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RESEARCH ARTICLE

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The effects of potassium and muscle homogenate on proprioceptive responses in crayfish and crab

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Many of the authors were students in a neurophysiology lab-based class who addressed authentic scientific-based questions in regards to the topic of examining how extra- and intracellular pH would influence proprioception. This course project is part of a new trend in teaching science to undergraduates (Linn, Palmer, Baranger, Gerard, & Stone, 2015). Course-based undergraduate research experiences (CUREs) are relatively new and an approach being adopted by science educators in high schools and colleges(Bakshi, Patrick, & Wischusen, 2016).

Abstract

Proprioception of limbs and joints is a basic sensory function throughout most of the animal kingdom. It is important to understand how proprioceptive organs and the associated sensory neurons function with altered environments such as increased potassium ion concentrations ($[K^+]$) from diseased states, ionic imbalances, and damaged tissues. These factors can drastically alter neuronal activity. To assess this matter, we used the chordotonal organ in a walking leg of a blue crab (*Callinectes sapidus*) and the muscle receptor organ of the crayfish (*Procambarus clarkii*). These organs serve as tractable models for the analysis of proprioception. The preparations can help serve as translational models for these effects, which may be observed in other invertebrate species as well as mammalian species (including humans). When extracellular potassium concentration ($[K^+]_o$) is increased to 20 mM in both preparations, mixed results are observed with activity increasing in some preparations and decreasing in others after mechanical displacement. However, when $[K^+]_o$ is increased to 40 mM, activity drastically decreases in all preparations. Additionally, proprioceptor sensory activity declines upon exposure to a diluted muscle homogenate, which contains a host of intracellular constituents. The robust effects of altered $[K^+]$ on proprioception in these models illuminate the potential detriments on neuronal function in cases of severe tissue damage as well as altered $[K^+]_o$.

1 | INTRODUCTION

The treatment of tissue injury by health care providers is complex depending on the type of injury, tissue type, and location. Treatment and care for healing goes beyond focusing on the injured site itself since other body systems and healthy tissue can be indirectly affected (Brancaccio, Lippi, & Maffulli, 2010; Cintra-Francischinelli et al., 2010). This is particularly an issue with large amounts of tissue injury due to the spillage of intracellular constituents into extracellular fluid (ECF) and entrance into the blood stream or into the hemolymph in the case of invertebrates. Compartmentation of dense tissue can reduce the effect to the rest of the body but may have an increased effect on the neighboring cells within the compartment. The acute and long-term effects on healthy tissue, which is exposed to cellular debris, are varied. The initial tramatome can have a mild to large effect on surrounding tissue depending on the amount of tissue initially damaged, degree of compartmentation, amount of ionic spillage, carbon dioxide (CO_2) accumulation, and resultant

alterations in pH (Astrup, Symon, Branston, & Lassen, 1977; Dreier et al., 2017).

Much of the focus on the acute effects of damaged tissue on healthy cells is the rapid depolarization of surrounding cells as a result of the rise in extracellular [K^+]. In addition high proteinemia is a consideration. The associated depolarization of most cells with varied extracellular $[K^+]_o$ is due to the delicate balance in the permeability of sodium ions (Na^+) and potassium ions (K^+) across the membrane at rest (Bernstein, 1902) and the activity of the Na^+/K^+ pump (Skou, 1965,1998). Feng, in the 1930s, recognized the effects of $[K^+]_o$ on sensory neurons and the consequences of raised $[K^+]_o$ has been a key factor to focus on for the systemic and direct effects on non-damaged cells following an injury. The uncontrolled excitation of cardiac and skeletal muscle as well as neurons can result in rapid death of an animal. However, even under physiological conditions with heightened electrical activity and efflux of K^+, depolarization in the surrounding cells can result from small changes in $[K^+]_o$ (Astrup et al., 1977; Baylor & Nicholls, 1969; Frankenhaeuser & Hodgkin, 1956; Orkand, Nicholls, & Kuffler, 1966).

Since muscle and neurons have a relatively high K^+ permeability (through leak channels to K^+), an increase in extracellular $[K^+]_o$ will depolarize the membrane. A slow depolarization can lead to an increasing number of voltage gated Na^+ channels opening and then inactivating, thus raising the threshold needed to initiate an action potential. If action potentials are initiated along with the $[K^+]_o$ maintaining a depolarized state, then the cells cannot repolarize and the voltage gated Na^+ channels will remain inactivated (Hodgkin & Huxley, 1952). The electrogenic Na^+/K^+ pump is more active in a depolarized state and would try to regain homeostatic ionic regulation of cells. Neurons with voltage gated Ca^{2+} channels, which are opened by the maintained depolarization, can be sensitive to the loading of Ca^{2+} ions and trigger cellular processes leading to cellular damage and cell death (Kuo, Siddique, Fu, & Heckman, 2005). A maintained depolarized cell tends to alter membrane properties and causes the membrane to become leakier and then irreversible damage to the cell occurs due to osmotic shock as well as to organelles within the cell which can release toxic substances (Kristensen, 1994). The K^+ and enzymes as well as spillage of other proteins from damaged cells can impact neighboring cells that can enlarge the tramatome. The associated cells, such as muscle spindles, Golgi tendon organs, pain endings, or neurons within a nerve next to or within the fascia of damaged skeletal muscle can be affected. If systemic level of K^+ and protein rise, this can affect tissues throughout an entire animal.

In addition to K^+ efflux, other intracellular constituents (i.e., amino acids and enzymes), as well as substances contained within intracellular organelles, can also promote more indirect tissue damage from the initial injury. Depending on the tissue in question, particular constituents within the cytoplasm of cells will have different effects. For example, the amino acid glutamate can bind to glutamate receptors on synaptic sites within the central nervous system (CNS) and result in glutamate induced toxicity. If an injury was in the vicinity of the brain or spinal cord, or even more in a more distant location, the glutamate surge can be transported from the blood to the CNS (Abdel-Salam, 2014; Camien, Sarlet, Duchateau, & Florkin, 1951; Simpson, Allen, & Awapara, 1959). Free glutamate can also rise in the blood from a substantial amount of skeletal muscle damage. Thus, glutamate can travel to distant sites, including the CNS, to cause alterations in physiological function.

The ability for an animal to have coordinated locomotion is in part due to the sensory feedback from proprioceptive neurons. In mammals, muscle spindles (i.e., intrafusal muscles), which are embedded within the much larger extrafusal muscle fibers, provide limb proprioception. Thus, one would predict that damage to a subset of extrafusal muscle fibers within a muscle would alter the function of the healthy neighboring muscle fibers and associated sensory neurons monitoring the muscle spindles. The muscle receptor organ (MRO) is analogous to the mammalian muscle spindle and is found within the crayfish abdomen (Kuffler, 1954; Rydqvist, Lin, Sand, & Swerup, 2007). We utilized the model crayfish MRO to examine both the effects of raised $[K^+]_o$ and a saline mixed with a homogenate of crayfish skeletal muscle on the function of the MRO. The sensory endings are embedded within the thick skeletal muscle fibres of the MRO, which are neurally innervated.

For comparative purposes, we also investigated the effects of raised $[K^+]_o$ and a crab muscle homogenate on the joint proprioceptor in the crab walking leg. The joint proprioceptors in the limbs of crustaceans are similar to those in all arthropods. These joint receptors are a type of mechanoreceptor with sensory endings embedded within chordotonal organs (COs). The COs are composed of an elastic strand, which monitors the joint movements. The sensory endings of neurons monitoring this movement are embedded within the elastic strand. The neurons detect the direction and rate of joint movement as well as static positions of the joint (Bush, 1965; Cooper, 2008; Cooper & Hartman, 1999; Wiersma, 1959). Alexandrowicz (1967) named the COs by the joint they are monitoring (i.e., PD is a CO between the propodus and dactylus). Alexandrowicz (1958, 1967, 1972) described the gross anatomy of the limb proprioceptive organs in the limbs of a variety of crustaceans and Whitear (1962, 1965) as well as others (Lowe, Mill, & Knapp, 1973; Mill, 1976; Mill & Lowe, 1973) described the fine structure of the COs. In this study, the PD organ in the walking leg of a blue crab (*Callinectes sapidus*) was used to measure functional changes, over a range of movements, for altered levels of $[K^+]_o$ and the influence of a homogenate of skeletal muscle from the same species of crab. The PD organ was chosen for use in these experiments as it is one of the better described COs in Crustacea (Burke, 1954; Cooper, 2008; Cooper & Govind, 1991; Cooper & Hartman, 1999; Hartman & Boettiger, 1967; Hartman & Cooper, 1994). The PD organ preparation is devoid of muscle directly associated with the sensory ending. Thus, changing the bathing environment can assess the direct actions on the activity of the PD organ.

The contribution of this study is that it serves as a model for teaching purposes as well as fundamental research in the influence of raised $[K^+]_o$. It also relates the effects of a known $[K^+]_o$ to that of a diluted muscle homogenate for drawing parallels to conditions, which arise for other animals with tissue injury. The use of muscle homogenate serves to provide analyses on the role of intracellular constituents on sensory nerve function in addition to K^+ alone. The effect of raising $[K^+]_o$ on the resting membrane potential for teaching purposes is commonly demonstrated in a classic student physiology laboratory

exercise (Atwood & Parnas, 1968; Baierlein, Thurow, Atwood, & Cooper, 2011). However, the novelty of this study is addressing the effects of raising $[K^+]_o$ and cellular homogenate on the function of proprioceptors in two organisms that serve as basic models in addressing neurobiological principles.

2 | METHODS

2.1 | Crab

Blue crabs (*C. sapidus*) were obtained from a local supermarket in Lexington, KY, which were delivered from a distribution center in Atlanta, GA. They were bought and maintained in a seawater aquarium for several days prior to use in order to assess their health. The crabs were adults and in the range of 10–15 cm in carapace width (from point to point). All crabs used were alive and were very active upon autotomizing a leg for experimentation. While holding the crab with a net or large tongs across the carapace from behind (avoiding the claws) a pinch across the merus of the walking leg with a pair of pliers would induce the leg to be autotomized. The leg was then placed in the Sylgard-lined dissecting dish and covered with crab saline at room temperature (21°C).

The CO in the propodite-dactylopodite joint (PD) of the first or second walking legs of the crab was used (Figure 1A). The details of the dissection and procedures were described in video and text by Majeed, Titlow, Hartman, and Cooper (2013). In brief, the first or second walking leg is induced to autotomize. The leg was then cut between the propus and carpus segments. A patch of cuticle on both sides was removed with a scalpel. The joint condyle was left intact. The preparation was pinned into a Sylgard-lined dissecting dish with fresh saline throughout the dissection so that the neurons stay alive. The opener tendon from its attachment at the distal end was cut and the opener muscle and tendon was removed. PD organ was identified and then the PD nerve was carefully separated from the main leg nerve with glass needles. After exposing the PD nerve and pulling the nerve into a suction electrode for recording the nerve activity, the dactyl was moved throughout the extended and flexed positions for several cycles with the aid of a wooden probe to ensure the nerve was not pulling on the chordotonal strand. A length of the nerve was left out of the suction electrode to provide slack. The experimental conditions consisted of moving the dactyl from a flexed 90° angle from the propus to a full 0° in an extended position (or open position) and then released. When the dactyl was released the joint would obtain a partial flexed position. Prior to the next displacement, the joint was flexed to the same starting position. The rates of movements from a 90° angle to a 0° angle were performed within 0.5 s (rate of 180°/s) and 4 s (rate of 22.5°/s) with 5 s between displacements. In other studies with crab (*Cancer magister*) COs in more proximal joints, reproducibility in repetitive movements at 1 Hz produced consistent firing rates as indexed by an eta^2 value (Cooper & Hartman, 1999). Thus, we assumed a 5-s interval to be sufficient to avoid any habituation for the PD neuron in blue crab (*C. sapidus*) but this was not explicitly examined. The analysis consisted of counting the number of spikes of the nerve within the periods of displacement. The joint was also extended in 1 s (rate of 90°/s) and held in the extended position for another 9 s to assess static responses of the neurons in an extended joint. The physical movements performed are described below in the section "*To ensure reproducibility in experimentation.*"

2.2 | Crayfish

Crayfish (*Procambarus clarkii*), measuring 6–10 cm in body length, were used throughout this study (Atchafalaya Biological Supply Co., Raceland, LA). They were housed individually in indoor tanks. The details of the dissection and procedures are described in video and text (Leksrisawat, Cooper, Gilberts, & Cooper, 2010). In brief, the crayfish was beheaded and the abdomen was then cut away from the thorax. With scissors the abdomen was cut along the lateral border on both sides to the telson. The muscles and gastrointestinal tract were pushed away from the dorsal side of the preparation as not to damage the muscles of interest. The deep extensor medial (DEM) muscle can be located by its fibers twisted in a helix, and the deep extensor lateral muscles, with linear fibers can be distinguished (see Leksrisawat et al., 2010). The preparation was then splint down the dorsal midline and the two halves were placed in a Sylgard-lined dissecting dish with fresh saline. The preparation was pinned so the muscle is not taut and the joints were able to be moved. The MRO nerve to either abdominal segment 2 or 3 was used in this study. The displacements used were from a relaxed position (similar to an extended abdomen in the intact animal) to a stretched position (similar to a flexed abdomen in the intact animal). The displacement rates were 0.5 and 4 s. In addition, a 1 s stretch and hold was used to obtain the static position sensitive response. The same electrode and signal recording technique was used as for the crab PD. The physical movements performed are described below in the section "*To insure reproducibility in experimentation.*"

2.3 | Saline and pharmacology

The salines used were the normal salines described previously (Leksrisawat et al., 2010; Majeed et al., 2013) with slight exceptions in the use of varied $[K^+]_o$ and saline containing homogenized skeletal muscle. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2 \cdot 2H_2O$, 2.45 $MgCl_2 \cdot 6H_2O$, and 5 HEPES adjusted to pH 7.4). All bathing and experimental solutions were kept at the experimental room temperature of 21°C. All chemical compounds were obtained from Sigma (St. Louis, MO). Skeletal muscle was diluted with the species-specific saline with one part muscle to three parts saline by volume. The muscle was then homogenized and let to settle for 5–10 min before using. The supernatant of the homogenized skeletal muscle was from the same species as the proprioceptors examined. For the crayfish, skeletal muscle was taken from the claws as well as the abdomen. For the crab, muscles were taken from both claws, which consisted mostly of closer muscle.

The procedure used for the various bathing environments was to first obtain recordings in normal saline, then replace the bathing medium with three exchanges from the recording dish with 20 mM $[K^+]$ and let the saline stand for 2 min before recording the neural activity to displacements. The media was then replaced to one containing

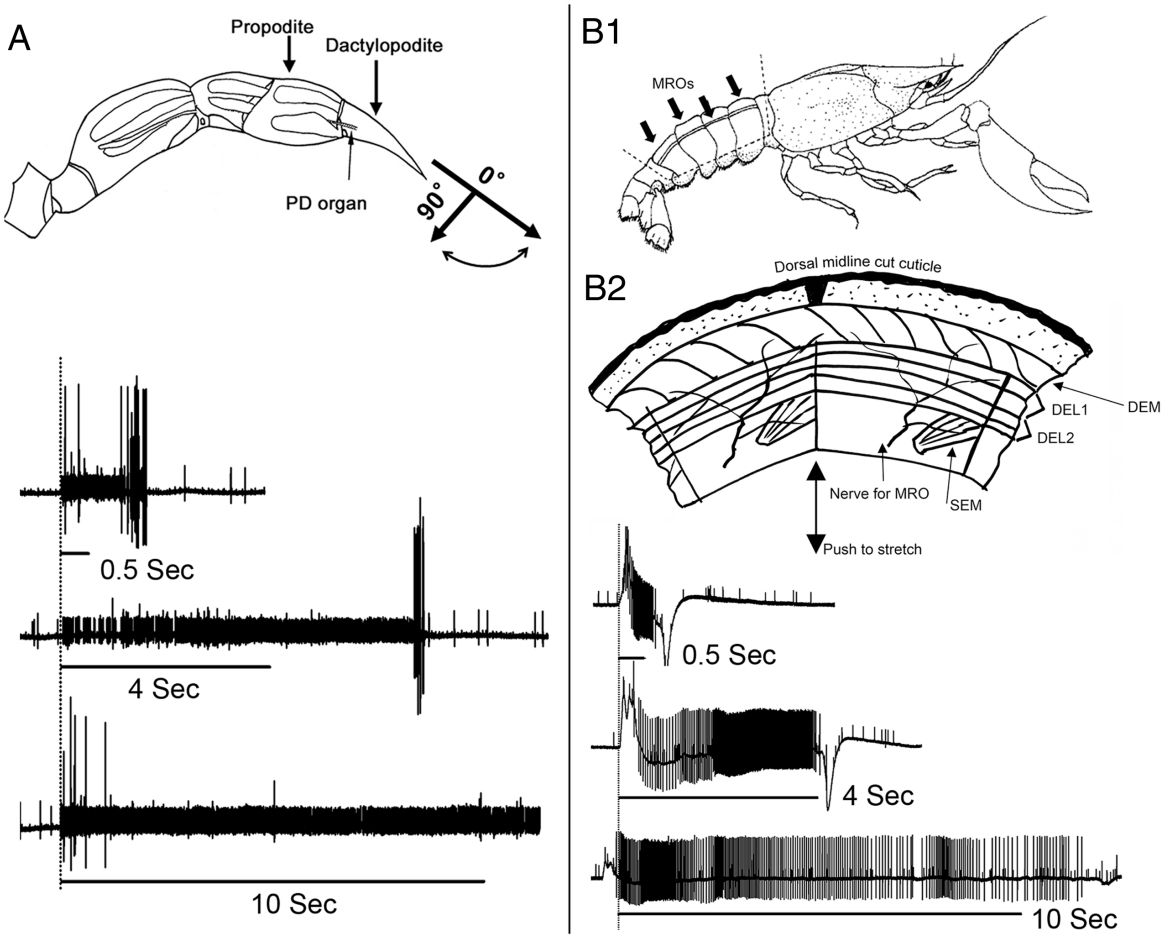

FIGURE 1 Anatomical arrangement of the displacements is used for the PD organ of the crab walking leg (A) and the MRO of the crayfish abdomen (B). Either a stop pin or an anatomical position is used for consistency in the displacements. Rates of displacement for the crab joint are 0.5 and 4 s from 90° to full extension (0°) and then held for a short time before returning the joint to 90°. The joint was also rapidly stretched and held for 10 s. (B1) The MROs are located on the dorsal aspect of the abdomen. Movements for the MRO consist of bending a joint in the hemi-longitudinal segment of the abdomen to a set location at a rate of 0.5 s or 4 s and then held for a short time before returning to starting position. The joint was also rapidly stretched and held for 10 s. (B2) Two abdominal segments are illustrated. A schematic view of the deep extensor muscles (looking from ventral to dorsal) is provided (see Sohn, Mykles, & Cooper, 2000). The crab limb is shown from the side with the position of the tendons and PD organ as they would be in transparency (A1; Majeed et al., 2013; Whitear, 1960). The particular muscles identified are: deep extensor medial (DEM) muscles, which have a spiral fiber pattern; DEL1, which is the first lateral group followed by the DEL2 muscles; the superficial extensor medial muscle (SEM), which lies directly dorsal to DEL2, and the two MRO muscles, which are more dorsal to the DEL1. The joint between the abdominal segments would be displaced at various rates to a set position while recording from the MRO nerve (the double arrow indicates where the joint between segments is located). Typical firing activity of the nerves is shown for a PD and a MRO preparation at each of the displacement rates

40 mM [K^+] with three more bath exchanges and a 2 min waiting period before recording. The supernatant of the homogenized skeletal muscle was performed on fresh preparations, which were only exposed prior to normal saline. The normal saline was removed and the diluted supernatant of the homogenized skeletal muscle was introduced to the bath by gently swishing it around in the bath to ensure exposure to the PD or the MRO tissue. Afterwards the bathing media was replaced with three to five exchanges of normal saline and the displacements recorded again.

2.4 | Electrophysiology

Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracelluar signals from the cut nerves (details of making the suction electrodes is provided in Baierlein et al., 2011). A P-15 amplifier (Grass Instruments, Astro-Med, West Warwick, RI) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO) obtained the signals to be recorded on a computer at a 10 or 20 kHz sampling rate.

2.5 | Statistical analysis

All data are expressed as a mean (±SEM). The rank sum pairwise test was used to compare the difference of frequency of neural activity after exchanging solution with saline containing each varied [K^+] or saline with homogenized muscle. This analysis was performed with Sigma Stat software. P of ≤ 0.05 was considered statistically significant.

2.6 | Experimental paradigm for displacements

The crab PD organ was used to model the effects on neurons directly since the skeletal muscle associated with the organ was removed (Figure 1A). The neuronal sensory endings are embedded within the elastic strand to detect the movement of the strand. However, the crayfish MRO is closer to modeling the mammalian muscle spindle as the terminals of motor neurons are still attached to the muscle and any force exerted on the sensory endings will result in inducing activity of the stretch-activated channels (SACs) (Figure 1B). They are within the sensory endings of the PD organ and the MRO and initiate ionic flux and depolarization of the neuron when they are deformed by the mechanical forces placed on them. The neurons within the PD organ and MRO responded differently depending on the rate and direction of movement as well as the static position of the joint. Schematic diagrams of the movements used in this study are shown along with the representative neural activity recorded from the whole nerve (Figure 1). The PD joint was displaced from 90° to 0° at various rates (0.5 and 4.0 s). The same rates of movements were used for the MRO to also provide a fast and slow displacement. However, the anatomical arrangement is different so a direct correlation in firing rates of the neurons cannot be made between the two preparations. The general responses to the same environmental conditions can be compared. The displacement for the MRO was to a set position that mimicked flexion of the abdomen. A static position of flexion (stretching of the MRO) or extension of the PD, which was held for 10 s, was used to index the neural activity and the effects of changing the bathing $[K^+]$ or exposure to skeletal muscle homogenate.

2.7 | To ensure reproducibility in experimentation

The data collected in the classroom with all the students using eight different physiological rigs were preliminary data in order to obtain an idea of what to expect for the different experimental conditions. The students made the recordings and analyzed the data. In addition, all the students contributed to compiling information and content for the manuscripts. For standardizing the rate of the movements and analysis, all the data presented in the manuscript were obtained by two people (one conducting the movements and one marking the files on the computer). One individual (V.D.) analyzed all the data sets so analysis would be consistent. The movements of the joints were performed by the same individual (R.C) for all trials. The movements were made by physically moving the joint and counting out loud: one- Miss (0.5 s), one-Mississippi (1 s), two- Mississippi (2^{nd} s), and so on. We timed the counting on a stopwatch for several trials to be consistent in the speed of counting. Each time a movement was started or stopped, a mark on the file with a tap on the keypad would be recorded. To be sure the static holds were correctly measured, a set time of 10 s were analyzed as indicated by a time stamp on the acquisition software. The velocity throughout the movement was kept as constant as possible by manual movement from the starting position to the end position for the crab PD and the crayfish MRO. Each movement was performed one time and repeated in each of the various adjusted saline conditions.

3 | RESULTS

3.1 | The effect of $[K^+]_o$

Three concentrations of bathing K^+ were examined in relation to the neuronal activity for the displacements of the joints related to the PD and MRO preparations. The normal physiological saline used for the crab preparations was 10.8 mM $[K^+]$. As a result exchanging the bath with 20 and 40 mM $[K^+]$ represented a doubling in concentration for each exposure. Representative responses from a crab PD preparation are shown (Figure 2) for each of the displacements in normal saline at a rate of 0.5 s (Figure 2A1), 4 s (Figure 2B1), and stretch and hold for 10 s (Figure 2C1). After changing the bathing media to a saline containing 20 mM $[K^+]$, the activity generally increased for each displacement (0.5 s, Figure 2A2; 4 s, Figure 2B2; and stretch and hold for 10 s, Figure 2C2). For the 4-s displacement, there was an increase of activity for 5 of the 6 PD preparations, although one preparation only slightly decreased in activity (Figure 7). However, exchanging the media to one containing 40 mM $[K^+]$ the activity was drastically reduced for the same displacements (0.5 s, Figure 2A3; 4 s, Figure 2B3; and stretch and hold for 10 s, Figure 2C3). To ensure the preparations were not permanently damaged from the high $[K^+]$ exposure, the bathing saline was returned to the normal physiological saline with three complete rinses of the saline bath. All six preparations showed some recovery upon replacing the bathing media to normal saline and continued to respond to a range of displacements (Figure 2A4, B4, and C4).

The same experimental paradigm was also conducted for six crayfish MRO preparations; however, the joint in between the abdominal segments to was bent to mimic flexion. The A1, B1, and C1 series of Figure 3 is the activity that occurs with the normal physiological saline for crayfish at $[K^+]$ 5.4 mM. The crayfish saline was adjusted to 20 mM and 40 mM $[K^+]$ and used as bathing media for examining the effects on the MRO activity. At 20 mM $[K^+]$, the crayfish MRO activity varied with some preparations increasing in activity and others decreasing in activity (Figure 3A2, B2, and C2). The exposure of 20 mM $[K^+]$ for the crayfish preparations was almost four times the normal $[K^+]$ in crayfish saline. For the crab, the concentration was only doubled to 20 mM $[K^+]$. The majority of the preparations (four out of six) did decrease the frequency of spiking with the displacements; however, two preparations increased in neural activity with displacements. Similar to the crab, the activity was nearly silenced at 40 mM $[K^+]$ in all six preparations (Figure 3A3, B3, and C3). As for the crab PD, a saline rinse was performed to verify the preparations were still viable. All six preparations responded well in exchanging back to normal saline although the activity did not fully recover (Figure 3A4, B4, and C4).

3.2 | Effects of muscle homogenate

To simulate an authentic situation of a skeletal muscle injury on the effects of joint proprioception, a homogenate of skeletal muscle was applied to the exposed crab PD and crayfish MRO preparations. The species-specific homogenate was used for each preparation. Since it was unlikely that a 100% muscle homogenate would occur in an injury to expose the healthy neighboring tissues, a dilution of the injured cells

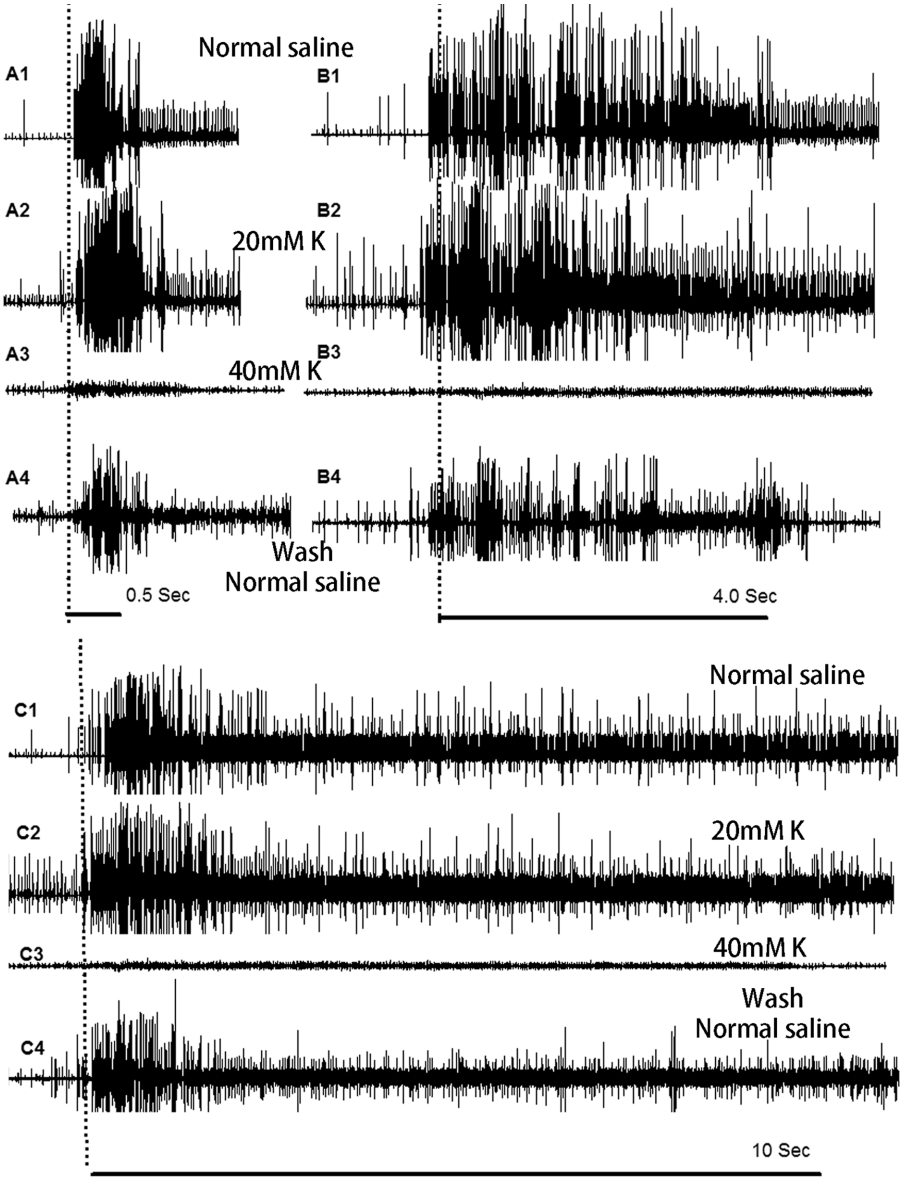

FIGURE 2 Representative traces in neuronal spiking for the different displacement rates and response to varying exposures in [K^+] for the crab PD organ. The 0.5 s displacement is shown in **A**, while the 4 s is shown in **B** and the static held displacement of 10 s shown in **C**. The responses in normal saline (**A1, B1, C1**), during exposure to 20 mM [K^+] (**A2, B2, C2**) and during exposure to 40 mM [K^+] (**A3, B3, C3**) as well as wash out with a return to normal saline (**A4, B4, C4**) are shown. The *y*-axis scale is the same throughout

was modeled with one part muscle homogenate to three parts species-specific saline. Thus, the cellular constituents would be diluted approximately to a 1/4 of the value within the cells. The muscle homogenate solution was made and used immediately on the preparations (less than 2 h for all preparations).

A representative crab PD preparation trace is shown in Figure 4 with exposure to saline, followed by muscle homogenate and then back to normal saline after several rinses in normal saline. The same displacement rates were used for examining the effect of the muscle homogenate as for examining the effects of varied [K^+]. The 0.5 s (Figure 4A series), 4 s (Figure 4B series), and 10 s stretch and hold (Figure 4C series) are shown for normal saline, exposure to muscle homogenate, and after returning to normal saline. Note that the muscle homogenate silenced the PD organ activity except for some very small spikes in the recording. These small spikes might have arisen from the very small axons of the static position sensitive neurons. However, the majority of the signal is completely absent with exposure to muscle homogenate. The muscle homogenate did not damage the preparations within the 5-min exposure as all six preparations returned to higher activity than the muscle homogenate upon rinsing out the muscle homogenate with normal physiological saline.

The crayfish MRO preparation showed a similar trend with the diluted muscle homogenate exposure. The preparations did not become completely silenced in neural activity with the displacements but in all six experiments the activity was drastically reduced (Figure 5). As for the crab PD, rinsing of the preparations four to five times with fresh normal saline returned the activity back to normal levels or to even higher levels of activity for each of the displacements.

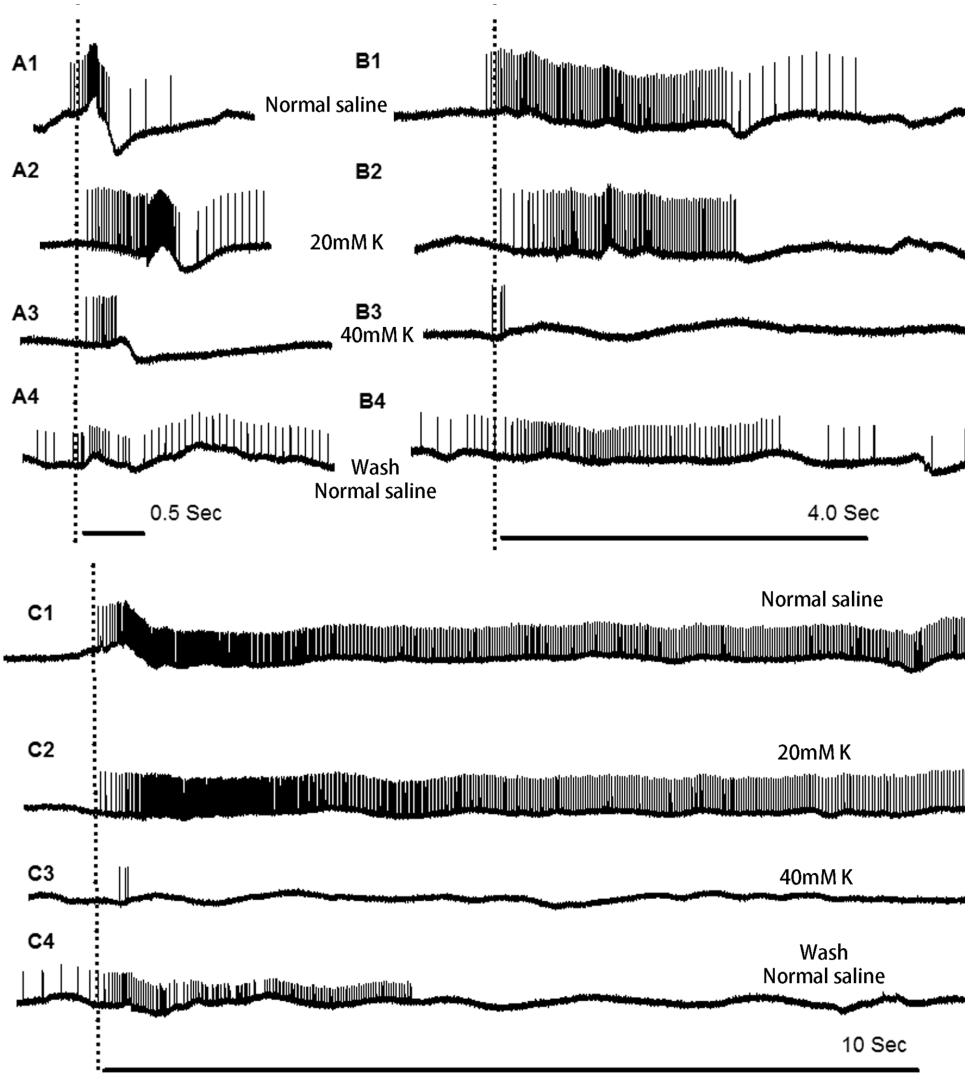

FIGURE 3 Representative traces in neuronal spiking for the different displacement rates and response to varying exposures in [K^+] for the crayfish MRO. The 0.5 s displacement is shown in **A**, while the 4 s is shown in **B** and the static held displacement of 10 s is shown in **C**. The responses in normal saline (**A1, B1, C1**), during exposure to 20 mM [K^+] (**A2, B2, C2**) and during exposure to 40 mM [K^+] (**A3, B3, C3**) as well as wash out with a return to normal saline (**A4, B4, C4**) are shown. The *y*-axis scale is the same throughout

The same displacement rates were used for examining the effects of muscle homogenate as were used for examining the effects of the varied [K^+].

To compare the overall effects of 20 mM [K+], 40 mM [K^+], and muscle homogenate on the activity of the proprioceptors for the crab and crayfish, the average change in percentage from the initial saline exposure was determined and is shown in Figure 6 for each of the displacement rates. Neuronal activity was reduced when both 40 mM [K^+] and muscle homogenate were applied ($N = 6$, $P < 0.05$ non-parametric sign rank sum) for a 0.5 s, 4 s, and 10 s hold displacements for the crab PD organ (Figure 6A). The same is true for the crayfish MRO preparation, because the neuronal activity for the 40 mM [K^+] exposure as well as muscle homogenate revealed a statistically significant reduction in activity ($N = 6$, $P < 0.05$, non-parametric rank sum) for the 0.5 s, 4 s, and 10 s hold displacements (Figure 6B). The activity profile for the 20 mM [K^+] exposure produced the greatest variability among the other exposure conditions. The same amount of time was provided for the experimental exposure; however, the activity increased in some preparations while it decreased in others. To better illustrate the changes in activity for the 20 mM [K^+] exposure the number of spikes counted within each displacement paradigm for saline and the 20 mM [K^+] exposure is shown in Figure 7. This was determined for the crab PD (Figure 7A) as well as for the crayfish MRO (Figure 7B).

The change in activity profiles was not consistent for all the preparations or for the different displacements. Only one out of six crab PD decreased in activity for the 0.5 s displacement. In addition, only one out of six for the four second displacement as well as for the 10 s displacement decreased in activity (Figure 7A). The pattern for the crayfish MRO was more erratic, as four out of the six preparations decreased for each displacement rate (Figure 7B). All of the 40 mM [K^+] exposures and muscle homogenates showed the same trends in decreasing activity after 2–5 min of exposure for both the crab PD organ and the crayfish MRO. However, the MRO preparation showed an initial difference upon exchanging saline for muscle homogenate,

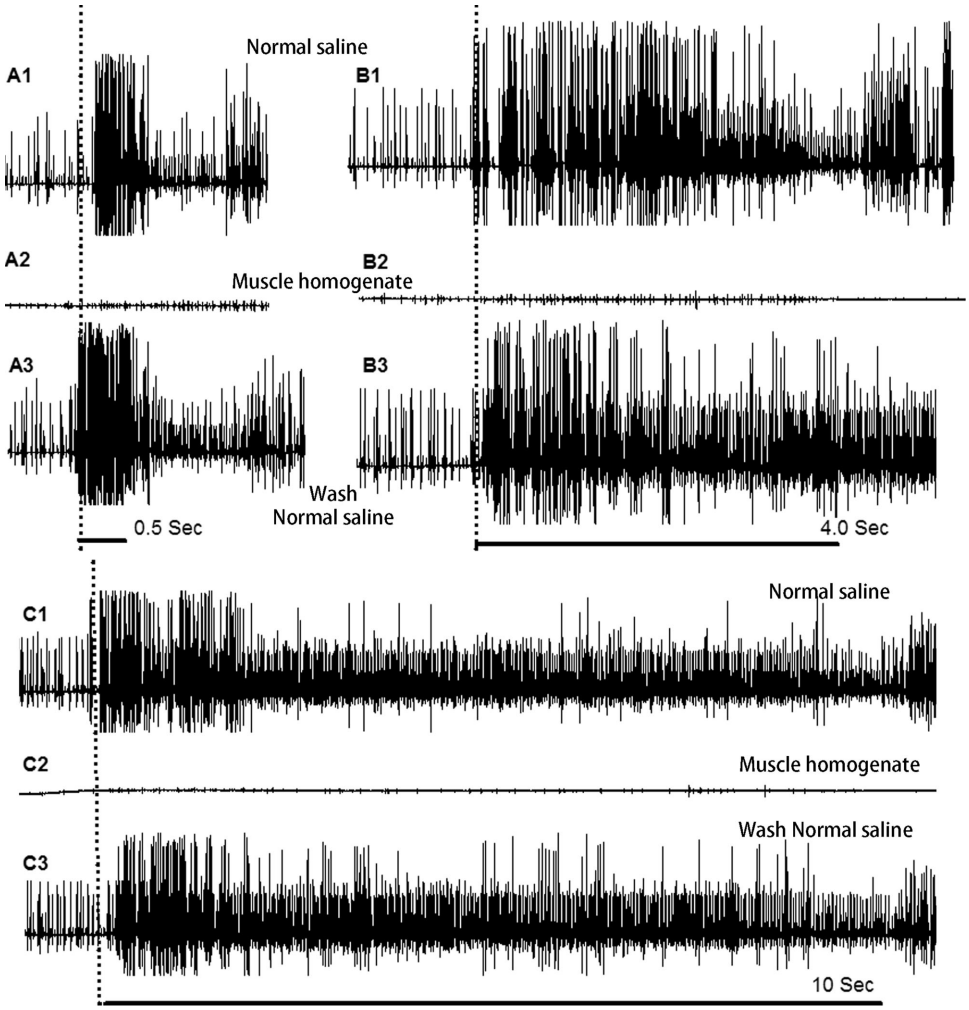

FIGURE 4 Representative traces in neuronal spiking for the different displacement rates and response to normal saline and muscle homogenate for the crab PD organ. The 0.5 s displacement is shown in **A**, while the 4 s is shown in **B** and the static held displacement of 10 s shown in **C**. The responses in normal saline (**A1, B1, C1**), during exposure to diluted muscle homogenate (**A2, B2, C2**) as well as wash out with a return to normal saline (**A3, B3, C3**) are shown. The *y*-axis scale is the same throughout

whereas the crab PD organ did not. The activity after the initial bath exchanges to muscle homogenate increased within the first minute (six of six preparations, $P < 0.05$, non-parametric sign rank sum). The activity then rapidly decreased for the time point at 5 min of exposure as shown for the average responses (Figure 6, 6six out of six preparations, $P < 0.05$, non-parametric sign rank sum).

4 | DISCUSSION

In this study, we demonstrated the proprioceptive neurons associated with the PD organ in the walking leg in the crab and the crayfish MRO preparation are viable for examining the effects of raised $[K^+]$ in the bathing media as well as the effects of muscle damage (or other tissue) on healthy proprioceptive function. The preparations can help serve as models for these effects, which may be observed in other invertebrate species as well as mammalian species, including humans. The rise in $[K^+]_o$ to 20 mM for both preparations reveals mixed results. Some preparations show an increase in activity, whereas others show a decrease in activity. The higher $[K^+]_o$ of 40 mM drastically decreased activity in all preparations, which was similar for exposure to the diluted muscle homogenate.

The consequences of raised extracellular K^+ ions on the resting membrane potential of cells are well established. However, the manner in which the activity of the neurons within a unit such as a mammalian muscle spindle or an intact proprioceptive organ is altered remains under investigation. Earlier studies reported that $[K^+]_o$ had an effect on membrane potential (Bernstein, 1902), but an interest in the effects of cellular leakage on surrounding tissue arose much later. The classic studies on the activity of sensory neurons in the skin of frogs with tissue damage, induced by scratching the skin, bring to the forefront an understanding and intrigue surrounding the implications of tissue damage on healthy cells. These studies show that the decrease in tactile responses resulting from tissue damage could also be reproduced by raised $[K^+]_o$ (Feng, 1933). This early report is likely the first instance of explaining a mechanism to account for the increase in tissue damage to healthy cells within an initial site of a tramatome (Astrup et al., 1977; Dreier et al., 2017). How concentration gradients of charged ions result

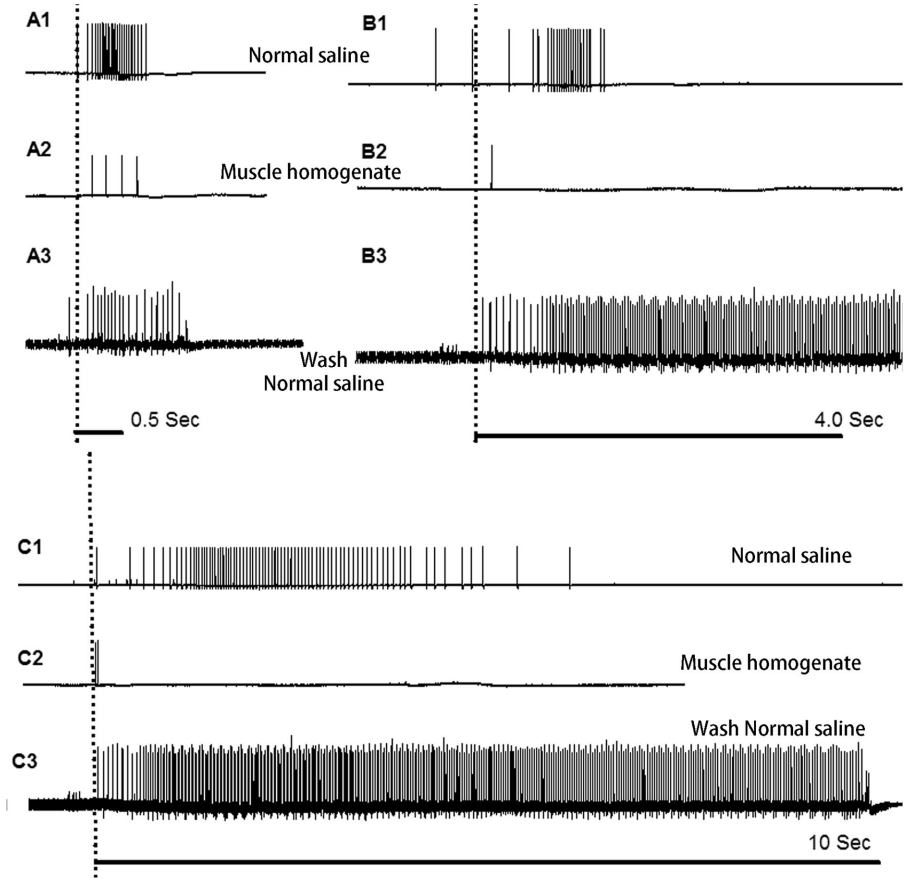

FIGURE 5 Representative traces in neuronal spiking for the different displacement rates and response to normal saline and muscle homogenate for the crayfish MRO. The 0.5 s displacement is shown in **A**, while the 4 s is shown in **B** and the static held displacement of 10 s shown in **C**. The responses in normal saline (**A1, B1, C1**), during exposure to diluted muscle homogenate (**A2, B2, C2**) as well as wash out with a return to normal saline (**A3, B3, C3**) are shown. The *y*-axis scale is the same throughout

in potential differences was being addressed as early as the late 1800s (Nernst, 1888). Scientists are now under the realization that cells are permeable to K^+ at rest and that slight alterations in the $[K^+]_o$ have an impact on both the resting membrane potential and axon excitability. As a result, scientists have a better understanding regarding the sensitivity of the cell to $[K^+]_0$ (Goldman, 1943; Hodgkin & Huxley, 1952; Hodgkin & Katz, 1949; see review Atwood, 1982). About two decades later, in the leech nervous system, it was shown that neural activity in one cell can depolarize resting neighboring cells as a result of a rise in $[K^+]_o$(Baylor & Nicholls, 1969; Orkand et al., 1966). We are not aware of any studies addressing the heightened activity of cells within envelopes of mammalian muscle spindles influencing each other. This may be of interest to researchers, as the potential for this to occur in diseased states certainly exists (i.e., neuronal and muscle spasticity, fibromyalgia, and ion channel pathologies).

The depolarization induced by raising the saline to 20 mM $[K^+]$ is substantial considering $[K^+]$ in normal crayfish saline is 5.4 mM (Cooper & Cooper, 2009; Fatt & Katz, 1953; van Harreveld, 1936). Interestingly this nearly four times increase compared with the physiological level does not result in a persistent desensitization of voltage-gated Na^+ channels in the neurons. This increase in the $[K^+]$ for crab saline from 10.8 mM (normal) to 40 mM results in a decrease of activity for all six PD preparations. Only one preparation decreased a slight amount in activity. In this one case, the 40 mM solution was exchanged with a quick exposure to 60 mM $[K^+]$, which resulted in a very substantial decrease in activity. The recovery of activity for the one preparation being exposed to 60 mM $[K^+]$ was rapid with a return to normal saline after a 2-min exposure. The doubling of $[K^+]$ to 20 mM from 10.8 mM also results in most crab PD (five out of six) preparations decreasing in activity. Given that the species of crayfish used in this study is found in fresh water to estuarine water, perhaps this particular species is not as sensitive to fluctuations in $[K^+]$ within the hemolymph compared to exclusively freshwater crayfish. Additionally, the blue crab used in this study ranges from the Chesapeake Bay (fresh water) to the open ocean. It would be of interest to know how well these animals can regulate $[K^+]_o$ in their hemolymph when exposed to varying salinities. A range for $[K^+]$ in the cerebral spinal fluid for non-diseased state humans in one study was found to be 2.4–3.0 mmol/L and 3.5–4.70 mmol/L in plasma (Pye & Aber, 1982). A twofold increase in plasma $[K^+]$ to 7 or 8 mmol/L in humans will likely lead to death, unless rapidly reduced (Conway, Creagh, Byrne, O'Riordan, & Silke, 2015; Gennari, 1998; Nyirenda, Tang, Padfield, & Seckl, 2009). A recent study measured the $[K^+]$ in abscesses in the human brain and found the levels to have an average 10.6 ± 4.8 mmol/L (Dahlberg, Ivanovic, Mariussen, & Hassel, 2015). The leakage of such an abscess would be severely detrimental to the surrounding healthy tissue. In the referenced study,

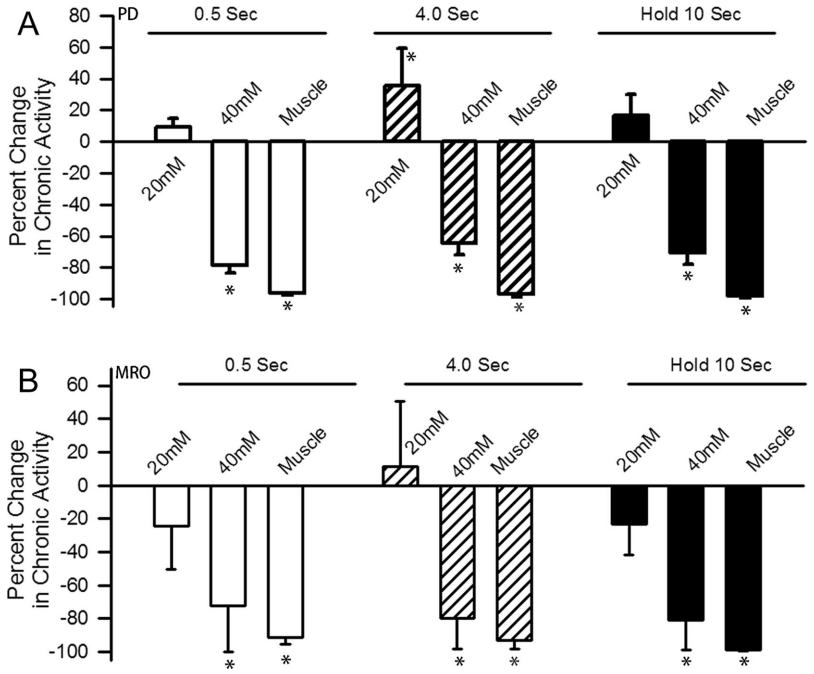

FIGURE 6 A percent change from saline is used to compare among the PD (A) and MRO (B) preparations for the effects of $[K^+]$ exposure (20 mM and 40 mM) as well as the diluted muscle homogenate for the various displacement rates and static held position. (*six out of six preparations illustrating the same trend ($P < 0.05$ non-parametric sign rank sum)

it is implied the $[K^+]$ within the abscess is a result of tissue breakdown contained in the location of the abscess. Given the slightly varied responses of excitation and depression of activity of the PD and MRO preparations and considering the activity was not totally abolished, it can be suggested that the depolarization by 20 mM $[K^+]$is not likely sustained long enough and to a large enough degree to result in Na^+ channel inactivation induced by the depolarization. The 40 mM $[K^+]$ exposure may have resulted in the neurons ceasing to fire due to Na^+ channel inactivation from a sustained depolarized state. Future studies with intracellular recordings of the neurons within the crayfish MRO and crab PD organ will be able to address this matter. However, in the exciter motor neuron of the opener muscle in the walking leg of crayfish, it was determined that at 23 mM $[K^+]$, the axon will stop firing due to voltage-gated Na^+ channel inactivation (Smith, 1983). This is likely a similar concentration at which the sensory neurons of the MRO cease firing. We doubled the $[K^+]$ from 20 to 40 mM so as to exceed what was previously determined to result in voltage-gated Na^+ channel inactivation (Smith, 1983) and we used the same 20 and 40 mM extracellular concentrations to compare the effects between the crayfish and crab preparations. It would be of interest to know if a different $[K^+]_o$ is required to have the same effect for the neurons in the crab PD organ.

Alterations in activity for intact proprioceptors within the animal may be a result of raised $[K^+]_o$ directly on sensory neurons, skeletal muscles, and/or motor neurons innervating the muscle. The activity of motor neurons may lead to contractions of skeletal muscle, which can have an impact on the firing frequency of the proprioceptors. Muscle contraction itself can occur with raised $[K^+]_o$ (Hodgkin & Horowicz, 1960a; Prosser, 1940). The individual tissues (motor nerve terminal, synaptic responses, muscle and sensory neurons) as well as intact preparation can be assessed in these models preparations to understand the integrative nature of proprioception by alterations in $[K^+]_o$ as well as factors resulting from tissue injury. The crayfish MRO is similar to an isolated mammalian skeletal muscle spindle, since the motor nerve terminals remain on the muscles associated with the sensory neurons. However, the crayfish neuromuscular junctions are unique in that glutamate is the transmitter for the excitatory motor neuron and inhibitory GABA-ergic motor neuron innervation is also present (Kuffler 1954; Elekes & Florey, 1987a,b; Swerup & Rydqvist, 1992).

The experiments with the diluted muscle homogenate are intriguing as the estimated $[K^+]_o$ is approximately 30 mM considering the crayfish skeletal muscle maintains a intracellular $[K^+]_i$ of close to 120 mM. As far as we know the $[K^+]_i$ has not been *precisely* determined for the skeletal muscle of crayfish or crab; however, the giant axons within the ventral nerve cord of the crayfish maintained a $[K]_i$ of 233 mM for an upper limit (Strickholm & Wallin, 1967). If this concentration is used as the $[K^+]_i$ for muscle, the estimated level will be around 59 mM for the diluted muscle homogenate (one part muscle to three parts saline). Considering the activity profile for the crayfish MRO with 40 mM $[K^+]$ exposure and muscle homogenate is quite similar, the free K^+ levels with the diluted muscle homogenate may be fitting for the observed effects. For the crab PD, the muscle homogenate generally shut down activity. This may likely be due to the fact that marine invertebrates are known to contain a higher $[K^+]_i$. Estimates are in the range of 370 mM for $[K^+]_i$ in squid axons (Caldwell & Keynes, 1960). If the same is true for the skeletal muscle of the seawater blue crab, a diluted muscle homogenate will be close to 90 mM $[K^+]$. This high concentration will result in maintaining inactivation of voltage-gated Na channels after their opening (Hodgkin & Horowicz, 1959,1960a,b). As for humans, skeletal muscle contains about 80% of the body's total potassium,

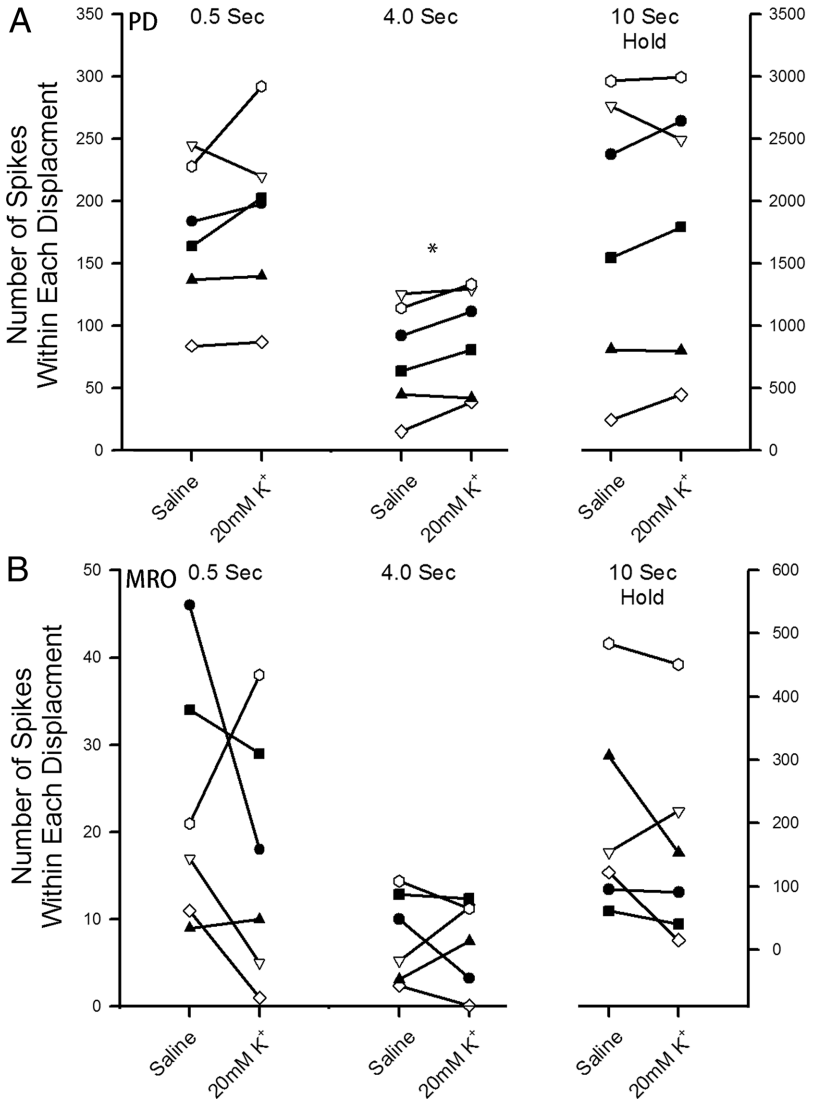

FIGURE 7 The number neuronal spikes measured for each preparation for each displacements condition before and during exposure to 20 mM $[K^+]$. The measures for the crab PD organ (A) and the crayfish MRO (B) for 0.5 s and 4.0 s displacements are shown on the left. The 10 s static hold positions are shown on the right ordinate. Only the PD for the 4 s displacement had a consistent response. (*six out of six preparations illustrated the same trend, $P < 0.05$ non-parametric sign rank sum)

which is not surprising considering muscle makes up the majority of mass for a healthy human (Cheng, Kuo, & Huang, 2013; Sjøgaard, Adams, & Saltin, 1985). The $[K^+]_i$ is around 160 mM, which will mean about 40 mM if the same dilution of skeletal muscle to saline is used. Human plasma is normally within the range 3.5–5 mM (Cheng et al., 2013). Considering damage to a large muscle in a human, this could raise plasma $[K^+]$ substantially.

Other constituents within the muscle cytoplasm, besides K^+, can also have an impact on the function of the proprioceptors. The free amino acids may impact some ion channels directly on the sensory neurons. The MRO may have more potential targets given the presence of glutamate and GABA receptors on the muscles embedded with the sensory endings (Robbins, 1959; Thieffry, 1984). Glutamate is known to be present in the homogenized crustacean skeletal muscle (Abdel-Salam, 2014; Camien et al., 1951; Simpson et al., 1959). The observed initial increase in activity upon exposure to muscle homogenate in the MRO can be the result of glutamate receptor activation and associated muscle contraction. This muscle contraction may stimulate SACs within the proprioceptors and enhance firing. Lowered responses over time are consistent with the hypothesis that continued depolarization likely induces Na^+ channel inactivation. The relative isolation of sensory endings in the crab PD preparation allows the circumvention of muscle-derived influence of SAC activation and an initial increase in activity. Neural activity is also influenced by ionic strength. The muscle homogenate combined with the saline has likely changed the ionic strength. In addition, the free Ca^{2+} concentration in the saline is likely lower than the total Ca^{2+} concentration because of binding of Ca^{2+} to divalent anions and likely the proteins with the muscle homogenate. This binding effect may be considerably higher in the hemolymph of the animal than in the Van Harreveld saline solution or in the saline combined muscle homogenate.

Unlike these crustacean preparations, the muscle spindle in mammals will likely behave differently with exposure to free amino acids, since the motor neurons are cholinergic. The effects on neurons in the

CNS by local tissue damage is heavily focused on the toxicity induced by free glutamate by activation of glutamate receptors (Doyle, Simon, & Stenzel-Poore, 2008; Yamamoto et al., 1999) or K^+ depolarization of neurons with little attention given to other amino acids such as cysteine, homocysteine, and glycine (Boldyrev & Johnson, 2007; Eaglig, Piez, & Levyi, 1961). The osmotic shock of cytoplasmic fluid, which has high protein content compared to the ECF, may also have an impact on the function of ion channels. In addition, an alteration in cytoplasmic pH in healthy cells surrounding tissue damage may arise, as cytoplasm is slightly more basic than ECF in general (Galler and Moser, 1986). It is noted that organelles often maintain a relatively acidic environment, so leakage of organelle-derived H^+ may influence ECF acidity and subsequently the cytoplasmic pH in healthy cells (Bevensee & Boron, 1998; Moody, 1981). This alteration in cytoplasmic pH may have a number of influences on synaptic transmission. Body wall muscle in crustaceans is known to be able to buffer intracellular pH relatively rapidly by ion exchange mechanisms (Galler and Moser, 1986). We did not address the osmotic effects with the application of the muscle homogenate, but this could indeed impact neuronal excitability. The effect of osmotic shock, free amino acids and duration in exposure to raised $[K^+]_i$ on primary neurons can readily be addressed in these model invertebrate preparations, which may provide some insight into addressing similar consequences in mammalian systems.

The compact CNS in vertebrates will likely amplify the effects of neighboring cellular damage on healthy cells. If swelling is present, which can dampen vascular flow, the damaging effects on healthy cells may even be more pronounced due to osmotic shock, changes in pH, ionic/amino acid spillage, and CO_2 accumulation (Dreier et al., 2017; Hartings et al., 2017). Slight imbalances in ionic state, specifically $[K^+]$ and pH/CO_2, may contribute to the onset of epilepsy and other neurological diseases (Kaila & Ransom, 1998; Nedergaard, Kraig, Tanabe, & Pulsinelli, 1991; Mellergard & Siesjo, 1998; Mahad, Trapp, & Lassmann, 2015; Tregub, Kulikov, Motin, Bespalov, & Osipov, 2015; Andrianopoulos et al., 2016). In such individuals, additional insults may have an additive or synergistic effect. It would be interesting to assess the susceptibility to changes in neural activity in response to hypokalemia or hyperkalemia in individuals with chronic obstructive pulmonary disease, who experience systemic reduction of plasma pH (Adrouge & Madias, 1981). Both signaling within the CNS as well in sensory neurons, including those found in muscle spindles (Bewick & Banks, 2015), are likely affected (Meves & Volkner, 1958). A firm understanding of the role of intracellular constituents released from tissue damage on healthy cells is in its early stage, and these model preparations can be used to spur additional analyses that can be translated to mammalian models.

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