Neuroscience Research 83 (2014) 54–63

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Anatomical and genotype-specific mechanosensory responses in *Drosophila melanogaster* larvae

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ARTICLE INFO

Article history:
Received 27 November 2013
Received in revised form 8 April 2014
Accepted 9 April 2014
Available online 21 April 2014

Keywords:
Mechanosensory
Behavior
Drosophila melanogaster larva
Crawling
Electrophysiology

ABSTRACT

Afferent neurons commonly exhibit a somatotopic arrangement in the central nervous system that organizes spatially discrete sensory input. We are interested in how that spatial input gets integrated into motor commands. With resources for screening genes and neural circuits, and given that the cells and ion channels that transduce tactile stimuli in *Drosophila* larvae are remarkably well-characterized, larval mechanosensation is an ideal system for investigating how specific behaviors emerge from localized sensory input. We observed that crawling larvae are more reactive to a 20 mN tactile stimulus on the head than on the tail or abdomen. Behavioral responses that were evoked by the stimuli also depended on where the stimulus was delivered. Differences in relative sensitivity were observed in different genotypes, e.g., a null *white* mutant and hypomorphic *smn* mutant are significantly more reactive to tail touches than Canton-S larvae. Responses were inhibited by silencing chemical transmission in a combination of multidendritic and chordotonal neurons, but not by inhibiting any specific subset of neurons. Extracellular recordings from segmental nerves revealed that sensory-evoked responses exhibit spike-timing dependence at the neural circuit level. Tactile stimuli reduced endogenous firing frequency and increased bursting periods when applied during periods of motor activity. The same stimulus initiated bursts of activity when applied during inactive periods. Together, these data depict the spatial and temporal complexity of mechanosensation as it applies to action selection, and provide a foundation for addressing how neural circuits in the CNS adjust their activity to afferent input.

1. Introduction

Experience, environment, metabolic state, and genotype are factors that influence how an animal responds to different stimuli. We want to know how these factors are weighted for responses to a given stimulus, and how the information is integrated in neural circuits. There are a number of sensory systems that could be used to address this problem; we chose mechanosensation in *Drosophila melanogaster* larvae because the detection of tactile stimuli in this organism has been characterized down to the molecular level (Kim et al., 2012; Yan et al., 2013), the specific subsets of neurons that detect mechanical stimuli have been identified (Hwang et al., 2007; Yan et al., 2013), and genetic tools can be used to control neural activity in these neurons (Venken et al., 2011). The purpose of this study was to further characterize behavioral and physiological responses to localized tactile stimuli as a prelude to investigations on plasticity in this system.

Given that some mechanical stimuli will likely have a different meaning to the larva in the sense of impending danger or harm to the animal, one would expect varying sensitivity in responsiveness. Such differences in the behavioral responses indicate the uniqueness of the neural circuitry involved in eliciting these behaviors. Sensory input is known to influence the development of larval sensorimotor circuits (Kohsaka et al., 2012; Fushiki et al., 2013). Sensory commands for responses to mechanosensory stimuli and the central pattern generator (CPG) for locomotion are known to communicate with each other (Hughes and Thomas, 2007; Song et al., 2007), however, the precise synaptic and neural architectural details remain to be determined. Modulation of this circuitry is of

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http://dx.doi.org/10.1016/j.neures.2014.04.003
0168-0102/

interest as it can provide insight into the finer behavioral regulation the organism possesses, regulatory elements which are likely to be common among animals that have a brain (Strausfeld and Hirth, 2013).

In *Drosophila* larvae, mechanosensory input evokes a few relatively stereotyped behaviors that depend on the intensity and location of the stimulus. Behaviors typically observed in response to a light brush on the thoracic segments while the animal is crawling include: pause, reverse contraction restricted to the anterior segment, full reverse contraction and subsequent change in crawling direction, or several consecutive reverse contractions (Kernan et al., 1994). These behaviors have been quantified with a numerical score based on intensity of the response. More forceful touches to the abdomen evoke a nocifensive rolling behavior that is performed by the animal to evade parasitoid wasps (Hwang et al., 2007; Robertson et al., 2013). Though the sensory and motor neurons are clearly arranged in a somatotopic CNS configuration, how the different behaviors are selected with respect to the stimulus location or pre-stimulus activity levels is not known.

In this study we present a series of behavioral and electrophysiological experiments that show how responses to tactile stimuli vary with respect to anatomical region, genotype, and specific subtypes of sensory neurons. By stimulating crawling larvae with moderately harsh touches to the head, abdomen, and tail, we show that sensitivity to mechanosensory stimuli in the three general regions varies, is highly consistent among genotypes, but can vary between genotypes. The effect of tactile stimuli on neural activity was studied during fictive crawling and when motor circuits were inactive.

2. Materials and methods

2.1. Fly maintenance and stocks

D. melanogaster stocks were kept in standard cornmeal fly food medium at 23 °C and 75% humidity on a 12 h light/dark cycle. The following strains were used: *Canton S* (wild type); w^{1118}, smn^{E33}, *D42*-GAL4 (all motor neurons), *elav*-GAL4 (pan-neuronal), *ppk*-GAL4 (class IV and class III dendritic arborization neurons), *nompC*-GAL4 (class III sensory and chordotonal neurons), *iav*-GAL4 (chordotonal neurons in the pattern of the *iav* gene (Gong et al., 2004; Kwon et al., 2010), and GAL4 driver specific for all multidendritic neurons and chordotonal neurons (Pw[+mW.hs] = GawB109(2)80, Pw[+mC] = UAS-mCD8::GFP.LLL5), which all were obtained from the Bloomington *Drosophila* Stock Center. UAS-shi^{ts} line was from the Kitamoto lab (Kitamoto, 2001).

2.2. Larva touch assay

2.2.1. General procedure

Larvae (5–10 at a time) were placed on an 8 cm agar dish (1% agar, 33% apple juice to stimulate crawling). Crawling larvae (early third instar) were prodded three times with an insect pin (Fine Science Instruments, 0.2 mm diameter), once on the tail, abdomen, and then head. All touches were directed to the dorsal midline at a 45° angle. Head and tail touches were aimed 0.5 mm from the end of the animal, and abdominal touches were in the longitudinal center of the animal. While watching the larvae through a stereomicroscope at 20× magnification, an observer recorded all behavioral responses evoked by the stimulus, e.g., no response (NR), pause, etc. When crawling speed was measured before performing the sensory assay, the number of peristaltic waves was counted for 15 s. Those data are reported as the number of body wall contractions per minute.

2.2.2. Force calibration

Three approaches were combined to measure the stimulus force. First, a third instar larva was secured to a glass microscope slide with double-sided tape. The prod (insect pin fixed to a writing pen with modeling clay) was then advanced toward the animal using a micromanipulator. We measured the rotation length and speed required to indent the cuticle a distance comparable to indentations generated manually by an experimenter. This stimulus was then applied with the preparation resting on an analytical balance to measure the force. Applying the stimulus with the micromanipulator, or manually, generated forces in the range of 0.5–2.0 g (4.5–19.6 mN). Lastly, to determine that our force measurements were accurate, we constructed a Von Frey-like instrument to match previously described specifications (Zhong et al., 2010). With this fiber, measurements made manually and with the micromanipulator were in the range of 4.5–5.5 g (44.1–53.9 mN), indicating that our apparatus is accurate. Using filaments in the 5–20 mN range or the insect pin yielded data that were statistically indistinguishable.

2.2.3. Inactivation of specific neurons using the temperature labile $shibire^{ts1}$ allele

Females from the UAS-$shibire^{ts}$ line were crossed with males expressing GAL4 in specific subsets of sensory neurons or motor neurons (positive control, *D42*-GAL4, which expresses the $shibire^{ts1}$ in all motor neurons and paralyzes the animal at restrictive temperature). To inhibit chemical transmission in specific neurons, the animals were incubated in a water bath above the restrictive temperature for the $shibire^{ts1}$ (dynamin) dominant-negative allele. Five larvae were placed in standard fly vials with 0.5 mg standard fly food and 0.5 mL distilled water. Vials were incubated for ten minutes in a 37 °C water bath, and then the animals were transferred to an agar-lined petri dish (8 cm diameter) resting on a hot plate that maintained an agar temperature of 37 °C.

2.3. Extracellular field potential recordings

Electrophysiology was performed on a partially dissected early third instar larva preparation. The dissection is a modified version of the flat filet preparation (Parton et al., 2010) in which the anterior and posterior segments are left fully intact, i.e. (1) the animal is pinned on the lateral edges and (2) the dorsal midline dissection is stopped before reaching the anterior or posterior ends. This allows for visceral organs to be removed and gives access to the ventral ganglion and nerves with minimal damage to the CNS or dorsal PNS structures where the stimulus is delivered. Dissections and recordings were made in modified HL3 saline, NaCl 70 mM, KCl 5 mM, $MgCl_2 \cdot 6H_2O$ 20 mM, $NaHCO_3$ 10 mM, trehalose 5 mM, sucrose 115 mM, $CaCl_2 \cdot 2H_2O$ 1 mM, and BES 25 mM and pH 7.1 (Stewart et al., 1994). All saline components were purchased from Sigma Aldrich.

Glass micropipette suction electrodes (7–10 μm inner diameter) were filled with saline and attached to an AxoClamp 2B amplifier. Signals were sampled at 10 kHz, digitized with a Powerlab 4SP A/D board (ADI), and visualized on a PC running LabChart7 (ADI). Mechanical stimulations were applied with the same insect pin described above by advancing the instrument with a micromanipulator. Electrical stimulations were delivered to posterior segmental nerves in filet preparations with the CNS intact as described in earlier work (Dasari and Cooper, 2004). A 10-pulse (40 Hz) stimulus was applied to the nerve and intracellular recordings were obtained from m6. Firing frequency and duration were measured in LabChart7. Spontaneous bursting period was defined as the time from the first peak of one burst to the first peak of the next burst. Burst duration was defined as the time from the first peak of one burst to the last peak of that burst. Burst frequencies were measured with the cyclic measurement tool in LabChart7,

with counts being detected based on a threshold height set above the noise level.

2.4. Analysis

Sample sizes (n) represent the number of individual animals tested for each genotype. The fraction of animals responding to each type of stimulus was compared between genotypes and between stimulus locations using Chi-square analysis of the proportion of animals responding and not responding to the stimulus. Post hoc analysis of the Chi-square results was performed using the COMPPROP procedure for multiple comparisons in SAS (v9.3). This is a Tukey-type test that compares proportions from a $2 \times c$ contingency table as Zar (1996) and SAS (2013). When significant differences were observed in the fraction of flies responding to a stimulus, the frequency of specific behavioral responses was compared. This was done using one-way ANOVA because the Chi-square test was not valid when the number of specific responses was below 5 for multiple groups. The Kruskal–Wallis one-way ANOVA test was used to compare groups that were determined to have non-normal distributions (Shapiro–Wilk test). Multiple comparisons were performed with Tukey's method when sample sizes were identical, with Dunn's method when sample sizes were different. This ANOVA procedure was also used to compare crawling speed between different genotypes, and to compare normalized neural activity levels in response to stimuli delivered to different regions. All ANOVA procedures were performed with SigmaPlot (v12.3). Mean differences in neural activity before and after tactile stimuli were compared using paired Student's t-test. Those tests, and linear regression analysis of the data in Fig. 4 were performed with Microsoft Excel-Plus (2013).

3. Results

3.1. Larval behavioral responses to sharp mechanosensory stimuli

Previous authors have described the behavioral repertoire evoked by mechanosensory stimuli in *D. melanogaster* (Kernan et al., 1994). Light brushes moving from anterior to poster on the lateral thoracic segment cause the animal to stop crawling, then either turn and continue crawling, crawl in the reverse direction, or simply resume crawling in the same direction. Stronger stimuli (50 mN) applied to the dorsal midline near abdominal segment 4 can evoke a rolling behavior described as "nocifensive" (Zhong et al., 2010). Here we applied a lighter tactile stimulus (20 mN) to the tail, abdomen, and head regions using the sharp end of an insect pin (Fig. 1A). Touching the tip of the tail evokes an escape behavior that causes the animal to increase crawling speed. We delivered stimuli to the last body segment, at the base of the tail. Fewer than 30% of Canton S larvae respond to this stimulus, whereas more than 60% respond to abdominal touches, and over 95% respond to head touches (Fig. 2A). The responses were indistinguishable when the stimulus was applied tail first, head first, or in a random order.

When comparing the types of behaviors evoked by stimuli at different locations, it is clear that the responses are specific to the location of the stimulus. In addition to the pause, turn, reverse, and rolling behaviors that have been described, we also observed a full body bend, which we call a c-bend, and a posterior bend, which we call a tail flip. The c-bend is observed almost exclusively in response to head stimuli (Fig. 2B), whereas the tail flip was mostly evoked by tail stimulations. Head touches also evoke reverse contractions and turns. In general, the head region is more sensitive and generates a greater variety of response behaviors than the abdomen or tail. To further characterize these mechanosensory response behaviors we delivered the gentle brush stimulus described by Kernan

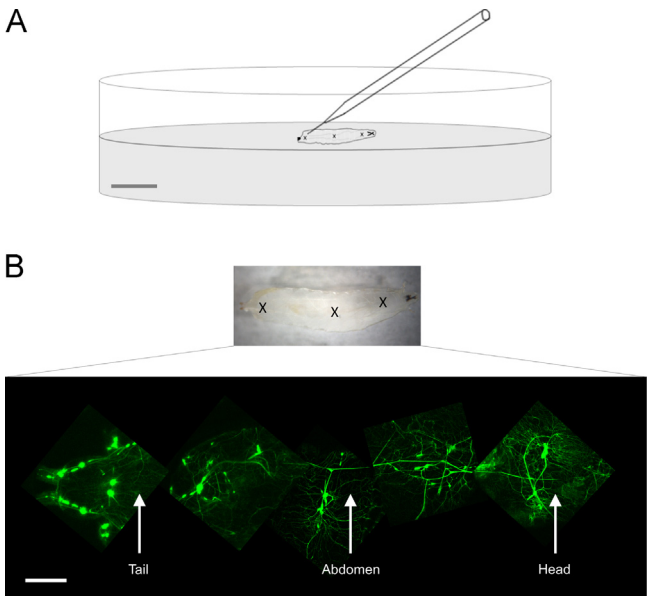

Fig. 1. Overview of the mechanosensory assay and larval mechanosensation. (A) Mechanical stimuli are delivered to the dorsal cuticle near the midline as the fly crawls on an agar-lined dish. The type of response, pause, roll, etc., was recorded from each stimulation. (B) Peripheral nervous system of a third instar larva. The three localized regions that receive stimuli are shown (white arrows). The image is from a live animal expressing GFP tagged to a membrane-bound protein (elav > mCD8::GFP; 40× objective). Scale bars, 5 mm (A) and 100 μm (B).

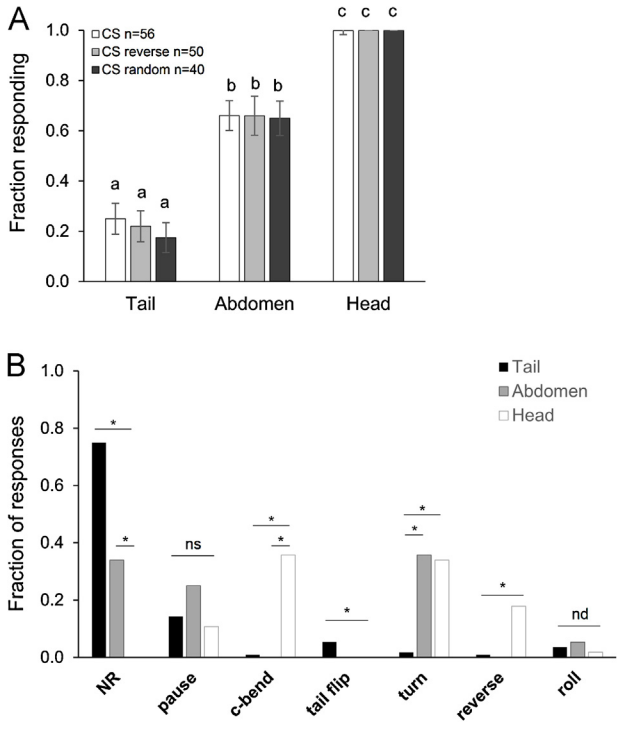

Fig. 2. Canton S (CS) larvae are more sensitive in the anterior and abdominal regions than in the posterior region. (A) Responsiveness is independent of the stimulus order (mean ± SEM; letters indicate similarity). (B) Evoked behavior depends on location of the stimulus. No response (NR) is most commonly observed when the tail is touched. C-bends and reverse contractions are observed almost exclusively in response to head stimuli. Turns were observed more frequently in response to head and abdomen stimulations than tail stimulations. Pauses and rolling behavior were observed at relatively similar frequencies in response to all stimuli ($^*p < 0.05$).

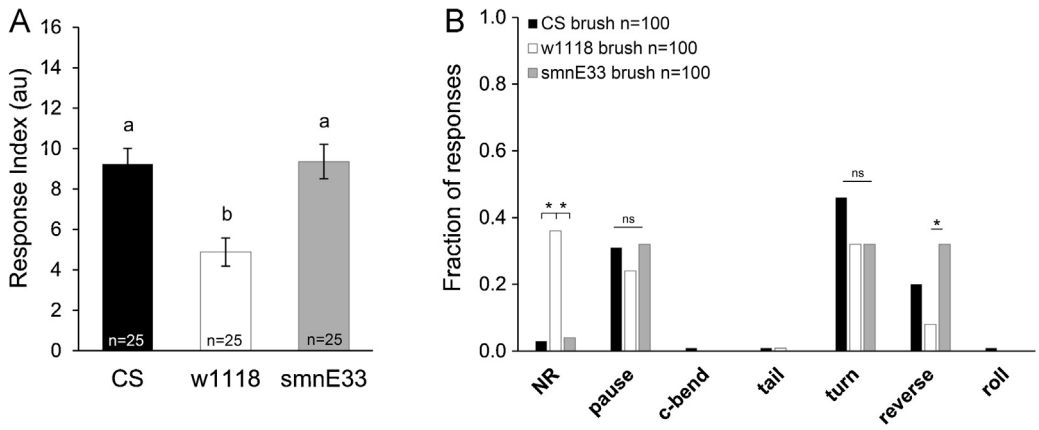

Fig. 3. Mechanosensory responses to gentle anterior brushes in *white*, and *smn* mutants. (A) Mechanosensory responses to gentle touch are shown using the scoring index from Kernan et al. (1994). Overall, *white* mutants were less responsive to these stimuli than CS or *smn* mutants (mean ± SEM; letters indicate similarity; n = number of animals tested). (B) Distribution of responses to gentle innocuous stimuli. *Smn* mutants responded with reverse contractions more frequently (*$p < 0.05$; n = number of brushes, 4/animal).

et al. (1994). The response index we measured was comparable to the scores reported in that study (Fig. 3A), and the same limited repertoire of responses was observed, i.e., pause, turn, and reverse (Fig. 3B). Thus the diversity of response behaviors to a tactile stimulus is positively correlated with the force of the stimulus.

Next we began to assess different genotypes, beginning with a mutant that is homozygous for a hypomorphic allele of the *smn* gene, smn^{E33} (Rajendra et al., 2007). *Smn* is an RNA binding protein that is involved in splicing and RNA localization. In flies, as in humans, mutations in *smn* cause early lethality through defects in RNA processing (Chang et al., 2008). Motor neurons are particularly susceptible to *smn* mutations and degeneration of motor neurons leads to muscle atrophy and motor defects. In flies, *smn* loss of function mutants exhibit a decrease in locomotion but an increase in synaptic output at the larval NMJ (Imlach et al., 2012). We observed slower crawling speeds in the smn^{E33} mutants (Fig. 4A), and compared to Canton-S larvae, the smn^{E33} mutants were also more responsive to tail stimuli (Fig. 4B). Within the greater number of responses there were more tail flips specifically (Fig. 4C_1). Compared to larvae with a loss of function mutation in the *white* allele (w^{1118}; smn^{E33} allele is in a $w^{-/-}$ background), smn^{E33} mutants are only slightly more responsive to tail touches, but execute nearly twice as many tail flips. There were no differences in the types of behavioral responses to head or abdomen touches (Fig. 4$C_{2,3}$).

The mechanosensory phenotype in smn^{E33} was rescued by crossing it with the w^{1118} line, but crawling speed in smn^{E33}/w^{1118} crosses was not restored to wild type levels, or to levels that were observed in smn^{E33}/CS larvae (Fig. 4). We also observed that smn^{E33} larvae are more sensitive than w^{1118} to the light touch paradigm used by Kernan et al. (1994), in this case responding with more reverse contractions (Fig. 3). To test for a correlation between baseline motor activity and mechanosensory responsiveness, we used linear regression to analyze the responses to tactile stimuli with respect to crawling speed within genotypes. No correlation was observed between the rate of crawling and the probability of responding to the stimulus at any of the regions tested (Fig. 5).

Three conclusions can be drawn from these results. First is that mutations can affect mechanosensory responses to tactile stimuli in specific anatomical regions. *White* mutations, which are commonly used as a selectable marker in mutagenesis or transgenesis, behave differently than the common wild type strain, Canton-S. This was true for responses to 20 mN tail stimuli and light brushes to the anterior segments. *White* mutants and *smn* mutants had similar responses to 20 mN head stimuli, but the *smn* mutant was more sensitive to light head brushes. These results show that mutations can affect mechanosensory responsiveness at different thresholds, though these experiments do not determine whether threshold for detecting the stimulus, or threshold for activating the motor output is affected. Lastly, the results suggest that *smn* mutants have an increased responsiveness to mechanosensation, which could be due to enhanced excitability in motor neurons that innervate the body wall muscles (Imlach et al., 2012).

3.2. Anatomical localization of specific sensory neuron subtypes that transduce tactile stimuli

Sensory neurons that tile the larval body wall are remarkably well-characterized in terms of function and morphology. For gentle touch and noxious stimulus assays, the stimulus is typically delivered to the regions where the arrangement of sensory neurons is highly ordered between segments. However, notice that the arrangement in the head and tail regions is highly irregular compared to the abdominal region (Fig. 1B). Our aim here was to determine which sets of sensory neurons are needed to respond to tactile stimuli in the head and tail regions. We used a temperature sensitive dominant negative dynamin mutant (UAS-$shibire^{ts}$) to block chemical neurotransmission, and disrupt specific subsets of sensory neurons with publicly available GAL4 lines. To determine if high temperature influences mechanosensation in general, we tested our Canton-S line. Elevated temperature only affected *Canton S* responses to abdominal touches, where sensitivity was slightly increased (Fig. 6A). As a negative control for heat inactivation of neural activity, we tested the parental UAS-$shibire^{ts}$ line at restrictive temperature in each experiment (Fig. 6B). Also, we tested the sensory neuron driven UAS-$shibire^{ts}$ lines at permissive temperature (Fig. 6C_{1-3}). For a positive control we observed that the UAS-$shibire^{ts}$ line crossed to a motor neuron GAL4 driver (*D42*-GAL4) caused paralysis.

This approach was used to inhibit class III and class IV multidendritic neurons, chordotonal neurons, a combination of class III and chordotonal neurons, and a combination of all multidendritic neurons and chordotonal neurons. Compared to the UAS-*shibire* background controls, only inhibition of all multidendritic neurons and chordotonal neurons had a significant effect on mechanosensory behavior (Fig. 6B). Inhibiting chordotonal neurons alone, or in combination with class III neurons slightly reduced responses to abdominal touches, compared to UAS-*shibire* at restrictive temperature (Fig. 6B), and to the isogenic controls tested at permissive temperature (Fig. 6$C_{1,2}$). However the only statistically significant reduction in mechanosensation was observed when inhibiting

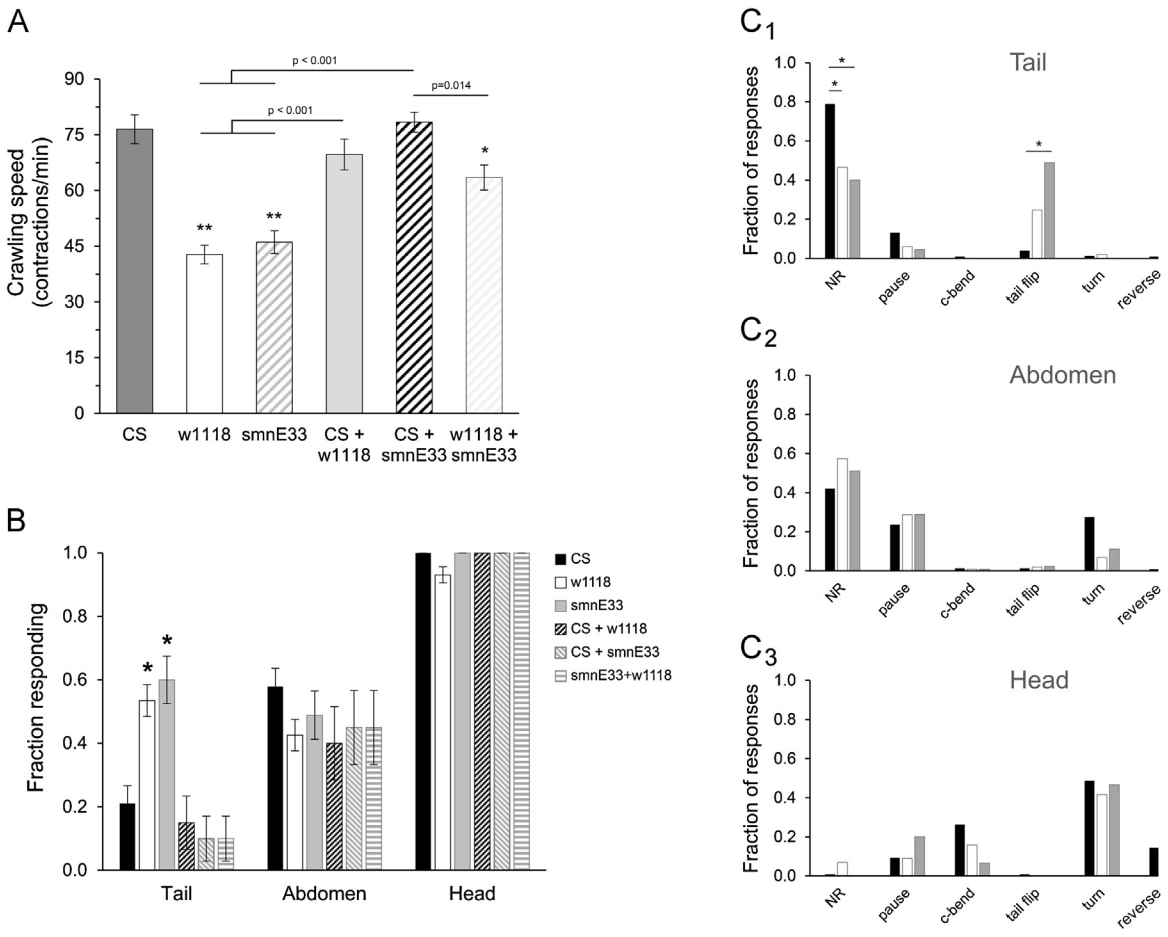

Fig. 4. Mechanosensory responses and crawling speed in *white* and *smn* mutants. (A) The *white* and *smn* mutants crawl slower than CS larvae. Combining either mutant with CS completely rescues the phenotype. Combining the *white* and *smn* mutations only partially rescues the phenotype. (B) *Smn* and *white* mutants are more responsive to tactile stimuli on the tail. (C_{1-3}) Distribution of mechanosensory responses in w^{1118} and smn^{E33}. In response to tail stimuli, *smn* mutants perform more tail flips than *white* mutants or CS larvae ($^{*}p < 0.05$, $^{**}p < 0.005$; $n = 25$ larvae for each genotype).

chordotonal neurons in combination with multiple subsets of multidendritic neurons (Fig. 6C_3). Therefore response to 20 mN tactile stimulation on the dorsal midline requires a combination of chordotonal and multidendritic sensory neurons.

The majority of responses to abdominal touches in MD + CH > shi^{ts} larvae were pauses (Fig. 6D_2). Therefore it appears that the phenotypic mechanosensory response at restrictive temperature is caused by reduced input to inhibitory neurons that stop crawling, rather than a direct synapse on motor neurons that would normally evoke a response behavior. This may not be the case in the head region as head touches evoke reverse contractions, turning behavior, and pauses at permissive temperature, each of which is reduced at restrictive temperature (Fig. 6D_3).

3.3. Neural activity associated with mechanosensory stimuli

To extend our characterization of mechanosensory responses to the cellular level, we used *en passant* electrophysiological recordings from posterior segmental nerves (Fox et al., 2006). In partially dissected preparations (Fig. 7A) we observed several minutes of bursting activity that resembles fictive crawling in other animals. The period between bursts ranged from 10 to 25 s. Presentation of tactile stimuli was often followed by a pause in the endogenous rhythm (Fig. 7B). The average spontaneous burst period after the stimulus was significantly longer than the average of the three previous periods, regardless of whether the stimulus was delivered to the head, abdomen, or tail (Fig. 7C_1). The change in bursting interval

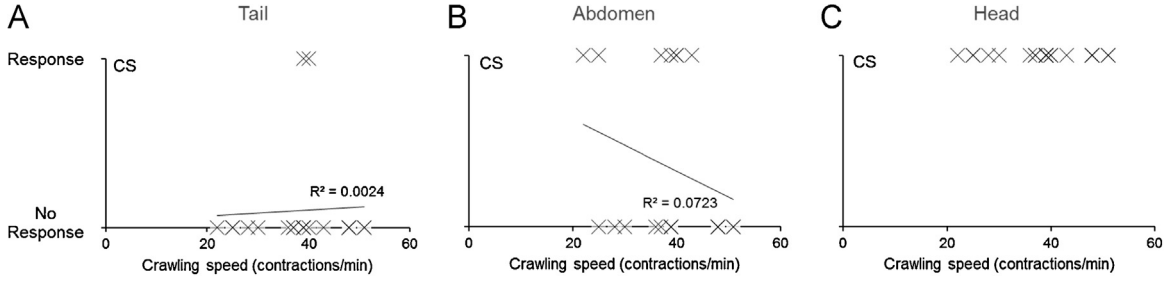

Fig. 5. Mechanosensory responses are not correlated with crawling speed. (A–C) Data points indicate the crawling speed (*x*-axis) and whether or not the larva responded to 20 mN tactile stimuli (*y*-axis). Linear regression analysis shows that there is no correlation between crawling speed and responsiveness in CS. The same was also true for *smn* and *white* mutants (not shown).

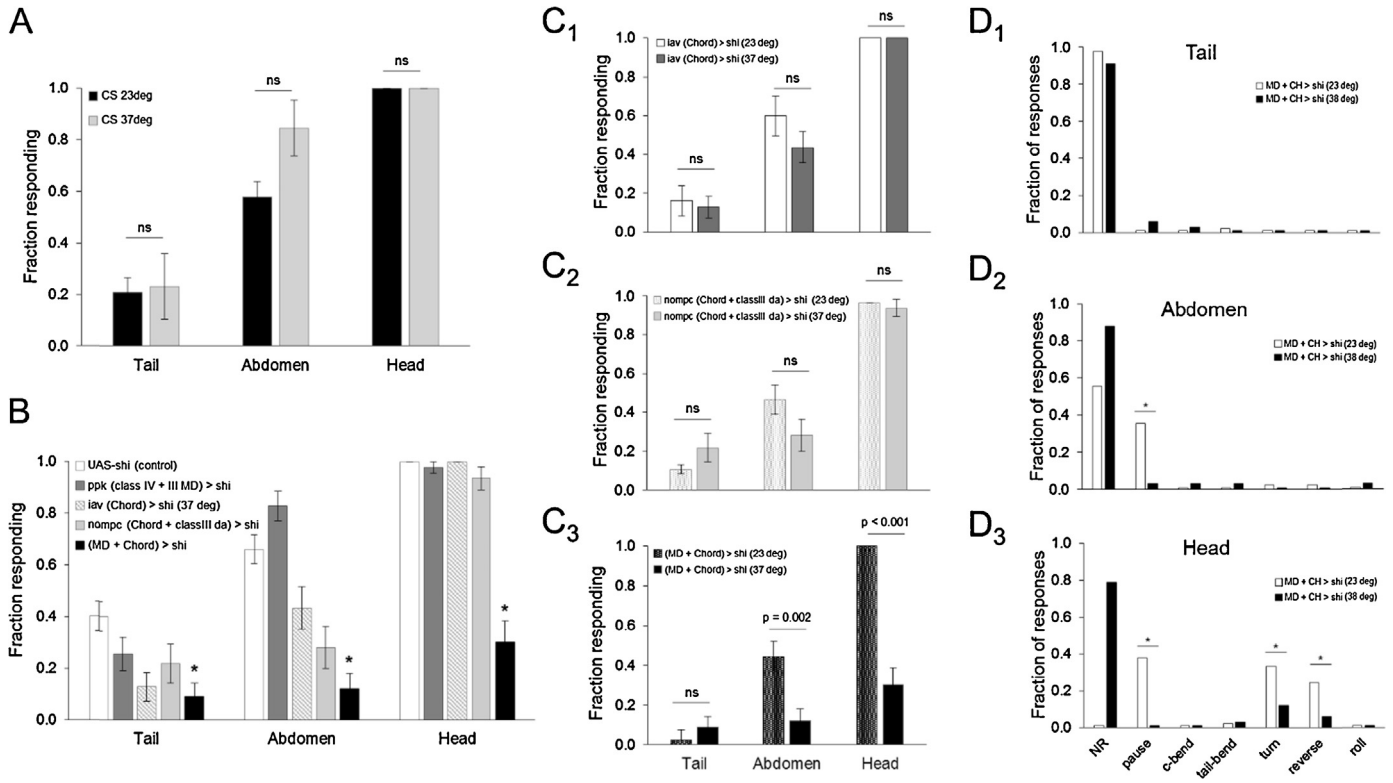

Fig. 6. Neuronal silencing reveals specific types of neurons involved in mechanosensation at different anatomical regions. (A) At high temperature (37 °C), larvae are slightly more sensitive to tactile stimuli in the abdominal region, but not the head or tail region. (B) Inhibiting chemical transmission in specific subsets of sensory neurons has distinct effects on mechanosensory responsiveness. Response probability is significantly reduced by silencing multidendritic and chordotonal neurons ($p < 0.05$ relative to UAS-shi^{ts1}). (C_{1-3}) When the sensory neuron driven GAL4 lines are compared at permissive and restrictive temperature, the only statistically significant effect was observed when silencing multidendritic and chordotonal neurons (mean ± SEM; UAS-shi^{ts1} $n = 77$ larvae, $ppk > shi^{ts1}$ $n = 47$ larvae, $iav > shi^{ts1}$ $n = 39$ larvae, $nompC > shi^{ts1}$ $n = 32$ larvae, MD + Ch > shi^{ts1} $n = 33$ larvae). (D_{1-3}) In response to abdominal touches in the MD + Ch > shi^{ts}, pauses were significantly reduced, whereas pauses, turning behavior, and reverse contractions were also reduced in response to head touches (*$p < 0.05$).

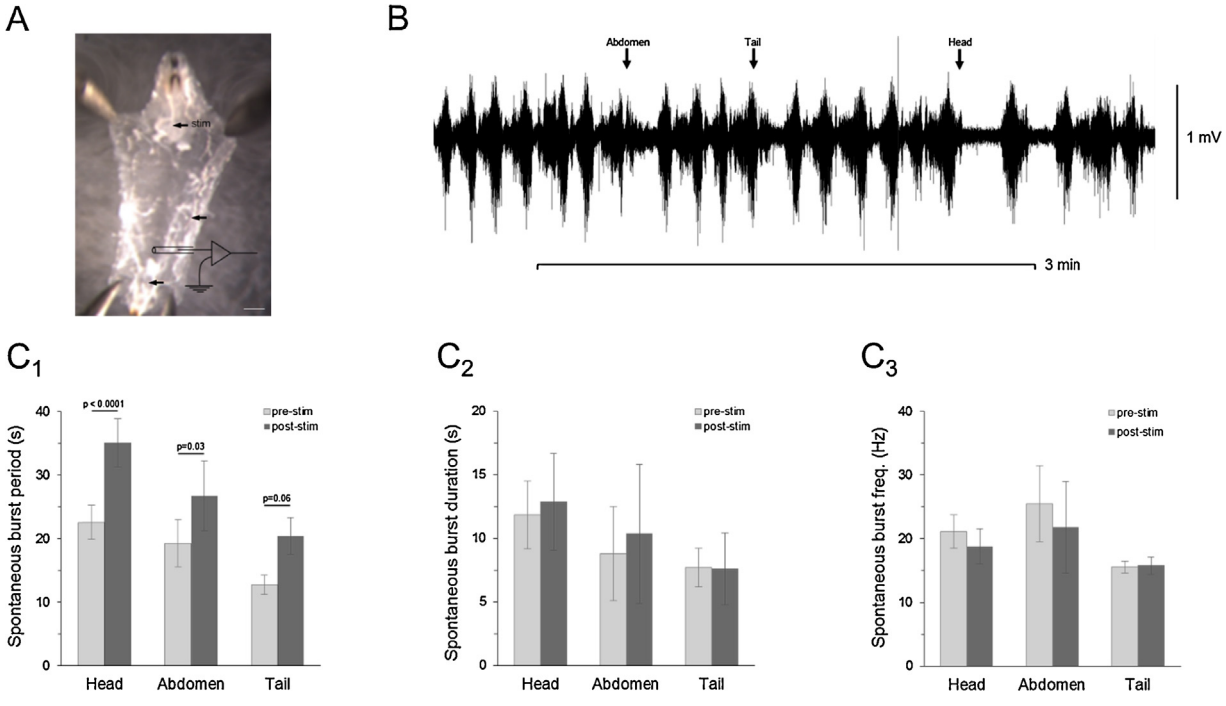

Fig. 7. Extracellular recordings from a segmental nerve during fictive crawling. (A) By not extending the longitudinal incision during dissection to the most anterior and posterior regions, the peripheral nervous system remains largely intact. *En passant* extracellular recordings were taken from a posterior segmental nerve, and stimuli were applied to the head, abdomen, and tail (black arrows) by advancing the insect pin prod with a micromanipulator. (B) Extracellular recordings show rhythmic activity in the nerve. Stimulations (black arrows) typically caused the period between bursts to increase. (C_1) The average of three intervals before the stimulus (pre-stim) was compared to the interval following the stimulus (post-stim). The interval increased in response to touches in each region. There were no significant changes in spontaneous burst duration (C_2) or burst frequency (C_3). Scale bar = 500 μm. Tail stimuli $n = 6$, abdomen stimuli $n = 6$, head stimuli $n = 19$.

did not vary between anatomical regions (data not shown), and the stimulus did not affect the duration or frequency of activity during the bursts (Fig. 7$C_{2,3}$).

In preparations that did not exhibit rhythmic bursting, tactile stimulations were delivered either during spontaneous activity (Fig. 8) or between bursts of spontaneous activity (Fig. 9). For stimuli delivered during activity we compared the frequency over 5 s prior to the stimulus, to the frequency measured 5 s after the stimulus. Consistent with the bursting data, spontaneous activity decreased following tactile stimuli (Fig. 8A). The decrease was statistically significant for all three anatomical regions (Fig. 8B), but there was no difference in the change between regions (Fig. 8C). Tactile stimuli that were delivered when the nerve was inactive were followed by a burst of activity (Fig. 9A). The average frequency of bursts in response to stimuli in the three different regions were, head: 9.3 ± 1.0 Hz, abdomen: 5.7 ± 0.6 Hz, and tail: 8.9 ± 1.2 Hz. The average duration of the bursts were, head: 14.1 ± 1.8 s, abdomen: 8.2 ± 1.7 s, and tail: 7.22 ± 1.5 s. To compare the response to touches in different regions, values from each animal were normalized to the average response to head stimuli in that animal, then the normalized values were compared using one-way ANOVA. The duration (Fig. 9B) and frequency (Fig. 9C) in response to abdominal stimuli were lower than responses to head and tail stimuli ($p < 0.05$).

Sensory-motor responses in *Drosophila* larvae can also be evoked by stimulating segmental nerves with an electrode (Dasari and Cooper, 2004). This paradigm mimics sensory input to the CNS and is used to investigate CNS output to a single muscle cell. A 10-pulse stimulus (40 Hz) typically evokes a burst of activity that is comparable in duration to the responses evoked by tactile stimuli (Fig. 10A). The average frequency of bursts recorded in seven larvae was 5.4 ± 1.1 Hz, which is comparable to the firing frequency of the phasic motor neuron MNSNb/d-Is observed during fictive crawling (Chouhan et al., 2010). This frequency was also comparable to the extracellular frequencies that were evoked by tactile stimuli (of course the extracellular recordings contain compound action potentials from several neurons firing out of phase). All of these frequencies were below the average frequency of bursts recorded during fictive crawling (Fig. 10B). These data suggest that the sensory-motor response is mediated by the type Is motor neurons, though these experiments alone do not rule out the possibility that input also comes from MNSNb-d-Ib neurons firing below their maximum frequency.

4. Discussion

The neural circuitry for larval motor output consists of segmentally reiterative motor neurons in the ventral nerve cord that send axons away from the CNS to body wall muscle fibers, and segmentally reiterative sensory neurons located in the body wall, which send axons back to the CNS. Given this symmetry, it was surprising to observe robust differences in mechanosensory responsiveness along the anteroposterior axis (Fig. 2). However, the results are ethologically relevant, as observations of wasp attacks on *D. melanogaster* larvae revealed that responses varied depending on the location of the attack (Robertson et al., 2013).

As one might predict, the head region is the most sensitive to 20 mN tactile stimulation, and head touches generated the biggest diversity in behavioral responses. The c-bend and roll behaviors were observed in response to 20 mN tactile stimuli (Fig. 2B), but not light brushing to the anterior segments (Fig. 3B). Rolling behavior is known to be a response to noxious stimuli (Zhong et al., 2010), it would appear that the bending behavior is also a nociceptive response. However, the frequency of bending behavior did not decrease significantly when the nociceptive neurons (*nompC*-GAL4, Fig. 6C_2) were inhibited. One possible explanation is that those neurons are fatigued or habituated from the heat stimulus that was used to acutely inhibit chemical transmission. A transient receptor potential (TRP) ion channel that detects heat is also expressed in those neurons (Tracey et al., 2003), and the bend behavior does appear to be an abbreviated form of the nocifensive roll.

Zhou et al. (2012) show that the degree of turning is also correlated with stimulus intensity, i.e., stronger stimuli cause a greater turning angle. This suggests a graded or analog relationship between afferent and efferent neurons for this behavior, but the process of switching to a different behavior in response to a tactile stimulus is not understood. Without input from the brain, larvae can crawl and react to light, but are unable to perform goal-directed movements like chemotaxis (Berni et al., 2012). Inhibiting a small subset of neurons in the brain changes the turning direction in response to an innocuous head touch (Zhou et al., 2012). It is unclear how descending input modulates mechanosensory input from the abdomen and tail, and whether it modulates motor circuits for rolling, reverse, or tail flex behaviors. It is also unclear how the circuits underlying these behaviors overlap with the central pattern generating circuits for crawling behavior. That is something that we attempted to address by measuring changes in neural activity in response to tactile stimulations during fictive locomotion. The pause in bursting after the stimulus was to be expected, in vivo the larvae stopped crawling before executing a response behavior. After the delay, rhythmic activity resumed with the same burst waveforms observed during crawling (Fig. 7C). This could mean that the posterior nerves do not convey impulses involved in other response behaviors, or that those behaviors are inhibited when the animal is pinned down and submerged in saline. To be sure, one would need to record from more anterior nerves during bursting activity.

Another feature of mechanosensory response behavior that became apparent from extracellular recordings was that the response to tactile stimulation depends on whether or not the nerve is active when the stimulus is delivered. This phenomenon is similar to spike-timing dependent plasticity observed at single synapses (Feldman, 2012), but it occurs on the neural circuit level. When the circuit was active, tactile stimuli caused the frequency of activity to decrease (Fig. 8), when inactive, tactile stimuli generated bursts of activity (Fig. 9). The duration of the bursts ($\sim$10 s) is similar to the duration of bursts recorded in single muscle fibers after evoking CNS activity through the segmental nerve with an electrode (Dasari and Cooper, 2004). The latter approach is now being used to identify the neurons that mediate this response and characterize plasticity in the circuit.

Response to input from the environment is a prominent behavioral characteristic that can have an immediate impact on an animal's survival. Ultimately we want to describe the physiological basis of these responses with the goal of understanding how they are affected by experience, genetic variation, and other factors. To investigate how neural circuits process information, the *Drosophila* larval nervous system is ideal because of the tools for controlling and measuring neural activity (Pfeiffer et al., 2008), and the extensive literature on behavioral genetics (Sokolowski, 2001). Mechanosensation and its associated behaviors in larvae are an attractive system because of the abundance of research that has been done on the NMJ and peripheral nervous system. Deciphering the CNS circuits that integrate this information has been a more difficult task (Iyengar et al., 2011), and will require genetic techniques to control and measure neural activity, in combination with electrophysiology and pharmacology to identify cellular and molecular features of decision-making. The simple decision addressed in this study was how to react to a tactile stimulus. It is not surprising that afferent neurons in different regions show different sensitivity and evoke different behaviors, but what makes the larva choose

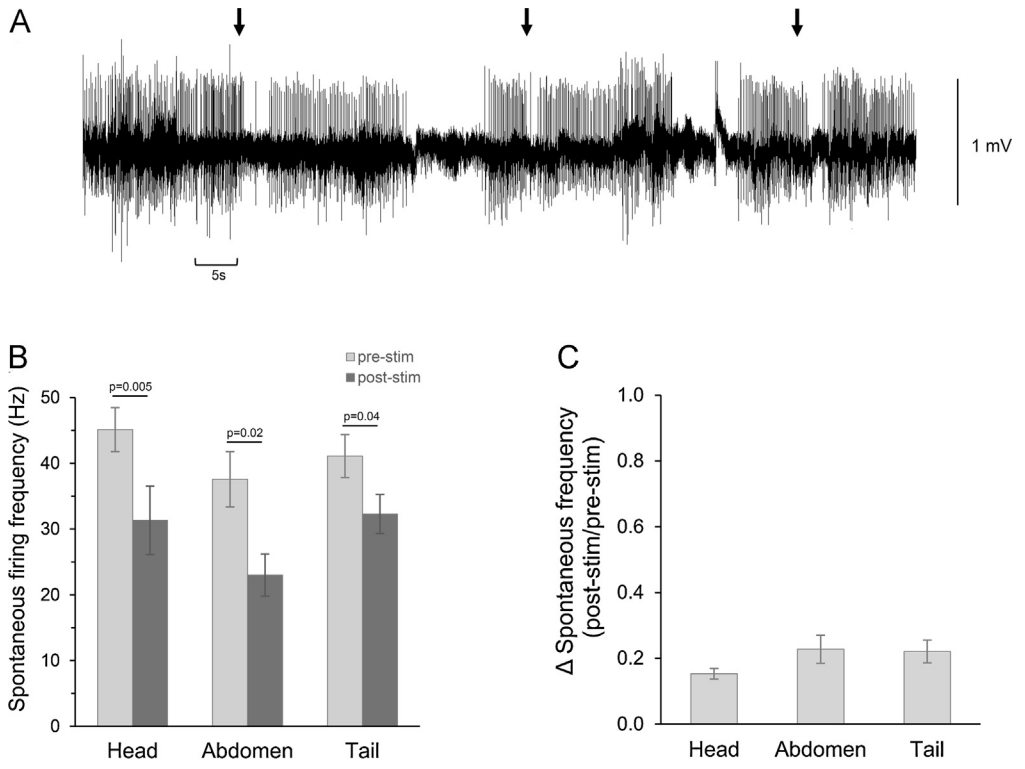

Fig. 8. Extracellular recordings from a segmental nerve during non-rhythmic spontaneous activity. (A) Extracellular recording that shows a typical decrease in spontaneous activity following mechanical stimulations (black arrows). (B) Spontaneous activity decreased in response to tactile stimuli delivered in each anatomical region. Firing frequency was measured for 5 s before (pre-stim) and 5 s after (post-stim) the stimulus was delivered. (C) The change in spontaneous frequency was the same for each anatomical region. Tail stimuli $n = 27$, abdomen stimuli $n = 19$, head stimuli $n = 15$.

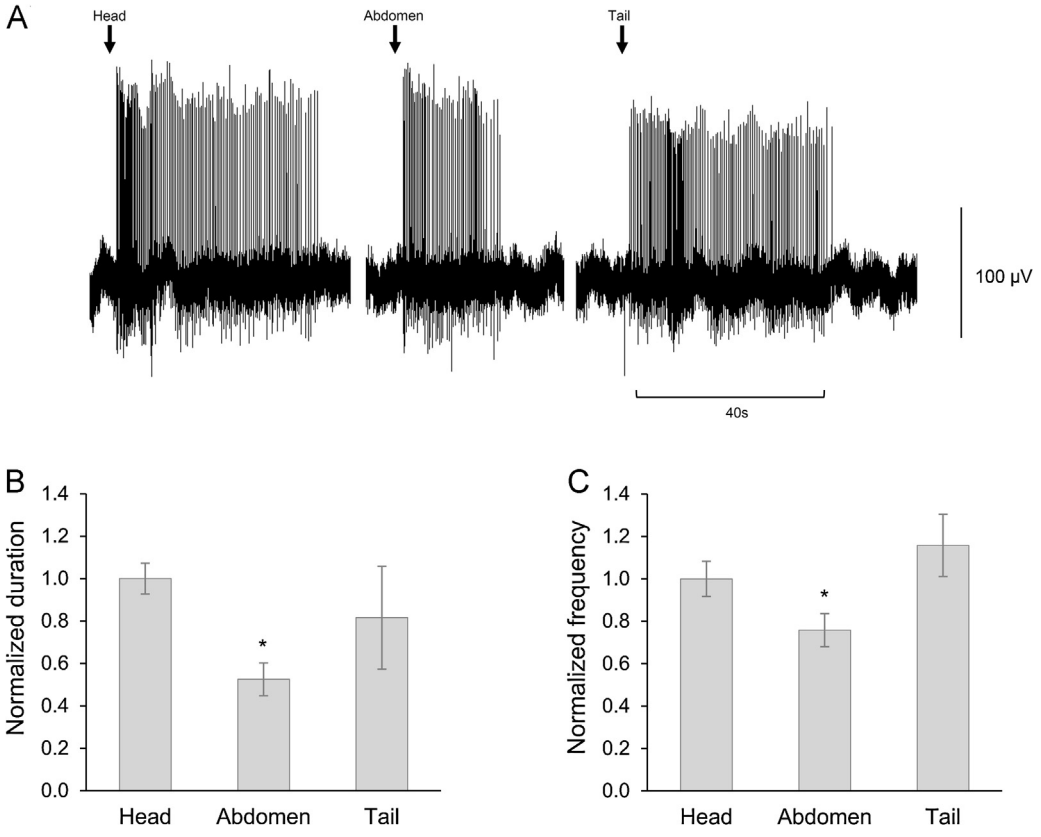

Fig. 9. Extracellular recordings from a segmental nerve during inactive periods. (A) Extracellular recording shows bursts of activity following three different stimuli (black arrows) in the same preparation. The duration and frequency of these bursts were normalized to the response to head stimulations in each prep. Normalized values were used to compare responses to stimuli delivered to the specific anatomical regions. On average, abdominal responses had a shorter duration (B) and slower frequency (C) than head and tail responses (*$p < 0.05$). Tail stimuli $n = 27$, abdomen stimuli $n = 30$, head stimuli $n = 31$.

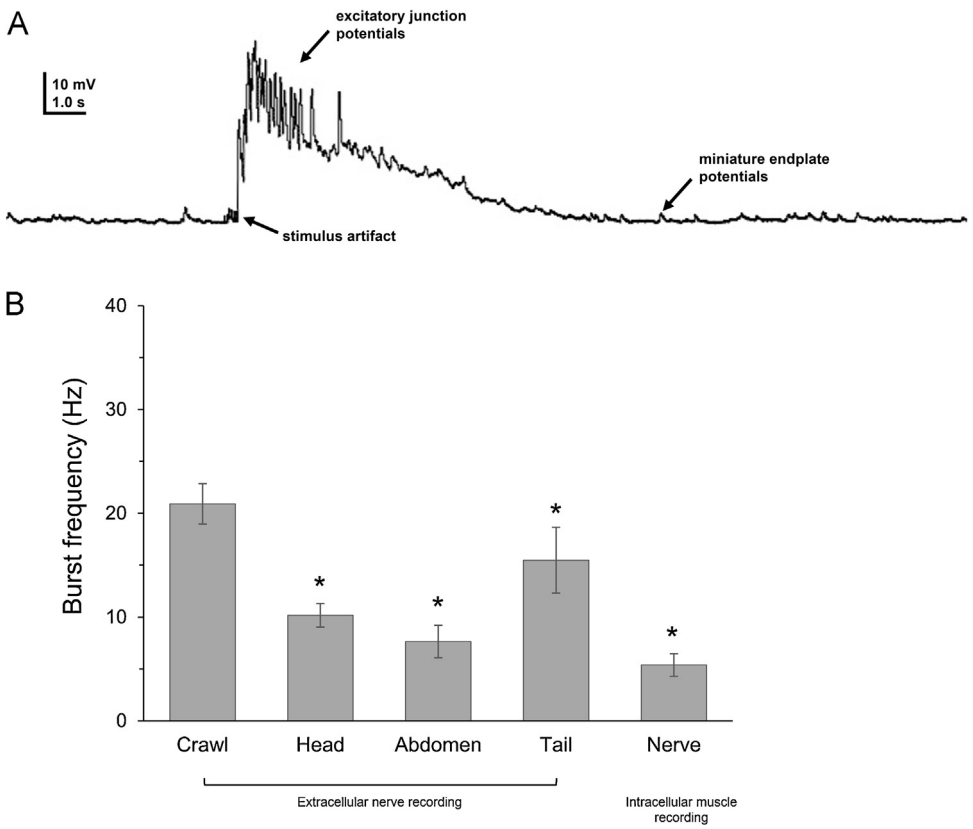

Fig. 10. Differences in frequency between endogenous bursting and mechanosensory responses. (A) Intracellular recording from muscle 6, segment 3, representative of activity that is evoked by stimulating segmental nerves in the posterior region with an electrode. (B) Average frequency of endogenous activity and mechanosensory activity. Note that crawling and mechanosensory activity were recorded with an extracellular electrode, whereas nerve-evoked activity was a single cell recording from muscle 6. Endogenous bursts $n = 31$, tail stimuli $n = 27$, abdomen stimuli $n = 31$, head stimuli $n = 31$, nerve-evoked bursts $n = 7$ ($^*p < 0.05$).

one behavior instead of another in response to the same stimulus? Our results show that when motor circuits are active, their output in response to afferent stimulation is not the same as when motor circuits are inactive. We are trying to determine if this difference is due to intrinsic properties of cells within the circuit or modulatory input from other cells.

5. Conclusion

Though the sensory and motor neurons of the larval peripheral nervous system are symmetrically arranged into segments, behavioral responses to mechanical stimuli in the anterior and posterior regions are quite different. The motor response recorded in segmental nerves also varies between anatomical regions, and depends on whether motor circuits are active when the stimulus is delivered.

Acknowledgements

Funding for this work was provided by a KBrIN summer research fellowship from the University of Kentucky (JR). We are grateful to Toshihiro Kitamoto, Doug Harrison, and the Bloomington *Drosophila* Stock Center for providing fly stocks.

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