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RESEARCH ARTICLE

Nociceptive neurons respond to multimodal stimuli in *Manduca sexta*

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ABSTRACT

The caterpillar *Manduca sexta* produces a highly stereotyped strike behavior in response to noxious thermal or mechanical stimuli to the abdomen. This rapid movement is targeted to the site of the stimulus, but the identity of the nociceptive sensory neurons are currently unknown. It is also not known whether both mechanical and thermal stimuli are detected by the same neurons. Here, we show that the likelihood of a strike increases with the strength of the stimulus and that activity in nerves innervating the body wall increases rapidly in response to noxious stimuli. Mechanical and thermal stimuli to the dorsal body wall activate the same sensory unit, suggesting it represents a multimodal neuron. This is further supported by the effects of rapidly repeated thermal or mechanical stimuli, which cause a depression of neuronal responsiveness that is generalized across modalities. Mapping the receptive fields of neurons responding to strong thermal stimuli indicates that these multimodal, nociceptive units are produced by class γ multidendritic neurons in the body wall.

KEY WORDS: Nociception, Caterpillar, Mechanosensation, Strike, Thermosensation, Multidendritic

INTRODUCTION

To avoid bodily damage, animals detect potentially harmful stimuli with a specialized sensory system called nociception. This involves peripheral sensory neurons (nociceptors) that have a high threshold for activation by thermal, mechanical, chemical or light stimuli (Burrell, 2017). These neurons typically project to specialized regions of the nervous system where they initiate avoidance or nocifensive behaviors and, at least in vertebrates, also activate pain sensation (Walters and de C Williams, 2019). Insects have been informative models for studying the mechanisms and neural circuits involved in these behaviors. For example, in response to noxious stimuli, *Drosophila melanogaster* larvae undergo a series of stereotyped avoidance movements including curling and rolling (Tracey et al., 2003). The underlying neural pathways in *D. melanogaster* have been studied extensively and shown to involve multiple parallel circuits and the integration of different sensory modalities (Chin and Tracey, 2017; Follansbee et al., 2017; Ohyama et al., 2015).

Similar behaviors are seen in other species. Tobacco hawkmoth larvae (*Manduca sexta*) respond to noxious stimuli to anterior segments by quickly moving their head away from the source of stimulation in an avoidance behavior known as withdrawal (Walters et al., 2001). Conversely, in response to noxious stimuli to the posterior abdomen, larvae respond with a rapid head swing towards the site of stimulation in a nocifensive strike. This movement can be used to startle a predator or directly remove the source of irritation (Walters et al., 2001). Strike behavior is targeted, and its spatial accuracy depends on the location of the stimulus (van Griethuijsen et al., 2013). Additionally, strong (e.g. pinching) repetitive noxious stimuli or chronic infection also causes a long-term increase in behavioral responsiveness (sensitization), which has been the focus of several studies (Adamo and McMillan, 2019; McMackin et al., 2016; Mukherjee and Trimmer, 2019; Tabuena et al., 2017; Walters et al., 2001).

Despite these studies, the sensory neurons mediating nocifensive behaviors in *Manduca* are unknown. In *D. melanogaster* larvae, the nociceptors have been identified as a subset of multidendritic (MD) sensory neurons that tile the body wall (Grueber et al., 2002, 2003; Mauthner et al., 2014; Terada et al., 2016). These class IV dendritic arborization (c4da) neurons are polymodal and can be activated by strong mechanical stimuli, high temperatures, strong light or noxious chemicals to elicit avoidance behaviors (Al-Anzi et al., 2006; Hwang et al., 2007; Robertson et al., 2013; Zhong et al., 2010). These neurons are sufficient for nociception, as the nocifensive behavior can be elicited by directly activating c4da neurons with optogenetics (Burgos et al., 2018; Ohyama et al., 2015; Terada et al., 2016; Yoshino et al., 2017).

Similar MD neurons are found in larval *M. sexta* where 12–16 primary neurons tile the body wall in each hemi-segment (Grueber and Truman, 1999). These neurons have been categorized by their tiling and branching patterns into three classes (α, β and γ) (Grueber and Truman, 1999; Grueber et al., 2001). The γ MD neurons have widely branching, non-overlapping dendritic fields that closely resemble the c4da neurons in *D. melanogaster* but their role in nociception has not yet been established.

Identifying nociceptive neurons in *Manduca* is expected to be helpful for comparative studies of nociceptive mechanisms and avoidance behaviors. The lifecycle and anatomy of *D. melanogaster* and *M. sexta* are superficially similar, making many comparisons relatively straightforward; however, the two species are separated by at least 260 million years of evolution (Misof et al., 2014). Hence, similarities and differences between the nociceptive transduction mechanisms and neural circuits in these two species can distinguish between fundamental and species-specific traits. This may also have implications for clinical research in nociception, pain and neuropathologies where human genetic approaches have led to convoluted and conflicting findings (Burrell, 2017).

In this paper, we used *M. sexta* as a behavioral and neural model for studying nociception. We show that localized mechanical and thermal stimuli can both evoke strike behavior. The same stimuli evoke sensory neuron activity in nerves supplying the body wall. At

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least part of this response is mediated by multimodal nociceptive sensory neurons whose receptive fields correspond to γ MD neurons.

MATERIALS AND METHODS

Experimental animals

Manduca sexta (Linnaeus 1763) larvae were reared in individual containers on an artificial diet (Bell and Joachim, 1976) in a colony at Tufts University. They were grown at 27°C on a 17 h:7 h light: dark cycle. Larvae of both sexes between the first and fourth days of the fifth instar were used in all experiments. Across these experiments, there were no discernible effects of age on strike threshold or neural responsiveness.

Stimuli

Stimulus duration was controlled using square-wave signals generated by a USB analog voltage output module (USB-9269, National Instruments, Austin, TX, USA) operated in MATLAB (MATLAB 2018b, The MathWorks, Inc., Natick, MA, USA). During electrophysiological experiments, square-wave signals were simultaneously recorded to synchronize stimulus timing with neural recordings.

Thermal stimuli

A small area of the body wall was heated using short pulses of infrared radiation generated by a low power (400 mW) laser (diode laser, 808 nm, Lilly Electronics, Hubei, China) (Bell and Joachim, 1976). The laser was mounted to a manipulator to position and focus each stimulus. The horizontal laser beam was reflected onto the preparation using a 45 deg dichroic short-pass filter (69-219, Edmonds Optics Inc., Barrington, NJ, USA) that allowed the target location on the body wall to be safely observed using a binocular microscope positioned above the preparation. The laser beam was focused to a diameter of 650–700 µm onto the body wall, which was coated with a thin layer (approximately 200–300 µm) of black paint (flat black, oil modified alkyd, Rust-Oleum, Vernon Hills, IL, USA). Temperatures varied with stimulus duration and were calibrated with a bare small diameter (0.002 mm) type T thermocouple (Omega Engineering, Inc., Stamford, CT, USA) using a National Instruments USB-9211 in LabVIEW software (National Instruments). For behavioral experiments, stimulus durations of 10–130 ms were used, reaching peak temperatures of 27–55°C. During electrophysiological experiments, the laser was passed through a series of glass filters (12-545-100, Fisherbrand, Hampton, NH, USA) so that a 1 s stimulation delivered a peak temperature of 70°C. Behavioral responses to this stimulus were also tested.

Mechanical stimuli

Strong mechanical stimuli were delivered to the body wall using stainless steel filaments 0.4 mm in diameter (Malin Co., Cleveland, OH, USA). As the filaments bent, they applied a constant tip force which could be varied by changing the length of the filament. Lower forces were achieved by attaching a short length of nylon fishing line to the filaments. Filaments were created to deliver forces between 2.5 and 100 mN and calibrated on an electronic balance. During behavioral experiments, stimuli were applied by hand. During electrophysiological experiments, 100 mN stimuli lasting 1 s were applied to the external surface of the cuticle using a solenoid-powered lever. The tip of this filament was capped with a droplet (diameter 0.8 mm) of glue to electrically isolate it from the preparation.

In experiments testing for neurons with a multimodal response, alternating thermal and mechanical stimuli (each 1 s duration) were delivered at 0.1 Hz. Sensory depression was evoked by either short (0.1 s) mechanical stimuli delivered at high frequency (3 Hz) or using repeated thermal stimuli (1 s) at 0.8 Hz.

Receptive field mapping

In initial receptive field mapping, the area between the spiracle and the dorsal midline (measuring 40–44 mm×64–72 mm) was defined by a 4×4 grid map. Thermal stimuli were applied to four distinct points within each box in the grid. The order of stimulus locations was randomized across preparations (n=3). Firing frequencies during stimuli were compared with basal firing frequencies 1 s prior to the laser pulse. The strength of the response was categorized by standard deviations above the basal firing frequencies. The average across the four stimulus locations in each box was reported.

In further mapping, preparations were stimulated on the fifth and sixth annuli of a segment with alternating thermal and mechanical noxious stimuli, first delivered dorsally and then, after a brief (<30 s) pause, stimulated laterally. Evoked spikes were sorted and classified as described below.

Behavior

The behavioral effects of thermal and mechanical stimuli of different intensities were tested on larvae grasping a rounded wooden dowel. Each larva was left undisturbed for 10–20 min to adjust to the new environment. Thermal or mechanical stimuli were then applied in a randomized order with an inter-stimulus interval >1 min to minimize sensitization effects (Mukherjee and Trimmer, 2019). The stimuli were delivered within a 1.5 mm radius of the spiracle of segment A4. Stimuli were tested on either the left or right side, or both. Behavioral responses were graded as no response, twitching of the body segment or nocifensive strike response.

Dissection and electrophysiological recordings

Larvae were anesthetized in CO_2 for 12–20 min prior to dissection. Segments anterior to A2 and posterior to A5 were excised, and a lateral incision was made on the ventral–lateral side of the body wall, between the spiracles and the prolegs (Fig. 1A). This incision was extended longitudinally and the gut was removed. The body was washed with a modified Miyazaki saline at 4°C (Trimmer and Weeks, 1989). Larvae were pinned cuticle-side down on a Sylgard dish containing saline at 4°C. The trachea, fat body and muscles of segment A3 between the ganglion and the spiracle were excised to reveal the dorsal nerve and its lateral branch (Fig. 1B). The anterior and posterior branches of the dorsal nerve were ablated. A window of cuticle below the ganglion was removed to allow access to the dorsal nerve. After a wash with 24°C saline, the preparation was flipped and re-pinned on the dish with the exterior cuticle exposed for stimulation (Fig. 1C).

A polished glass electrode was used to suck onto the dorsal nerve until a tight seal was formed. The dorsal nerve was severed close to the ganglion to remove all efferent spike activity, leaving only afferent spikes from peripheral sensory neurons. Recordings were amplified with a differential AC amplifier (Model 1700, A-M Systems, Sequim, WA, USA) with filtering for frequencies below 100 Hz and above 10,000 Hz. The signal was digitized by Powerlab 4/35 processed in Labchart 8 Pro software (ADInstruments Inc., Colorado Springs, CO, USA) at a sampling frequency of 40 kHz. Although neural signals continued for hours after the dissection, all recordings were collected within 15–120 min of the start of the dissection.

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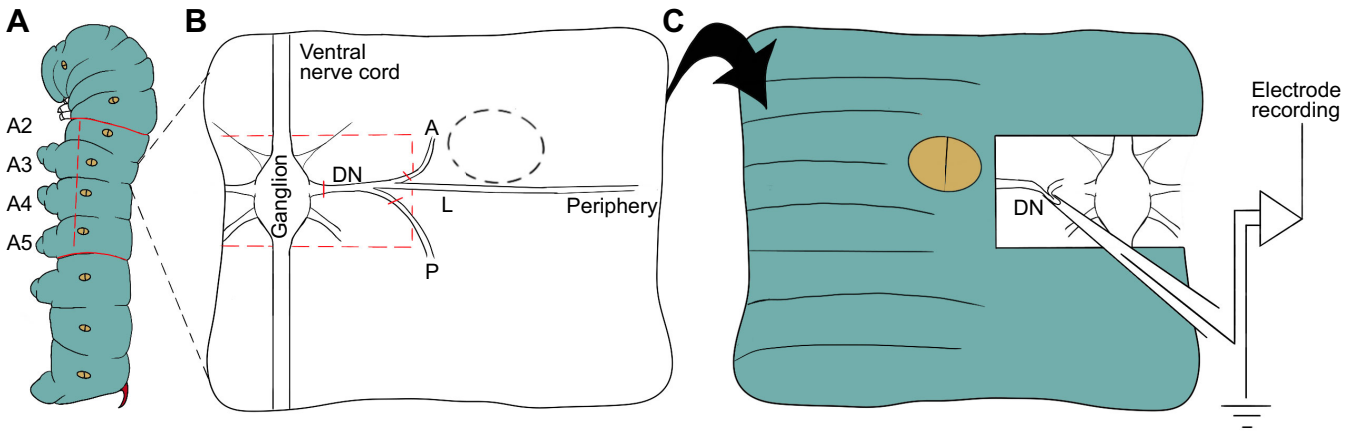

Fig. 1. Dissection and recording methods. (A) *Manduca sexta* larvae were dissected with a lateral incision between the spiracles and the prolegs, as shown by the red dashed line. Segments A2–A5 are labeled. (B) The lateral (L) branches of the dorsal nerve (DN) were dissected out. The dorsal nerve was disconnected from the ganglion and the posterior (P) and anterior (A) branches. A window was cut (dashed red lines) to allow access to the dorsal nerve. (C) The preparation was flipped over, and a glass suction electrode was attached to the dorsal nerve. Differential extracellular recordings recorded action potentials sent by peripheral sensory neurons while the cuticle was stimulated.

Analysis

Electrophysiological recordings were digitally filtered (low-pass cutoff at 1500 Hz and high-pass cutoff at 500 Hz), which improved the signal to noise ratio without distorting spike shapes. Spikes were detected using an amplitude threshold above the baseline noise. In most recordings, the threshold exceeded double the amplitude of the noise. Spikes were aligned and sorted using the spike sorting wizard in DataView (Heitler, 2007). Principal component analysis (PCA) was run on features of spike shape such as amplitude, rise time and width (n>1000 spikes minimum; most had n>5000 spikes). The three principal components that explained the most orthogonal variance were extracted and clustered by an unsupervised algorithm. The total number of clusters was calculated by Rissanen's minimum description length, and cluster means were determined using an expectation maximum equation.

Although spikes identified in the multimodal nociceptive cluster often had the second largest amplitude of spikes in peripheral recordings of the lateral branch of the dorsal nerve (larger amplitude spikes are usually generated by the stretch receptor organ), relative amplitude and shape of spikes changed with different recording properties. Thus, this cluster was defined by its response to both noxious thermal and noxious mechanical stimuli, which was consistent across preparations. Spike shape diagrams were created in DataView, and spike times, principal components and stimulation times were exported to R (http://www.R-project.org/) for statistical analysis.

To analyze the change in firing frequency across time points in depression trials, we used a one-way repeated measures analysis of variance (RM-ANOVA). This test assumes that the residuals are normally distributed and spherical. Mechanical responsiveness during depression with mechanical stimuli met the assumptions of normal distribution (Shapiro–Wilk, W=0.96, P=0.0974) and sphericity (Mauchly, χ^2=0.25, P=0.0608). Thermal responsiveness during depression with mechanical stimuli violated the assumptions of normal distribution (Shapiro–Wilk, W=0.94, P=0.0127), but square-root transformed data did not violate the assumptions of normal distribution (Shapiro–Wilk, W=0.96, P=0.0574) or sphericity (Mauchly, χ^2=0.93, P=0.8579). Mechanical responsiveness during depression with thermal stimuli violated the assumptions of normal distribution (Shapiro–Wilk, W=0.95, P=0.0434), but log-transformed data did not violate the assumptions of normal distribution (Shapiro–Wilk, W=0.96, P=0.1548) or sphericity (Mauchly, χ^2=0.27, P=0.1432). Thermal responsiveness during depression with thermal stimuli violated the assumptions of normal distribution (Shapiro–Wilk, W=0.92, P=0.0068), but square-root transformed data did not violate the assumptions of normal distribution (Shapiro–Wilk, W=0.98, P=0.5309) or sphericity (Mauchly, χ^2=0.88, P=0.8186). Multiple comparisons testing on significant RM-ANOVA models was performed using Tukey's HSD.

RESULTS

Stimuli above a threshold trigger a nocifensive strike response

To establish stimulus thresholds for thermal and mechanical activation of strike behavior (Fig. 2A), stimuli of different intensity were applied to the fourth abdominal segment (A4). The responses to brief, local, thermal stimulation were tested between the peak temperatures of 27 and 55°C (Fig. 2B, n=9). It was found that larvae could sense temperatures between 27 and 32°C, often responding with brief twitches. Temperatures above 41°C predominantly resulted in strikes, and temperatures above 50°C consistently elicited strike behavior. Because local twitching could not be reliably observed during mechanical stimulation (the stimulus itself causes cuticular deformation), all responses to mechanical stimuli were classified as strike or no strike. Applied forces below 6 mN did not evoke strikes but the forces above 46 mN consistently triggered strike behavior (Fig. 2C, n=18). In subsequent experiments, peak temperatures of 70°C and peak forces of 100 mN were used to ensure that noxious stimuli were always suprathreshold.

Spike sorting reveals a single multimodal neuron

Sensory spike activity in the dorsal nerve increased in response to both noxious thermal and noxious mechanical stimuli (Fig. 3A,B) and quickly returned to basal levels at the end of the stimulus.

To determine whether the same neuron was responding to both modalities, alternating mechanical and thermal stimuli were applied to a preparation for 5 min. Spike sorting by clustering on principal components (Fig. 3C) revealed that a sensory unit forming one cluster fired in response to both thermal and mechanical stimuli (Fig. 3A,B). Further analysis of this cluster showed that the spike waveforms in response to each modality were indistinguishable by shape and principal components (Fig. 3D). Although the total number of clusters varied, a multimodal cluster of spikes was

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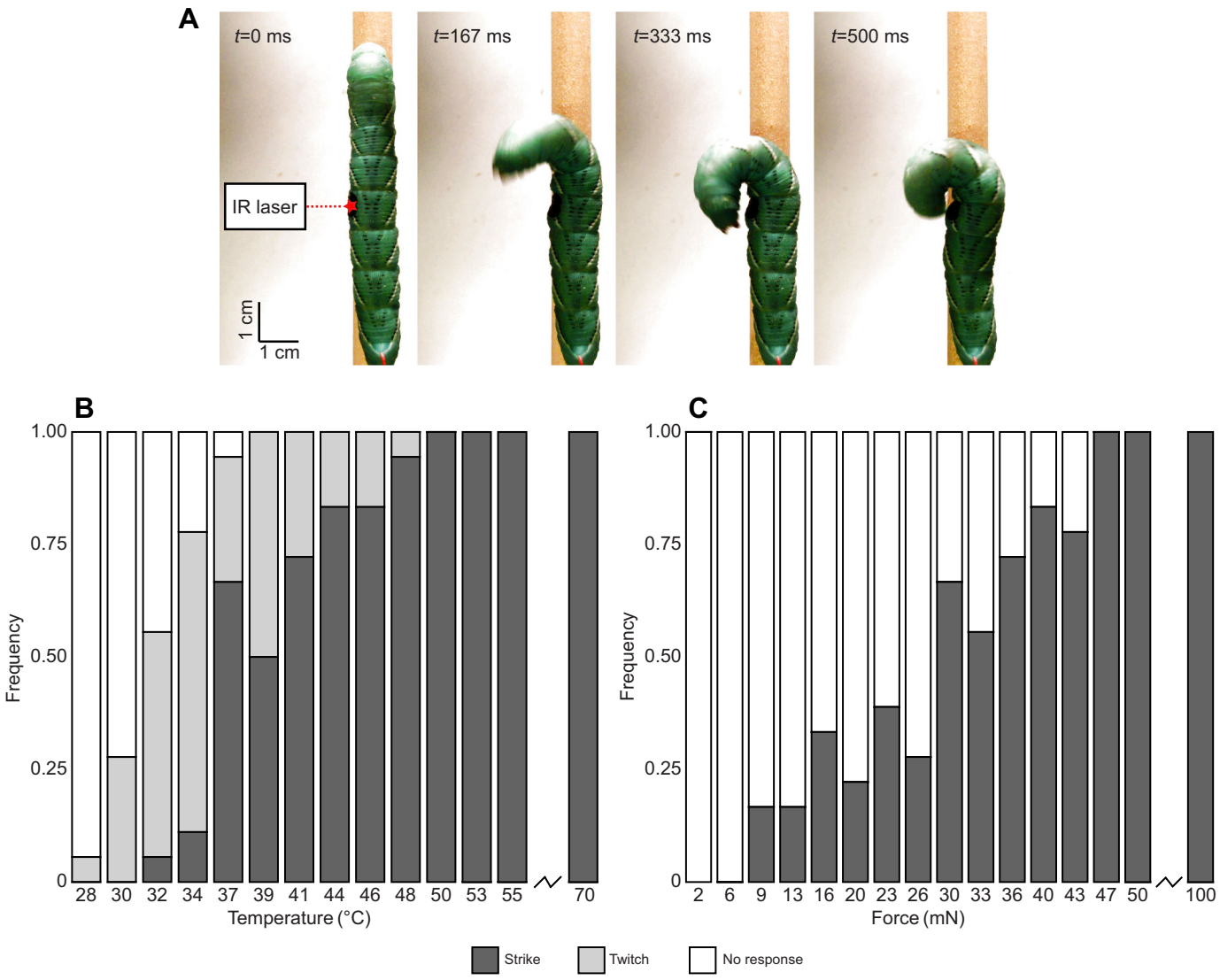

Fig. 2. Stimuli above a noxious threshold produce a stereotypical strike response. (A) Frames of a *M. sexta* larva strike response. Segment A4 was painted black for thermal absorption. (B) Responsiveness of the larvae to temperatures between 28 and 70°C classified as strike, twitch or no response (n=9 caterpillars, 18 total trials). (C) Responsiveness of the larvae to forces between 2 and 100 mN classified as strike or no response (n=18).

consistently found across all preparations (n>25). Because this multimodal unit fired primarily in response to noxious thresholds of stimuli, we have described it as nociceptive.

This multimodal unit responded tonically to noxious thermal stimuli, with little change in the firing frequency of other units (Fig. 4A). Noxious mechanical stimuli evoked phasic responses in the multimodal unit as well as in other units, suggesting that these neurons adapt quickly (Fig. 4B). In addition to MD neurons, it is likely that other mechanoreceptor neurons in the body wall, including those associated with sensory hairs, contribute to this phasic response (Peterson and Weeks, 1988). Occasionally during mechanical stimulation there was a tonic increase in firing frequency of a single unit (not shown), presumably caused by the stretch receptor organ, which has a known tonic response to cuticular deformation (Simon and Trimmer, 2009).

Nociceptive depression is independent of modality

To further examine whether this multimodal nociceptive unit represents a single neuron, we analyzed how responsiveness to mechanical and thermal stimuli was affected by repetition. Repeatedly applying a thermal stimulus at 0.8 Hz for 1–3 min, or repeatedly stimulating with mechanical stimuli at 3 Hz for 2–8 min, decreased or completely suppressed the nociceptive response to both stimuli. This depression of the nociceptive response is unlikely to result from localized tissue damage because other neurons continued to fire throughout the experimental protocol (Fig. 5A). The depression was short lived, and after 6 min, there was a partial or complete recovery of responsiveness to both types of stimulus. Depression took longer to develop and lasted longer in response to mechanical stimuli compared with thermal stimuli. Mean responsiveness to thermal and mechanical stimuli differed across time points during repeated thermal stimulation (RM-ANOVA on square root-transformed thermal responsiveness, $F_{2,8}$=22.67, P=0.0005 and RM-ANOVA on log-transformed mechanical responsiveness, $F_{2,8}$=27.66, P=0.0003) (Fig. 5B). Thermal and mechanical responsiveness after repeated thermal stimulation was significantly lower than initial responsiveness (Tukey's HSD, P=0.0009 and P=0.0111, respectively). Thermal and mechanical responsiveness was significantly increased from depressed levels

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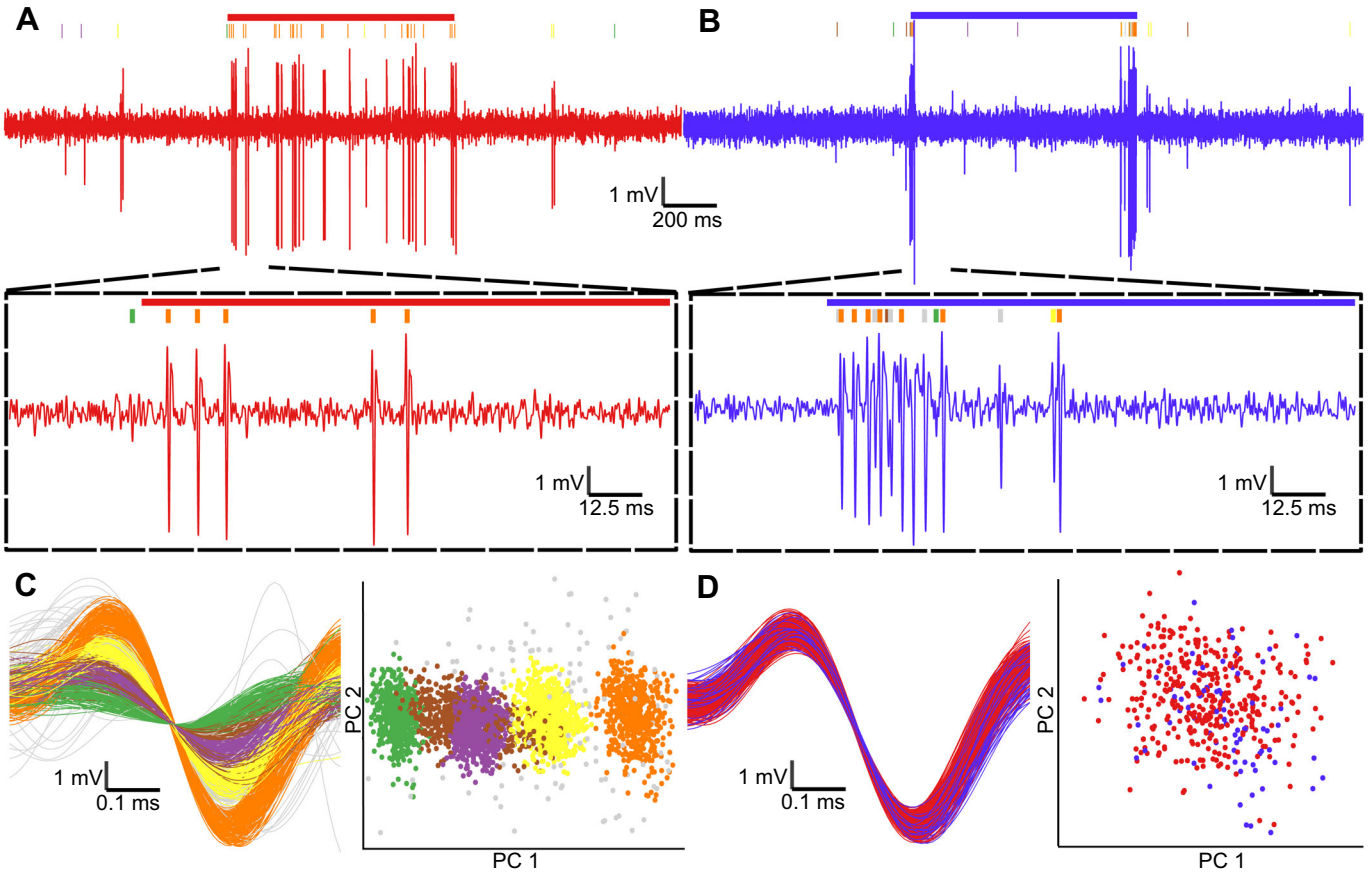

Fig. 3. Thermal and mechanical responses include a common spike shape. (A,B) Extracellular recordings of the dorsal nerve during noxious thermal (A) and noxious mechanical (B) stimulation (thick horizontal bars). The spike raster is shown above the recording, colored by clusters from principal component analysis (PCA). The nociceptive spike (orange) is common to thermal and mechanical responses. (C) Traces of ~3200 spikes from a 5 min recording of 0.1 Hz alternating stimulation colored by cluster. Clusters are separated by the first two principal components (PC). (D) Traces of the ~500 nociceptive spikes in response to thermal or mechanical stimuli recolored by stimulus. Recolored nociceptive spikes do not separate by the first two principal components.

after 6 min of recovery (Tukey's HSD, P=0.0001 and P=0.0029, respectively).

Similarly, mean responsiveness to thermal and mechanical stimuli differed across time points during repeated mechanical stimulation (RM-ANOVA on square root-transformed thermal responsiveness, $F_{2,10}$=25.52, P=0.0001 and RM-ANOVA on mechanical responsiveness, $F_{2,10}$=8.35, P=0.0074) (Fig. 5C). Thermal and mechanical responsiveness after repeated thermal stimulation was significantly lower than initial responsiveness (Tukey's HSD, P=0.0222 and P=0.0130, respectively). Thermal and mechanical responsiveness was significantly increased from depressed levels after 6 min of recovery (Tukey's HSD, P=0.0314 and P=0.0123, respectively). The onset and duration of depression also differed between preparations but in all cases depression and recovery co-varied for the two modalities.

The receptive fields of nociceptive units correspond to γ MD neurons

Nociceptors in larval *D. melanogaster* have been identified as c4da neurons that are among a group of sensory neurons tiling the body wall. In *M. sexta*, similar MD neurons have been classified into three classes (α, β and γ) with non-overlapping receptive fields (Fig. 6A). The nociceptive receptive field in the dorso-lateral region of the body wall was mapped using thermal stimuli while recording from the dorsal nerve (Fig. 6B). Responses could be elicited across the entire region, with only small differences in the magnitude of the evoked response (Fig. 6B). This broad receptive field implicates the MD neurons as possible nociceptors but does not correspond to the α subtype, whose dendrites arborize in a narrow region (Fig. 6A). To distinguish γ MD and β MD neurons, spikes were sorted and classified in response to alternating thermal and mechanical stimuli applied to the dorsal and lateral positions on the hemi-segment (Fig. 6C). Two different sensory units were found to be nociceptive, corresponding to the dorsal and lateral receptive fields of the γ MD neurons (Fig. 6D).

DISCUSSION

The role of mechanical and thermal sensation in nociceptive and avoidance behavior in insect larvae

Both withdrawal and strike behavior in *M. sexta* are elicited by strong mechanical or thermal stimuli applied to the body wall. However, there is substantial variability in the timing and magnitude of these movements and they are known to be affected by context and previous experience (van Griethuijsen et al., 2013; McMackin et al., 2016; Tabuena et al., 2017; Walters et al., 2001). This variability suggests that noxious stimuli do not simply trigger reflexive motor programs, but instead involve integration of multiple sensory inputs and a variety of motor pathways. Avoidance behaviors in *D. melanogaster* larvae are also affected by the type and magnitude of sensory stimulation (Hu et al., 2017) and there are numerous parallel circuits mediating these responses

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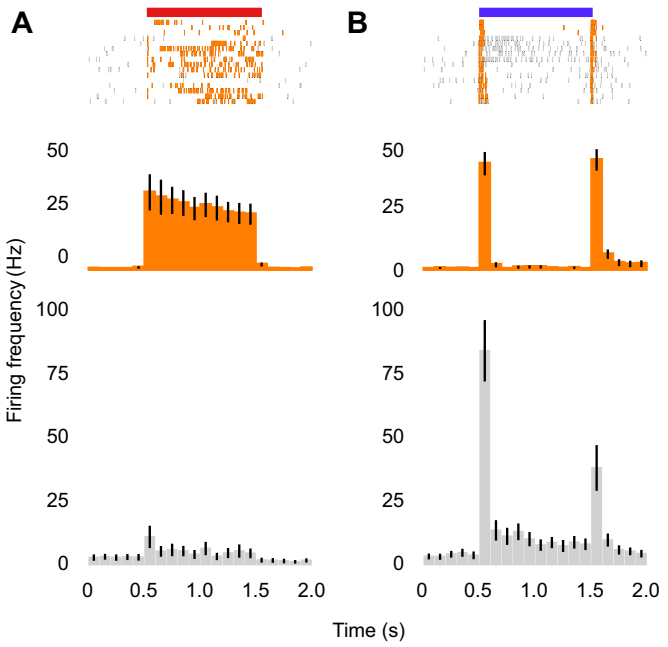

Fig. 4. Firing patterns of thermal and mechanical nociception. (A,B) Example rasters of the reaction to thermal (A) or mechanical (B) stimuli. Orange, mean firing frequency of the nociceptive spike in reaction to either stimulus. Gray, mean firing frequency of other spikes in reaction to either stimulus. Firing frequencies were calculated with windows of 100 ms and averaged over 90 stimulations from five preparations; error bars represent 95% confidence intervals.

(Burgos et al., 2018; Chin and Tracey, 2017; Ohyama et al., 2015; Yoshino et al., 2017).

Previous studies on nociception in *M. sexta* have used mechanical stimuli to evoke strikes. *Manduca sexta* caterpillars have numerous mechanical sensors that could respond to these stimuli. Primary among these are filiform sensilla (Peterson and Weeks, 1988), chordotonal organs (Simon and Trimmer, 2009) and MD neurons in the body wall (Grueber and Truman, 1999; Grueber et al., 2001). Gentle touch can evoke local reflexes such as proleg withdrawal by activating filiform hairs and it is presumed these hairs are the primary mechanism for sensing physical contact with the environment (Weeks and Jacobs, 1987).

Internal mechanoreceptors, such as stretch receptors, are thought to act as proprioceptors, providing information about internal stresses and body position. However, soft animals deform in response to low forces so the distinction between touch sensing and proprioception might not be as marked as it is for adult insects and vertebrates. This certainly appears to be the case for *M. sexta* in which the response properties of stretch receptors are not well matched to movement or position encoding (Simon and Trimmer, 2009). The role of the MD neurons is not well characterized in *M. sexta*. Apart from mechanical responses of an α or β MD neuron, and higher threshold mechanical responses of a γ MD neuron (Grueber et al., 2001), little is known about the sensory properties of the primary or secondary plexus of MD neurons (Grueber and Truman, 1999).

Thermal receptors in Lepidoptera are even less well known. Temperature sensing has been detected in butterfly wing veins (Schmitz and Wasserthal, 1993) and moth antennae (Gödde and Haug, 1990), but until recently the only evidence for thermal sensing in caterpillars was confined to antennal receptors (Schoonhoven, 1967) and anecdotal behavioral observations (Frings, 1945). It has now been shown that strong localized thermal stimuli, delivered using an infrared laser, can evoke strike behavior in *M. sexta* (Mukherjee and Trimmer, 2019). What is not known is how this stimulus is detected, or how it relates to mechanical nociception.

Manduca sexta responses to thermal and mechanical stimuli

Although strike responses are elicited by strong mechanical and thermal stimuli, we found that *M. sexta* can also respond to relatively weak temperature changes on very small patches of the body wall. Local muscular contractions were elicited with peak temperatures as low as 30°C. With stimulus temperatures above 40°C, these local responses were quickly superseded by an increased tendency to strike. It is possible that the twitches evoked by low-temperature stimulation are caused by direct activation of motor neurons or muscles, but this is unlikely given the small size of the stimulus. We made measurements of the temperature at various distances from the center of the stimulus and estimate that at a peak surface temperature of 30°C, the underlying tissues 100–200 µm away would remain close to room temperature. It is more likely that some sensory neurons in the body wall can respond to small changes in temperature. *Drosophila melanogaster* larvae have specialized low-temperature thermal sensors in the terminal organs and dorsal organs (Dillon et al., 2009), and many neurons in the body wall are thermo-responsive (Liu et al., 2003).

Noxious thermal and mechanical stimuli to a single location activate the same neuron

In contrast to the responses to low-temperature stimuli, thermal pulses above 50°C evoked strike behavior. Similarly, strong mechanical pokes above 47 mN evoked similar nocifensive behavior. Recordings from the dorsal nerve innervating the stimulus site showed that a single firing unit responded to both types of stimulus, which strongly suggests that a single neuron responds to both modalities (Rey et al., 2015). We identify this multimodal cluster as nociceptive as it responds to these noxious stimuli, but it is possible that other sensory neurons below our threshold of detection fire in response to these stimuli and mediate nocifensive behavior.

Repeated noxious stimulation at high frequencies causes depression of the nociceptor

Manduca sexta's response to noxious stimulation is affected by prior stimulation. For example, after brief but intense mechanical stimulation, larvae become more sensitive to gentle mechanical stimulation (allodynia) and they have increased sensitivity to repeated noxious stimuli (hyperalgesia) (Tabuena et al., 2017; Walters et al., 2001). Hyperalgesia has also been demonstrated in response to long-interval (10 min) repetition of thermal stimuli. This form of sensitization involves both peripheral and central components (Mukherjee and Trimmer, 2019).

We have now demonstrated a second form of nociceptive plasticity. When very brief mechanical and thermal stimuli are delivered at high frequency for an extended period, the nociceptive neuron becomes less responsive or even completely suppressed. This does not appear to be caused by permanent damage as nociceptive responses can be recovered after a stimulus-free period. Although nociceptive adaptation has been described in nematodes (Hilliard et al., 2005), to our knowledge, this is the first time that neural depression in nociceptors has been explicitly demonstrated in insects. The behavioral relevance of this neural depression is

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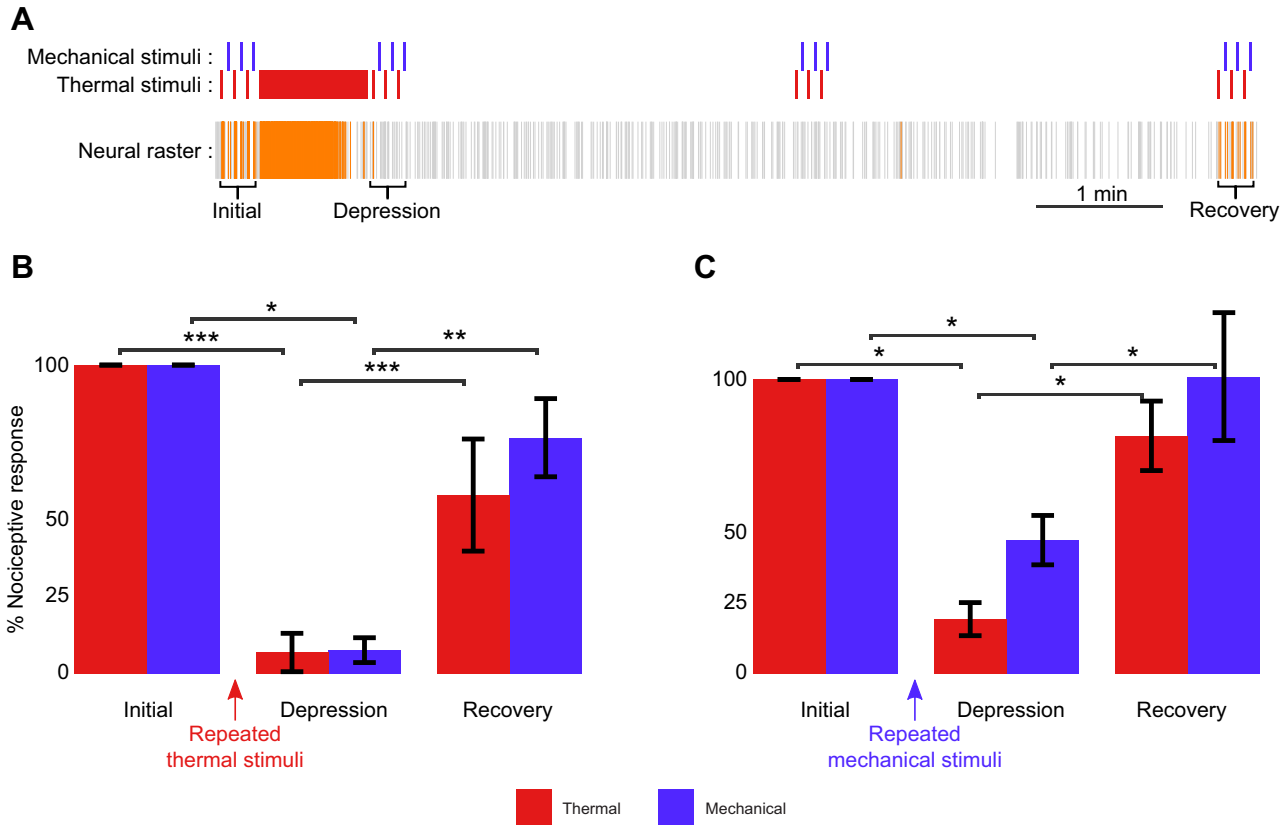

Fig. 5. Cross-thermal and mechanical depression. (A) Example raster plot of repeated thermal stimulation and depression. Thermal (red) and mechanical (blue) stimuli are shown above. Nociceptive spikes are shown in orange, non-nociceptive spikes are shown in gray. (B,C) Bar charts of mechanical depression (n=6) and thermal depression (n=5). Mean percentage responsiveness was measured as the average number of nociceptive spikes from three samples at each time point relative to 100% of initial responsiveness. Responsiveness at each time point was compared using repeated measures ANOVA with a pairwise Tukey test. *P<0.05, **P<0.01 and ***P<0.001. Error bars represent s.e.m.

unknown; in this study, the phenomenon was elicited with extreme conditions of rapidly repeated stimuli on sensory neurons detached from the central nervous system. We have used this new finding to demonstrate that depression evoked by mechanical or thermal stimuli is multimodal, always affecting the other modality in the same way. This strongly supports the results of spike sorting and

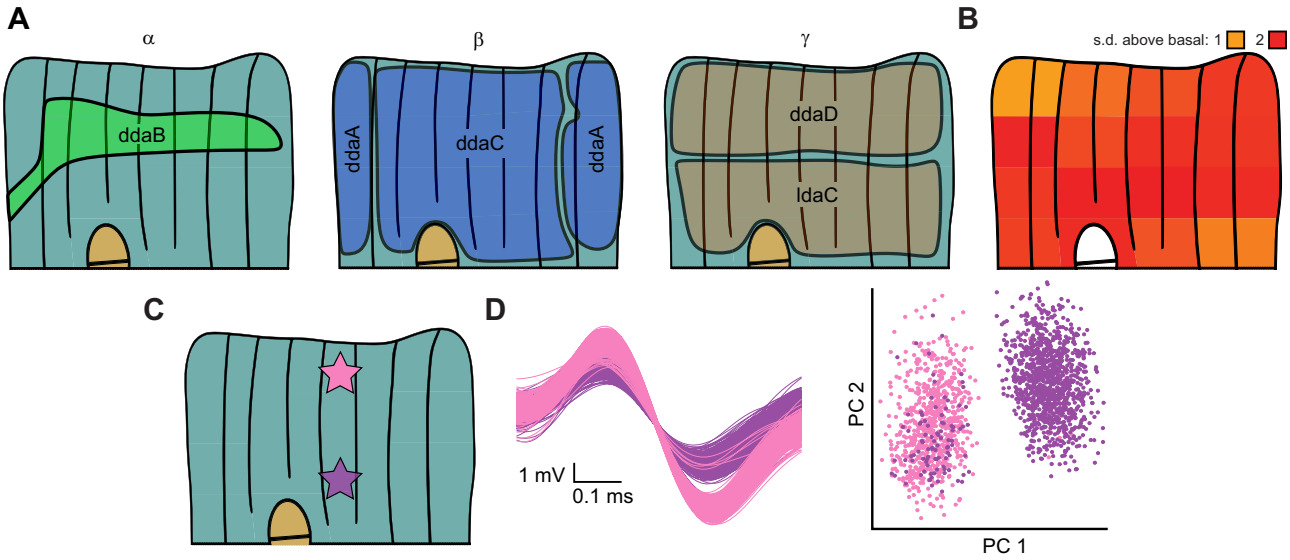

Fig. 6. Neural response of nociceptive neuron conforms to receptive fields of class γ multidendritic (MD) neurons. (A) Potential receptive fields of identified MD neurons (ddaA–D) in a single hemi-segment. Modified from Grueber et al. (2001). Dorsal is up, anterior is left. (B) General sensitivity to noxious heat stimulation. Responses were graded by standard deviation above the mean basal firing frequency recorded immediately preceding the stimulus. (C) Location of dorsal (pink) and lateral (purple) stimuli. (D) Spike sorting of recordings, colored by stimulus position.

confirms that a single neuron responds to both thermal and mechanical stimuli.

These multimodal nociceptors are likely γ MD neurons

MD neurons in *M. sexta* have been hypothesized to mediate nociception. Here, we systematically demonstrated that the dorsal hemi-segment is generally receptive to noxious stimuli, further implicating MD neurons. The divisions in the receptive fields correspond to the dendritic fields of γ MD neurons. Other observations also point to a role of γ MD neurons in nociception. First, c4da neurons in *D. melanogaster*, which have a known role in nociception, and γ MD neurons in *M. sexta* have similar dendritic branching patterns that are more complex than branching of other classes (Grueber et al., 2001, 2002). Second, one of the γ MD neurons (ddaD) responds phasically at the start and end of intense mechanical stimulation (Grueber et al., 2001), much like the nociceptive neurons shown here.

Ability to distinguish modalities

Although no behavioral difference has been observed between nocifensive strikes in response to mechanical and thermal stimuli, this has not been tested empirically. In this model, we propose that γ MD neurons respond to both noxious heat and noxious poking, but it is still possible that *M. sexta* larvae can distinguish these modalities. In this study, mechanical stimuli triggered both the nociceptive neuron and other sensory neurons responsive to mechanical stimulation. In a population encoding model, the simultaneous firing of these nociceptive and mechanoreceptive neurons may be processed differently in the central nervous system (Ma, 2010).

Moreover, the responses to mechanical stimuli were phasic, while the responses to thermal stimuli were tonic. This may also allow for discrimination. It has been found that noxious light and noxious thermal stimulation are encoded differently in *D. melanogaster* c4da neurons to produce different nocifensive behaviors (Terada et al., 2016). This should be further tested with more controlled stimuli and various intensities. For example, it is also possible that this nociceptive neuron responds to changing intensities of stimuli, and that tonic responses were triggered by the dynamically increasing thermal stimulus, while phasic responses resulted from rapid adaptation once the mechanical stimulus reached a constant level.

A new model for multimodal nociception

These findings pave the way for future studies into *Manduca* larvae as a model for multimodal nociception. More stimulus modalities, such as noxious cold and chemical stimuli, should be explored to identify other overlaps in nociceptive pathways. Comparisons between the encoding properties of γ MD neurons in *M. sexta* and c4da neurons in *D. melanogaster* may help to reveal more generalizable principles in multimodal nociceptive signaling. The large size of *M. sexta* larvae may also allow for a better understanding of how the spatial resolution of nociception is established. *Manduca sexta* also have a secondary plexus of MD neurons which is not found in *D. melanogaster*. Their roles in sensation should now be explored.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.P.C., B.A.T.; Methodology: D.P.C., M.R.; Formal analysis: D.P.C.; Investigation: D.P.C., M.R.; Writing - original draft: D.P.C.; Writing - review & editing: D.P.C., M.R., A.E.S., B.A.T.; Visualization: D.P.C.; Supervision: A.E.S., B.A.T.; Project administration: B.A.T.; Funding acquisition: B.A.T.

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