophilic (Mori and Ohshima 1995).

Recently the way AFD neuron responds to temperature has begun to be elucidated. Kimura et al. used *in vivo* calcium imaging to show that AFD neurons respond to warming with increased intracellular Ca^{2+} (Kimura, Miyawaki et al. 2004). Interestingly, this response is not to absolute temperature per se but to directional change of temperatures. Also the threshold of this response is established by the

cultivation temperature of the worm: the AFD neurons respond to warming only above the cultivation temperature (Kimura, Miyawaki et al. 2004). In addition, it was shown that AFD can sense temperature changes as small as 0.05°C via short-term adaptation. They also have a bidirectional thermosensory response by increasing intracellular calcium to warming and lowering intracellular calcium to cooling, allowing the AFD neurons to phase-lock their calcium dynamics to oscillatory thermosensory inputs (Clark, Biron et al. 2006).

It's not known what the actual temperature sensor in AFD neuron is yet. Genetic studies suggest that cGMP-gated ion channel encoded by the *tax-2* and *tax-4* genes function downstream of the temperature sensor as the double mutants display identical thermotactic defects observed in AFD ablated animals (Coburn and Bargmann 1996; Komatsu, Mori et al. 1996). Tax-4 and Tax-2 form channels that are highly Ca^{2+} permeable that may account for the intracellular Ca^{2+} increase in AFD neurons in response to warming above cultivation temperature (Komatsu, Jin et al. 1999). Indeed, this response is absent in a tax-4 null mutant (Kimura, Miyawaki et al. 2004).

Recent work by Inada et al. suggests a subfamily of guanylyl cyclase genes, *gcy-8, gcy-18,* and *gcy-23* may function upstream of *tax-2* and *tax-4* by increasing cGMP level. Interestingly, these guanylyl cyclase genes are functionally redundant (Inada, Ito et al.

2006). However, the mechanism for how external temperature information is converted into guanylyl cyclase activity remains unknown.

In addition to the bilateral AFD neurons, other thermosensory neurons have also been predicted to play a role in regulating thermotactic behaviors (Mori and Ohshima 1995; Clark, Biron et al. 2006). Recently, AWC olfactory neurons were shown to respond to temperature. Unlike AFD neurons that only respond to temperature changes above the cultivation temperature, the AWC neurons are responsive to temperature changes both below and above cultivation temperature. Calcium imaging in these neurons revealed stochastic calcium responses in a cultivation temperature dependent manner. Animals lacking the AWC neurons or with hyperactive AWC neurons are defective in the regulation of reorientation rate in thermotactic behavior. The different responses of AFD and AWC neurons to temperature change suggest that different strategies may be used by *C. elegans* for thermal information coding and behavior regulation (Biron, Wasserman et al. 2008).

1.2.2 Thermosensation in *Drosophila*

1.2.2.1 *Drosophila* sensory system

The *Drosophila* sensory neurons can be divided into two classes: Type I and Type II. Type I neurons are external sensory neurons that terminate in a single ciliated

dendrite as part of a specialized sensillum (Hartenstein 1988; Tobin and Bargmann 2004). Each of these sensory organs is thought to be derived from a single ectodermal precursor (sensory organ precursor) which gives rise to one or several monodendritic neurons and several support cells. Type I sensory organs can be categorized into two major groups. The first group includes mechano- or chemosensory organs that have external sensory structures in the cuticle such as bristles, campaniform, and basiconical sensilla. The second group is comprised of internally located stretch receptors, such as chordotonal organs in larvae for proprioception and Johnston's organ in adults for hearing (Brewster and Bodmer 1995). The dendrites of these neurons are bathed in high K^+, low Ca^{2+} endolymph secreted by supporting cells (Kernan and Zuker 1995). The deflection of the sensory bristles causes rapid depolarization of these neurons (Corfas and Dudai 1989). The latency for the depolarization is so short (200 μs) that second-messenger-dependent pathway is not likely to be involved (Walker, Willingham et al. 2000). Instead it was proposed that the depolarization is caused by the direct activation of a mechanosensitive channel. Type I neurons are mostly involved in various aspects of mechanosensation and chemosensation (Tobin and Bargmann 2004). Recently it was also shown that Type I innervated terminal organ can detect temperature and respond to cooling (Liu, Yermolaieva et al. 2003).

Type II sensory neurons, the multiple dendritic (md) neurons, on the contrary, do not have cilia, but extend multiple dendrites with naked nerve endings. In most cases, they are not associated with support cells. Based on the morphology, md neurons fall into three subclasses: md-da neurons have extended multiple branched dendrites beneath the larval epidermis, md-bd neurons have bipolar dendrites and md-td neurons extend their dendrites along the tracheal branches (Bodmer and Jan 1987). I will discuss the classification and functions of these md-da neurons in detail in chapter 3.

1.2.2.2 Molecular and cellular basis of thermosensation in *Drosophila*

Drosophila melanogaster has been used as a robust model system to study the cellular and molecular basis of sensory functions. Indeed, the powerful genetic and molecular tools available in *Drosophila* have provided insight into vision, taste and olfaction. Recently, the cellular and molecular mechanisms for nociception and thermosensation are also beginning to be elucidated.

Drosophila larvae prefer 18 °C versus either 11 °C or 30 °C (Liu, Yermolaieva et al. 2003). By pan-neuronally expressing a genetically encoded calcium indicator yellow cameleon 2.1 (YC2.1), Liu et al. observed increased intracellular Ca^{2+} in the Type I neurons innervated terminal organ in response to cooling from 18 °C to 10 °C. Extracellular recording in the terminal organ also detected increased spiking with cooling. Blocking the synaptic transmission in terminal organ using the tetanus toxin

light chain (TeTxLC) reverses the preference of 18 °C over 11 °C in larvae (Liu, Yermolaieva et al. 2003).

Chordotonal neurons, which also belong to Type I neurons, have been implicated to have a role in thermosensation as well. *Drosophila* larvae choose their preferred temperature of 17.5°C over slightly cooler temperatures (14–16°C) through activation of chordotonal neurons. Inactive (Iav), a Drosophila TRPV family channel is expressed specifically in chordotonal neurons and mutations in Iav eliminates larvae's ability to choose 17.5°C over 14~16°C. It's suggested that lower temperatures activate the chordotonal neurons via Iav and induces repulsive behaviors(Kwon, Shen et al. 2010). It's not known how temperature activates the chordotonal neurons.

Type II neurons have also been found to be thermosensitive. Electrophysiological recording from abdominal nerve in third instar larvae detected two types of response. One type of neurons increase firing with a threshold of 38 °C, which is the same as the nocifensive behavior threshold. Another type of neurons increase firing rate above 28 °C and stops firing above 41 °C, indicating a function in sensing innocuous warm temperatures (Tracey Jr, Wilson et al. 2003). Calcium imaging in Type II md neurons during cooling and heating produced mixed results (Liu, Yermolaieva et al. 2003).

In a different experimental paradigm, larvae showed innate thermotaxis towards the coolest zone on a thermal gradient of 27 °C to 41 °C (Rosenzweig, Brennan et al.

2005). It was found that *dTrpA1* RNAi knockdown prevented larvae from avoiding the temperatures. The specific site of action for dTRPA1 is unknown. An antibody against dTRPA1 labeled primarily a small subset of central brain neurons, neuroendocrine cells of the corpus cardiacum and some unidentified cells near the mouth hooks as well as in the digestive system. This antibody did not detect any expression in md neurons or chordotonal neurons and silencing md neurons using TeTxLC or lacking chordotonal neurons did not cause defective thermotactic behavior in this setting. This suggests that md neurons and chordotonal neurons are not required for larval thermotaxis behavior within this temperature range. When using a dTRPA1 reporter to drive expression of TeTxLC or the cell apoptosis gene *hid*, the larvae exhibited a partial phenotype of *dTrpA1 RNAi* injected animals. These results suggest that dTRPA1 mediates avoidance of innocuous warm temperatures and may function in a subset of previously unknown thermosensory neurons in the larvae.

Drosophila adults shows a preference for specific temperatures. Sayeed et al. showed that adult flies had a strong preference for 24 °C on a linear thermal gradient ranging from 18 °C to 31.5 °C (Sayeed and Benzer 1996). Unlike nematodes, the cultivation temperature of flies does not reset their temperature preference. The thermal sensor for this preference was located to the 3rd antennal segment as flies with their 3rd antennal segments removed became thermoblind below 31.5 °C. This suggests that the

3rd antennal segments contain temperature sensing neurons for temperature discrimination and preference between 18 °C to 31.5 °C. Flies with both sides of their 3rd antennal segments removed still avoided temperatures above 31.5 °C, indicating additional thermal sensors detecting higher threshold temperatures.

dTRPA1 was also found to be involved in adult thermotaxis behavior (Hamada, Rosenzweig et al. 2008). On a thermal gradient from 18 °C to 32 °C, *dTrpA1* mutants failed to avoid warm region between 28 °C to 32 °C. A *dTrpA1-GAL4* reporter (different from a previous *dTrpA1-GAL4* reporter made by (Rosenzweig, Brennan et al. 2005)) and dTRPA1 anti-sera co-labeled a small set of anterior cells (AC neurons) located in the *Drosophila* brain. Calcium imaging in these neurons revealed intracellular calcium increase in response to warming with an average threshold of 24.9 °C and this response was missing in dTRPA1 mutants. The activation threshold of these neurons was consistent with the activation threshold of dTRPA1 channel. A *dTrpA1* genomic minigene rescue restored the *dTrpA1* expression in the AC neurons and also restored the thermotactic behavior. Using *dTrpA1-GAL4* to express *UAS-dTrpA1RNAi* phenocopied the mutant behavior. These results suggest that dTRPA1 is essential for adult flies to avoid innocuous warm temperatures and that it functions in internal brain AC neurons.

Another TRPA channel gene, *pyrexia*, has been shown to be involved in protecting flies from thermal stress. *pyrexia*-null flies have significantly reduced

tolerance to 40 °C and would paralyze. It was shown that *in vitro*, temperatures above 40 °C evoked currents in cells expressing Pyrexia and it primarily conducted potassium over sodium. This suggests that the activation of this channel does not lead to depolarization but hyperpolarization of the neuron (Lee, Lee et al. 2005).

In addition to TRP channels that are involved in thermosensation in flies (*painless*, *dTrpA1*, *pyrexia*), another type of molecule was recently identified as potential player in thermosensation. Several genes encoding proteins that function in histamine signaling were identified from a P-element screen for thermotaxis mutants on a thermal gradient from 15 °C to 45 °C, including a subunit of a histamine-gate ion channel and histamine decarboxylase. These mutants display a shifted preference for temperature of 27 °C instead of 24 °C in wild type and rescued animals (Hong, Bang et al. 2006)

Recently, Gallio et al. found a small set of neurons located in the 3rd segment of the antenna and three cells in the arista expressing a *Drosophila* TRPP channel *brv1* to be activated by cold temperatures around 17 °C. The rest three neurons in the arista respond to warm temperatures around 27 °C. Interestingly, the cold sensing neurons and the warmth sensing neurons project to two distinct glomeruli in the proximal antennal-protocerebrum (PAP). The two populations of cells function independently as blocking the activities of individual population undermines either cold avoidance or

warmth avoidance, but not both. This result suggests a “labeled line” organization for temperature sensing (Gallio, Ofstad et al. 2011).

1.2.3 Thermosensation in mammals

In mammals, the skin forms a protective layer around the body, serving as a physical barrier as well as a sensory organ for detecting environmental information, such as mechanical, chemical and thermal changes, so that appropriate behaviors can be initiated.

While many aspects of temperature sensation remain unknown, recent studies on TRP channels are starting to elucidate some of the mechanisms. As I discussed earlier, DRG afferents contain thermosensory nerve endings. Based on their molecular expression markers, they can be divided into four non-overlapping subgroups: TRPV1 expressing , TRPV1 and TRPA1 co-expressing, TRPM8 expressing and TRPV2 expressing (Jordt, McKemy et al. 2003; McKemy 2007; Basbaum, Bautista et al. 2009) (Peier, Reeve et al. 2002; Plant and Strotmann 2007). In addition to the sensory afferents from the DRG, other tissues/organs have also been found to be involved in thermosensation. The hypothalamus is known to be a center for body temperature regulation. TRPV4, a warmth activated TRP channel is broadly expressed in both neural and non-neural tissues. Recently, expression of TRPV4, has been detected in the

hypothalamus (Plant and Strotmann 2007). Another warmth activated TRP channel, TRPV3, is detected in keratinocytes, which have also been proposed to have a direct role in temperature sensation as well. More details of TRP channel involved in temperature sensation and their site of action will be discussed in Chapter 1.3.2.

1.3 Molecular basis of temperature sensation –TRP channels

1.3.1 Introduction to TRP channels

Transient receptor potential (TRP) ion channels are six transmembrane cation ion channels that are involved in various sensory functions, including vision, taste, smell, hearing, mechanosensation and thermosensation (Caterina, Schumacher et al. 1997; Colbert, Smith et al. 1997; Caterina, Rosen et al. 1999; Guler, Lee et al. 2002; Peier, Reeve et al. 2002; Xu, Ramsey et al. 2002). So far, 33 TRP channel genes have been identified in mammals, about 60 in zebra fish, 30 in sea squirts, 24 in nematodes, 16 in fruit flies and 1 in yeast (Christensen and Corey 2007). The TRP superfamily is composed of seven subfamilies: TRPC, TRPV, TRPM, TRPN, TRPA, TRPP and TRPML. Six of the subfamilies have members conserved from worms, flies, mice to human. The remaining subfamily, TRPN, have only been identified in invertebrates and zebra fish with no mammalian homologue discovered yet (Montell 2005). TRP channels typically have varying number of ankyrin repeats, a predicted coiled coil region and a putative caveolin-binding domain at the N-terminus. The C-terminus contains a TRP signature

motif (EWKFAR), a proline-rich motif, a calmodulin/inositol 1, 4, 5-trisphosphate (IP_3) receptor-binding (CIRB) domain, and a predicted coiled coil region (Montell 2001).

The founding member of the TRP family, *trp*, was found to be critical for the light response in *Drosophila* photoreceptor cells. Mutants in *trp* had transient instead of sustained response to light and the light-induced Ca^{2+} influx is dramatically decreased (Cosens and Manning 1969; Hardie and Minke 1992; Montell 2005). The cloning and characterization of *trp* revealed a new ion channel with four N-terminal ankyrin repeats and six transmembrane domains that is similar to many of the voltage-gated and second messenger-gated ion channels (Montell and Rubin 1989).

Two other TRPC channels were also identified to be involved in *Drosophila* photo reception, TRPL and TRPγ. Same as TRP, both of TRPL and TRPγ are highly enriched in photoreceptor cells (Montell and Rubin 1989; Niemeyer, Suzuki et al. 1996; Xu, Chien et al. 2000). Double mutants of *trp* and *trpl* had no response to light (Niemeyer, Suzuki et al. 1996). Expression of a dominant negative form of TRPγ also eliminated the light response in *trp* mutants (Xu, Chien et al. 2000).

Since then, a superfamily of ion channels has been discovered. Some members of the TRPC family are store-operated channels (SOC), some are activated by diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PIP_2) (Zhu, Jiang et al. 1996; Chyb, Raghu et al. 1999; Vannier, Peyton et al. 1999), yet

others through an exocytotic mechanism (Montell 2005). The TRPV subfamily functions in several sensory modalities, including temperature sensation, chemosensation, osmolarity and mechanosensation (Caterina, Schumacher et al. 1997; Colbert, Smith et al. 1997; Caterina, Rosen et al. 1999; Guler, Lee et al. 2002; Peier, Reeve et al. 2002; Xu, Ramsey et al. 2002). Some members of the TRPM family are ion channels containing C-terminal enzyme domains (chanzymes) (Nadler, Hermosura et al. 2001; Runnels, Yue et al. 2001). One of the TRPM members TRPM8 can be activated by cooling as well as menthol, the effective ingredient in mint (McKemy, Neuhausser et al. 2002; Peier, Moqrich et al. 2002). Both the TRPN and TRPA family members have characteristic long ankyrin repeats. TRPN members mostly function in mechanosensation and no vertebrate homologue has been identified yet (Walker, Willingham et al. 2000; Sidi, Friedrich et al. 2003). TRPA members are involved in thermosensation, mechanosensation and chemosensation and will be discussed in more details in latter chapters. TRPP and TRPML families are more distantly related to the other 5 subfamilies. Mutations in these genes have been associated with a couple of human diseases, such as polycystic kidney diseases (Mochizuki, Wu et al. 1996; Qian, Germino et al. 1997; Bargal, Avidan et al. 2000; Sun, Goldin et al. 2000).

1.3.2 TRP channels involved in temperature sensation

Several TRP channels have been shown to be directly temperature activated. In mammals, six warmth/heat-activated (TRPV1, TRPV2, TRPV3, TRPV4, TRPM4 and TRPM5) and two cool/cold-activated (TRPM8 and TRPA1) has been identified.

Initially coming out of a screen for capsaicin responsiveness, TRPV1 was the first TRP channel found to be temperature gated (Caterina, Schumacher et al. 1997). TRPV1 is activated by noxious temperatures with a threshold of 42 °C and is expressed in a subset of nociceptive neurons in the DRG and trigeminal ganglion, probably in the cell bodies of unmyelinated C-fibers, which also have a thermal activation threshold around 42 °C. Cultured neurons from TRPV1 knockout mouse completely lack response to capsaicin and heat (>42 °C). However, somewhat unexpectedly, the TRPV1 mutant mouse only displayed very mild defects in responding to noxious heat (>42 °C), suggesting the existence of other noxious heat temperature sensors that are functionally redundant (Caterina, Leffler et al. 2000; Davis, Gray et al. 2000). Although the dTRPA1-/- mice only showed mild defects in responding to heat, they have severe deficits in inflammation-induced thermal hyperalgesia. Indeed, TRPV1 activity is potentiated by many components of the inflammatory soup, such as NGF, bradykinin, prostaglandins, PKA, PKC , ATP and proton (Tominaga, Caterina et al. 1998; Premkumar and Ahern 2000; Chuang, Prescott et al. 2001; Tominaga, Wada et al. 2001; Bhave, Zhu et al. 2002; Hu,

Bhave et al. 2002). Collectively, these studies indicate that TRPV1 is not only a noxious heat sensor but its activity is also modulated by many other molecules.

TRPV2 is activated by even higher temperature with a threshold of >52 °C (Caterina, Rosen et al. 1999). TRPV2 is not activated by the compounds that potentiate TRPV1. Expressed in medium- to large-diameter cells in rat DRG neurons, TRPV2 may account for the high thermal threshold of type I AMH nociceptors of Aδ fibers, which has a threshold of ~53 °C. The function of TRPV2 expression in Aβ fibers is unknown.

TRPV3 and TRPV4 are both activated by warm temperatures in heterologous expression systems with thresholds of ~34-38 °C for TRPV3 and ~27-35 °C for TRPV4 (Smith, Gunthorpe et al. 2002; Watanabe, Vriens et al. 2002). Instead of being expressed in DRG neurons, TRPV3 is detected in keratinocytes in the skin (Peier, Reeve et al. 2002; Plant and Strotmann 2007) and TRPV4 is broadly expressed in both neural and non-neural tissues, including in the hypothalamus, which is a center for body temperature regulation(Plant and Strotmann 2007).

TRPM4 and TRPM5 have also found to be temperature sensitive , mediating inward currents at temperatures between 15 and 35 °C (Talavera, Yasumatsu et al. 2005). TRPM5 mediates the enhanced gustatory nerve response to sweet compounds as temperature increases between 15 °C and 35 °C. The strong temperature sensitivity of

TRPM5 may underlie some known effects of temperature on perceived taste in humans (Talavera, Yasumatsu et al. 2005).

Two cold activated TRP channels have been identified so far, TRPM8 and TRPA1. TRPM8 can be activated by cool temperatures of 25~28 °C as well as by menthol, the effective ingredient in mint and a bunch of other cooling compounds, such as menthone, eucalyptol, and icilin (McKemy, Neuhausser et al. 2002; Peier, Moqrich et al. 2002).

TRPM8 is expressed in a subset of small-diameter C fibers in the DRG and TG neurons, and is not co-expressed with TRPV1 (McKemy, Neuhausser et al. 2002; Peier, Moqrich et al. 2002). Although the activation temperatures for TRPM8 is between 25 ~28°C, TRPM8 deficient mice have severe defects in their behavioral and physiological responses to cold temperatures above 10 °C (Bautista, Siemens et al. 2007; Colburn, Lubin et al. 2007), which is unexpected.

TRPA1 was initially cloned as a noxious cold activated ion channel with a broad range of activating temperatures (12 °C~24 °C, average threshold 17 °C) (Story, Peier et al. 2003). However, the involvement of TRPA1 in noxious cold sensation remains controversial as different groups have had different observations (Story, Peier et al. 2003; Jordt, Bautista et al. 2004; Bautista, Jordt et al. 2006; Kwan, Allchorne et al. 2006; Karashima, Damann et al. 2007; Zurborg, Yurgionas et al. 2007; Karashima, Talavera et

al. 2009; Knowlton, Bifolck-Fisher et al. 2010). In heterologous expression systems, cold activation of TRPA1 was observed by some groups (Reid 2005), but not by others (Jordt, Bautista et al. 2004; Nagata, Duggan et al. 2005). It was also proposed that the cold activation of TRPA1 is indirectly caused by cold evoked intracellular Ca2+ increase (Zurborg, Yurgionas et al. 2007). Studies in TRPA1 knockout mice have also generated contradicting results, with some groups observing cold sensing defects (Kwan, Allchorne et al. 2006; Karashima, Talavera et al. 2009) while others not (Bautista, Jordt et al. 2006; Knowlton, Bifolck-Fisher et al. 2010). In general, more evidence is supporting a role of TRPA1 in cold sensation. The discrepancies are likely due to different experimental designs and preparations that caused some group fail to observe cold induced effects. However, it's not known if TRPA1 is directly activated by cold since no single channel recording is done and it's not known which motif is required for cold sensation of this channel.

However, TRPA1 is required for nociception. It's expressed in a subset of TRPV1 expressing DRG and TG neurons(Story, Peier et al. 2003). TRPA1 is activated by irritant chemicals such as mustard oil, allicin and tear gas as well as inflammatory mediators such as bradykinin and cannabinoids(Bandell, Story et al. 2004; Jordt, Bautista et al. 2004). Indeed, dTRPA1 deficient mice have decreased inflammatory pain (Bautista, Jordt et al. 2006). These animals also have defects in punctate mechanical stimuli (Kwan,

Allchorne et al. 2006). More details of TRPA1's role in mechanosensation will be discussed in later chapter 1.4.2.2.

1.3.3 Potential mechanisms for temperature sensation of TRP channels

With the identification of more and more TRP channels that can be activated by different temperatures, a pressing question arises as what the molecular mechanisms for the temperature sensitivities are. While the mechanisms are still largely unknown, some research started to shed light onto this mystery. One study showed that different C-terminal truncations of TRPV1 change the functional properties of this channel. Up to 72 aa truncation of the C terminus progressively reduce the temperature threshold of TRPV1 from 41.5 °C to 28.6 °C. The voltage-induced currents of the truncated mutants exhibited a slower onset, markedly reduced outward rectification, and significantly smaller peak tail current amplitudes. At the same time, the capsaicin-, pH- sensitivities are also reduced, which raise the question whether the reduction to temperature is specific. Truncation of the entire TRPV1 C-terminal domain (155 residues) caused the channel to be not functional (Vlachová, Teisinger et al. 2003). In another study, swapping the C-terminus of TRPV1 with TRPM8 exchanges their temperature threshold. Interestingly, the C terminus is not essential for TRPV1 capsaicin responsiveness or TRPM8 menthol responsiveness (Vlachová, Teisinger et al. 2003). These findings suggest that the C-terminus may contain the thermal sensor in these two channels.

Recently the pore regions of TRP channels have also been suggested to be critical for temperature activation. Residues in the 6th transmembrane and pore region of TRPV3 are required for its heat activation (Grandl, Hu et al. 2008). Consistent with this, mutations in the outer pore region of TRPV1 specifically impair temperature activation (Grandl, Kim et al. 2010).

Three possible mechanisms have been proposed for the gating of temperature sensitive channels. The channel protein may undergo temperature-dependent structural change that leads to channel opening. Alternatively, the changes in temperature may cause the production of some molecule that can bind to the channel and activate the channel. Finally, temperature change may cause rearrangements of the lipid bilayer that exert tension to the channel and lead to the activation of the channel (Clapham 2003). Both TRPV1 and TRPM8 can be activated by temperature changes in excised patches, suggesting that intracellular factors are not required for activation (Tominaga, Caterina et al. 1998; Reid and Flonta 2002). Another thermoTRP, TRPV4, however, cannot be activated in excised inside-out membrane patches, suggesting the requirements of some intracellular components (Watanabe, Vriens et al. 2002). Another study showed that temperature activation of TRPV1 and TRPM8 channels are tightly linked to voltage-dependent gating. Both channels are activated upon depolarization and changes in temperature result in shifts of their voltage-dependent activation curves. Ligands

binding (capsaicin for dTRPA1 and menthol for TRPM8) shifts temperature sensitivity of these two channels as well as membrane potential activating curve (Voets, Droogmans et al. 2004). With these findings, a thermodynamic principle is proposed, whereby thermosensitivity arises from the difference in activation energies associated with voltage-dependent opening and closing. One study suggests that there are separate activation domains for temperature, PIP2 and voltage activation domain at the C-terminus by studying chimeric TRPM8 protein with different lengths of the C-terminus replaced by the equivalent regions of TRPV1 (Brauchi, Orta et al. 2007).

1.4 Molecular basis of mechanosensation

The ability to sense mechanical force is critical for virtually all living organisms. It ranges from osmoregulation to regulation of vascular tone and to more specialized hearing and touching. In many cases, it was suggested that channels directly gated by mechanical forces mediate the current as the latency between the occurrence of the current and the application of the mechanical force is less than 5 milliseconds (ms), shorter than the time required for second-messenger mediated signal transduction. Examples of this include the activation of bristle neurons by bristle deflection in adult flies (<200 us) and chordotonal neurons activation upon acoustic stimulus in Johnston's organ in flies (< 1.2 ms). In many other cases, such as with osmotic stimuli, the latency is

longer than what is expected for a channel that is directly mechanically gated as reviewed in (Christensen and Corey 2007).

Christensen et al. proposed several criteria for judging whether the receptor current is directly activated by a mechanical stimuli and whether a particular candidate protein participates in that channel.

1) Does mechanosensation involve direct activation of a channel? 2) Does the candidate protein participate in mechanical transduction? 3) Is the candidate protein mechanically sensitive? 4) Is the candidate protein a pore-forming subunit? 5) Is the candidate protein a force-sensitive subunit? (Christensen and Corey 2007).

Three types of ion channels have been implicated in mammalian somatosensory mechanotransduction: Transient Receptor Potential (TRP) channels, Degenerin/Epithelial Na^+ Channels (DEG/ENaC) channels and two-pore potassium channels (KCNK). In the following chapters, I will focus on the TRP channel family and DEG/ENaC channel family. In addition, studies in bacterial mechanosensitive channel MscL and MscS have provided insight into a lipid tension model for mechano-gating of ion channels.

1.4.1 Prokaryotic mechanosensitive channels and lipid tension model

Mechanosensation is universal and has existed as early as single cellular organisms have been in existence for the detection and response to osmotic changes.

Studies in bacterial mechanosensitive channel (MS) provide mechanisms on mechanotransduction channel gated by forces generated by the lipid bilayer.

MscL (mechanosensitive channel of large conductance) and MscS (mechanosensitive channel of small conductance) were identified from mutant studies of bacteria. Mild osmotic down shift lyses the MscL/MscS double mutant bacteria but not wild type (Levina, Totemeyer et al. 1999) reviewed in (Anishkin and Kung 2005). Patch clamp results indicate that the unitary conductance of MscL is ~3 nanosiemens (ns) and ~1 ns for MscS, both of which are significantly larger than those of most other ion channels. The resolved X-ray crystallographic structure of closed MscL shows that it forms homopentamer with two transmembrane domains in each subunit (Chang, Spencer et al. 1998). Significantly, purified channel proteins reconstituted into pure lipid bilayer can conduct current upon stretch force, indicating that these channels respond directly to the change of force coming from the lipid bilayer (Perozo, Cortes et al. 2002; Perozo, Kloda et al. 2002).

Since there is no other proteins involved in this process, a lipid tension model has been proposed (reviewed in (Anishkin and Kung 2005; Kung 2005). In this model, the interaction of the lipid bilayer and the channel is critical to determine the opening probability of the channel. Indeed, several lines of evidence support this model. First, the hydrophobic components on the N-terminus of the first transmembrane domain

(TM1) of MscL are important for the interaction of the channel and lipid bilayer. Mutating some of these critical residues changes channel property that lead to either gain or loss of function. This result suggests that the hydrophobic region of MscL is critical for the gating of the channel (Li, Wray et al. 2004). Secondly, the force profiles of the lipid bilayer have been examined and the amplitudes of these forces are estimated to be in the order of hundreds of dyne cm^{-1}, which is much larger than the lysis tension of the bilayer (Cantor 1997; Cantor 1999; Lindahl and Edholm 2000; Gullingsrud and Schulten 2003). In addition, the composition of the lipid bilayer has been modified to change the curvature of the lipid bilayer as the force profile is significantly affected by the bilayer's geometry. As a result, the activation threshold of MscL and MscS can also be changed (Sheetz and Singer 1974). Recently, there has also been some study on the interaction of the channel and the lipids (Ollila, Risselada et al. 2009). However, major work in this field has been lacking.

1.4.2 Eukaryotic mechanosensitive channels and tethered model

1.4.2.1 Mechanosensation of TRP channels:

Many members of the TRP channel family have been shown to be involved in mechanosensation such as TRPV1, TRPV4, TRPV2, TRPC1, TRPC3, TRPC6, TRPM4, TRPM7, TRPN1, TRPA1, TRPY1, TRPP1, TRPP2 (Yin and Kuebler 2010).

TRPV4 has been implicated in a wide variety of mechanosensory processes and the most well studied role is probably in the systemic response to changes in the osmolarity of the body. However, it's not known whether TRPV4 is directly gated or indirectly activated by mechanical stimuli. So far most evidence suggests that TPRV4 functions downstream of an osmotic sensor rather than a direct mechanotransduction channel (Christensen and Corey 2007).

In *C. elegans*, *osm-9*, an invertebrate homologue of TRPV family is required for nose touch sensitivity in the cilia of nose touch neurons. Interestingly, expressing TRPV4 with the only pore region can rescue the sensitivity of nose touch and hyperosmolarity, suggesting OSM-9 is part of conduction pathway for this mechanosensory channel but is not required for gating (Liedtke, Tobin et al. 2003).

Several TRP channels have been implicated to be required for mechanosensation in fruit flies. The bristles of adult flies detect deflection. The current is believed to be produced by directly gated mechanotransduction channels because the latency between the stimulus and the current is too short (less than 200 us) to involve a second messenger. NOMPC (no mechanoreceptor potential C), a member of the TRPN family is required for bristle touch sensitivity. However, it's likely that NOMPC simply functions as an amplification channel for another truly mechanically gated ion channel as there is still remaining non-adapting current in the mutants (Walker, Willingham et al. 2000).

Fruit flies use the Johnston's organ to detect sound. The latency between the acoustic stimulus and the receptor current is less than 1.2 ms, which also implies the presence of a mechanotransduction channel (Christensen and Corey 2007). Three TRP channels have been shown to mediate mechanotransduction in Johnston's organ. *nompC* mutants reduce auditory nerve firing by 50%, which is consistent with a role for NOMPC in signal amplification(Gopfert, Albert et al. 2006; Sun, Liu et al. 2009). However, recently, the idea that NOMPC might still function as a direct transducer in flies' sound receptor is re-gaining interest. Calcium imaging shows that the remaining nerve potentials in nompC mutants are associated with the activity of gravity/wind receptors, but not from the sound receptors. The sound receptors of the mutants actually fail to respond to sound. Thus, NompC is required for *Drosophila* sound receptors in mechanical signal detection and amplification (Sun, Liu et al. 2009; Effertz, Wiek et al. 2011).

Two other members belong to the TRPV family – *nanchung* (Nan) and *inactive* (Iav), when either of which is mutated, auditory nerve response is completely abolished (Kim, Chung et al. 2003; Gong, Son et al. 2004). Both Nan and Iav is located in the ciliary outer segment of the chordotonal organ, which would be expected for a channel mediating auditory transduction. The two proteins depend on each other for correct subcellular localization, suggesting the two proteins form a heteromultimeric channel

(Gong, Son et al. 2004; Gopfert, Albert et al. 2006). In heterologous system, both Nan and Iav mediate current upon hypo-osmotic stimulation, suggesting a possible role in direct mechanotransduction. However, more work needs to be done to establish Nan and Iav as the mechanotransduction channel in fly hearing.

Painless, a *Drosophila* TRPA gene, has been shown to be required for both mechanical and thermal nociception in *Drosophila* larvae. While heterologously expressed Painless is activated by noxious heat, it's not activated by hypertonic or hypotonic solutions (Sokabe, Tsujiuchi et al. 2008).

To date, no TRP channel has been demonstrated to be directly mechanically gated. However it was proposed that the long ankyrin repeats present in some of the TRP family members, such as TRPN and TRPA, may function as a gating spring (Sotomayor, Corey et al. 2005; Lee, Abdi et al. 2006). The polyankyrin repeats may curve like a nano-spring and have elastic properties. Steered molecular dynamic simulations and atomic force microscopy illustrated that the spring constant of these polyankyrin domains is close to that of an expected physiological gating spring. Changing the ankyrin repeats in the genes described before and test to see how this change can alter the physiological properties of channel conductance would be an important way to test this nano-spring hypothesis and to show that the channel is indeed mechanically gated.

1.4.2.2 DEG/ENaC channels

DEG/ENaC (Degenerins/Epithelial Na^+ channel) channels are likely the first group of ion channels to be implicated in mechanosensation in eukaryotes. They got their name from the study of touch transduction in *C. elegans* and Na^+ reabsorption in specialized kidney and lung epithelia in human (Canessa, Horisberger et al. 1993). Many members of the DEG/ENaC channel super family have been identified in *C. elegans*, *Drosophila* and mammals. They have been shown to be involved in a wide range of biological functions including touch sensation, pain sensation, proprioception, heat sensitivity, taste and learning (Bianchi and Driscoll 2002).

DEG/ENaCs form homo- and heteromeric cation channels that are usually highly selective for Na^+ and these channels can usually be inhibited by amiloride. DEG/ENaC subunits are typically comprised of two transmembrane domains (TM1 and TM2), intracellular N- and C- termini and an extracellular central loop containing several cysteine-rich domains (CRDs). TM2 forms the channel pore. Some highly conserved sequence in the N-terminus and near TM1 determines the open probability, ion permeation and Na^+ selectivity. So far diverse stimuli has been found to activate DEG/ENaC channels, including protons, neuropeptides and mechanical forces (Bianchi and Driscoll 2002).

Two of the founding members of DEG/ENaC channel family are well studied for their roles in touch transduction in *C. elegans*. They came out of screens for mechano-sensory abnormal (mec) mutants of *C. elegans* among many other *mec* genes (Chalfie and Au 1989). The mechanoreceptor channel complex contains at least five proteins that are needed for transducing the touch stimulus, with MEC-4 and MEC-10 being the pore forming components.

Both MEC-4 and MEC-10 are expressed in all of six touch neurons of *C. elegans* (ALML, ALMR, PLML, PLMR, AVM and PVM (Huang and Chalfie 1994)). In the touch neurons, MEC-4 localized to discrete puncta along the process where mechanosensory proteins are believed to exist as a complex (Chelur, Ernstrom et al. 2002). Mutations in either gene produced touch insensitivity (Chalfie and Sulston 1981) and gain-of function mutations of either gene caused touch neuron degeneration (Chalfie and Wolinsky 1990; Driscoll and Chalfie 1991). Interestingly, when MEC-4(d), a dominant mutation of MEC-4, was co-expressed with MEC-10 in Xenopus oocytes, they formed Na^+ selective channels and the currents was blocked by amiloride. The mutant phenotype of this particular allele caused swelling and death of touch neurons and that's why the *C. elegans* subunits were named "degenerins" (DEG). When wild type MEC-4 and MEC-10 were co-expressed in Xenopus oocytes, hypotonic solutions failed to induce currents. Interestingly, co-expression of MEC-2, a stomatin-like protein with MEC-4 and MEC-10

(both wild type and (d) variants) in Xenopus oocytes increases current amplitude up to 40 times without changing channel density at the cell surface (Goodman, Ernstrom et al. 2002). MEC-2 is an intracellular protein that interacts with MEC-4 and changes its permeability to lithium (Goodman, Ernstrom et al. 2002). MEC-2 mutants are insensitive to touch stimuli. UNC-24 is similar to MEC-2 that encodes PHB (prohibit homology) domain that is found in Stomatin and several other proteins such as flotillin, prohibitin, and podocin in vertebrates. However, in heterologous expression system, UNC-24 has no effect on MEC-4d current and decreases it by 30% when MEC-2 is present (Zhang, Arnadottir et al. 2004). Another *mec* mutant, *mec-6*, is a single-pass transmembrane protein that is similar to mammalian. Mutations in *mec-6* produce touch insensitivity, eliminate MEC-4 puncta and suppress *mec-4(d)* and *mec-10(d)* induced neuronal degeneration (Chalfie and Sulston 1981; Huang and Chalfie 1994; Chelur, Ernstrom et al. 2002). MEC-6 increases Na^+ currents produced by MEC-4(d)/MEC-10(d) by ~30-fold, and functions synergistically with MEC-2 to increase the currents by 200-fold (Chelur, Ernstrom et al. 2002). *In vivo*, both MEC-2, MEC-6 and UNC-24 expression overlaps with MEC-4 puncta. They also physically interact with MEC-4, MEC-10 and other subunits. Electrophysiology recordings and calcium imaging of the touch neurons have provided better understanding of the mechanosensitivity of the touch receptor. In one study, the PLM, one of the six touch neurons in *C. elegans*, was recorded using patch clamp while

mechanical forces were applied to the posterior body wall. The onset of the mechanical force triggers a mechanoreceptor current (MRC) with a latency of less than 0.5 ms. This current quickly decays if the force is continuously applied. However, if the force is removed, an additional MRC of equal amplitude is generated. The force required for generating MRC can go as low as 100nN and the amount of force and the amplitude of the current are positively correlated. The current can be blocked by amiloride (O'Hagan, Chalfie et al. 2005).

Electrophysiological recordings of the touch neurons in different mutants shed light onto the function of individual genes. *mec-2*, *mec-4* and *mec-6* are required for channel function as null mutations in any of these genes completely eliminated MRCs.

In addition, point mutations in MEC-4 and MEC-10 that change the selectivity of the channel alter the properties of the MRC. This combined with the rapid onset of the current suggests that the MEC-4/MEC-10 complex transduces mechanical force in these neurons (O'Hagan, Chalfie et al. 2005).

While MEC-4, MEC-10, MEC-2, MEC-6 and UNC-24 comprise the mechanoreceptor channel complex, several other genes have also been implicated to be required for touch sensitivity in *C. elegans.* These genes include extracellular matrix and intracellular cell skeletons and so on (Du, Gu et al. 1996; Fukushige, Siddiqui et al. 1999).

Since both intracellular microtubules and extracellular matrix have both been implicated to modify touch sensitivity in *C. elegans*, initially a dual-tether model was proposed for the mechanotransduction of the MEC-4/MEC-10 channel complex (Huang, Gu et al. 1995). However, since null mutations in *mec-7* and *mec-12*, β and α tubulins, do not eliminate MRCs, it argues against them being part of the direct gating (O'Hagan, Chalfie et al. 2005). Currently, a model of MEC-4/MEC-10 channel complex being only tethered extracellularly is favored (Bounoutas and Chalfie 2007).

Acid-sensing ion channels (ASICs) also belong to the DEG/ENaC family. They share the same overall structure with this family and are composed of two TMs and a large extra-cellular domain in between (Waldmann, Champigny et al. 1997). Several ASICs have been cloned from mammals, toadfish, lamprey, shark and zebrafish. 4 ASIC genes and 7 isoforms have been cloned in mammals so far, ASCI1-4. ASICs are widely expressed in both neuronal and non neuronal tissues (Lingueglia 2007).

So far the only known activator of ASICs is extracellular proton. Like other DEG/ENaC members, ASICs are voltage-independent and highly selective for Na^+. ASICs have been shown to play a role in nociception. ASIC1, ASIC2 and ASIC3 are expressed in C and Aδ fibers which are the polymodal nociceptive afferents. ASIC2 and ASIC3 are also expressed in Aβ fibers that are low threshold mechanoreceptors. It's believed that mild acidic pH elicits non-adapting pain through ASICs, while TRPV1,

which can also be activated by proton mediates detection of acidic environment below pH 6 (Jones, Slater et al. 2004). In addition to the defects in sensing acid, ASICs knockout mice also have defects in mechanosensation. ASIC2 and ASIC3 deficient mice have subtle defects in cutaneous mechanosensitivity. ASIC3 -/- mice also have mild defects in mechanical nociception. Interestingly, a mammalian homologue of MEC-2 called stomatin-like protein 3 (SLP3) is critical for mechanosensitivity in Aβ and low threshold mechanosensory Aδ afferents. Mice lacking SLP3 show profound defects in texture sensation and do not prefer smooth surfaces over texture floor like wild type animals do (Wetzel, Hu et al. 2007). It is not known, however, whether SLP3 interacts with ASICs *in vivo* like MEC-2 interacts with MEC-4/MEC-10 channel complex (Tsunozaki and Bautista 2009).

1.4.2.3 Tethered model of mechanosensitive channels

The tethered model hypothesized a mechanosensitive ion channel attaches extracellular or intracellular, (or both) tethers in order to transduce force. The studies on the mechanoreceptor complex in *C. elegans* support this model. A screen for gentle touch defective mutants led to the identification of the candidate mechanotransducer *mec-4* , *mec-10* degenerin / epithelial sodium channel (DEG/ENaC) transduction complex.

In addition to *mec-4* and *mec-10,* that form the pore forming subunits of the ion channel complex, many other genes were identified that are required for the light touch

behavior. The mechanotransducing complex, is composed of several components: MEC-4 and MEC-10 are thought to be the pore forming subunits of the ion channel complex (Chelur, Ernstrom et al. 2002; Goodman, Ernstrom et al. 2002); other components include a paraoxonase-like protein MEC-6 and stomatin-like proteins MEC-2 and UNC-24 (reviewed in (O'Hagan and Chalfie 2006) (Goodman and Schwarz 2003) (Ernstrom and Chalfie 2002). Mutations in *mec-4*, *mec-2*, and *mec-6* eliminate mechanoreceptive currents and touch sensitive Ca^{2+} transient currents (Chalfie and Au 1989) (Chalfie and Sulston 1981) (O'Hagan, Chalfie et al. 2005) (Suzuki, Kerr et al. 2003). Stomatin is a component in red blood cell membranes and is involved in cation transport regulation. Hereditary stomatocytosis results in a disease with altered cell permeability (Stewart, Argent et al. 1993). The stomatin-like domain of MEC-2 is likely to be responsible for its association with the transduction complex (Goodman, Ernstrom et al. 2002; Zhang, Arnadottir et al. 2004).

Other genes identified from the gentle touch screen suggest that other potential "tethers" may be part of the transduction complex. Many of these genes were shown to colocalize with the MEC-4 expressing puncta and can be immunoprecipitated with the pore forming channel complex MEC-4/MEC-10 complex. A mec-4::YFP reporter labels punctate structures along the touch receptor neuron process. The extracellular matrix proteins (ECM) MEC-1 and MEC-5 co-localize with mec-4::YFP puncta *in vivo,* and

formation of the puncta is affected by mutations in *mec-1* and *mec-5* (Du, Gu et al. 1996; Emtage, Gu et al. 2004). Mutation in either of these genes results in touch insensitivity. Genes that are components of the cytoskeleton also came out of the screen, such as *mec-12* (alpha-tubulin) and *mec-7* (beta tubulin) (Chalfie and Thomson 1982) (Fukushige, Siddiqui et al. 1999); (Savage, Hamelin et al. 1989). *mec-7* null mutation results in touch insensitivity and a reduced mechanoreceptor current, suggesting that these cytoskeletal components play a role in mechanotransduction (O'Hagan, Chalfie et al. 2005). There are still residual mechanosensory currents in *mec-7* mutants, suggesting it's not essential for mechanotransduction. Since other tubulins are present in the same touch neurons, the residual response may due to functional redundancy. Identification of these cytoskeletal and ECM components in touch screens has given rise to a "tethered model" where the transduction channel interacts with components of the cytoskeleton and/or extracellular matrix to detect force (O'Hagan, Chalfie et al. 2005) (Goodman 2006).

Chapter 2 General Methods

2.1 Thermal nociception behavioral assay

The thermal nociception behavioral tests were performed as described previously (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010), with slight modifications.

On the first day, crosses were established with six female and three male flies in each vial with standard cornmeal food. For each genotype, six vials were established and maintained at 25 °C and 75% humidity.

On the sixth day of the experiment, wandering third-instar larvae from vials were rinsed out of the vial with distilled water and into a plastic 60 mm Petri dish. Excess water in the dish was aspirated such that the larvae remained moist but were not floating in the water that was remaining in the dish.

The temperature of the noxious heat probe (a soldering iron sharpened to a chisel tip shape that was 0.6 mm wide) was controlled by the adjustment of the voltage. A digital thermocouple (Physitemp, BAT-12) welded to the tip of the probe was used so that its temperature could be precisely measured. The stimulus was delivered by the gentle touching of the larvae laterally, in abdominal segments four, five, or six. Each

larva was tested only once and discarded after the test. Analysis of videotaped behavior was performed offline. The response latency was measured as the time interval from the point at which the larva was first contacted by the probe until it initiated the rolling movement (the beginning of the first, complete 360° roll).

2.2 Mechanical nociception behavioral assay

The mechanical nociception behavioral tests were performed as described previously (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010), with slight modifications.

Wandering third-instar larvae were collected as described for thermal nociception assays and then stimulated with a calibrated Von Frey filament. Von Frey filaments were made from Omniflex monofilament fishing line Shakespeare (6 lb test, diameter 0.009 inch [0.23 mm]). Fibers were cut to particular lengths and attached to a glass pipette such that specific length of the fiber protruded from the end and 10mm anchored the fiber. In our hands, 4 mm overhang gives 100 mN force, 8mm overhang gives 50 mN force and 10 mm overhang gives 30 mN force. Noxious mechanical stimuli were delivered by the rapid depression and release of the fiber on the dorsal side of a larva. The stimulus was delivered to abdominal segments four, five, or six. A positive

response was scored if at least one nocifensive roll occurred after the first mechanical stimulus.

2.3 Gentle touch behavioral assay

The gentle touch behavioral tests were performed as described previously (Tracey Jr, Wilson et al. 2003; Zhong, Hwang et al. 2010), with slight modifications. Wandering third-instar larvae were collected as described for thermal and mechanical nociception assays and were stimulated with an eye lash. The eye lash was fixed on the tip of a stick and gently touched the head of the larvae in the middle of normal locomotion. If there is no response to the gentle touch and the larva continued its normal locomotion, it was scored as 0. If the larva paused, it was scored as 1. If the larva paused and turned its head, it was scored as 2. If the larva produced one reverse peristaltic wave, it was scored as 3 and if multiple reverse peristaltic waves were produced, it was scored 4. Each larva was tested 4 times with the eye lash and the larva was allowed to fully recover between each test until they start to produce normal forward locomotion. All four scores of the larva were added up to generate a total score for that larva. Between 15 and 30 larvae were tested for each genotype.

2.4 Optogenetic activation of neurons

The optogenetic activation of *ppk-GAL4* neurons was performed as described previously (Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010). However, the strain utilized here (*ppk-GAL4 UAS-ChR2::eYFP/CyO; UAS-dicer-2*) harbored the *UAS-dicer-2* transgene, which enhances RNAi (Dietzl, Chen et al. 2007). When crossed to the *UAS-ppkRNAi* strain, only half of the progeny inherit the *ppk-GAL4 UAS-ChR2::eYFP* chromosome. These larvae were identified following exposure to blue light by visualizing their YFP fluorescence. The YFP-negative (CyO) larvae were used as in internal negative control because they never produce nocifensive escape locomotion in response to blue light.

Optogenetic activation in this work refers to a technique that uses blue light to depolarize neurons expressing a blue-light activated ion channel, channelrhodopsin-2 (ChR2). Four to six virgin female flies were crossed to 3 male flies to make the progeny of the right genotype. Females were allowed to lay eggs for 24 hours on apple juice agar with a dollop of yeast paste (either all-trans retinal [atr+, 500 μM] or atr- [0.5% ethanol]. The larval progeny were allowed to develop and feed on the yeast paste for 72 additional hours. For behavioral analysis the larvae were transferred to 60 mm plastic Petri dishes containing 1-2 ml deionized H_2O. Larvae were then stimulated with blue light (460-500 nm) using the Hg light source of a Leica MZ16 FA stereomicroscope

(14,000 lux). Blue light pulses were manually controlled and lasted for two to three seconds. Each larva was given three pulses of blue light. Behavioral responses were videotaped and analyzed off line. A positive nocifensive roll was scored if the larva completed at least 1 revolution (360 degrees) in response to any of the three blue light pulses. A positive accordion phenotype was scored if segmental muscle contractions occurred in response to the blue light.

2.5 Thermotaxis behavioral assay

To study thermosensation, the temperature preference of *Drosophila* was examined on a linear thermal gradient (Figure 6-5A1). The gradient was produced by resting an aluminum slab (27 X 18 X 2.5 cm) on a cold plate [Teca Corp. (Chicago) model LHP-800CP] under one end and a hot plate (Corning model PC320) under the other, with the thermal joint compound [Thermalloy (Dallas) no. 249] applied to the interfaces to enhance thermal conductance. To keep the relative humidity uniform along the gradient, the aluminum slab was covered with a moist sheet of chromatography paper (Whatman 3 mm) that was marked into 10 observation fields (Figure 6-5A2) dashed lines in top view) for recording the distribution of flies. Temperature along the gradient, measured on the paper with a thermocouple, was stable, reproducible, and linear. A 0.5-cm height Plexiglass cover (27 X 18 cm) confined the flies to the gradient. The cover included three plastic strips (Figure 6-5A2), horizontal lines in top view) providing

channels to test four groups simultaneously. To prevent flies from escaping the temperature gradient by resting on the walls or roof of the cover, the latter was coated with rain-X. Tests were conducted in darkness. When the temperature reaches equilibrium, it ranges from 16.5 °C to 32.5 °C from one end to the other. Adult flies were aged between 3-10 days old. 40 flies (20 females and 20 males) were used in one trial. Flies were aspirated from vials to the thermogradient under the plexiglass cover through a hole which is centered in the thermogradient (the hole is later covered by tape). Once in the lane, the flies can move freely for 15 min in the dark. At the end of the 15 min, the positions of the flies were determined by taking a picture of the thermal apparatus so that the distribution of the flies can be determined for this particular trial. In most cases, two trials were run side by side in the two centered lanes. After the trials, flies were anesthetized by CO_2 and removed from the thermogradient.

2.6 Live calcium imaging in 3rd antennal segment of adult Drosophila

UAS-YC3.3; ;dTrpA1-B-GAL4/+; and *UAS-YC3.3;; dTrpA1-B-GAL4/+, dTrpA1*^{W903*}*/Df(3L)ED4415* flies were aged between 8 and 12 days old. An individual fly was first anesthetized on a CO_2 pad. It was then removed from the pad with its wings being held using tweezers to let it recover from the CO_2 anesthetization for 3 min. Then the fly head was removed using a razor blade at the point where it is connected to the thorax. The fly head was then placed immediately in an imaging chamber (Warner

Instrument Series 20 Chamber platform P-2) between two cover slips. The fly 3rd antennal segments were placed facing the bottom cover slip and was directly imaged through the objective lens. The temperature change was controlled by a water perfusion system. The perfusion system was set up so that the water passed through an inline solution heater (Warner Instrument Model SH-27B) before flowing through the imaging chamber and into the aspirator. A thermocouple that was connected to a data acquisition board (Warner Instrument Corporation TC-324B) was placed in the water reservoir of the imaging chamber to measure the temperature in real time. Since the fly head was placed below the imaging chamber, it remained dry throughout the experiment. Microscopy was performed on a Zeiss LSM 5 Live Confocal System with 20X objective lens and 440 nm laser. Images were acquired as time series at 1 frame/sec.

2.7 Calcium imaging in cell culture

Drosophila cell line S2R+ cells were maintained in Schnyder medium with 10% FBS. Cells growing on round cover slips (Warner Instruments #1.5 Glass Coverslip 25mm Round) in 6 well tissue culture plates (Falcon) were transfected with 0.75 μg of *Ubiquitin-GAL4*, 0.5 μg of *UAS-GCaMP3.0* and the gene of interest, either 0.25 μg of *UAS-dTrpA1-A* or 0.25μg of *UAS-dTrpA1-B* per well using cellfectin (Invitrogen). Imaging was conducted on the 4th day after the transfection. The round cover slip is assembled into an imaging chamber with the cells facing up and gently rinsed with HL3

saline. HL3 saline goes through a through an inline solution heater (Warner instrument) before it flows through the imaging chamber and goes into the vacuum. A thermal couple that is connected to a data acquisition board is placed in the water reservoir of imaging chamber to measure the temperature in real time. To increase the saline temperature, we turn on the inline solution heater; to cool down the temperature, we turn off the inline solution heater as well as add ice-chilled HL3 saline to the reservoir. Microscopy was performed on a Zeiss LSM 5 Live Confocal System using 20X lens. Images were acquired as time series at 1 frame/sec.

Chapter 3 Identification of *Drosophila* Larval Nociceptors

3.1 Introduction

Relatively little is known about the molecular and neural circuits that encode somatosensory information in *Drosophila*. The circuits used by the brain to encode information about external temperature, touch, and body position are virtually unknown. However, for other sensory systems, studies of *Drosophila* have led to an exquisitely detailed understanding, and to the realization that some of the basic principles that are used to encode sensory information are shared between mammalian and insect brains. For example, the glomerular architecture of the olfactory system is evolutionarily conserved (Ressler, Sullivan et al. 1994; Vassar, Chao et al. 1994; Mombaerts, Wang et al. 1996; Gao, Yuan et al. 2000; Vosshall, Wong et al. 2000). Thus, by analogy, the study of somatosensation in *Drosophila* may provide significant insight into basic and evolutionarily conserved mechanisms of somatosensory processing.

To study this problem we previously developed a behavioral paradigm for the study of nociception (pain sensing) in the *Drosophila* larva (Tracey, Wilson et al. 2003). This paradigm was based upon the simple observation that *Drosophila* larvae produce a stereotyped defensive behavior in response to noxious mechanical, chemical or thermal

stimuli. The pattern of locomotion elicited by noxious stimulation causes the larvae to roll in a highly stereotyped corkscrew-like fashion (Tracey, Wilson et al. 2003). This defensive motor output is completely distinct from the well-known rhythmic peristaltic locomotion that is used for other larval behaviors such as foraging. To our knowledge, the rolling pattern of locomotion occurs only following noxious stimulation (heat, mechanical or chemical). The behavior is therefore nocifensive (defensive behavior that is elicited by sensory stimuli that have the potential to cause injury).

Although the nocifensive behavior can be elicited readily in the laboratory, the relevance of the behavior to stimuli that might be encountered in the natural environment remains unclear. For this behavior to have evolved, and for it to be innately encoded in the genome, the behavior presumably provides a selective advantage. This advantage must exceed the energetic costs of maintaining the neuronal circuits that mediate the response.

Predation is one possible selective pressure that may have favored the evolution of the rolling escape behavior. For example, many small parasitoid wasps of the superfamilies *Chalcidoidea* and *Ichneumonoidea* attack *Drosophila* larvae (Carton, Bouletreau et al. 1986). The female wasp penetrates the *Drosophila* larval cuticle with a sharp ovipositor and lays its egg. The larval wasp then devours the *Drosophila* from within and an adult wasp eventually emerges rather than a fly. The cellular immune

system of the *Drosophila* larvae has the ability to encapsulate and destroy the wasp egg so that all wasp infections do not result in the death of the larva (Rizki, Rizki et al. 1990). An evolutionary arms-race causes the wasp to evolve its own strategies to disrupt the host immune system (Rizki and Rizki 1984; Russo, Brehelin et al. 2001; Morales, Chiu et al. 2005). Wasp parasitism is believed to impose strong selective pressures on *Drosophila.* Studies of natural populations of *Drosophila* have found rates of infection that exceed sixty percent (Powell 1997). The ecological importance of wasp parasitism is not limited to *Drosophila.* Greater than 300,000 species of parasitic wasps exist in nature, and they in turn affect many more species of plants and insects (Quicke 1997).

Several previous lines of evidence suggest that multidendritic (md) sensory neurons of the larva might function as nociceptors. First, they morphologically resemble vertebrate nociceptive neurons since they have multiply-branched (Bodmer, Carretto et al. 1989; Merritt and Whitington 1995; Gao, Brenman et al. 1999; Williams and Shepherd 1999; Moore, Jan et al. 2002; Anderson, Ressegui et al. 2005; Williams and Truman 2005; Shima, Kawaguchi et al. 2007) naked nerve endings attached to epidermal cells. Second, larvae with genetically silenced md neurons are completely insensitive to noxious stimulation and fail to produce the nocifensive response (Tracey, Wilson et al. 2003; Rosenzweig, Brennan et al. 2005). Third, the Painless TRP channel is required for

nociception and is expressed in md neurons (Tracey, Wilson et al. 2003). Based on these lines of evidence we have previously proposed that md neurons function as nociceptors.

However, the morphology of md neurons suggests that these neurons are not a uniform population of cells. Rather, at least four morphological subtypes have been identified. According to the complexity of the dendritic arbors and other morphological features, the neurons are termed class I–IV. The class I neurons have the simplest dendritic arbors while the class IV are the most complex (Grueber, Jan et al. 2002). Central projections of each of the md neurons have been analyzed in detail (Merritt and Whitington 1995; Schrader and Merritt 2000; Grueber, Ye et al. 2007). The projections were consistent with the possibility that the neurons within a morphological class might provide similar informational content to the brain (i.e. suggesting a similar function). The central axonal projections were found to vary among classes of md neurons, suggesting functional specialization among the classes (Grueber, Ye et al. 2007). Class I neurons project to motor neuropile of the dorsal abdominal ganglion and have been proposed to provide feedback to motor neurons. However, class II, class III and class IV neurons all project to ventral neuropile and, by analogy to other insects, are predicted to have somatosensory functions.

Several studies have suggested non-nociceptive functions for md neurons (Ainsley, Pettus et al. 2003; Hughes and Thomas 2007; Song, Onishi et al. 2007). For

example, deletion of *pickpocket,* which encodes a DEG/ENaC type ion channel that is expressed solely in the class IV md neurons, results in an interesting larval locomotion phenotype. *pickpocket* mutant larvae move more rapidly than the wildtype larvae and turn less frequently (Ainsley, Pettus et al. 2003). More recently, Hughes and Thomas found that the synaptic output of class I md neurons and the dendritic (dbd) neurons, were required for propagation of peristaltic muscle contraction that occurs during normal larval locomotion (Hughes and Thomas 2007).

The various central projection patterns and dendritic morphologies of multidendritic neurons led us to ask: are all multidendritic neurons nociceptors or is nociception function restricted to a particular subtype? In addition, we have begun to investigate selective advantages that may have led to the evolution of rolling locomotion. We propose that selective pressures imposed by parasitoid wasps may have played a role in the evolution of this behavior.

3.2 Methods and materials

3.2.1 Molecular cloning

To generate UAS-Channelrhodopsin2-EYFP the ChR2-YFP fusion gene was PCR amplified from the pLECYT template using the forward primer (5'CACCATGGATTATGGAGGCGGCCTGAGT3') and the reverse primer (5'CTATTACTTGTACAGCTCGTC3'). To generate UAS-Channelrhodopsin2-mCherry

the humanized ChR2-mCherry fusion gene was PCR amplified from the pFCK-hChR2-mCherry-W template using the forward primer (5'CACCATGGACTATGGCGGCGCTTTGTC3') and the reverse primer (5'TTACTTGTACAGCTCGTCCATGCCGAG3'). The PCR products were then cloned into pENTR/D-TOPO (Invitrogen) and then into the *Drosophila* pUASt-W Gateway destination vector (Murphy 2003) using Clonase II (Invitrogen). The UAS-ChR2-EYFP and UAS-Chr2-mCherry constructs were used to generate transgenic animals by transposase mediated transformation of w^{1118}.

3.2.2 Fly strains

yw; *GAL4109(2)80* (*md-GAL4*), *w*; *c161-GAL4*, *w*;*1003.3-GAL4*, *ppk-GAL4*, *w*; *2-21-GAL4*, *w;UAS-mCD8::GFP, w; UAS-Channelrhodopsin-2::YFP* The *UASChR2YFP* inserts of Line C and Line 1 are on chromosome II. The insertion of Line2 is found on chromosome II. In Line AB two insertions are present: one on chromosome II and the other on chromosome III. The *w; UAS-Chop2* line (Schroll, Riemensperger et al. 2006) was a third chromosome insertion. *Drosophila* stocks were raised on standard cornmeal molasses fly food medium at 25°C. Where possible balancers containing the *Tb* marker were used to follow inheritance of the UAS insertion(s). Alternatively, YFP fluorescence provided a means to follow *UAS-ChR2YFP* inheritance.

3.2.3 Confocal microscopy

For visualization of GAL4 driver patterns, larvae heterozygous for the indicated driver *and UAS-mCD8-GFP* were filleted, fixed in 4% paraformaldehyde. For immunostaining, mCD8-GFP was detected with rabbit anti-GFP (Invitrogen 1:1000) and the secondary anti-rabbit Alexa Fluor 568 (Molecular probes 1:1000) and the neurons were counterstained with FITC conjugated goat anti-HRP (Cappel 1:200). Similar methods were used for detection of YFP tagged ChR2. Microscopy was performed on a Zeiss LSM 5 Live Confocal System. Images shown are maximum intensity projections of confocal Z-stacks, brightness and contrast were adjusted using Adobe Photoshop.

3.2.4 Behavioral assays

The thermal nociception behavioral tests were performed as described previously (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010), with slight modifications. On the first day, crosses were established with six female and three male flies in each vial. For each genotype, six vials were established and maintained at 25 °C and 75% humidity. On the sixth day of the experiment, wandering third-instar larvae from vials were rinsed out of the vial with distilled water and into a plastic 60 mm Petri dish. Excess water in the dish was aspirated such that the larvae remained moist but were not floating in the water that was remaining in the dish.

The temperature of the noxious heat probe (a soldering iron sharpened to a chisel tip shape that was 0.6 mm wide) was controlled by the adjustment of the voltage. A digital thermocouple (Physitemp, BAT-12) welded to the tip of the probe was used so that its temperature could be precisely measured. The stimulus was delivered by the gentle touching of the larvae laterally, in abdominal segments four, five, or six. Each larva was tested only once and discarded after the test. Analysis of videotaped behavior was performed offline. The response latency was measured as the time interval from the point at which the larva was first contacted by the probe until it initiated the rolling movement (the beginning of the first, complete 360° roll).

In mechanical nociception assays, wandering third-instar larvae were collected as described for thermal nociception assays and then stimulated with a 100 mN calibrated Von Frey filament. Von Frey filaments were made from Omniflex monofilament fishing line Shakespeare (6 lb test, diameter 0.009 inch [0.23 mm]). Fibers were cut to a length of 14 mm and attached to a glass pipette such that 4 mm of the fiber protruded from the end and 10 mm anchored the fiber. Noxious mechanical stimuli were delivered by the rapid depression and release of the fiber on the dorsal side of a larva. The stimulus was delivered to abdominal segments four, five, or six. A positive response was scored if at least one nocifensive roll occurred after the first or second mechanical stimulus.

3.2.5 Optogenetic activation

Four to six virgin female flies of the GAL4 driver strain were crossed to male flies of the UAS-ChR2YFP strain. Females were allowed to lay eggs for 24 hr on apple-juice agar with a dollop of yeast paste (either atr+ [500 μM] or atr−). The larval progeny were allowed to develop and feed on the yeast paste for an additional 72 hr. For behavioral analysis, the larvae were transferred to 60 mm plastic Petri dishes containing 1–2 ml deionized H_2O. Larvae were then stimulated with blue light (460–500 nm) with the Hg light source of a Leica MZ16 FA stereomicroscope (14,000 lux). Blue light pulses were manually controlled and lasted for several seconds. Each larva was given three pulses of blue light. Behavioral responses were videotaped and analyzed offline. A positive nocifensive roll was scored if the larva completed at least one revolution (360°) in response to any of the three blue light pulses. A positive accordion phenotype was scored if segmental muscle contractions occurred in response to the blue light.

3.3 Results

3.3.1 GAL4 drivers that target different subsets of multidendritic neurons

The *Drosophila* GAL4/UAS system allows for targeting of gene expression to precise cells of the animal (Brand and Perrimon 1993; Gao, Brenman et al. 1999). Several GAL4 drivers have been identified that allow for targeting of gene expression to arborizing md neurons. We first examined these drivers to determine if they would be useful for targeting specific subsets of md neurons in behavioral experiments. We planned to cross the drivers to UAS-tetanus toxin light chain (*UAS-TnT-E*) lines (Sweeney, Broadie et al. 1995) and then to test animals that were trans-heterozygous for the GAL4 driver and *UAS-TnT-E* in nociception behavioral assays. Since the tetanus toxin light chain cleaves the v-snare synaptobrevin, it reduces evoked synaptic vesicle release in the neurons which express the GAL4 driver, effectively silencing them.

Four GAL4 drivers were identified that would be useful for our purposes. The *GAL4109(2)80* driver (*md-GAL4*) (Gao, Brenman et al. 1999) was expressed in all four classes of md neurons (Figure 3-1a$_{1-4}$) whose projections decorated the entire epidermis. The class I and II neurons, whose relatively unbranched dendrites tile only a subset of the entire epidermis, were strongly targeted by the *c161-GAL4* driver (Smith and Shepherd 1996; Williams and Truman 2005) (Figure 3-1b$_{1-4}$). As mentioned above, based on central projections, and on behavioral evidence, the class I neurons have been

previously proposed to function as proprioceptors (Grueber, Ye et al. 2007; Hughes and Thomas 2007). The function of class II md neurons is not known.

The class III and IV md neurons possess more complex dendrites that tile the entire epidermis. There is no region that lacks endings from these cells, a feature that would be expected for nociceptive neurons. The class III neurons (as well as class II neurons) were targeted by the recently described *1003.3-GAL4* driver (Figure 3-1c$_{1-4}$) (Hughes and Thomas 2007). Finally, the *pickpocket1.9*-GAL4 (*ppk-GAL4)* (Ainsley, Pettus et al. 2003) driver targets class IV md neurons (Figure3-1d$_{1-4}$).

3.3.2 Silencing of class IV multidendritic neurons eliminates thermal nociception behavior

As shown previously, when *md-GAL4* was used to drive the expression of *UAS-TnT-E* in all four classes of md neurons, the behavioral response to noxious heat was completely abrogated (Tracey, Wilson et al. 2003) (Figure 3-2 B) compared to controls without a driver *UAS-TnT-E*/+ (Figure 3-2 A).

We next assayed the *c161-GAL4* driver which targeted to all neurons of the class I and II subtypes. Compared to the control (*UAS-TnT-E*/+), larvae expressing *UAS-TnT-E* under control of *c161-GAL4* showed only a slight (although statistically significant, Wilcoxon Rank-Sum Test, $p<0.05$) delay in their initiation of nocifensive responses (Figure 3-2 C) to the noxious heat probe which contrasts with the results seen with *md-*

GAL4 (Figure 3-2 B). However, the rolling behavioral output did not appear to be as coordinated as in the wild type and it took these larvae longer than control lines to achieve a complete roll. This result is consistent with previous studies that implicated the class I md neurons in proprioceptive feedback that plays a role in peristaltic locomotion. Our results may indicate that the class I (or possibly the class II) md neurons also provide proprioceptive feedback necessary for completion of rolling behavior.

We also inactivated the class II and class III neurons using the *1003.3-GAL4* driver to drive *UAS-TnT-E*. These larvae appeared normal in their initiation of the behavioral response compared to the control *UAS-TnT-E*/+ (Wilcoxon Rank-Sum Test, p=0.32) (Figure 3-2 D). The rolling response of larvae with silenced class II and class III neurons appeared coordinated and we did not observe any obvious defects in the quality of the behavior. This result also suggests that the coordination defects seen with the *c161-GAL4* driver were unlikely to be due to inhibition of class II neurons.

The data shown above indicate that blocking the output of class I and class II, or of class II and class III neurons did not strongly affect thermal nociception responses. This suggested that the class IV neurons might be the relevant neurons involved in the thermal nociception response. To test this we were able to specifically inactivate the class IV neurons using the *ppk-GAL4* driver. Indeed, larvae of the *ppk-GAL4*/*UAS-TnT-E* genotype showed a dramatically impaired thermal nociception response compared to

the control *UAS-TnT-E*/+ (Figure 3-2E, Wilcoxon Rank-Sum Test $p<0.00001$). The frequency of larvae that failed to perform nocifensive behavior even after 10 seconds of stimulation was significantly increased relative to control genotypes. The rare larvae of this genotype that did produce the rolling behavior appeared to be coordinated. Although blocking the class IV neurons did not completely eliminate thermal nociception, the remaining response could be due to an incomplete block of the synaptic output in these cells by tetanus toxin light chain. Alternatively, parallel processing may occur through sensory neurons that we have yet to identify as nociceptive.

Our prior studies indicated that strong mechanical stimuli elicited the same nocifensive behavior that can be elicited by noxious heat. We thus tested which of the various subtypes of md neurons were required for mechanical responses (Figure 3-2 F). Blocking the class I and class II neurons did impair the behavioral responses to mechanical stimuli. However, this result is difficult to interpret given that these larvae also appeared uncoordinated as described above. Blocking the output of classes II and III had a milder effect on mechanical nociception. Finally, as with thermal nociception, blocking class IV neurons most strongly diminished the response to mechanical stimuli (Figure 3-2F) suggesting the possibility that these cells have polymodal nociceptive functions.

Combined, these results suggested that the synaptic output of class IV md neurons plays a major role in initiating nocifensive behavioral responses to noxious heat or mechanical stimulation. However, there are caveats to this interpretation of the data. For example, although our data suggested that the class IV md neurons are required for the nocifensive behavior they did not prove that these neurons are nociceptors. Instead, it was possible that these neurons were required for the central nervous system to efficiently control the behavior. For example, they could merely provide proprioceptive feedback.

3.3.3 Optogenetic activation of class IV neurons elicits nocifensive behavior

Therefore, we next tested whether activation of md neurons would be sufficient to elicit nocifensive behaviors. If activating these neurons could be shown to trigger the nocifensive behavior, it would place them upstream of the rolling behavioral output in this neuronal pathway.

Targeted photo-activation of neurons has been achieved using Channelrhodopsin-2 (Boyden, Zhang et al. 2005; Li, Gutierrez et al. 2005; Schroll, Riemensperger et al. 2006; Arenkiel, Peca et al. 2007; Wang, Peca et al. 2007; Zhang, Wang et al. 2007) (ChR2) a light activated cation channel (Nagel, Szellas et al. 2003) from green algae. Importantly, ChR2 has been shown to be capable of causing light induced

action potentials in *Drosophila* motor neurons (Schroll, Riemensperger et al. 2006). Further, GAL4 lines were used to drive *UAS-Channelrhodopsin-2* in dopaminergic or octopaminergic neurons. Remarkably, illumination with blue light resulted in specific associative learning effects in larvae that were dependent on feeding of larvae all *trans*-retinal (which forms the chromophore for Channelrhodopsin-2) (Schroll, Riemensperger et al. 2006).

We generated flies that express fluorescently tagged Channelrhodopsin-2 (*ChR2-YFP* or *ChR2-mCherry*) under control of the GAL4/UAS system (*UAS-ChR2-YFP, UAS-ChR2-mCherry*) (Boyden, Zhang et al. 2005; Zhang, Wang et al. 2007). The fluorescent tags allowed us to identify *a priori*, lines that generated detectable expression levels of ChR2 in the md neurons (Figure 3-3 A, B). Expression of identical ChR2-YFP and ChR2-mCherry proteins has been previously shown to render rat hippocampal neurons light sensitive (Boyden, Zhang et al. 2005; Zhang, Wang et al. 2007).

We selected several of the YFP tagged lines for behavioral analysis. The lines selected showed detectable fluorescence in peripheral sensory neurons when we crossed them to appropriate GAL4 drivers (Figure 3-3B). We chose a more strongly expressing line (AB) with UAS insertions on both the second and third chromosomes, for behavioral analysis and crossed it to the GAL4 strains described above. The larval progeny from these crosses were raised to third instar fed on yeast paste that either

contained *all-trans retinal (atr+)* or on yeast paste that did not contain *all-trans retinal (atr-)*; the latter contained the diluent alone (0.5% EtOH). Individual third instar larvae were then transferred to a small droplet of water in a Petri dish so that they could be viewed on a fluorescent stereomicroscope that was equipped with a video recorder (allowing the behavioral response to illumination of blue light to be recorded).

The *atr+* yeast paste had no effect in control larvae that lacked a GAL4 driver (*UAS-ChrR2-YFP/+).* However, in the presence of GAL4 drivers we indeed observed behaviors to pulses of blue light (460-500nm) that were strongly dependent on feeding of *all-trans retinal.* Even in the presence of GAL4 drivers, *ChR2-YFP* expressing larvae that were fed the *atr-* yeast paste only rarely produced behaviors in response to blue light (Figure 3-3 C, D, Figure 3-4A).

We first tested the effects of expressing *ChR2-YFP* using the class I-IV (*md-GAL4*) driver. In response to blue light pulses we found that the larvae performed one of two distinct behavioral responses. In the most prevalent response, the blue light caused the larvae to simultaneously contract the muscles of all body segments and to scrunch like a compressed accordion (Figure 3-3 C).

In a more rarely occurring response, the larvae rolled using a motor pattern that appeared quite similar to the nocifensive rolling behavior (Figure 3-3 D). However in these larvae, the light-induced rolling was eventually followed by the accordion like

muscle contraction behavior. The latter behavior is never seen in response to noxious heat or mechanical stimuli.

We hypothesized that the accordion like behavior and the rolling behavior might reflect activation of distinct behavioral pathways, as a consequence of activating multiple neurons with distinct functions. For example, it seemed possible that some md-neurons might trigger segmental muscle contractions while others might trigger nocifensive rolling. If this were the case, then the behaviors described above could be the result of competition for these two pathways by light induced activation of the relevant triggering sensory inputs. We thus further tested whether activation of the two behavioral pathways could be separated, by more precise targeting of the ChR2-YFP to distinct md-neuron subsets.

Indeed, when we expressed ChR2-YFP in the Class I and II neurons (*c161-GAL4*), or in the class II and III neurons (*1003.3-GAL4*) we never observed rolling behavior in response to illumination with blue light (Figure 3-3 D). Instead, the blue light caused the larvae to perform the accordion response with high penetrance (Figure 3-3 C, Figure 3-4B). That we did not observe rolling behavior in response to activation of the class I, II, or III neurons is consistent with the inactivation studies described above since we did not find evidence that the synaptic output of these neuronal types was strongly required for the initial steps of thermal nociception behavior.

We hypothesize that the accordion phenotype reflects a role for the class II and/or class III md neurons in propagating the wave of muscle contraction during peristaltic locomotion. During normal locomotion, activation of the class II or the class III neurons may occur via muscle contraction within a segment. This may produce a signal coordinating the contraction of muscles in the next segment. The accordion phenotype likely represents a manifestation of this process, but in an abnormal situation where the signal is sent to all segments simultaneously via optogenetic activation.

In contrast, optogenetic activation of class IV md neurons (*ppk-GAL4/UAS-ChR2-YFP*) caused robust nocifensive-like rolling behavior and never resulted in the accordion like behavior (Figure 3-3 C, D, Figure 3-4 C). The penetrance of the nocifensive response to the blue light pulse was impressive, with 87% of the larvae responding with rolling behavior in lines strongly expressing ChR2-YFP. With lines that expressed ChR2-YFP more weakly, the same behavior was observed, but with a reduced frequency (Figure 3-3 E).

Qualitatively, this light induced nocifensive behavior appeared very similar to thermally and mechanically induced rolling behavior. However, the light induced behavior was initiated very rapidly (<100 ms after the light was turned on. This was more rapid than the rolling induced with our standard nociception stimulus of 47 ˚C where the larvae often require thermal stimulation of several seconds to elicit the

response. The rapid behavioral responses with ChR2-YFP likely reflect the extremely rapid kinetics of light activated channel relative to slower kinetics of thermal nociception at 47 °C. Indeed, very rapid thermal nociception responses can also be observed (rolling that initiates <100ms following contact with the thermal probe) but these require a stronger thermal nociception stimulus (53 °C probe) (W.D.T, R.Y.H, L.Z., unpublished). Combined, these data conclusively demonstrate that activation of class IV md neurons of the third instar *Drosophila* larva is sufficient to trigger a nocifensive-like motor output. In addition, we have found that the output of the class IV neurons is necessary for triggering normal nocifensive behavior in thermal and mechanical nociception assays. We therefore propose that the class IV multidendritic neurons function as nociceptors.

3.3.4 Paradoxical directionality of rolling behavior

We have used a thermal stimulus as a convenient method for the identification of neurons and mutants that affect the nociception pathway. To perform the rolling escape response, the larva uses its muscles to move in a highly coordinated fashion that is distinct from peristaltic locomotion. This suggests the existence of multiple central pattern generators in the larval brain (Figure 3-7). Rolling locomotion causes larvae to move at a significantly higher velocity (3-5 mm/sec) (R.H., L.Z., unpublished) than typical peristaltic locomotion (1 mm/sec). This increased velocity presumably provides

a selective advantage in a rapid escape from a potentially damaging thermal insult. However, it is unlikely that the rolling behavior evolved solely for thermal nociception since strong mechanical stimuli also elicit the rolling response.

Indeed, the following observations suggest that thermal nociception is unlikely to be the only selective pressure that drove the evolution of this behavior. Surprisingly, we have found that *Drosophila* larvae have a genetically encoded tendency to initially roll towards the noxious heat probe rather than to roll away from it. When animals were stimulated on the right side of the body, the preferred initial direction of rolling was to the right (ie. rolling clockwise) (Figure 3-5 A). When stimulated on the left the preferred direction of rolling was towards the left (rolling counter-clockwise) (Figure 3-5 A). The result was that the animals tended to initially roll in a direction that pushed them against the thermal probe rather than in a direction that carried them away. After failing to escape following several revolutions, the larvae did reverse direction of rolling. The choice of roll direction was not random, indicating that the larval brain was capable of sensing the direction from which the stimulus was coming. Yet, paradoxically, the circuitry that controlled rolling was strongly biased to produce the initial direction of motor output towards the side of the body that had been stimulated.

In natural populations of *Drosophila* then, there must be a strong selective advantage in directional rolling towards the side of the body in which the nociceptors

have been activated. It is not obvious to us how this would be the case if noxious heat were the sole selective pressure driving the evolution of this behavior.

3.3.5 Third instar larvae escape from wasp attacks by rolling

We sought an explanation for this paradox in the natural ecology of *Drosophila*. Diverse species of parasitoid wasps require a *Drosophila* host in order to complete their life cycle. For example, the figitid wasp, *Leptopilina boulardi* is an obligate and ubiquitous parasitoid of *Drosophila melanogaster* (Carton 1984). Female *Leptopilina* lay their eggs within the *Drosophila* by penetrating the larval cuticle and epidermis using a sharp ovipositor. It has been reported that larval hosts of sufficient size (third instar) can behaviorally defend from wasp attack with vigorous movements (van Lenteren, Isidoro et al. 1998). Inexperienced wasps have been reported to be more susceptible to larval defenses while experienced wasps eventually learn to attack smaller, more defenseless, animals (van Lenteren, Isidoro et al. 1998). Thus, we wished to observe larval behavioral responses to wasp attack in order to determine whether the defensive behaviors triggered by wasps was similar to the responses that were triggered by activation of class IV neurons via Channelrhodopsin-2YFP.

We reasoned that the very fine and highly branched dendrites of the Class IV multidendritic neurons might be capable of detecting attacks from the wasp ovipositor. If this were the case, wasp attack should elicit the rolling response. We further reasoned

that the bias in rolling direction that we observed experimentally might be of benefit in evading a wasp attack. Rolling in the direction counter to the side of the attack might actually assist the wasp in its attempts to penetrate the cuticle (Figure 3-5 B). In contrast, rolling towards the direction of wasp attack might deflect the ability of the wasp ovipositor to penetrate the cuticle and allow the larva to escape (Figure 3-5 C). We hypothesized that such a mechanism would favor the evolution of neuronal circuits that encoded a behavioral tendency to roll towards the side of the body in which nociceptors had been activated, rather than away.

As we had predicted, we found that third instar *Drosophila* larvae did respond to attack by *Leptopilina boulardi* by producing the rolling response (Figure 3-6). The wasp triggered rolling resembled both mechanical and optogenetically triggered rolling. Also, as expected, we observed larvae that rolled towards the side of the body from which the wasp attack had come. However, the overall outcome of the larval responses was somewhat unexpected to us. Surprisingly, as a consequence of the larva rolling towards the wasp, the thin threadlike ovipositor of the wasp became wrapped around the larva (Figure 3-6). The rolling behavior of the larva thus acted like a spool winding a thread. After the larva had performed several revolutions, the wasp ovipositor eventually reached the limits of its length. The larva continued to roll and finally carried the wasp through the air and onto its back. At this point the wasp, clearly in danger of getting

stuck in the medium, appeared to prematurely break off its attack. The larva then quickly left the area as the female wasp retracted its ovipositor.

3.4 Discussion

In this study, we have provided strong evidence that at least one of the classes of *Drosophila* multidendritic neuron is nociceptive. These are the Class IV neurons which express the *pickpocket* gene. First, we have shown that blocking the Ca^{2+} evoked synaptic output of class IV md neurons significantly impairs thermal and mechanical nociception behavior. Second, we have shown that optogenetic activation of these neurons is sufficient to trigger the stereotyped rolling response.

It is important to note that our data do not rule out the possibility that the class IV neurons might have polymodal functions. It is possible, for example, that the firing

rate triggered by nociceptive stimuli exceeds a critical threshold that triggers rolling while lower firing rates could be used to regulate turning or rate of locomotion. The latter possibility would be consistent with prior studies which proposed that the class IV md neurons function as proprioceptors. This hypothesis was based on the fact that larvae mutant for *pickpocket* move rapidly and turn less frequently than wild type larvae and by the fact that *pickpocket* is solely expressed in class IV neurons (Ainsley, Pettus et al. 2003). It will be interesting to further investigate the *pickpocket* mutant phenotypes in light of the nociceptive function for the class IV neurons that we have presented here. The differences in the central projections of distinct types of md neurons are notable in light of the functions that we have observed. Of the four classes of multidendritic neurons, only the class IV md neurons have projections which cross the midline to innervate contralateral post-synaptic targets (Grueber, Ye et al. 2007). This has similarity to pain processing in vertebrate brains in which ascending tracts of the contralateral spinal cord carry painful sensory information to higher order neurons of the brain (Purves, Augustine et al. 2001). Future studies will allow us to determine whether the brain of the larva is involved in perception of the noxious stimulus or whether lower level processing in the abdominal or thoracic ganglion plays a role.

Perhaps the most interesting question related to this issue is whether input from the class IV neurons of larvae, or of adult flies, results in a negative associative valence.

In larvae, this might allow avoidance of regions of fruits whose odor is associated with the presences of wasps. Interestingly, the class IV neurons persist through metamorphosis and are present in adult flies (Williams and Truman 2005) where they are unlikely to activate a rolling central pattern generator. Furthermore, noxious heat is known to be an effective unconditioned stimulus in adult *Drosophila* operant learning paradigms (Wolf and Heisenberg 1991; Wustmann, Rein et al. 1996; Liu, Wolf et al. 1999; Zars, Wolf et al. 2000). Can optogenetic activation of the class IV md neurons be used as a substitute for an unconditioned stimulus in operant negative associative learning paradigms? Does electric shock used in olfactory learning paradigms activate overlapping neuronal circuits?

Our unambiguous identification of the class IV md neurons as nociceptors opens the door to further analysis of these interesting questions and will allow us to dissect this neuronal circuit, from the molecule to the behavior. In addition, our identification of parasitoid wasp attack as an ecologically relevant stimulus that elicits rolling demonstrates an evolutionarily important adaptive function for this fascinating behavior.

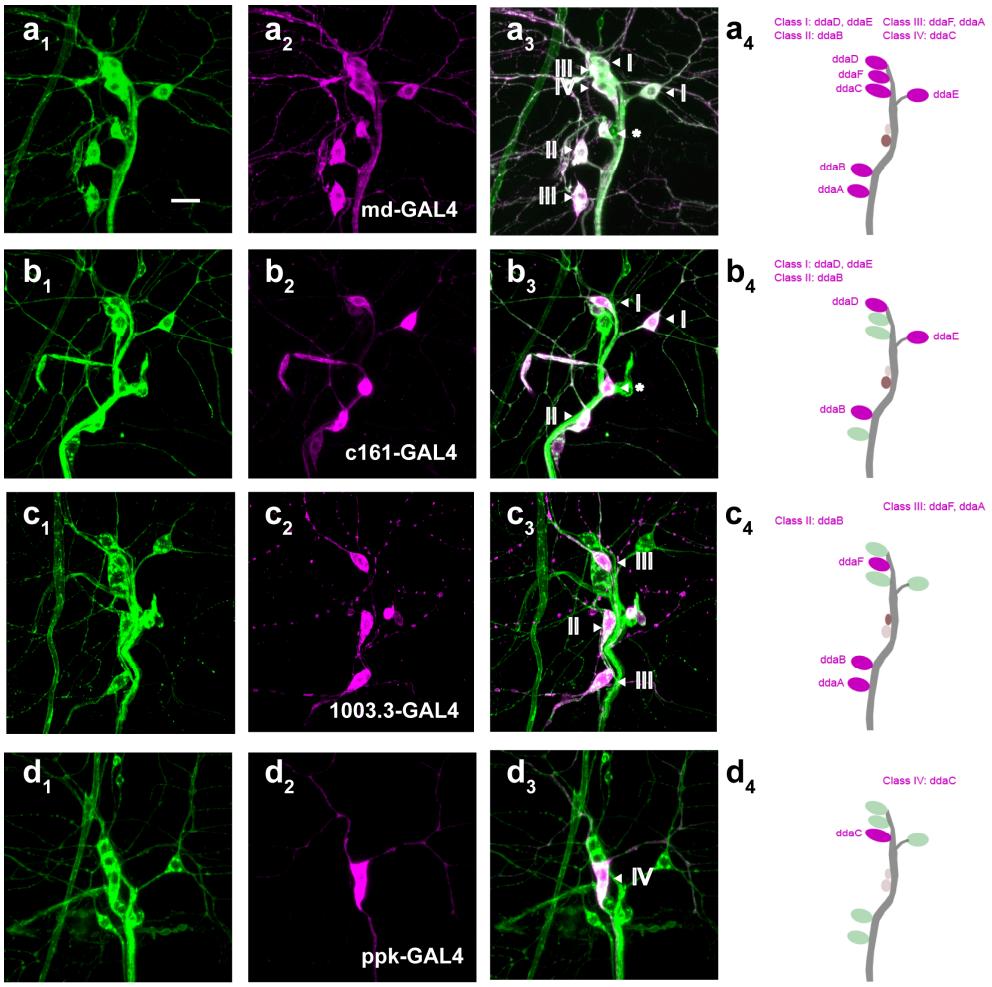


Figure 3-1: GAL4 drivers which target distinct subset of multidendritic neurons

(A-D) Confocal microscope images of third instar larval multidendritic neurons (dorsal cluster labeled with *UAS-MCD8-GFP*). **(A_1-D_1)** Immunostaining with the pan-neuronal marker anti-HRP-FITC (green). **(A_2-D_2)** Co-immunostaining with anti-GFP (magenta). **(A_3-D_3)** Merge. **(A_4-D_4)** Schematic diagrams of a dorsal cluster of MD neurons labeled by class and name for each GAL4 driver. **(A)** Expression pattern of *md-GAL4* (class I-IV). **(B)** Expression pattern of *c161-GAL4* (class I-II). **(C)** Expression pattern of *1003.3-GAL4* (class II-III). **(D)** Expression pattern of *ppk1.9-GAL4* (class IV). Roman numerals represent multidendritic neuron class. Asterisk indicates the dmdI neuron. Scale bar is 10 μm.

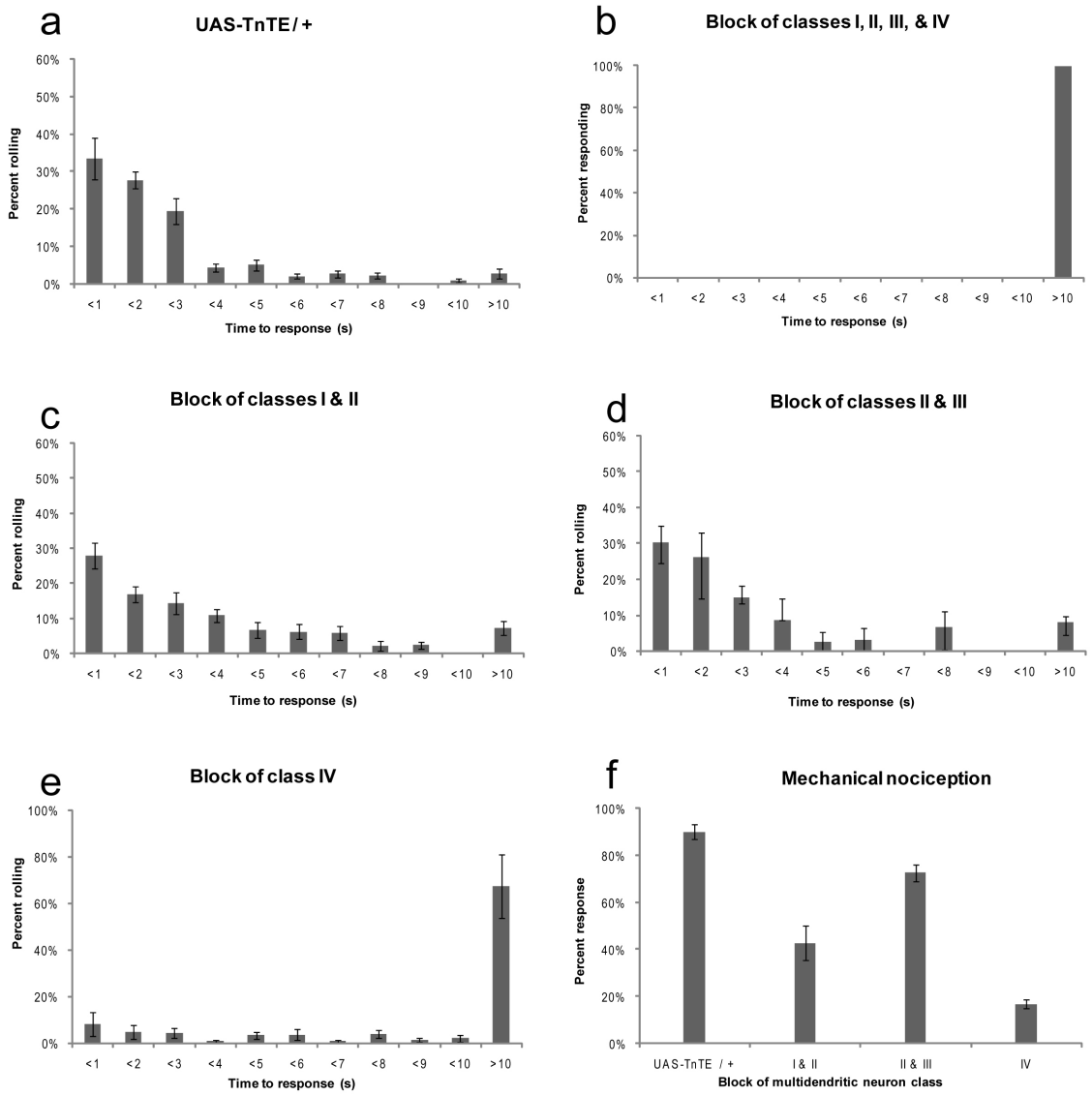


Figure 3-2: Class IV multidendritic neurons are necessary for nocifensive behavior

Figure 3-2: Class IV multidendritic neurons are necessary for nocifensive behavior
(A-F) The distribution of latencies of thermal nocifensive behavior in third instar larvae that were lightly touched with a 47°C probe. (A) Nocifensive response of the *UAS-TnTE*/+ larvae without a GAL4 driver (n=112). (B) Blocking the synaptic output of all four classes of multidendritic neurons completely blocked thermal nocifensive responses (n=27). (C) Blocking the output of class I and II md neurons using the *C161-GAL4* driver slightly increases the latency of thermal nociception behavior (n=195). (D) Blocking the output of class II and III md neurons using the *1003.3-GAL4* driver did not affect thermal nociception behavior (n=63). (E) Silencing of class IV multidendritic neurons dramatically impaired thermal nociception behavior (n=139). (F) Effects of blocking different subsets of md neurons upon the frequency of mechanical nociception behavior (*UAS-TnTE*/+ n=179, *c161-GAL4* n=79, *1003.3-GAL4* n=61, *ppk-GAL4* n=65). In all panels, error bars indicate standard error of mean.

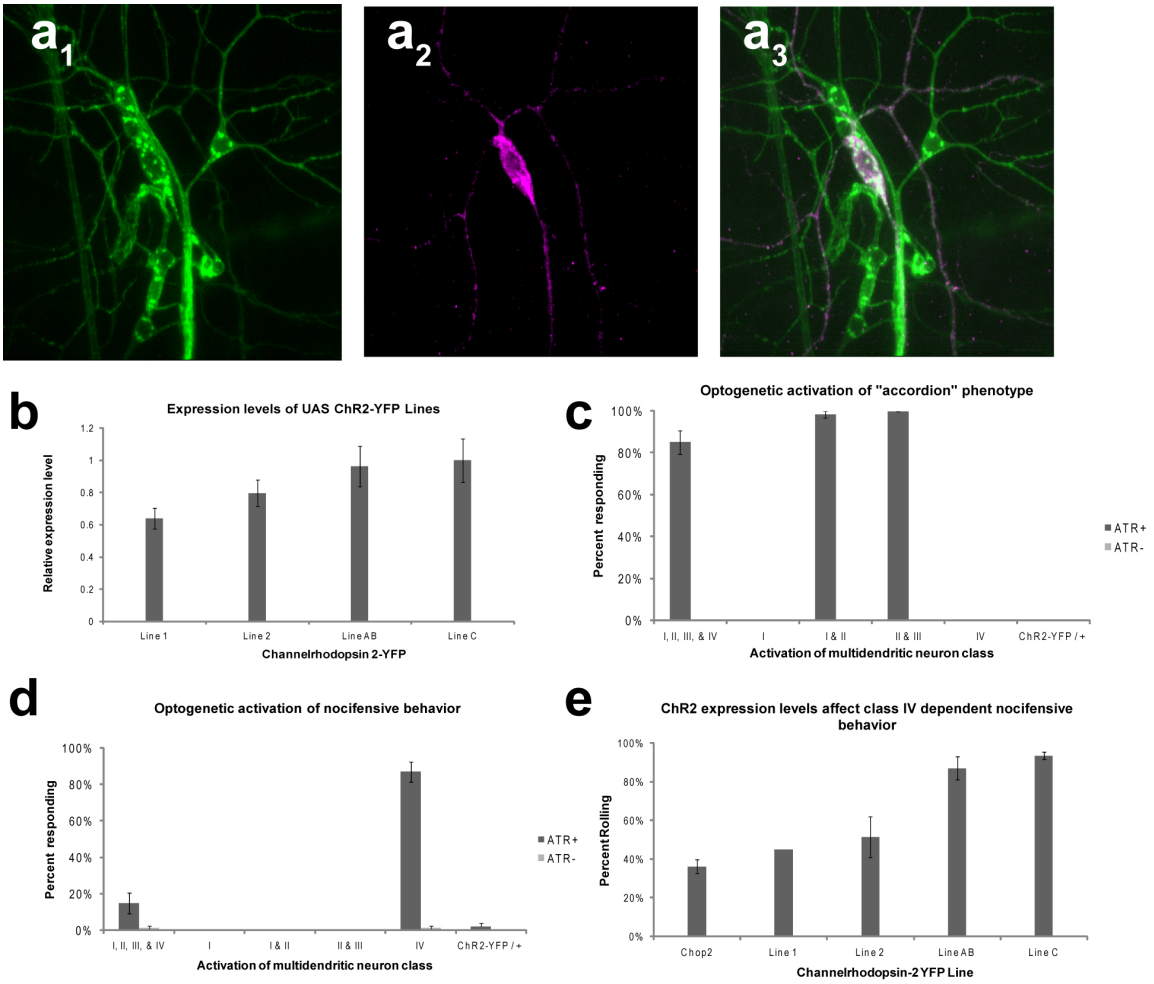


Figure 3-3: Optogenetic activation of class IV multidendritic neurons is sufficient to elicit the nocifensive response

Figure 3-3: Optogenetic activation of class IV multidendritic neurons is sufficient to elicit the nocifensive response (A_{1-3}) Expression of Channelrhodopsin2-YFP under control of ppk-GAL4. (A_1) pan-neuronal marker anti-HRP (green). (A_2) anti-GFP (magenta). (A_3) Merge of a_1 and a_2. (B) Expression levels of *UAS-ChR2-YFP* lines measured by pixel intensity of confocal images and normalized to staining intensity of line C. (C) Optogenetic activation of various subsets of multidendritic neurons triggers the "accordion" phenotype at high frequency. (D) Optogenetic activation of class IV multidendritic neurons elicited nocifensive rolling behavior at high frequency. Rolling behavior with activation of classes I-IV was also elicited (15%). (E) ChR2-YFP expression levels are correlated with efficiency of nocifensive behavior. Sample sizes for c and d (class I neuron driver *2-21-GAL4*: *atr+* n=54, *atr-* n=43), (class I-IV neuron driver md-GAL4: *atr+* n=103, *atr-* n=92), (classes I & II driver *c161-GAL4*: *atr+* n=117, *atr-* n=21), (class II & III driver *1003.3-GAL4*: *atr+* n=46, *atr-* n= 52), (class IV *ppk-GAL4 atr+* n=181, *atr-* n=112). Sample sizes for e (Chop2 n=173, Line 1 n=20, Line 2 n=84, Line AB n=181, Line C n=80). UAS-Chop2 is an untagged (no fused fluorescent protein) Channelrhodopsin-2 line from the Fiala laboratory(Schroll, Riemensperger et al. 2006) with an insertion on the third chromosome. Error is standard error of the mean.

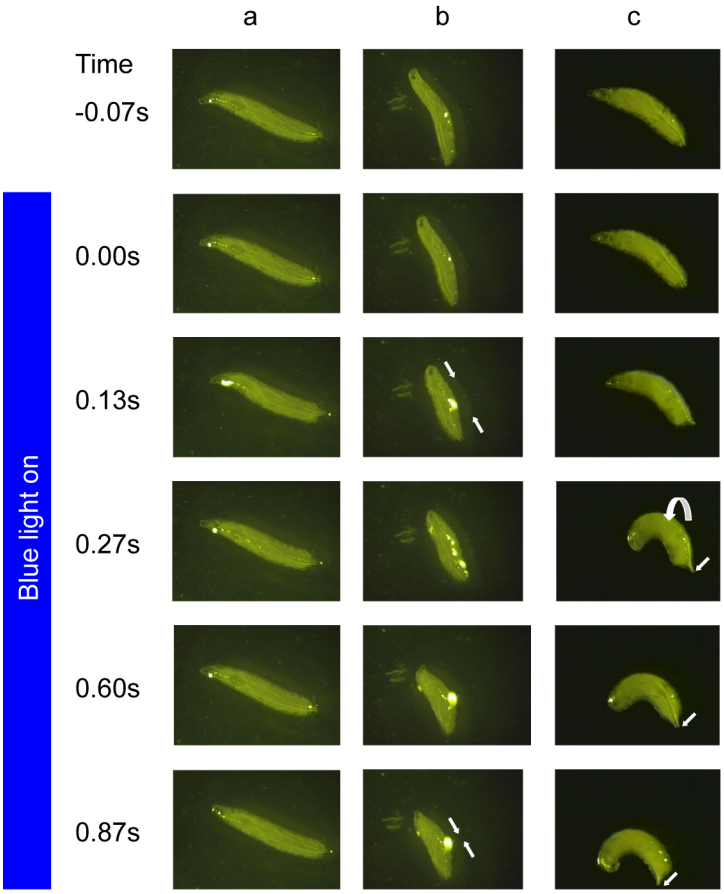


Figure 3-4: Optogenetic activation of class IV md neurons triggers nocifensive response

Figure 3-4: Optogenetic activation of class IV md neurons triggers nocifensive response

(A-C) Still images of third instar larvae and optogenetically activated behaviors extracted from videotaped responses. (A,C) *ppk-GAL4* UAS ChR2-YFP larvae that been fed atr- yeast (A) or atr+ yeast and illuminated with blue light. (B) *c161-GAL4 UAS ChR2-YFP* larva fed ATR+ and illuminated with blue light. (A) A larva expressing ChR2YFP in class IV md neurons showed little response to the blue light when fed yeast paste lacking *all-trans retinal*. (B) A larva expressing ChR2YFP in class I and II md neurons simultaneously contracts muscles of every segment to produce the accordion like behavior (straight white arrows denote reduction in length of larva from contractions). (C) A larva fed atr+ yeast and also expressing ChR2YFP in the class IV md neurons produced a rolling motor response (curved arrow) that was indistinguishable from nocifensive rolling behavior. Note the net lateral direction of movement (straight white arrows). The time point of the sequence is shown in the left with illumination occurring at time zero.

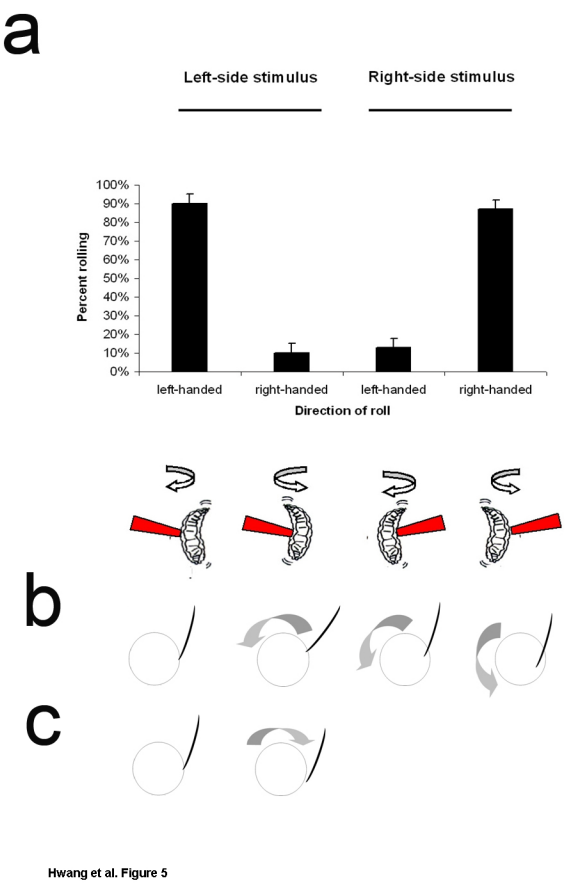


Figure 3-5: Paradoxical directionality of rolling behavior

(A) Directionality of larval rolling is biased. Top: Larvae had a strong tendency to roll towards the heat stimulus. When stimulated on the right (n=114), they predominantly rolled to the right. When stimulated on the left (n=102) they had a strong tendency to roll to the left. Roll direction was determined according to the first complete (ie. 360°) roll. Bottom: schematic representation of roll direction. (B) Hypothetical effects of rolling away from a wasp attack causes increased penetration. (C) Hypothetical effects of rolling towards the side of the body where attacked. In B and C the cross section of the larva is depicted as a circle and the ovipositor of the wasp attacking from above is depicted as a curved line.

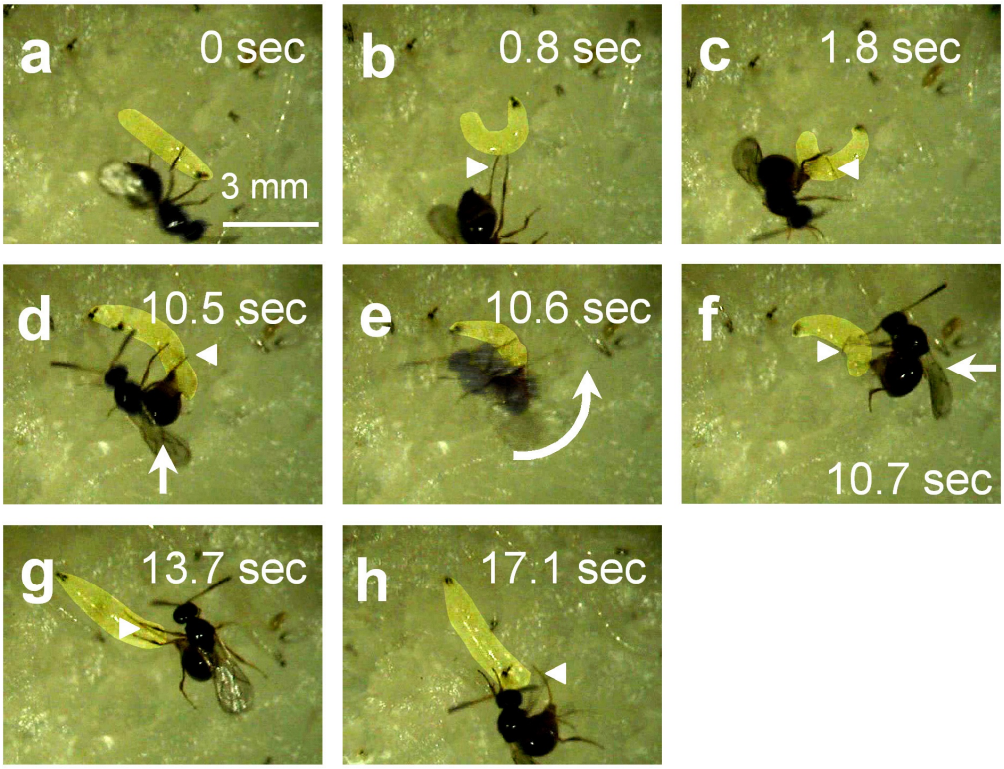


Figure 3-6: Rolling behavior allows *Drosophila* larvae to escape from parasitoid wasp attack

Leptopilina boulardi female and an early third instar *Drosophila melanogaster* larva. (a) Wasp ovipositor penetrates larval cuticle and epidermis; (b) Nocifensive rolling behavior is triggered. The long threadlike ovipositor of the wasp is clearly visible (arrowhead). (c) Nocifensive rolling results in the ovipositor being wrapped around the larva. (d-f) Rolling of larva flips parasitoid wasp onto her side; (note that the image of the wasp in panel e is blurred due to its rapid movement during the exposure). (g) Larva moves quickly away from parasitoid wasp; (h) larva is freed from ovipositor. Note: Images have been digitally adjusted to increase the contrast of the larva relative to the background (so that it could be easily seen).

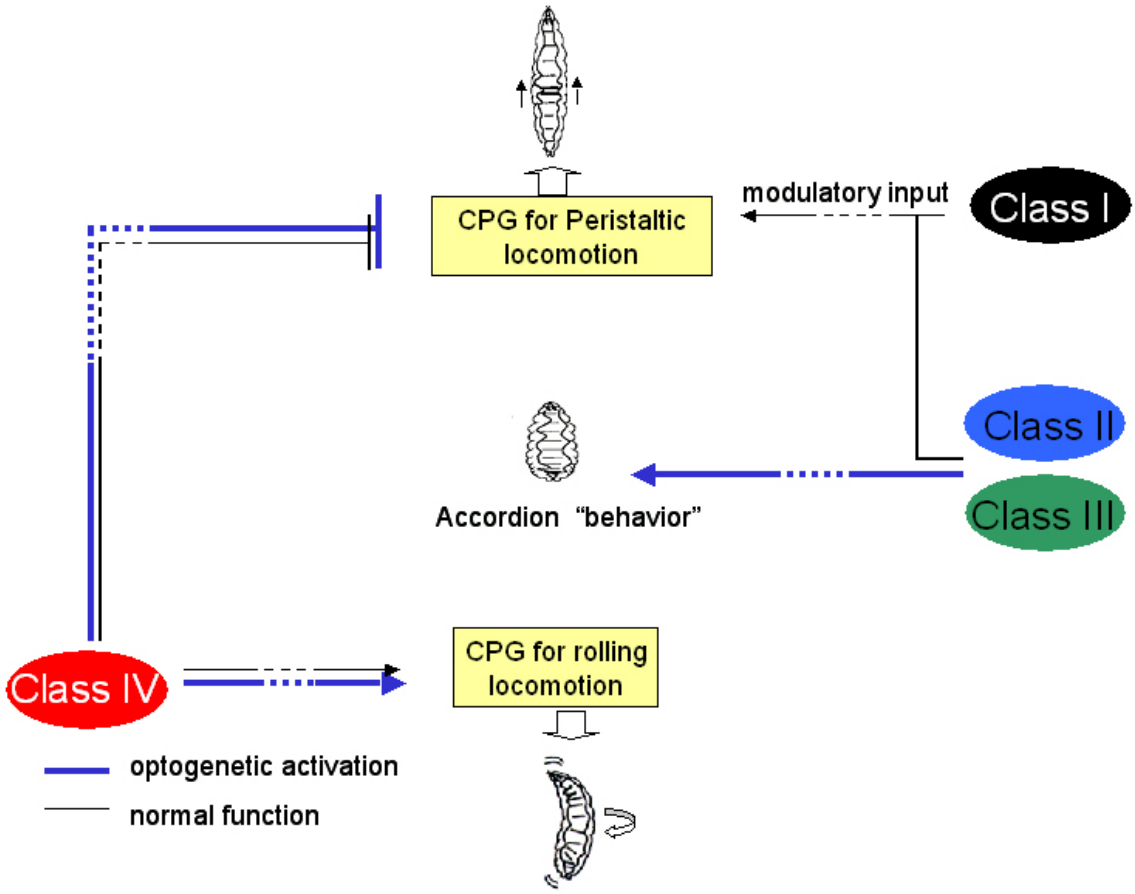


Figure 3-7: Model for sensory control of alternate patterns of larval locomotion

The class IV neurons are both necessary and sufficient to trigger rolling escape behavior. Other classes of md-neurons modulate peristaltic locomotion through a distinct Central Pattern Generator. When inappropriately activated via Channelrhodopsin the accordion phenotype is manifested.

Chapter 4 Pickpocket is a DEG/ENaC Protein Required for Mechanical Nociception in *Drosophila melanogaster* Larvae

4.1 Introduction

Mechanotransduction, the molecular detection of force, plays a critical role in the survival of living organisms. In sensory systems, mechanotransduction is necessary for the sense of touch, hearing, the detection of osmotic changes, and proprioception. Mechanosensory cells are diverse even within an organism. How do various eukaryotic cells detect these somewhat distinct mechanical forces? A common theme that has emerged from mechanosensation studies in disparate organisms is that ion channels play a central role in mechanosensation. In several models of neurosensory mechanotransduction, force gated ion channels transduce mechanical energy into an electrical current which depolarizes the sensory neuron. It is thought that the transduction channels detect the force without the need for second messengers making neurosensory mechanotransduction orders of magnitude faster than sensory systems that rely on signaling cascades (such as phototransduction). Despite the central role and importance of mechanotransduction channels few have been molecularly identified.

Some mechanotransduciton channels have been found in prokaryotes, where they are important for surviving in extreme osmotic conditions. The mechanosensitive channel of large conductance (MscL) opens in response tension in the membrane. This is true even in biochemical preparations of MscL reconstituted in artificial liposomes. This has led to a model where force is transmitted through the lipid membrane to the mechanosensitive channel which responds directly to tension without the need for additional protein co-factors (reviewed in (Kung 2005) (Anishkin and Kung 2005)).

In *C. elegans,* mechanotransduction channels have been identified that require multiple proteins for efficient detection of force. The required genes include *mec-4* and *mec-10* which encode founding members of the Degenerin / Epithelial Sodium Channel (Deg/ENaC) family. In addition to the pore forming MEC-4 and MEC-10 proteins, other components include a paraoxonase-like protein (MEC-6), stomatin-like proteins (MEC-2 and UNC-24), extracellular matrix (ECM) proteins (MEC-1 and MEC-5), and alpha and beta tubulin (MEC-12 and MEC-7) (reviewed in (O'Hagan and Chalfie 2006); (Garcia-Anoveros and Corey 1997); (Goodman and Schwarz 2003) (Ernstrom and Chalfie 2002).

Direct electrophysiological evidence strongly supports the idea that the *mec-4* / *mec-10* channels encode pore forming subunits of the mechanoreceptor transduction channel of body wall touch neurons (O'Hagan, Chalfie et al. 2005). Although null alleles of *mec-4* or *mec-10* completely eliminated transduction currents (receptor potentials), the

ion selectivity of the receptor potential was altered by missense mutations of *mec-4* and *mec-10* (O'Hagan, Chalfie et al. 2005). Because these mutations alter the quality of the transduction current rather than eliminating them, *mec-4* and *mec-10* must be pore - forming subunits of the transduction channel.

Despite the very strong evidence from *C. elegans*, DEG/ENaCs have yet to be clearly implicated as mechanotransducers in other species. A number of studies have been performed on the mammalian acid sensing ion channels (ASICs) which are related to DEG/ENaC but a clear role in mechanosensation has not been demonstrated (reviewed in (Wemmie, Price et al. 2006) (Lingueglia 2007). Studies with ASIC knockout mice have resulted in a mixed consensus on the roles for these channels in mechanosensation. For example, ASIC2 null mice were reported to have reduced sensitivity in low-threshold rapidly adapting mechanoreceptors (Price, Lewin et al. 2000) but another study reported no change in mechanosensation with an independently generated ASIC2 null (Roza, Puel et al. 2004). ASIC1a null mice do not have any phenotype for cutaneous mechanosensation (Page, Brierley et al. 2004) whereas ASIC3 null mice have been reported to have an increased sensitivity to light touch but reduced sensitivity to noxious pinch (Price, McIlwrath et al. 2001). In contrast to cutaneous mechanosensation, studies have found that ASICs (ASIC1, ASIC2, and ASIC3) are required for visceral mechanosensation. For example, the disruption of ASIC3 results in

decreased mechanosensitivity in almost all visceral afferents except gastroesophogeal mucosal receptors (Page, Brierley et al. 2004); (Jones, Xu et al. 2005);(Page, Brierley et al. 2004). Thus although ASICs have been implicated in a number of mechanosensory functions, it is unclear how they are involved or whether they participate in a mechanotransduction complex like that of *C. elegans*.

Little is known about DEG/ENaCs and mechanosensation in *Drosophila melanogaster*. The most studied *Drosophila* DEG/ENaC channel was named *pickpocket (ppk).* The name *pickpocket,* derives from the precise expression pattern of this gene in a highly branched class of sensory neurons that innervate the larval (and adult) epidermis. The many neurites of the sensory neurons that express *ppk* suggested that the cells expressing it might be exquisitely sensitive to touch, such as those that might protect an unsuspecting victim from a pickpocket (Adams, Anderson et al. 1998). However, when genetic mutants for *pickpocket* were eventually identified, no role for the channel in the larval detection of gentle touch was identified (Ainsley, Pettus et al. 2003).

Consistent with this, we have found that the *ppk* expressing sensory neurons, known as class IV multidendritic neurons, have no detectable role in larval responses to gentle touch. Instead, these neurons are required for behavioral responses to harsh (noxious) mechanical stimuli. The behavioral output of the *Drosophila* larvae to noxious mechanical stimuli is completely distinct from gentle touch responses. In response to

noxious mechanical stimulation, the *Drosophila* larvae use their muscles to rotate around the long body axis in a corkscrew like motion. This escape locomotion behavior is also triggered by noxious heat stimulation of the larval body wall and therefore represents a common behavioral output to nociceptive sensory stimuli. Using genetic silencing techniques, we have shown that synaptic output of the class IV md neurons is necessary for eliciting the larval nocifensive behavior to noxious mechanical stimuli as well as noxious heat stimuli. In addition, we have found that activation of the class IV neurons with the light activated ion channel (channelrhodopsin-2) protein is sufficient to trigger the nocifensive larval escape response (Hwang, Zhong et al. 2007). Thus, these cells function as polymodal nociceptors.

Painless, a TRPA channel expressed in multidendritic neurons, has been shown to be required for thermal nociception in *Drosophila* larvae and adults as well as for mechanical nociception in larvae. *painless* mutants when stimulated with a heat probe raised to the noxious temperature of 46°C have a delayed time until initiation of nocifensive rolling at 46°C compared with wild type larvae. Similarly, *painless* mutants require more than three times the mechanical force (100 mN) to elicit an equivalent response in wild type larvae (30 mN) (Tracey, Wilson et al. 2003).

While *painless* mutants have been shown to be defective in both thermal and mechanical nociception, the nociceptive behavior is not completely eliminated,

suggesting that other genes, channels, or pathways still persist. In addition, since the *painless* channel is required for both thermal and mechanical nociception, it may not function as a direct mechanosensor but might play a more general role in the excitability of the nociceptive neurons. Expression of Painless protein in heterologous cells shows that it is temperature sensitive but responses to osmotic challenge were not observed. In addition, *painless* expression in gustatory neurons is required for detection of isothiocyanate compounds but the channel is not directly activated by isothiocyanates in heterologous cells.

Thus, with the identification of class IV MD neurons as larval nociceptors, we asked whether *ppk* is involved in nociception (Adams, Anderson et al. 1998), (Ainsley, Pettus et al. 2003). Our results indicate that *ppk* is required for mechanical nociception although it is not required for thermal nociception. Larvae mutant for *ppk* show a dramatically reduced behavioral response to a nociceptive mechanical stimulus. In addition, RNAi knockdown of *ppk* in class IV MD neurons phenocopies the effects of the mutant and produces a similar defect in larval nocifensive responses to a mechanical stimuli. These results genetically separate mechanical harsh nociception from thermal nociception in *Drosophila*. Furthermore, our results suggest an evolutionarily conserved role for DEG/ENaC channels in mechanotransduction.

4.2 Materials and methods

4.2.1 Fly strains and husbandry

The following fly strains were used: *pickpocket1.9-GAL4, UAS-ppkRNAi (III) (Xu, Bogert et al. 2004), Df(2L)A400, Df(2L)b88h49, UAS-dicer2 (III)* (from VDRC), *UAS-Channelrhodopsin2-EYFP* (lineC, II), *elB*^{8}. The *ppk elB* mutant larvae were transheterozygous for the two deficiencies: Df(2L)A400/*Df(2L)b88h49*. *elB* mutants consisted of *elB*^{8}/*Df(2L)A400* or *elB*^{8}/*Df(2L)b88h49*. Channelrhodopsin experiments used virgins of the genotype *pickpocket1.9-GAL4*, *UAS-ChR2eYFP* Line C/Cyo; *dicer2* crossed to males of the corresponding *UAS-RNAi* line. Isogenic white males were used for channelrhodopsin experimental controls and in both groups, only fluorescent larvae were tested. If not specifically mentioned, fly lines were obtained from Bloomington Stock Center. Flies were raised on standard cornmeal medium. All stocks were maintained at 25 °C and 75% humidity and on a 12 hr light 12 hr dark circle.

4.2.2 Mechanical nociception and gentle touch behavioral assays

The mechanical nociception assays were performed as described previously (Hwang et al., 2007), with slight modifications. Wandering third-instar larvae were stimulated with a 50 mN calibrated Von Frey filament. Omniflex monofilament fishing line Shakespeare (6 lb test, diameter 0.009 inch [0.23 mm]) was cut to a length of 18 mm

and attached to a glass pipette such that 8 mm of the fiber protruded from the end of the pipette and 10 mm anchored the fiber.

4.2.3 Molecular characterization of genetic aberrations of *ppk* deficiency

Genomic DNA was extracted from adult flies using QIAGEN DNeasy Tissue Kit. The following primers were used to amplify ppk and its neighboring genes. *SpeI:* forward 5' - TCA AGC AAC TGG ACC TAA ACC G - 3', reverse 5' - GTT ATC TCG TGA AAA TGA GTG GCG - 3'. *ppk:* forward 5' - AGC ACG ACC ATT CAC GGC ATA C - 3' reverse 5' - CCA AAG TTC ACT CAC TGG GCA TC - 3'; *elB* C-terminus: forward 5' - CGC AAT ACG ACG CTT TTC CG - 3', reverse 5' - TTG ATA GTT CCC CCT GTG AGG TC - 3'; *elB* : Forward primer 5' - AGC AGT CAT TCA TCC AGC GTT AG - 3', reverse primer 5' - TTC ATT CGG GCG TAG TTC AGA TAC - 3'; *Pburs* : forward primer 5' - CAA CCA TCG GTG ATA ACG CC – 3', reverse primer 5' - TCG CCA CAT TTG AAG CAC TTG - 3'.

4.2.4 Optogenetic activation of class IV neurons

ChR2 induced nociception behavior was similar to that seen in (Hwang, Zhong et al. 2007) with the modification that a blue light of 17,000 lux was used. In addition, ten females and five males were used in each cage. Crosses were between virgins of the

genotype (*pickpocket1.9-GAL4; UAS-dicer-2*) crossed to the appropriate RNAi line (or isogenic white control).

4.3 Results

4.3.1 Two overlapping deficiencies remove *pickpocket* and a neighboring gene *elbowB*

We have previously found that ppk expressing sensory neurons (Figure 4-1A and B) (class IV multidendritic neurons) are required for behavioral responses to harsh (noxious) mechanical stimuli. The *pickpocket* locus is located on the left arm of chromosome 2 at the cytological position 35B1. *ppk* is flanked by *elbow B*, a transcription factor involved in tracheal development (Dorfman, Glazer et al. 2002), and *spel1*, a gene involved in DNA mismatch repair (Flores and Engels 1999). Two overlapping deficiencies Df(2L)b88h49 and Df(2L)A400 have been shown (Ainsley, Pettus et al. 2003) to overlap and to completely remove *ppk.* Thus larvae and flies transheterozygous for these deficiencies are DNA nulls for the *ppk* gene. It was previously reported that the 44kb overlapping deficiency specifically removed *ppk;* leaving *spel1* and *elB* intact (Ainsley, Pettus et al. 2003). However, the interval between *spel1* and *elbow B* which contains *ppk* is approximately 20kb suggesting that at least one other gene (*spel1* or *elbow B*) must be removed if these deficiencies truly overlap by 44kb. Therefore we reexamined these deficiencies first to confirm the absence of *ppk*, but also to test whether other genes are removed in the transheterozygous combination. Polymerase chain

reaction amplification of genomic DNA from transheterozygotes *Df(2L)b88h49/Df(2L)A400* indicated that the overlap of these two deficiencies actually removes two genes, *ppk1* and *elB* (Figure 4-2). For the purpose of this discussion, we will refer to transheterozygous larvae of this genotype as *ppk elB*$^{A400/B88}$.

4.3.2 Gentle touch responses are intact in *pickpocket* mutants

Although the class IV MD neurons that are known to express *ppk* are nociceptive, it seemed possible that *ppk* could be expressed at low levels in non-nociceptive neurons. Therefore we first tested whether *ppk* was required for responses to gentle touch in *Drosophila* larvae (as DEG/ENaCs are in *C. elegans*). Using the gentle touch assay previously developed by Kernan et al (Kernan, Cowan et al. 1994), we tested *ppk elB*$^{A400/B99}$ mutant larvae for their behavioral response. The *ppk elB*$^{A400/B99}$ mutant larvae were indistinguishable from wild type larvae (Figure 4-3). Our results confirm a previous report that found no requirement for *ppk* in gentle touch responses (Ainsley, Pettus et al. 2003).

4.3.3 Thermal nociception responses are normal in *pickpocket* mutants

Our prior studies indicate that class IV MD neurons are required for nocifensive avoidance of noxious heat and harsh mechanical stimuli. Therefore to examine the function of *ppk* in nociception, we first tested whether *ppk* was required for avoidance of noxious heat. *ppk elB*$^{A400/B88}$ mutant larvae were tested for thermal nociception by

stimulating them with a probe heated to 46 °C and their latency to initiation of larval nocifensive escape behavior was recorded. Wild type larvae initiate escape locomotion within the first 3 seconds of stimulation with the noxious heat probe. Similarly, *Df(2L)A400*/+ heterozygotes showed a response that was indistinguishable from wild type. The normal thermal nociception response of *Df(2L)A400*/+ demonstrates that removal of one copy of *ppk* does not cause a thermal nociception phenotype (Figure 4-4A). However, *Df(2L)b88h49*/+ heterozygotes did show an impaired response to noxious heat (Figure 4-4B). Thus, the *Df(2L)b88h49* strain shows a dominant impairment in thermal nociception. Complete removal of *ppk* in the *ppk elB*$^{A400/B88}$ genotype, if anything, showed improved thermal nociception relative to *Df(2L)b88h49*/+ (Figure 4-4C). Since removal of an additional copy of *ppk* does not enhance the *Df(2L)b88h49* phenotype and since Df(2L)A400/+ shows wild type thermal nociception behavior, our results do not support a role for *ppk* in thermal nociception.

4.3.4 Mechanical nociception phenotypes

Given the lack of a clear thermal nociception phenotype in *ppk elB*$^{A400/B88}$ mutants, we next tested the *ppk* mutants for defects in mechanical nociception. To examine mechanical nociception behavior, *ppk elB*$^{A400/B88}$ mutant larvae were examined for the nociceptive response to harsh mechanical stimuli delivered by 50 mN Von Frey fibers. In this assay, wandering third instar larvae were prodded with the Von Frey fiber on the

dorsal midline between segments A4-A6. Following such a stimulus, wild type larvae were seen to respond with nocifensive escape locomotion after prodding. During escape locomotion, the larva rotates around its long body axis causing it to roll in a corkscrew fashion (Tracey, Wilson et al. 2003). To quantify this behavioral response, larvae were prodded a single time with the Von Frey fiber and the larva's behavioral response was recorded. The percentage of larvae that responded to this stimulus with nocifensive rolling was calculated and averaged over trials.

Given the dominant thermal nociception phenotype observed for the *Df(2L)b88h49*/+ deficiency, we first examined larvae that were heterozygous for either *Df(2L)b88h49* or *Df(2L)A400*. Both *Df(2L)b88h49*/+ and *Df(2L)A400*/+ showed robust responses (Figure 4-5A). In contrast, the transheterozygous *ppk elB*$^{A400/B88}$ mutant larvae showed a significant reduction in nocifensive responses to the noxious mechanical stimuli (Figure 4-5A). The *ppk elB*$^{A400/B88}$ mutant larvae responded with nocifensive escape locomotion in only 27% of the trials while the heterozygous controls showed escape locomotion in 77% of trials. Instead of escape locomotion, the mutant larvae often paused locomotion following the noxious mechanical stimulus, a behavior that resembles the wild type response to gentle touch.

4.3.5 *elbowB* mutants complement mechanical nociception phenotypes

Although *ppk elB*$^{A400/B88}$ mutant larvae were defective for mechanical nociceptive behavior, it was possible that the mutant defect was due to the absence of *elB* rather than absence of *ppk*. *elB* is a zinc-finger containing protein that is involved in several developmental processes. Expression of *elB* has been detected in a subset of tracheal trunks and *elB* mutants develop aberrant migration of dorsal and lateral tracheal branches in embryos (Dorfman, Glazer et al. 2002). In addition, *elB* is expressed in the leg, wing, eye, and head primordia where it is involved in appendage formation via the repression of body wall genes (Weihe, Dorfman et al. 2004). Finally *elB* plays a role in the regulation of the size of the eye-head primordium (Luque and Milan 2007). Although *elB*'s expression has not been found in larval nociceptors, a non-cell autonomous effect on nociception seemed possible. In order to test this possibility, we performed complementation tests with the *elB*^{8} allele, a γ ray induced mutation of *elB*, and the deficiencies that remove *ppk* (*Df(2L)b88h49* and *Df(2L)A400*). The defective mechanical nociception phenotypes of the two deficiencies were fully complemented by *elB*^{8} (Figure 4-5B) indicating that the nociception defects of *ppk elB*$^{A400/B88}$ mutant larvae are unlikely to be a consequence of *elB* deficiency.

4.3.6 RNAi knockdown of *pickpocket* in class IV multidendritic neurons re-produces genetic mutants phenotypes

In order to further determine whether the mechanical nociception defect observed in *ppk elB*$^{A400/B88}$ larvae was due to removal of *ppk,* we used the GAL4-UAS system to drive expression of *ppk*-RNAi in class IV multidendritic neurons. Indeed, there was a statistically significant reduction in the larval responses to harsh mechanical stimulation when the RNAi knockdown was performed using a single copy of the *ppk-GAL4* driver and a single copy of the *UAS-ppk-RNAi* (*w; ppk-GAL*4/+; *UAS-ppk-RNAi*/+). An even more severe phenotype was observed with increased expression levels of the RNAi transgene (using a strain that was homozygous for the *ppk-GAL4* driver and for *UAS ppk-RNAi*). Indeed, the defect in mechanical nociception in this strain was nearly as severe as was observed in the *ppk elB*$^{A400/B88}$ null background (34%, Figure 4-5C). Since *ppk* RNAi in the class IV md neurons phenocopies the mechanical nociception defect seen in *ppk elB*$^{A400/B88}$, it is likely that the defect of the *ppk elB*$^{A400/B88}$ mutant is due to the absence of *ppk*.

The clearly observable mechanical nociception defects observed with *ppk RNAi* expressed in nociceptive neurons gave us an additional opportunity to test whether or not *ppk* plays a role in thermal nociception. If for example the *ppk* channels have a housekeeping function in the class IV md neurons, we would also expect to observe a phenotype in thermal nociception phenotype caused be RNAi knockdown of the gene.

On the other hand, if *ppk* functions specifically in mechanotransduction, then *ppk* RNAi would be unlikely to affect behavioral responses to noxious heat.

To distinguish among these possibilities we examined the thermal nociception behaviors in larvae bearing a single copy of the *ppk-GAL4* and a single copy of *UAS-ppk-RNAi*. These larvae showed wild type thermal nociception behavior (Figure 4-4F). In addition, normal responses to noxious heat were seen in larvae that had the higher dose of RNAi (Figure 4-4G). Thus, thermal nociception pathways are insensitive to *ppk*-RNAi which contrasts with the results seen for mechanical nociception.

4.3.7 Optogenetic activation of class IV neurons expressing *pickpocket* RNAi

The specific requirement for *ppk* in mechanical nociception behavior suggests a possible role for *ppk* at the mechanotransduction step in these polymodal nociceptive sensory neurons. Nevertheless, we wished to further test the possibility that PPK might affect the general excitability of these neurons. We have previously shown that expression of UAS-channelrhodopsin-2 (ChR2-YFP) under control of *ppk-GAL4* can be used to activate class IV md neurons with blue light which in turn triggers nocifensive escape locomotion (Hwang, Zhong et al. 2007). Blue light activation of the larval nociceptors via ChR2YFP should therefore bypass mechanotransduction, but it should still depend on factors that control the general excitability of the neuron. Thus, if *ppk* has

a non-specific function in class IV multidendritic neurons then the blue light triggered escape locomotion should be impaired by *ppk* knockdown. As a positive control, to confirm that blue light triggered escape locomotion indeed depends on a known sodium channel, we tested whether class IV specific RNAi knockdown of *para* had an effect on blue light triggered behavior. The *para* gene encodes an NaV type sodium channel that is essential for action potentials propagation in *Drosophila* (Wu and Ganetzky 1980), (Siddiqi and Benzer 1976). Indeed, *para* knockdown with *UAS-para-RNAi*, resulted in a nearly complete block to optogenetically triggered escape locomotion. In control larvae that expressed Chr2-YFP in nociceptors, escape locomotion was seen in response to 81% of blue light pulses. This was reduced to only 4% when *para-RNAi* was targeted to the same cells that expressed Chr2-YFP. In contrast, knockdown of *ppk* did not significantly affect the ChR2-YFP triggered nocifensive rolling responses (Control: 81%, SEM 3% vs *ppk* knockdown: 74% SEM 7%, p=0.19, Figure 4-6). Combined with the results described above, these data suggest a specific mechanosensory role for *pickpocket* in larval nociceptors.

4.4 Discussion

In this study, we have provided evidence that the *ppk* gene, which is specifically expressed in polymodal nociceptors of the *Drosophila* larva (class IV md neurons), is

required for mechanical nociception but not for thermal nociception in *Drosophila* larvae. Genetic null mutants of *ppk* showed severely impaired mechanical nociception behavior and RNAi knock-down of *ppk* gene expression in Class IV md neurons phenocopied this defect. Unfortunately, a dominant thermal nociception defect in the *Df(2L)b88h49*/+ larvae does not allow us to completely rule out a role for *ppk* in thermal nociception. Nevertheless, several lines of evidence argue against a role for *ppk* in thermal nociception. First, *Df(2L)A400*/+ shows wild type thermal nociception behavior which suggests that the dominant phenotype of *Df(2L)b88h49*/+ is not linked to removal of *ppk.* Second, the dominant phenotype of *Df(2L)b88h49*/+ is not enhanced by further removal of *ppk* in *Df(2L)b88h49*/ *Df(2L)A400* animals. Finally, RNAi knockdown of *ppk* did not result in a defect in thermal nociception (despite the fact that mechanical nociception was phenocopied).

The situation for *ppk* is distinct from that of the *painless* gene which is required for both thermal and mechanical nociception responses (Tracey, Wilson et al. 2003). In heterologous cells, Painless currents can be activated by heat but not by osmotic pressure. This supports a direct role for Painless in transduction of thermal nociception stimuli but a direct role as a mechanosensor remains unproven. Interestingly, heat activated Painless currents are strongly affected by intracellular calcium ions. This sensitivity to calcium suggests that Painless in mechanical nociception could feasibly act

downstream of Pickpocket. For example, if Pickpocket functions as a direct mechanosensor, calcium influx through voltage gated channels might indirectly activate Painless. In this model, the function of Painless would be as an amplifier of mechanically gated Pickpocket currents. Although expression of Pickpocket in heterologous cells has also not been found to produce mechanically activated currents, the absence of mechanical currents by heterologously Pickpocket channels may indicate that factors present *in vivo* are needed for the mechanical sensitivity of these channels. A model where Pickpocket functions as a component of mechanotransduction complex seems likely based on the well established functional role of DEG/ENaCs in the *C. elegans* mechanotransduction complex. The sub-cellular localization of the Pickpocket protein is found in discrete varicosities on the dendrites (Adams, Anderson et al. 1998) which is reminiscent of the punctate localization of the mechanotransduction complex of the touch receptor neuron processes in *C. elegans* (Chelur, Ernstrom et al. 2002).

Interestingly, although the mechanical nociception stimulus used in our assays seems qualitatively distinct from the gentle touch used in *C. elegans* studies, our results suggest that the molecular machinery involved may be similar. This is somewhat surprising since the sensory neurons that utilize Deg/ENaC channels for gentle touch detection in *C. elegans* (Chalfie, Sulston et al. 1985) (Goodman 2006); (Hall and Altun 2008) are not involved in harsh touch responses. Harsh touch along the body wall is

mediated by sensory neuron PVD and harsh touch on the tail is mediated by neuron PVC (Hall and Altun 2008) (Chalfie and Sulston 1981). Similarly, gentle touch to the body wall of the *Drosophila* larva is thought to be detected by ciliated chordotonal neurons which utilize the TRP channels NOMP-C, Iav, and Nan in mechanotransduction.

Our identification of Pickpocket as a potential mechanotransducer in the nociceptive neurons suggests an evolutionarily conserved role for Deg/ENaCs in neurosensory mechanotransduction. Furthermore, the *pickpocket* mutant phenotype genetically separates mechanical nociception from thermal nociception in *Drosophila.*

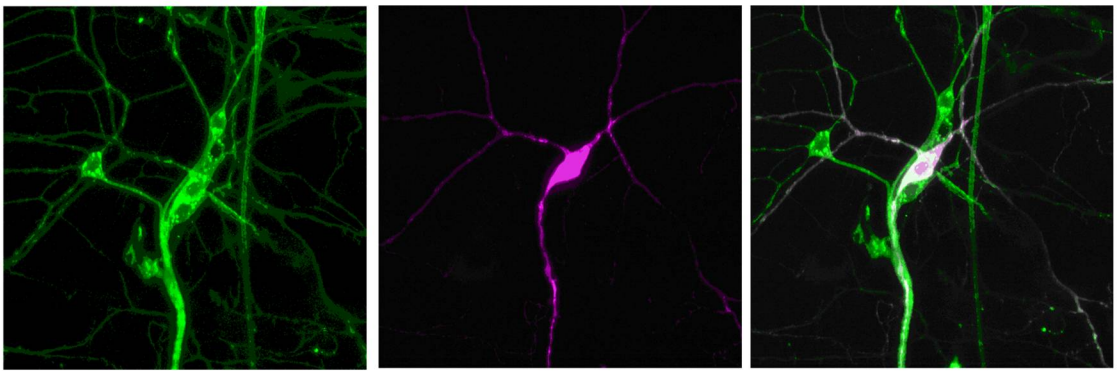


Figure 4-1: Expression of *pickpocket* in class IV multidendritic neurons

Expression of *pickpocket* in class IV multidendritic neurons by using *ppk-GAL4* driving *UAS-mCD8::GFP* expression. Confocal images of dorsal cluster peripheral neurons were taken in third instar larva. (A) Immunostaining with a pan-neuronal marker (anti-HRP-FITC). (B) Immunostaining with anti-GFP (magenta) in the ddaC neuron. (C) Merge of A and B.

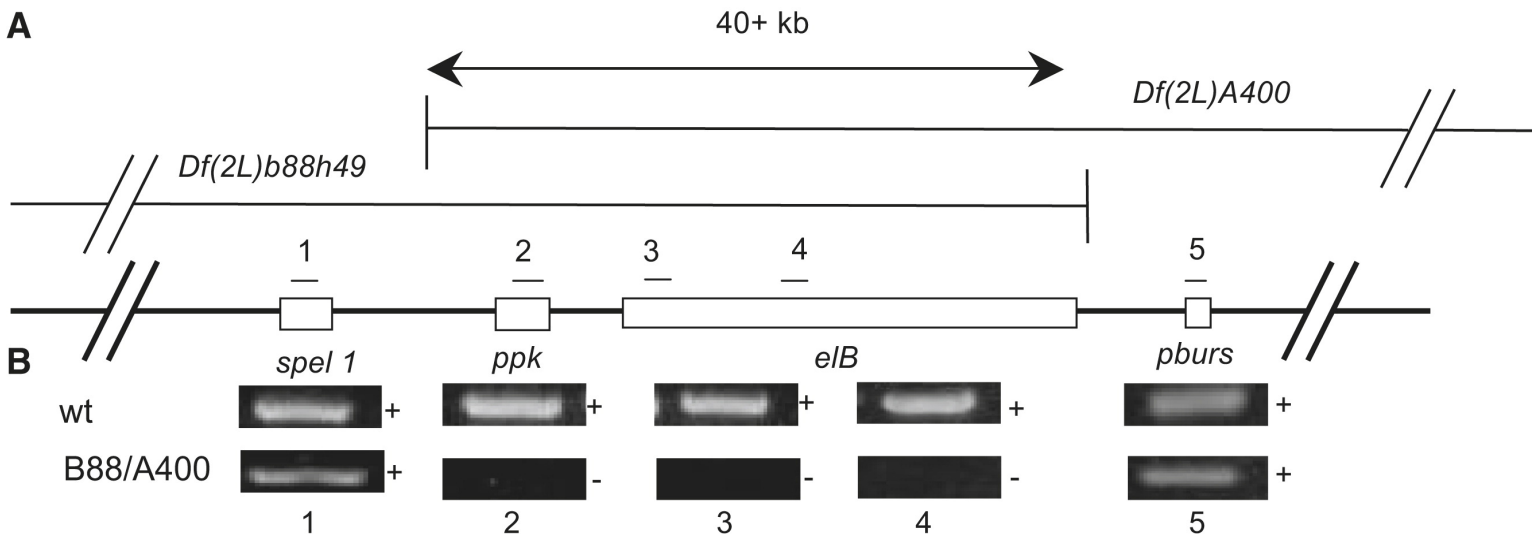


Figure 4-2: Genomic region containing the *pickpocket* gene.

(A) Schematic representation of Df(2L)B88h49 and Df(2L)A400. Double arrow indicates region overlap as determined by results Polymerase Chain Reaction (see panel B). Df(2L)B88h49/ Df(2L)A400 are deficient for both pickpocket and elB. (B) Results of PCR on genomic DNA from wildtype (wt) or Df(2L)B88h49/Df(2L)A400 (B88/A400). On the distal side of pickpocket, spel1 is not deleted. On the proximal side, pburs is present. Within the overlap of the deficiencies, pickpocket and elB are both deleted.

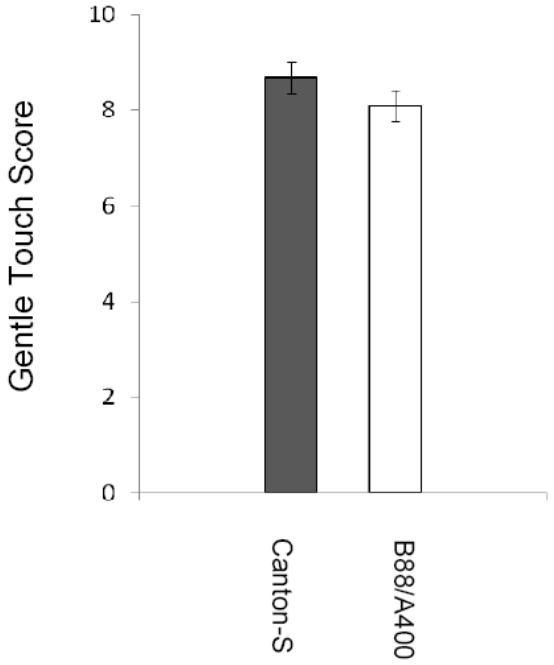


Figure 4-3: Behavioral responses to gentle touch in *pickpocket* mutants.

Kernan gentle touch assay (Kernan, Cowan et al. 1994). *Df(2L)A400/Df(2L)b88h49* (pickpocket null mutant, n=32) larvae do not appear different from control larvae (Canton-S, n=39).

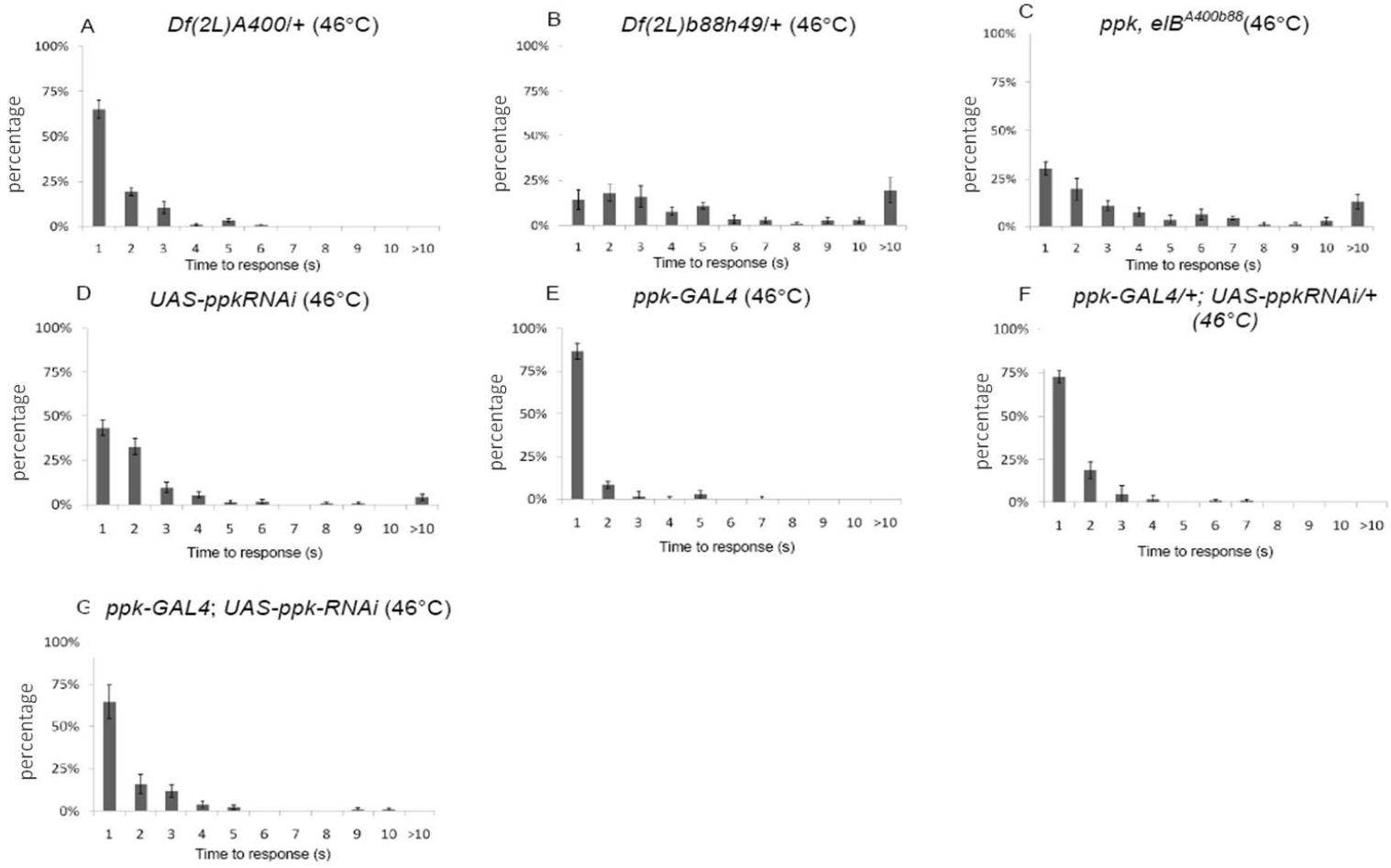


Figure 4-4: ***pickpocket*** **is not required for thermal nociception**

Figure 4-4: *pickpocket* is not required for thermal nociception

Distribution latencies of thermal nocifensive rolling behavior in third-instar larvae that were lightly touched with a 46°C probe. All Y axes are percentage of rolling. All X axes are time in seconds until rolling. All error bars are standard error of the mean. (A) *Df(2L)A400*/+ (n=173) (B) *Df(2L)B88h49*/+, (n=80) (C) *Df(2L)A400/Df(2L)B88h49, pickpocket* null (n=100). (D) *UAS ppk RNAi*/+ (n=207) (E) *ppk-GAL4*/+ (n=153) (F) *ppk-GAL4 UASppk RNAi*/+ (n=73). (G) *ppk-GAL4 UAS-ppkRNAi* (n=96)

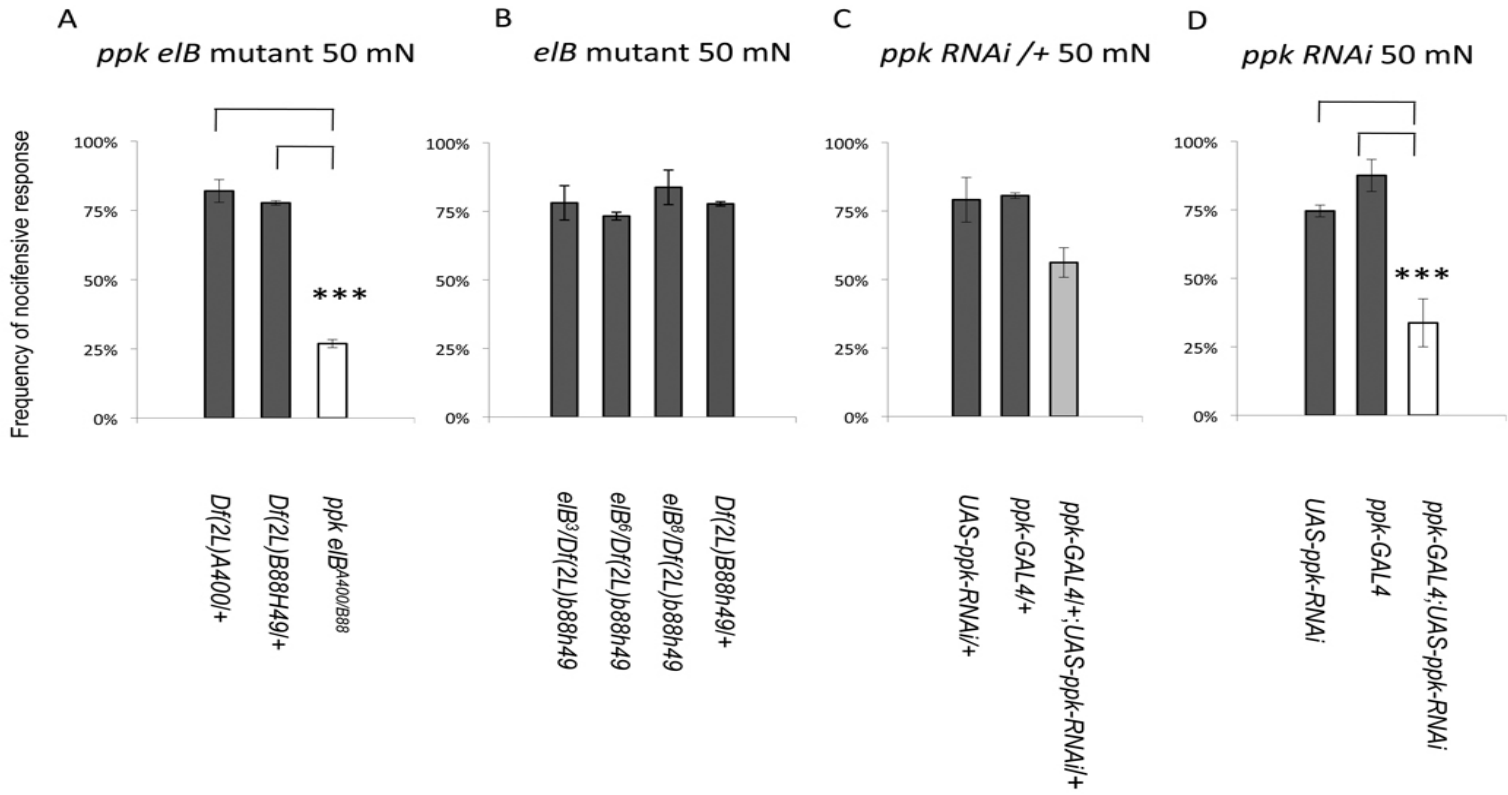


Figure 4-5: ***pickpocket*** **is required for mechanical nociception**

Figure 4-5 *pickpocket* is required for mechanical nociception (A) Response of deficiency lines (*Df(2L)A400*/+ (5 trials, n=181), *Df(2L)b88h49*/+ (5 trials, n=186), and *Df(2L)A400*/*Df(2L)B88h49* (*ppk* null mutant, 3 trials, n=156)). *Df(2L)A400*/*Df(2L)B88h49* animals showed a severe reduction in nocifensive responses ($p<0.001$ (for both one-way ANOVA and Sheffe's post-hoc test). (B) Nociception responses in *elB* mutants and control (*elB3*/*Df(2L)B88h49* (3 trials, n=86) , *elB6*/*Df(2L)B88h49*(3 trials, n=122), *elB8*/*Df(2L)B88h49* (3 trials, n=98) and *Df(2L)B88h49*/+ (5 trials, n=181)) ($p=0.39$ one-way ANOVA). (C) Nociception responses with heterozygous *ppk-RNAi* strains (*UAS-ppk-RNAi*/+ (3 trials, n=92), *ppk-GAL4*/+ (3 trials, n=132) and *ppk-GAL4*/+; *UAS-ppk-RNAi*/+ (3 trials, n=223)). Although a statistically significant difference was found among the groups ($p<0.05$ one-way ANOVA) Sheffe's post-hoc analysis did not detect a significant difference among the means in pair-wise comparisons. (D) Nociception behavior with homozygous *ppk-RNAi* strains (*UAS-ppk-RNA*i (7 trials, n=415), *ppk-GAL4* (5 trials, n=219), *ppk-GAL4*; *UAS-ppk-RNAi* (3 trials, n=96)). ($p<0.001$ (for both one-way ANOVA and Sheffe's post-hoc test.

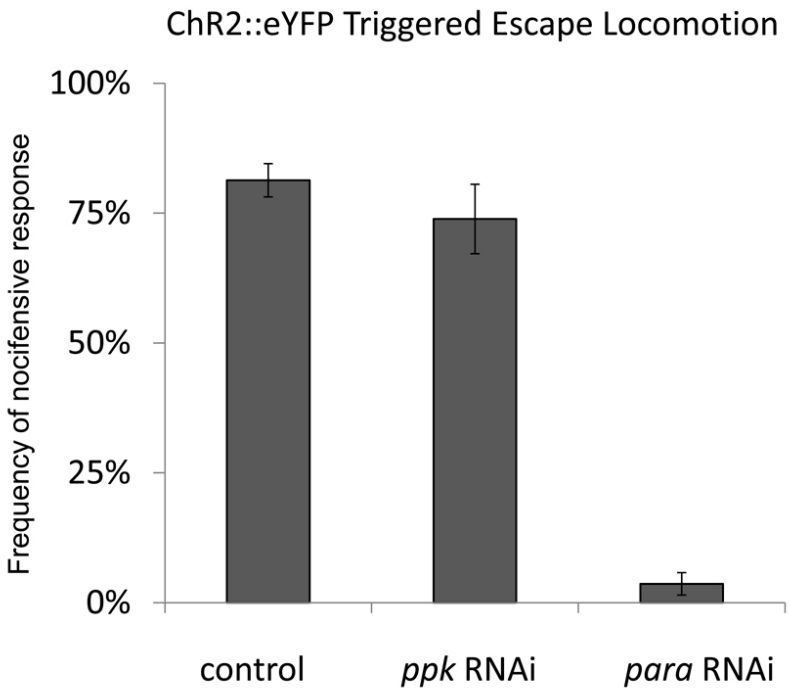


Figure 4-6: Knockdown of *ppk* did not affect ChR2 triggered nocifensive behavior

Blue light triggered activation of nociception behavior was seen in 81%(SEM 3%) of animals of the Control optogenetic activation genotype (*ppk-GAL4 UAS-ChR2::eYFP* Line C / +, *UAS-dicer-2*/+ (8 trials, n=245)). Knockdown of *ppk* did not significantly reduce the frequency of escape locomotion in response to blue light (note that *UAS-dicer-2* [35]was utilized in this experiment to enhance the effectiveness of RNAi) (*ppk-GAL4, UAS-ChR2::eYFP* Line C / *UAS-ppk-RNAi* ; *UAS-dicer-2* / +(3 trials, n=103) ((74% SEM 7%) t-test relative to control, p=0.32). In contrast, knockdown of *para* dramatically reduced the response to blue light (*ppk-GAL4 UAS-ChR2::eYFP* Line C / *UAS-para-RNAi*; *UAS-dicer-2* / +, 4 trials, n=115).

Chapter 5 *dTrpA1* is required for both thermal and mechanical nociception in *Drosophila melanogaster* larvae

5.1 Introduction

The ability to sense potential or actual tissue damaging stimuli, such as noxious temperature, mechanical stimuli and irritant chemicals, is critical for the survival of animals. Members of transient receptor potential (TRP) channels have been shown to play an important role for living organisms to sense environmental changes (Montell 2005). Several members of this family have been shown to be involved in nociception, i.e. the neural processes of encoding and processing noxious stimuli.

In mammals, TRPA1 has been implicated to be a key player in nociception. Recently, the identification of a new heritable Familial Episodic Pain Syndrome (FEPS) in a Colombian family represents the first human pain-related syndrome to be linked to the TRP gene superfamily (Kremeyer, Lopera et al. 2010; Waxman 2010). The mutation causes single amino acid aberration (N855S) in the S4 transmembrane segment of TRPA1. The mutant channel showed a normal pharmacological profile but altered biophysical properties, with a 5-fold increase in inward current on activation at normal resting potentials (Kremeyer, Lopera et al. 2010). In mice, TRPA1 is detected in a subset of TRPV1 expressing DRG C fibers and Aδ fibers, which are the nociceptive afferents in

mammals (Story, Peier et al. 2003). TRPA1 has been found to be required for chemical , mechanical and noxious cold nociception, although the latter remains controversial(Bautista, Jordt et al. 2006; Kwan, Allchorne et al. 2006; Karashima, Talavera et al. 2009; Knowlton, Bifolck-Fisher et al. 2010). TRPA1 is activated by many irritant chemicals, such as cinnamaldehyde, allyl isothiocyanates (mustard oil), allicin (garlic) and acrolein (tear gas) (Bautista, Movahed et al. 2005; Bautista, Jordt et al. 2006). Many of these chemicals are reactive electrophiles that are thought to activate TRPA1 through covalent modification of cysteines (Bautista, Jordt et al. 2006; Macpherson, Dubin et al. 2007). Indeed, TRPA1 knockout mice have defects in avoiding these noxious compounds (Bautista, Jordt et al. 2006; Kwan, Allchorne et al. 2006).

TRPA1 has also been shown to be required for mechanical nociception. Knockout mice were less sensitive to transient punctate mechanical stimuli applied to their hind paw (Kwan, Allchorne et al. 2006). C fibers from TRPA1-/- mice or treated with HC-030031, a selective TRPA1 antagonist, displayed significantly reduced mechanically-evoked action potential firing compared to wild type mouse C fibers (Kerstein, del Camino et al. 2009; Kwan, Glazer et al. 2009). Recently, rat TRPA1 was shown to be activated by hypertonic solutions when heterologously expressed in HEK293 cells, supporting a role for TRPA1 in mechanosensation (Zhang, Chen et al. 2008).

TRPA1 was initially cloned as a noxious cold activated ion channel with a broad range of activating temperatures (12 °C~24 °C, average threshold 17 °C) (Story, Peier et al. 2003). However, the involvement of TRPA1 in noxious cold sensation remains controversial as different groups have had different observations (Story, Peier et al. 2003; Jordt, Bautista et al. 2004) (Bautista, Jordt et al. 2006; Kwan, Allchorne et al. 2006; Karashima, Damann et al. 2007; Zurborg, Yurgionas et al. 2007; Karashima, Talavera et al. 2009; Knowlton, Bifolck-Fisher et al. 2010). In heterologous expression system, cold activation of TRPA1 was observed by some groups (Reid 2005), but not by others (Jordt, Bautista et al. 2004; Nagata, Duggan et al. 2005). It was also proposed that the cold activation of TRPA1 is indirectly caused by cold evoked Ca^{2+} increase (Zurborg, Yurgionas et al. 2007). Studies in TRPA1 knockout mice have also generated contradicting results, with some groups observing cold sensing defects (Kwan, Allchorne et al. 2006; Karashima, Talavera et al. 2009) while others not (Bautista, Jordt et al. 2006; Knowlton, Bifolck-Fisher et al. 2010). In general, more evidence is supporting a role of TRPA1 in cold sensation. The discrepancies are likely due to different experimental designs and preparations that caused some group fail to observe cold induced effects.

In addition, TRPA1 can be activated by the inflammatory peptide bradykinin when co-expressed with its receptor (Bandell, Story et al. 2004). This activation is likely

to be mediated by phospholipase C (PLC) (Bandell, Story et al. 2004; Jordt, Bautista et al. 2004; Wang, Dai et al. 2008). TRPA1 knockout mice have pronounced deficits in bradykinin-evoked nociceptor excitation and pain hyper sensitivity (Bautista, Jordt et al. 2006). TRPA1 can also be activated by intracellular Ca^{2+} via an EF-hand domain. This provides a potential mechanism for activation downstream of other signaling pathways that lead to elevated intracellular Ca2+ level (Zurborg, Yurgionas et al. 2007).

Drosophila melanogaster has recently emerged as a system to study the molecular signaling of nociception (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Neely, Hess et al. 2010; Zhong, Hwang et al. 2010). *painless*, a *Drosophila* homologue of mammalian TRPA1, was identified in a forward genetic screen for nociception mutants; it has been shown to play a role in larval and adult nociception (Tracey Jr, Wilson et al. 2003; Xu, Cang et al. 2006). At the larval stage, *painless* is required for both thermal and mechanical nociception. *painless* is expressed in multidendritic (md) neurons that include the nociceptors of *Drosophila* (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007). In heterologous expression systems, Painless is activated by noxious heat (~42 °C), suggesting that Painless functions as a heat transduction channel in nociceptor neurons (Sokabe, Tsujiuchi et al. 2008).

Distinct from the requirement of *painless* in nociception, *dTrpA1*, The *Drosophila* orthologue of mammalian TRPA1, has been assigned a role in detecting innocuous warm temperatures. In both heterologous expression systems and ectopic expression *in vivo*, dTRPA1 can be activated by warm temperatures, with a threshold of between 24-29 °C (Viswanath, Story et al. 2003; Pulver, Pashkovski et al. 2009). *dTrpA1* has been implicated to function in thermotaxis behavior in both larvae and adult flies (Rosenzweig, Brennan et al. 2005; Hamada, Rosenzweig et al. 2008; Kwon, Shim et al. 2008). A small set of anterior cells located in the *Drosophila* brain (AC neurons) express dTRPA1 and have been suggested to function as internal sensors for avoidance of warm temperatures between 28-32 °C through direct warmth activation of dTRPA1(Hamada, Rosenzweig et al. 2008). In addition to direct temperature activation, *dTrpA1* has also been shown to be required for discriminating subtle temperature differences between the optimal temperature of 18 °C and19 °C~24 °C, likely going through a phospholipase C-dependent signaling cascade (Kwon, Shim et al. 2008).

dTrpA1 has also been shown to be involved in chemosensation. As in thermosensation, direct and indirect mechanisms have been suggested for dTRPA1 to function in gustatory neurons. One study showed that *dTrpA1* was required for detection of aristolochic acid and the insect repellent citronellal downstream of G

protein (Gq)/ phospholipase C (PLC) activation (Kim, Lee et al. 2010; Kwon, Kim et al. 2010). Another study shows that dTRPA1 functions as a molecular sensor for reactive electrophiles to protect flies from ingesting noxious compounds(Kang, Pulver et al. 2010).

Here we show that like its mammalian homologue, *dTrpA1* is also required for nociception. We have identified a new isoform of *dTrpA1* that is specifically expressed in the nociceptors of *Drosophila* larvae and is required for both mechanical and thermal nociception. Our results suggest a conserved and ancient role of *dTrpA1* in nociception. This new isoform is not a direct temperature sensor per se and is required downstream of sensory transduction.

5.2 Materials and methods

5.2.1 Fly strains and husbandry

The following fly strains were used*: yw; GAL4109(2)80 (md-GAL4), ppk-GAL4, UAS-dTrpA1-A; UAS-dTrpA1 RNAi* (VDRC GD collection transformant ID 37249); *Df(3L)ED4415; dTrpA1* ^{W903*} (from TILLING project), w;*UAS-mCD8::GFP, UAS-dicer2, UAS-ChannelRhodopsin-2::YFP line C (UAS-ChR2YFP)*. Where possible, balancers containing the Tb marker (TM6b Tb or T(2; 3) K87CyTbRoi) were used so that the inheritance of the insertion(s) could be followed at larval stage. Alternatively, YFP

fluorescence provided a means to follow *UAS-ChR2YFP* inheritance. Flies were raised on standard cornmeal medium. All stocks were maintained at 25 °C and 75% humidity on a 12-12 light dark cycle.

5.2.2 Thermal nociception assay

The thermal nociception behavioral tests were performed as described previously (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010), with slight modifications.

On the first day, crosses were established with six female and three male flies in each vial with standard cornmeal food. For each genotype, six vials were established and maintained at 25°C and 75% humidity.

On the sixth day of the experiment, wandering third-instar larvae from vials were rinsed out of the vial with distilled water and into a plastic 60 mm Petri dish. Excess water in the dish was aspirated such that the larvae remained moist but were not floating in the water that was remaining in the dish.

The temperature of the noxious heat probe (a soldering iron sharpened to a chisel tip shape that was 0.6 mm wide) was controlled by the adjustment of the voltage. A digital thermocouple (Physitemp, BAT-12) welded to the tip of the probe was used so that its temperature could be precisely measured. The stimulus was delivered by gently

touching the larvae laterally, in abdominal segments four, five, or six. Each larva was tested only once and discarded after the test. Analysis of video-recorded behavior was performed offline. The response latency was measured as the time interval from the point at which the larva was first contacted by the probe until it initiated the escape locomotion (the beginning of the first, complete 360° roll).

5.2.3. Mechanical nociception assay

The mechanical nociception behavioral tests were performed as described previously (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010), with slight modifications.

In mechanical nociception assays, wandering third-instar larvae were collected as described for thermal nociception assays and then stimulated with a 30 mN calibrated Von Frey filament. Von Frey filaments were made from Omniflex monofilament fishing line Shakespeare (6 lb test, diameter 0.009 inch [0.23 mm]). Fibers were cut to a length of 20 mm and attached to a glass pipette such that 4 mm of the fiber protruded from the end and 10 mm anchored the fiber. Noxious mechanical stimuli were delivered by the rapid depression and release of the fiber on the dorsal side of a larva. The stimulus was

delivered to abdominal segments four, five, or six. A positive response was scored if at least one nocifensive roll occurred after the first or second mechanical stimulus.

5.2.4 Gentle touch assay

The gentle touch behavioral tests were performed as described previously (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010), with slight modifications.

Wandering third-instar larvae were collected as described for thermal and mechanical nociception assays and were stimulated with an eye lash. The eyelash was fixed on the tip of a stick and gently touched to the head of the larvae during normal locomotion. If there was no response to the gentle touch and the larva continued its normal locomotion, it was scored as 0. If the larva paused, it was scored as 1. If the larva paused and turned its head, it was scored as 2. If the larva produced one reverse peristaltic wave, it was scored as 3 and if multiple reverse peristaltic waves were produced, it was scored 4. Each larva was tested 4 times with the eyelash and the larva was allowed to fully recover between each test until they started to produce normal forward locomotion. All four scores of the larva were added up to generate a total score for that larva. 15 to 30 larvae were tested for each genotype.

5.2.5 Confocal microscopy

For the visualization of GAL4 patterns, *dTrpA1-B-GAL4*; UAS-mCD8-GFP larvae were anesthetized with ether until immobile (~15 minutes exposure to ether). Microscopy was performed on a Zeiss LSM 5 Live Confocal System using 488nm laser. Images shown are maximum-intensity projections of confocal Z stacks. For calcium imaging in S2R+ cells transfected with GCaMP3.3, single slice of time series were taken using a 488 nm laser.

5.2.6 Cell culture and transfection

Drosophila cell line S2R+ cells were maintained in Schneider's *Drosophila* medium (Invitrogen) with 10% FBS. Cells growing on round cover slips (Warner Instruments #1.5 Glass Coverslip 25mm Round) in 6 well tissue culture plates (Falcon) were transfected with 0.75 μg of *Ubiquitin-GAL4*, 0.5μg of *UAS-GCaMP3.0* and the gene of interest, either 0.25 μg of *UAS-dTrpA1-A* or *UAS-dTrpA1-B* using cellfectin (Invitrogen). Imaging was conducted on the 4th day after the transfection. The round cover slip was assembled into an imaging chamber (Warner Instrument Corporation Series 20 Chamber Platform P-2) with the cells facing up and gently rinsed with HL3 saline. HL3 saline was delivered through an inline solution heater (Warner Instrument Corporation Model SH-27B) before it flowed through the imaging chamber and went into the vacuum. A thermal

couple (Warner Instrument Corporation TA-29) that was connected to a data acquisition board (Warner Instrument Corporation TC-324B) was placed in the water reservoir of imaging chamber to measure the temperature in real time. The inline solution heater was used to raise the temperature of the saline. To cool down the temperature, the inline solution was turned down and ice-chilled HL3 saline was added to the reservoir. Microscopy was performed on a Zeiss LSM 5 Live confocal system using 20X lens. Images were acquired as time series at 1 frame/sec.

5.2.7 Molecular cloning

For the generation of *dTrpA1-B-GAL4*, polymerase chain reaction (PCR) from a BAC clone template (GH03996) was with the forward primer 5′-CACCCCATTCCACTTGAGTGAGGACTAC-3′ and the reverse primer 5′-GACCGCTGTAGACTCCGTTG-3′. The resulting PCR product was then cloned into pENTR/D-TOPO (Invitrogen) and then into the *Drosophila* pCaSpeR-DEST6 (DGRC stock no. 1032) Gateway destination vector with Clonase II (Invitrogen). This construct was used for the generation of transgenic animals by the transposase-mediated transformation of *w1118*. Two independent transformants were analyzed and showed similar expression patterns.

For the cloning of *dTrpA1-B*, reverse transcription polymerase chain reaction (RT-PCR) was performed on RNA extraction of 1st and 2nd instar Canton S larvae. Oligo (dT)12-18 (Invitrogen) and primer

5′-CTACATGCTCTTATTGAAGCTCAGGGCG-3′ mix were used as primers for reverse transcription and first-strand cDNA synthesis used SuperScript TM II RT (Invitrogen). PCR was performed using the forward primer 5′-ATGCCCAAGCTCTACAACGGAGTCTA-3′ and the reverse primer: 5′-CTACATGCTCTTATTGAAGCTCAGGGCG-3′ to amplify the cDNA. The PCR product was re-amplified using the same pair of primers. dTrpA1-B PCR product was cloned into TOPO-XL (Invitrogen) and fully sequenced. TOPO-XL-dTrpA1-B was further subcloned into pUAST construct using the EcoRI restriction site.

5.2.8 Transgenic flies

The pCaSpeR-DEST6-dTRPA1B-GAL4 construct was injected by the Duke Transgenic fly facility for P-element mediated transformation. This particular line used in this research is an insertion on the 2nd chromosome. Injections for dTRPA1 BAC CH322-154N09 were performed by Genetivision for PhiC31 mediated chromosome integration with VK37(2L)22A3 as the docking site.

5.2.9 Optogenetic activation of class IV md neurons

The optogenetic activation of *ppk-GAL4* neurons was performed as described previously (Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010) . When crossed to the *UAS-dTrpA1RNAi* strain, only half of the progeny inherit the *ppk-GAL4 UAS-ChR2::eYFP* chromosome. These larvae were identified following exposure to blue light by visualizing their YFP fluorescence. The YFP-negative (CyO) larvae were used as in internal negative control because they never produce nocifensive escape locomotion in response to blue light.

Optogenetic activation in this work refers to a technique that uses blue light to depolarize neurons expressing a blue-light activated ion channel, channelrhodopsin-2 (ChR2). Four to six virgin female flies were crossed to 3 male flies to make the progeny of the right genotype. Females were allowed to lay eggs for 24 hours on apple juice agar with yeast paste containing all-trans retinal [atr+, 500 μM] or atr- [0.5% ethanol]. The larval progeny were allowed to develop and feed on the yeast paste for 72 additional hours. For behavioral analysis the larvae were transferred to 60 mm plastic Petri dishes containing 1-2 ml deionized H2O. Larvae were then stimulated with blue light (460-500nm) using the Hg light source of a Leica MZ16 FA stereomicroscope (14,000 lux). Blue light pulses were manually controlled and lasted for two to three seconds. Each

larva was given three pulses of blue light. Behavioral responses were videotaped and analyzed off line. A positive nocifensive response was scored if the larva completed at least 1 revolution (360°) in response to any of the three blue light pulses.

5.3 Results

5.3.1 Tilling screen for *dTrpA1* identified a mutant allele of *dTrpA1* with premature stop codon.

We performed a screen for EMS-induced mutations of the *dTrpA1* locus with the *Drosophila* Tilling Project (http://tilling.fhcrc.org:9366/fly/). Nineteen fly strains were identified that harbored mutations in *dTrpA1* (Figure 5-1A). While most of the lines carried synonymous mutations or conservative missense changes, one line stood out as a potentially loss-of-function allele of *dTrpA1* may be a functional null allele of *dTrpA1*. As in all TRP channels, dTRPA1 is predicted to have 6 transmembrane domains with a pore loop between the 5th and 6th transmembrane domain. In this allele $dTrpA1^{W903*}$, the codon for a tryptophan was mutated to a premature stop codon. As this residue was located upstream of the pore loop, this stop codon in the fourth transmembrane domain of dTRPA1was likely to lead to the production of a non-functional dTRPA1 channel (Figure 5-1B). In addition, *Df(3L)ED4415*, an existing deficiency removing 210kb that included *dTrpA1* (as well as 25 other genes), was available for our studies. As expected for a large deficiency *Df(3L)ED4415* is not homozygous viable. In order to separate the

dTrpA1 mutation from other unlinked EMS-induced mutations that might be present in the strain, we out-crossed the *dTrpA1*^{W903*}mutant chromosome to the *Df(3L)ED 4415* strain for six generations.

5.3.2 *dTrpA1* mutants are defective for both thermal and mechanical nociception

We next tested the resulting out-crossed *dTrpA1* mutant animals in behavioral assays for nociception. *Drosophila* larvae produce stereotyped nocifensive escape locomotion (NEL) behavior in response to noxious thermal or mechanical stimuli (Tracey Jr, Wilson et al. 2003; Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010). In wild type animals gently touched with a 46 °C probe the initiation of NEL behavior was seen within 3 seconds (Figure 5-2 A, B & C). In contrast, the *dTrpA1*^{W903*} mutant larvae had a significant delayed response latency. Many of the mutants failed to initiate the escape behavior within 10 seconds (Figure 5-2D). The *dTrpA1*^{W903*}/+ larvae showed normal response to noxious heat, indicating a recessive mutant phenotype. To confirm that the mutant phenotype was mapped to the *dTrpA1* locus, we performed complementation tests with *Df(3L)ED4415*. *Df(3L)ED4415* failed to complement *dTrpA1*^{W903*} as the transheterozygous larvae showed severe defects in responding to noxious heat (Figure 5-2E). The *dTrpA1* mutants were also tested for mechanical nociception responses (Figure 5-3A). Compared to wild type and heterozygous controls

that robustly produced nocifensive response to stimulation with a 30 mN von Frey fiber, both the *dTrpA1*^{W903*}/*Df(3L)ED4415* and *dTrpA1*^{W903*} larvae were significantly less responsive (Figure 5-3 A). In contrast to nociception behaviors, *dTrpA1* mutant larvae showed normal responses to gentle touch (Figure 5-3B). In addition, gross motor functions of *dTrpA1* mutants appeared normal.

5.3.3 Genomic rescue of *dTrpA1* mutants

Complementation tests with *Df(3L)ED4415* indicated that the mutation responsible for the nociception defects mapped within the 210 kb region deleted by *Df(3L)ED4415*. To further test whether the mutation in *dTrpA1* was indeed causally responsible for the nociception defects, we performed genetic rescue experiments with a 21kb piece of genomic DNA that included the entire *dTrpA1* locus and the neighboring gene, *mcm7*. The results indicated a complete rescue of the thermal and mechanical nociception defects (Figure 5-4) which further narrowed down the genetic aberration causing the nociception defects to one of the two genes, *dTrpA1* and *mcm7*. Combined, these data constitute formal genetic proof that the EMS-induced mutation in *dTrpA1* and /or *mcm7* caused pronounced defects in behavioral responses to both thermal and mechanical nociception stimuli.

5.3.4 Knockdown of *dTrpA1* expression in class IV md neurons

Evidence suggests that class IV multidendritic (md) neurons function as nociceptors in *Drosophila* larvae. However, *dTrpA1* reporters are not expressed in these cells. Nevertheless, to test whether *dTrpA1* was functionally required in the nociceptors themselves, we used the nociceptor specific *pickpocket-GAL4* driver to express *UAS-dTrpA1-RNAi*. We also incorporated *UAS-dicer2* in our cross to enhance the RNAi effect. The *pickpocket-GAL4/+; UAS-dTRPA1RNAi/UAS-dicer2* larvae showed reduced responses to both noxious thermal and mechanical stimuli, indicating that dTRPA1 is likely to function in the nociceptors for its role in nociception (Figure 5-5).

5.3.5 Ectopic expression of *dTrpA1-A* in class IV md neurons

The heat activation threshold of the known dTRPA1 (which we will refer to as dTRPA1-A) is between 24 °C and 29 °C (Viswanath, Story et al. 2003). Thus, although our results suggested a site of action for *dTrpA1* in nociceptor neurons, the biophysical properties of dTRPA1-A seemed inconsistent with this possibility. Given the biophysical properties of dTRPA1-A, if it was expressed in nociceptors then a behavioral threshold for nociception should be 29 °C. In contrast, both the *Drosophila* larval nociception behavior and the activation of larval nociceptor have a threshold around 39 °C (Tracey Jr, Wilson et al. 2003; Xiang, Yuan et al. 2010). Indeed, the thermal nociception behavior

of larvae with forced expression of *dTrpA1-A* in the nociceptor neurons (*ppk-GAL4/+; UAS-dTRPA1/+*) had a dramatically lowered thermal nociception threshold. Greater than ninety percent of these larvae responded to a 30 °C heat stimuli with NEL in less than one second (Figure 5-6 D) which is in dramatic contrast to the behavior of wild type larvae which never produced NEL in response to 30 °C heat (Figure 5-6 A, B, C).

Several important conclusions can be drawn from these results. First, this result further confirms that class IV md neurons are indeed nociceptors, since expression of *dTrpA1* in class IV md neurons altered the thermal threshold of the nocifensive behavior. Second, this result is intriguing as it argues against the possibility of *TrpA1-A* being expressed in these neurons. This is in contrast with the results of *dTrpA1* mutant behavior, genomic rescue and RNAi knockdown experiments which when combined suggest that *dTrpA1* is required in class IV md neurons for nociception.

5.3.6 Cloning of a new *dTrpA1* isoform

A potential explanation for our findings was found when we examined the genomic region surrounding the *dTrpA1* locus and identified two highly conserved exons upstream of the known transcriptional start site of *dTrpA1-A*. These two exons were annotated as part of the neighboring gene *misfire* (*mfr*). However, none of the many *misfire* cDNA clones included these two exons. Thus, we hypothesized that these exons might be part of the *dTrpA1* locus but incorrectly annotated as *misfire* exons. To test this

hypothesis we designed primers against the novel exons and performed reverse transcriptase PCR with downstream primers against *dTrpA1*. We successfully amplified, cloned and sequenced PCR products from these reactions, identifying a novel *dTrpA1* transcript. The DNA sequence of plasmid clones derived from the RT-PCR reaction confirmed the incorporation of the two upstream exons into a novel *dTrpA1* transcriptional splice variant. This newly identified transcript initiates from a promoter 4.2 kb of *dTrpA1-A* and skips the first exon of the known *dTrpA1* transcript. In addition, this new transcript was also alternatively spliced through removal of the 10th exon of *dTrpA1-A* and the incorporation of a novel exon (Figure 5-7 A,B). We refer to this new upstream transcript and splice variant as *dTrpA1-B* (Figure 5-7B) to distinguish it from the known transcript, *dTrpA1-A* (Figure 5-7A).

The conceptually translated dTRPA1-B protein differs from dTRPA1-A. At the N-terminus, 101 amino acids are encoded by exons 1 and 2 of *dTrpA1-B* and these amino acids are not easily aligned with the 68 amino acids that are encoded by the first exon of *dTrpA1-A*. Similarly, the *dTrpA1-B* exon 11 encodes 31 amino acids that align poorly with the 30 amino acids encoded by the exon 10 of *dTrpA1-A* (Figure 5-8). The amino acid sequence changes are located to the N- and C- termini of the ankyrin repeats domain before the transmembrane domain (Figure 5-7 A, B). At the N-terminus of the

ankyrin repeats dTRPA1-B is proline and serine rich relative to dTRPA1-A. At the C-terminus of the ankyrin repeats, dTRPA1-B is basic relative to dTRPA1-A (Figure 5-8).

5.3.7 Nociceptor neuron expression of *dTrpA1-B*

Existing GAL4 reporter strains for *dTrpA1-A* (Rosenzweig, Brennan et al. 2005; Hamada, Rosenzweig et al. 2008) are not expressed in the class IV md nociceptor neurons (L.Z., W.D.T unpublished observation). However, these reporters utilized genomic DNA from regions downstream of the transcriptional start site of *dTrpA1-B* and therefore likely lack important upstream enhancer elements for *dTrpA1-B*. Therefore, to investigate the expression pattern of the new transcript we cloned the 2.1kb genomic interval between *dTrpA1-B* and *misfire* into a GAL4 reporter transformation vector and generated transgenic GAL4 reporter strains (Figure 5-9A). Remarkably, this *dTrpA1-B-GAL4* reporter drives nearly exclusive expression in class IV nociceptor neurons in larvae (Figure 5-9B). This expression pattern led us to hypothesize that dTRPA1-B might be the functional isoform of the class IV md-da nociceptor neurons.

5.3.8 Heterologous expression of the new dTRPA1 isoform

The novel exons of *dTrpA1-B* cause important changes in amino acid sequence of dTRPA1-B relative to dTRPA1-A (Figure 5-8). To test how these changes affect the biophysical properties of dTRPA1-B, we expressed the cloned cDNA in *Drosophila* S2R+

cells that also expressed the genetically encoded Ca^{2+} sensor GCaMP 3.0. For comparison, we also investigated the properties of the dTRPA1-A isoform. Consistent with the known properties of the dTRPA1-1A warmth activated channel, we observed a dramatic increase of intracellular calcium between temperature range of 25-30 °C (as indicated by increased fluorescent intensity of GCaMP 3.0) in cells transfected with *dTrpA1-A* (Figure 5-10 A, C, D). In contrast, S2R+ cells transfected with *dTrpA1-B* did not respond to temperature changes (Figure 5-10 B, C, D). These results indicate that the amino acid changes of dTRPA1-B change the properties of the channel. Since the mammalian TRPA1 is activated by cold with a threshold of 17 °C, we also tested whether the new isoform could respond to noxious cold. Between the temperature ranges of 22 °C and 15 °C, no Ca^{2+} response was detected (data not shown). The lack of a positive response to temperatures in dTRPA1-B transfected cells was not due to dTRPA1-B being a non-functional channel. Application of the dTRPA1 agonist allyl isothiocyanate (AITC) at concentration of 0.033mM, caused robust Ca^{2+} responses in the dTRPA1-B transfected cells but not in non-transfected cells. The response to AITC in dTRPA1-B cells resembled AITC responses in dTPRA1-A transfected cells, in contrast to the temperature responses. These results show that dTRPA1-B, unlike dTRPA1-A, does not function as a thermoTRP.

5.3.9 *dTrpA1* mutants are defective in NEL triggered by optogenetic activation of class IV md neuron.

Our results suggested dTRPA1-B may not function as a direct temperature sensor. This raised the possibility that dTRPA1-B might affect excitability of nociceptors downstream of transduction. To test this, we asked whether *dTrpA1* mutants would affect behavioral responses to optogenetic activation of the class IV neurons. We have previously shown that blue light triggered NEL in animals expressing ChR2::eYFP in class IV nociceptive neurons (optogenetic activation) (Hwang, Zhong et al. 2007; Zhong, Hwang et al. 2010). Blue light activation of the larval nociceptors via ChR2::eYFP bypass mechanotransduction/thermotransduction, but still depends on factors that control the general excitability of the neuron. For example, the *para* gene, that encodes a NaV type sodium channel, is essential for action potential propagation in *Drosophila* and is required in class IV md neurons for blue light-triggered NEL (Zhong, Hwang et al. 2010). If dTRPA1 functions downstream of transduction in class IV md neurons, then the ChR2 activation triggered response might be impaired by dTRPA1 knockdown.

The genetic mutants of dTRPA1 showed a mild yet significant decrease in behavioral responses to ChR2 activation of class IV md neurons compared to wild type larvae (Figure 5-11 A). We confirmed this by knocking down of dTRPA1 in class IV md neurons, which also impaired the nocifensive response triggered by ChR2 activation. In

control transgenic larvae expressing ChR2::eYFP in class IV md neurons, escape locomotion behavior was seen in 85% of the larvae tested. 63% of larvae expressing dTRPA1-RNAi in the same neurons that express Chr2::eYFP responded to ChR2 activation, which is significantly different from the controls (Figure 5-11 B). This indicates that dTRPA1is required downstream of ChR2 activation in the class IV md neurons in this scenario, which could also explain the requirement of dTRPA1 in nociception pathways. However, the defects of the mutants and RNAi knockdown in the ChR2 experiments are much milder compared to the thermal and mechanical nociception defects. This may imply that in addition to a role in amplification, dTRPA1 may have more specific functions close to the transduction step in these neurons.

Taken together, we have cloned a new isoform of the *dTrpA1* that is transcribed from an alternate promoter and shows alternative splicing relative to *dTrpA1-A*. The two isoforms show distinct expression patterns and are involved in different sensory functions. The newly identified *dTrpA1-B* isoform is expressed in nociceptors in larvae and is required for nociception. It does not function as a thermoTRP on its own. Rather, dTRPA1-B may function downstream of other transduction mechanisms in nociceptors and is partially required for general excitability of the class IV md neurons.

5.4 Discussion

In this research, we have found that dTRPA1-B is required for both thermal and mechanical nociception in *Drosophila* larvae. Genetic mutants of *dTrpA1* showed severe defects in thermal and mechanical nociception. Tissue specific RNAi knockdown of *dTrpA1* in class IV md neurons phenocopied these defects. dTRPA1-B is encoded by a novel transcript of *dTrpA1*, and an isoform specific reporter indicates it is specifically expressed in class IV md neurons.

Interestingly, this new isoform, does not respond to temperature changes within 15-42 °C temperature range but does respond to AITC. *dTrpA1* mutants and RNAi knockdown in class IV md neurons show minor reduction in optogenetically triggered nocifensive behavior, indicating that dTRPA1 is at least partially involved in general excitability functions in these neurons.

Two other ion channel genes have been found to be required for nociception in *Drosophila* (*painless* and *pickpocket* (*ppk*)). Like *dTrpA1*, *painless* is required for both thermal and mechanical nociception. In heterologous expression system, Painless can be activated by heat with a threshold of 42.6 °C, close to the behavioral threshold for thermal nociception as well as the heat activation threshold of class IV md neurons. All of these findings point to a direct role for Painless as the transduction channel for thermal nociceptive stimuli. Whether or not Painless can also function as a

mechanosensor remains unknown; heterologously expressed Painess is not activated by osmotic pressure (Sokabe, Tsujiuchi et al. 2008). We have proposed that a DEG/ENaC protein coding gene *pickpocket* (*ppk*) as a direct mechanosensor or part of a mechanosensor in these nociceptors (Zhong, Hwang et al. 2010). *ppk* mutants show defects in mechanical nociception, but not in thermal nociception. *ppk* RNAi animals show no defects in optogenetic activation triggered NEL, suggesting Pickpocket function can be bypassed by Channelrhodopsin activation. These findings place the function of Pickpocket upstream of the general excitability of these neurons, suggesting a possible role in mechanotransduction.

dTRPA1-B is required for both mechanical and thermal nociception. However, heat- or cold- induced intracellular calcium increases did not occur in S2R+ cells expressing dTRPA1-B. This suggests that dTRPA1-B is not a direct temperature sensor. It's not known though, if dTRPA1 can form heteromeric channel with other TRP channel subunits as has been seen for the TRP/TRPL channel complex (Gillo, Chorna et al. 1996; Sotomayor, Corey et al. 2005). TRPA1-B and Painelss are co-expressed in the nociceptor neurons and it's possible that the two could form heteromeric channels. If this is the case the thermosensor could reside on Painless subunits.

Alternatively, dTRPA1-B may function downstream of Painless and Pickpocket. The human TRPA1 has been shown to be activated by intracellular Ca^{2+} via an EF-hand

domain (Zurborg, Yurgionas et al. 2007). dTRPA1-B may be activated similarly by Ca^{2+} influx through Painless or Pickpocket activation. dTRPA1-B may also be activated downstream of other signaling molecules such as PLC as shown in thermotaxis behavior and chemosensory functions (Kwon, Shim et al. 2008; Kim, Lee et al. 2010; Kwon, Kim et al. 2010).

This naturally occurring dTRPA1-B isoform provides great opportunity for studying the structure function of this gene. dTRPA1-A is activated by temperature range of 24-29 °C while the dTRPA1-B does not respond to temperature changes within the range of 15-42 °C. Since the only differences in sequence between the two protein isoforms are at the N-terminus and in the downstream exon of the ankyrin repeat domain, these amino acids in dTRPA1-A must be critical for heat activation of the channel. Very little is known about the temperature sensing motif(s) in thermo TRPs. While the mechanisms are still largely unknown, some research has begun to shed light onto this mystery. Some studies suggest that the C termini of TRP channels are important for temperature activation. Different C-terminal truncations of TRPV1 change the functional properties of this channel (Vlachová, Teisinger et al. 2003). Swapping the C-terminus of TRPV1 with TRPM8 exchanges the temperature responses of the channels. Interestingly, the C terminus is not essential for TRPV1 capsaicin responsiveness or TRPM8 menthol responsiveness (Vlachová, Teisinger et al. 2003).

These findings suggest that the C-terminus may contain the thermal sensor in these channels.

Recently the pore regions of TRP channels have also been suggested to be critical for temperature activation. Residues in the 6th transmembrane and pore region of TRPV3 are required for its heat activation (Grandl, Hu et al. 2008). Consistent with this , mutations in the outer pore region of TRPV1 specifically impair temperature activation (Grandl, Kim et al. 2010).

Our result suggests that the ankyrin repeats containing N- terminus of TRP channel also contain elements that are required for thermosensation. Making chimeric channels containing these elements will allow us to further investigate the specific functions of these motifs.

Our findings that *dTrpA1* functions in nociceptive pathway in *Drosophila* suggest an evolutionary conserved role for TRPA family members in nociception. Furthermore, the identification of a novel dTRPA1 isoform may shed light onto the temperature sensing mechanisms of thermoTRPs.

A

Individual	Sequence	Effect	SIFT (IC)	Screened	Stock
d16G2	c1070y	L1079=		Het	Z3_2480
d3B8	c951y	T1059I	0.54 (3.18)	Het	Z3_5333
d34H3	g397r	V892=		Het	Z3_1344
d41C8	c486y	S922F	0.03 (3.18)	Het	Z3_5500
d53E1	a1201r	K1123R	0.16 (3.18)	Het	Z3_4005
d55E3	g597r	W903*		Het	Z3_4362
d65A1	a742w	L1007=		Het	Z3_1987
d67C4	a1270t	K1146M	0.01 (3.43)	Hom	Z3_1713
d71G4	g1124a	K1097=		Hom	Z3_0798
d77H8	g310a	P863=		Hom	Z3_2640
d78G2	c919t	T1048=		Hom	Z3_2930
d8B3	c1174y	P1114L	0.00 (3.41)	Het	Z3-PMM_0118
d86E5	g581r	A954T	0.43 (3.18)	Het	Z3_0546
d88A2	g397r	V892=		Het	Z3_1363
d91E7	g893r	D1040N	0.75 (3.18)	Het	Z3_4499
d92D1	c1146y	L1105F	0.00 (3.18)	Het	Z3_3942
d92G2	a564w	Y948F	0.33 (3.18)	Het	Z3_2585
d93D1	ccc814c	P1014= (up		Het	Z3_5939
d95A3	a697t	V992=		Hom	Z3_1476

Truncated

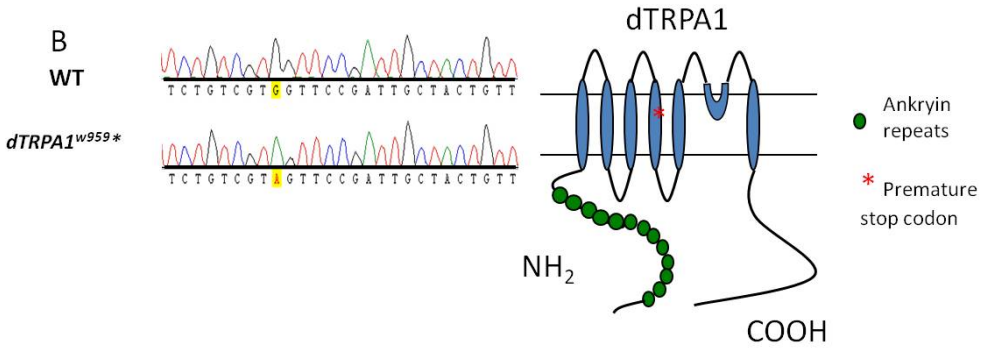


Figure 5-1: TILLING screen for *dTrpA1* identified an allele with a premature stop codon

Figure5-1: TILLING screen for *dTrpA1* identified an allele with a premature stop codon. (A) A list of lines from *dTrpA1* TILLING screen. "Effect" column indicates the amino acid change caused by the mutation. One of the lines, as indicated by the red box, has a premature stop codon. (B) Diagram of dTRPA1 structure and location of the premature stop codon in $dTrpA1^{W903*}$ strain. dTRPA1 is predicted to have 13 ankyrin repeats at the N-terminus and six transmembrane domains. The point mutation (G ->A) was verified by sequencing the corresponding genomic region. It changes a tryptophan to a premature stop codon that is located in the fourth transmembrane domain, before the predicted pore loop of the channel.

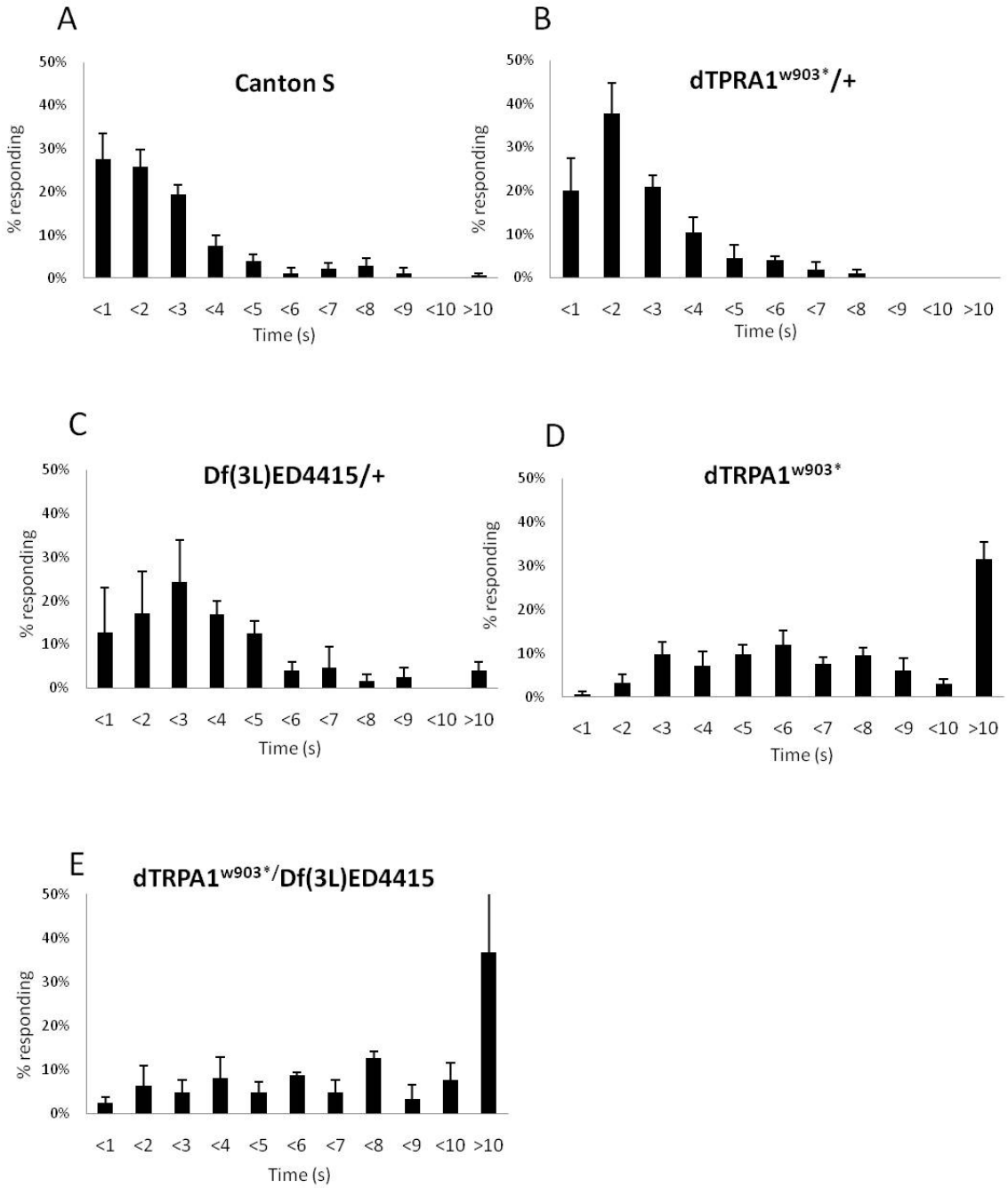
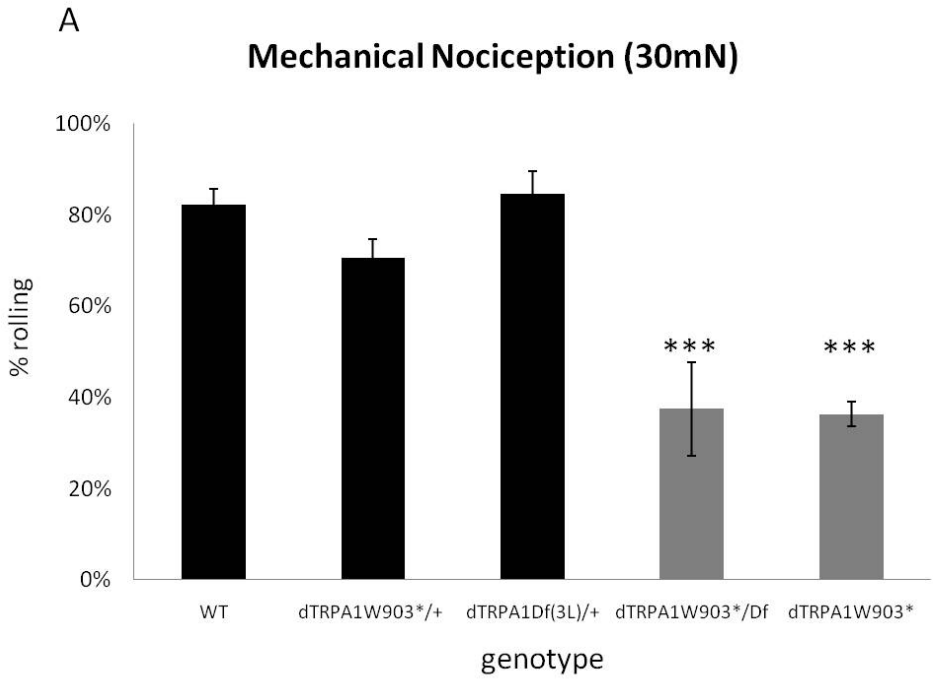


Figure 5-2: *dTrpA1* is required for thermal nociception.

Figure5-2: *dTrpA1* is required for thermal nociception. (A-G) shows the distribution of latencies of thermal nocifensive behavior in wondering third-instar larvae that were lightly touched with a 46 °C probe. (A)Thermal nocifensive response of wild type canton S larvae (n=105). (B) Thermal nocifensive response of *dTrpA1*^{w903*}/+ larvae (n=80). (C) Thermal nocifensive response of *Df(3L)ED4415*/+ larvae (n=54). (D) *dTrpA1*^{W903*} homozygous larvae are defective for their thermal nocifensive behavior. (n=149). $P < 0.001$. (E) *Df(3L)ED4415/dTRPA1*^{W903*} transheterozygous larvae defective for their thermal nocifensive behavior (N=67). $P< 0.001$.



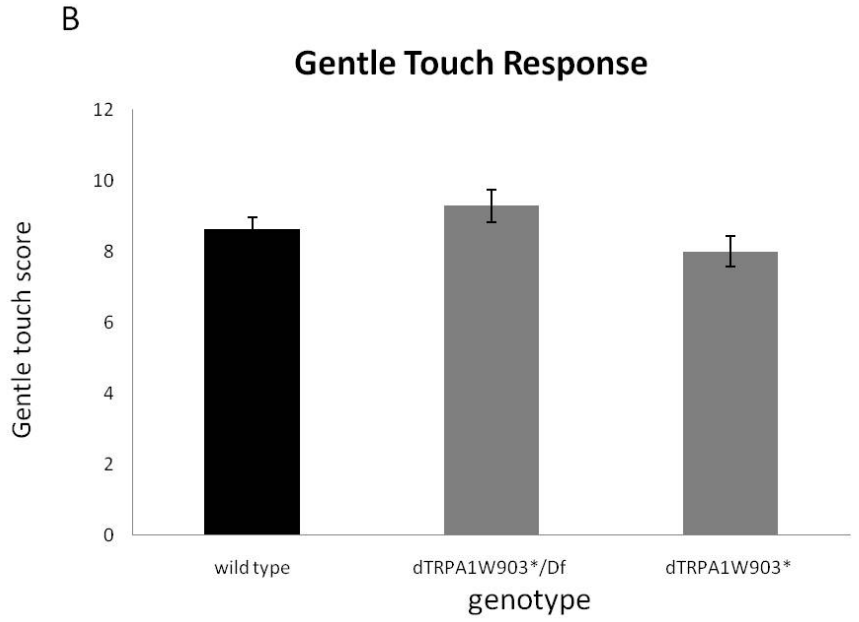


Figure 5-3: *dTrpA1* is required for mechanical nociception but not for gentle touch response

Figure 5-3: *dTrpA1* is required for mechanical nociception but not for gentle touch response (A) shows the percentage of larvae produced nocifensive behavior upon a von frey fiber stimulation of 30 mN force. Both the *dTrpA1*^{W903*} and Df(3L)ED4415/*dTrpA1*^{W903*} produced significantly less response compared to wild type and heterozygous animals ($P<0.001$) . (B) Gentle touch response is normal in *dTrpA1* mutants $P=0.25$ for *dTrpA1*^{W903*} and $P=0.26$ for *Df(3L)ED4415/dTrpA1*^{W903*}.

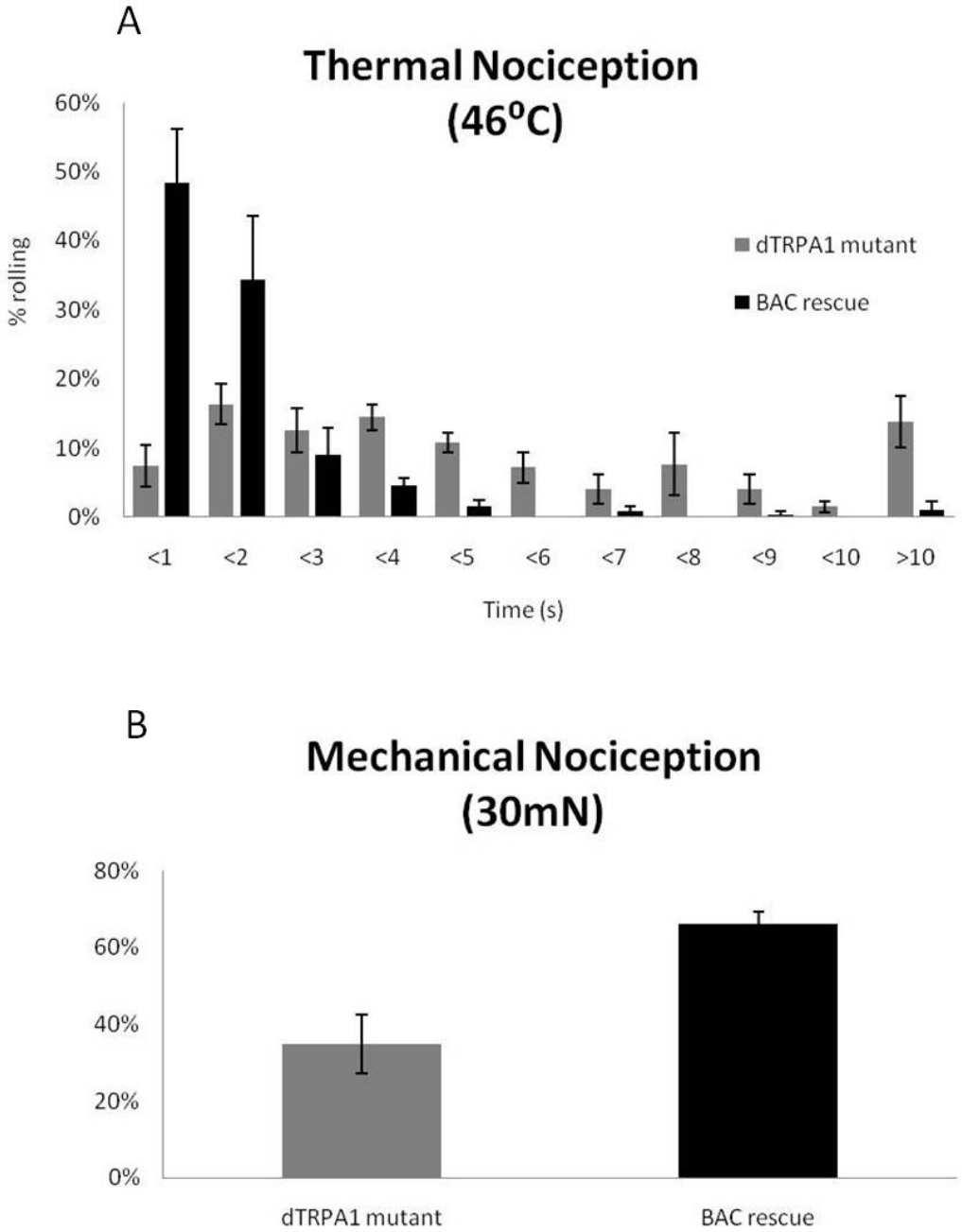


Figure 5-4: Genomic rescue of *dTrpA1* restored both the thermal and mechanical nociception defects

Figure5-4: Genomic rescue of *dTrpA1* restored both the thermal and mechanical nociception defects. (A) In the thermal nociception assay the *dTRPA1-BAC*/+; *Df(3L)ED4415/dTRPA1*^{W903*} (n=143) were able to produce nocifensive behavior with a much shorter latency compared to the mutant flies (n=180). (B) In the mechanical nociception assay, significantly more *dTRPA1-BAC*/+; *Df(3L)ED4415/dTRPA1*^{W903*} (n=139) larvae were able to produce nocifensive rolling upon the stimulation of a 30 mN mechanical force compared to the mutant (n=111). $P < 0.05$.

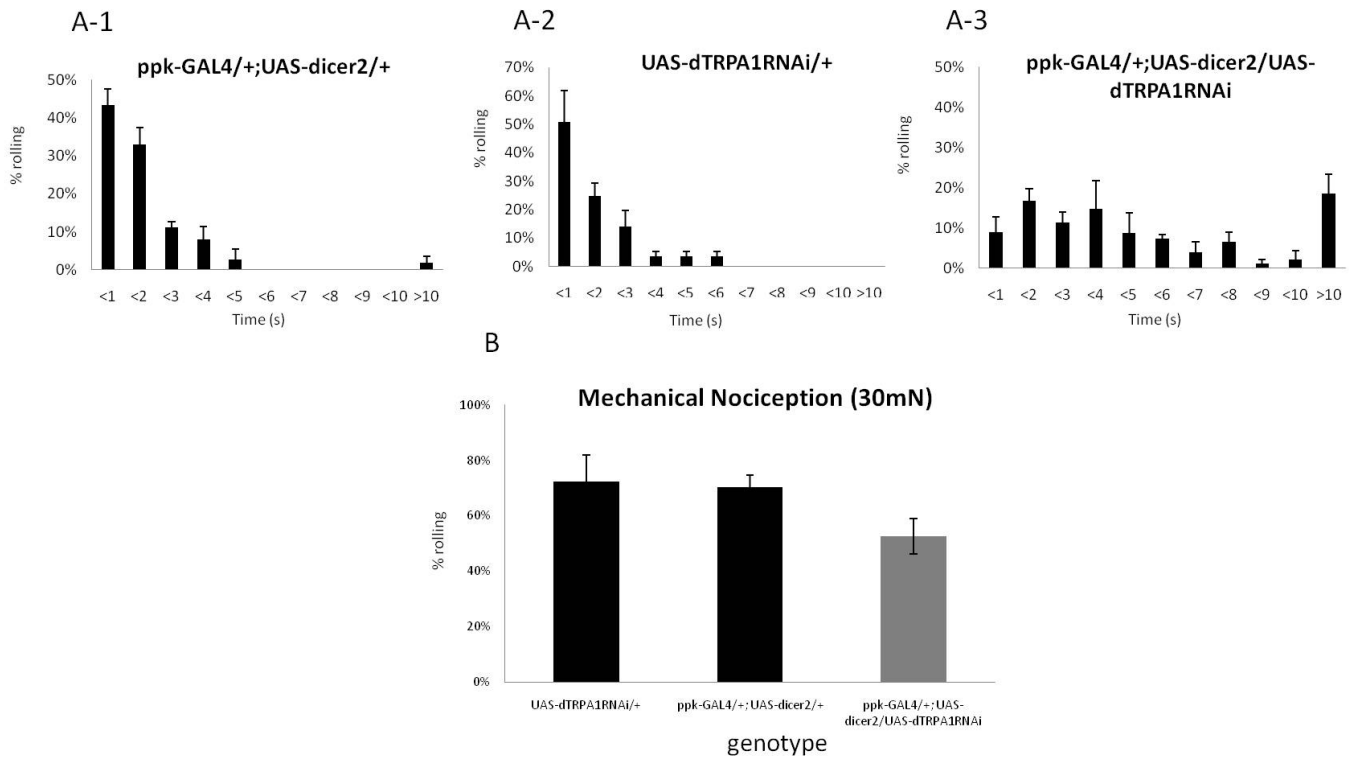


Figure 5-5: RNAi knockdown of *dTrpA1* in class IV md neurons impairs both thermal and mechanical nociception

Figure 5-5: RNAi knockdown of *dTrpA1* in class IV md neurons impairs both thermal and mechanical nociception

(A-1, 2, 3) The response latencies of larvae with *dTrpA1RNAi* knockdown in class IV md neurons (*ppk-GAL4*/+; *UAS-dicer2*/*UAS-dTrpA1RNAi*, n=112) were significantly longer compared to the control groups (*ppk-GLA4*/+; *UAS-dicer2*/+, n=113; *UAS-dTrpA1RNAi*/+, n=49). $P < 0.05$. (B) *dTrpA1RNAi* larvae (*ppk-GAL4*/+; *UAS-dicer2*/*UAS-dTrpA1RNAi*, n=124) had less frequent response rate to a 30 mN von frey fiber stimulation compared to the control groups (*ppk-GLA4+; UAS-dicer2*/+, n=110; *UAS-dTrpA1RNAi*/+, n=84). P=0.07.

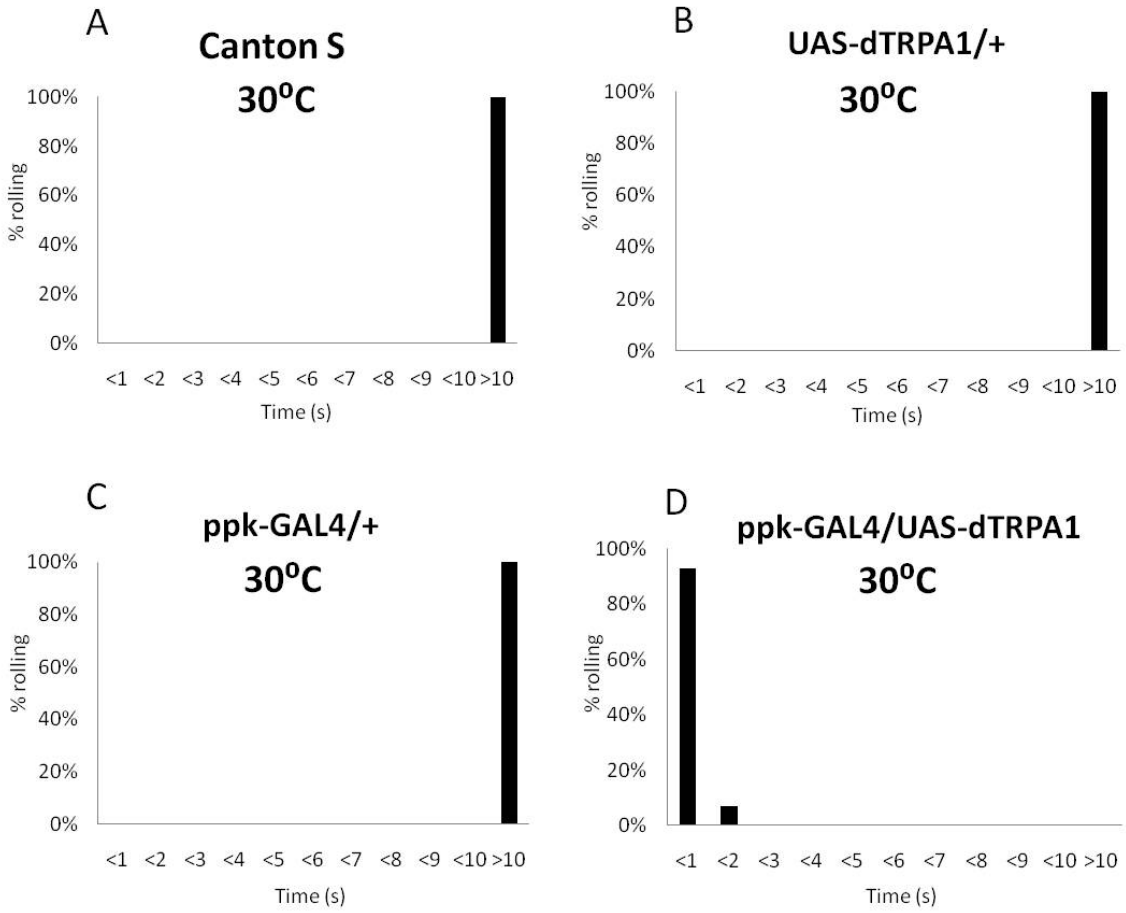


Figure 5-6: Ectopic expression of dTRPA1 in Class IV md neurons lowers the thermal nociception threshold.

(A) Wild type Canton S flies (n=86) did not show nocifensive responses to 30 °C stimuli. (B) *UAS-dTRPA1/+* larvae (n=54) did not show nocifensive responses to 30 °C stimuli. (C) *ppk-GAL4/+* larvae (n=37) did not show nocifensive responses to 30 °C stimuli. (D) *UAS-dTRPA1/ppk-GAL4* larvae (n=28) showed robust nocifensive rolling responses to 30 °C stimuli. P<0.001.

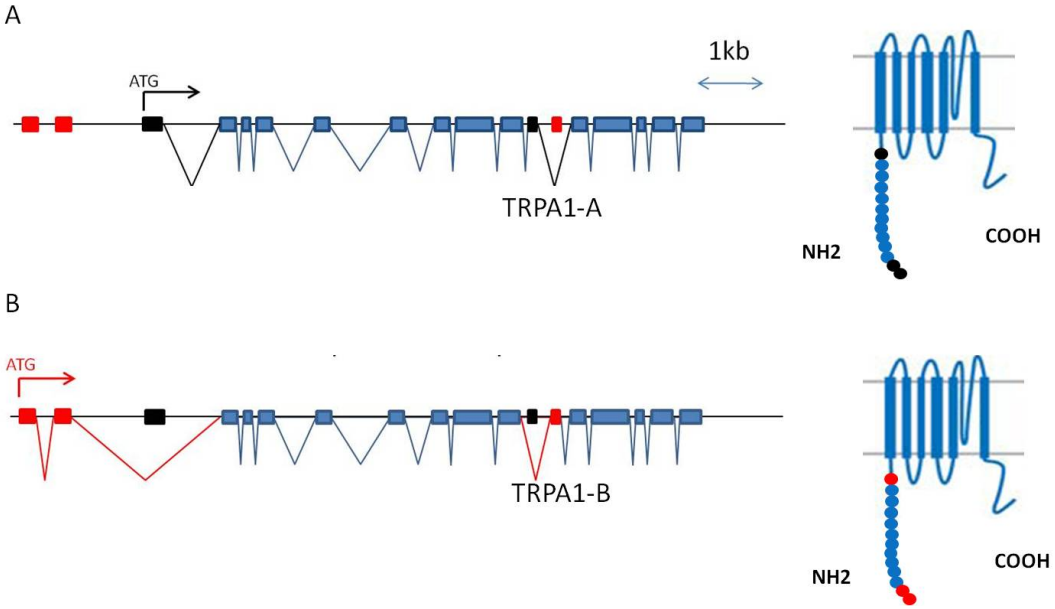
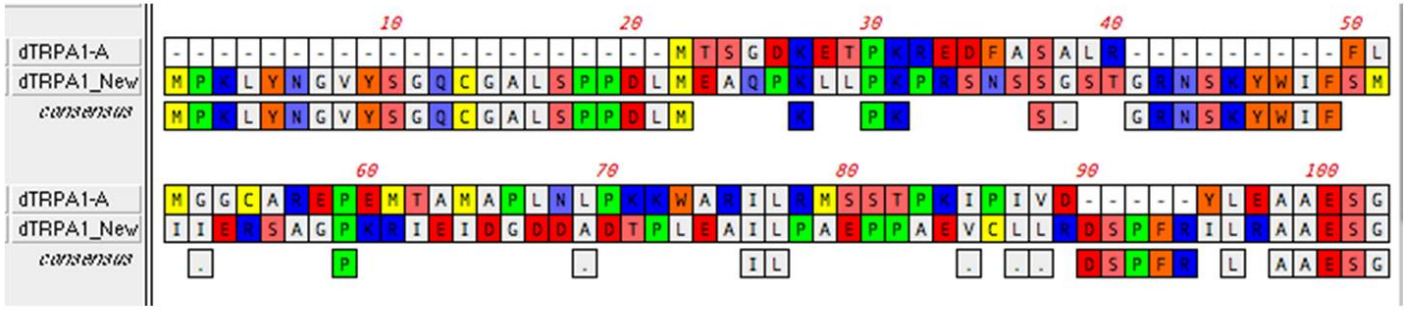


Figure 5-7: Gene structures of the two different isoforms of *dTrpA1* and the corresponding changes in the two channel proteins

(A) The canonical warmth activated dTRPA1-A. (B) The newly cloned isoform of dTRPA1-B alternatively spliced out the exons labeled in red and incorporates exons labeled in black. The changes are located at the N- and C- termini of the channel proteins.

A Exon 1 of dTRPA1-A aligned with exons 1 and 2 of dTRPA1-B

(dTRPA1-B is proline and serine rich relative to dTRPA1-A)



B Exon 10 of dTRPA1-A aligned with exon 11 dTRPA1-B

(dTRPA1-B is basic relative to dTRPA1-A)

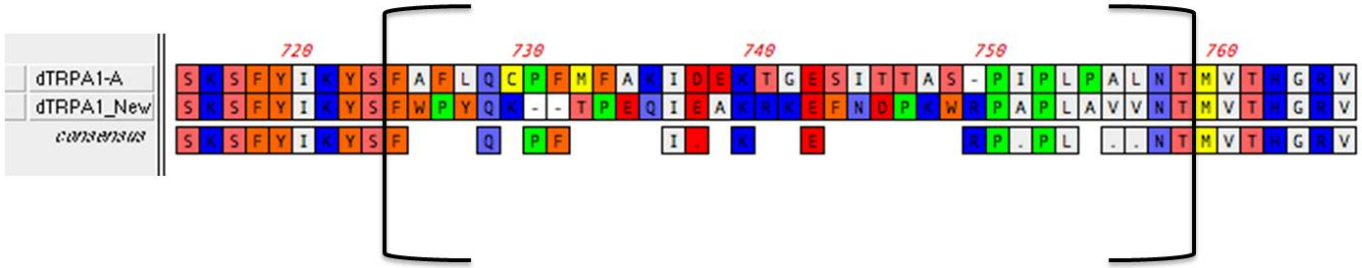


Figure 5-8: Sequence alignment of the two alternatively spliced regions of the two dTRPA1 isoforms

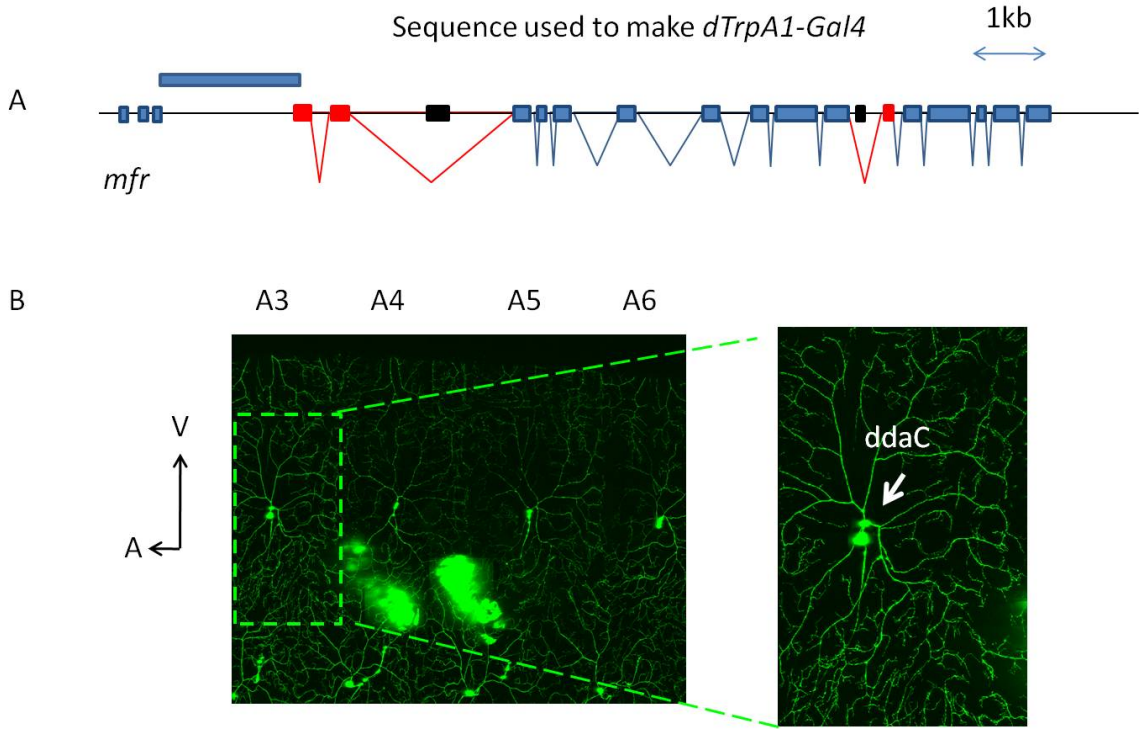


Figure 5-9: *dTrpA1-B-GAL4* is specifically expressed in Class IV md neurons

(A) The genomic sequence used to generate the *dTrpA1-B-GAL4* reporter is located in the intergenic region between the first exon of *dTrpA1-B* and *mfr,* as labeled with the blue bar above the gene structure. (B)The left image is the maximum-intensity projection of four dorsal segment of *dTrpA1-B-GAL4*>>UAS-mCD8GFP 3rd instar larvae. The right image is a zoomed in view of the ddaC neuron, which is a dorsal neuron of the three class IV neurons in each hemi-segment.

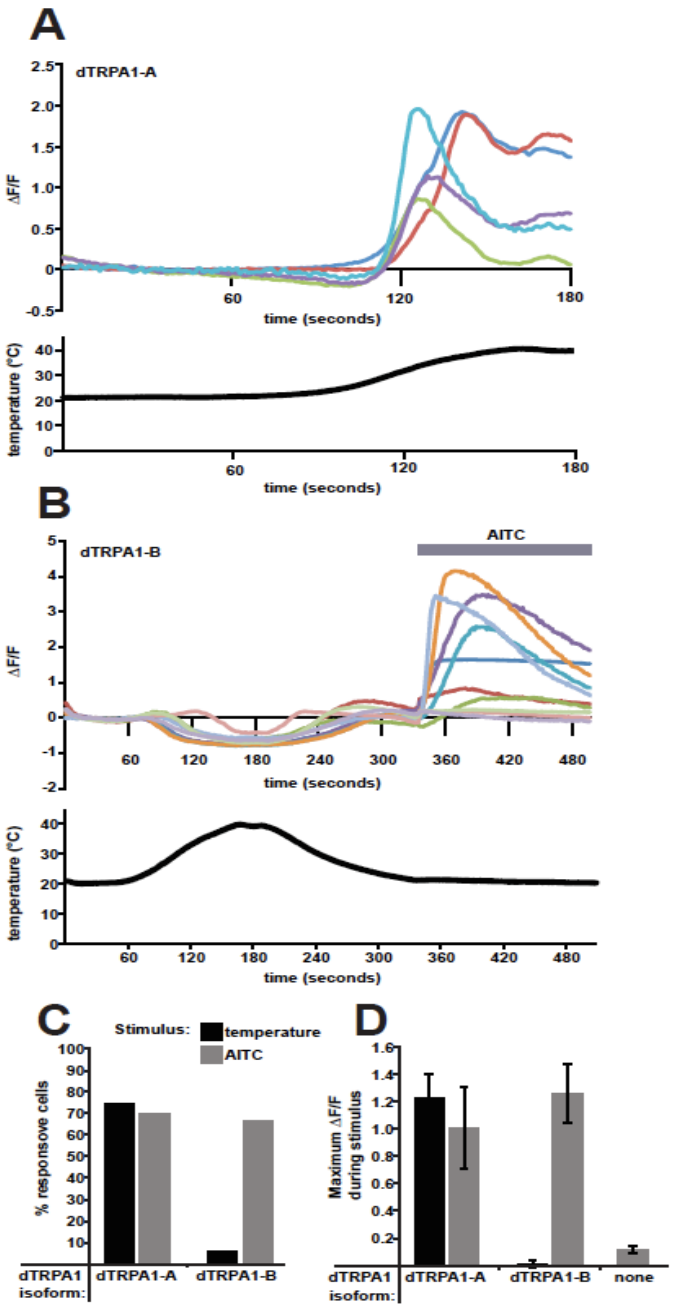
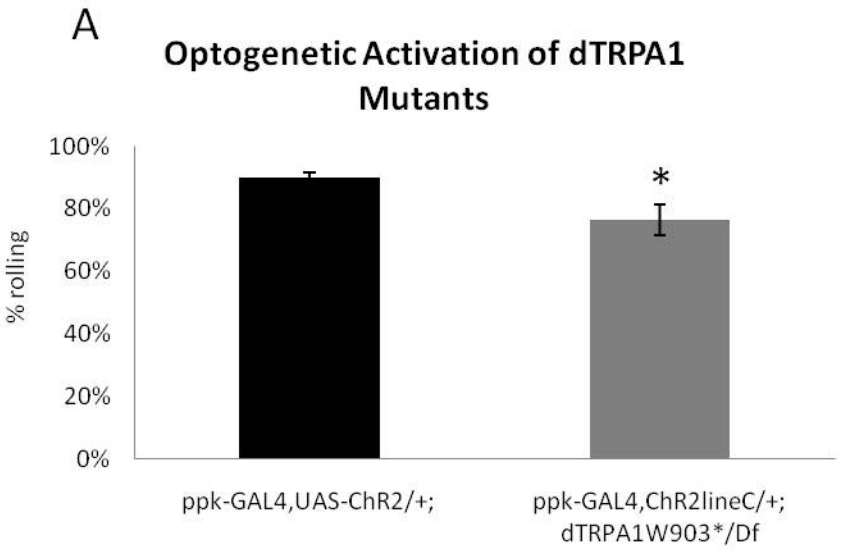


Figure 5-10: dTRPA1-B is not activated by temperature in a heterologous expression system but responds to allyl isothiocyanate (AITC)

Figure 5-10: dTRPA1-B is not activated by temperature in a heterologous expression system but can respond to allyl isothiocyanate (AITC)

S2R+ cells were cotransfected with either dTrpA1-A or dTrpA1-B and genetically encoded Ca2+ indicator GCaMP 3.0. (A) Cells transfected with the canonical dTRPA1-A can be activated by warmth (25 °C ~30 °C) as indicated by the increase of intracellular Ca2+ level. (B) Cells transfected with the new dTRPA1-B do not have Ca2+ response to temperatures between 22 °C~42 °C. These cells show robust responses to application of 0.033mM AITC. (C) 74% of dTRPA1-A transfected cells (as indicated by green fluorescence of cotransfected GCaMP3.3) showed response to warmth as compared to only 6% of the dTRPA1-B transfected cells (black bars). 70% of dTRPA1-A and 67% of dTRPA1-B transfected cells showed responses to 0.033mM AITC (gray bars). Cells showing a $\Delta F/F$ of 0.5 or greater during temperature ramp or AITC were considered to be responsive.(D) The average peak $\Delta F/F$ during the heat ramp for all dTRPA1-A transfected cells was 1.2 as compared to 0.01 for dTRPA1-B transfected cells (black bars). These responses are significantly different with a $p<0.001$. The average peak $\Delta F/F$ during 0.033mM AITC application for all dTRPA1-A transfected cells was 1.0 as compared to 1.3 for dTRPA1-B transfected cells and 0.1 for untransfected cells (gray bars).



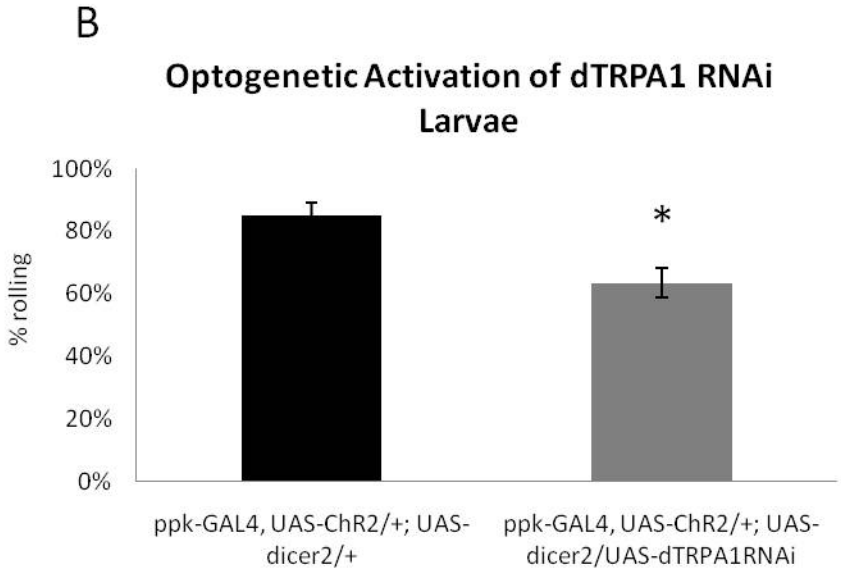


Figure 5-11: *dTrpA1* is required for normal optogenetic activation of class IV neurons triggered nocifensive behavior.

Figure 5-11: dTrpA1 is required for normal optogenetic activation of Class IV neurons triggered nocifensive behavior (A) *dTrpA1* mutant larvae were defective in optogenetic activation of class IV neurons triggered nocifensive behavior ($P < 0.05$). (B) RNAi knockdown of dTRPA1 expression in Class IV md neurons also leads to reduced responses to optogenetic activation triggered nocifensive behavior. ($P < 0.05$).

Chapter 6 Identification of two types of thermal sensory cells in the fly antennae

6.1 Introduction

Thermotaxis refers to the locomotion behavior that animals use to locate to their preferred living temperatures within an innocuous temperature range. This is especially critical for heterothermic animals, whose body temperatures are largely determined by the external environment temperature, as the environmental temperature will directly determine the metabolism and body function. Different species apply different mechanisms and strategies for thermotaxis.

C. elegans exhibits characteristic thermotaxis behavior. They have a strong preference for the temperature at which they are cultivated and well fed between 16 °C and 25 °C. When placed on a thermal gradient, they will migrate to the growth temperature and move isothermally. Conversely, when cultivated at a temperature and food deprived, they will avoid that temperature (Hedgecock and Russell 1975; Mori and Ohshima 1995). Bilateral sensory neurons located near the tip of the nose, AFD, can mediate most aspects of temperature sensation. When AFD neurons are ablated, animals no longer display preference for their cultivating temperature (Mori and Ohshima 1995). Recently, AFD neurons have been shown to respond to warming with increased intracellular Ca^{2+}. Interestingly, this response is not to absolute temperature

per se but to the directional change of temperatures (Kimura, Miyawaki et al. 2004). This neuron also has bidirectional intracellular Ca^{2+} response to warming and cooling (Clark, Biron et al. 2006). However, it is not known what the actual temperature sensor is in AFD neurons that directly transduce temperature information to neuronal activity.

Drosophila shows a preference for specific temperatures. Adult flies have a strong preference for 24 °C on a linear thermal gradient raging from 18 °C to 31.5 °C (Sayeed and Benzer 1996). Unlike nematodes, the cultivation temperature of flies does not influence their temperature preference. The thermal sensor for this preference is localized to the 3rd antennal segment as flies with both sides of their 3rd antennal segments removed became thermo-blind below 31.5 °C (Sayeed and Benzer 1996). However, the identities and properties of the temperature sensing neurons in the antennae remain largely unknown.

Recently, a small set of neurons located in the 3rd antennal segment of the antenna and three cells in the arista expressing a *Drosophila* TRPP channel *Brv1* were found to be activated by cold temperatures around 17 °C. The three other neurons in the arista respond to warm temperatures around 27 °C. The cold sensing neurons and the warmth sensing neurons project to two distinct glomeruli in the proximal antennal-protocerebrum (PAP) (Gallio, Ofstad et al. 2011).

Thermosensory neurons have also been found in the brain. A pair of anterior cell (AC) neurons expressing dTRPA1 were shown to be warmth activated and *dTrpA1* was found to be required for thermotaxis behavior of adult flies (Hamada, Rosenzweig et al. 2008). On a thermal gradient from 18 °C to 32 °C, *dTrpA1* mutants failed to avoid warm region between 28 °C to 32 °C as wild type flies did. A *dTrpA1-A-GAL4* reporter and dTRPA1 anti-sera co-labeled a small set of anterior cells (AC neurons) located in the *Drosophila* brain. Calcium imaging in these neurons revealed intracellular calcium increase in response to warming with an average threshold of 24.9 °C and this response was missing in *dTrpA1* mutants. The activation threshold of these neurons is consistent with the activating threshold of dTRPA1 channel. Flies that selectively express dTRPA1 in the AC neurons select normal temperatures, whereas flies in which dTRPA1 function is reduced or eliminated choose warmer temperatures. These results suggest that *dTrpA1* is essential for adult flies to avoid innocuous warm temperatures and it functions in the internal brain AC neurons (Hamada, Rosenzweig et al. 2008).

Drosophila larvae also exhibit thermotaxis behavior. In one experimental setting, larvae prefer 18 °C versus either 11 or 30 °C (Liu, Yermolaieva et al. 2003). This avoidance of cold (11 °C) is likely to be mediated by Type I neurons innervating the terminal organ, which is located at the anterior part of the larva. Increased intracellular Ca^{2+} was observed in these neurons in response to cooling from 18 °C to 10 °C (Liu,

Yermolaieva et al. 2003). Extracellular recording in the terminal organ also detected increased spiking with cooling. Blocking the synaptic transmission in terminal organ abolishes the cold avoidance (Liu, Yermolaieva et al. 2003; Rosenzweig, Kang et al. 2008).

In addition, several TRP channels have been shown to be required for normal thermotaxis behavior although their sites of action are not clear. TRP and TRPL have been found to be required for cold avoidance in larvae (Rosenzweig, Kang et al. 2008). The mechanisms for the two TRP channels' involvement in cold sensation seem to be different from their function in the *Drosophila* visual system, which is dependent on the phospholipase C NORPA. In a different experimental paradigm, larvae preferred to migrate to the coolest zone on a thermal gradient of 27 °C to 41 °C. This warmth avoidance is mediated by dTRPA1 through some unspecified neurons (Rosenzweig, Brennan et al. 2005). *dTrpA1* is also required for discriminating subtle temperature differences between the optimal temperature of 18 °C and 19 °C~24 °C. The activation of dTRPA1 in this case is downstream of phospholipase C-dependent cascade and site of action is not clear (Kwon, Shim et al. 2008).

Here, using a GAL4-reporter of the new dTRPA1 isoform TRPA1-B, I have identified a population of cells in the 3rd antennal segment of adult flies that specifically respond to cooling, but not to warming. *dTrpA1* mutants have reduced intracellular

calcium level in these cells at temperatures below 22 °C and also have defects in cool avoidance. In addition, I have unexpectedly identified another population of cooling sensing neurons that are known to be olfactory neurons. The olfactory co-receptor, Or83b, plays an important role in cool avoidance.

6.2 Materials and methods

6.2.1 Fly strains and husbandry

The following strains were used: *dTRPA1^{W903*}/Df(3L)ED4415*, *dTrpA1-B-GAL4*, *ΔOr84b*, *Or83b-GAL4 VP16*, *UAS-Cameleon3.3*, *UAS-Cameleon3.3;;Df(3L)ED4415/TM6b*, *dTrpA1-B-GAL4*; *dTRPA1^{W903*}/k87*, *dTrpA1-B-GAL4,UAS-nsyb-GFP*, *dTrpA1-B-GAL4*; *UAS-mCD8GFP*. Flies were raised on standard cornmeal medium. All stocks were maintained at 25 °C and 75% humidity on a 12-12 light dark cycle.

6.2.2 Adult fly thermotaxis assay

Adult fly thermotaxis assays were performed as described previously with slight modifications (Sayeed and Benzer 1996). The same thermal gradient apparatus was used as in (Sayeed and Benzer 1996). A thermal gradient was established using one aluminum slab (56cm X18cm X2.5cm) on top of one heat block (Corning model PC320) on one side and a cold block (Teca Corp. (Chicago) Model LHP-800CP) on the other side. A moisturized Whatman paper (3mm) was placed on top of the aluminum slab which the

flies are in direct contact with. A plexiglass cover with 4 lanes (27cm X 18cm) was used to keep the flies underneath. The temperature on the thermogradient ranged from 16.5 °C to 32.5 °C on the surface of the moisturized Whatman paper. Before each experiment, the inner side of the plexiglass cover was first lightly coated with rain-X so that flies would remain on the thermogradient without climbing onto the plexiglass lid during the experiment. Adult flies were aged between 3-10 days old. 40 flies (20 females and 20 males) were used in each trial. Flies were aspirated from vials to the thermogradient under the plexiglass cover through a hole which was centered in the thermogradient. The hole was later covered by tape after the flies were loaded. Once in the lane, the flies were allowed to move freely for 15 min in the dark. At the end of the 15 min, a picture was taken so that the distribution of the flies could be determined. In most cases, two genotypes were run side by side in the two centered lanes. After the trials, flies were anesthetized with CO_2 and removed from the thermogradient.

6.2.3 Calcium imaging of cells in the 3rd antennal segments of flies in response to temperature changes

UAS-YC3.3; ; dTrpA1-B-GAL4/+; and *UAS-YC3.3;; dTrpA1-B-GAL4/+, dTrpA1^{W903*}/Df(3L)ED4415* flies were aged between 8 and 12 days old. An individual fly was first anesthetized on a CO_2 pad. It was then removed from the pad with its wings being held using tweezers to let it recover from the CO_2 anesthetization for 3 min. Then the fly head was removed using a razor blade at the point where it is connected to the

thorax. The fly head was then placed immediately in an imaging chamber (Warner Instrument Series 20 Chamber platform P-2) between two cover slips. The fly 3rd antennal segments were placed facing the bottom cover slip and was directly imaged through the objective lens. The temperature change was controlled by a water perfusion system. The perfusion system was set up so that the water passed through an inline solution heater (Warner Instrument Model SH-27B) before flowing through the imaging chamber and into the aspirator. A thermocouple that was connected to a data acquisition board (Warner Instrument Corporation TC-324B) was placed in the water reservoir of the imaging chamber to measure the temperature in real time. Since the fly head was placed below the imaging chamber, it remained dry throughout the experiment. Microscopy was performed on a Zeiss LSM 5 Live Confocal System with 20X objective lens and 440 nm laser. Images were acquired as time series at 1 frame/sec.

6.2.4 Immunostaining in the 3rd antennal segment and adult brain

3rd antennal segments were dissected and fixed for 10 minutes in 4% paraformaldehyde (PFA). The antennae were then permeabilized in 0.4% *Triton* X-100 PBS for 2 hours. For immunostaining, the antennae were stained with anti-Or83b antibody (mouse) and anti-GFP antibody (Rabbit) followed by anti-mouse 568 and anti-rabbit 488 secondary antibodies. Antennae were mounted in Vectashield (H-1000 Vector laboratories, Inc.). Microscopy was performed on a Zeiss LSM 5 Live Confocal System.

Adult fly brain were dissected and fixed for 15 minutes at room temperature in 4% paraformaldehyde. The brains were then permeabilized and blocked in 0.2% *Triton* X-100 PBS with 2% BSA for 1 hour. For immunostaining, the brains were stained with primary anti-nc82 antibody (mouse, 1:200) and primary anti-GFP antibody (rabbit, 1:1000) at 4°C overnight. Anti-mouse Alexa 568 (1:1000) and anti-rabbit Alexa 488 (1:1000) were used for secondary antibody staining at 4°C overnight. For the flies with their antennae or both antennae and maxillary palps removed, the cuts were done between 1-4 days after *eclosion* of the flies and the flies were aged for 2 additional weeks before dissecting their brains. The intact flies were also aged for the same period of time when their brains were dissected. Brains were mounted in Vectashield. Z-stack confocal images were taken on a Zeiss LSM 5 Live Confocal System.

6.3 Results

6.3.1 *dTrpA1-B-GAL4* reporter is expressed in a subset of cells in the 3rd antennal segment

After building a *UAS-mCD8GFP*; *dTrpA1-B-GAL4* line to visualize the expression pattern of *dTrpA1-B-GAL4*, I observed strong expression in cells (estimated to be between 140 and 200 cells) in the 3rd antennal segment (Figure 6-1 A). Since the 3rd antennal segment is best known for its function in olfaction, I first hypothesized that

these cells were olfactory receptor neurons (ORNs). To test this, I stained the *UAS-mCD8GFP*; *dTrpA1-B-GAL4* antennae with anti-Or83b antibody, which is expressed in about 80% of the olfactory receptor neurons (Larsson, Domingos et al. 2004) (Figure 6-1-B_{1-4}). I found that the *dTrpA1-B-GAL4* cells and the *Or83b* neurons do not overlap and that the *dTrpA1-B-GAL4* cells are relatively large and flat compared to the *Or83b* expressing neurons. The anti-Or83b stained long and thin dendrites that are characteristics of olfactory sensilla. In contrast, the *dTrpA1-B-GAL4* expressing cells did not possess dendrites that innervated chemosensory sensilla.

6.3.2 *dTrpA1-B-GAL4* expressing antennal neurons project to glomeruli in the antennal lobe

ORNs located in the 3rd antennal segments project to the antennal lobe (AL), which is the primary olfactory association center in *Drosophila*. The antennal lobe is composed of glomeruli that are synaptic modules between the terminal branches of olfactory receptor neuron axons and the dendritic arborizations of their target neurons (Hildebrand and Shepherd 1997). The general validity of the two principles of olfaction hold true in both mammals and insects: each ORN expresses a single olfactory receptor and each glomerulus receives inputs from ORNs that expressing the same olfactory receptor (Hildebrand and Shepherd 1997; Couto, Alenius et al. 2005).

The *dTrpA1-B-GAL4* expressing cells do not overlap with Or83b expressing odorant neurons. Nevertheless, I decided to examine whether the *dTrpA1-B-GAL4*

expressing cells also project to the antennal lobe. To investigate that, we have generated *dTrpA1-B-GAL4*, *UAS-nsyb-GFP* flies for visualizing glomeruli expression. Indeed, expression in several specific glomeruli was observed. In intact flies, expression in 11 glomeruli of each antennal lobe were detected. Strong expression was detected in 4 glomeruli: VA7l, VA6, VL2p and VM4. In addition, relatively weaker expression was detected in 7 other glomeruli: VA1d, DA2, DA4, VA4, DL3, VL2a and VM7 (Figure 6-2 A_{1-3})(Laissue, Reiter et al. 1999). To confirm that the glomeruli projections are from the terminal branches of *dTrpA1-B-GAL4* expressing neurons, both sides of the 3rd antennal segments were ablated and see how this would affect the glomeruli pattern. Eliminating both sides of the 3rd antennal segments resulted in the disappearance of fluorescence in most glomeruli, with only three glomeruli remaining: VA7l, VA4 and VM7 (Figure 6-2 B_{1-3}). These three glomeruli are known to be from projections of ORNs in the maxillary palps (Couto, Alenius et al. 2005). Indeed, when both of the 3rd antennal segments and maxillary palps were removed, all of the glomeruli projections disappeared (Figure 6-2 C_{1-3}). Four of the antennal lobe glomeruli have odorant receptors mapped to them yet four others (VL2p, VL2a, VM4, DA4) remain unassociated with identified odorant receptors (Table 6-1) (Couto, Alenius et al. 2005; Vosshall and Stocker 2007). It's interesting to note that *dTrpA1-A* expressing AC neurites elaborated within two such

unassociated glomeruli, VL2a and VL2p (Hamada, Rosenzweig et al. 2008), which are also targeted by *dTrpA-B-GAL4*.

6.3.3 *dTrpA1-B-GAL4* expressing antennal neurons have elevated intracellular calcium level in response to cooling

It was shown previously that *dTrpA1* mutant flies were defective in avoiding warm temperatures between 28 °C and 32 °C in a thermotaxis assay (Hamada, Rosenzweig et al. 2008). Since the 3rd antennal segment was shown to be required for flies to distinguish temperatures on a thermogradient below 31.5 °C (Sayeed and Benzer 1996), the expression in the 3rd antennal segment made us wonder if *dTrpA1-B-GAL4* expressing neurons could have temperature sensing properties. To test this hypothesis, I used *dTrpA1-B-GAL4* to drive expression of UAS-YC3.3, a genetically encoded calcium indicator, for calcium imaging of the temperature response in the antennae. Calcium imaging was carried out on freshly cut fly heads that were exposed to temperature ramps (Figure 6-3 A, B). Indeed, I observed increased intracellular calcium levels in response to temperature changes.

The temperature ramps incorporates two cycles of temperature changes, with each one going from room temperature 22 °C to high temperature 33 °C and then back to room temperature (Figure 6-3 C). We observed one major calcium increase in each temperature cycle (Figure 6-3 C). Surprisingly, the dTRPA1 neurons responded to

directional change of temperatures, but not to absolute temperatures. Although the fly head had experienced the same temperature range during the warming phase, there is no major calcium increase. Both the onset and the peak of the major calcium increase only happened during the cooling phase (Figure 6-3 C). This calcium signal during the cooling phase was not due to delayed response to the warming because with a temperature protocol where the heads were held at 30 °C and then cooled, a single peak of calcium increase during the cooling phase was still observed (L.Z. D.T.W unpublished data).

6.3.4 *dTrpA1* mutants show an altered response to cooling in *dTrpA1-B-GAL4* antennal neurons

To investigate how *dTrpA1* may be involved in this cooling-triggered response, I performed calcium imaging of these neurons in *dTrpA1* mutant background (*UAS-YC3.3;; dTrpA1-B-GAL4*/+, *dTrpA1*^{W903*}/*Df(3L)ED4415*). In the mutants, these neurons still showed increased intracellular calcium in response to cooling. However, the mutants showed a premature decay of the response. Wild type (n=12) initiated the calcium increase shortly after the cooling onset and the response maintained at relatively high level during the cooling process before it reached 22 °C (Figure 6-4). On the other hand, *dTrpA1* mutants' response (n=12) to cooling was more narrowly tuned to temperature range. Intracellular calcium level was significantly lower compared to wild type at temperatures below 22 °C while the onset and peak calcium signal intensity remained

the same (Figure 6-4). Both wild type and mutant flies did not show calcium response during the warming process. This result indicates that there is dTRPA1 independent cooling sensor in these neurons. However, dTRPA1 functions to maintain this signal over a wider range of temperatures. It's important to note that this GAL4 driver of *dTrpA1* should reflect the expression pattern of the new isoform dTRPA1-B. This result is consistent with the fact that dTRPA1-B is not a temperature sensitive channel in a heterologous expression system. Its function in these neurons is likely to be the same as in the nociceptive neurons, downstream of sensory transduction.

6.3.5 *dTrpA1* mutants show defects in avoiding cool temperature zone in thermotaxis

After identifying a new population of neurons located in the 3rd antennal segment that respond to cooling and that *dTrpA1* mutants have decayed response at relatively higher temperatures, we set out to test how this difference at cellular levels may affect thermotaxis behavior. To examine this effect, we designed our thermogradient to cover the temperature range of our calcium imaging experiment with a high end at 32.5 °C and with a low end at 16.5 °C (Figure 6-5 A-1, 2). Wild type flies showed a bell-shaped distribution centered around 25 °C and avoided temperatures below 19.5 °C and above 31.5 °C (Figure 6-5 B-1, 2). *dTrpA1* mutants, however, failed to avoid temperatures both below 19.5 °C and above 31.5 °C (Figure 6-5 B-1, 2). The warmth avoidance defect is consistent with previous observations by Hamada et al.

(Hamada, Rosenzweig et al. 2008). The defects in cool temperature avoidance between 16.5 °C and 19.5 °C, on the other hand, have not been observed before. This is likely due to the fact that the previous study used a slightly different thermal gradient with the low end temperature being slightly higher (18 °C) than ours (16.5 °C). Since mutants have reduced intracellular Ca^{2+} response in these neurons below 22 °C, we propose that the *dTrpA1-B- GAL4* expressing cooling sensing cells mediate this cooling avoidance behavior in adult fly thermotaxis.

6.3.6 *Or83b* antennal neurons show slow calcium oscillations in response to cooling

In addition, we have identified another population of neurons that can respond specifically to cooling. Using Or83b-GAL4 as a control to demonstrate the specificity of the response we have observed in *dTrpA1—B-GAL4* cells, we unexpectedly observed altered intracellular calcium level in these neurons in response to cooling (Figure 6-6 A). Instead of a single Ca^{2+} peak, the intracellular calcium level slowly oscillated once triggered by the cooling and this effect lasted for minutes. The onset of the oscillation was between 22-25 °C, slightly below the optimal temperature flies normally prefer. These oscillations typically had cycles between 30 and 50s. Oscillations increased the baseline level of calcium in these cells as indicated by the calcium indicator. Oscillations

were stopped by the opposite change of temperature, warming, and can be triggered again by cooling (Figure 6-6 B).

6.3.7 *Or83b* is required for flies to avoid cool temperature zone in thermotaxis assay

We then tested *Or83b* mutants for their thermotaxis behavior. The mutant flies (Or83b-GAL4VP16/+; ΔOr83b & UAS-Or83b/+; ΔOr83b) showed striking defects in avoiding the cool region of the thermogradient or were strongly attracted to the cool region of the thermal gradient (Figure 6-7 A, B). 68% of the *Or83b-GAL4VP16/+; ΔOr83b* flies and 44% of *the UAS-Or83b/+; ΔOr83b* flies stayed in the 16.5 °C ~17.5 °C temperature zone at the end of the 15 min assay, while wild type flies completely avoided this temperature zone. To show that *Or83b* is indeed required for normal thermotaxis behavior, we then performed rescue experiment expressing *Or83b* with *Or83b-GAL4VP16* in *ΔOr83b* mutant background (*Or83b-GAL4VP16/+; ΔOr83b & UAS-Or83b/+; ΔOr83b*). A significantly smaller proportion of the rescued flies were seen in the cool temperature zone, confirming *Or83b* is required in the *Or83b* neurons for the flies to avoid non-optimal cool temperatures.

6.4 Discussion

Taken together, we have identified two populations of antennal cells that respond to cooling in different ways. The *dTrpA1-B-GAL4* expressing cells had single calcium spike triggered by cooling while the *Or83b* neurons initiated intracellular

calcium oscillations that lasted for minutes as long as the temperature does not go back up. Accordingly, both the *dTrpA1* and *Or83b* mutants showed defects in cool avoidance on a thermogradient.

The sense of environmental temperatures in order to respond properly is of vital importance to all living organisms. How the signals of temperatures are coded by the nervous system is largely unknown. In recent years, the discovery of temperature sensitive TRP channels that can be activated by different range of temperatures has greatly expanded our knowledge of the temperature sensation modality.

TRPV1 is activated by noxious heat of above 42 °C and in mice it is expressed primarily in small to medium-diameter sensory DRG neurons (C and Aδ fibers). TRPV2 is activated by even higher temperature with a threshold of 52 °C and is detected in medium to large diameter DRG neurons (Aδ and Aβ fibers).

TRPM8 is expressed in a subset of small-diameter C fibers in the DRG and TG neurons, but is not co-expressed with TRPV1 (McKemy, Neuhausser et al. 2002; Peier, Moqrich et al. 2002). In heterologous expression systems, TRPM8 is activated by cool temperatures between 23~28 °C. TRPM8 deficient mice have severe defects in their behavioral and physiological responses to cold temperatures above 10 °C (Bautista, Siemens et al. 2007; Colburn, Lubin et al. 2007).

Another TRP channel TRPA1 respond to lower temperature that is referred to as noxious cold. In heterologous expression systems, TRPA1 is activated by cold temperatures with a threshold of about 17 °C (Story, Peier et al. 2003; Karashima, Talavera et al. 2009). TRPA1 is expressed in a subset of TRPV1 expressing DRG cells. TRPV3 and TRPV4 are both activated by warm temperatures in heterologous expression systems with thresholds of ~34-38 °C for TRPV3 and ~27-35 °C for TRPV4 (Smith, Gunthorpe et al. 2002; Watanabe, Vriens et al. 2002). Instead of being expressed in DRG neurons, TRPV3 is detected in keratinocytes in the skin (Peier, Reeve et al. 2002; Plant and Strotmann 2007) and TRPV4 is broadly expressed in both neural and non-neural tissues, including in the hypothalamus, which is a brain region controlling body temperature regulation (Plant and Strotmann 2007). Keratinocytes have also been proposed to have a direct role in temperature sensation.

So far, in mammals, available evidence suggests that temperature coding may be orchestrated by different cell types and that each express different sets of temperature sensitive ion channels to respond to a defined temperature range (Lumpkin and Caterina 2007). If this model is true, it explains how animals are able to distinguish discrete temperatures. However, with our current knowledge of the temperature activation spectrum of TRP channel, this model would suggest a relatively low resolution for temperature sensation. On the other hand, animals can distinguish

relatively subtle change of temperatures that are within the range of temperature that activate one TRP channel. How does the nervous system code for such accurate and dynamic temperature information? One efficient way would be to add a second set of temperature information, the directional change of temperature to the system. When the information of absolute temperature range and the information of the directional change of temperature are combined, the brain can assess the current temperature, the change of temperature and predict the temperature in a near future point.

Our discovery of such cooling-sensing neurons may shed light onto the complex temperature coding system. Several temperature sensors have already been identified in the adult fly, including the warmth sensing AC neurons in the brain, the warmth sensing cells in the arista and the cold sensing cells in the 3rd antennal segment (Hamada, Rosenzweig et al. 2008; Gallio, Ofstad et al. 2011). Sayeed et al. has found the 3rd antennal segment to be of critical importance for temperature sensation as flies with their 3rd antennal segments ablated completely lost temperature preference below 31.5 °C. Our findings of two populations of cooling sensing neurons in the 3rd antennal segment confirm the importance of the 3rd antennal segment in temperature sensation. The unique activating mode of these neurons combined with other temperature sensing elements will give the flies continuous temporal and spatial map (combined with other

environmental cues such as visual cues) of temperature as they navigate their environment.

Both the *dTrpA1-B-GAL4* expressing cells and *Or83b* neurons project to the antennal lobes, suggesting some of the temperature information may be processed in the antennal lobe. The *dTrpA1-B-GAL4* expressing neurons project to several glomeruli (11), including VL2a and VL2p, two glomeruli that temperature-sensing AC neurons project to. It would be of great interest to find out how these individual glomeruli responds to cooling: do all of them respond or only a subset respond, are their responses synchronized or do different glomeruli respond at different temperature ranges in a orchestrated way?

It's intriguing to see two cooling sensing population of neurons in the antenna. The two populations have different characteristics. The *dTrpA1-B GAL4* cells have a higher temperature onset, almost as soon as the temperature started to drop in our temperature setting. On the other hand, the *Or83b* neurons tend to have a lower temperature onset, typically below 25 °C. In addition, the response of the two populations is quite different. The *dTrpA1-B-GAL4* cells give a single intracellular calcium spike while the *Or83b* neurons have prolonged slow calcium oscillations that are stopped by warming. It's not known how the two types of response might interact with

each other. When we imaged the *dTrpA1-B-GAL4* cells in *Or83b* mutants, we didn't see an obvious difference in the population calcium response to cooling.

Cells respond to directional change of temperature have also been reported in other systems. In *C. elegans*, AFD neurons respond to warming with increased intracellular Ca^{2+} and this response is not to absolute temperature per se but directional change of temperatures. The threshold of this response is established by the cultivation temperature; the AFD neurons respond to warming only above the cultivation temperature (Kimura, Miyawaki et al. 2004). In addition, it was shown that AFD can sense temperature changes as small as 0.05 °C via short-term adaptation. They also have a bidirectional thermosensory response by increasing intracellular calcium to warming and lowering intracellular calcium to cooling, allowing the AFD neurons to phase-lock their calcium dynamics to oscillatory thermosensory inputs (Clark, Biron et al. 2006).

In *Aedes aegypti*, a pair of antagonistic thermoreceptive cells associated with two peg-in-pit sensilla have been found on the antennal tip. One of the cells responds to rapid warming (warm cell) and the other cell respond to cooling (cold cell). Both the warm cell and cold cell respond not only to the actual temperature at particular instance in time but also to the rate with which temperature changes (Gingl, Hinterwirth et al. 2005).

It's also interesting that neurons known to be olfactory neurons displaying responses to temperature changes. Whether this means these neurons are polymodal or temperature sensation is integrated into olfaction is unknown. Or83b is a co-receptor expressed in most of antennal olfactory neurons (70% ~80%) and *Or83b* mutants are defective in detecting most odors (Larsson, Domingos et al. 2004). A very interesting hypothesis for the latter possibility is that the calcium oscillations boost the excitability of the olfactory neurons to lower concentration of odors. Indeed, at lower temperatures, compounds are less volatile compared to at higher temperatures. The elevated baseline calcium level during oscillation may facilitate the excitation of these neurons when lower concentrations of odors bind to the receptors.

Glomerulus	Odorant Receptors
VA7l (maxillary palp)	Or46a
VA6	Or82a
VL2p	N/A
VM4	N/A
VA1d	Or88a
DA2	Or56a
DA4	N/A
VA4 (maxillary palp)	Or85d
DL3	Or65a, b & c
VL2a	N/A
VM7	Or42a

Table 6-1: List of *dTrpA1-B-GAL4* expression positive glomeruli and their known associated odorant receptors

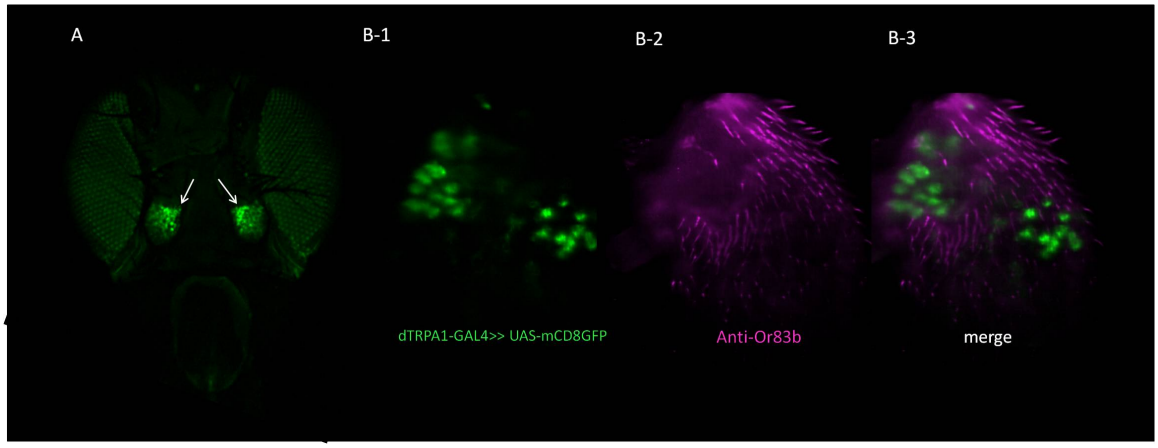


Figure 6-1: *dTrpA1-B-GAL4* is expressed in the 3rd antennal segment of adult flies

(A) Expression in the 3rd antennal segment is detected with *dTrpA1-B-GAL4>>UAS-mCD8GFP.* (B1-B3) *dTrpA1-B-GAL4* expression did not colocalize with Or83b expressing neurons in the 3rd antennal segment. (B-1) Cells expressing *dTrpA1-B-GAL4>>UAS-mCD8GFP* (Green). (B-2) The dendrites of Or83b expressing neurons were labeled by the anti-Or83b antibody (magenta). (B-3) Merge of B-1 and B-2. *dTrpA1-B-GAL4* expressing cells did not overlap with Or83b expressing neurons.

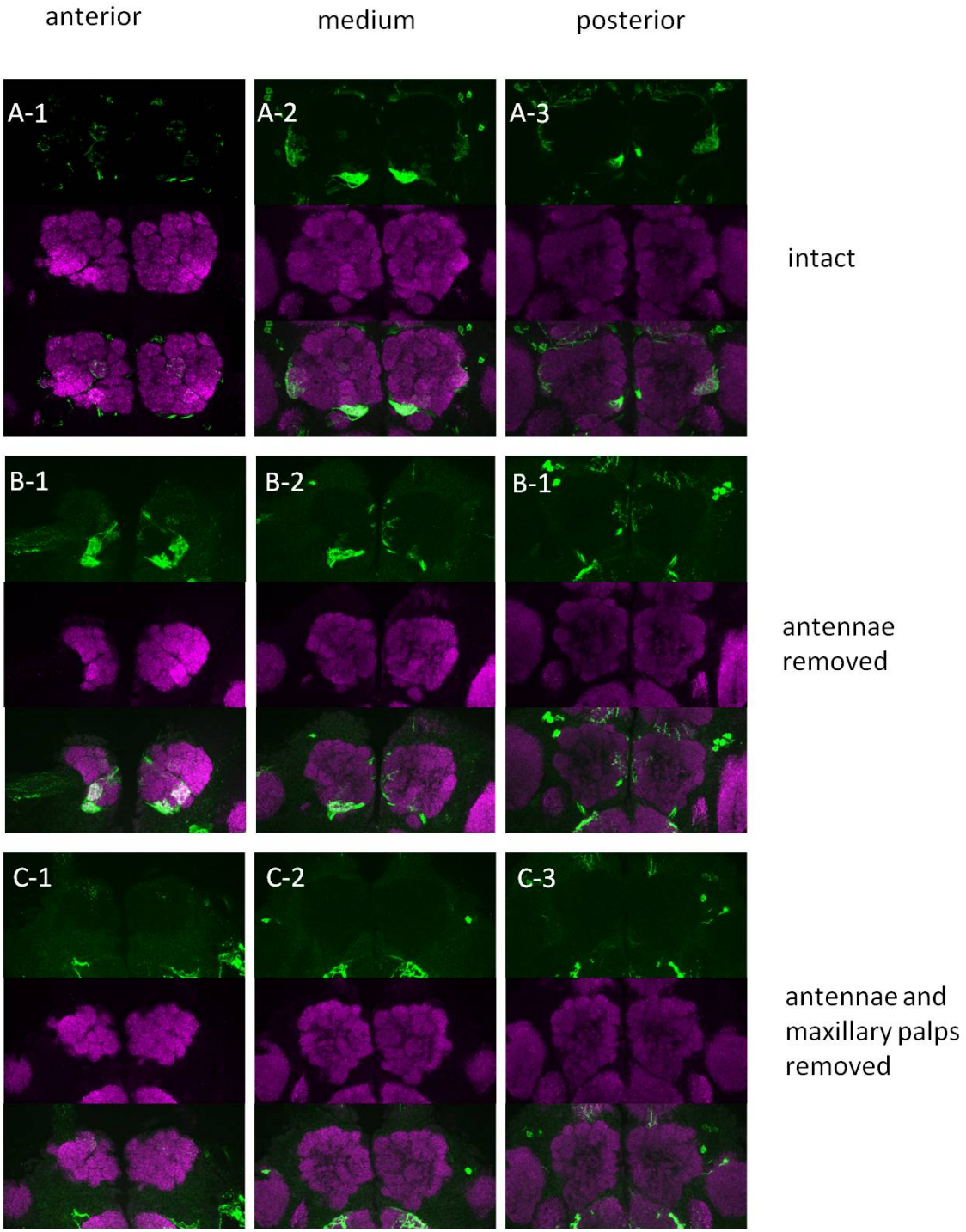


Figure 6-2: ***dTrpA1-B-GAL4*** **antennal lobe expression pattern**

Figure 6-2: *dTrpA1-B-GAL4* antennal lobe expression pattern

dTrpA1-B-GAL4 expressing antennal lobe glomeruli in *TrpA1-B-GAL4 >>UAS-nsyb-GFP* flies. Expression positive glomeruli were visualized using anti-GFP staining (green, top panel); individual glomerulus was visualized using nc82 antibody staining (magenta, middle panel). The merged images are shown in the bottom panel. The anterior images were maximum intensity projections of 5 slides of 6-12 μm from the anterior part of the antennal lobe; the medium images were maximum intensity projections of 5 slides of 17-23 μm from the anterior part of the antennal lobe and the posterior images were maximum intensity projections of 5 slides of 28-34 μm from the anterior part of the antennal lobe (A_{1-3}) *dTrpA1-B*-GAL4 antennal lobe expression pattern in intact flies. Expression was detected in four glomeruli with relative strong expression: VA7l, VA6, VL2p and VM4 and seven other glomeruli with relative weak expression: VA1d, DA2, DA4, VA4, DL3, VL2a and VM7. (B_{1-3}) *dTrpA1-B*-GAL4 antennal lobe expression pattern in flies with both sides of the 3rd antennal segments removed. Most of the GFP positive glomeruli disappeared with only 3 remaining: VA7l, VA4 and VM7. (C_{1-3}) removing both the 3rd antennal segments and the maxillary palps completely eliminated GFP-positive glomeruli.

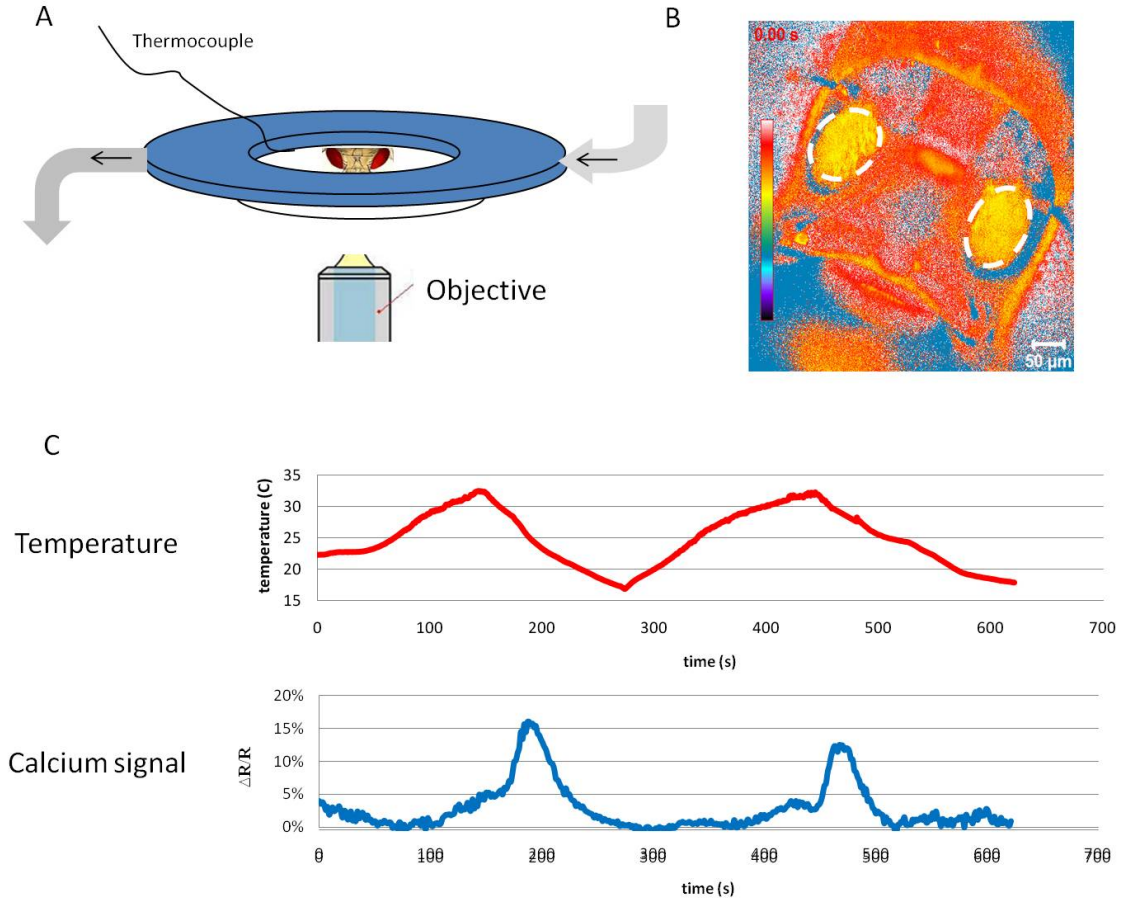


Figure 6-3: Identification of cooling sensing cells using calcium imaging of *dTrpA1-B-GAL4* expressing cells

(A) Experimental set up. See more details in the Methods. (B) Pseudocolored picture of a fly head during calcium imaging. The two circled areas are the two 3rd antennal segments. (C) A representative trace of cooling triggered intracellular Ca^{2+} increase. The top panel is the temperature trace. The bottom panel is the calculated ΔR/R, which corresponds to the intracellular Ca^{2+} level. Both of the two spikes of Ca^{2+} increase started during the cooling phases of the temperature ramp.

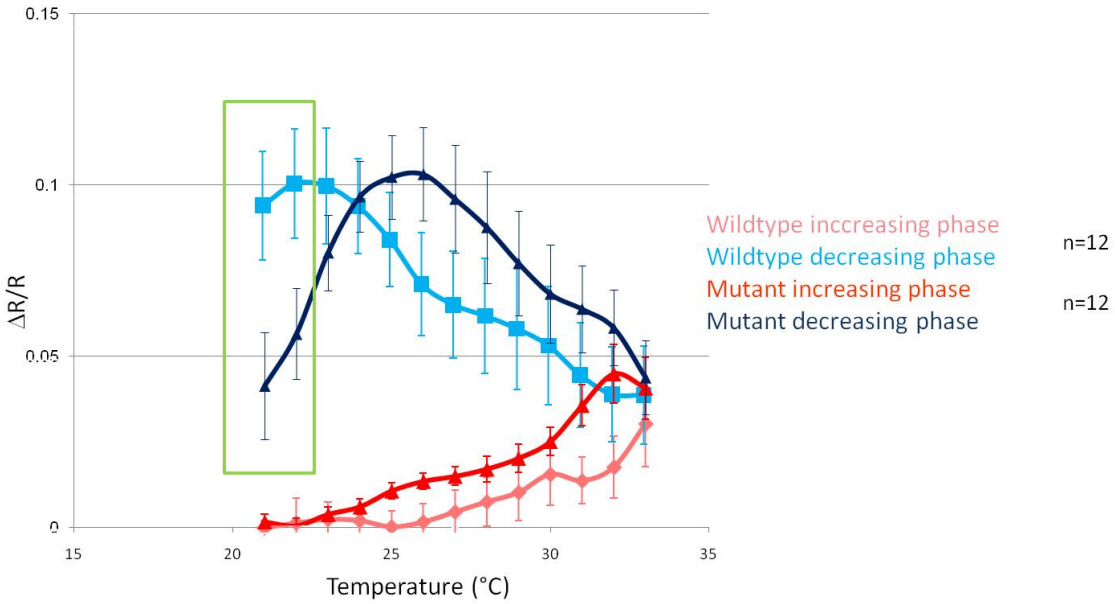


Figure 6-4: *dTrpA1* mutant flies have a premature decay in the antennal cooling response

The average ΔR/R responses in *dTrpA1-B-GAL4* expressing 3rd antennal segment cells in both wild type and mutant flies. Both the wild type (light blue line) and *dTrpA1* mutant (dark blue line) flies showed significantly increased intracellular Ca^{2+} during the cooling phase but not during warming phase (pink line and red line). *dTrpA1* mutants have a premature decay of the Ca^{2+} response at temperatures below 22 °C (green box).

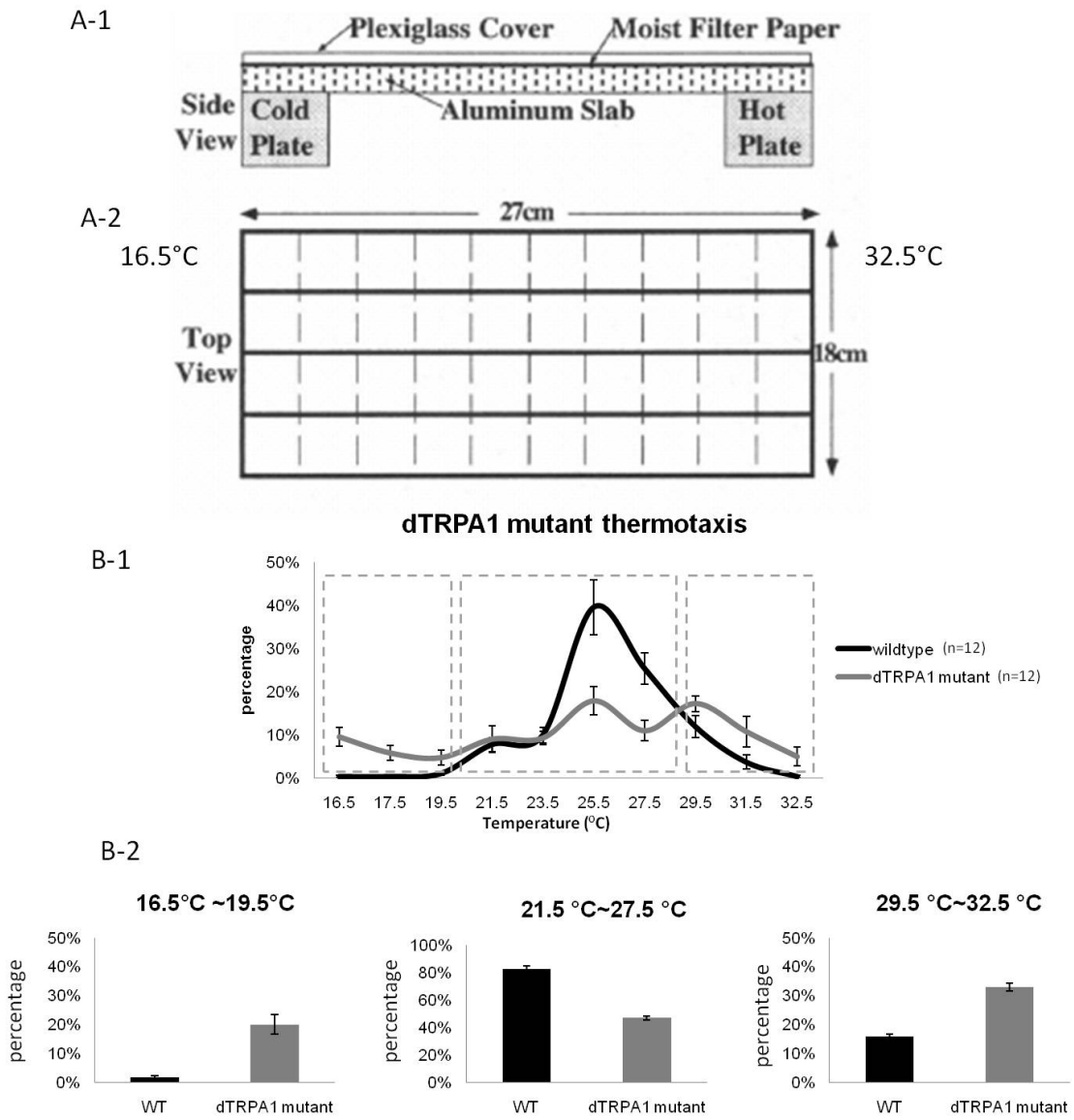


Figure 6-5: *dTrpA1* mutants are defective in thermotaxis behavior

Figure 6-5: *dTrpA1* mutants are defective in thermotaxis behavior

(A1-A2) Thermotaxis apparatus set up (adapted from (Sayeed and Benzer 1996). (A) The thermogradient was established by placing one aluminum slab on top of one heat block on one side and a cold block on the other side. For more details, see Methods. (B1-B2) *dTrpA1* mutants were defective in avoiding both warm and cool region of the thermal gradient. (B-1) Distributions of wild type and dTrpA1 mutants on a thermogradient from 16.5 °C to 32.5 °C. Wild type flies had bell shaped distribution centered around 25 °C and avoided temperatures below 19.5 °C and above 29.5 °C. (B-2) Comparison of wild type and dTRPA1 mutants distribution in three different temperature zones: 16.5 °C~19.5 °C, 21.5 °C ~27.5 °C and 29.5 °C~32.5 °C. Compared to wild type flies, *dTrpA1*mutants were defective in avoiding both the cool (16.5 °C~19.5 °C) and warm (29.5 °C~32.5 °C) region of the thermogradient.

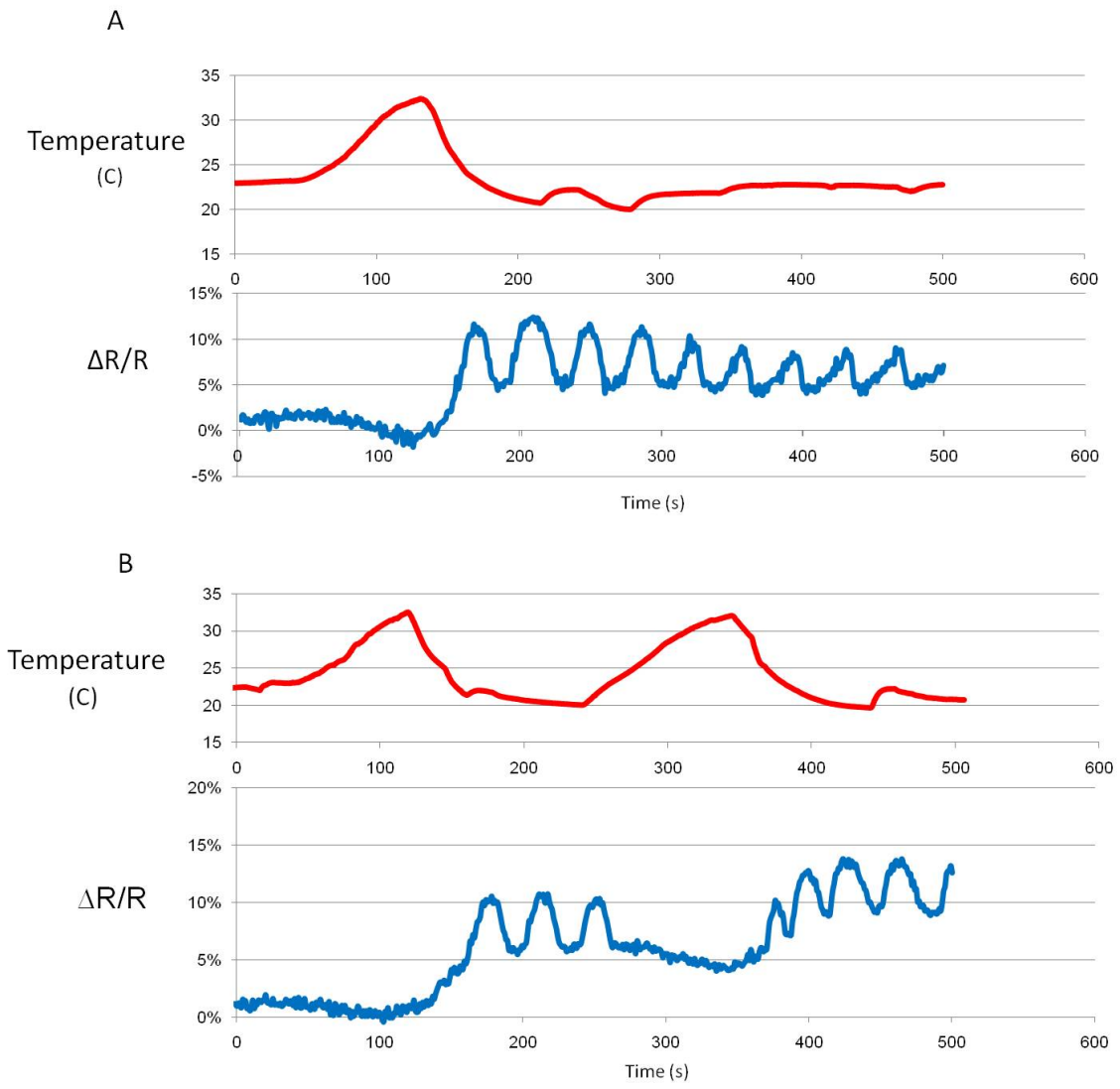


Figure 6-6: *Or83b* expressing neurons have cooling triggered intracellular Ca^{2+} oscillations.

Figure 6-6: *Or83b* expressing neurons have cooling triggered intracellular Ca^{2+} oscillations. (A) Cooling triggered prolonged Ca^{2+} oscillations in the *Or83b* neurons. The top panel is the temperature trace and the bottom panel is the calculated $\Delta R/R$. (B) Cooling triggered Ca^{2+} oscillations were terminated by warming. The top panel is the temperature trace and the bottom panel is the calculated $\Delta R/R$.

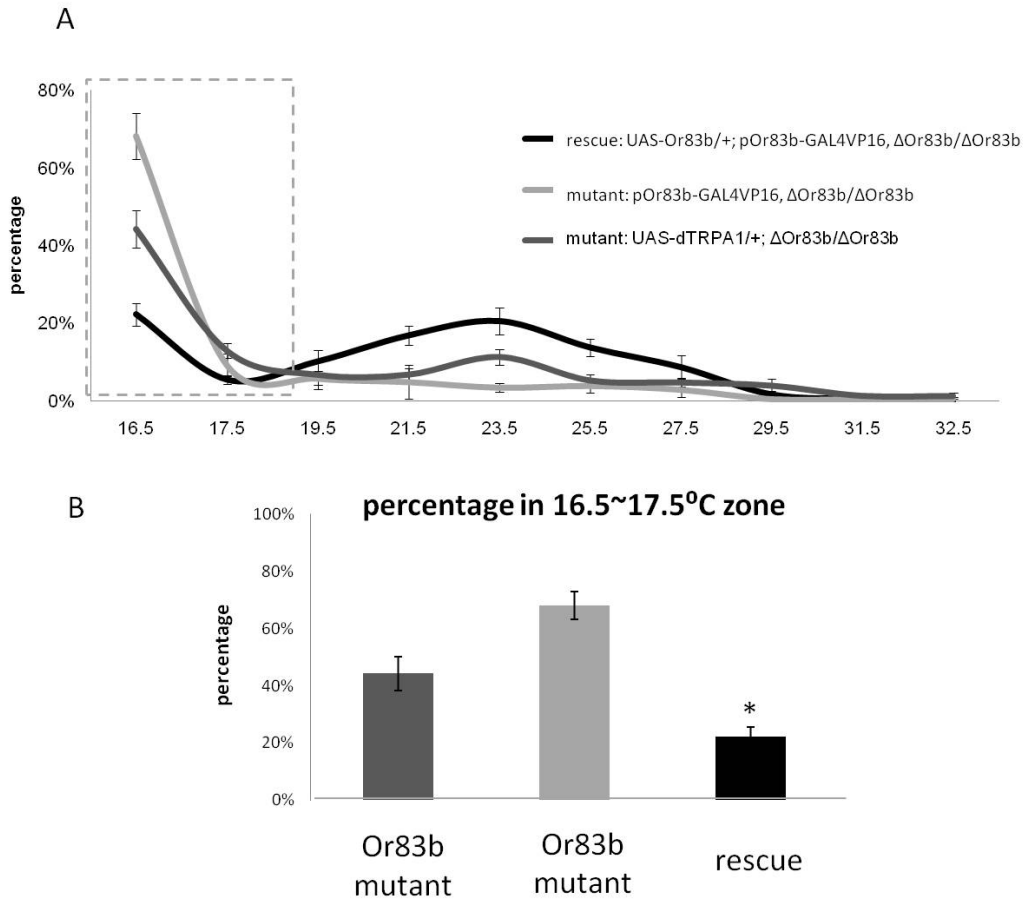


Figure 6-7: *Or83b* is required for flies to avoid cool temperatures

(A) Distributions of *Or83b* mutants *Or83b-GAL4vp16*, *ΔOr83b/Or83b* & *UAS-Or83b/+*; *ΔOr83b*) and rescued flies (*UAS-Or83b/+*; *Or83b-GAL4*, ΔOr83b/ ΔOr83b) on a thermogradient from 16.5 °C to 32.5 °C. (B) Comparison of the percentage of flies in the cool zone. The mutants had severe defects in avoiding the cool temperature zone between 16.5 °C and 17.5 °C. *Or83b-GAL4>>UAS-Or83b* in *ΔOr83b* background partially restored the cool avoidance behavior.

References

Adams, C. M., M. G. Anderson, et al. (1998). "Ripped pocket and pickpocket, novel Drosophila DEG/ENaC subunits expressed in early development and in mechanosensory neurons." J Cell Biol **140**(1): 143-152.

Ainsley, J. A., J. M. Pettus, et al. (2003). "Enhanced locomotion caused by loss of the Drosophila DEG/ENaC protein Pickpocket1." Curr Biol **13**(17): 1557-1563.

Al-Anzi, B., J. W. D. Tracey, et al. (2006). "Response of Drosophila to Wasabi Is Mediated by painless, the Fly Homolog of Mammalian TRPA1/ANKTM1." Current Biology **16**(10): 1034-1040.

Anderson, R., M. Ressegui, et al. (2005). "Calcium/Calmodulin-Dependent protein kinase II alters structural plasticity and cytoskeletal dynamics in Drosophila." Journal of neuroscience **25**(39): 8878-8888.

Anishkin, A. and C. Kung (2005). "Microbial mechanosensation." Current Opinion in Neurobiology **15**(4): 397-405.

Arenkiel, B. R., J. Peca, et al. (2007). "In vivo light-induced activation of neural circuitry in transgenic mice expressing channelrhodopsin-2." Neuron **54**(2): 205-218.

Bandell, M., G. M. Story, et al. (2004). "Noxious Cold Ion Channel TRPA1 Is Activated by Pungent Compounds and Bradykinin." Neuron **41**(6): 849-857.

Bargal, R., N. Avidan, et al. (2000). "Identification of the gene causing mucolipidosis type IV." Nat Genet **26**(1): 118-123.

Bargmann, C. I., E. Hartwieg, et al. (1993). "Odorant-selective genes and neurons mediate olfaction in C. elegans." Cell **74**(3): 515-527.

Basbaum, A. I., D. M. Bautista, et al. (2009). "Cellular and Molecular Mechanisms of Pain." Cell **139**(2): 267-284.

Basbaum, A. I. and T. M. Jessell (2000). Principles of Neuroscience New York, McGraaw-Hill.

Bautista, D. M., S.-E. Jordt, et al. (2006). "TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents." Cell **124**(6): 1269-1282.

Bautista, D. M., P. Movahed, et al. (2005). "Pungent products from garlic activate the sensory ion channel TRPA1." PNAS **102**(34): 12248-12252.

Bautista, D. M., J. Siemens, et al. (2007). "The menthol receptor TRPM8 is the principal detector of environmental cold." Nature **448**(7150): 204-208.

Bhave, G., W. Zhu, et al. (2002). "cAMP-Dependent Protein Kinase Regulates Desensitization of the Capsaicin Receptor (VR1) by Direct Phosphorylation." Neuron **35**(4): 721-731.

Bianchi, L. and M. Driscoll (2002). "Protons at the Gate: DEG/ENaC Ion Channels Help Us Feel and Remember." Neuron **34**(3): 337-340.

Bodmer, R., R. Carretto, et al. (1989). "Neurogenesis of the peripheral nervous system in Drosophila embryos: DNA replication patterns and cell lineages." Neuron **3**(1): 21-32.

Bodmer, R. and Y. N. Jan (1987). "Morphological differentiation of the embryonic peripheral neurons in <i>Drosophila</i&gt." Development Genes and Evolution **196**(2): 69-77.

Bounoutas, A. and M. Chalfie (2007). "Touch sensitivity in <i>Caenorhabditis elegans</i&gt." Pflügers Archiv European Journal of Physiology **454**(5): 691-702.

Boyden, E. S., F. Zhang, et al. (2005). "Millisecond-timescale, genetically targeted optical control of neural activity." Nat Neurosci **8**(9): 1263-1268.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development **118**(2): 401-415.

Brauchi, S., G. Orta, et al. (2007). "Dissection of the components for PIP2 activation and thermosensation in TRP channels." Proceedings of the National Academy of Sciences **104**(24): 10246-10251.

Brewster, R. and R. Bodmer (1995). "Origin and specification of type II sensory neurons in Drosophila." Development **121**(9): 2923-2936.

Canessa, C. M., J.-D. Horisberger, et al. (1993). "Epithelial sodium channel related to proteins involved in neurodegeneration." Nature **361**(6411): 467-470.

Cantor, R. S. (1997). "The Lateral Pressure Profile in Membranes: A Physical Mechanism of General Anesthesia." Biochemistry **36**(9): 2339-2344.

Cantor, R. S. (1999). "The influence of membrane lateral pressures on simple geometric models of protein conformational equilibria." Chemistry and Physics of Lipids **101**(1): 45-56.

Carton, Y. (1984). "Experimental-Analysis of Interactions between Drosophila-Melanogaster and Parasitic Wasp - Leptopilina-Boulardi (Sympatry, Allopatry, Xenopatry)." Genetics Selection Evolution **16**(4): 417-430.

Carton, Y., M. Bouletreau, et al. (1986). The *Drosophila* parasitic wasps. The Genetics and Biology of Drosophla. M. Ashburner, H. L. Carson and J. N. Thompson Jr.**:** 347-394.

Caterina, M. J., A. Leffler, et al. (2000). "Impaired Nociception and Pain Sensation in Mice Lacking the Capsaicin Receptor." Science **288**(5464): 306-313.

Caterina, M. J., T. A. Rosen, et al. (1999). "A capsaicin-receptor homologue with a high threshold for noxious heat." Nature **398**(6726): 436-441.

Caterina, M. J., M. A. Schumacher, et al. (1997). "The capsaicin receptor: a heat-activated ion channel in the pain pathway." Nature **389**(6653): 816-824.

Chalfie, M. and M. Au (1989). "Genetic control of differentiation of the Caenorhabditis elegans touch receptor neurons." Science **243**(4894 Pt 1): 1027-1033.

Chalfie, M. and J. Sulston (1981). "Developmental genetics of the mechanosensory neurons of Caenorhabditis elegans." Developmental Biology **82**(2): 358-370.

Chalfie, M., J. E. Sulston, et al. (1985). "The neural circuit for touch sensitivity in Caenorhabditis elegans." J Neurosci **5**(4): 956-964.

Chalfie, M. and J. N. Thomson (1982). "Structural and functional diversity in the neuronal microtubules of Caenorhabditis elegans." J Cell Biol **93**(1): 15-23.

Chalfie, M. and E. Wolinsky (1990). "The identification and suppression of inherited neurodegeneration in Caenorhabditis elegans. ." Nature **345**: 410-416.

Chang, G., R. H. Spencer, et al. (1998). "Structure of the MscL Homolog from Mycobacterium tuberculosis: A Gated Mechanosensitive Ion Channel." Science **282**(5397): 2220-2226.

Chelur, D. S., G. G. Ernstrom, et al. (2002). "The mechanosensory protein MEC-6 is a subunit of the C. elegans touch-cell degenerin channel." Nature **420**(6916): 669-673.

Christensen, A. P. and D. P. Corey (2007). "TRP channels in mechanosensation: direct or indirect activation?" Nat Rev Neurosci **8**(7): 510-521.

Chuang, H.-h., E. D. Prescott, et al. (2001). "Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition." Nature **411**(6840): 957-962.

Chyb, S., P. Raghu, et al. (1999). "Polyunsaturated fatty acids activate the Drosophila light-sensitive channels TRP and TRPL." Nature **397**(6716): 255-259.

Clapham, D. E. (2003). "TRP channels as cellular sensors." Nature **426**(6966): 517-524.
Clark, D. A., D. Biron, et al. (2006). "The AFD Sensory Neurons Encode Multiple Functions Underlying Thermotactic Behavior in Caenorhabditis elegans." J. Neurosci. **26**(28): 7444-7451.

Coburn, C. M. and C. I. Bargmann (1996). "A Putative Cyclic Nucleotide-Gated Channel Is Required for Sensory Development and Function in C. elegans." Neuron **17**(4): 695-706.

Colbert, H. A., T. L. Smith, et al. (1997). "OSM-9, A Novel Protein with Structural Similarity to Channels, Is Required for Olfaction, Mechanosensation, and Olfactory Adaptation in Caenorhabditis elegans." J. Neurosci. **17**(21): 8259-8269.

Colburn, R. W., M. L. Lubin, et al. (2007). "Attenuated Cold Sensitivity in TRPM8 Null Mice." Neuron **54**(3): 379-386.

Corfas, G. and Y. Dudai (1989). "Habituation and dishabituation of a cleaning reflex in normal and mutant Drosophila." J. Neurosci. **9**(1): 56-62.

Cosens, D. J. and A. Manning (1969). "Abnormal electroretinogram from a *Drosophila* mutant." Nature **224**(5216): 285-287.

Couto, A., M. Alenius, et al. (2005). "Molecular, Anatomical, and Functional Organization of the Drosophila Olfactory System." Current Biology **15**(17): 1535-1547.

Davis, J. B., J. Gray, et al. (2000). "Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia." Nature **405**(6783): 183-187.

Dietzl, G., D. Chen, et al. (2007). "A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila." Nature **448**(7150): 151-156.

Dorfman, R., L. Glazer, et al. (2002). "Elbow and Noc define a family of zinc finger proteins controlling morphogenesis of specific tracheal branches." Development **129**(15): 3585-3596.

Driscoll, M. and M. Chalfie (1991). "The mec-4 gene is a member of a family of Caenorhabditis elegans genes that can mutate to induce neuronal degeneration." Nature **349**(6310): 588-593.

Du, H., G. Gu, et al. (1996). "Extracellular proteins needed for C. elegans mechanosensation." Neuron **16**(1): 183-194.

Effertz, T., R. Wiek, et al. (2011). "NompC TRP Channel Is Essential for Drosophila Sound Receptor Function." Current biology : CB **21**(7): 592-597.

Emtage, L., G. Gu, et al. (2004). "Extracellular proteins organize the mechanosensory channel complex in C. elegans touch receptor neurons." Neuron **44**(5): 795-807.

Ernstrom, G. G. and M. Chalfie (2002). "Genetics of sensory mechanotransduction." Annu Rev Genet **36**: 411-453.

Flores, C. and W. Engels (1999). "Microsatellite instability in Drosophila spellchecker1 (MutS homolog) mutants." Proc Natl Acad Sci U S A **96**(6): 2964-2969.

Fukushige, T., Z. Siddiqui, et al. (1999). "MEC-12, an alpha-tubulin required for touch sensitivity in C. elegans." J Cell Sci **112**(3): 395-403.

Gallio, M., T. A. Ofstad, et al. (2011). "The Coding of Temperature in the Drosophila Brain." Cell **144**(4): 614-624.

Gao, F. B., J. E. Brenman, et al. (1999). "Genes regulating dendritic outgrowth, branching, and routing in Drosophila." Genes Dev **13**(19): 2549-2561.

Gao, Q., B. Yuan, et al. (2000). "Convergent projections of Drosophila olfactory neurons to specific glomeruli in the antennal lobe." Nat Neurosci **3**(8): 780-785.

Garcia-Anoveros, J. and D. P. Corey (1997). "The molecules of mechanosensation." Annu Rev Neurosci **20**: 567-594.

Gillo, B., I. Chorna, et al. (1996). "Coexpression of Drosophila TRP and TRP-Like Proteins in Xenopus Oocytes Reconstitutes Capacitative Ca2+ Entry." Proceedings of the National Academy of Sciences of the United States of America **93**(24): 14146-14151.

Gingl, E., A. Hinterwirth, et al. (2005). "Sensory Representation of Temperature in Mosquito Warm and Cold Cells." Journal of Neurophysiology **94**(1): 176-185.

Gong, Z., W. Son, et al. (2004). "Two Interdependent TRPV Channel Subunits, Inactive and Nanchung, Mediate Hearing in *Drosophila*." The Journal of Neuroscience **24**(41): 9059.

Goodman, M. B. (2006). Mechanosensation, Wormbook, ed. the C. elegans research community. E. M. Jorgensen and J. M. Kaplan.

Goodman, M. B., G. G. Ernstrom, et al. (2002). "MEC-2 regulates C. elegans DEG/ENaC channels needed for mechanosensation." Nature **415**(6875): 1039-1042.

Goodman, M. B. and E. M. Schwarz (2003). "Transducing touch in Caenorhabditis elegans." Annu Rev Physiol **65**: 429-452.

Gopfert, M. C., J. T. Albert, et al. (2006). "Specification of auditory sensitivity by Drosophila TRP channels." Nat Neurosci **9**(8): 999-1000.

Grandl, J., H. Hu, et al. (2008). "Pore region of TRPV3 ion channel is specifically required for heat activation." Nat Neurosci **11**(9): 1007-1013.

Grandl, J., S. E. Kim, et al. (2010). "Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain." Nat Neurosci **13**(6): 708-714.

Grueber, W., L. Jan, et al. (2002). "Tiling of the Drosophila epidermis by multidendritic sensory neurons." Development **129**: 2867-2878.

Grueber, W. B., B. Ye, et al. (2007). "Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology." Development **134**(1): 55-64.

Guler, A. D., H. Lee, et al. (2002). "Heat-Evoked Activation of the Ion Channel, TRPV4." J. Neurosci. **22**(15): 6408-6414.

Gullingsrud, J. and K. Schulten (2003). "Gating of MscL Studied by Steered Molecular Dynamics." Biophysical Journal **85**(4): 2087-2099.

Hall, D. H. and Z. F. Altun (2008). C. elegans atlas, CSHL Press.

Hamada, F. N., M. Rosenzweig, et al. (2008). "An internal thermal sensor controlling temperature preference in Drosophila." Nature **454**(7201): 217-220.

Hardie, R. C. and B. Minke (1992). "The trp gene is essential for a light-activated Ca2+ channel in Drosophila photoreceptors." Neuron **8**(4): 643-651.

Hartenstein, V. (1988). Development of Drosophila larval sensory organs: Spatiotemporal pattern of sensory neurones, peripheral axonal pathways and sensilla differentiation

Hedgecock, E. M. and R. L. Russell (1975). "Normal and mutant thermotaxis in the nematode Caenorhabditis elegans." Proc Natl Acad Sci USA **72**(10): 5.

Hensel, H. (1981). "Thermoreception and Temperature Regulation." Academic Press **London**.

Hildebrand, J. G. and G. M. Shepherd (1997). "MECHANISMS OF OLFACTORY DISCRIMINATION:Converging Evidence for Common Principles Across Phyla." Annual Review of Neuroscience **20**(1): 595-631.

Hong, S.-T., S. Bang, et al. (2006). "Histamine and Its Receptors Modulate Temperature-Preference Behaviors in Drosophila." J. Neurosci. **26**(27): 7245-7256.

Hu, H.-J., G. Bhave, et al. (2002). "Prostaglandin and Protein Kinase A-Dependent Modulation of Vanilloid Receptor Function by Metabotropic Glutamate Receptor 5: Potential Mechanism for Thermal Hyperalgesia." J. Neurosci. **22**(17): 7444-7452.

Huang, M. and M. Chalfie (1994). "Gene interactions affecting mechanosensory transduction in Caenorhabditis elegans." Nature **367**(6462): 467-470.
Huang, M., G. Gu, et al. (1995). "A stomatin-like protein necessary for mechanosensation in C. elegans." Nature **378**(6554): 292-295.

Hughes, C. L. and J. B. Thomas (2007). "A sensory feedback circuit coordinates muscle activity in Drosophila." Mol Cell Neurosci **35**(2): 383-396.

Hwang, R. Y., L. Zhong, et al. (2007). "Nociceptive Neurons Protect Drosophila Larvae from Parasitoid Wasps." Current Biology **17**(24): 2105-2116.

Hwang, R. Y., L. Zhong, et al. (2007). "Nociceptive neurons protect Drosophila larvae from parasitoid wasps." Curr Biol **17**(24): 2105-2116.

Inada, H., H. Ito, et al. (2006). "Identification of Guanylyl Cyclases That Function in Thermosensory Neurons of Caenorhabditis elegans." Genetics **172**(4): 2239-2252.

Jones, N. G., R. Slater, et al. (2004). "Acid-Induced Pain and Its Modulation in Humans." The Journal of Neuroscience **24**(48): 10974-10979.

Jones, R. C., 3rd, L. Xu, et al. (2005). "The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3." J Neurosci **25**(47): 10981-10989.

Jordt, S.-E., D. M. Bautista, et al. (2004). "Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1." Nature **427**(6971): 260-265.

Jordt, S.-E., D. D. McKemy, et al. (2003). "Lessons from peppers and peppermint: the molecular logic of thermosensation." Current Opinion in Neurobiology **13**(4): 487-492.

Julius, D. and A. I. Basbaum (2001). "Molecular mechanisms of nociception." Nature **413**(6852): 203-210.

Kang, K., S. R. Pulver, et al. (2010). "Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception." Nature **464**(7288): 597-600.

Karashima, Y., N. Damann, et al. (2007). "Bimodal Action of Menthol on the Transient Receptor Potential Channel TRPA1." The Journal of Neuroscience **27**(37): 9874-9884.

Karashima, Y., K. Talavera, et al. (2009). "TRPA1 acts as a cold sensor in vitro and in vivo." Proceedings of the National Academy of Sciences **106**(4): 1273-1278.

Kernan, M., D. Cowan, et al. (1994). "Genetic dissection of mechanosensory transduction: Mechanoreception-defective mutations of drosophila." Neuron **12**(6): 1195-1206.

Kernan, M. and C. Zuker (1995). "Genetic approaches to mechanosensory transduction." Current Opinion in Neurobiology **5**(4): 443-448.

Kerstein, P., D. del Camino, et al. (2009). "Pharmacological blockade of TRPA1 inhibits mechanical firing in nociceptors." Molecular Pain **5**(1): 19.

Kim, J., Y. D. Chung, et al. (2003). "A TRPV family ion channel required for hearing in Drosophila." Nature **424**(6944): 81-84.

Kim, S. H., Y. Lee, et al. (2010). "Drosophila TRPA1 channel mediates chemical avoidance in gustatory receptor neurons." Proceedings of the National Academy of Sciences **107**(18): 8440-8445.

Kimura, K. D., A. Miyawaki, et al. (2004). "The C. elegans Thermosensory Neuron AFD Responds to Warming." Current Biology **14**(14): 1291-1295.

Knowlton, W. M., A. Bifolck-Fisher, et al. (2010). "TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics in vivo." Pain **150**(2): 340-350.

Komatsu, H., Y.-H. Jin, et al. (1999). "Functional reconstitution of a heteromeric cyclic nucleotide-gated channel of Caenorhabditis elegans in cultured cells." Brain Research **821**(1): 160-168.

Komatsu, H., I. Mori, et al. (1996). "Mutations in a Cyclic Nucleotide-Gated Channel Lead to Abnormal Thermosensation and Chemosensation in C. elegans." Neuron **17**(4): 707-718.

Kremeyer, B., F. Lopera, et al. (2010). "A Gain-of-Function Mutation in TRPA1 Causes Familial Episodic Pain Syndrome." Neuron **66**(5): 671-680.

Kung, C. (2005). "A possible unifying principle for mechanosensation." Nature **436**(7051): 647-654.

Kwan, K. Y., A. J. Allchorne, et al. (2006). "TRPA1 Contributes to Cold, Mechanical, and Chemical Nociception but Is Not Essential for Hair-Cell Transduction." Neuron **50**(2): 277-289.

Kwan, K. Y., J. M. Glazer, et al. (2009). "TRPA1 Modulates Mechanotransduction in Cutaneous Sensory Neurons." The Journal of Neuroscience **29**(15): 4808-4819.

Kwon, Y., S. H. Kim, et al. (2010). "Drosophila TRPA1 Channel Is Required to Avoid the Naturally Occurring Insect Repellent Citronellal." Current Biology **20**(18): 1672-1678.

Kwon, Y., W. L. Shen, et al. (2010). "Fine Thermotactic Discrimination between the Optimal and Slightly Cooler Temperatures via a TRPV Channel in Chordotonal Neurons." Journal of Neurobiology **30**(31): 10465.

Kwon, Y., H.-S. Shim, et al. (2008). "Control of thermotactic behavior via coupling of a TRP channel to a phospholipase C signaling cascade." Nat Neurosci **11**(8): 871-873.

Laissue, P. P., C. Reiter, et al. (1999). "Three-dimensional reconstruction of the antennal lobe in Drosophila melanogaster." The Journal of Comparative Neurology **405**(4): 543-552.

Larsson, M. C., A. I. Domingos, et al. (2004). "Or83b Encodes a Broadly Expressed Odorant Receptor Essential for Drosophila Olfaction." Neuron **43**(5): 703-714.

Lee, G., K. Abdi, et al. (2006). "Nanospring behaviour of ankyrin repeats." Nature **440**(7081): 246-249.

Lee, Y., Y. Lee, et al. (2005). "Pyrexia is a new thermal transient receptor potential channel endowing tolerance to high temperatures in Drosophila melanogaster." Nat Genet **37**(3): 305-310.

Levina, N., S. Totemeyer, et al. (1999). "Protection of Escherichia coli cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity." EMBO J **18**(7): 1730-1737.

Li, X., D. V. Gutierrez, et al. (2005). "Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin." Proc Natl Acad Sci U S A **102**(49): 17816-17821.

Li, Y., R. Wray, et al. (2004). "Intragenic suppression of gain-of-function mutations in the Escherichia coli mechanosensitive channel, MscL." Molecular Microbiology **53**(2): 485-495.

Liedtke, W., D. M. Tobin, et al. (2003). "Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in Caenorhabditis elegans." Proceedings of the National Academy of Sciences of the United States of America **100**(Suppl 2): 14531-14536.

Lindahl, E. and O. Edholm (2000). "Spatial and energetic-entropic decomposition of surface tension in lipid bilayers from molecular dynamics simulations." Journal of Chemical Physics **113**(9).

Lingueglia, E. (2007). "Acid-sensing ion channels in sensory perception." J Biol Chem **282**(24): 17325-17329.

Liu, L., R. Wolf, et al. (1999). "Context generalization in Drosophila visual learning requires the mushroom bodies." Nature **400**(6746): 753-756.

Liu, L., O. Yermolaieva, et al. (2003). "Identification and function of thermosensory neurons in Drosophila larvae." Nat Neurosci **6**(3): 267-273.

Lumpkin, E. A. and M. J. Caterina (2007). "Mechanisms of sensory transduction in the skin." Nature **445**(7130): 858-865.

Luque, C. M. and M. Milan (2007). "Growth control in the proliferative region of the Drosophila eye-head primordium: the elbow-noc gene complex." Dev Biol **301**(2): 327-339.

Macpherson, L. J., A. E. Dubin, et al. (2007). "Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines." Nature **445**(7127): 541-545.

McKemy, D. (2007). "Temperature sensing across species." Pflügers Archiv European Journal of Physiology **454**(5): 777-791.

McKemy, D. D., W. M. Neuhausser, et al. (2002). "Identification of a cold receptor reveals a general role for TRP channels in thermosensation." Nature **416**(6876): 52-58.

Merritt, D. J. and P. M. Whitington (1995). "Central projections of sensory neurons in the Drosophila embryo correlate with sensory modality, soma position, and proneural gene function." J Neurosci **15**(3 Pt 1): 1755-1767.

Mochizuki, T., G. Wu, et al. (1996). "PKD2, a Gene for Polycystic Kidney Disease That Encodes an Integral Membrane Protein." Science **272**(5266): 1339-1342.

Mohri, A., E. Kodama, et al. (2005). "Genetic Control of Temperature Preference in the Nematode Caenorhabditis elegans." Genetics **169**(3): 1437-1450.

Mombaerts, P., F. Wang, et al. (1996). "Visualizing an olfactory sensory map." Cell **87**(4): 675-686.

Montell, C. (2001). "Physiology, Phylogeny, and Functions of the TRP Superfamily of Cation Channels." Sci. STKE **2001**(90): re1-.

Montell, C. (2005). "The TRP Superfamily of Cation Channels." Sci. STKE **2005**(272): re3-.

Montell, C. and G. M. Rubin (1989). "Molecular characterization of the drosophila trp locus: A putative integral membrane protein required for phototransduction." Neuron **2**(4): 1313-1323.

Moore, A. W., L. Y. Jan, et al. (2002). "hamlet, a binary genetic switch between single- and multiple- dendrite neuron morphology." Science **297**(5585): 1355-1358.

Morales, J., H. Chiu, et al. (2005). "Biogenesis, structure, and immune-suppressive effects of virus-like particles of a Drosophila parasitoid, Leptopilina victoriae." Journal of Insect Physiology **51**(2): 181-195.

Mori, I. and Y. Ohshima (1995). "Neural regulation of thermotaxis in Caenorhabditis elegans." Nature **376**(6538): 344-348.

Murphy, T. (2003). The Drosophila Gateway Vector Collection.
Nadler, M. J. S., M. C. Hermosura, et al. (2001). "LTRPC7 is a Mg[middot]ATP-regulated divalent cation channel required for cell viability." Nature **411**(6837): 590-595.

Nagata, K., A. Duggan, et al. (2005). "Nociceptor and Hair Cell Transducer Properties of TRPA1, a Channel for Pain and Hearing." The Journal of Neuroscience **25**(16): 4052-4061.

Nagel, G., T. Szellas, et al. (2003). "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel." Proc Natl Acad Sci U S A **100**(24): 13940-13945.

Neely, G. G., A. Hess, et al. (2010). "A Genome-wide Drosophila Screen for Heat Nociception Identifies [alpha]2[delta]3 as an Evolutionarily Conserved Pain Gene." Cell **143**(4): 628-638.

Niemeyer, B. A., E. Suzuki, et al. (1996). "The Drosophila Light-Activated Conductance Is Composed of the Two Channels TRP and TRPL." Cell **85**(5): 651-659.

O'Hagan, R. and M. Chalfie (2006). "Mechanosensation in Caenorhabditis elegans." Int Rev Neurobiol **69**: 169-203.

O'Hagan, R., M. Chalfie, et al. (2005). "The MEC-4 DEG/ENaC channel of Caenorhabditis elegans touch receptor neurons transduces mechanical signals." Nat Neurosci **8**(1): 43-50.

Ollila, O. H. S., H. J. Risselada, et al. (2009). "3D Pressure Field in Lipid Membranes and Membrane-Protein Complexes." Physical Review Letters **102**(7): 078101.

Page, A. J., S. M. Brierley, et al. (2004). "The ion channel ASIC1 contributes to visceral but not cutaneous mechanoreceptor function." Gastroenterology **127**(6): 1739-1747.

Peier, A. M., A. Moqrich, et al. (2002). "A TRP Channel that Senses Cold Stimuli and Menthol." Cell **108**(5): 705-715.

Peier, A. M., A. J. Reeve, et al. (2002). "A Heat-Sensitive TRP Channel Expressed in Keratinocytes." Science **296**(5575): 2046-2049.

Perozo, E., D. M. Cortes, et al. (2002). "Open channel structure of MscL and the gating mechanism of mechanosensitive channels." Nature **418**(6901): 942-948.

Perozo, E., A. Kloda, et al. (2002). "Physical principles underlying the transduction of bilayer deformation forces during mechanosensitive channel gating." Nat Struct Mol Biol **9**(9): 696-703.

Plant, T. and R. Strotmann (2007). TRPV4. Transient Receptor Potential (TRP) Channels. V. Flockerzi and B. Nilius, Springer Berlin Heidelberg. **179:** 189-205.

Powell, J. R. (1997). Progress and prospects in evolutionary biology : the Drosophila model. New York, Oxford University Press.

Premkumar, L. S. and G. P. Ahern (2000). "Induction of vanilloid receptor channel activity by protein kinase C." Nature **408**(6815): 985-990.

Price, M. P., G. R. Lewin, et al. (2000). "The mammalian sodium channel BNC1 is required for normal touch sensation." Nature **407**(6807): 1007-1011.

Price, M. P., S. L. McIlwrath, et al. (2001). "The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice." Neuron **32**(6): 1071-1083.

Pulver, S. R., S. L. Pashkovski, et al. (2009). "Temporal Dynamics of Neuronal Activation by Channelrhodopsin-2 and TRPA1 Determine Behavioral Output in Drosophila Larvae." Journal of Neurophysiology **101**(6): 3075-3088.

Purves, D., G. Augustine, et al. (2008). Neuroscience.

Purves, D., G. J. Augustine, et al. (2001). Neuroscience. Sunderland, MA, Sinauer Associates, Inc.

Qian, F., F. J. Germino, et al. (1997). "PKD1 interacts with PKD2 through a probable coiled-coil domain." Nat Genet **16**(2): 179-183.

Quicke, D. L. J. (1997). Parasitic wasps. London ; New York, Chapman & Hall.
Reid, G. (2005). "ThermoTRP channels and cold sensing: what are they really up to?" Pflügers Archiv European Journal of Physiology **451**(1): 250-263.

Reid, G. and M.-L. Flonta (2002). "Ion channels activated by cold and menthol in cultured rat dorsal root ganglion neurones." Neuroscience Letters **324**(2): 164-168.

Ressler, K. J., S. L. Sullivan, et al. (1994). "Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb." Cell **79**(7): 1245-1255.

Rizki, R. and T. Rizki (1984). "Selective destruction of a host blood cell type by a parasitoid wasp." PNAS **81**(19): 6154-6158.

Rizki, T. M., R. M. Rizki, et al. (1990). "Leptopilina-Heterotoma and L-Boulardi - Strategies to Avoid Cellular Defense Responses of Drosophila-Melanogaster." Experimental Parasitology **70**(4): 466-475.

Rosenzweig, M., K. M. Brennan, et al. (2005). "The Drosophila ortholog of vertebrate TRPA1 regulates thermotaxis." Genes Dev **19**(4): 419-424.

Rosenzweig, M., K. Kang, et al. (2008). "Distinct TRP channels are required for warm and cool avoidance in Drosophila melanogaster." Proceedings of the National Academy of Sciences **105**(38): 14668-14673.

Roza, C., J. L. Puel, et al. (2004). "Knockout of the ASIC2 channel in mice does not impair cutaneous mechanosensation, visceral mechanonociception and hearing." J Physiol **558**(Pt 2): 659-669.

Runnels, L. W., L. Yue, et al. (2001). "TRP-PLIK, a Bifunctional Protein with Kinase and Ion Channel Activities." Science **291**(5506): 1043-1047.

Russo, J., M. Brehelin, et al. (2001). "Haemocyte changes in resistant and susceptible strains of D. melanogaster caused by virulent and avirulent strains of the parasitic wasp leptopilina boulardi." Journal of Insect Physiology **47**: 167-172.

Savage, C., M. Hamelin, et al. (1989). "mec-7 is a beta-tubulin gene required for the production of 15-protofilament microtubules in Caenorhabditis elegans." Genes Dev **3**(6): 870-881.

Sayeed, O. and S. Benzer (1996). "Behavioral genetics of thermosensation and hygrosensation in Drosophila." Proceedings of the National Academy of Sciences of the United States of America **93**(12): 6079-6084.

Schrader, S. and D. J. Merritt (2000). "Central projections of Drosophila sensory neurons in the transition from embryo to larva." J Comp Neurol **425**(1): 34-44.

Schroll, C., T. Riemensperger, et al. (2006). "Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae." Curr Biol **16**(17): 1741-1747.

Scott, S. A. (1992). "Sensory Neurons: Diversity, Development, and Plasticity." Oxford University Press **New York**.

Sengupta, P., H. A. Colbert, et al. (1993). "The cellular and genetic basis of olfactory responses in Caenorhabditis elegans." Ciba Found Symp **179**.

Sheetz, M. P. and S. J. Singer (1974). "Biological Membranes as Bilayer Couples. A Molecular Mechanism of Drug-Erythrocyte Interactions." Proceedings of the National Academy of Sciences **71**(11): 4457-4461.

Sherrington, C. S. S. (1906). "The Integrative Action of the Nervous System." (New York: Scribner's Sons).

Shima, Y., S. Kawaguchi, et al. (2007). "Opposing roles in neurite growth control by two seven-pass transmembrane cadherins." Nature Neuroscience **10**(8): 963-969.

Siddiqi, O. and S. Benzer (1976). "Neurophysiological defects in temperature-sensitive paralytic mutants of Drosophila melanogaster." Proc Natl Acad Sci U S A **73**(9): 3253-3257.

Sidi, S., R. W. Friedrich, et al. (2003). "NompC TRP Channel Required for Vertebrate Sensory Hair Cell Mechanotransduction." Science **301**(5629): 96-99.

Smith, G. D., M. J. Gunthorpe, et al. (2002). "TRPV3 is a temperature-sensitive vanilloid receptor-like protein." Nature **418**(6894): 186-190.

Smith, S. A. and D. Shepherd (1996). "Central afferent projections of proprioceptive sensory neurons in Drosophila revealed with the enhancer-trap technique." J Comp Neurol **364**(2): 311-323.

Sokabe, T., S. Tsujiuchi, et al. (2008). "Drosophila Painless Is a Ca2+-Requiring Channel Activated by Noxious Heat." The Journal of Neuroscience **28**(40): 9929-9938.

Song, W., M. Onishi, et al. (2007). "Peripheral multidendritic sensory neurons are necessary for rhythmic locomotion behavior in Drosophila larvae." Proc Natl Acad Sci U S A **104**(12): 5199-5204.

Sotomayor, M., D. P. Corey, et al. (2005). "In Search of the Hair-Cell Gating Spring: Elastic Properties of Ankyrin and Cadherin Repeats." Structure **13**(4): 669-682.

Stewart, G. W., A. C. Argent, et al. (1993). "Stomatin: a putative cation transport regulator in the red cell membrane." Biochim Biophys Acta **1225**(1): 15-25.

Story, G. M., A. M. Peier, et al. (2003). "ANKTM1, a TRP-like Channel Expressed in Nociceptive Neurons, Is Activated by Cold Temperatures." Cell **112**(6): 819-829.

Sun, M., E. Goldin, et al. (2000). "Mucolipidosis type IV is caused by mutations in a gene encoding a novel transient receptor potential channel." Human Molecular Genetics **9**(17): 2471-2478.

Sun, Y., L. Liu, et al. (2009). "TRPA channels distinguish gravity sensing from hearing in Johnston's organ." Proceedings of the National Academy of Sciences **106**(32): 13606-13611.

Suzuki, H., R. Kerr, et al. (2003). "In vivo imaging of C. elegans mechanosensory neurons demonstrates a specific role for the MEC-4 channel in the process of gentle touch sensation." Neuron **39**(6): 1005-1017.

Sweeney, S., K. Broadie, et al. (1995). "Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects." Neuron **14**: 341-351.

Talavera, K., K. Yasumatsu, et al. (2005). "Heat activation of TRPM5 underlies thermal sensitivity of sweet taste." Nature **438**(7070): 1022-1025.

Tobin, D. M. and C. I. Bargmann (2004). "Invertebrate nociception: Behaviors, neurons and molecules." Journal of Neurobiology **61**(1): 161-174.

Tominaga, M., M. J. Caterina, et al. (1998). "The Cloned Capsaicin Receptor Integrates Multiple Pain-Producing Stimuli." Neuron **21**(3): 531-543.

Tominaga, M., M. Wada, et al. (2001). "Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia." Proceedings of the National Academy of Sciences of the United States of America **98**(12): 6951-6956.

Tracey, W. D., Jr., R. I. Wilson, et al. (2003). "painless, a Drosophila gene essential for nociception." Cell **113**(2): 261-273.

Tsunozaki, M. and D. M. Bautista (2009). "Mammalian somatosensory mechanotransduction." Current Opinion in Neurobiology **19**(4): 362-369.

van Lenteren, J. C., N. Isidoro, et al. (1998). "Functional anatomy of the ovipositor clip in the parasitoid Leptopilina heterotoma (Thompson) (Hymenoptera : Eucoilidae), a structure to grip escaping host larvae." International Journal of Insect Morphology & Embryology **27**(3): 263-268.

Vannier, B., M. Peyton, et al. (1999). "Mouse trp2, the homologue of the human trpc2 pseudogene, encodes mTrp2, a store depletion-activated capacitative Ca2+ entry channel." Proceedings of the National Academy of Sciences of the United States of America **96**(5): 2060-2064.

Vassar, R., S. K. Chao, et al. (1994). "Topographic organization of sensory projections to the olfactory bulb." Cell **79**(6): 981-991.

Viswanath, V., G. M. Story, et al. (2003). "Ion channels: Opposite thermosensor in fruitfly and mouse." Nature **423**(6942): 822-823.

Vlachová, V., J. Teisinger, et al. (2003). "Functional Role of C-Terminal Cytoplasmic Tail of Rat Vanilloid Receptor 1." The Journal of Neuroscience **23**(4): 1340-1350.

Voets, T., G. Droogmans, et al. (2004). "The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels." Nature **430**(7001): 748-754.

Vosshall, L. B. and R. F. Stocker (2007). "Molecular Architecture of Smell and Taste in Drosophila." Annual Review of Neuroscience **30**(1): 505-533.

Vosshall, L. B., A. M. Wong, et al. (2000). "An olfactory sensory map in the fly brain." Cell **102**(2): 147-159.

Waldmann, R., G. Champigny, et al. (1997). "A proton-gated cation channel involved in acid-sensing." Nature **386**(6621): 173-177.

Walker, R. G., A. T. Willingham, et al. (2000). "A Drosophila Mechanosensory Transduction Channel." Science **287**(5461): 2229-2234.

Wall, P. and R. Melzack (1999). Textbook of Pain.
Wang, H., J. Peca, et al. (2007). "High-speed mapping of synaptic connectivity using photostimulation in Channelrhodopsin-2 transgenic mice." Proc Natl Acad Sci U S A **104**(19): 8143-8148.

Wang, H. and C. J. Woolf (2005). "Pain TRPs." Neuron **46**(1): 9-12.
Wang, S., Y. Dai, et al. (2008). "Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a molecular mechanism of inflammatory pain." Brain **131**(5): 1241-1251.

Ward, S. (1973). "Chemotaxis by the Nematode Caenorhabditis elegans: Identification of Attractants and Analysis of the Response by Use of Mutants." Proc Natl Acad Sci U S A **70**(3): 817.

Watanabe, H., J. Vriens, et al. (2002). "Heat-evoked Activation of TRPV4 Channels in a HEK293 Cell Expression System and in Native Mouse Aorta Endothelial Cells." Journal of Biological Chemistry **277**(49): 47044-47051.

Waxman, S. G. (2010). "Channelopathic Pain: A Growing but Still Small List of Model Disorders." Neuron **66**(5): 622-624.

Weihe, U., R. Dorfman, et al. (2004). "Proximodistal subdivision of Drosophila legs and wings: the elbow-no ocelli gene complex." Development **131**(4): 767-774.

Wemmie, J. A., M. P. Price, et al. (2006). "Acid-sensing ion channels: advances, questions and therapeutic opportunities." Trends Neurosci **29**(10): 578-586.

Wetzel, C., J. Hu, et al. (2007). "A stomatin-domain protein essential for touch sensation in the mouse." Nature **445**(7124): 206-209.

Williams, D. W. and D. Shepherd (1999). "Persistent larval sensory neurons in adult Drosophila melanogaster." J Neurobiol **39**(2): 275-286.

Williams, D. W. and J. W. Truman (2005). "Cellular mechanisms of dendrite pruning in Drosophila: insights from in vivo time-lapse of remodeling dendritic arborizing sensory neurons." Development **132**(16): 3631-3642.

Wolf, R. and M. Heisenberg (1991). "Basic organization of operant behavior as revealed in Drosophila flight orientation." J Comp Physiol [A] **169**(6): 699-705.

Wu, C. F. and B. Ganetzky (1980). "Genetic alteration of nerve membrane excitability in temperature-sensitive paralytic mutants of Drosophila melanogaster." Nature **286**(5775): 814-816.

Wustmann, G., K. Rein, et al. (1996). "A new paradigm for operant conditioning of Drosophila melanogaster." J Comp Physiol [A] **179**(3): 429-436.

Xiang, Y., Q. Yuan, et al. (2010). "Light-avoidance-mediating photoreceptors tile the Drosophila larval body wall." Nature **468**(7326): 921-926.

Xu, H., I. S. Ramsey, et al. (2002). "TRPV3 is a calcium-permeable temperature-sensitive cation channel." Nature **418**(6894): 181-186.

Xu, K., B. A. Bogert, et al. (2004). "The fragile X-related gene affects the crawling behavior of Drosophila larvae by regulating the mRNA level of the DEG/ENaC protein pickpocket1." Curr Biol **14**(12): 1025-1034.

Xu, S. Y., C. L. Cang, et al. (2006). "Thermal nociception in adult Drosophila: behavioral characterization and the role of the painless gene." Genes, Brain and Behavior **5**(8): 602-613.

Xu, X. Z. S., F. Chien, et al. (2000). "TRP[gamma], a Drosophila TRP-Related Subunit, Forms a Regulated Cation Channel with TRPL." Neuron **26**(3): 647-657.

Yin, J. and W. Kuebler (2010). "Mechanotransduction by TRP Channels: General Concepts and Specific Role in the Vasculature." Cell Biochemistry and Biophysics **56**(1): 1-18.

Zars, T., R. Wolf, et al. (2000). "Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the engram." Learn Mem **7**(1): 18-31.

Zhang, F., L. P. Wang, et al. (2007). "Multimodal fast optical interrogation of neural circuitry." Nature **446**(7136): 633-639.

Zhang, S., J. Arnadottir, et al. (2004). "MEC-2 is recruited to the putative mechanosensory complex in C. elegans touch receptor neurons through its stomatin-like domain." Curr Biol **14**(21): 1888-1896.

Zhang, S., J. Arnadottir, et al. (2004). "MEC-2 Is Recruited to the Putative Mechanosensory Complex in C. elegans Touch Receptor Neurons through Its Stomatin-like Domain." Current biology : CB **14**(21): 1888-1896.

Zhang, X.-F., J. Chen, et al. (2008). "Transient receptor potential A1 mediates an osmotically activated ion channel." European Journal of Neuroscience **27**(3): 605-611.

Zhong, L., R. Y. Hwang, et al. (2010). "Pickpocket Is a DEG/ENaC Protein Required for Mechanical Nociception in Drosophila Larvae." Current Biology **20**(5): 429-434.

Zhu, X., M. Jiang, et al. (1996). "trp, a Novel Mammalian Gene Family Essential for Agonist-Activated Capacitative Ca2+ Entry." Cell **85**(5): 661-671.

Zurborg, S., B. Yurgionas, et al. (2007). "Direct activation of the ion channel TRPA1 by Ca2+." Nat Neurosci **10**(3): 277-279.

Biography

LIXIAN ZHONG

EDUCATION

Doctor of Philosophy (Ph.D.), Pharmacology 2004-2011
Duke University, Department of Pharmacology, Durham, North Carolina, USA

Master of Arts (M.A.), Economics 2008-2010
Duke University, Department of Economics, Durham, North Carolina, USA

Bachelor of Science (B.S.), Biological Sciences 1999-2003
Peking University, Beijing, China
Recipient of Sumitomo Corporation Scholarship and Academic Excellence Award

EXPERIENCE

GRADUATE STUDENT, Laboratory of Molecular Pain Signaling
Principle Investigator: Dan Tracey
Duke University, Durham, North Carolina, USA 2005- 2011
Dissertation research

INTERN, Department of Research Policy and Cooperation
the World Health Organization
Geneva, Switzerland Jun-Aug, 2010
Summer internship

GRADUATE STUDENT (rotation)
Principle Investigator: Rob Wechsler-Reya
Duke University, Durham, North Carolina, USA Oct-Jan, 2004
Worked on Sonic Hedgehog signaling in glioma cell lines

GRADUATE STUDENT (rotation)
Principle Investigator: Sally Kornbluth
Duke University, Durham, North Carolina, USA Aug-Oct, 2004
Used western blot to look at protein degradation in cell apoptosis process

GRADUATE STUDENT (rotation)
Principle Investigator: Eric Feron
University of Michigan, Ann Arbor, Michigan, USA Jan-May, 2004
Worked on APC/fl-catenin/Tcf signaling in colon cancer

GRADUATE STUDENT (rotation)
Principle Investigator: Anand Swaroop
University of Michigan, Ann Arbor, Michigan, USA Sep-Dec, 2003
Designed and initiated the cloning a construct for making knockout mice to study human X-linked retinitis pigmentosa (XLRP)

UNDERGRADUATE RESEARCH ASSISTANT,
Principle Investigator: Jun Gu
Peking University, Beijing, China 2002-2003
Compared the levels of expression of a serum factor Klotho in different age group of human population using western blot and ELISA

SELECTED PEER-REVIEWED PUBLICATIONS

Hwang RY*, Zhong L*, Xu Y, Johnson T, Zhang F, Deisseroth K, Tracey WD. Nociceptive Neurons Protect Drosophila larvae from Parasitoid Wasps. Current Biology. 2007; (17) 2105-2116. (* authors contributed equally to work)

Zhong L*, Hwang RY*, Tracey WD. Pickpocket is a DEG/ENaC protein required for mechanical nociception in Drosophila larvae. Current Biology 2010; (20), 429–434 (*authors contributed equally to work)

SELECTED CONFERENCE ABSTRACTS AND TALK

Zhong, L., Stearns, N.A., Holfine, A.M. & Tracey, W.D. Transmembrane topology of The TRPA channel Painless. Gordon Conference of Mechanotransduction & Gravity Signaling in Biological Systems, 2005.
Zhong, L. & Tracey, W.D. The Drosophila mutant oblivious is defective in nociception. 47th Annual Drosophila Research Conference, 2006.
Zhong, L., Hwang, R.Y., Johnson, T., Xu, Y. & Tracey, W.D. Optogenetic control of nociception behavior in Drosophila larvae. Cold Spring Harbor Neurobiology of Drosophila Conference, 2007.
Zhong, L., *, Hwang, R. Y.*, Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., W. Daniel Tracey Nociceptive Multidendritic Neurons Protect Drosophila Larvae From Parasitoid

Wasps (Authors contributed equally to work *). The Society for Neuroscience Annual Meeting, 2007.
Zhong, L., Tracey, W.D. The Drosophila TRPA1 Channel Is Required for Thermal and Mechanical Nociception. Howard Hughes Medical Institute Conference: Force-Gated Ion Channels From Structure to Sensation, 2008.
Zhong, L., Hwang, R.Y., Tracey, W.D Distinct Classes of Thermosensory Neurons in the Drosophila Antenna(selected talk) Cold Spring Harbor Neurobiology of Drosophila Conference, 2009.
Zhong, L.*, Hwang, R.Y.*, Tracey, W.D. Pickpocket is a DEG/ENaC Channel Required for Mechanical Nociception (Authors contributed equally to work *). 50th Annual Drosophila Research Conference, 2009.
Zhong, L., Tracey, W.D. The Drosophila TRPA1 Channel Is Required for Thermal and Mechanical Nociception. 51st Annual Drosophila Research Conference, 2010.