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Research article

An invertebrate model in examining the effect of acute ferric iron exposure on proprioceptive neurons

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ABSTRACT

Iron is an essential element for plant and animal life and is found in soil, fresh waters and marine waters. The Fe^{3+} ion is a vital prosthetic group and cofactor to mitochondrial electron transport complexes and numerous proteins involved in normal functioning. Despite its importance to life-sustaining processes, overexposure results in toxicity. For example, ferric iron (Fe^{3+}) accumulation in the mammalian central nervous system is associated with various neurological disorders. Although current literature addresses the long-term effects of Fe^{3+} overload, fewer studies exist examining the effects of acute exposure. Using the blue crab (*Callinectes sapidus*), we investigate the effects of acute Fe^{3+} overload on proprioception within the propodite-dactylopodite (PD) nerve. For proprioceptive studies, 10- and 20-mM ferric chloride and ferric ammonium citrate solutions were used at 5- and 20- min exposure times. Exposure to 20 mM concentrations of ferric chloride and ferric ammonium citrate reduced excitability in proprioceptive neurons. Thus, Fe^{3+} likely blocks stretch-activated channels or voltage-gated Na^{+} channels. The depressive effects of Fe^{3+} are partly reversible following saline washout, indicating cells are not acutely damaged. Gadolinium ($GdCl_3$, 1 and 10 mM) was used to examine the effects of an additional trivalent ion comparator. Gd^{3+} depressed PD nerve compound action potential amplitude while increasing the compound action potential duration. This study is relevant in demonstrating the dose-dependent effects of acute Fe^{3+} and Gd^{3+} exposure on proprioception and provides a model system to further investigate the mechanisms by which metals act on the nervous system.

1. Introduction

Iron is an essential metal ion in most organisms and all animals, maintaining normal mitochondrial, nuclear, and cellular functions as a protein cofactor (Zoroddu et al., 2019). Tight cellular regulation of iron transport and accumulation is required in the cytoplasm and mitochondria (Paul et al., 2017). However, as with other essential metals, iron overload is associated with pathological conditions. In humans, chronic exposure to high iron levels is linked to the development of Parkinson's disease due to long-term accumulation of the metal in the substantia nigra of the brain (Snyder and Connor, 2009). Age-related iron accumulation is also associated with Alzheimer's disease and Friedreich ataxia (Delatycki et al., 2000). In other mammals, exposure to iron, as in the case of aneurysms, leads to neuronal, glial, astrocytic, and microglial injury (Helbok et al., 2022; Li and Jia, 2023; Righy et al., 2016; Wang et al., 2023; Ward et al., 2022; Wilkinson et al., 2018). The inflammatory response that results from this injury complicates conclusions for direct toxicity in response to iron itself. Local cellular damage is likely due to the generation of free radicals by iron through the Fenton reaction and other cellular processes (Chen et al., 2011;

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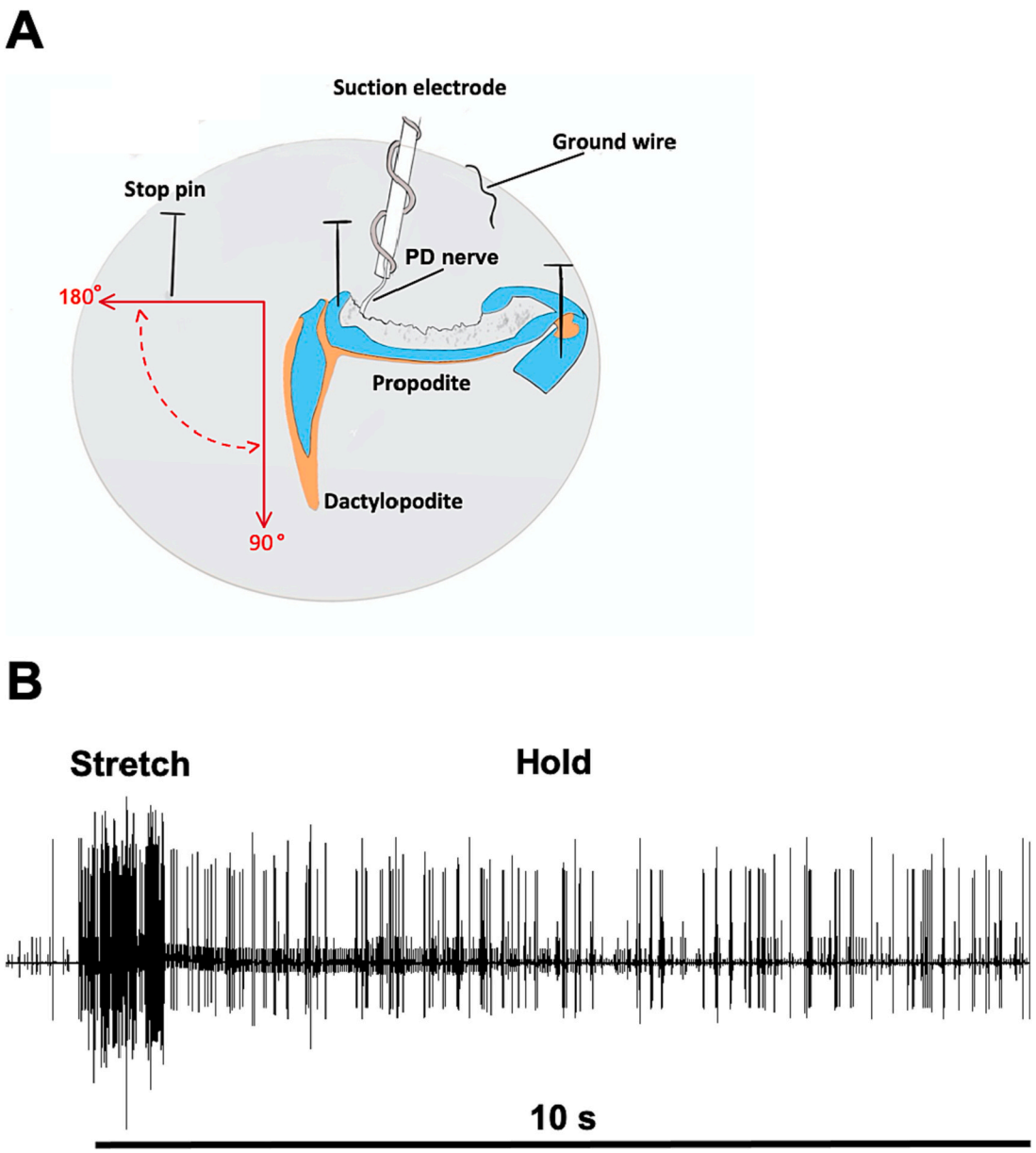


Fig. 1. The PD organ and associated nerve was exposed in the first or second walking leg of the crab. (A) The joint was initially bent at 90 degrees and then extended out to 180 degrees within 1 s and held for at least 9 additional seconds. (B) The entire 10 s was used for analysis of the number of spikes that occurred while being bathed in different solutions.

Nakamura et al., 2005).

It has now been clinically acknowledged that quickly reducing hemoglobin in patients with hemorrhagic strokes, along with additional sources of free iron from damaged RBCs, is vital to avoid acute and long-term damage to the affected areas of the brain (Almutairi et al., 2019). In cases of iron accumulation resulting from ingestion, it is vital to diminish free iron and the iron bound by proteins to avoid damage to various tissues. This decrease of iron levels is generally performed using systemic chelators, and such treatments have been shown to reduce ferroptosis-induced heart damage (Farr and Xiong, 2021; Fang et al., 2019).

In animals, iron is obtained through dietary means. Selective transport occurs through the digestive lining into the circulatory fluid, directly into cells and within the mitochondria, and through additional carrier-mediated processes in the circulatory system. The expression levels of iron channels and transporters, as well as soluble carriers, can vary in physiological and pathological conditions within animals. In the mammalian nervous system, the level of iron varies amongst cell types in their physiological states as well as when cellular injury occurs (Kress et al., 2002). Multiple reviews have been written on the subject of iron transport (Anderson and Vulpe, 2009; Liu et al., 2021; Wessling-Resnick, 2000).

Ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions can modulate a glycine-activated chloride current in the brain of rodents and thus, lead to hyperexcitability of neural circuitry in a short period (Solntseva et al., 2015). The specific effects of acute Fe^{3+} exposure on cells, particularly in binding or blocking ionotropic receptors and ion channels, have not been fully addressed, and it is an area of research which warrants further investigations (Jiang et al., 2019; Agarwal, 2001; Winegar et al., 1991). Fe^{3+} at 100–500 μM concentrations was shown to block K^+ currents in neurons of a snail (Bukanova et al., 2007). It is feasible that Fe^{2+} as well as Fe^{3+} may block other ion channels as well as ionotropic receptors, but such reports are scarce. This may be due to an incomplete investigation of the subject or a lack of data demonstrating the outcomes on ion

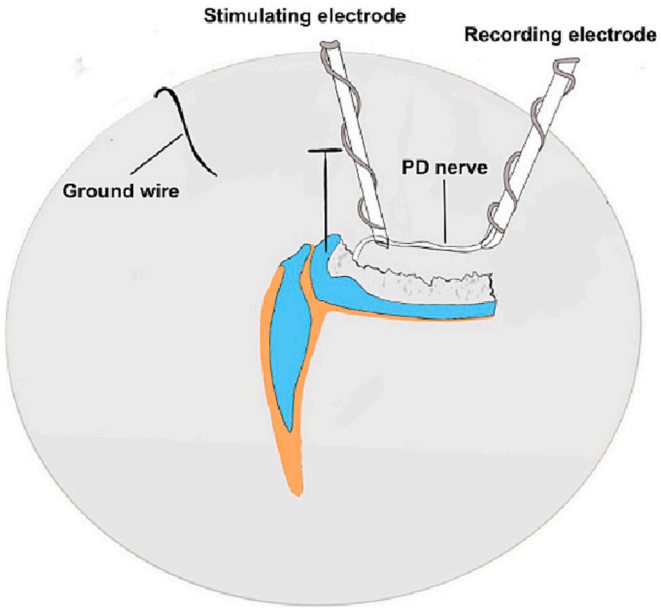


Fig. 2. Recording the evoked compound action potential (CAP) of the PD nerve in a crab first walking leg. The PD nerve is dissected away from the PD organ and leg. The proximal end is placed in a suction electrode and used to record the PD activity and induced CAP in different bathing media. The distal end of the nerve nearest to the PD organ was pulled into a suction electrode to induce CAPs. The preparation is pinned to secure the leg in place. A ground wire is placed in the bathing media.

channels, pumps, ion exchangers, and ionotropic receptors. In this study, the rapid (5 min) and prolonged (20 min) effects of iron exposure on neuronal activity in sensory neurons were addressed. In a previous study (Dayaram et al., 2017a), the effects of gadolinium chloride ($GdCl_3$) on stretch-activated channels (SACs) in the chordotonal organ of the blue crabs were examined to pharmacologically profile the subtype of SACs in the sensory endings of the proprioceptive neurons. Within 10 min, nerve activity was silenced, and no responses occurred while moving the leg joint. However, it was not conclusively determined if Gd^{3+} blocked the SACs or ion channels along the nerve. Thus, the present study assessed the effect of Gd^{3+} on electrically stimulated compound action potentials of the isolated proprioceptive nerves in blue crabs. This allowed for a comparison of the effects of Fe^{3+} and Gd^{3+} on electrical responses of the nerves independently of the sensory receptors. It is possible that both compounds of a 3+ ionic state share similarities in their effects on neuronal function or dysfunction.

Additionally, invertebrates have served as model organisms for numerous neurophysiological studies over the years (Sattelle and Buckingham, 2006). Many excised neuronal preparations from invertebrates are experimentally suitable due to their viability for hours in a simple saline solution at room temperature. Marine crustaceans, such as the blue crab, are relatively abundant and easily obtained. The proprioceptive sensory neurons in the blue crab are readily accessible to record neural activity. Thus, suction electrodes can be placed with ease to record neuronal behavior while moving a limb or electrically inducing compound action potentials to assess the effects of various compounds. The sensory neurons are sensitive to 4-AP (4-aminopyradine), TEA (triethanolamine) and show similar excitability with low Ca^{2+} and acidic conditions as in mammalian neurons (Dayaram et al., 2017a,b; Malloy et al., 2017; Wycoff et al., 2018; Stanley et al., 2020). With much of the similar pharmacological and ionic properties for electrical activity as mammalian neurons, the crab nerve can serve as a model to screen developing pharmacological compounds (i.e. Rizolue; Nethery et al., 2021), anesthetics (i.e., tricaine mesylate; Stanley et al., 2020), various ions such as metals (i.e., Mn^{2+}, Fe^{3+}, Gd^{3+}) and the effects of contaminants in the environment (Dayaram et al., 2017a; Pankau et al., 2022).

2. Methods

2.1. Animals

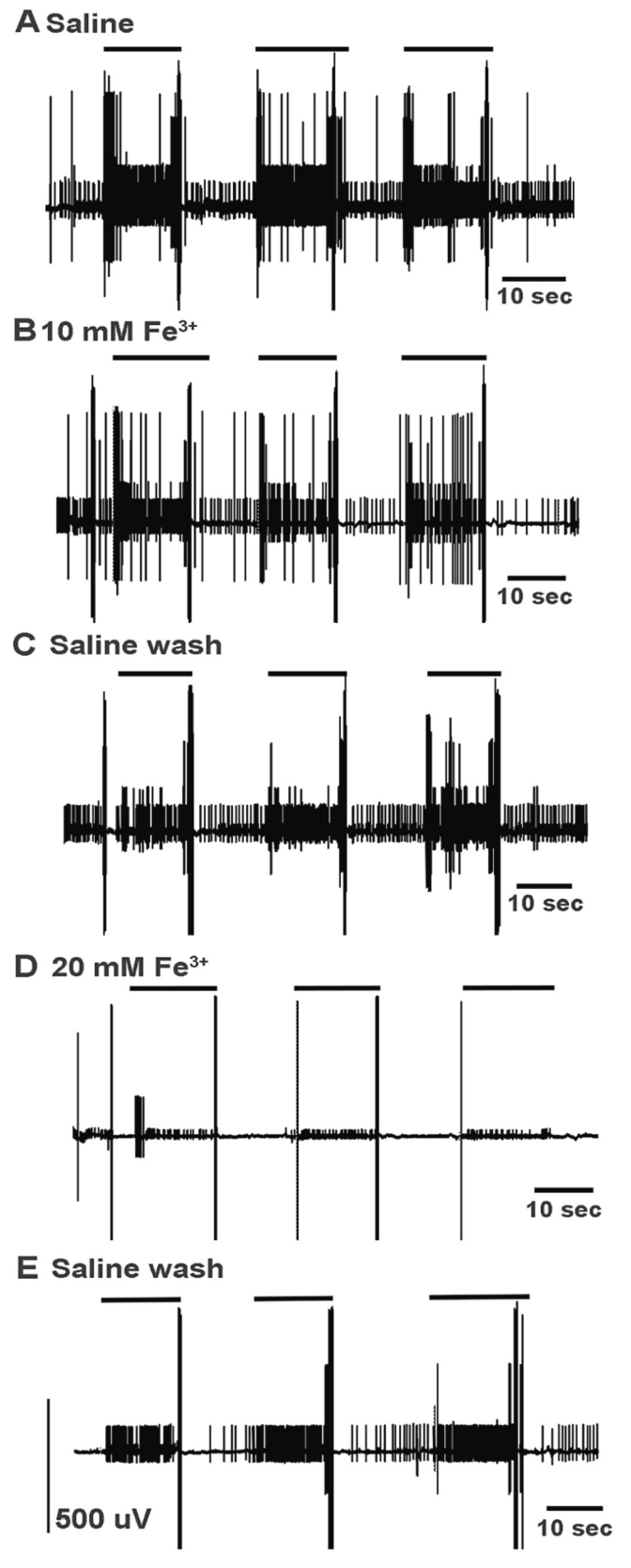
Adult blue crabs (*Callinectes sapidus*) were sourced from a local supermarket in Lexington, KY, USA. Crabs were wild-caught and delivered from a distribution center in Atlanta, GA. Prior to purchase, all animals were assessed for the presence of disease along with overall body condition. To limit the effects of size variability, well-conditioned crabs with a carapace width ranging from 10 to 15 cm were selected. Male and female crabs were housed together in a seawater tank in which temperatures were maintained at 14–16 °C and allowed to acclimate for 3 days. Prior to autotomy procedures, each crab was briefly inspected and confirmed to be alive and active.

2.2. Solutions

The 10- and 20-mM ferric chloride ($FeCl_3$) and ferric ammonium citrate ($(NH_4)_5[Fe(C_6H_4O_7)_2]$) solutions were created by dissolving the compounds in crab saline. Crab saline used to obtain recordings and maintain preparations consisted of (in mM): 470 NaCl, 7.9 KCl, 15.0 CaCl2·2H2O, 6.98 MgCl2·6H2O, 11.0 dextrose, HEPES acid and 5 HEPES base normalized to pH 7.5. Ferric ammonium citrate was used in place of ferric citrate due to its greater solubility, allowing for its full dissolution at 20 mM. In contrast, the $FeCl_3$ solution did not fully dissolve at 20 mM. In order to approximate a baseline pH for each solution, Hydrion pH test strips were used and revealed both solutions to be acidic (pH≈ 5.0 in the case for $FeCl_3$). To avoid the potential effects of acidity on nerve activity, both compounds were adjusted with 1.0 M NaOH until a pH of 7.5 was obtained for physiological recordings from the propodite-dactylopodite (PD) nerve of blue crabs. Because pH adjustments with NaOH contributed excess Na^+ to each treatment, a saline solution was created in order to control changes in nerve activity due to osmotic shock. As the 20 mM $FeCl_3$ solution required the greatest amount of NaOH to reach physiological pH (2.5 mL), the same volume of 1.0 M NaCl was added to saline to create the sodium control solution. This adjustment raised the overall NaCl concentration from 470 mM to 470.1 mM. Thus, no significant changes in the activity were anticipated. A 1 and 10 mM gadolinium chloride ($GdCl_3$) solution was created by directly dissolving $GdCl_3$ in saline. A previous study established that 1 mM $GdCl_3$ was sufficient to halt PD nerve activity in response to stretching the joint (Dayaram et al., 2017a). Therefore, a 1 and 10 mM concentration was used to examine the effect of Gd^{3+} on electrically induced nerve activity independent of the sensory endings and associated stretch-activated channels. The 10 mM Gd^{3+} solution was used to directly compare to the 10 mM Fe^{3+} solutions.

2.3. Recording crab PD organ activity

In the present experiment, procedures similar to the PD nerve isolation and electrophysiological recording techniques described by Pankau et al. (2022) and Majeed et al. (2013) were followed. In brief, crabs were induced to autotomize the first or second walking leg by using forceps to apply gentle pressure in the autotomy plane located within the merus of each limb. In order to expose the PD organ, the cuticle was excised from both lateral and medial sides of the propodite region through which the nerve spans with a No. 11 scalpel blade. Using a dissecting microscope, the PD nerve was isolated from the primary leg nerve with forceps. The perimeter of the leg was secured with stainless steel dissection pins in a Sylgard-lined dish filled with crab saline, and the isolated PD nerve was placed in a suction electrode to measure its activity.



(caption on next column)

Fig. 3. Representative responses from a preparation undergoing the experimental paradigm for acute iron exposure. In this preparation, the effect of ferric chloride was examined. (A) The baseline responses to movement of the PD joint in saline produced robust activity. (B) Exposure to 10 mM ferric chloride did not reduce the overall activity in comparison to exposure at 20 mM concentrations (D). (C) Saline washouts between exposures were performed to promote recovery prior to the 20 mM iron exposure. (E) The final saline wash and joint movements were helpful in demonstrating that preparations were not irreversibly damaged following exposure. The bars above the traces indicate the extension of the joint, which are generally longer than 10 s, but only the initial 10 s was analyzed.

Treatment groups consisted of: a saline control (N = 6), 10 and 20 mM $FeCl_3$ solutions at 5 min exposure time (10 mM: N = 6; 20 mM: N = 6), 10 and 20 mM $FeCl_3$ solutions at 20 min exposure time (10 mM N = 6; 20 mM N = 6), along with identical treatment groups and sample sizes for the ferric ammonium citrate solution. $GdCl_3$ was examined at 1 and 10 mM. For each treatment, the dactyl was moved from flexion to full extension in 1 s, held in the extended position for 9 s, then returned to its original position in 1 s. All 10s of data was used during analysis (Fig. 1A, B). The procedure described above was replicated three times for each treatment in the following order: saline control, Fe^{3+} or Gd^{3+} solution, saline washout. Throughout each trial, a dissecting pin was used as a stop point to ensure consistency in the maximum range of extension obtained. During saline washouts, the bathing media was exchanged a total of three times prior to data collection.

2.4. Analysis of the extracellular spikes

Analysis of nerve activity (spikes) over the 10 s of flexion and extension was accomplished using the cyclic measurement function in LabChart 7.0 and 8.0 (ADI Instruments). A sine wave fit of the trace with a selected standard deviation provided an index of which spike amplitudes would be detected. As spike amplitudes varied widely within a single preparation, utilizing a set standard deviation introduced limitations in the ability of the software to accurately differentiate small signals from electrical noise. Such software issues are detailed in a previous report (Tanner et al., 2022). Thus, counts were confirmed by eye. Additional spikes omitted by software along with erroneous inclusions of electrical noise were either added or subtracted from the count by modifying the standard deviation within the data. To maintain consistency, one individual was designated with the task of identifying which spikes would be included or excluded. In general, spikes with an amplitude approximately two times the baseline noise were included in final counts. To examine reproducibility, participants in a university course conducted the same experiments. The results of these additional blinded studies and analysis are presented in the Discussion.

2.5. Compound action potentials

Procedures similar to the PD nerve isolation and CAP recording techniques described by Pankau et al. (2022) were followed. In brief, the PD nerve was isolated by dissecting away the main leg nerve. The nerve was then placed to the side of the leg while the portion of shell preceding the propodite region was removed for ease of recording (Fig. 2). At the distal end of the PD nerve, a stimulating electrode was placed to induce CAPs. The proximal portion of the nerve extending from the propodite region was placed in a recording electrode to measure background proprioceptive activity as well as the induced CAPs (Fig. 2). A ground wire was placed along the side of the dish. Following electrode placement, both the distal and proximal ends of the nerve were packed with petroleum jelly to increase contact between the electrode and nerve surface, therefore maximizing the amplitude of the extracellular signals recorded. The nerve was stimulated until the voltage producing maximum CAP amplitude and duration was identified. Once a peak response was obtained, the voltage was reduced slightly below this

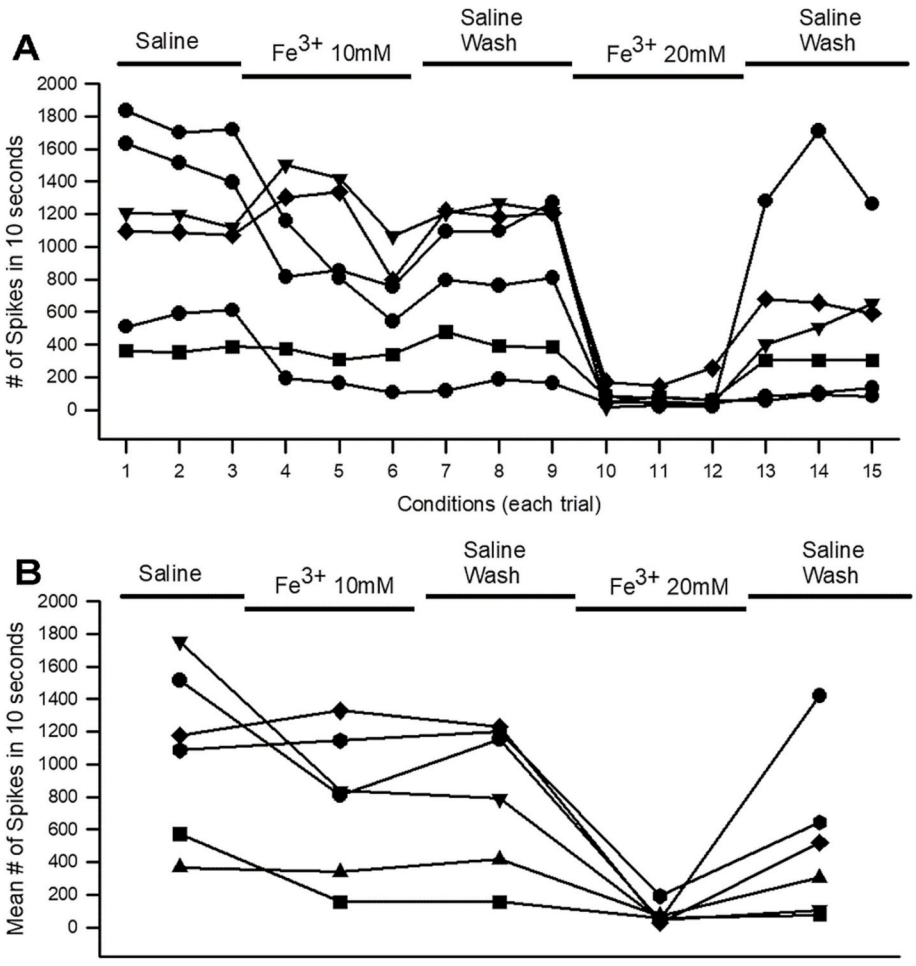


Fig. 4. The effect of ferric ammonium citrate on stimulated sensory nerve activity. (A) The number of spikes measured in a 10 second window in which the joint was moved from the starting position (bent 90 degrees) into full extension within 1 s and held in the extended position for 9 s. Each line represents a different preparation. Data was collected for three saline trials, three trials after switching the bathing media to ferric ammonium citrate (10 mM), three trials after changing the bath to fresh saline and three trials following ferric ammonium citrate (20 mM) exposure. The final exchange was performed to rinse the preparation once with fresh saline and to replace the bath with saline. Joint movements were then repeated three more times. Each movement was separated by a minimum of 10 s while the joint was held in a bent position. (B) The number of spikes in each of the three trails was averaged and graphed in the same manner as shown in (A).

maximum level. With the maximum CAP amplitude and duration serving as a point of reference, any further increase in the CAPs could be clearly identified upon exposure to the Fe^{3+} or Gd^{3+} solutions. Treatment groups were identical to the description provided above.

2.6. Statistical methods

For all crab proprioception data sets, a Shapiro-Wilks test was first used to determine if the obtained data fell within a normal distribution. For all passing data sets, a paired *t*-test or a Wilcoxon Signed-Rank Test was used with a significance level of 0.05. ANOVA tests were additionally performed as needed.

3. Results

3.1. Acute exposure of Fe^{3+} to the PD organ

To ascertain the effect of acute iron exposure on the PD organ, the activity of the PD nerve in response to movement at the joint was examined after a 5-minute incubation period in 10 mM ferric chloride. After three trials of joint movements were complete, the bathing media was exchanged twice with fresh saline followed by three trials of joint movements. The procedure was then repeated using a higher concentration of ferric chloride (20 mM). This same procedure was used for ferric ammonium citrate at 10 and 20 mM.

The saline containing elevated Na^{+} levels produced no additional effect on the overall activity of the PD organ during joint displacements. Therefore, the effects of a higher Na^{+} concentration on nerve activity within the pH-adjusted Fe^{3+} solutions were concluded to be negligible. A depression of nerve activity was observed at the 20 mM concentrations of iron. An exchange back to fresh saline was examined to determine if the organ was damaged by the iron exposure. Representative recordings of the three trails in each experimental condition are illustrated after exposure to iron chloride (Fig. 3). Bars above each trace of activity indicate a period of joint extension, and scale bars below each trace indicate time. Basal activity between joint movements was not analyzed.

Exposure to ferric ammonium citrate did not produce a consistent change in nerve activity at 10 mM concentrations. In contrast, 20 mM concentrations substantially depressed activity in response to joint movement. In four of the six preparations, a recovery of activity was observed following rinses with fresh saline. However, recovery was partial in nature and did not return to the baseline level observed prior to exposure to the 20 mM solution (Fig. 4A). The average of the responses following each exposure period reveals the overall trends (Fig. 4B). It is important to note that two of the six preparations displayed a slight increase in activity upon exposure to 10 mM ferric ammonium citrate, three preparations exhibited a pronounced decrease in activity and one preparation showed little change in activity. It is hypothesized that increasing exposure time would be beneficial in producing more consistent responses.

Exposure to ferric chloride revealed similar outcomes in comparison to ferric ammonium citrate exposure. No consistent change in nerve activity was observed upon exposure to the 10 mM concentrations. However, 20 mM ferric chloride substantially depressed activity when moving the joint. A recovery of activity following fresh saline washouts was only observed in two of the six preparations. As with the ferric ammonium citrate, activity was not restored to the baseline level prior to iron exposure at 20 mM concentrations (Fig. 5A). The average in the

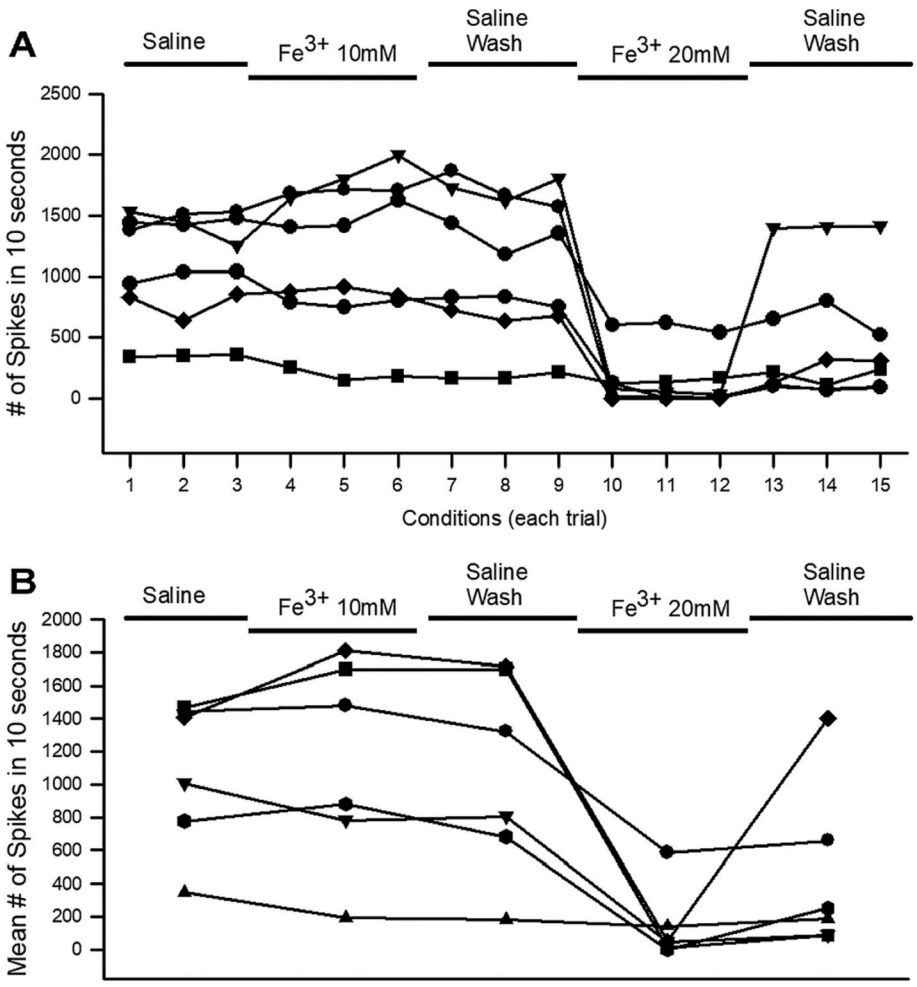


Fig. 5. The effect of ferric chloride on stimulated sensory nerve activity. (A) The number of spikes measured in a 10 second window in which the joint was moved from the starting position (bent 90 degrees) into full extension within 1 s and held in the extended position for 9 s. Each line represents a different preparation. Data was collected for three saline trials, three trials after switching the bathing media to ferric chloride (10 mM), three trials after changing the bath to fresh saline and three trials following ferric chloride (20 mM) exposure. The final exchange was performed to rinse the preparation once with fresh saline and to replace the bath with saline. Joint movements were then repeated three more times. Each movement was separated by a minimum of 10 s while the joint was held in a bent position. (B) The number of spikes in each of the three trails was averaged and graphed in the same manner as in (A).

responses for each exposure reveals the overall trends (Fig. 5B). Again, it is interesting to note that two of the six preparations displayed a slight increase in activity with 10 mM ferric chloride while three preparations exhibited a pronounced decrease in activity and one preparation showed little change in activity.

3.2. Twenty minutes of exposure to iron

To determine if a longer exposure time would be detrimental to the PD organ, a twenty-minute static exposure to ferric chloride or ferric ammonium citrate was used. Due to the acidity of the ferric chloride solution, a significant adjustment using NaOH was required to raise the pH to a physiological value of 7.5 (a precaution also taken for the five-minute treatment). Due to the introduction of excess Na^+ within the ferric chloride solution, a saline solution with an equal Na^+ concentration was created and utilized to address the potential for osmotic shock following pH adjustment. This adjustment raised the overall NaCl concentration from 470 mM to 470.1 mM. Thus, no significant changes in the activity were anticipated.

No consistent change in neural activity was observed upon exposure to the saline solution adjusted with additional Na^+ (Fig. 6 A1, A2). However, both ferric citrate (Fig. 6, B1 and B2) and ferric ammonium chloride (Fig. 6, C1 and C2) produced a net decrease in neural activity (Table 1). Statistical analysis is presented in Table 1.

Each condition was compared as before and during exposure. Both Paired *t*-tests and Wilcoxon Sign tests were utilized for each individual preparation after averaging the three trials in saline or during exposure to Fe^{3+}. The comparisons are of the 10 s of activity measured for extending the PD joint and holding in an extending position. * $P < 0.05$. In not all cases is there an equal variance in the data sets due to the lack of neural activity to measure; therefore, the Sign test (non-parametric) is more appropriate.

3.3. Effect of iron on compound action potentials

Because neural activity decreased upon exposure to 20 mM concentrations of either iron-containing compound, it was vital to investigate the potential mechanisms by which iron depresses nerve activity. Specifically, the present study sought to examine if iron affected the stretch-activated channels located within the sensory endings or the electrical conduction and activation of the neurons. Therefore, PD nerves were electrically stimulated to induce compound action potentials (CAPs) as detailed in the Methods. The voltage to induce a maximal response in both amplitude and shape of the CAP was determined by gradually increasing the voltage (Fig. 7, A series). After a maximal response was obtained, the bathing media was exchanged to 10 mM ferric chloride or ferric ammonium citrate (Fig. 7, B series). A decrease in CAP amplitude upon exposure to 10 mM ferric ammonium citrate was observed in five of the six preparations (Table 2). Statistical analysis is presented in Table 2. CAP did not decrease in amplitude with 10 mM ferric ammonium citrate in one preparation as shown in Fig. 7 (B series). The depressive effects of 10 mM ferric chloride were not as pronounced in comparison to ferric citrate (Table 2). However, after 20 mM exposure to ferric chloride or ferric ammonium citrate, the amplitudes and shapes of the CAPs were altered. In certain cases, no CAP was discernible (Table 2). A representative decrease in CAP amplitude upon exposure to

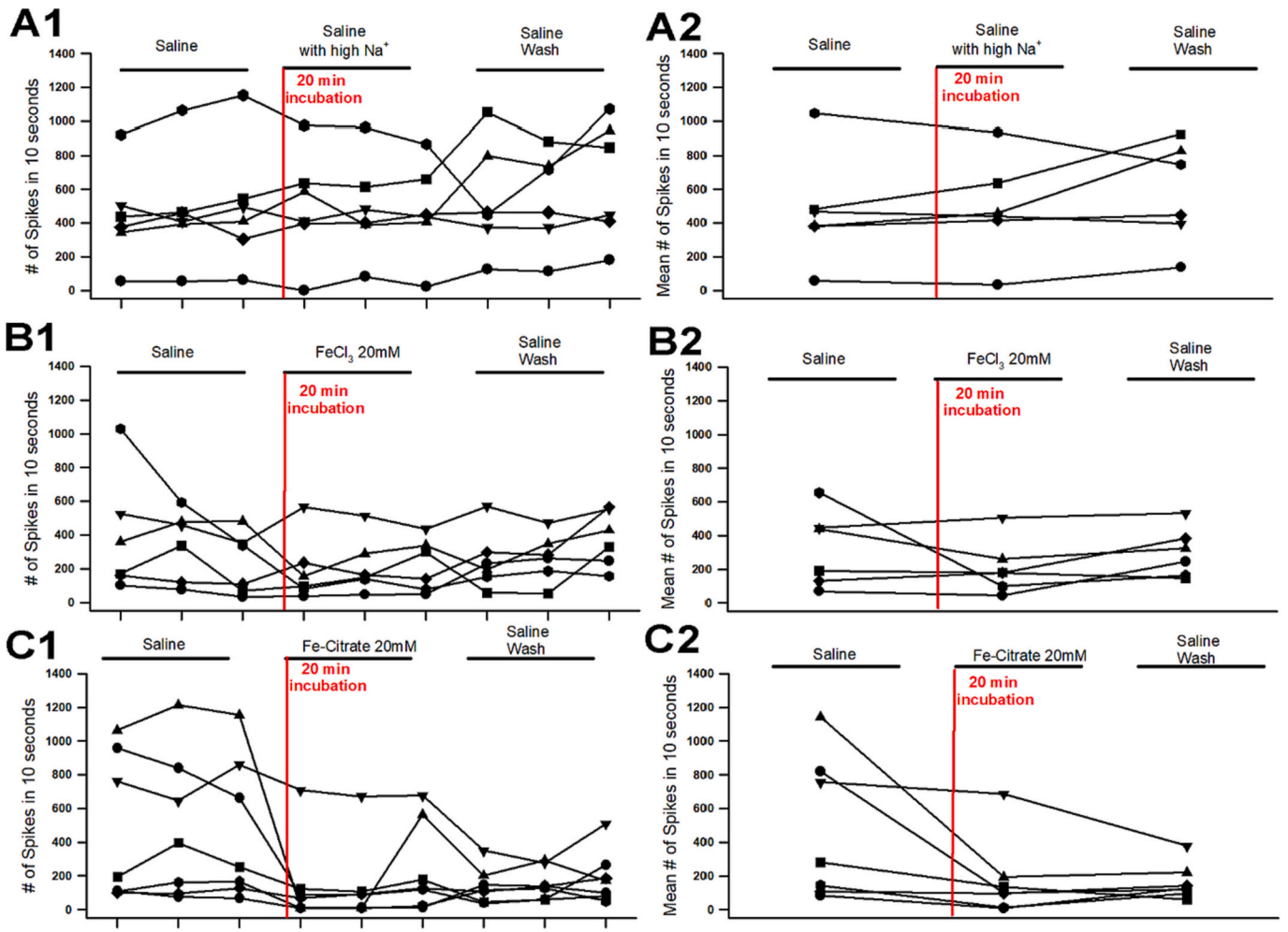


Fig. 6. The effect of 20-minute incubation in ferric chloride or ferric ammonium citrate on stimulated sensory nerve activity. (A1 and A2) Saline with 0.1 mM additional NaCl. Additional NaCl was added to determine if the excess Na^+ introduced from the addition of NaOH during pH corrections in the iron solutions resulted in altered neural activity. (A1) The number of spikes was measured in a 10 second window in which the joint was moved from the bent position (90 degrees) into full extension within 1 s and held in an extended position for the next 9 s. Each line represents a separate preparation. Three trials are performed in saline, after switching the bath to saline with additional Na^+ (0.1 mM) and following a final saline washout. (A2) The number of spikes in each of the three trails was averaged and graphed in the same manner as in (A1). (B1) The number of spikes for each of the three trials in each condition: saline, ferric chloride (20 mM) and a final saline. (B2) The number of spikes in each of the three trails was averaged and graphed in the same manner as in (B1). (C1) The number of spikes for each of the three trials in each condition: saline, ferric ammonium citrate (20 mM) and a final saline. (C2) The number of spikes in each of the three trails was averaged and graphed in the same manner as in (C1).

Table 1
Significant change in the neural activity before and during exposure to Fe of the PD organ.

	1 min	20 min
	Paired T-test (Sign test)	Paired T-test (Sign test)
10 mM $FeCl_3$	NS (NS)	NS (NS)
20 mM $FeCl_3$	* (*)	* (*)
10 mM FeCitrate	NS (NS)	NS (NS)
20 mM FeCitrate	* (*)	* (*)

20 mM ferric ammonium citrate is shown in Fig. 7 where CAPs are observable (Fig. 7, C series).

3.4. Effect of gadolinium on compound action potentials

Using the same experimental arrangement as described in the Fe^{3+} studies, the effects of $GdCl_3$ (1 mM and 10 mM) on compound action potentials was investigated. In all preparations (n = 6), exposure to 1 mM $GdCl_3$ resulted in an immediate reduction of CAP amplitude ($P < 0.05$ rank sum non-parametric). However, in order to directly compare Gd^{3+} to Fe^{3+} for the same concentrations, $GdCl_3$ was compared using 10 mM. The peak amplitude of the CAPs was reduced in four of the six preparations and was larger in 2 of the 6 preparations. Both examples are illustrated in Figs. 8 and 9. In four of the six preparations the CAP amplitude experienced a substantial reduction within 15 min of incubation (Fig. 8C). 10 mM $GdCl_3$ decreased the initial CAP to the baseline level. However, delayed spontaneous and evoked CAPs (synchronized in time) would appear later in the recording. These effects differed from those produced by Fe^{3+} exposure in that the duration of the CAPs was prolonged with Gd^{3+} exposure. When multiple CAPs occur with $GdCl_3$ exposure, quantitative measures in amplitude are not feasible. Additionally, the area under the curve for the CAPs with $GdCl_3$ became difficult to assess due to the occurrence of spontaneous CAPs. The spontaneous spikes and summating CAPs were common as compared to the original evoked CAP in the initial saline in 6 out of 6 preparations ($P < 0.05$ rank sum non-parametric, Table 2).

Six different preparations were used for each exposure of ferric ammonium citrate or ferric chloride. The overall amplitudes were

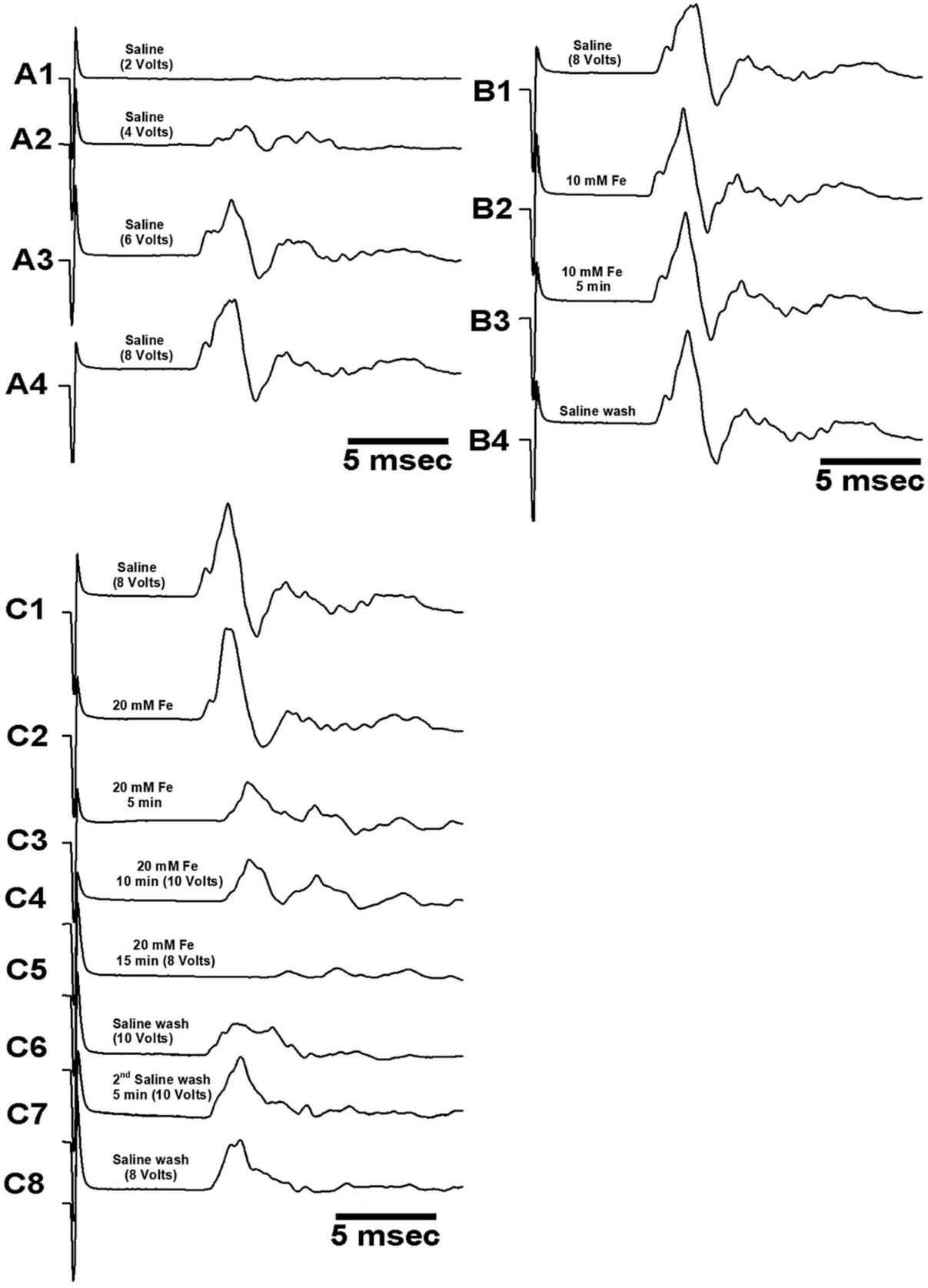


Fig. 7. Representative responses of the effects of iron exposure on the compound action potential (CAP) of the PD nerve. (A series) A stimulus voltage was incrementally increased to obtain a maximum amplitude and waveform shape of the CAP. (B series) With the stimulus voltage held constant, the media was exchanged from saline to ferric ammonium citrate (10 mM) and then exchanged back to saline with one rinse of the bath with fresh saline. (C series) With the same stimulus voltage used to obtain a maximal response, the CAP was induced in saline, during exposure to ferric ammonium citrate (20 mM) and again exchanged back to fresh saline. The same preparation was used in the A-C series.

Table 2
Changes in amplitude, delay and number of compound action potentials (CAPs) of the PD nerve with Fe^{3+} or Gd^{3+} exposure.

Preparation	Iron citrate		Iron chloride		Gadolinium chloride	
	10 mM	20 mM	10 mM	20 mM	1 mM	10 mM
1	↓	↓ Inhibited	No change	↓ Inhibited	↑Duration	↑# CAPS
2	↓	↓↓ Present	No change	↓	↑Duration	↑# CAPS
3	No change	↓ Inhibited	No change	↓↓ Present	↑Duration	↑# CAPS
4	↓	↓ Inhibited	No change	↓↓ Present	↑Duration	↑# CAPS
5	↓	↓ Inhibited	↓	↓↓ Present	↑Duration	↑# CAPS
6	↓	↓↓ Present	↓↓	↓ Inhibited	↑Duration	↑# CAPS

qualitatively assessed. A single arrow indicates a slight depression of no >50 % in amplitude. Two arrows indicates more than a 50 % overall depression in the amplitude of the largest of the waveforms within the complex waveform. A downward arrow indicates an inhibition with some amplitude still present or that no CAP was observed within the trace. Two arrows indicate a larger decrease than one arrow. An arrow with "present" indicates that a CAP was still discernible in the original location. For exposure to gadolinium (10 mM), the initial CAP amplitude appears to decrease while delay increases (slower conduction velocity as shown with an upward arrow). Additional CAPs appearing with 10 mM gadolinium are indicated by an upward arrow and # ($N = 6$ for each concentration and each compound; $P < 0.05$, Sign test, for 20 mM ferric ammonium citrate, ferric chloride, and gadolinium at both 1 mM and 10 mM).

In examining the reproducibility of the present studies, a neurophysiology class replicated the data collection process under identical experimental conditions in a single-blind study. Students were not made aware of which compound they were testing. As the rate of movements may have varied amongst groups, the results are qualitatively compared to the data previously collected with a single individual performing all joint movements and while another individual marked the traces on a computer and analyzed all data sets. The class participants replicated two conditions of 10 mM $FeCl_3$, two conditions of 20 mM $FeCl_3$, two conditions of 20 mM ferric ammonium citrate and two conditions of Na^+-adjusted saline (Supplementary data: S1).

4. Discussion

Extracellular Fe^{3+} introduced by two compounds, $FeCl_3$ and ferric ammonium citrate, depressed neuronal conduction as observed by the decrease in evoked CAP amplitudes. For both compounds, this depression was most notable at 20 mM concentrations. At 10 mM concentration, depression was only seen upon exposure to ferric ammonium citrate. It is possible that the depression in CAP amplitudes originates from an inability of the neurons to initiate action potentials. Additionally, Fe^{3+} may act to inhibit signal propagation along the axons. Thus, it is possible that Fe^{3+} reduces the observed number of spikes by blocking stretch-activated channels (SACs) as well as by reducing electrical activity monitoring within the PD nerve. To examine the effect of an additional trivalent metal, $GdCl_3$ was used. In PD nerves with intact sensory endings, activity was rapidly decreased in response to stretching the joint. However, depression of axonal excitability was less pronounced. With exposure to Gd^{3+}, CAP widths increase over time, indicating a change in conduction velocity along the axons. Changes in conduction velocity were not an action induced by Fe^{3+}. Instead, a decrease in the CAP amplitude indicated that fewer neurons were activated. The novelty of this study is in presenting the blue crab as a model for neurophysiology. The blue crab is an accessible model across North America from suppliers and naturally inhabits the Atlantic ocean along North America to the northern aspect of South America. Additionally, the blue crab is now found in the Mediterranean Sea. The accessibility, low maintenance and easily recordable neurons of this organism thus increase the prospect of its use as a model in future studies. Furthermore, the neurophysiological effect of Fe^{3+} from two different complexes (ferric chloride ($FeCl_3$) and ferric ammonium citrate ($(NH_4)_5[Fe(C_6H_4O_7)_2]$)) was investigated rather than using one form, as commonly presented in past studies. The potential action of Fe^{3+} blocking stretch-activated channels in proprioceptive neurons has not been addressed previously, nor has the widening of CAPs in nerves upon exposure to Gd^{3+}. The alteration in the CAPs could have implications in further demonstrating the effects of Gd^{3+} on voltage-gated ion channels as well as K2P channels responsible for maintaining electrical excitability and conduction. The findings presented in the present study serve as a model to encourage further research on the actions of Fe^{3+} and Gd^{3+}on neurophysiological function.

The rationale for examining two forms of Fe^{3+} was to exclude that the effects observed were due to the counter-ions within each solution. Following the addition of NaOH to the 20 mM ferric chloride solution, a small amount of precipitation was observed. Thus, the free Fe^{3+} in solution is lower than 20 mM. Therefore, it is possible that the depression of neuronal activity in $FeCl_3$ during 20-min exposure was disrupted due to increasing precipitation that continued to lower the free Fe^{3+} in the solution over time (see Fig. 6, B1 and B2). It is additionally possible that depression was less appreciable with 20 min exposure due to a compensatory mechanism within the neuronal cells in response to prolonged exposure. Because ferric ammonium citrate is more soluble, no precipitation was observed at 20 mM concentrations. Ferric ammonium citrate is widely used in many reports (Zeng et al., 2021; Irrsack et al., 2021; Che et al., 2021), most likely due to its full dissolution at physiological pH and lower acidity in comparison to ferric chloride. Ferric citrate can also be utilized to assess the effect of Fe^{3+}; however, it is less soluble than ferric ammonium citrate.

It is important to note that the hydrolysis of Fe^{3+} can produce a 'hard acid' in water due to binding with -OH and producing free H^+. As the pH from acidic conditions becomes more basic, Fe^{3+} becomes less soluble and forms a precipitate. This phenomenon is observed when adjusting the saline containing $FeCl_3$ to a physiological range (pH 7.4–7.5) for the nervous system of the crab model. In contrast, ferric ammonium citrate maintains a soluble iron (III) complex. Soluble Fe^{3+} is known to occur in complexes within biological systems, thus demonstrating the bioavailability of the compound under physiological conditions (Sánchez et al., 2017; Neilands, 1995; Hider and Kong, 2010). The addition of $GdCl_3$ in water does not produce such acidic conditions and remains soluble at a pH of 7.4 to 7.5 likely due to weaker hydrolysis. However, the saline with high $GdCl_3$ concentration does appear slightly milky. The hydrated form can be obtained as $GdCl_3 \bullet 6H_2O$. The valence electrons available with Fe^{3+}, Gd^{3+} and these metals within complexes will impact the solubility and hydrolysis. The size differences in the ions additionally impact their behavior and structure in water. However, when examining the physiological effects of these metals on tissues, as in this study, the other salts in the saline and buffers may alter the chemistry known for these metals from studies conducted with only water. Furthermore, the HEPES buffer used in the physiological saline may also act as a chelating agent by chemical reactions we are not aware of.

Based on the results of the present study, it is possible that Fe^{3+} blocks SACs in the sensory endings, thus decreasing responsiveness to bending at the PD joint. However, the proposed mechanism can only be confirmed by recording the sensory endings or directly through the

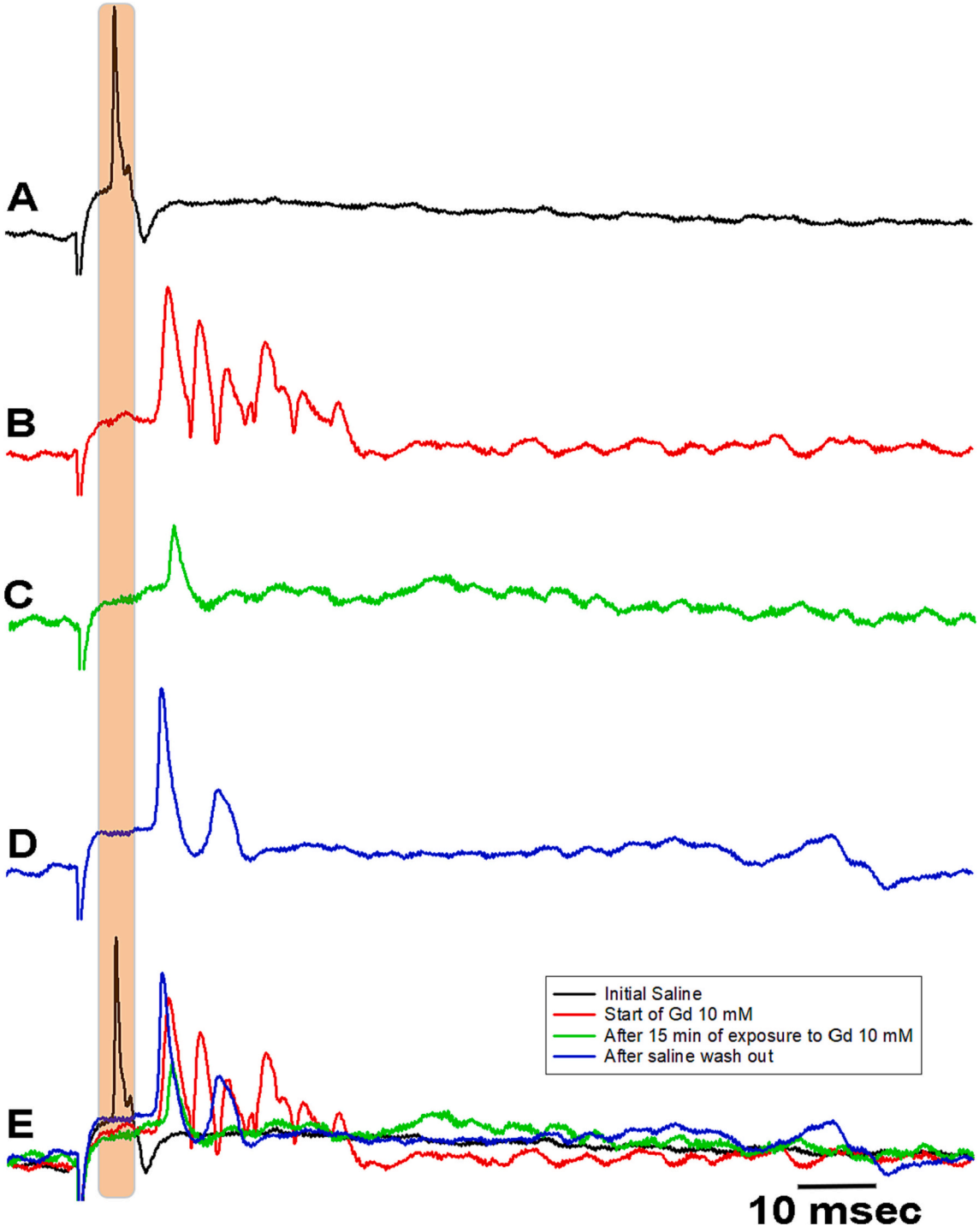


Fig. 8. Representative response of the effects of $GdCl_3$ exposure on the compound action potential (CAP) of the PD nerve. The media was exchanged from saline to one with $GdCl_3$ (10 mM) and then exchanged back to saline following three rinses with fresh saline. The same stimulus voltage was used to obtain a maximal response of a CAP in saline, during exposure to $GdCl_3$ and when exchanged back to fresh saline. Note that the CAP amplitude is reduced while the duration is prolonged. The shaded area indicates the region from the stimulus the initial CAP in saline was observed as a reference. (A) Saline. (B) After $GdCl_3$ (10 mM) exposure. Note that delayed CAPs occur. (C) After 15 min exposure to $GdCl_3$ (10 mM). (D) After exchanging the bath for fresh saline. (E) Superimposed traces, as all data is collected at the same scale.

channels with electrophysiological techniques. Such methods have not yet been accomplished in chordotonal organs of crustaceans. It may be possible to measure ion flux with ion sensitive indicators loaded into the neurons. The type of ionic currents passing through SACs in crustaceans and the molecular makeup of the SACs to determine which subtypes are present in crustacean chordotonal organs is an active area of research (McCubbin et al., 2020). Profiling the SAC subtype in the PD organ by pharmacological approaches indicates that the channels are not altered by traditional agonists or antagonists for SACs, such as amiloride, ruthenium red and streptomycin (Dayaram et al., 2017b; McCubbin et al., 2020).

Even if the SACs are decreasing in responsiveness to joint movements, the electrically evoked CAPs, which are independent of the sensory endings, further demonstrate a reduction in excitability with the

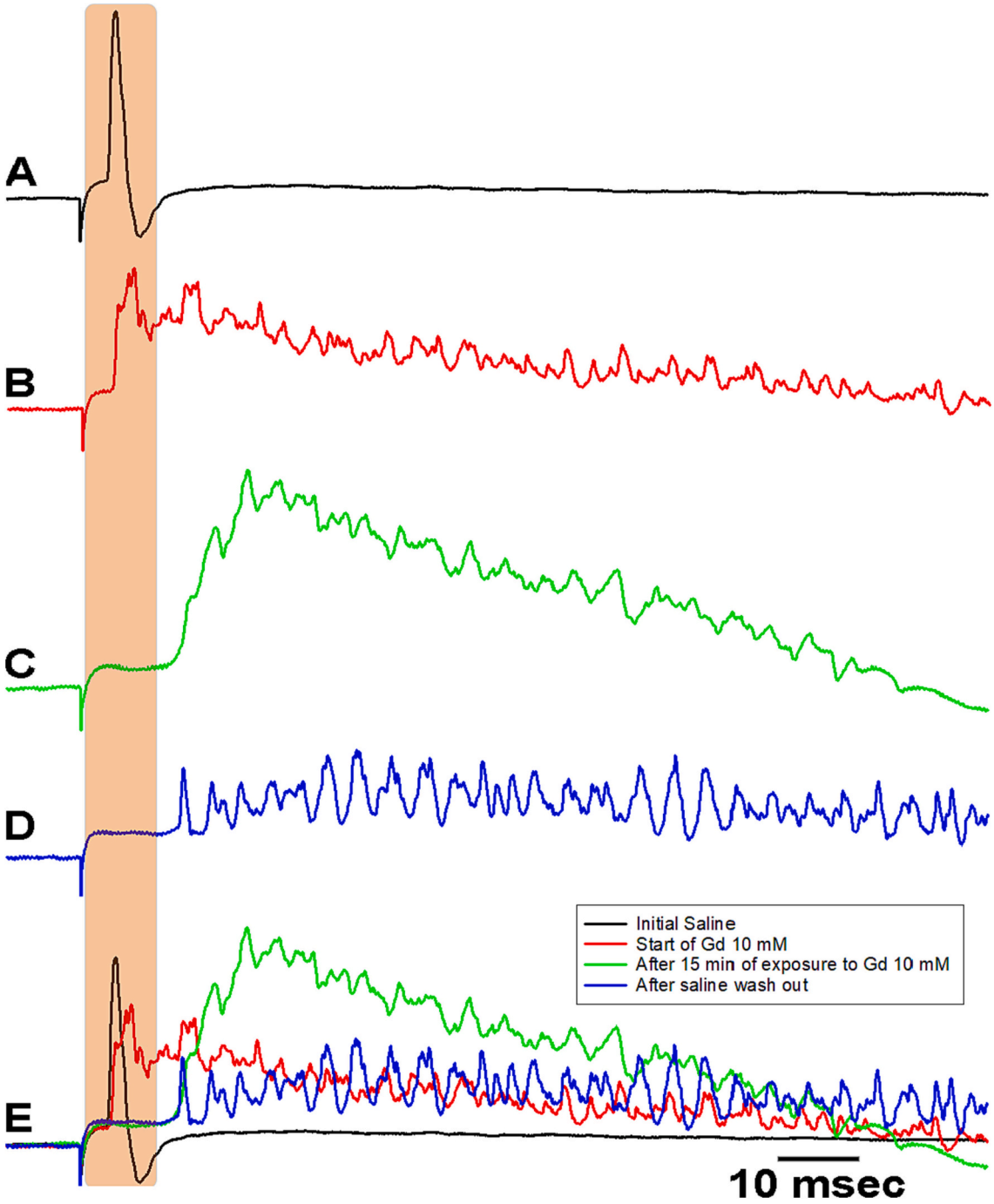


Fig. 9. Representative response of the effects of $GdCl_3$ exposure on the compound action potential (CAP) of the PD nerve. The media was exchanged from saline to one with $GdCl_3$ (10 mM) and then exchanged back to saline following three rinses of the bath with fresh saline. The same stimulus voltage was used to obtain a maximal CAP response in saline, during exposure to $GdCl_3$ and when exchanged back to fresh saline. Note that CAP amplitude is increased while the duration is prolonged. The shaded area indicates the region from the stimulus the initial CAP in saline was observed as a reference. (A) Saline. (B) After $GdCl_3$ (10 mM) exposure. Note that delayed CAPs occur. (C) After 15 min exposure to $GdCl_3$ (10 mM). (D) After exchanging the bath for fresh saline. (E) Superimposed traces, as all data is collected at the same scale.

higher concentrations of ferric chloride and ferric ammonium citrate. Thus, Fe^{3+} likely inhibits voltage-gated Na^+ or K^+ channels from initiating electrical responses. There is no precedence for Fe^{3+} blocking Na^+ channels; however, it was reported that Fe^{3+} blocks voltage-gated K^+ channels in snail neurons (Bukanova et al., 2007). If this were the case for the present crab preparation, the neurons may remain in a state of depolarization, unable to repolarize and induce the conformational change necessary to remove Na^+ channel inactivation. This would render the neurons unresponsive to bending at the joint or electrical stimulation in efforts to evoke CAPs. Thus, when the Fe^{3+} is removed by flushing with fresh saline, the neurons are likely able to repolarize and regain excitability.

It is of interest to address if the ferrous form of iron (Fe^{2+}) produces similar results to the ferric form. Other invertebrates such as freshwater crayfish and *Drosophila* preparations would be of interest to determine how the marine invertebrate investigated in the present study compares to their Fe^{3+} sensitivity. Studies addressing the effects Mn^{2+} on the same crab PD preparation revealed that Mn^{2+} also appeared to reduce the

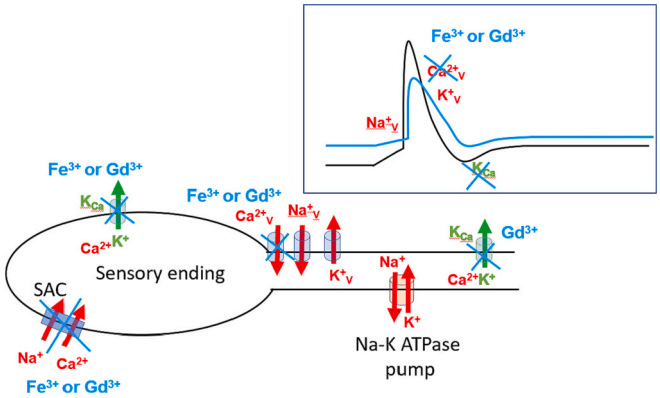


Fig. 10. Schematic model of the potential actions of Fe^{3+} and Gd^{3+} on the proprioceptive PD organ. Both ions appear to have a rapid action on the sensory ending, such as blocking the stretch-activated ion channels (SACs). The reduced Ca^{2+} entry would then reduce the activation of the calcium-activated potassium channels (K_{Ca}) in the sensory endings and in the axon. Both ions may also delay the function of the voltage-gated calcium channel (Ca^{2+}_V). It is also possible that Gd^{3+} may pass through the channel but not have an action on the K_{Ca}. Not illustrated is the potential of Gd^{3+}, but not Fe^{3+}, in also retarding the function of the voltage-gated potassium channel (K^+_V). As illustrated in the inset, the resting membrane potential may be in a depolarized state closer to threshold and prolonged in duration which would thus produce a compound action potential longer duration. The Ca^{2+} component in action potentials of crustacean neurons may be blocked by reducing the amplitude and blocking the repolarization by reducing the action of the K_{Ca} channel.

number of spikes at 30 mM with the use of two different compounds ($MnSO_4$ and $MnCl_2$). At 15 mM, $MnSO_4$ and $MnCl_2$ produced no significant acute affect (Pankau et al., 2022). However, CAPs were still obtained by electrically stimulating the nerve. Thus, it appears Mn^{2+} blocks the SACs in the sensory endings (Pankau et al., 2022). This is likely the same mechanism of action for Gd^{3+} (Dayaram et al., 2017a,b). Although conduction velocity was decreased upon Gd^{3+} exposure, the CAPs remained present while nerve activity in response to joint movement was greatly depressed in the intact PD organ.

Following the addition of 1 and 10 mM Gd^{3+}, spontaneous electrical activity resulted and persisted well beyond the stimulation producing the evoked CAP. Previous literature has established that lowering the extracellular Ca^{2+} concentration in the bathing media can cause spontaneous electrical activity in neurons (Atkins et al., 2021). Such activity was believed to result from a reduction in the function of calcium-activated potassium channels along the axon, thus contributing to excitability by reducing after hyperpolarization (Atkins et al., 2021). However, the definitive mechanism by which this phenomenon occurs is still debated (Atkins et al., 2021). If the voltage-gated Na^+ channels were blocked, one would expect the CAPs to gradually become smaller in amplitude as known for anesthetics when examining CAPs with amphibian and crustacean nerves (Saelinger et al., 2019). As Gd^{3+} is known to block Ca^{2+} channels, the neuron may behave in a similar manner as when being bathed in a low Ca^{2+} solution (Filipovic and Sackin, 1991). The effect of Gd^{3+} on Ca^{2+} channels, including calcium-activated potassium channels, may explain the spontaneous CAPs but does not entirely address the increased CAP amplitude and wide duration observed. If Gd^{3+} blocked the SAC in the sensory endings, the activity from bending of the joint would cease. However, in the axon, Gd^{3+} may pass through the voltage-gated Ca^{2+} channel in these crustacean neurons and maintain depolarization for a longer period of time. Thus, a prolonged CAP would occur as observed. It should also be noted that Gd^{3+} may not activate the calcium-activated potassium channel intracellularly, which would additionally contribute to prolonged depolarization, production of the additional CAPs and altered conduction velocity over time. If the membrane cannot repolarize well due to K(Ca) dysfunction, in addition to the blocking of voltage-gated K^+ channels, then conduction velocity over time could be altered. A summary of the present discussion is schematically shown in the model below (Fig. 10).

To understand the cellular mechanisms of the whole-nerve responses shown in this study, future experiments are needed to obtain single-channel recordings of the SACs and to identify the subtype of ion channels in the axon that are affected by exposure to Fe^{3+} and Gd^{3+}. Additionally, it would be of interest to record from single neurons rather than the entire nerve to examine the effects of these metals on the evoked action potentials and conduction velocity. The sensory axons of the crab PD nerve are small in diameter, which would make their isolation experimentally challenging. However, such procedures may be feasible in the larger motor neurons or by using lobster or crayfish sensory neurons within the muscle receptor organ (MRO) (He et al., 1999; Cooper et al., 2003; Leksrisawat et al., 2010).

5. Conclusions

Acute exposure to high extracellular concentrations of both Fe^{3+} and Gd^{3+} results in a reduction of proprioceptive activity. While 1 mM $GdCl_3$ was sufficient to consistently depress nerve activity, the effects of ferric chloride and ferric ammonium citrate were most notable at 20 mM concentrations. Due to the observed reduction in spike activity and CAP amplitude, it is possible that Fe^{3+} acts to inhibit SACs within the sensory units as well as signal conduction along the axon. As Gd^{3+} also reduced spike activity, it may act to inhibit SACs in a manner similar to that of Fe^{3+}. Elongation of the CAP duration and the occurrence of spontaneous electrical activity were not seen during acute exposure to Fe^{3+} as was the case for Gd^{3+} exposure. Thus, it is likely that Gd^{3+} additionally blocks Ca^{2+} channels within the PD organ, resulting in spontaneous activity. The discoveries on the effects of acute Fe^{3+} and Gd^{3+} exposure are vital in further understanding the specific mechanism of action as well as identifying a window of time in which neuronal recovery can be promoted following exposure. Such discoveries offer much potential in improving clinical knowledge and treatment of patients exposed to these compounds, such as in the case of intracranial hemorrhages or acute Gd^{3+} toxicity resulting from the frequent use of contrast dyes in imaging. In the future, it would be of interest to investigate the role of ferrous iron (Fe^{2+}) on proprioception and to further examine the cellular mechanisms by which Fe^{3+} and Gd^{3+} produce the whole-nerve responses observed in the present study.

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Institutional Review Board statement

Not applicable for invertebrates.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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