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ORIGINAL ARTICLE

The effects of tricaine mesylate on arthropods: crayfish, crab and *Drosophila*

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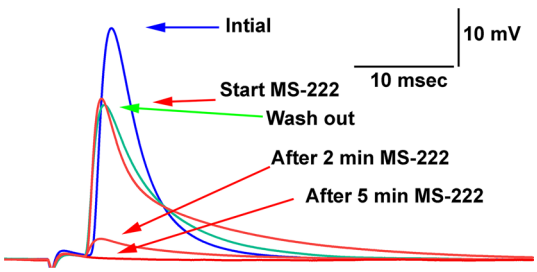
Abstract

Tricaine mesylate, also known as MS-222, was investigated to characterize its effects on sensory neurons, synaptic transmission at the neuromuscular junction, and heart rate in invertebrates. Three species were examined: *Drosophila melanogaster*, blue crab (*Callinectes sapidus*), and red swamp crayfish (*Procambarus clarkii*). Intracellular measures of action potentials in motor neurons of the crayfish demonstrated that MS-222 dampened the amplitude, suggesting that voltage-gated Na + channels are blocked by MS-222. This is likely the mechanism behind the reduced activity measured in sensory neurons and depressed synaptic transmission in all three species as well as reduced cardiac function in the larval *Drosophila*. To address public access to data, a group effort was used for analysis of given data sets, blind to the experimental design, to gauge analytical accuracy. The determination of a threshold in analysis for measuring extracellular recorded sensory events is critical and is not easily performed with commercial software.

Graphic abstract



Keywords Sensory · Anesthetics · Invertebrate · Crustacean · Insect · Cardiac · Neuromuscular · MS-222

Introduction

MS-222 (also known as tricaine mesylate [TMS]) is an anesthetic commonly used to relax and anesthetize fish for tagging, assessment, or experimental studies (Collymore et al. 2016; Gilbert and Wood 1957). MS-222 is similar in chemical structure to benzocaine, another common anesthetic. Given that MS-222 is used in aquatic facilities in which fish are injected with or held in a bath containing MS-222, it is of interest to examine if the mechanism of action may be similar for invertebrates such as aquatic

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crustaceans. Crayfish, crabs, and lobsters are also used for food consumption (SOFIA 2018), and the slaughter of them has raised ethical concerns on sedation prior to slaughter for human consumption (Roth and Øines 2010; Diggles 2018; Weineck et al. 2018).

It appears that MS-222 is a voltage-gated sodium channel blocker, which is the mechanism of its anesthetic action as examined in vertebrates (Butterworth and Strichartz 1990; Ramlochansingh et al. 2014). Given that the family of voltage-gated sodium channels in neurons share a high degree of protein sequence similarity among animals, it would be expected that MS-222 would potentially block neural activity in invertebrates (Bagnéris et al. 2014; Lee et al. 2012; Zakon 2012). The voltage-gated sodium channel blocker tetrodotoxin (TTX) is effective in mammals as well as crustaceans and insects (Catterall et al. 2007; Davies et al. 2007). Similarly, the active compound in essential oil from cloves (i.e., eugenol) appears to block voltage-gated sodium channels in mammals, crustaceans, and insects (Wycoff et al. 2018). Since literature is scarce on the examination of MS-222 and its effects on neuronal function in crustaceans and insects, we sought to examine its action on sensory and motor systems in model crustaceans (red swamp crayfish, *Procambarus clarkii*; blue crab, *Callinectes sapidus*) as well as on motor unit function in the model larval fruit fly (*Drosophila melanogaster*). While its action as a sodium channel blocker may have predictable responses in modulating neuronal and muscular activity, we wished to characterize and confirm its effects in these models.

A readily accessible sensory organ in crayfish is the abdominal muscle receptor organ (MRO) and in crabs is a joint receptor organ (i.e., chordotonal organ) within the limbs. These two sensory systems have been intensively studied anatomically and physiologically for many years. The MRO is analogous to the muscle spindle in mammalian skeletal muscle (Bewick and Banks 2015), which is responsible for proprioception of the limbs; it is composed of two types of sensory neurons (i.e., rapidly adapting and slow adapting), each associated with a single muscle fiber (Kuffler 1954; see Rydqvist et al. 2007 for a review). The sensory endings of the crab leg chordotonal organ are embedded within a strand composed of elastin and collagen that stretches and relaxes with movement of the joint as a result of muscle contraction (Alexandrowicz 1967; de Castro; Hartman and Boettiger 1967; Leksrisawat et al. 2010; Whitear 1960; Wiersma 1959; Wiersma and Boettiger 1959). The PD chordotonal organ is named by the joint in which it detects the movement; in this case, it is the propodite-dactylopodite, the distal two most segments in the walking leg (Whitear 1960). The PD organ is readily accessible and the stimulus of extending or flexing the joint is easily reproducible among each preparation (Dayaram et al. 2017; Stanback et al. 2019). There are in the range of 50–80 sensory neurons in the adult crab PD organ, whereas an adult lobster can have more than 4500 neurons in a PD organ (Cooper and Govind 1991; Hartman and Cooper 1994). Some neurons within the PD organ sense dynamic movement of the joint, while others are static position-sensitive. As with the crayfish MRO, the static position-sensitive neurons show some accommodation over time (Cooper 2008; Cooper and Hartman 1999; Dayaram et al. 2017; Hartman and Boettiger 1967). Monitoring the frequency of the extracellular spikes from the nerves associated with the MRO and PD organ allows one to examine the effect of pharmacological compounds on the activity of primary sensory neurons.

The neuromuscular junction of the crayfish opener muscle in the walking leg and the body wall muscles in the larval fruit fly are common models for investigating synaptic transmission. The *Drosophila* model also has the added benefit of being conducive to genetic manipulation for more in-depth studies involving slight alterations in protein sequence of channels to further elucidate the mechanisms behind the action of MS-222 in the nervous system. If MS-222 causes the neurons to fail to generate depolarizing signals large enough to open voltage-gated calcium channels in the presynaptic motor nerve terminals, synaptic communication is blocked which can then result in immobility and an anesthetic effect. The examination of primary sensory neurons as well as motor function helps not only to demonstrate the commonality in the mechanism of action but also to show that a compound is not just providing a paralytic effect. This investigation aids in increasing knowledge of these model physiological systems as well in understanding the action of MS-222 in invertebrate models.

Materials and methods

Animals

The maintenance and animals used were the same as mentioned in previous reports (Dayaram et al. 2017; Malloy et al. 2017; Stanback et al. 2019). In brief, blue crab (*C. sapidus*) and red swamp crayfish (*P. clarkii*) were obtained from a distribution center in Atlanta, GA, and delivered to and bought from a local supermarket in Lexington, KY, USA.

The crayfish (6–10 cm in body length and 12.5–25 g in body weight) were housed in individual standardized plastic containers with weekly exchanged dry fish food and oxygenated water (20–21 °C). The blue crabs were maintained in a seawater aquarium prior to use for three to five days. All experiments were implemented in female adults with a carapace width (from point to point) of 10–15 cm. The crabs were fed with frozen squid, and the water temperature was maintained between 14 and 16 °C. The crabs and crayfish were caught from the wild, and the crabs as well as

the crayfish were most likely two to three years old. Similar dissection procedures and electrophysiological measures for these preparations are described in detail with text and video format. They are described in brief below.

Crab chordotonal organ (PD)

The dissection and recording procedures are described in (Majeed et al. 2013a, b). In brief, the animal was induced to autotomize the first or second walking leg by lightly pinching at the base of the leg with pliers. The propodite-dactylopodite (PD) chordotonal organ spans the last segment of the leg (Fig. 1a1). The PD organ was exposed by cutting a window in the cuticle on both sides of the leg in the propodite segment. The leg was pinned in a Sylgard-lined dish and covered with crab saline. The PD nerve was then exposed and pulled into a suction electrode for recording (Fig. 1a2 and a3). During the experiment, the dactyl was moved from a flexed position to an open position in a one-second time frame, held for 10 s, and then moved back to the starting position (Fig. 1a1). An insect dissecting pin was used to mark the displacement range, and each displacement was marked on the computer recording file. The crab saline used during recordings of the sensory nerves consisted of (in mM) 470 NaCl, 7.9 KCl, 15.0 CaCl2 2H2O, 6.98 MgCl2 6H2O, 11.0 dextrose, 5 HEPES acid and 5 HEPES base adjusted to pH 7.4.

Crayfish muscle receptor organ (MRO)

The dissection and recording procedures are described in Leksrisawat et al. (2010). In brief, the isolated crayfish abdomen was placed in a Sylgard-lined dish filled with crayfish saline (Fig. 1b1). The MRO was moved using a wooden dowel from a relaxed position to a stretched position in a one-second time frame, held for 10 s, and then moved back to the starting position. An insect dissecting pin was used to mark the displacement range, and each displacement was marked on the computer recording file. The segmental nerve to the segment of interest is pulled into a suction electrode for recording the extracellular spikes (Fig. 1b1 and b2).

The displacement rates were the same as for the crab PD organ. The crayfish saline used was a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 CaCl2 2H2O, 2.45 MgCl2 6H2O, and 5 HEPES adjusted to pH 7.4).

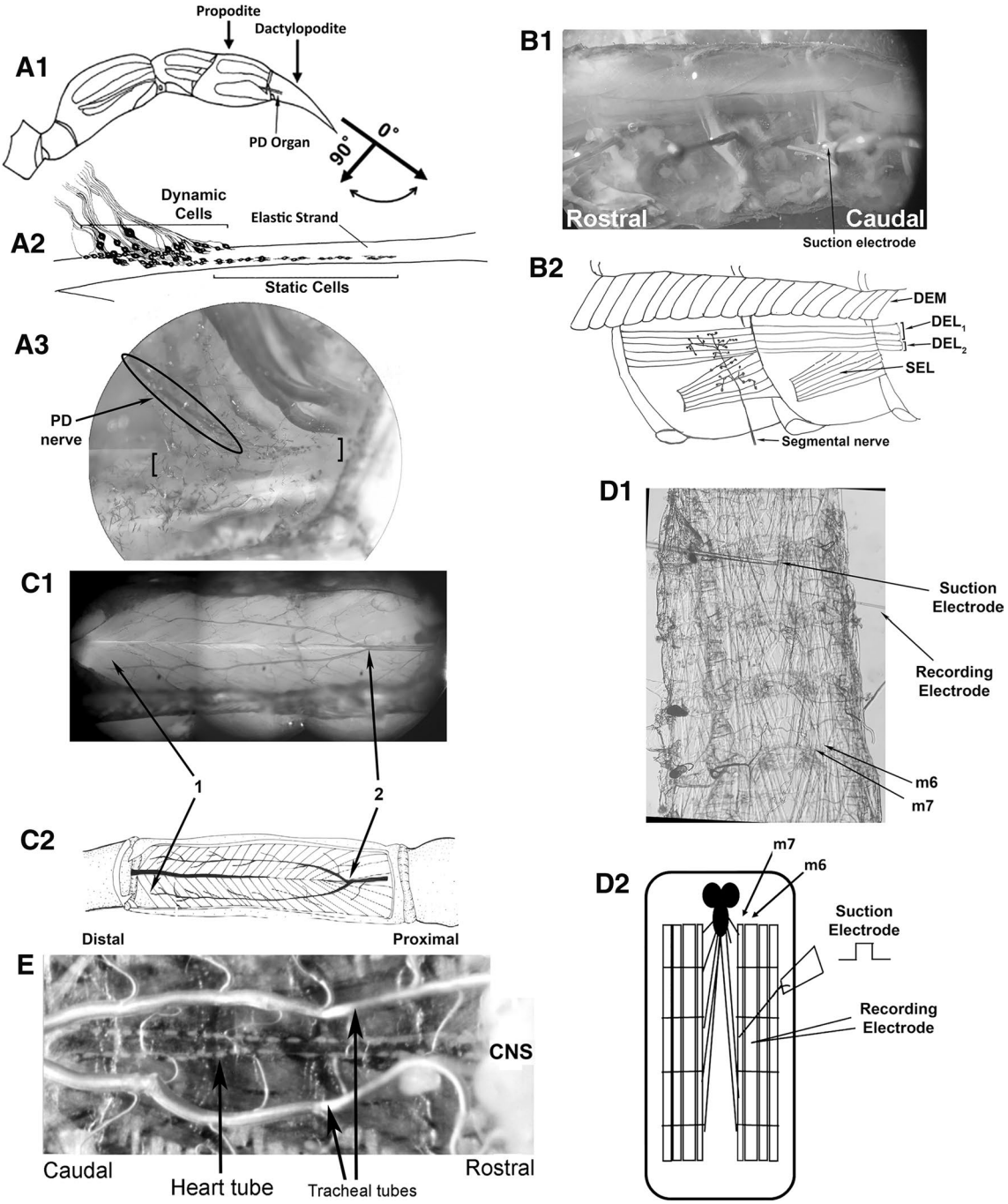
The crayfish walking leg opener neuromuscular preparation

The dissection and recording procedures are described in Cooper and Cooper (2009). In brief, the ventral cuticle of the propodite and the closer muscle is removed to expose the ventral surface of the opener muscle in the propodite cavity (Fig. 1c1). The cuticle over the flexor muscle in the meropodite segment is removed. The apodeme (tendon) at the meropodite-carpopodite joint is cut for the flexor, exposing the extensor muscle and the leg nerve. The main leg nerve and the extensor muscle are exposed so the most dorsal branch of the main leg nerve in the proximal end of the meropodite segments contains the excitatory motor neuron to the opener muscle. This nerve branch is then pulled into a suction electrode for stimulation.

To evoke action potentials in the excitatory axon, it is selectively stimulated by a Grass stimulator. The distal muscle bundles (Fig. 1c2) were impaled with a sharp intracellular electrode (20–30 MOhm resistance) filled with 3 M KCl. The excitatory junction potentials (EJPs) were recorded from the muscle fiber of interest. Short-term facilitation (STF) in the EJPs was obtained by stimulating at 40 Hz for 25 stimuli within a train and repeated every 250 ms. To record action potentials within the excitatory motor neuron, a microelectrode was placed into the excitatory axon of the opener muscle close to the axon bifurcation (Fig. 1c1, c2) (He et al. 1999). A standard head stage and amplifier for intracellular recording of the muscle and axon was used (Axonclamp 2B, and 1 X LU head stage, Molecular Devices, Sunnyvale, CA, USA). The crayfish saline used was the same as described above for the MRO preparation.

The larval *Drosophila* neuromuscular preparation

The dissection and recording procedures are described in Kurdyak et al. (1994) and Li et al. (2001). In brief, third instar Canton S larvae were used in these studies. Early third instars were developmentally staged so that all larvae were 50–70 h post-hatching. Flies were maintained at room temperature (21–23 °C) on a 12-h light/dark cycle in vials partially filled with a cornmeal–agar–dextrose–yeast medium. The larval dissections were performed beginning with a longitudinal dorsal midline cut to expose the CNS. The segmental nerves were cut and one segmental nerve was sucked into a suction electrode filled with the bathing saline and stimulated. Muscle 6 (m6) was utilized for intracellular recordings (Fig. 1d1 and d2). The EJPs were measured by intracellular recordings with a sharp glass electrode (3 M KCl, 40 megaohm resistance) and AxoClamp 2B amplifier. Preparations were used immediately after dissection. Electrical signals were recorded online to a computer via a PowerLab/4 s interface (ADI Instruments, Colorado Springs, CO, USA). The segmental nerves were stimulated at 0.5 Hz (S88 Stimulator, Astro-Med, Inc., Grass Co., West Warwick, RI, USA). The modified HL3 saline was used for physiological measures at a pH of 7.1 (de Castro et al. 2014; Stewart et al. 1994). Saline solution (in mM): 1.0 $CaCl_2{\cdot}2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose,



25 5 N, N-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES).

The larval *Drosophila* heart

The dissection and recording procedures are described in (Cooper et al. 2009). In brief, the fileted larva preparations were dissected ventrally and pinned on four corners. Guts and visceral organs were removed, leaving the heart intact and still attached to the rostral and caudal ends of the dermis (Fig. 1e). Dissection time was 3–6 min. The preparation was allowed to recover in saline for 3–5 min after dissection. Heartbeats were counted in the caudal region of the heart by manual inspection through a dissecting microscope (de Castro et al. 2014). The bathing saline was exchanged to one containing MS-222 (0.1%) and the heart rate was counted again, followed by removal of the MS-222 and flushing the preparation with fresh saline and counting the heart rate after the washout.

◂**Fig. 1** Preparations used to examine the effects of MS-222. **a1** A schematic diagram of the first walking leg of the blue crab, *C. sapidus*, containing the propodite-dactylopodite (PD) chordotonal organ. **a2** An enlarged diagram of the dynamic and static cells of the PD nerve embedded in the elastic strand. **a3** A view of the PD organ stained with 0.05% solution of methylene blue. The brackets enclose the length of the elastic strand where the dynamic and static cell bodies are embedded. **b1** A hemi-section of the crayfish abdomen viewed from ventral to dorsal after removal of the ventral muscle. The segmental nerve containing the nerves associated with the MRO is taken up by a suction electrode. **b2** A schematic diagram of the preparation seen in **b1** with muscles labeled. The deep extensor muscles (DEL1, DEL2, and DEM) and the superficial lateral extensor muscle (SEL) are labeled. The MRO organ is beneath the DEL1 muscle alongside the DEM. **c1.** A view of the crayfish walking leg opener muscle and axon stained with 0.05% solution of methylene blue. **c2.** A schematic diagram of the preparation seen in **c1**. The muscle excitatory junction potentials were recorded from the distal muscles of the preparation, marked as '1′ in the figure. The axon recordings were taken from the 'Y' portion of the axon toward the proximal end of the preparation, marked as '2′ in the figure. **d1** Dissection exposing the body wall muscles of a 3rd instar larva for recording synaptic responses in identifiable muscles. The synaptic excitatory junction potentials were initiated by stimulating the transected segmental nerve with a suction electrode. Recordings were obtained from the m6 muscle fiber with an intracellular electrode. **d2** A schematic diagram of **d1. e** The filleted larva preparation for exposure of the heart to MS-222 and measuring heart rate. Heartbeats were counted by manual inspection through a dissecting microscope before and after switching to the compound of interest. Heart rates were measured from the caudal end of the preparation close to where the two tracheal tubes come together

Data analysis

The analysis of the electrical signals from the PD was processed by measuring the number of spikes within the first second, which covered the dynamic movement of the joint to the stretched position. The stretched position was then held for another ten seconds. The 8 s following the initial one second of dynamic activity was used to measure the activity of the static position-sensitive neurons as indicated in Fig. 6. Three trials were performed for each time point. The activity from the set of three trials was averaged for each time point for both the one and eight second activity measures. Measures were made during initial saline exposure, after 15 min of exposure to MS-222 and after 20 min of a saline washout. Control experiments were performed with exchange of saline to saline without MS-222 for the same time periods. The control data of saline exchanged with saline is presented in previous reports and was obtained in the same laboratory as the current study (Stanback et al. 2019).

The analysis of the electrical signals from the MRO was processed by measuring the number of spikes within the first 10 s of activity, which included the one second of dynamic activity as the MRO was stretched and an additional 9 s of static activity.

The rank sum pairwise test or a sign test was used to compare the differences in responses before and after exchanging solutions. When the assumption of normality held, a paired *t* test was conducted to analyze the data. In some cases, synaptic responses were nonexistent with exposure to MS-222 which did not allow for parametric analysis. The analysis was performed with Sigma Stat software. A p-value < 0.05 was considered statistically significant. To examine the consistency and reproducibility of the data, groups of participants blinded to the specific settings of the analysis software were asked to supply their interpretations of the number of spikes for some of the same data sets.

Results

Crab chordotonal organ

A representative preparation of the crab PD organ illustrates the robust dynamic activity within the first second of joint movement (Fig. 2a1). After being held in a static position of leg extension the dynamic activity returns when the joint is moved back to the original flexed position. The static activity is monitored over the next 8 s after the initial one second of dynamic activity from extending the joint. The static activity generally shows some accommodation over time. The dynamic (1 s) and static (8 s) activities both substantially decreased after incubation for 15 min in MS-222 (0.1%) (Fig. 2a2). Twenty minutes after removal of MS-222 by washing out the preparations with three fresh saline exchanges and flushing the saline around the preparation, the dynamic and static activities gradually return (Fig. 2a3). Averaging the three repetitive trials for each exposure (initial saline, exposure to MS-222, and saline wash) for the six preparations demonstrates the trend that MS-222 decreases the neural activity for both the dynamic and static activities (Fig. 2b and c; $N = 6$, $p < 0.05$, sign test).

Crayfish muscle receptor organ (MRO)

A representative preparation of the crayfish MRO illustrates the activity within the abdominal joint movement (Fig. 3A1). After being held in a static position for 10 s, the joint is returned to the original position. Given that the MRO is comprised of only two neurons, there is not a recruitment of additional neurons as is the case for the crab PD organ. The dynamic and static activities were analyzed together over 10 s. The 10 s included the one second of movement to the static position and 9 s of holding at the stretched position (Fig. 3a). The neural activity substantially decreased after incubation for 15 min with MS-222 (0.1%) (Fig. 3a2). Twenty minutes after removal of MS-222 by washing the preparations with three fresh saline exchanges and flushing the saline around the preparation, the activity gradually returns (Fig. 3a3). Averaging the three repetitive trials

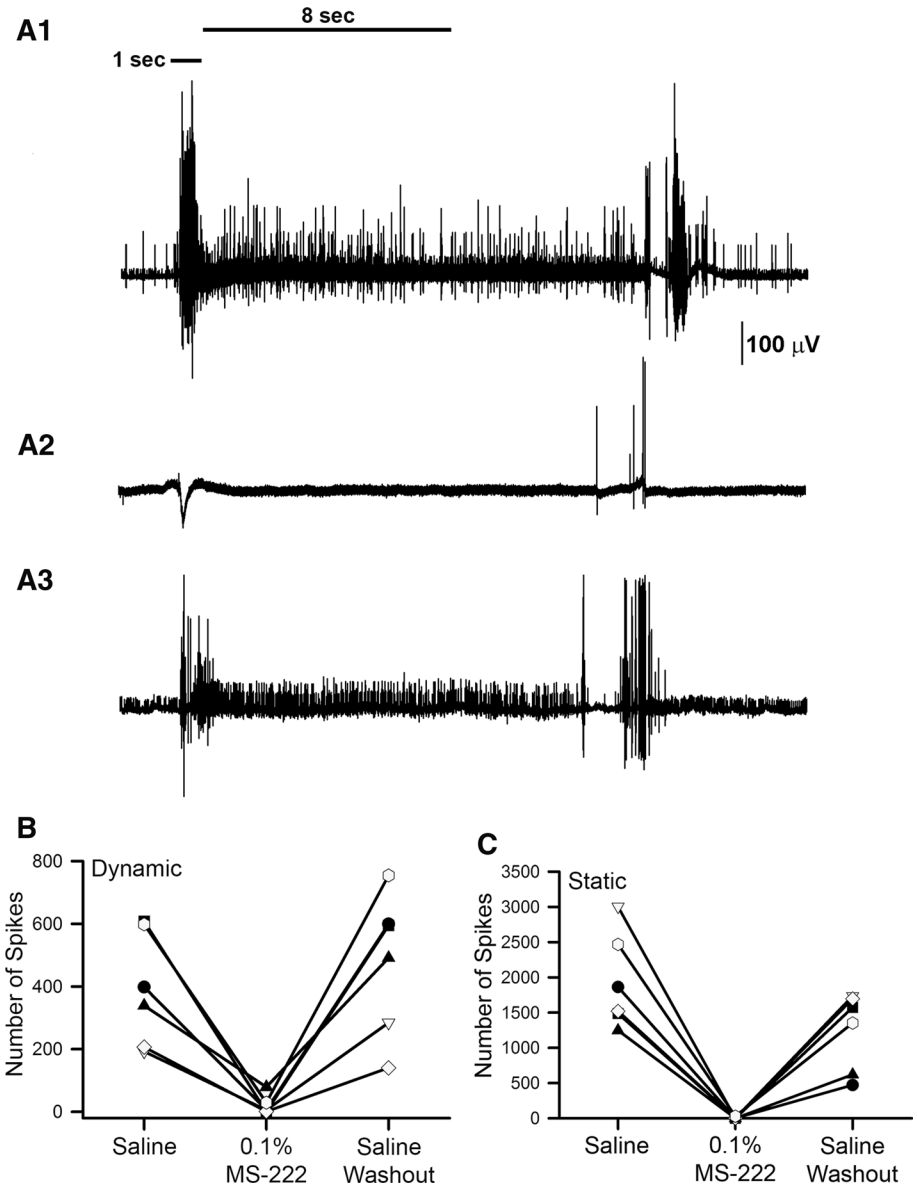


Fig. 2 **a1** Representative trace of the extracellular one-second dynamic and eight-second static activities of the PD nerve in saline while extending the joint to the fully extended position and held statically for more than 10 s prior to returning the joint to its starting position. **a2**. Representative trace of the extracellular one-second dynamic and eight-second static activities of the PD nerve in MS-222 (0.1%) while moving the limb from a flexed to extended position. **a3**. Representative trace of the extracellular one-second dynamic and eight-second static activities of the PD nerve after three saline washouts with the same movement paradigm as A and B. The traces are longer than 8 s to ensure that static activity is captured prior to returning the joint to the starting position. **b** The mean number of spikes for the extracellular one-second dynamic activity for each PD organ preparation in saline, after 15 min of incubation in MS-222 (0.1%), and 20 min after three saline washouts. **c** The mean number of spikes for the extracellular 8 s static activity for each PD organ preparation in saline, after 15 min of incubation in MS-222, and 20 min after three saline washouts. The MS-222 had a significant effect in reducing the neural activity for both the dynamic and static sensitive neurons ($N=6$, $p<0.05$, sign test)

for each exposure (initial saline, exposure to MS-222, and saline wash) for the six preparations demonstrates the trend that MS-222 decreases the neural activity (Fig. 3b; $N=6$, $p<0.05$, sign test).

The crayfish walking leg opener neuromuscular preparation

The evoked EJP responses on the opener muscle rapidly facilitate with repetitive stimulation as illustrated in a representative preparation (Fig. 4a). The amplitudes of the EJPs tend to reach a plateau by the 25th stimuli when the nerve is stimulated at 40 Hz (Crider and Cooper 2000; Desai-Shah et al. 2008). Thus, the amplitude of the 25th EJP within the stimulus train is used for assessment for the effect of MS-222 on synaptic responses. Within 20 min of incubation in saline containing MS-222 (0.1%), the responses are greatly attenuated but return after exchanging and flushing the preparation with fresh saline not containing MS-222 (Fig. 4b). A similar trend was observed for all six preparations (Fig. 4c; $N=6$, $p<0.05$, paired t test).

The action potential within the excitatory motor neuron innervating the opener muscle is depressed after exposure to MS-222 (0.1%); however, the amplitude returns after removal of MS-222 as shown for a representative preparation (Fig. 5a). This same trend occurred for all six preparations (Fig. 5b; $N=6$, $p<0.05$, sign test).

The larval *Drosophila* neuromuscular preparation

The EJPs recorded on m6 of third instar larval preparations showed a similar trend as for the crayfish opener

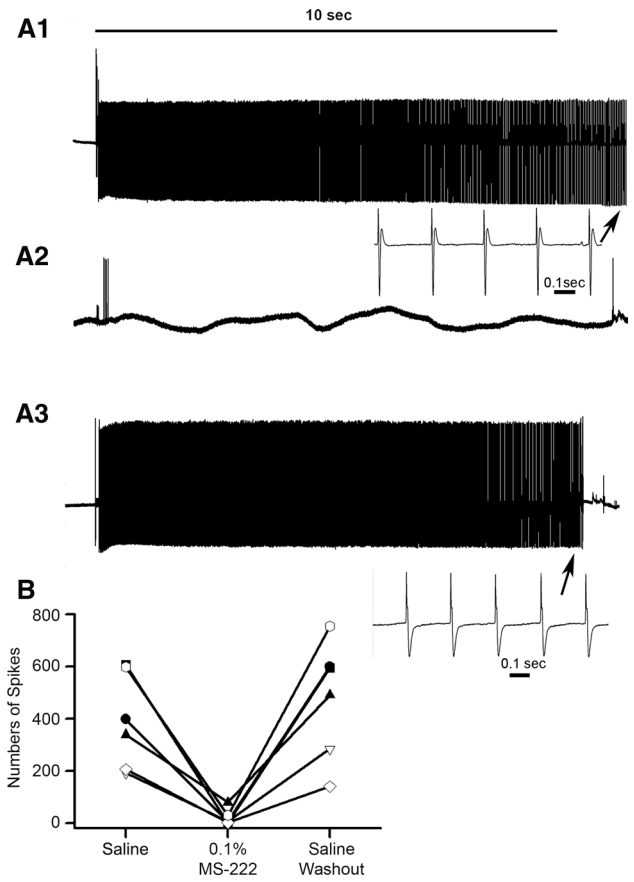


Fig. 3 **a1** Representative trace of the extracellular ten second activity of the MRO segmental nerve in saline. **a2** Representative trace of the extracellular ten second activity of the MRO segmental nerve after a 15-minute incubation in MS-222 (0.1%). **a3**. Representative trace of the extracellular ten-second activity of the MRO segmental nerve 20 min after three saline washouts. **b** The mean number of spikes for the ten-second activity for each MRO preparation in saline, after 15 min of incubation in MS-222, and 20 min after three saline washouts. Enlarged insets show the individual spikes recorded. The MS-222 had a significant effect in reducing the neural activity ($N=6$, $p<0.05$, sign test)

neuromuscular junction in being depressed by exposure to MS-222 (0.1%), as shown in a representative preparation (Fig. 6a and b). The synaptic innervation on m6 is of higher output, recruiting more synaptic vesicles to fuse than for the crayfish opener muscle (Atwood and Cooper 1996). The EJP responses are thus robust enough such that they are not required to facilitate to obtain good measures of the EJP amplitude. Thus, only a single stimulus at 0.5 Hz is sufficient to measure EJP responses. This same trend occurred for all six preparations (Fig. 6c; $N=6$, $p<0.05$, paired t test; * indicates a significant difference).

The larval *Drosophila* heart

The larval heart also showed sensitivity to MS-222 (0.1%), as the heart stopped beating when exposed to the compound. The heartbeat returned following the removal of MS-222 and flushing the preparation with fresh saline. This same trend occurred for all 6 preparations (Fig. 7; $N=6$, $p<0.05$ sign test).

For the participants in the classroom setting, MS-222 was applied to a larval heart preparation and washed out with saline for all 14 participants in a class to observe the heart rate. The class data set is not included as some students found it hard to accurately count the very fast rates. The course participants concurred that MS-222 stopped the heartbeat and that flushing the preparations with saline returned the heart to beating again. The data used for quantification were obtained from two advanced students (CS and RA) not in the course who were familiar with counting larval heart rates.

Reproducibility in analysis of number of spikes

Measuring the number of spikes for the crab PD and crayfish MRO with various participants in a classroom setting illustrated that, even with providing the same data set and a movie on the analysis procedure, variation in the measured number of spikes resulted. The class was told that the crab PD recordings needed to be separated into sections of the first second and the following 8 s for analysis and to analyze the entire 10 s of the MRO data sets. The student results are compared for two crab PD and two crayfish MRO preparations for the different conditions. The analysis is compared for eight independent groups of participants, with four groups each analyzing the same data sets (Tables 1 and 2).

As shown in Tables 1 and 2, the variability in the interpretations of the number of spikes with the groups and the control is in some cases extremely high. In cases where the variability was low, the number of spikes in the data set was extremely small which shows consistency in less noisy data sets. In most cases, the control group observation of the number of spikes was vastly different, on the higher side, especially in the PD data sets.

In examining the files where large discrepancies in numbers occurred, it was apparent that the ability to set the standard deviation in the software of Chart 7 or Chart 8 (ADI instruments) for the trace to count the spikes resulted in erroneous measures unless it was adjusted throughout the data gathered in the region of interest. If the standard deviation was set for the region of the trace where there are a high number of spikes occurring and that same standard deviation was used when no or very few spikes were occurring, then spurious spikes are detected in what one's eye would determine as noise in the baseline. This is highlighted in

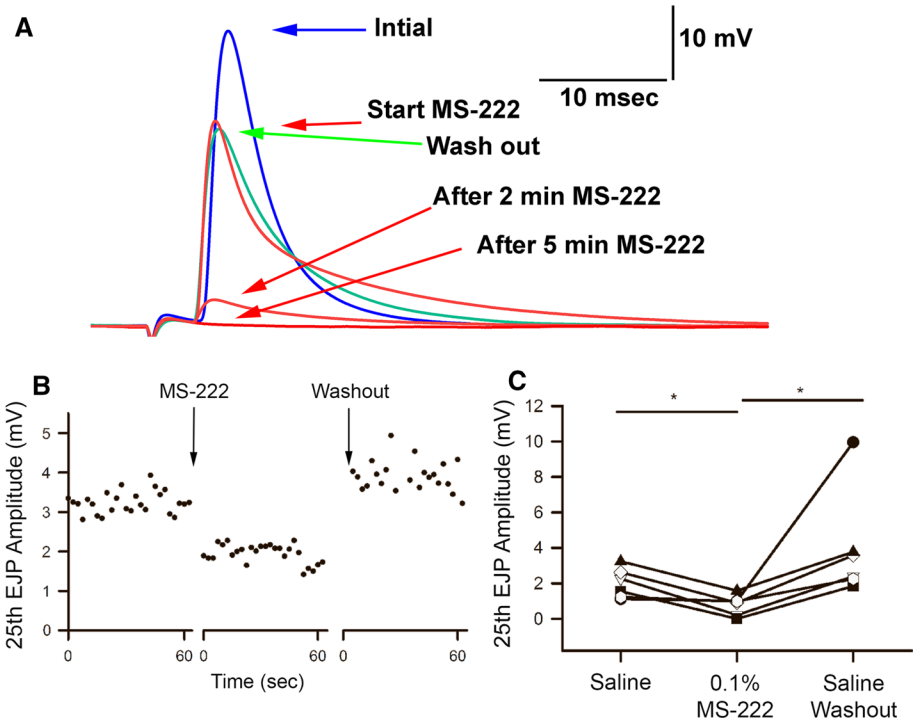


Fig. 4 **a** Representative traces of the excitatory junction potentials (EJPs) recorded with an intracellular electrode from the distal muscle fibers in opener muscle of a crayfish walking leg. The responses show a marked facilitation that occurs throughout the stimulation train delivered at 40 Hz for 25 stimuli. The responses are shown before and during MS-222 exposure (0.1%) as well as after exchanging the bathing media with fresh saline to remove the MS-222. **b** The 25th EJP amplitudes (mV) for a representative opener muscle preparation in saline, after 15 min of incubation in MS-222, and 20 min after three saline washouts. **c** The mean 25th EJP amplitude (mV) for each opener muscle preparation in saline, after 15 min of incubation in MS-222, and 20 min after three saline washouts. ($N=6$, $p<0.05$, paired t test; * indicates a significant difference)

Fig. 8 with the same standard deviation used in two different regions of the same continuous file over the experiment as compared to adjusting the standard deviation for each region being measured to determine the number of spikes. Note in Fig. 8a, the standard deviation of the trace is set at 1.2396 and what appears to be spikes as determined by eye appears to be reasonably different from the baseline to be detected by the software as spikes for this response from a PD preparation. However, in Fig. 8b, the same standard deviation was used when no spikes are observed by eye in the 8 s to be analyzed (the range shown with line with double arrow), but yet a large number of spikes was determined by the software (see green boxes highlighting with lines and dots the measured spikes). In Fig. 8b, the number of spikes is erroneously large as the deflections in the baseline are being determined as events.

Having the participants re-examine the data set shown in Fig. 8a and b, it was then determined that indeed no spikes should have been counted in Fig. 8b for the eight-second time frame. This same issue in determining the number of spikes from a set standard deviation of the trace within different regions of the same file was the reason for the discrepancies for the other participants.

Discussion

This study demonstrated that MS-222 substantially reduced electrical activity in the sensory neurons of the crab PD organ and the crayfish MRO. The PD and MRO are primary sensory neurons, being an indication that sensory input in other sensory systems is likely also attenuated with MS-222. The electrical activity responses are able be recovered after exposure to 0.1% MS-222 with substantial flushing of the tissue with fresh saline.

The sensory endings for the MRO are embedded within the muscle and may not be as accessible for large pharmacological compounds within the saline; this is also a possibility for the sensory endings of the PD organ. However, the endings of the sensory neurons where the mechanical–electrical transduction occurs are surrounded by a scolpale cap (Whitear 1960, 1962, 1965). Each scolopidium contains the endings of a pair of sensory cells, and the action potential of the neurons is initiated in the sensory endings within the scolopidium (Hartman and Boettiger 1967; Mendelson 1963). Compounds applied to the preparations, such as eugenol or others that block sodium voltage-gated channels, will decrease axonal electrical activity and the ability of the

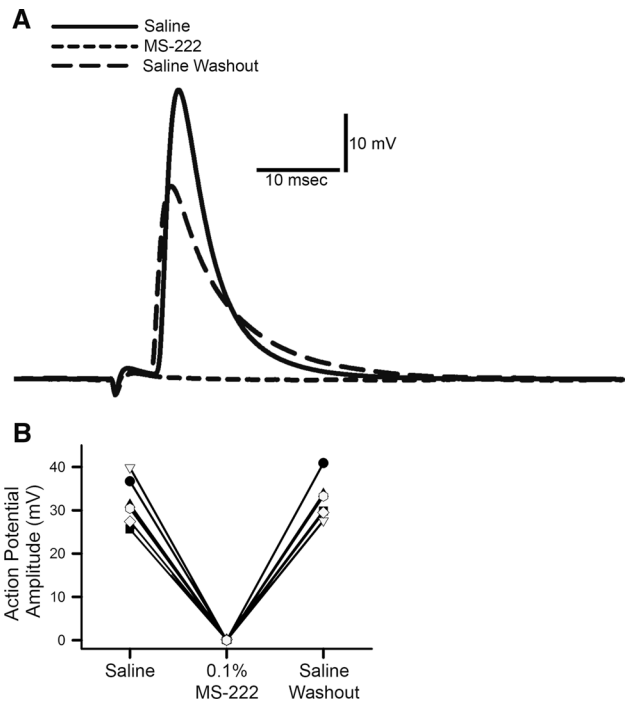


Fig. 5 **a** A representative trace of the action potential from the excitatory motor neuron to the opener muscle in saline, after 15 min of incubation in MS-222, and 20 min after three saline washouts. An average of 5 responses in each condition was used. **b** The mean action potential amplitudes (mV) for each preparation in saline, after 15 min of incubation in MS-222, and 20 min after three saline washouts. ($N=6$, $p<0.05$, sign test)

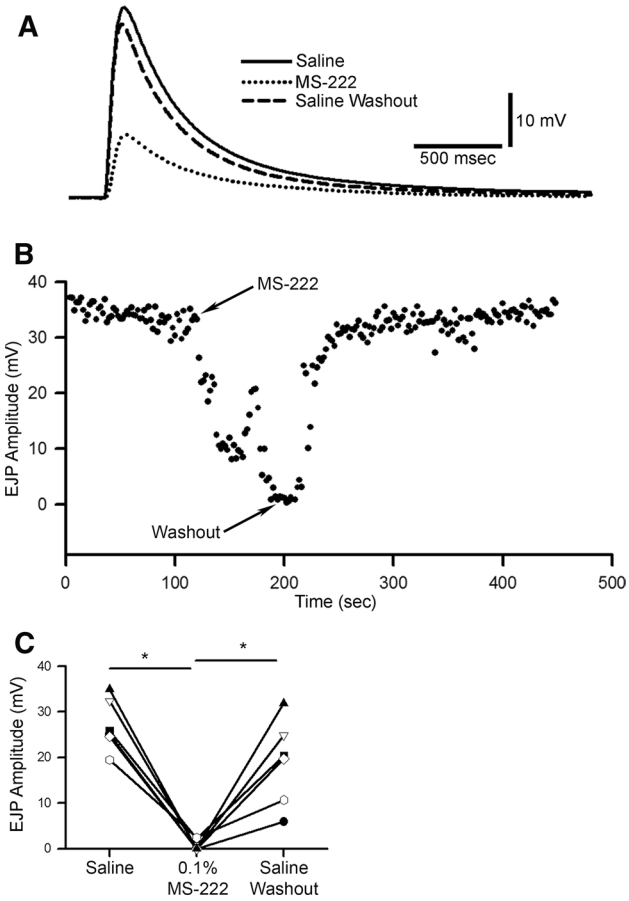


Fig. 6 **a** Representative traces of the excitatory junction potentials (EJPs) recorded with an intracellular electrode from m6 muscle in saline, during exposure to MS-222 (0.1%), and after three saline washouts. **b** The EJP amplitudes (mV) for a representative larval NMJ preparation in saline, upon exposure to MS-222, and after three saline washouts. Stimulations were delivered at 0.5 Hz. **c** The mean EJP amplitude (mV) for each fly NMJ preparation in saline, after incubation with MS-222, and after three saline washouts ($N=6$, $p<0.05$, paired t test; * indicates a significant difference)

nerve to reach threshold, but may not have any effect on the mechanical–electrical transduction process itself producing the graded sensory response.

Measures of synaptic responses showed that MS-222 did not totally depress the EJP amplitude to zero for the crayfish opener unlike what occurred for the *Drosophila* larval NMJ using the same incubation time. The *Drosophila* larval NMJ is exposed on the surface of the single muscle fibers as compared to the muscle fibers of the crayfish which are housed within bundles composed of multiple fibers. It is possible that with longer incubation or higher concentrations of MS-222, the EJPs on the crayfish opener NMJ would be completely suppressed. Motor nerves of both preparations have glial sheaths wrapping around them, but these are likely loosely packed. Raising potassium rapidly stops electrical conduction in crayfish axons which is likely due to depolarization and inactivation of voltage-gated sodium channels (Malloy et al. 2017). Perhaps these sheaths reduce the exposure of the nerve to the same concentration that is in the bathing solution.

The in situ larval heart tube preparation is very exposed to compounds in the bathing saline and is rapidly affected by the MS-222 as soon as it is applied, similar to the larval NMJ. The myogenic properties of the larval heart to

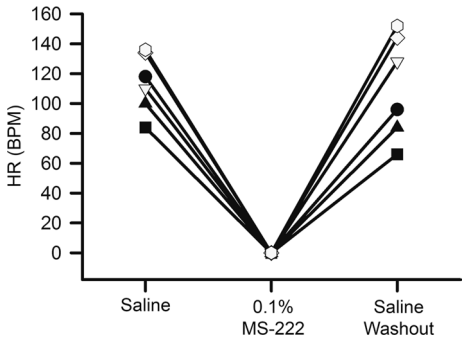


Fig. 7 The heart rate for each larval preparation in saline, after exposure to MS-222 (0.1%), and after three saline washouts without MS-222. ($N=6$, $p<0.05$, sign test)

Table 1 The number of spikes counted in two PD organs for different participating groups conducting analysis

Data set	Trial	Number of spikes											
		First second						Seconds 2-9					
		G1	G2	G3	G4	C	SD	G1	G2	G3	G4	C	SD
1	1	139	140	148	191	231	40.33	455	381	378	1503	2194	686.79
	2	113	94	111	202	227	60.53	465	254	366	1218	1785	659.94
	3	147	94	130	217	281	74.77	447	269	371	1291	1611	610.20
	4	1	1	0	2	0	0.84	346	30	0	310	0	175.07
	5	3	2	0	3	1	1.30	339	14	0	300	0	173.09
	6	0	0	0	0	2	0.89	351	17	0	261	2	167.32
	7	81	72	53	69	82	11.72	350	329	250	473	403	83.33
	8	31	72	30	90	116	37.47	425	423	169	648	511	174.75
	9	98	98	71	87	121	18.26	499	499	157	1468	502	494.10
2	1	84	102	253	262	232	86.37	468	867	1621	2043	1503	627.70
	2	127	180	205	315	207	68.55	674	995	1111	2648	999	778.98
	3	98	199	201	275	188	62.99	289	410	603	1412	1242	505.36
	4	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0
	7	68	143	135	67	117	36.39	453	536	774	422	586	139.08
	8	101	133	157	97	143	26.29	370	351	728	351	390	162.92
	9	93	126	176	88	195	48.30	466	381	730	438	886	217.31

The first one second and the second to ninth seconds were separated for analysis of the traces. *G1–G4* are four different participant groups. *C* is the control group. *SD* is the standard deviation of the observational measurements. The control group was composed of the same two individuals who analyzed all the previous data sets presented in the study. Trials 1–3 were of saline exposure only. Trials 4–6 were for MS-222 (0.1%) exposure, and trials 7–9 were for wash out of MS-222 with saline only. Two different data sets from different experimental preparations were used for comparisons

induce pacing continue to be a model for understanding the ionic properties involved. The heart rate is also rapidly modulated by serotonin, dopamine and octopamine (de Castro et al. 2019; Majeed et al. 2013a, b, 2014; Malloy et al. 2015; Titlow et al. 2013a) and other pharmacological agents (Gu and Singh 1995) as well as changes in the extracellular and intracellular calcium concentration (Desai-Shah et al. 2010; Zhu et al. 2016a, b). As with mammalian hearts, there appears to be modulation by stretch activated channels in the larval *Drosophila* heart (de Castro et al. 2019; Sénatore et al. 2010). Given that eugenol as well as MS-222 stops the larval heart rate and the heart regains function after removal of the compounds, this potentially suggests both compounds are similar in their mechanism of action (Weineck et al. 2019). It is known that eugenol blocks the human *N*-type calcium channels (Lee et al. 2005). MS-222 may influence a multitude of ion channels and its effects on the channels may be concentration-dependent. Given that MS-222 gradually reduced the synaptic responses at the NMJs, it is feasible that both voltage-gated sodium and calcium channels may be altered. It is known that MS-222 does prevent action potentials in neurons of fish (Attili and Hughes 2014), and in squid axons tricaine can block both Na + and K + currents (Frazier and Narahashi 1975). Further studies with single channel recordings of various channel types would help resolve this issue.

The reduced action potential amplitude in the opener neuromuscular preparation in the presence of MS-222 supports its function as a voltage-gated sodium channel blocker. Additionally, the amplitude in the action potential would gradually return following removal of MS-222, further supporting the notion. Thus, this is likely a key mechanism of action in reducing movements of an animal as well as its other effects that are similar to those of other anesthetics such as benzocaine, which is similar in molecular structure (Cakir and Strauch 2005; Ramlochansingh et al. 2014).

This study suggests that MS-222 can potentially be used as an anesthetic in crustaceans and insects similar to its anesthetic action in fish. Anesthetics and sedatives are commonly used in human and veterinarian medical practices, experimental animal research, and during transportation of animals. There are a variety of anesthetics available, and the type of treatment, species of animal, and homeostatic conditions of the subject in question must be taken into careful consideration when selecting a proper sedative. While anesthetic characterization for various mammalian, avian,

Table 2 The number of spikes counted in two MROs for different participating groups conducting analysis

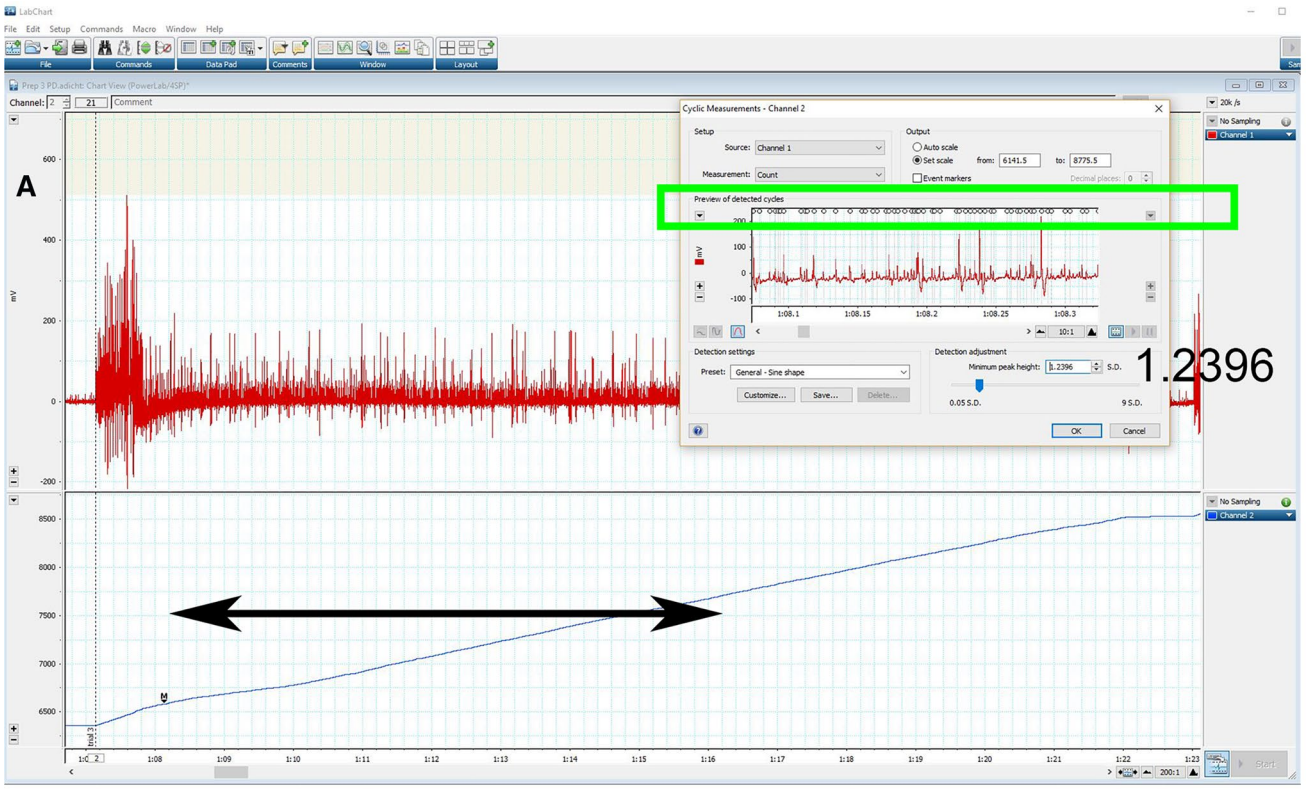
Data set	Trial#	Number of spikes					
		Ten seconds					
		G1	G2	G3	G4	C	SD
1	1	660	497	497	662	495	90.20
	2	705	680	689	695	686	9.51
	3	653	641	644	663	644	9.03
	4	6	2	0	7	0	3.32
	5	6	4	0	7	0	3.29
	6	13	2	10	22	0	8.88
	7	876	475	874	864	439	227.34
	8	1094	617	1096	1094	547	281.89
	9	1598	795	1598	1582	798	436.13
2	1	304	326	171	300	160	79.86
	2	475	473	485	481	476	4.90
	3	552	664	1115	1105	559	287.37
	4	8	266	72	244	7	126.79
	5	6	320	15	316	5	169.48
	6	4	8	10	11	7	2.74
	7	611	620	633	615	650	15.87
	8	536	534	402	542	548	61.96
	9	592	601	602	595	601	4.44

The ten seconds from the start of the movement were for analysis of the traces. *G1–G4* are four different participant groups. *C* is the control group. *SD* is the standard deviation of the observational measurements. The control group was composed of the same two individuals who analyzed all the previous data sets presented in the study. Trials 1–3 were of saline exposure only. Trials 4–6 were for MS-222 (0.1%) exposure and trials 7–9 were for washout of MS-222 with saline only. Two different data sets from different experimental preparations were used for comparisons

amphibian, and fish species is well-known, there are numerous taxonomic groups that have yet to be examined thoroughly (Brodbelt 2009; Bowker et al. 2014; Brown et al. 1996; Flecknell 1984; Gaynor et al. 1999; Lierz and Korbel 2012). Arthropoda is one such group where there has been much debate over standard procedures in sedation and nerve response (Diggles 2018; EFSA 2005; Håstein et al. 2005; Sømme 2005; Weineck et al. 2018). Arthropoda, specifically crustaceans and insects, are typically used as research models for addressing physiological and ecological topics. Implanted monitoring devices are sometimes used in various species, which can result in the need to restrain the animal (Barron 2000; Listerman et al. 2000; Weineck et al. 2018). Such handling may lead to stressful conditions for an animal that could have lingering effects, which may impact the animal negatively and ultimately alter the data one is interested in gathering (Davis et al. 2015; Javahery et al. 2012; Wilkens et al. 1985).

There are few published reports in the use of MS-222 on invertebrates (sea urchin: Applegate et al. 2016; Coelenterates: Stoskopf 2006; Freshwater prawn: Coyle et al. 2005; Horseshoe crab: Archibald et al. 2019; freshwater ostracod, a benthic crustacean: Schmit and Mezquita 2010). Other anesthetics, such as benzocaine, have been examined in crustaceans (Lea 1996); however, there are only scant reports of examining MS-222 and benzocaine on insects (Guttman 1967; Wayson 1976). Future studies examining behavioral measures following application of MS-222 will be beneficial in elucidating its practical use as an alternative method of anesthesia in these models. Hopefully, this report will encourage an increase in research of anesthetic use on insects and crustaceans.

In this study, exposed in situ preparations were used for examining the direct effect of a compound on neurons and cardiac function in model invertebrate preparations. These preparations are also common ones used for experimental procedures as well as teaching in neurobiology and physiology. For classroom use, since there is an increase interest in animal welfare, using reversible anesthetics might be more appealing for student participants conducting the common crustacean and insect teaching laboratory protocols (Baierlein et al. 2011; Bierbower and Cooper 2009; Cooper and Cooper 2009; Cooper et al. 2009, 2011;Dayaram et al. 2017; Holsinger and Cooper 2012; Johnson et al. 2014; Leksrisawat et al. 2010; Majeed et al.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Cyclic Measurements - Channel 2
Setup
Source: Channel 1
Measurement: Count
Output
Auto scale
Set scale from: 6141.5 to: 8775.5
Event markers
Preview of detected cycles
Detection settings
Preset: General - Sine shape
Customize... Save... Delete...
Detection adjustment
Minimum peak height: 1.2396 S.D.
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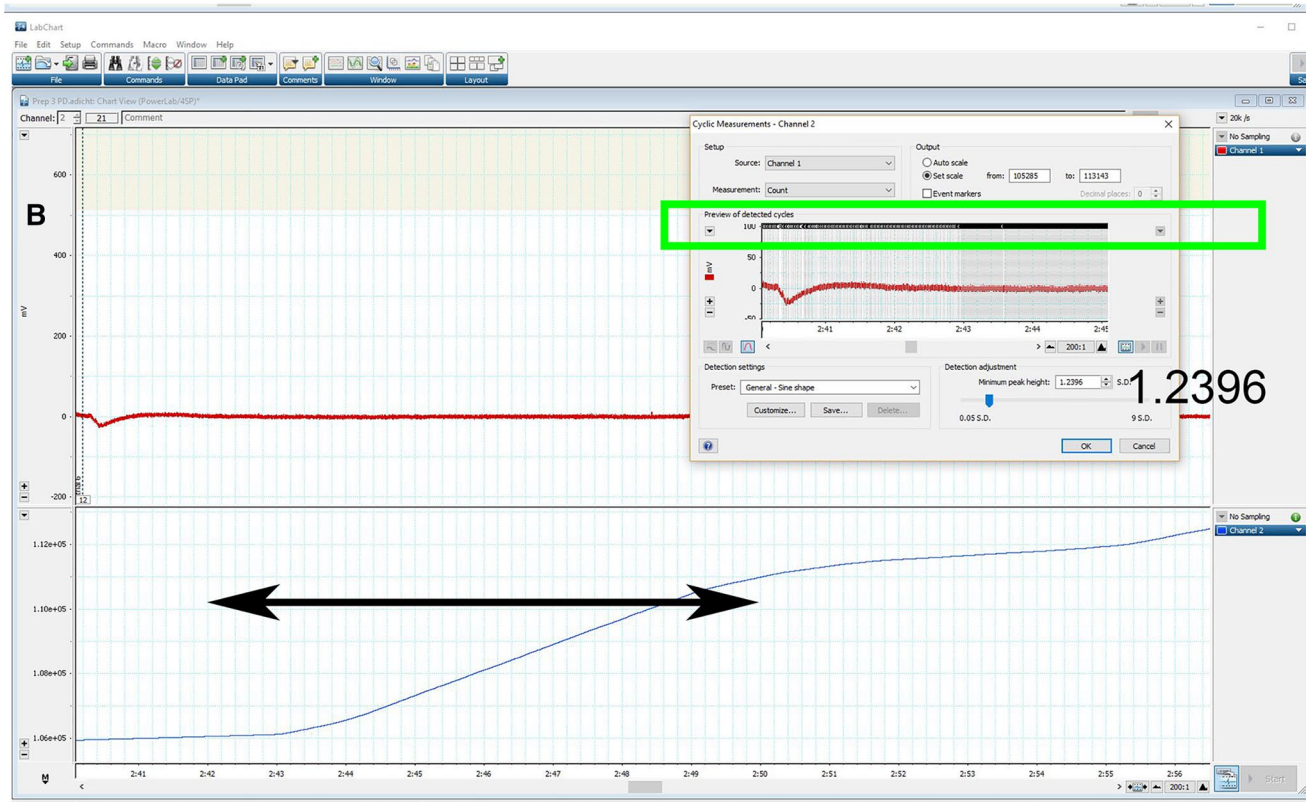
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Cyclic Measurements - Channel 2
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Source: Channel 1
Measurement: Count
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Auto scale
Set scale from: 105285 to: 113143
Event markers
Preview of detected cycles
Detection settings
Preset: General - Sine shape
Customize... Save... Delete...
Detection adjustment
Minimum peak height: 1.2396 S.D.
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◂**Fig. 8** Analysis in the number of responses with a consistent criterion of standard deviation of the mean. Computer screen capture in two regions of a file where one has a prominent number of spikes (**a**) and a second where there are no spikes (**b**) during the movement and hold in an extended angle of the PD joint for the crab model. Each panel (**a** & **b**) has a top region showing the raw data along with a superimposed screen of the window to determine the parameters for counting the number of spikes above the mean of the baseline responses. Note the analysis is set at the same standard deviation of the mean (i.e., 1.2396) for both regions of the file. The bottom aspect of each panel (**a** & **b**) depicts the continuous running value in the number of counts. The number of spikes counted is also noted by the open circles above the trace in the green boxes of the highlighted analysis windows superimposed on panels for both **a** and **b**. In B the open circles are so close together it appears as a line

2017; Malloy et al. 2017; Robinson et al. 2011; Thenappan et al. 2019; Titlow et al. 2013b).

The species of crab and crayfish used in this study are also of commercial importance as eatable crustaceans. Using MS-222 as an anesthetic for crustaceans that are intended for eating may not be feasible, as MS-222 may potentially remain in the tissue after slaughter. The fact that 0.1% MS-222 did wash out and the activity returned in neurons and cardiac tissue is promising. However, an anesthetized crustacean would likely need to be able to circulate the hemolymph and have respiratory function (i.e., gills) to remove the compound. Such whole animal experiments are needed to examine the retention of MS-222 in tissue if the animal is to be used for animal or human consumption. Crayfish can be anesthetized with isoeugenol or tricaine methanesulfonate (MS-222) (Håstein et al. 2005) as an option for avoiding pain and distress prior to being killed for food processing. However, in the USA isoeugenol is not allowed as it is a potential carcinogen. MS-222 requires a withdrawal period of 21 days which might be feasible for reducing transport stress but not for use prior to slaughter. Future studies in varying the dosage in exposure to whole animals or injection into animals as well as in isolated preparations could help in establishing if MS-222 could be more widely utilized for a variety of purposes.

Given that raw and sometimes compiled data are now commonly made available along with published reports, it is important to detail how that data are analyzed. Even with a careful description with one representative data set, the explanation may not be applicable to all the data sets as we have highlighted herein. Even with an explanation in video format, this may not be sufficient for comprehension, and it is hard for others to completely reproduce the analysis to the same degree, although they may obtain similar trends in the analysis. This issue is also of concern when implementing citizen science or crowdsourcing projects to analyze data sets (Clare et al. 2019). A large number of repetitive measures needs to be undertaken to screen for accuracy (Lea et al. 2017).

The investigation of authentic scientific investigations with participating students in a classroom setting is a trend which is being promoted to expose students to research as a group. This ACURE (authentic course-based undergraduate research experiences; Malloy et al. 2017; Stanback et al. 2019; Wycoff et al. 2018) approach builds on the CURE (course-based undergraduate research experiences) philosophy (Bakshi et al. 2016; Linn et al. 2015); however, it aids students in undergoing a more complete research experience. In addition, utilizing participants within a course setting to analyze data sets blind to the experimental conditions provides an additional level for interpretation of the findings.

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Compliance with ethical standards

Conflict of interest None.

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