ARTICLE
Quantifying the Effects of Two Local Anesthetics on the Crayfish Stretch Receptor Organ: An Integrated Neurophysiology Lab

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The crayfish stretch receptor organ (SRO) preparation represents a robust experimental model for undergraduate laboratory experiences. For example, this preparation may be included as part of a course-based undergraduate research experience (CURE), where students work independently to plan and carry out their own experiments. In the current paper, we provide an example of how local anesthetics may be used to manipulate the SRO preparation and to perform quantitative analyses of SRO action potential firing rates.

Local anesthetics provide interesting tools for manipulating physiological responses within the nervous system. A variety of inexpensive anesthetics are available for student use and each of these is expected to inhibit neurophysiological responses. While specific anesthetics exhibit subtle differences in chemical organization, they are generally understood to block voltage gated sodium channels. In the current study, we investigated the effects of two local anesthetics, MS-222 and procaine, on the action potential firing rate from the crayfish SRO. Using quantitative analyses of SRO action potential generation, we determined that each anesthetic has unique inhibitory effects on action potential firing rate that may be explained by their neuropharmacological properties. This manipulation may thus be utilized as an interesting experimental tool in undergraduate teaching laboratories. Local anesthetics applied to crayfish SRO preparations can thus be used to deepen student understanding of local anesthetics, exercise quantitative analyses, and provide experimental tools for independent experimental design.

Key words: stretch receptors; muscle receptor organ; MS-222; procaine; local anesthetics; crayfish; neurophysiology

Increasingly, science educators are accepting the need to implement hands-on, active learning strategies in our curriculum. Examples of common models that fulfill the goals of active learning include course-based undergraduate research experiences (CURE) (Auchincloss et al., 2014) and project-based learning (PBL) (Zwick, 2018). The State University of New York (SUNY) at Fredonia is a small liberal arts university on the shores of Lake Erie in western New York. In the Fredonia Biology Department we began offering research oriented courses as part of our upper level elective offerings several years ago. One of these courses is *Advanced Neurophysiology Lab*, which focuses on the fundamental principles of neurophysiology. During the first ~2/3 of the course students are introduced to preparations that permit the recording of action potentials, sensory responses, and motor system physiology. After students have had opportunities to work with these different preparations, they develop independent research projects that aim to test additional hypotheses, using the techniques they were introduced to earlier in the semester. What we present in the current paper is one example of an experimental approach students may employ to extend their observations of a robust sensory physiology preparation and to apply quantitative analyses to their experiments.

Crayfish are common subjects for neurophysiology experiments, with paired segmental nerve structures that are easily accessible by both students and researchers. While simple to dissect, the diversity of neural structures contained within crayfish demonstrate a wide variety of important neuroscience concepts. For example, pairs of stretch receptors found in each segment of the crayfish abdomen are ideal for the demonstration and manipulation of the mechanotransduction of proprioceptive information (Florey & Florey, 1955). These stretch receptors receive input from specialized muscle receptor organs to encode dynamic changes in muscle length (Alexandrowicz 1951; Purali, 2005; Rydqvist, 2007). Muscle receptor organs run in parallel with superficial extensor muscles and convert mechanical stimulation into neural signals (Alexandrowicz 1951; Purali, 2005; Rydqvist, 2007). They are functionally analogous to human intrafusal muscle spindles, which also provide proprioceptive muscular feedback Rydqvist, 2007; (Leksrisawat et al., 2010). Additionally, these muscle receptor organs are conserved across various decapod crustacea and are similar in a variety of other crustaceans (Pilgrim, 1960; Wallis et al., 1995). These features make crayfish stretch receptors a useful preparation for understanding how muscle stretch is converted into neural activity.

The crayfish stretch receptor organ (SRO) preparation is widely used in neurophysiology teaching labs, where action potentials can be recorded in response to tail flexion (Leksrisawat et al., 2010; Wyttenbach et al., 2011). They provide a useful method of study due to the robust nature of the preparation and the differentiation between the two types of receptor neurons. The rapidly adapting neuron mediates the phasic response, which is responsible for encoding changes in muscle stretch. The slowly adapting

neuron continues to fire as the muscle remains stretched, representing absolute muscle stretch in a complementary tonic signal. These receptors differ in ion channel types, channel distribution across the receptor membrane, and viscoelastic properties of their associated muscle receptor organs (Rydqvist et al., 2007). Therefore, each stretch receptor type exhibits unique ion currents in response to mechanical stimulation. Both receptors transmit signals within the same abdominal nerve and provide information about distinct aspects of muscle stretch.

Local anesthetics act on voltage gated sodium channels, which are integral to generating the neural responses recorded from stretch receptor neurons (Fozzard et al., 2011). Many local anesthetics are used interchangeably because of this common action on sodium channels. However, their effects on action potentials are not always uniform, indicating that they vary in specific mechanisms of inhibition. This is supported by previous research showing that local anesthetics differentially affect sodium channels through action on their open, closed, or inactivated states (Butterworth & Striachartz, 1990).

In the current study, we were able to quantify the normal phasic and tonic stretch receptor responses to mechanical stimulation. Additionally, by applying MS-222 and procaine to the stretch receptor preparation, we measured the different effects of local anesthetics. By recording these unique responses, we also evaluated these anesthetics as a potential tool for teaching labs. Finally, we examined the consequences of these local anesthetics in the context of neurophysiology through analysis on both whole crayfish and stretch receptor preparations.

Laboratory Objectives

1. Provide opportunity for students to demonstrate knowledge of neuroanatomy through crayfish dissection
2. Master commonly used extracellular electrode neurophysiological recording techniques
3. Observe and quantitate action potential frequency in response to mechanical stimulation with stretch receptors
4. Plot action potential frequency as a function of time and fit these data with power decay curves
5. Design individual experiments with different local anesthetics to modulate action potentials
6. Apply statistical analyses to compare different experimental conditions

MATERIALS AND METHODS

Crayfish (*Procambarus clarkii*) were obtained live from Carolina Biological Supply Company and held in a freshwater tank until use. Animals were anesthetized in ice for 10 minutes prior to dissection, then quickly decapitated (Leksrisawat et al., 2010; Wyttenbach et al., 2011). Crayfish preparations were immersed in a standard crayfish saline composed of: 205mM NaCl, 5.3mM KCl, 13.5 mM $CaCl_2$ *$2H_2O$, 2.45mM $MgCl_2$ *$6H_2O$, 5mM HEPES, and adjusted to a pH of 7.4. Both anesthetics (MS-222; tricaine methanesulfonate; Sigma Chemical, St. Louis, MO), procaine (procaine hydrochloride; MP Biomedicals, Solon, OH) were mixed to the desired concentration in either crayfish saline (for SRO preparations) or DI water (whole animal immersion) and adjusted to a pH of 7.4.

Whole Crayfish Anesthesia

Two different methods of crayfish anesthesia were tested during this experiment: immersion and injection. We first immersed the whole crayfish in 1.0 L of an anesthetic solution. All anesthetic solutions were made at 0.1% (0.1g/100mL). This concentration was chosen as an intermediate dosage, as it has proven effective as an amphibian anesthetic (Medler, 2019). Crayfish were immersed in the solution for a 30-minute period, over which we monitored them continuously for anesthetic effects. Once they were removed from the anesthetic and any effects had worn off, they were returned to their tank for future use.

To test anesthetic injections, we used 0.1% MS-222 or procaine solutions mixed in crayfish saline, which represented a moderate dosage. 1.0 mL of the anesthetic was injected by inserting a 28 gauge hypodermic needle through the carapace and into the heart of the crayfish, which were then placed in a 1.0 L container of pond water. The crayfish were then monitored for 30 minutes to observe the effects of each anesthetic. Physical stimulation of the crayfish (reaching in as if to grasp the animal) was repeated with any cessation of movement and any decrease in aggressive response noted. After any potential anesthetic effects wore off, they were returned to their tank for reuse following a minimum of 5 days of recovery.

Stretch Receptor Preparation

The crayfish stretch receptor was prepared according to written and video instructions from Crawdad Online (Wyttenbach et al., 2011). We first anesthetized the crayfish on ice as described above. Using scissors, we separated the abdomen from the thorax, then made a lateral cut along each side of the abdomen. To expose the nerve containing both stretch receptors, we pushed back and removed the flexor muscles, leaving the extensor muscles intact. This preparation was pinned to a petri dish lined with Sylgard and immersed in 50 mL of crayfish saline.

Extracellular recordings were made from the severed end of the nerve emerging from the SROs within extensor muscles. The desired nerve was located bilaterally, anterior to each set of ribs. The preparation was viewed under a stereomicroscope to locate the correct nerve and the end of the nerve was carefully drawn into a suction electrode. Once the nerve was situated in the pipette tip, we were able to record its response using an AM Systems Model 3000 AC/DC differential amplifier. For all trials, we quickly flexed the tail to 90° and held it for 20 seconds to observe the resulting action potentials. Further description of this laboratory preparation can be found in the following video by Leksrisawat et al. (2010) that introduces the crayfish stretch receptors: https://www.jove.com/video/2323/muscle-receptor-organs-crayfish-abdomen-student-laboratory-exercise. All experiments were carried out at room temperature (22° C) in a physiology teaching lab.

Anesthetics were applied to the stretch receptor

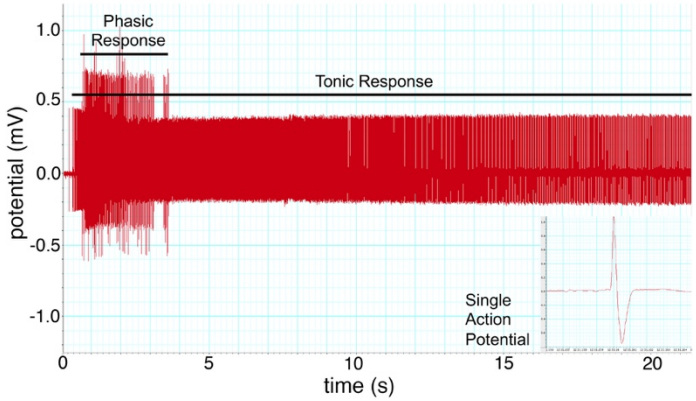

Figure 1. Control stretch receptor recording. This recording includes both tonic and phasic portions of a normal stretch receptor response to 90° tail flexion. An individual action potential is displayed as an example of the pattern observed on a more expanded time scale. At the time scale shown in the main part of the recording, each action potential appears as a vertical line.

preparation after a high frequency train of action potentials in response to abdominal flexion was consistently elicited for at least 5 minutes. The nerve remained in the pipette tip while half of the crayfish saline (25 mL) was removed from the dish with a pipette and replaced with an anesthetic solution. For a similarly intermediate dosage as used on whole crayfish, anesthetic solutions were mixed to 0.1% in crayfish saline. The time the anesthetic was added was recorded in each trial, then the tail was flexed in the same 20 second intervals as controls. The time all nerve response ceased was also recorded, as well as whether any recovery of response occurred following removal of the anesthetic (typically after 5 – 10 minutes). Each preparation was used for multiple tail flexion trials over a period of up to several hours, so long as it was still responsive.

We recorded and analyzed neural responses using AD Instruments Lab Chart 8 for Mac software. Lab Chart's Cyclic Measurements mode allowed us to measure firing rate as a function of time, with a simple threshold set at an appropriated level to discriminate the desired response from external noise. For several responses in each condition, the firing rate at 0, 10 and 20 seconds were recorded and averaged by treatment. To further analyze firing rates, the maximum sustained firing rate over each 0.5 second interval of selected responses were measured and plotted in Excel. The data from each trial were fitted with a power curve of best fit, with significant gaps and cessations in firing excluded to find the most accurate equation.

Statistical Analyses

Action potential firing frequencies were determined at the immediate onset of tail flexion (0 seconds), and subsequently after 10 and 20 seconds of flexion. The firing rates were compared among control, MS-222 treated, and procaine treated preparations using an analysis of variance (ANOVA) test. When significant differences were detected among the mean firing rates a Tukey-Kramer post-hoc test was used to determine which of the means were different from one another. Differences were considered to be significant if they were < 0.05.

In addition to testing for differences among mean firing rates, we analyzed the rate of decay of action potential firing rates between control and MS-222 preparations. Data were log-transformed to convert the curvilinear decay rates into a linear plot. The firing of action potentials was too erratic after procaine treatment to plot a predictable rate of decay. To test whether the rate of decay differed between control and MS-222 treated preparations, we used an analysis of covariance (ANCOVA) approach. When applying ANCOVA, the first step is to test for significant differences between the slopes of two or more lines (Neter et al., 1990; see also Parris J, 2011: https://www.youtube.com/watch?v=mApbp1RDy-U). In our case, we tested the interaction between treatment (control vs. MS-222) and action potential frequency. If this interaction is statistically significant it means that the slopes are different from one another and this is the end of the analysis. If the slopes are not different, then the next step is to determine whether the elevation of the lines are significantly different from one another. All statistical analyses of these data were performed using JMP Pro 14 statistical software.

RESULTS

Whole Crayfish Anesthesia

To test the effects of local anesthetics on whole crayfish, live animals were placed in a 1.0L bath of 0.1% anesthetic solutions for 30 minutes. For both MS-222 and procaine trials, there was no indication of anesthesia over the 30-minute period (n=3 animals per anesthetic). When occasionally given tactile stimulation, crayfish responded equally robustly throughout the entire experiment. Therefore, immersion in anesthetics at this dosage was not effective for either MS-222 or procaine.

Additionally, we injected 0.1% anesthetic solutions into the hearts of live crayfish and monitored the effects. Crayfish injected with MS-222 showed no anesthetic effects over the 30-minute monitoring period (n=3). Crayfish were given tactile stimulation, and there was no change in the robustness of their response following injection. However, procaine injections resulted in mild, short-term sedation of the live crayfish. Approximately 2 minutes following injection, they exhibited altered behaviors and could be flipped onto their backs without resistance. While sedated, subjects pulled their claws close together, tucked their tails firmly under their bodies, and did not respond aggressively to being picked up. Across the procaine trials, this sedation had a mean duration of 2 minutes (n=3 animals, sd=1.3). Following this time period, crayfish regained normal activity levels and responsiveness for the remainder of the experiment. Despite this small effect, injection was unsuccessful in inducing full anesthesia for MS-222 or procaine at this concentration. For the purposes of animal anesthesia, we recommend using standard cold immersion.

Stretch Receptor Preparation

To observe the electrophysiological effects of anesthetics on the crayfish stretch receptor, crayfish were prepared as previously described. Control recording occurred prior to the application of any anesthetics, with the preparation

immersed in regular crayfish saline. The phasic and tonic portions of the neural response to 90° tail flexion are shown in Figure 1.

Flexion of the tail immediately activates both phasic and tonic SROs within the superficial extensor muscles. Trains of action potentials are elicited from both SROs simultaneously and these overlap within a recording (Figure 1). Analyses of multiple control recordings determined that the rapidly adapting (phasic) neuron fired for an average of 3.75 seconds (n=4 trials, sd=1.5) in response to tail flexion. The slowly adapting (tonic) neuron continued to fire for the entire 20 seconds of stimulation across all control trials. The maximum firing rate averaged 131.1 Hz (n=4 trials, sd=36.2) for the phasic receptor and 117.6 Hz (n=5 trials, sd=40.8) for the tonic receptor. This maximum rate occurred immediately upon flexion of the tail, then gradually decreased as this position was held. As shown in Figure 2, the responses of both stretch receptors followed power decay curves, which matches previously findings by Nakajima & Onodera (1969). The rate of decay is demonstrated by the exponential term in the power equation. This decay rate of the action potential frequency is greater in the phasic ($s^{-0.685}$) than in the tonic ($s^{-0.290}$) SRO (see Figure 2 legend for decay equations).

When MS-222 was applied to the stretch receptor preparation, there was a change in the observed neural response. Across 5 trials using 0.05% MS-222 solution, the anesthetic caused variable responses, with occasional normal responses interspersed with disruption to tonic firing. As displayed in Figure 3, a characteristic gap in tonic firing occurred in 35.8% of responses analyzed during MS-222 trials (n=95 trials). This disruption in firing, which occurred between the initial phasic response and the beginning of sustained tonic firing, lasted an average of 2.2 seconds (n=34 trials, sd=1.6). In some responses, no action potentials from the tonic stretch receptor were observed in response to tail flexion. The average maximum firing rate following treatment with MS-222 was 137.1 Hz (n=4 trials, sd=23.3), which was not significantly different from the control maximum (Figure 4; $p>0.07$). In all trials, the complete cessation of nerve response occurred an average of 44.8 minutes following MS-222 application (n=5 trials sd=6.1). After a rinse with normal crayfish saline, all MS-222 treated nerves regained neural activity.

Since MS-222 had a relatively weak effect on the stretch receptor, we hypothesized that calcium-mediated currents might contribute to the observed responses. The stretch activated ion channels in the SRO are permeable to sodium, potassium, and calcium ions (Rydqvist, 2007). Once voltage gated sodium channels were disrupted by the local anesthetic, the continuation of responses could be attributed to unaffected calcium influx. To test this possibility, we mixed crayfish saline as stated above, but with the $CaCl_2$ replaced with an equal concentration of $CoCl_2$. The substitution of cobalt acted to both eliminate extracellular calcium and block any calcium channels. Therefore, the effect on the firing of the stretch receptors indicated whether calcium played a role in the continuation of response after MS-222 was applied.

When the cobalt-substituted crayfish saline was applied to the stretch receptor preparation, the tonic neuron was strongly affected. Both tonic and phasic receptor responded almost identically, with high frequency tonic responses that had an average duration of only 1.8 seconds (n=8, sd=0.7). Additionally, a baseline firing rate of approximately 12 Hz

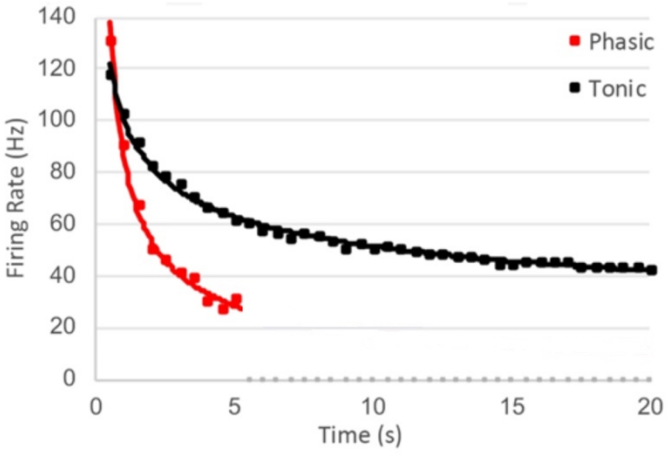

Figure 2. Firing rate decay for phasic and tonic stretch receptors. Average control phasic (n=4) and tonic (n=5) responses are shown for 20 seconds of tail flexion. Phasic firing rate is initially greater than tonic, but decays more rapidly and stopped after ~5 seconds. Each response type is fitted with a power decay curve. Tonic decay: rate (Hz) = $100.08s^{-0.290}$ ($R^2 = 0.99$). Phasic decay: rate (Hz) = $85.657s^{-0.685}$ ($R^2 = 0.98$).

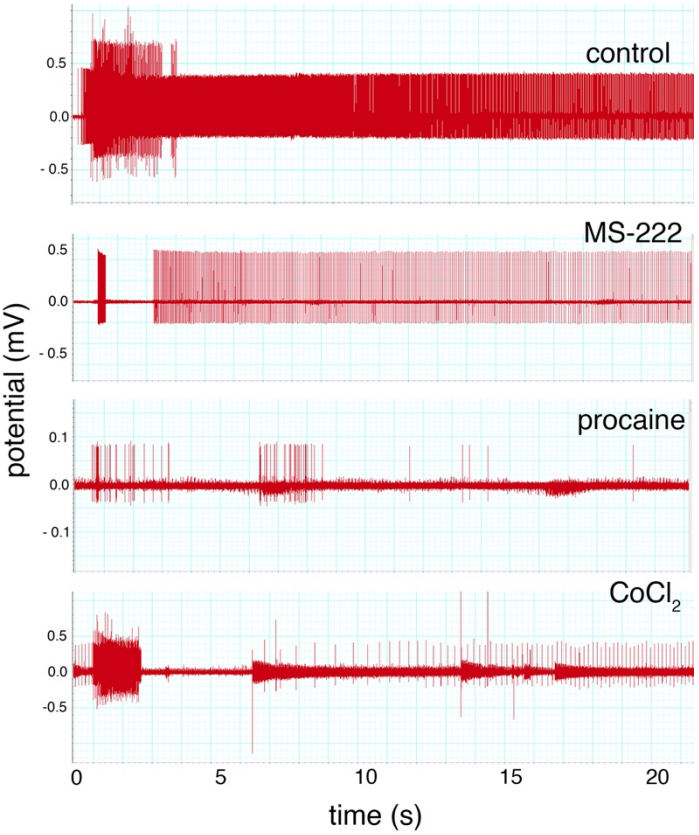

Figure 3. Characteristic nerve responses by treatment. Panels show the typical firing patterns recorded from the stretch receptor preparation when treated with MS-222, $CoCl_2$, and procaine in response to 90° tail flexion at approximately time 0. Each panel displays ~20 seconds of recording.

was observed across 3 trials. The lack of calcium clearly had an effect on adaptation in the tonic receptor, as well as overall firing rates. One possibility is that calcium entry contributes to the depolarization that triggers actionpotentials. When we attempted to apply MS-222 in the cobalt containing crayfish saline, we were unable to obtain any responses.

When preparations were treated with a 0.05% procaine solution, a rapid inhibitory response was observed. Instead of the gap observed after MS-222 treatment, a dramatic decrease in the maximum action potential rate was observed, as seen in the burst pattern shown in Figure 3. The average maximum firing rate decreased to 54.5 Hz under the effects of this anesthetic, which was significantly lower ($p<0.03$) than the control (Figure 4) ($n=4$, $sd=10.0$). Nerve activity ceased an average of 12 minutes following procaine application ($n=5$, $sd=4.4$). The stretch receptor responses did not return in any of the five trials, even after prolonged rinses with regular crayfish saline.

We determined the average firing rate at 0, 10, and 20 seconds following tail flexion for the control, MS-222, and procaine treatments. To determine whether the treatment had an effect on average firing rate at each time point, we used a one-way ANOVA. This demonstrated a statistically significant effect of treatment on firing rate at all three times. Therefore, we used a Tukey-Kramer HSD test to determine significant differences between treatment pairs at each time interval. The averages for each condition, as well as their significance, are shown in Figure 4. These comparisons confirm the general observations we made while recording from the SRO. Namely, firing frequency of MS-222 treated preparations was similar to control at the initial response, but declined significantly over time. Procaine treatment significantly suppressed firing frequency at each time point of the trial.

To demonstrate typical changes in tonic firing patterns, the maximum sustained firing rate in each 0.5 second interval of the 20 second tail flexion was measured for selected responses. These data were plotted as a function of time for control, MS-222, procaine, and $CoCl_2$ trials. From these graphs, we were able to observe the different firing patterns in each experimental group. A typical response for each experimental condition compared to the control is shown in Figure 5 below.

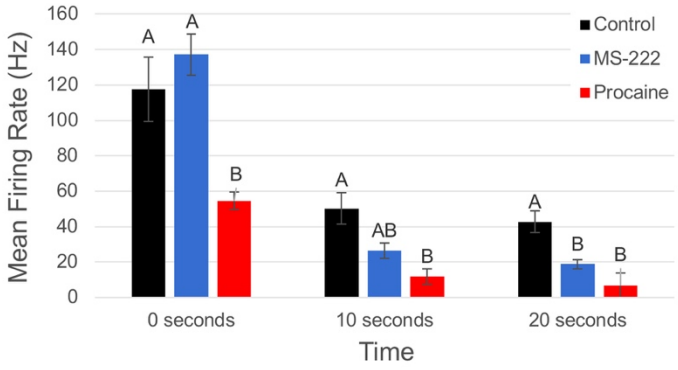

Figure 4. Mean firing rate at 0, 10, and 20 seconds of tonic stretch receptor response. Bars show the average firing rate for control ($n=5$), MS-222 ($n=4$), and procaine ($n=4$) responses at three different time intervals +/- standard error. Means with the same letter at each time point are not significantly different from one another as determined with a Tukey-Kramer HSD test.

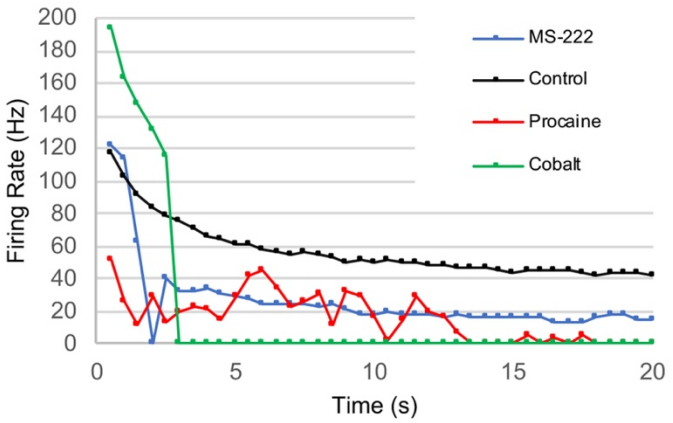

Figure 5. Characteristic firing pattern of tonic stretch receptor for each condition. One example of a common response from control, MS-222, procaine, and $CoCl_2$ treatments is shown over the 20 second period recorded.

Excluding frequent gaps in MS-222 firing, the tonic portions of both MS-222 and $CoCl_2$ treated nerves fit power decay curves similar to the control. However, procaine resulted in intermittent bursts of action potentials which did not match any decay curve. To determine if MS-222 tonic responses followed a different power decay curve than the controls, responses from each condition were graphed on a log-log plot. With this transformation, each power decay curve was represented as a negative linear plot, with a slope equal to the rate of decay. Linear representations of the average response for each treatment are shown in Figure 6.

Once in a logarithmic form, the slope of firing rate of the MS-222 treated preparation was tested relative to the control. According to an ANCOVA slope comparison, the slope was significantly different from the tonic control ($p<0.0001$; $df=1$; $F=39.0$). This indicates that MS-222 alters the rate of decay for the power curve of the tonic stretch receptor, demonstrating that MS-222 treatment does inhibit the rate of action potential firing. The decay rates from the procaine and $CoCl_2$ treated preparations were quite erratic and therefore not included in this analysis.

DISCUSSION

The crayfish stretch receptor preparation used for this experiment is relatively simple, yet robust. The initial dissection is simple enough for students to learn and the desired nerve is repeated in each segment of the abdomen, allowing for multiple attempts (Leksrisawat et al., 2010). However, beyond just demonstration, the treatments used in this study provide students an opportunity to experiment with stretch receptors and explore the various effects of local anesthetics. Local anesthetics are inexpensive, and a variety of different compounds are easily obtained from common chemical suppliers. While complex neuropharmacology underlies the differential reactions of these anesthetics, their results on the firing of stretch receptors are easily observable. Experiencing how neurons

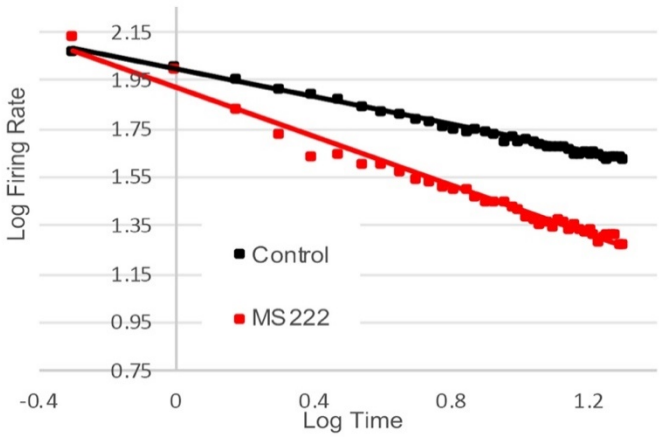

Figure 6. Log-log plot of firing rate decay as a function of time. This transformation demonstrates the linear view of power decay functions for tonic responses from control and MS-222 treated preparations. Control: y = -0.2897x + 2.0004 (R^2 = 0.99). MS-222: y = -0.5016x + 1.9186 (R^2 = 0.98). The rate of decay (slope) was significantly greater in the MS-222 treated preparation ($p < 0.0001$).

may be disrupted by anesthetics is important in developing a strong understanding of nervous system function. As such, recording the inhibitory responses of these local anesthetics will deepen understanding of action potentials and the voltage gated sodium channels responsible for them.

Helping students with the transition from qualitative to quantitative data analysis can be a particular challenge in physiology labs. The decline in action potential frequency can be discerned from collection of data without quantifying the frequencies (Figures 1 and 3). Students often want to stop
at this descriptive level, but working through quantitative analyses may reveal patterns that would otherwise be missed. For example, the power decay curves for tonic stretch receptors can be plotted as shown in Figure 2. Plotting the data in this way demonstrates that the phasic SRO fires at a higher frequency initially, but then declines more rapidly than the tonic SRO. The adaptation mechanism of the slowly adapting neuron is an excellent example of quantitative modeling of neural activity – a goal which researchers are still investigating in more complex nervous structures. Firing rates from anesthetized SROs can also be compared with controls and analyzed with statistics (Figure 4) and log-transformed data reveal that decay rates have different slopes (Figure 6). These examples should provide students with ideas about how they might analyze their own recorded data. Applying different anesthetics across a range of concentrations could offer additional comparisons.

Anesthetics

All local anesthetics used in this experiment are known to inhibit neural activity by blocking voltage gated sodium channels (Fozzard et al., 2011, Attili & Hughes, 2014). Although they share this overarching mode of action, these anesthetics can act through subtly different mechanisms. The effectiveness of different local anesthetics, their speed of action, and specific effects on neural activity vary as a function of their biochemical properties. These differences can be clearly observed through the differing time course and unique firing patterns resulting from the application of MS-222 and procaine (Figures 3-5).

Local anesthetics act on voltage gated sodium channels following diffusion through the lipid bilayer (Lin & Rydqvist, 1999b). Therefore, examining their biochemistry is extremely important in understanding the effects of these anesthetics. Hydrophobicity is a key component of effectiveness, since local anesthetics must enter through the cell membrane before blocking sodium channels (Butterworth & Strichartz, 1990). When using local anesthetics in aqueous forms, there is a tradeoff between effectiveness and solubility in water. For example, benzocaine is very hydrophobic and therefore readily taken up by the cell membrane, but it is also quite difficult to dissolve in water (Ávila & Martínez, 2002).

Although most local anesthetics share basic structural characteristics, MS-222 and procaine still have important biochemical differences. MS-222 contains the ionic (+1) form of tricaine and is therefore relatively hydrophilic, as seen in Figure 7. In contrast, procaine has a typical anesthetic structure and is more hydrophobic than MS-222. This has been quantified through topological polar surface area, which is much greater in MS-222 than procaine (National Center for Biotechnology Information, https://pubchem.ncbi.nlm.nih.gov/compound/). This matches our stretch receptor results, where the more hydrophobic local anesthetic (procaine) had a stronger and more rapid inhibitory effect than MS-222. Additionally, stretch receptor preparations treated with procaine never resumed neural activity, in contrast to the rapid recovery shown following MS-222 treatment. Therefore, the more hydrophobic anesthetic had a more potent effect on the stretch receptors at equal concentrations. The reversibility of MS-222 may make it a better choice for experimental use with the SRO.

The specific pattern of disruption of the firing of receptor neurons also varied across anesthetics. MS-222 often elicited a distinctive gap in firing rate, as seen in one of the example responses in Figure 3. Since the pattern was distinctly different from any procaine results, this suggests some level of diversity in mechanism across local anesthetics. Further research into the specific mechanisms

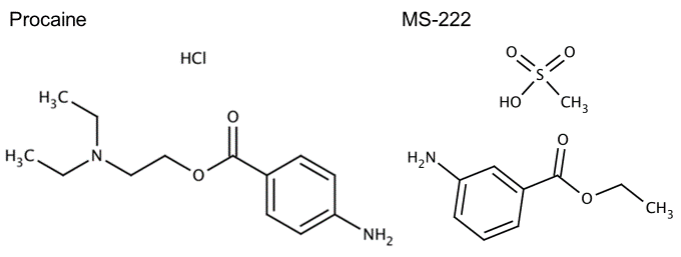

Figure 7. Molecular structures of procaine and MS-222. Procaine's longer hydrocarbon chains make it more hydrophobic than MS-222. Images from Discovery Fine Chemicals (https://discofinechem.com/).

of action for MS-222 and procaine could provide insight into these varying effects. The more polar nature of MS-222 indicates that structural differences could be responsible for its altered action on stretch receptors.

Contrary to our results on the stretch receptor preparation, application of the same local anesthetics did not have an effect on live crayfish. The limited effects with MS-222 immersion have previously been reported for crayfish (Obradovic, 1986; Brown et al., 1996). These results are quite different from their strong anesthetic effects on fish and amphibians, where MS-222 in particular is often used (Downes, 1995; Mitchell, 2009). A possible explanation could be the physiological differences between invertebrates and vertebrates, resulting in differential anesthetic action. For example, frog nerve and muscle preparations are highly sensitive to MS-222 (Medler, 2019). We routinely observe that immersion in 0.1% MS-222 completely anesthetizes frogs in approximately 5 – 10 minutes. Concentration is likely not the problem, since a previous study on crayfish determined that bath anesthesia in MS-222 at concentrations up to 1.0 g/L were not sufficient to induce anesthesia (Brown et al., 1996).

The lack of effect from immersion in an anesthetic bath could stem from limited absorption across the exoskeleton and cuticle of these crustaceans. If the local anesthetics were not properly absorbed by the crayfish in bath form, our results may not indicate that these anesthetics are ineffective. However, our injection of anesthetics into the blood showed a similar lack of anesthetic effect. Therefore, it is possible that the effects of MS-222 and procaine on stretch receptors are specific and not widespread across the crayfish nervous system. The study by Brown et al. (1996) indicated that lidocaine needed to be injected intramuscularly at a minimum concentration of 300 µg/g body mass to have an effect. Overall, the effects of chemical anesthetics on crustaceans have not been carefully studied.

There has been growing attention given to the welfare of aquatic animals, including crustaceans (Weineck et al., 2018; Browman et al., 2019; Diggles, 2019). A dilemma for researchers is how to effectively anesthetize animals in a humane way, without disrupting the physiological signals of interest. The variability in the effects of whole animal immersion versus direct application to neuronal tissues in the current paper illustrates some of the challenges encountered with using chemical anesthetics. There has recently been attention focused on the idea that hypothermia can be an effective means of anesthesia for amphibians and reptiles (Lillywhite et al., 2016). Immersion in ice has been the standard method of anesthesia for crustaceans and still seems to provide the simplest and most effective approach for anesthesia and euthanasia.

Decay Curves

The firing rates of both tonic and phasic control responses to mechanical stimulation were fit by separate power decay curves (Figure 2). This is consistent with previous research which found that current and firing rate of the slowly and rapidly adapting neurons decayed at different rates (Nakajima & Onodera, 1969). On a single channel level, the open probabilities of the tonic mechanoreceptor can be described mathematically using a Boltzmann equation (Swerup & Rydqvist, 1992). While this single channel modeling has been directly related to current, its direct connection to firing rate is less clear.

Although both anesthetics had significant inhibitory effects 20 seconds into tail flexion, the MS-222 treated nerves displayed a similar tonic power decay curve to the control responses. This was not true for procaine, since it caused unpredictable bursts of action potentials. However, the rate of decay was significantly increased in MS-222 trials compared to controls (Figures 4-6). Therefore, although the maximum firing rates for both conditions began at similar levels, the MS-222 treated tonic response decayed more rapidly. Although MS-222 was much slower acting than procaine, both local anesthetics eventually inhibited the stretch receptor neurons.

Quantifying the firing rates of the tonic and phasic stretch receptors clearly demonstrated the differences in adaptation between the two mechanoreceptors (Figure 2). These differences can be attributed to the combined action of multiple factors, including the physical distribution of voltage gated sodium channels, the voltage thresholds of these channels, and the viscoelastic properties of mechanical transduction (Krnjevíc & van Gelder, 1961; Lin & Rydqvist, 1999a). As a result of these factors, the rapidly and slowly adapting neurons have distinct signals which transduce the phasic and tonic aspects of muscle stretch, respectively.

To remove extracellular calcium from the stretch receptor preparation, the calcium chloride in the crayfish saline was replaced with cobalt chloride. Both types of receptor initially fired for a similar duration following this treatment, but then were greatly reduced in their response (Figures 3 and 5). Rydqvist (2007) previously suggested that Ca^{2+}-activated potassium channels may play a role in the process of slow adaptation, which could explain the observed results. The initial increased firing rates in the low calcium experiments (Figures 3 and 5) may seem counterintuitive, but are consistent with well know effects of hypocalcemia on the nervous system (Han et al., 2015). The precise mechanisms leading to this increased activity are not fully understood, but are discussed in detail in the review by Han et al. (2015).

CONCLUSIONS

Our experimental results clearly show the effects of local anesthetics on crayfish stretch receptor preparations. While both anesthetics had inhibitory effects on the firing of the receptor neurons, their specific effects on stretch receptor responses were distinct. MS-222 increased the tonic decay rate, sometimes producing distinctive gaps in firing. In contrast, procaine caused erratic bursts of neural activity in response to tail flexion, which were significantly inhibited compared to control responses. These differential effects may be attributed to differences in structure which affect these anesthetics' neuropharmacological properties. Additionally, the substitution of cobalt chloride caused hyperexcitability and rapid tonic adaptation. This result could potentially demonstrate the importance of calcium currents in slow adaptation, or simply the effect that low calcium levels have on sodium channels.

The differential effects of MS-222 and procaine on the SRO preparation demonstrate that local anesthetics can be useful tools for student research experiments. One important characteristic of CUREs and project-based student learning is to build upon and extend knowledge reported by other students or scientists. Experimentation with a variety of local anesthetics provides one mechanism for students to expand upon and refine what we know about the crayfish SRO. Additionally, the application of quantitative analyses to observational data gives students opportunities to develop skills used by practicing scientists.

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Received February 19, 2020; revised May 22, 2020; accepted May 27, 2020.

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