Topic Introduction

Nociception in *Drosophila* Larvae

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Nociception is the sensory modality by which animals sense stimuli associated with injury or potential tissue damage. When *Drosophila* larvae encounter a noxious thermal, chemical, or mechanical stimulus, they perform a stereotyped rolling behavior. These noxious stimuli are detected by polymodal nociceptor neurons that tile the larval epidermis. Although several types of sensory neurons feed into the nociceptive behavioral output, the highly branched class IV multidendritic arborization neurons are the most critical. At the molecular level, *Drosophila* nociception shares many conserved features with vertebrate nociception, making it a useful organism for medically relevant research in this area. Here, we review three larval assays for nociceptive behavior using mechanical stimuli, optogenetic activation, and the naturalistic stimuli of parasitoid wasp attacks. Together, the assays described have been successfully used by many laboratories in studies of the molecular, cellular, and circuit mechanisms of nociception. In addition, the simple nature of the assays we describe can be useful in teaching laboratories for undergraduate students.

BACKGROUND

Nociception is the sensory process for detecting noxious stimuli that indicate actual or potential tissue damage. In humans, nociception evokes the feeling of pain. Pain treatment is a critical public health issue, and understanding the molecular underpinnings of nociception can contribute to the discovery of new drug targets. To assess nociception in other animals, researchers rely on observations of behaviors that promote avoidance or protection from harm (nocifensive behaviors). *Drosophila* larvae offer an advantage in the study of nociception, because their nocifensive behavior is very clear and distinct from other behaviors, and easy to quantify in highly reproducible experimental paradigms.

When *Drosophila* larvae encounter a noxious stimulus, they bend their body into a characteristic "c" shape (c-bend), and then rotate around their long body axis in a corkscrew-like manner (Tracey et al. 2003). This rolling behavior, also known as nocifensive escape locomotion, is distinct from typical forward and backward locomotion, head casting and turning during exploration, or the pause-and-turn response after innocuous (gentle) touch (Kernan et al. 1994). Therefore, rolling is very easy to score with minimal training, and provides a clear behavioral readout to assess nociceptive function.

With their relatively simple nervous system and the availability of genetic tools, *Drosophila* have been an invaluable system for studying the sensory input into neural circuits that drive behavior. Understanding the mechanisms by which fly larvae sense and process noxious

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stimuli has broad implications for the fields of medicine and neuroscience (Caldwell and Tracey 2010). Furthermore, studying nociception provides insights into general mechanisms of somatosensory processing, including the contribution of genes, molecules, cell morphology, circuits, and plasticity.

NOCICEPTOR NEURONS

Nociception relies on specialized, high-threshold sensory neurons (nociceptors) that rapidly respond to noxious stimuli. Signals from these selectively tuned neurons are transmitted to defined downstream neural circuits responsible for escape and avoidance behaviors. *Drosophila* larvae have peripheral somatosensory neurons with soma and dendrites embedded in the epidermis and axons projecting to the ventral nerve cord (Grueber et al. 2002, 2007; Tracey et al. 2003; Gerhard et al. 2017). The neurons that sense and transmit noxious information belong to the group of dendritic arborization (da) neurons, which have branching dendrites that are located just below the epidermal cells of the body wall. Class IV da (cIV-da) neurons are polymodal nociceptors, and can be activated by multiple types of noxious stimuli, including harsh mechanical touch, high temperature, and potentially damaging chemicals (Hwang et al. 2007; Zhong et al. 2012; Lopez-Bellido et al. 2019). cIV-da neurons are the main transducers of noxious information, and are both necessary and sufficient to elicit nocifensive rolling (Hwang et al. 2007). Class II and class III da neurons, which are mainly mechanosensitive (Tsubouchi et al. 2012; Yan et al. 2013), also contribute to mechanical nociception but to a lesser extent (Hwang et al. 2007; Hu et al. 2017).

NOXIOUS STIMULI

Larval rolling can be triggered by multiple types of potentially damaging stimuli, including harsh mechanical touch, high temperature, and noxious chemicals and acids. Methods have been developed to assess larval sensitivity to each of these modalities (Honjo et al. 2014). In our associated protocol, we describe a mechanical nociception assay that involves poking larvae with a von Frey filament that exerts a standardized force (see Protocol: **Mechanical Nociception Assay in *Drosophila* Larvae** [Mauthner and Tracey 2024a]; Tracey et al. 2003; Zhong et al. 2010; Hoyer et al. 2018). Another commonly used assay tests thermal nociception by locally applying a thermal stimulus to larvae with a calibrated heat probe (Tracey et al. 2003; Petersen et al. 2018). Thermal nociception can also be tested by immersing larvae in a drop of water that is heated to a noxious temperature (Oswald et al. 2011); however, in this case, the rate of temperature change influences larval behavior, and thermosensitive neurons in the central nervous system (CNS) contribute to sensing the stimulus (Luo et al. 2017). Chemical nociception can be assessed by immersing larvae in a bath solution containing noxious chemicals such as allyl isothiocyanate or hydrochloric acid (Lopez-Bellido et al. 2019). In each assay, sensitivity is quantified on a population level by measuring the percentage of larvae that respond by rolling or by calculating the average latency to roll. Combined with genetic manipulations (mutations or RNA interference), these assays have revealed genetic determinants of nociceptive sensitivity (Christianson et al. 2016; Honjo et al. 2016; Walcott et al. 2018). Although cIV-da neurons are polymodal, molecular determinants of cIV-da sensitivity can be modality-specific. For example, the receptor ion channel dTRPA1 responds to heat and chemicals (Kang et al. 2011; Neely et al. 2011; Zhong et al. 2012), whereas the heteromeric receptor ion channel Pickpocket/Balboa (ppk26) responds to mechanical stimuli (Zhong et al. 2010; Gorczyca et al. 2014; Guo et al. 2014; Mauthner et al. 2014).

Although the artificial stimuli described in the assays above are useful because they can be applied in a precise and repeatable manner, they may not accurately mimic stimuli that larvae encounter in their natural ecological context. A naturally occurring dangerous encounter in which larvae use their

rolling escape behavior is a parasitoid wasp attack (Hwang et al. 2007; Robertson et al. 2013). Parasitoid wasps of the genus *Leptopilina* attack fruit fly larvae with their ovipositor, and lay eggs within the larvae. The wasp offspring then develops within the larva, consuming fly tissues during the pupal stage. Eventually an adult wasp emerges from the pupal case instead of a fly. Parasitoid wasps can be maintained in a laboratory setting, and wasp attacks and initial larval responses can be observed (see Protocol: **Assaying Nociception Behaviors in *Drosophila* Larvae During Parasitoid Wasp Attacks** [Mauthner et al. 2024]). An attack with the wasp's sharp ovipositor is a noxious mechanical insult, and successful penetration of the larval cuticle likely results in polymodal signals including damage to the epidermis and nociceptor dendrites and the injection of venom along with the wasp egg.

NOCICEPTION CIRCUITS

Molecular tools for *Drosophila* include methods to manipulate neuronal activity, which makes it possible to investigate the contribution of specific neuronal cell types in sensory–motor circuits. For example, optogenetic activation with nociceptor-specific expression and light-triggered activation of ion channels such as channelrhodopsin (*ppk1.9-Gal4 UAS-ChR2::YFP/+*) trigger larval rolling after exposure to blue light (see Protocol: **Optogenetic Stimulation of Nociceptive Escape Behaviors in *Drosophila* Larvae** [Mauthner and Tracey 2024b]; Hwang et al. 2007; Honjo et al. 2012). Other methods to activate neurons include the red-shifted optogenetic channel CsChrimson (Klapoetke et al. 2014; Ohyama et al. 2015; Meloni et al. 2020) or thermogenetic activation with the heat-activated ion channel dTRPA1 (Kang et al. 2011; Zhong et al. 2012). Conversely, blocking nociceptor output with expression of tetanus toxin (*ppk1.9-Gal4/UAS-TNTE*), which cleaves synaptobrevin and disrupts neurotransmitter release, prevents rolling in response to noxious stimuli (Sweeney et al. 1995; Hwang et al. 2007).

The nociception circuit in the CNS integrates sensory information and produces the motor-command output for the rolling behavior. A combination of functional studies and structural connectomics (tracing of connections in electron microscopy volumes of the larval brain) have identified components of the nociception circuit (Ohyama et al. 2015; Hu et al. 2017; Takagi et al. 2017; Yoshino et al. 2017; Burgos et al. 2018; Dason et al. 2020). cIV-da neurons project to the ventral nerve cord and synapse with secondary interneurons, many of which receive multisensory inputs from other somatosensory neurons (Ohyama et al. 2015; Hu et al. 2017; Takagi et al. 2017; Burgos et al. 2018). Optogenetic activation of some secondary interneurons, as well as downstream command-like Goro neurons, is sufficient to trigger rolling (Ohyama et al. 2015; Hu et al. 2017; Yoshino et al. 2017; Burgos et al. 2018; Dason et al. 2020). However, the neural encoding of the motor command and how it coordinates the sequential activation of muscle groups is not yet understood, although some progress has been made toward this goal (He et al. 2022; Cooney et al. 2023).

NOCICEPTIVE PLASTICITY

In addition to feedforward sensory–motor pathways, there are local connections between interneurons and motorneurons (Ohyama et al. 2015; Takagi et al. 2017; Burgos et al. 2018), as well as modulatory feedback loops. The nociception circuit exhibits plasticity, which changes the larva's nociceptive sensitivity, depending on environmental conditions or internal state. Exposure to a constant noxious stimulus leads to desensitization of the nociception circuit (Kaneko et al. 2017). In contrast, ultraviolet light–induced injury heightens noxious sensitivity (Babcock et al. 2009, 2011; Im et al. 2018). Changes in nociceptive processing can occur though cellular signaling pathways in nociceptive sensory neurons (Honjo and Tracey 2018), through descending feedback loops that modulate synaptic transmission from nociceptors to secondary interneurons, or through descending

neuromodulatory mechanisms that inhibit the nociception circuit (Hu et al. 2017, 2020; Kaneko et al. 2017; Oikawa et al. 2023).

NOCICEPTION IN ADULT FLIES

Adult flies also exhibit escape and avoidance behaviors in response to noxious stimuli, which can be measured with various behavioral assays. Existing paradigms primarily rely on the jumping reflex or the avoidance of a noxious heat source like a hot plate, an infrared laser beam, a heated ring barrier, or a heated chamber (Xu et al. 2006; Aldrich et al. 2010; Neely et al. 2010). The jumping escape response has also been used to investigate nociceptive sensitization pathways after injury in adult flies (Khuong et al. 2019). Investigation of chemical nociception in adult flies relies on feeding assays that measure the consumption or avoidance of noxious compounds mixed into food or water (Al-Anzi et al. 2006; Kang et al. 2010; Kim et al. 2010; Li et al. 2020). A caveat to these experimental conditions is that these types of escape or avoidance responses are not specific to nociception behaviors, as they can also be elicited by nonnoxious stimuli like a puff of air, light, sound, or chemosensory cues. Therefore, carefully controlled experimental conditions need to be maintained to ensure that other sensory modalities are not being measured.

CONCLUSION

Drosophila larvae offer a useful model with which to study many aspects of nociception, including conserved genes and molecules, neuronal morphology, neuronal circuits, and mechanisms of plasticity. This is made possible by the clear and quantifiable larval nocifensive escape behavior. Each behavioral assay introduced here involves simple tools that are easy for any laboratory to obtain and requires only a short training phase with a certain degree of manual dexterity and patience.

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