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Pokes, Sunburn, and Hot Sauce: *Drosophila* as an Emerging Model for the Biology of Nociception

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Abstract

The word “nociception” is derived from the Latin “nocere,” which means “to harm.” Nociception refers to the sensory perception of noxious stimuli that have the potential to cause tissue damage. Since the perception of such potentially harmful stimuli often results in behavioral escape responses, nociception provides a protective mechanism that allows an organism to avoid incipient (or further) damage to the tissue. It appears to be universal in metazoans as a variety of escape responses can be observed in both mammalian and non-mammalian vertebrates, as well as diverse invertebrates such as leeches, nematodes, and fruit flies (Sneddon [2004] *Brain Research Review* 46:123–130; Tobin and Bargmann [2004] *Journal of Neurobiology* 61:161–174; Smith and Lewin [2009] *Journal of Comparative Physiology* 195:1089–1106). Several types of stimuli can trigger nociceptive sensory transduction, including noxious heat, noxious chemicals, and harsh mechanical stimulation. Such high-threshold stimuli induce the firing of action potentials in peripheral nociceptors, the sensory neurons specialized for their detection (Basbaum et al. [2009] *Cell* 139:267–284). In vertebrates, these action potentials can either be relayed directly to a spinal motor neuron to provoke escape behavior (the so-called monosynaptic reflex) or can travel via spinal cord interneurons to higher-order processing centers in the brain. This review will cover the establishment of *Drosophila* as a system to study various aspects of nociceptive sensory perception. We will cover development of the neurons responsible for detecting noxious stimuli in larvae, the assays used to assess the function(s) of these neurons, and the genes that have been found to be required for both thermal and mechanical nociception. Along the way, we will highlight some of the genetic tools that make the fly such a powerful system for studies of nociception. Finally, we will cover recent studies that introduce new assays employing adult *Drosophila* to study both chemical and thermal nociception and provide an overview of important unanswered questions in the field.

Keywords

Drosophila sensory neurons; thermal nociception; mechanical nociception; chemical nociception; nociceptive sensitization; tissue damage; fly behavioral response; dendritic arborization neurons; allodynia; hyperalgesia; neuroscience; behavioral assay

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DEVELOPMENT OF *DROSOPHILA* LARVAL NOCICEPTORS

Vertebrate nociceptors are characterized by “free” nerve endings that contact barrier epithelial tissues such as the skin, oral mucosa, or gut (Koltzen-burg et al., 1997; Brierley et al., 2004). This architecture is optimized for the rapid sensory detection of high-threshold stimuli capable of producing tissue damage (Lumpkin and Caterina, 2007). The afferent peripheral axons extend to the cell bodies of vertebrate nociceptors, which are localized in the dorsal root or trigeminal ganglia. For sensory neurons innervating the skin of the body, efferent axons extend into various laminae of the dorsal horn of the spinal cord where the detection of noxious stimuli can be relayed to the brain.

How are *Drosophila* nociceptive sensory neurons specified, what is their architecture, and to what structures in the CNS do they project? Developing fly larvae have two major types of peripheral sensory neurons positioned below the barrier epidermis: type I and type II. Type I neurons are associated with the bristle-type and chordotonal sensory organs and have a single ciliated dendrite. As their morphology suggests, the type I neurons are more associated with mechanosensory function, such as light touch (Kernan et al., 1994). By contrast, the type II neurons that we will mostly focus on here have many fine dendritic extensions and are structurally similar to mammalian nociceptors, which have naked dendritic projections to the epidermis (Hartenstein, 1988; Gao et al., 1999; Grueber et al., 2002). The type II neurons have a diverse lineage, with some of them arising from external bristle-type sensory organs, some from chordotonal organs, and some from a lineage unrelated to sensory organs (Brewster and Bodmer, 1995).

The type II neurons are also called multidendritic (Md) sensory neurons or dendritic arborization (da) sensory neurons, as their elaborate dendritic projections make contacts with nearly every epidermal cell of the larval barrier epidermis. They can be grouped into four subtypes based on their branching morphology (Fig. 1; Grueber et al., 2002). In each hemi-segment, there are three Class I, four Class II, five Class III, and three Class IV Md neurons. Class I and II dendritic fields are relatively sparse and compact, whereas Class III and IV neurons have more complex branching patterns that cover a wider territory with no overlapping of branches, a phenomenon known as tiling (Grueber et al., 2002, 2003). Each class of Md neurons also has a distinct axonal projection to a specific medial-lateral position in the ventral nerve cord of the CNS, suggesting that the second-order neurons to which each class connects may be different, both spatially and functionally (Grueber et al., 2007). Although the Md neuron cell bodies are positioned just underneath the epithelium and above the body-wall musculature (Bodmer and Jan, 1987), the exact spatial location of the free nerve endings—whether they run beneath, burrow within, or run above the epidermal sheet —remains unclear.

THERMAL NOCICEPTIVE FUNCTION OF *DROSOPHILA* LARVAL MD NEURONS

The elaborate arborization of larval multidendritic sensory neurons over the barrier epidermis is highly suggestive of a function in sensory perception. But does each class of Md neurons respond to different sensory inputs such as touch and temperature? Class I neurons, together with bipolar dendrite neurons, function in a proprioceptive sensory feedback circuit for rhythmic locomotion (Hughes and Thomas, 2007; Song et al., 2007), whereas class IV Md neurons are involved in avoidance behavior from a very bright light (Xiang et al., 2010). Are there also designated nociceptive neurons among the various classes of Md neurons? If so, which classes are nociceptive, which modalities does each neuronal class perceive, and what genes are required for this perception? One could imagine a model where each neuronal class subserves a particular nociceptive function, for instance

detection of noxious heat, noxious cold, harsh touch, and noxious chemicals. Alternatively, one could imagine a model where each neuronal class makes a partial contribution to the perception of each modality. Finally, there could be a single multimodal class of Md neurons wholly responsible for perception of all nociceptive modalities. As we will see below, the data so far suggest that class IV neurons are remarkably multimodal. However, data have not yet been obtained for all classes of neurons for each nociceptive modality (see Fig. 1). Full answers to the questions posed above await the development of functional assays for each nociceptive modality, the development of Gal4 drivers specific for each class of neuron, and the old *Drosophila* standby, genetic screening.

There were earlier observations on aversive behavioral responses in the insect phyla (Wigglesworth, 1980). The first modern genetic study in the field of *Drosophila* nociception was done by Tracey and colleagues (2003). In this landmark study, *Drosophila* larvae were presented with either noxious mechanical or thermal stimuli and a characteristic aversive withdrawal behavior was described that is distinct from both their normal locomotory movements and from their response to light touch (Kernan et al., 1994). The corkscrew-like rolling behavior provoked by noxious temperatures (42°C and up) or a harsh poke indicated that fly larvae, like other metazoans (Kavaliers, 1988), respond to potentially damaging stimuli through “nocifensive” escape behaviors.

Using this behavioral response, Tracey et al. (2003) screened a collection of 1,500 larval-viable P-element insertion mutants for those that cause insensitivity to noxious heat. Such an unbiased approach would have been unthinkable in vertebrate systems where nociception assays are more complex and time-consuming, and mutant collections are vastly more expensive and space-intensive to maintain. This screen identified a number of insensitive mutants, including a gene they named *painless*. Importantly, *painless* was found to encode a transient receptor potential (TRP) channel. Members of this gene family had well-established roles in sensory transduction (Montell, 2005) and had also been the subject of intense scrutiny in the nociception field since some of them are known to gate upon exposure to noxious temperatures or harsh mechanical stimulation (Caterina et al., 1997). The discovery of *painless* in flies indicated that, as for other sensory modalities, there is a conserved molecular basis for the perception of noxious stimuli.

To identify the neurons in which Painless might act, Tracey and his colleagues took advantage of the *Drosophila* GAL4/UAS system for tissue-specific transgene expression (Brand and Perrimon, 1993). Using a Gal4 driver that expresses in all multidendritic sensory neurons (*md-GAL4*; Gao et al., 1999), they drove expression of the tetanus toxin light chain (*UAS-TeTxLC*) (Tracey et al., 2003), which blocks the release of synaptic vesicles (Sweeney et al., 1995). While this manipulation blocked aversive withdrawal to noxious heat and harsh touch, it left open which class of multidendritic neuron was primarily responsible for the response. The effort to map the expression pattern and presumed functional location of Painless included RNA in situ hybridization, anti-Pain antibody staining, and an enhancer trap GAL4 line in the *painless* coding region (Tracey et al., 2003). These approaches found Pain to be expressed in a subset of peripheral neurons, including multidendritic neurons, and likely localized in the dendritic processes of these neurons. In 2007, Tracey and colleagues used a newly developed driver specific for Class IV md neurons (*ppk1.9-Gal4*; Ainsley et al., 2003) and the *UAS-TeTxLC* transgene to show that these neurons are required for both thermal and mechanical nociception. Moreover, an optogenetic gain-of-function experiment using class IV–specific expression of Channelrhodopsin (Nagel et al., 2003; Boyden et al., 2005) demonstrated that light-mediated neuronal activation of these neurons was sufficient to provoke nocifensive escape behaviors and reinforced the conclusion that Class IV neurons are the primary nociceptive sensory neurons in these animals (Hwang et al., 2007).

Whether and to what extent class I–III neurons also contribute to nociception, and which modalities of aversive stimuli these neurons might perceive, remains unclear.

MECHANICAL NOCICEPTIVE FUNCTION OF *DROSOPHILA* LARVAL MD NEURONS

Drosophila larvae have a light touch (< 10 mN) response mediated by Type 1 bipolar sensory neurons that involves a brief pause in their normal peristaltic locomotion (Kernan et al., 1994). These same larvae exhibit a nocifensive withdrawal response to a Von Frey filament calibrated to deliver a 50 mN local stimulus (Tracey et al., 2003; Hwang et al., 2007; Zhong et al., 2010). The behavioral response to such a noxious mechanical stimulus is similar to that observed with noxious heat; the fly larvae exhibit an escape motion consisting of rolling around their body axis. As with thermal nociception, when neuronal activity is blocked via expression of tetanus toxin in class-IV Md neurons, the mechanically provoked behavioral response was curtailed (Hwang et al., 2007). However, blocking the activities of class-I and -II multidendritic sensory neurons also resulted in a mild reduction in the aversion response to a harsh mechanical stimulus (Hwang et al., 2007). This is different from thermal nociception where blocking class IV almost completely abolished nociception and suggests that there could be some overlap of function between Class-I/II and Class-IV neurons for mechanical nociception. Alternatively, Class-I and -II neurons may somehow modulate the output of Class-IV neurons in response to harsh mechanical touch.

painless is not the only gene found to be required for perception of noxious mechanical stimuli. In a study by Zhong et al. (2010), the Gal4/UAS system was used to direct tissue-specific expression of *UAS-RNAi* transgenes (Dietzl et al., 2007) that could potentially knock down genes likely to mediate aversive withdrawal to harsh touch. The DEG/ENaC (degenerin/ epithelial Na+ channel) gene family encodes sodium channels that have been extensively studied in *Caenorhabditis elegans* (for review, see Tobin and Bargmann, 2004) and in vertebrates for their functions in mechanosensation (Price et al., 2000) and nociception (Price et al., 2001). In *Drosophila*, a member of the DEG/ ENaC family, *pickpocket1*, is expressed in the nociceptive multi-dendritic neurons (Ainsley et al., 2003; Hwang et al., 2007). RNAi-mediated gene knockdown of *pickpocket1* and a transheterozygous combination of deletions that remove *pickpocket1* caused a significant reduction in the percent of larvae exhibiting nocifensive responses toward mechanical stimuli (Zhong et al., 2010). The function of Pickpocket1 appears limited to mechanical nociception because its knockdown did not affect aversive withdrawal to thermal stimuli even though it is expressed in the class IV multidendritic neurons. Hwang and Tracey argued that Pickpocket1 probably functions upstream of Painless, which mediates both mechanical and thermal nociception. Further epistatic analysis should clarify whether this is the case or whether the genes act in parallel.

NOCICEPTIVE SENSITIZATION OF Md NEURONS FOLLOWING TISSUE DAMAGE

The discovery that TRP channels mediate nociceptive responses to heat and mechanical stimuli in *Drosophila* larvae and in *C. elegans* (Wittenburg and Baumeister, 1999) suggested that the molecular basis for baseline nociceptive responses may be conserved across the animal kingdom. However, these results raised the question of whether nociceptive responses in invertebrates would show the same types of complexity and modulation that are observed in vertebrates. One aspect of this complexity is the ability of nociceptive behaviors to sensitize in the presence of tissue damage. Peripheral sensitization could in theory result from (1) additional neurons becoming responsive to the stimulus, (2) a reduced threshold of

nociceptive neurons, or (3) increased output from the nociceptive sensory neurons. Sensitization can be divided into two types that differ by the strength of the input stimulus (Sandkühler, 2009). In allodynia, nociceptive responses are observed in the presence of subthreshold stimuli that would not normally cause aversive responses. A good example is the pain accompanying a tepid shower after sunburn. In hyperalgesia, exaggerated responsiveness to normally noxious stimuli is observed. The sensitization that accompanies tissue damage during the transient healing process is thought to foster protective behaviors that prevent further damage. Normally, sensitization returns to normal levels following healing but in some cases, hypersensitivity is prolonged and results in chronic pain. Since our understanding of chronic pain is very limited, genetically tractable models of the acute-to-chronic nociceptive transition are urgently needed.

So, do insects exhibit nociceptive sensitization? In a behavioral study, Walters et al. (2001) showed that *Manduca sexta* larvae have stronger escape responses following a repeated noxious mechanical stimulation. Moving into *Drosophila*, Babcock et al. (2009) developed an assay to genetically dissect nociceptive sensitization in fly larvae. To induce epidermal damage, early third instar larvae were exposed to acute UV radiation (a mimic of sunburn) and then tested for their nociceptive responses to both sub- and supra-threshold thermal stimuli. Normally, larvae do not sense 38°C as noxious. However, after UV-induced tissue damage, this temperature (and even lower ones down to ~34°C) now caused aversive withdrawal in the majority of larvae, indicating the development of thermal allodynia. Irradiated larvae also developed thermal hyperalgesia, where a normally noxious 45°C stimulation resulted in an increase in the percentage (90% from 20–30%) of animals displaying escape responses in less than 5 sec. In the Babcock et al. (2009) study, allodynia peaked at 24 hr and lasted less than 48 hr, and hyperalgesia peaked at 8 hr post-UV irradiation and returned to baseline before 24 hr. The transient nature of the sensitization response upon acute injury nicely parallels what has been found in vertebrate studies (Hucho and Levine, 2007).

Using markers specific for the damaged epidermis and the class-IV Md neurons that mediate thermal nociception, Babcock et al. (2009) observed that the gross structure of the nociceptors remained intact whereas the epidermis underwent a profound morphological deterioration likely caused by caspase 3 (Dronc)-mediated cell death. By testing candidate genes suspected of roles in vertebrate nociceptive sensitization, Babcock and colleagues (2009) found that sensitization required a TNF-like ligand (Eiger) produced by the dying epidermal cells and a TNF-receptor-like protein (Wengen) expressed on the nociceptive sensory neurons. This result suggested that not only is the basic nociceptive machinery (TRP channels) conserved in *Drosophila*, but so are the signaling pathways that can somehow modulate this machinery following tissue damage. Curiously, the authors found that macrophage-like blood cells are dispensable for both types of sensitization, indicating that there are some profound differences between the fly and vertebrate sensitization responses, at least at the level of the cell types that provide sensitization signal. Understanding how sensitization arises at a mechanistic level awaits both further genetic analysis and also the development of methods to perform electrophysiological analysis (Xiang et al., 2010) on the affected sensory neurons.

THERMAL NOCICEPTION IN ADULT *DROSOPHILA*

Although the initial molecular/genetic nociception studies were performed with *Drosophila* larvae, perhaps because the neurons involved had been so well described at the developmental/anatomical levels, in recent years a number of groups have begun to use adult *Drosophila* to assay responses to noxious thermal and chemical stimuli. As with larvae (Rosenzweig et al., 2005, 2008), adult flies prefer certain temperatures in the ambient range

(~24°C; Sayeed and Benzer, 1996). Temperatures higher than 40°C are recognized as noxious and can provoke withdrawal responses (Wolf and Heisenberg, 1991). To test the latency of nociceptive behavior to noxious heat in adult flies, Xu et al. (2006) developed an assay in which a single fly is glued to a fixture and exposed to a laser beam centered on the fly's abdomen. Aldrich et al. (2010) used the same assay in a separate study and reported the surface abdomen temperature was ~40°C on exposure to the beam. To test for aversive withdrawal, a small piece of cotton was given to the fly to hold. The latency to dropping of the piece of cotton upon laser stimulation was used as a behavioral readout of nociception. A second assay developed by Xu et al. (2006) monitored a jumping response by a fly tethered to a fixture above and lowered onto a hot plate heated to 47°C. In this assay, the latency from contact with the hot plate to jumping was measured.

Xu et al. (2006) used the laser and hot plate assays to test whether *painless* mutant flies show an increased withdrawal latency upon thermal stimulation. They do. This result indicates that the role of Painless in thermal nociception is not restricted to the larval stage. Based on the *pain-GAL4* enhancer trap expression pattern, Xu et al. (2006) suggested that neurons in the peripheral nervous system and thoracic ganglia comprise part of the thermal nociception circuit. Expression was also observed in the mushroom bodies (MB) in the brain but removal of this structure, by either chemical treatment (hydroxyurea) or gene mutations that miniaturize it (*mbm1*), did not affect thermal nociception (Xu et al., 2006). Additionally, *pain-GAL4* and anti-Pain antibody staining showed Painless expression in gustatory neurons, in the anterior wing margin, and cells in various parts of the brain (Al-Anzi et al., 2006; Xu et al., 2006). Whether the adult network of tiling body wall sensory neurons (Shimono et al., 2009) is the primary locus of action of Painless remains unclear.

While both the laser beam and hot plate assays provide useful tools to test individual adult flies for thermal nociceptive responses, both are rather cumbersome to scale up to a population level, and the behavioral readouts can include a higher than desired proportion of false positives. Sayeed and Benzer's work testing temperature preference in the adult flies utilized a band heater placed in one side of a T-maze (Sayeed and Benzer, 1996). This assay system was later adapted by Manev and Dimitrijevic (2004) who placed the band heater onto the countercurrent apparatus developed by Symour Benzer in 1967 in the landmark study on phototactic behavior in flies (Benzer, 1967). To test if flies can be used for pharmacological studies of nociception, Manev and Dimitrijevic (2004) tested if injection of 3-APMPA, an agonist for the $GABA_B$ receptor, increases the threshold for heat avoidance. They found, consistent with mammalian studies (Thomas et al., 1996), that this drug had antinociceptive effects in flies (Manev and Dimitrijevic, 2004).

Later Aldrich et al. (2010) modified this assay system further and tested for genes required for adult thermal nociception. The authors placed 50–70 flies into the apparatus and challenged them in two consecutive trials. A calculated performance index (PI) reflects the proportion of flies failing to avoid the heat at the end of two consecutive trials; when the temperature of the heat band was in the comfortable range (25–35°C), most flies crossed the barrier and gave a PI near-maximum value of 2, whereas noxious heat ranging from 40 to 50°C blocked flies from crossing the heat band and resulted in lower PI scores (Aldrich et al., 2010). Using this assay system, Aldrich et al. (2010) identified a function for the *amnesiac* gene in thermal nociception. *amnesiac* is predicted to encode a neuropeptide precursor, and has known functions in memory retention (Quinn et al., 1979; Feany and Quinn 1995), associative learning (Tempel et al., 1983), non-associative learning (Quinn et al., 1979), courtship conditioning (Sakai et al., 2004), and sensitivity to ethanol (Moore et al., 1998). *amnesiac* mutant flies also have defects in non-noxious temperature preference assays (Hong et al., 2008). Using the light-driven heat avoidance and laser beam assays described above, the authors found reduced responsiveness and increased latency to noxious

heat in *amnesiac* mutant flies (Aldrich et al., 2010). They also tested larval thermal nociceptive behavior in *amnesiac* mutants, and found defects equivalent to those exhibited by *painless* mutants. At the cellular level, it is not yet clear where *amnesiac* functions for thermal nociception in both larvae and adults. In adults, anti-AMN predominantly stains two dorsal paired medial (DPM) neurons, which innervate the whole mushroom bodies in the adult brain (Waddell et al., 2000). Less is known about the expression pattern of *amn* in other areas in adult flies, or in larvae although DeZazzo et al. (1999) reported some expression in embryonic peripheral neurons and unidentified cells in the third instar larval CNS. Tissue-specific knockdown of *amnesiac* will be needed to identify the functionally relevant cells for its role in thermal nociception.

In the latest study on thermal nociception in adult flies, Neely et al. (2010) used a temperature-controlled chamber to give adult flies a choice of a non-noxious and a noxious temperature. When the bottom surface of the chamber is heated to 46°C, wild-type flies respond by avoiding that surface and resting on a nonnoxious surface, whose temperature was maintained at or near 31°C. To identify genes involved in thermal nociception, Neely et al. (2010) combined this high-throughput behavioral assay with a genome wide *UAS-RNAi* screen. Using a pan-neuronal Gal4 driver, they screened 11,664 genes for poor avoidance of the noxious heat surface. The screen netted 580 candidate genes for thermal nociception.

straightjacket (*stj*) was one of the genes found in this assay. Knockdown of *stj* resulted in a low percentage of flies avoiding noxious heat (46°C), and also a more profound failure than *painless* mutants to elicit thermal nociceptive escape responses in larvae (Neely et al., 2010). *stj* mutant (stj^2) larvae display a normal response to light touch but have not yet been analyzed for responses to harsh touch (Neely et al., 2010). The gene encodes a member of the α*2*δ family of voltage-gated Ca^{2+} channels, which have been implicated in the development and function of synapses (Catterall, 2000; Dickman et al., 2008; Ly et al., 2008; Kurshan et al., 2009). It is expressed in the central and peripheral nervous system in adult flies as well as multidendritic neurons in the larval peripheral nervous system, suggesting a functional role in larval nociceptive neurons. Knockdown of *stj* in particular subsets of central or peripheral neurons has not yet been tested at either stage. For such studies, the *pars intercerebralis* (PI) and the subesophageal ganglion in the adult brain, the sensilla of the fly leg, the ventral nerve cord (VNC) in the larval central nervous system, and the multi-dendritic neurons in larval peripheral nervous system (Ly et al., 2008; Neely et al., 2010) would be promising tissues to start with as they all show Stj expression.

The study by Neely et al, (2010) is very encouraging in the sense that a gene found in a fly pain study also appears to function in vertebrates. The mammalian ortholog of *stj* is α*2*δ*3*, a protein that is closely related to α*2*δ*1*, a known target of the prominent analgesic drugs gabapentin and pregabalin (Field et al., 2006). Tellingly, mice lacking α*2*δ*3* display a defect in acute thermal nociception. More interestingly, $\alpha 2\delta 3^{-/-}$ mice showed delayed thermal hyperalgesia in a peripheral inflammatory sensitization model, even though inflammation occurred normally and mechanical hyperalgesia remain normal (Neely et al., 2010). Thus, it seems that α*2*δ*3* has specific and limited roles in thermal nociception. In Neely et al.'s study of single nucleotide polymorphism (SNP) associated with heat pain variance in humans, they identified minor SNPs at the α*2*δ*3* locus that were associated with reduced thermal pain sensitivity and less chronic pain after surgery (Neely et al., 2010).

CHEMICAL NOCICEPTION IN ADULT *DROSOPHILA*

Chemical nociception is the detection of tissue-damaging chemicals or environmental irritants by nociceptors. Examples of irritants include acids, plant-derived compounds like capsaicin and menthol, or electrophiles found in pungent compounds, like isothiocyanates

(ITC) such as wasabi and allicin from garlic. To test chemical nociception in adult flies, Al-Anzi et al. (2006) developed a two-choice preference test. In this assay, the authors marked control or irritant-containing food with red and blue dyes. After a 1-hr feeding session with starved flies, the color of the fly abdomens was examined. Al-Anzi et al. (2006) tested for aversive behavior to allyl and benzyl isothiocyanate (AITC and BITC), and found that the flies avoid these chemicals in a dose-dependent manner. As an alternative assay, Al-Anzi et al. (2006) and later K. Kang et al. (2010) measured an actual physical aversion to these compounds by examining proboscis extension upon contact with food containing them. The proboscis extension response (PER) is based on observation of hungry flies encountering unadulterated food; when a droplet of sugary solution is touched on the forelegs of a fly, the fly extends its proboscis to drink (Dethier, 1976). Al-Anzi et al. (2006) tested AITC and BITC in their proboscis extension test, whereas K. Kang et al. (2010) tested three electrophiles: AITC, N-methyl maleimide (NMM), and Cinnamaldehyde (CA). Adding these compounds to the sucrose solution offered to the flies resulted in a decreased PER score after the first ingestion. Both groups argue that this indicates that the pungent taste caused by these compounds is sensed by the organism as a potentially harmful environmental agent, and thus it results in avoidance of further consumption. There is ambiguity in these assays, however, as to whether they can distinguish a painful experience from an unpleasant taste. The adverse sensory experience of humans with these same compounds suggests that flies may perceive them as genuinely noxious although data on whether they can directly cause tissue damage in flies is needed.

Which genes and molecules play a role in chemical nociception? The transient receptor potential (TRP) family encodes cation permeable channel proteins with six transmembrane domains. There are 13 members in *Drosophila* TRP family, and they can be divided into seven subfamilies based on amino acid sequence comparison (Montell, 2005). Members of TRP family genes have been extensively investigated for their roles in sensory transduction pathways. For example, *Trp* and *TrpL* function in phototransduction (Montell et al., 1985; Niemeyer et al., 1996), *nanchung* and *inactive* in hearing (Kim et al., 2003; Gong et al., 2004), and *nompC* in mechanosensation (Walker et al., 2000). Two members of TRP family were investigated for their roles in chemical nociception: *painless* and *TrpA1* (Al-Anzi et al., 2006; K. Kang et al., 2010). Both of them belong to the TRPA subfamily.

Al-Anzi et al. (2006) found that *painless* is required in adult chemical nociception using the proboscis extension and two-dye food preference assays. *painless* mutants failed to avoid AITC and BITC. K. Kang et al. also tested *painless* mutant flies for their avoidance to AITC and NMM, and found a partial aversion (K. Kang et al., 2010). This difference in the degree of *painless* phenotype could be due to differences in experimental procedure (ingestion was allowed in K. Kang et al., 2010), strength of the specific alleles tested, or differences in how the assay was scored (Al-Anzi et al. measured the response on the first offering, whereas K. Kang et al. averaged the responses from the second to the fifth offerings). Al-Anzi et al. (2006) also tested whether *painless* mutants showed a normal set of preferences among foods that are not noxious to the fly. Normal wild-type flies avoided NaCl and Quinine, and were attracted to sugar solutions; this is also true of *painless* mutants suggesting that baseline gustatory function is normal in these animals. Interestingly, capsaicin, a plant-derived irritant that elicits burning sensations in mammals, did not provoke nociceptive responses; rather it attracted the flies. However, a second group in another study found no preference for capsaicin (Marella et al., 2006).

In addition to *painless*, K. Kang et al. (2010) tested if *trpA1* is required in chemical nociception in adult *Drosophila* using the PER as the behavioral readout. Flies homozygous for *trpA1* null alleles failed to avoid uptake of electrophiles (AITC, NMM). Earlier *in vitro* studies reported that TrpA1 does not respond to electrophiles (Bandell et al., 2004; Sokabe

et al., 2008; Xiao et al., 2008), but K. Kang et al. (2010) found a mutation in the original trpA1 cDNA and found robust responses to electophiles in Xenopus oocytes upon expressing the corrected dTrpA1 cDNA. It is interesting that mammalian TrpA1 also responds to electrophiles with very similar persistent activation after withdrawal, suggesting a shared mechanism of chemical-mediated channel activation (Hinman et al., 2006; Macpherson et al., 2007). *TrpA1* mutants also fail to avoid other insect repellents such as citronellal (Kwon et al., 2010) and aristolochic acid (Kim et al., 2010) although TrpA1 does not appear to be directly gated by these compounds. The genetic and cellular specificity of TrpA1 for the chemical nociceptive response was verified by a rescue experiment, where TrpA1 expression in peripheral chemosensors using *Dll-GAL4, MJ94-GAL4*, or *Gr66a-GAL4*, restored sensitivity to electrophiles (K. Kang et al., 2010).

It remains a bit unclear whether gustatory neurons in the adult fly serve a dual role as nociceptors for noxious chemicals or whether there are other sensory neurons that initially detect these compounds. Al-Anzi et al. (2006) proposed that chemical nociceptors in their study are the sensory neurons located in the labial palpus and the leg tarsus based on the expression pattern of Painless. The authors used co-labeling of *Painless-GAL4*, an enhancer trap line, with markers for the gustatory neurons including Gr66a, Gr47a, or Gr32, and concluded that the main nociceptive sensory neurons are largely gustatory neurons. In case of K. Kang et al. (2010), the authors suggested, based on TrpA1 antibody staining, that sensory neurons that innervate sensilla numbers 8 and 9 in the labral sense organ (LSO) in the mouthparts function as chemical nociceptors. Testing if optogenetic activation of these neurons can elicit the same behavioral responses without chemical stimuli or whether blocking the activity of these neurons fails to elicit aversive behavior would help resolve this issue.

PERSPECTIVES FOR FUTURE WORK

The study of nociception and nociceptive sensitization in *Drosophila* is still in its early stages. The advantages of the experimental organism are clear: its unparalleled resolving power for genetic analysis and the relatively simple anatomy of its peripheral and central nervous systems. The pioneer studies reviewed here provide a platform to identify and investigate genes, neurons, and circuits that underlie basic nociception and its modulation. As shown in Figures 1 and 2, however, assays have not yet been developed for all nociceptive sensory modalities at each stage and even the functions of many sensory neurons presumed to be nociceptive in larvae remain unclear. Nevertheless, the findings of functional roles for TRP channels, DEG/ENaC channels, straightjacket, and TNF and its receptor (see Table 1) in various aspects of nociception suggest strongly that the molecular basis of pain sensing is highly conserved at the evolutionary level. However, one point that should not be lost is that the studies we have covered so far have yet to identify genes that were not previously suspected at some level of a role in vertebrate nociception. This is changing. A recent study on nociceptive sensitization in *Drosophila* larvae showed that components of the Hedgehog (Hh) signaling pathway are required for both thermal allodynia and hyperalgesia (Babcock et al., 2011). This critical developmental pathway had not previously been suspected of a role in nociception in any system. Importantly, a role for Hh in modulation of nociception is conserved in vertebrates (Babcock et al., 2011). For the field to remain viable over the long term, work in the fly will need to continue to reveal new players in nociceptive biology that have conserved roles in vertebrates. Below we outline a few major biological questions (beyond further gene discovery) that are likely to preoccupy the field over the next several years.

Neurons and Circuits: Who Receives Nociceptive Input and How Is the Information Processed?

The identity of the neurons that of the neurons that receive nociceptive input is to date most clear in larvae. As shown in Figure 1, the class-IV multidendritic neurons are known to mediate the initial sensation of noxious heat (Tracey et al., 2003; Hwang et al., 2007), harsh touch (Hwang et al., 2007; Zhong et al., 2010), and even bright blue and UV-spectrum light (Xiang et al., 2010). This remarkable multi-modality of Class-IV neurons raises the interesting processing question of how (or whether) the neuron “knows” how it is being stimulated. It also raises the question of what the precise role of Class-I–III multidendritic neurons are. Do these neurons receive other types of input (noxious cold? chemical?) or do they play a role in modulating the activity of Class-IV neurons that receive the primary input? In the adult, for all of the possible nociceptive modalities, more anatomical work needs to be done to pinpoint the relevant afferent neurons.

It remains an open question in the fly whether the CNS plays a major (or any) role in modulating the organismal response to different types of nociceptive input. In other words, is there a neural circuit mediating pain responses in the fly and what is the architecture of this circuit? Do flies exhibit the same types of neuromodulation, such as endorphin-mediated dampening of nociception, which can occur in vertebrates under conditions of stress or trauma? To resolve these questions, the field will need to use the powerful new tools available for neuronal circuit-mapping (Pfeiffer et al., 2008; Potter et al., 2010; Hadjieconomou et al., 2011; Hampel et al., 2011) and apply them to pinpointing both the peripheral and central neurons that are required for nociceptive behaviors.

Modalities and Pathways

Another interesting question that has not been tackled systematically is how different modes of stimulation can be resolved at the level of intracellular signaling. An interesting example is Painless. Painless can mediate three different modes of aversive stimulation: thermal (larval and adult), mechanical (larval), and chemical (adult). Further, it can mediate two of these in the same neuron, the larval Class-IV multidendritic neuron. Although Painless can directly gate in the lower noxious temperature range (Sokabe et al., 2008), it has not yet been tested if it can gate mechanically as is the case for other TRP channels (L. Kang et al., 2010). One possibility is that thermal sensation involves direct gating of Painless whereas mechanical sensation involves gating of Pickpocket1 either with or without gating of Painless and this is how the cell distinguishes the initial input. A further question is whether the signaling downstream of Painless is shared by these two modes of stimuli. Given that both modes of stimulation cause similar nocifensive responses, it seems possible that the same downstream network could be utilized by both. What about the chemical nociceptive function of Painless? Since chemical nociception has not yet been tested in larvae (Fig. 2) but only in adults, it is possible that there is a different signaling cascade downstream of Painless activation by noxious chemicals that is unique to chemical nociceptors.

A similar processing conundrum exists for TrpA1, which is involved in thermal preference (Rosenzweig et al., 2005,d 2008; Kwon et al., 2008) and chemical nociception, albeit in different neurons. At the electrophysiological level, the dynamics of the channel activation is different with these two stimuli: transient with thermal stimulation versus long lasting with chemical (AITC) stimulation. In the case of the insect chemorepellents citronellal and aristolochic acid, TrpA1 does not gate directly, suggesting that its role in mediating aversion to these compounds is indirect and mechanistically distinct from its roles in thermal preference and response to electrophiles.

Open Questions Related to Nociceptive Sensitization

The recent demonstration that thermal nociceptive responses in *Drosophila* larvae can sensitize in response to tissue damage is encouraging for viewing *Drosophila* nociceptive biology as a complex phenomenon possessing multiple levels of regulation. However, to date, sensitization has only been demonstrated in response to one type of tissue damage, UV irradiation, and only for one nociceptive modality, noxious heat, and only in larvae. Clinically, mechanical sensitization is a much more serious problem for patients with chronic pain syndromes and it will be interesting to determine whether tissue damage can also cause mechanical allodynia and/or hyperalgesia. Such experiments will require a more precise knowledge of the actual threshold between light touch and harsh touch behavioral responses. If mechanical sensitization exists, it will be interesting to see whether TNF (Babcock et al., 2009) or Hh (Babcock et al., 2011) mediates it as for thermal sensitization.

A second important question is whether different modes of tissue damage cause sensitization(s) of similar magnitude and utilizing similar inductive pathways. For instance, would physical wounding cause Dronc- and TNF-dependent thermal sensitization? Or Hh-dependent sensitization? A final question is whether an individual larva or fly can habituate to repeated exposure to a noxious stimulus of any modality. Most of the experiments in the field to date have involved population studies where each individual is only stimulated once. These experimental paradigms do not allow one to test whether these sensory responses habituate or adapt as has been shown for other sensory modalities (Wang et al., 2010).

For over 30 years, *Drosophila* has been one of the main drivers in finding genes that are important in an incredible array of developmental processes. The expanding focus on medically relevant physiological processes such as nociception reviewed here represents a new avenue for using *Drosophila* as a research tool. Our hope and expectation is that these studies will yield a similar trove of riches in the years to come as the field expands to explore more varied and diverse aspects of nociceptive biology.

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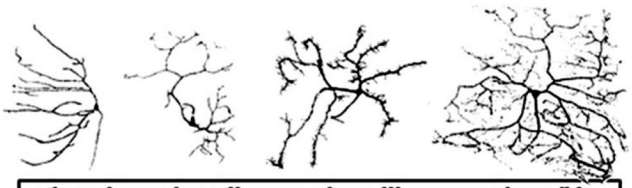

	class I	class II	class III	class IV
Noxious Heat	-	-	-	+++
Mechanical	+	+	-	+++
Chemical	?	?	?	?
Noxious Cold	?	?	?	?

Fig. 1.

Classes of larval multidendritic neurons and the nociceptive sensory modalities they mediate. The top diagrams show the characteristic dendritic morphologies of the four classes of Multidendritic (Md) neurons that arborize over the larval barrier epidermis (adapted from Grueber et al., 2007 with the permission of the publisher). Below is a table that indicates which nociceptive sensory modalities each neuronal class subserves (data derived from Hwang et al., 2007). Boxes with question marks indicate where the assays for these modalities (noxious cold and chemical) have yet to be developed with *Drosophila* larvae and thus the neurons that mediate these potential responses have yet to be determined. +++, fully required for responsiveness. +, partially required for responsiveness. −, not required for responsiveness. See text for a discussion of the possible roles of the Class-I–III Md neurons.

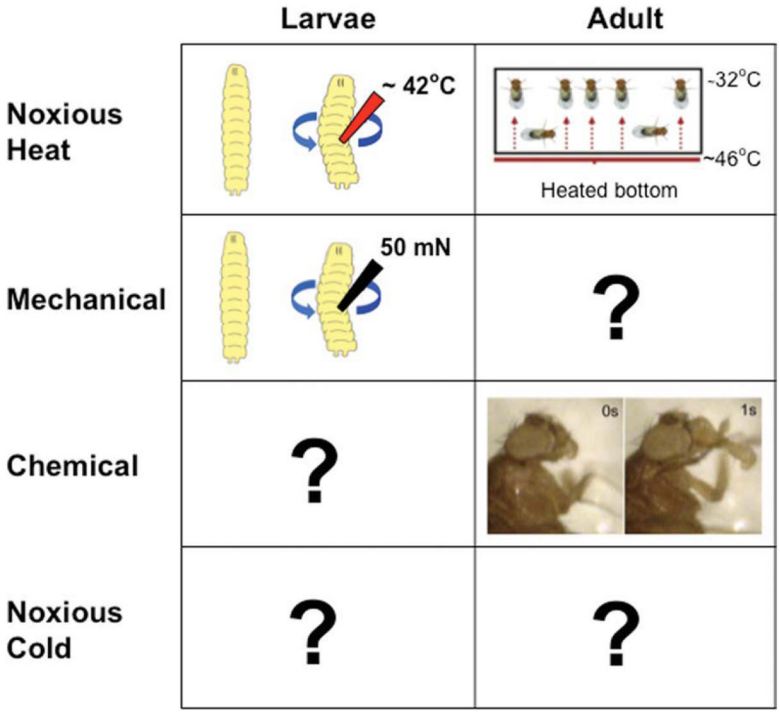

Fig. 2.

Assays for the different nociceptive sensory modalities in *Drosophila* larvae and adults. The table shows the different nociceptive sensory modalities and representative assays for assessing behavioral responses. Boxes with question marks indicate modalities/stages where assays have yet to be developed. Only noxious heat has been studied so far at both stages, using a custom-designed heat probe that can be dialed to a particular setpoint temperature (larvae) or a heated chamber that allows adults to choose between a noxious 46°C or non-noxious 32°C temperature range (diagram adapted from Neely et al., 2010, with permission from the publisher). Other thermal assays have been developed and are discussed in the text. The mechanical nociception assay in larvae involves a quick poke with a stiff fiber that delivers a ~ 50-mN force. The behavioral response to this stimulus, a corkscrew-like body roll, is very similar to that observed upon focal presentation of a noxious heat probe. For chemical nociception, one adult-based assay involves presenting the adult with a liquid laced with a presumably noxious compound (see text for details) and measuring the willingness of the adult to extend its proboscis and sample and resample the proffered nourishment. Other adult-based assays are discussed in the text. Proboscis extension pictures were adapted from Gordon and Scott (2009) with permission from the publisher.

TABLE 1

Summary of Genes and Their Involvement in Nociceptive Behavior

	Nociceptive stimuli				
	Larval nociception			**Adult nociception**	
Genes	**Thermal**	**Mechanical**	**Sensitization**	**Thermal**	**Chemical**
painless	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
TrpA1	**Yes**	?	**Yes**	?	**Yes**
ppk1	No	**Yes**	?	?	?
stj	**Yes**	?	?	**Yes**	?
amnesiac	**Yes**	?	?	**Yes**	?
TNF/TNFR	No	?	**Yes**	?	?
Hh signaling	No	?	**Yes**	?	?

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