The Effect of Lipopolysaccharides on Primary Sensory Neurons in Crustacean Models

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Many types of gram-negative bacteria are responsible for serious infections, such as septicemia. Lipopolysaccharides (LPS), the endotoxins released from these bacteria, are responsible for inducing the immune response of organisms such as crustaceans, who have well-conserved Toll-like receptors. Little is known about the direct impact LPS has on primary sensory neurons apart from this immune reaction. Previous studies have demonstrated that motor neurons increase both spontaneous and evoked firing frequencies with LPS, but differences have been observed across species. Here, the effects of LPS from two strains of gram-negative bacteria (*Serratia marcescens* and *Pseudomonas aeruginosa*) on firing frequency of primary sensory proprioceptors in the crab propodite-dactylopodite (PD) organ and crayfish muscle receptor organ (MRO) is examined. These sensory organs correlate to mammalian proprioception, as the MRO is analogous to the mammalian muscle spindle, and the PD organ allows for the separation of motor nerve function from sensory neuronal transduction. The neuronal function of the two model organisms was studied through the stretch-activation of rapidly-adapting and slowly-adapting sensory neurons. Results indicated that there is no statistically significant impact on sensory transduction through the application of LPS; however, in the crab PD organ, the application of LPS from both strains decreased the nerve activity except when the LPS from both bacteria was applied together. In the crayfish MRO, there usually was an increase in nerve activity. In saline controls, there was also an increase in firing of the neurons in both preparations, but this also was not statistically significant. Interestingly, the MRO muscle fibers often contracted upon the addition of LPS, perhaps indicating that the known impact LPS has on motor nerve function is partially responsible for the results obtained.

Abbreviations: MRO-muscle receptor organ; PD-propodite-dactylopodite joint;

Keywords: Sensory, proprioception; crustacean, endotoxin

Introduction

Various types of gram-negative bacterial strains are commonly involved in human and veterinary hospital-acquired infections and septicemia. It is also known that gram-negative bacteria, such as S*erratia marcescens* (*S. m.*) and *Pseudomonas aeruginosa* (*P. a.*), which are fairly ubiquitous, not only infect humans and other land animals but also freshwater and seawater organisms (Lorenzon et al., 2002; Pien et al., 2010; Carl et al., 2014; Kim et al., 2015; Palavutitotai et al., 2018). The bacteria can also be transferred through the food chain (Özogul and Hamed, 2018). Crustaceans, as other animals,

live in environments which require an immune system to protect themselves from bacterial infections. The hemolymph of crustaceans can be invaded by bacteria which may cause septicemia. The actions of the immune system in crustaceans have been investigated over the years (see review by Hauton, 2012).

As in mammals, the endotoxin lipopolysaccharide (LPS), which is released from gram-negative bacterial strains, induces an immune response in crustaceans. A recently described prophenoloxidase activating system, which is an important immune response for arthropods, is known to be activated by bacterial endotoxins. The immune response can result in cells releasing factors (such as cytokines and nitric oxide) which cause tissues to respond (Chai et al., 2018; Rodríguez-Ramos et al., 2016). In mammals, some of these substances are responsible for the major aspects of the immune response. It is apparent that not all of the immune responses are beneficial to the host, such as altered neural and cardiac function (Eidelman et al., 1996; Wilson and Young, 2003; Friedrich et al., 2015; Tong and Zhou, 2017). However, even LPS itself can have direct actions on target tissue apart from, and in addition to, the immune response, such as skeletal muscle, the heart, and some neurons (Anyagaligbo et al., 2018; Cooper etal., 2019).

Several innate immune signal transduction pathways are evolutionarily preserved between species and encode for proteins involved in antimicrobial and antiviral processes. Toll-like receptors, possessed by both crustaceans and humans, have been broadly conserved across malacostracan, the largest crustacean class. Specifically, the Toll-interacting protein (TOLLIP) in malacostraca shares analogous features with mammalian TOLLIPs. In contrast, the Immune deficiency (Imd) signaling pathway, activated by the peptidoglycan layer in gram-negative bacteria, is thought to be reduced in crustaceans, but is still not well understood (Lai and Aboobaker, 2017).

While the immune response of crustaceans has been well examined, it is unknown if LPS itself has direct actions on sensory neurons in crustaceans or in mammals apart from the immune response. One study reported direct actions on motor neurons in crayfish by LPS from *S. m.* (2 µg/ml). This resulted in a Ca^{2+} ion leak into the motor nerve terminal, causing an increased frequency of the spontaneous fusion of synaptic vesicles and an increase in evoked (i.e., nerve stimulated) synaptic transmission (Parnas et al. 1971). If LPS can have an effect on motor neurons in crayfish, it is reasonable to postulate that sensory neurons may also be directly affected, causing altered responsiveness to stimuli and transmittance of electrical activity. In addition, the effects noted in a freshwater crustacean (i.e., crayfish) may also occur in seawater crustaceans (crabs, lobsters, sea water shrimp, and krill), despite increased ion concentrations in the hemolymph of seawater invertebrates. The efficiency and type of innate immune system may also vary among crustaceans. Thus, different responses to direct actions of LPS of a given bacteria may occur in various species of crustaceans. An earlier study on an amphibian (i.e., frog) neuromuscular preparation revealed the direct action of LPS from *Salmonella typhimurium* (10 µg/ml) on motor neurons. This also showed an increase in the occurrences of spontaneous vesicle fusion events, as in crayfish, but blocked the nerve evoked synaptic transmission (Person, 1979). The implication was that the membrane was leaky to Ca^{2+} ions but that voltage-gated Ca^{2+} channels were blocked by LPS. Thus, physiological differences occur on motor neurons in relation to exposure of LPS among these species. Recently, the larval neuromuscular junction of *D. melanogaster* was directly exposed to LPS from *S. m.* and *P. a.* Both the evoked synaptic responses and the amplitudes of the spontaneous quantal events were depressed, suggesting an inhibition of the postsynaptic glutamatergic receptors on the body wall muscle (Cooper et al., 2019). In addition, the body wall muscle and heart muscle transiently

hyperpolarized in response to LPS, potentially reducing the ability of the striated muscles from being activated (Anyagaligbo et al., 2018; Cooper et al, 2019).

Since investigations of the direct action of LPS on sensory neurons in mammals and in crustacean species is lacking, we tested this possibility in two model preparations used to study sensory neuronal function which have some functional correlation to mammalian proprioception. Crabs contain joint proprioceptors in their legs that possess sensory endings rooted within elastic strands that form a chordotonal organ. For this study, the propodite-dactylopodite (PD) chordotonal organ was used due to its accessibility in the leg and allowance for the most reproducible stimulus, as it monitors joint activity by the rate of movement and static position. The PD organ contains both dynamically sensitive neurons, meaning that they fire only throughout the primary movement, and statically sensitive neurons, which fire during the various joint positions and show range fractionation (Hartman and Boettiger, 1967; Cooper and Hartman, 1999; Cooper 2008; Dayaram et al., 2017; Malloy et al., 2017). These static position sensitive neurons, over time, demonstrate minor accommodation. This, along with the fact that the PD organ contains sensory endings separate from muscle fibers, enables for the exploration of the direct effects of LPS contained in a saline bath on the different categories of sensory neurons involved in proprioception apart from the complexities of motor innervation.

Crayfish contain the complex muscle receptor organ (MRO), which is analogous to the human muscle spindle. It is comprised of sensory endings rooted within muscle fibers. These endings are exposed as the fibers stretch, opening stretch-activated ion channels. Two categories of sensory neurons, the rapidly adapting and the slow adapting, are present and associated with their separate muscle fibers (Kuffler 1954; see Rydqvist et al., 2007 for a review). In dissected preparations, both the cell bodies and axons are exposed, enabling for direct contact with the saline solution containing LPS (Cooper et al., 2008). The effects of LPS exposure on the crayfish MRO can potentially be inferred to the effects one might obtain for a muscle spindle in vertebrates, although the receptors for LPS, such as the Imd and Toll-like, are likely of different types (Beutler 2004; Lai and Aboobaker, 2017). These sensory organs, which are isolated from the whole body, allow one to address the direct action of LPS on the function of the system without initiating an interfering immune response.

Material and Methods

Animals

The maintenance and animals used were the same as mentioned in previous reports (Malloy et al., 2017; Dayaram et al., 2017). In brief, Blue Crab (*Callinectes sapidus*) and Red Swamp Crayfish (*Procambarus clarkii*) were obtained from a distribution center in Atlanta, GA, and delivered to and bought from a local supermarket in Lexington, KY, USA. The crayfish (6-10 cm in body length and 12.5-25 g in body weight) were housed in individual standardized plastic containers with weekly exchanged dry fish food and oxygenated water (20-21°C). The Blue Crabs were maintained in a seawater aquarium prior to use for three to five days. All experiments were implemented in female adults with a carapace width (from point to point) of 10-15 cm. The crabs were fed with frozen squid and the water temperature was maintained between 14-16°C. The crabs and crayfish were caught from the wild, and most likely the crabs, as well as the crayfish, were two to three years old.

Electrophysiological recordings of proprioceptive sensory nerves

Procedures for dissection and preparation of the crab PD organ can be found in detail in

Majeed et al. (2013), Malloy et al. (2017) and Wycoff et al. (2018). Briefly, the Blue Crabs were obtained and checked for response to stimuli prior to autotomizing the first or second leg and placing it in a Sylgard-lined dish with crab saline. The PD nerve was then exposed and pulled into a suction electrode for recording. During the experiment, the dactyl was moved from a flexed position to an open position in a 1 s time frame, held for 10 s, and then moved back to the starting position. An insect dissecting pin was used to mark the displacement range, and each displacement was marked on the computer recording file. The crab saline used during recordings of the sensory nerves consisted of (in mM) 470 NaCl, 7.9 KCl, 15.0 $CaCl_2 \cdot 2H_2O$, 6.98 $MgCl_2 \cdot 6H_2O$, 11.0 dextrose, 5 HEPES acid and 5 HEPES base adjusted to pH 7.4.

Procedures for dissection and preparation of the crayfish MRO preparation can be found in Leksrisawat et al. (2010) and Dayaram et al. (2017). In brief, the dissected crayfish abdomen was placed in a Sylgard-lined dish filled with crayfish saline. The MRO was moved using a wooden dowel from a relaxed position to a stretched position in a 1 s time frame, held for 10 s, and then moved back to the starting position. An insect dissecting pin was used to mark the displacement range, and each displacement was marked on the computer recording file. The displacement rates were the same as for the crab PD organ. The crayfish saline used was a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2 \cdot 2H_2O$, 2.45 $MgCl_2 \cdot 6H_2O$, and 5 HEPES adjusted to pH 7.4). The concentrations of LPS for the various preparations are stated in the Results and were set based on the concentrations used in other literature. The two forms of LPS were chosen because of their ubiquitous nature and virulence in hospital-acquired bacterial infections like septicemia (Pien et al., 2010; Carl et al., 2014; Kim et al., 2015; Palavutitotai et al., 2018). LPS from *S. m.* and *P. a.* and all chemical compounds were obtained from Sigma (St. Louis, MO, USA).

Electrophysiology

Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracellular 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, Rhode Island, USA) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO, USA) obtained the signals to be recorded on a computer at a 10 or 20 kHz sampling rate.

The analysis of the electrical signals from the PD and MRO was processed by measuring the number of spikes within the first second, which covered the dynamic movement of the joint. The movement to the stretched position was made within 1 s. The stretched position was then held for another 10 s. The 8 s following the initial 1 s were used to measure the activity of the static position sensitive neurons as indicated in Figure 1. Three trials were performed for each time point. The activity from the set of three trials was averaged for each time point for both the 1 s and 8 s activity measures. Measures were made during saline exposure prior to saline tainted with LPS and after 20 minutes of exposure to LPS. Control experiments were performed with exchange of saline to saline without LPS for the same time periods.

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 LPS or saline as a control for exchanging the bathing media. An ANOVA was used for comparing the percent differences among treatments. The analysis was performed with Sigma Stat software. $P \leq 0.05$ was considered statistically significant. To examine the consistency and reproducibility of analysis, comparisons in analysis among different participants were performed on some of the same data sets.

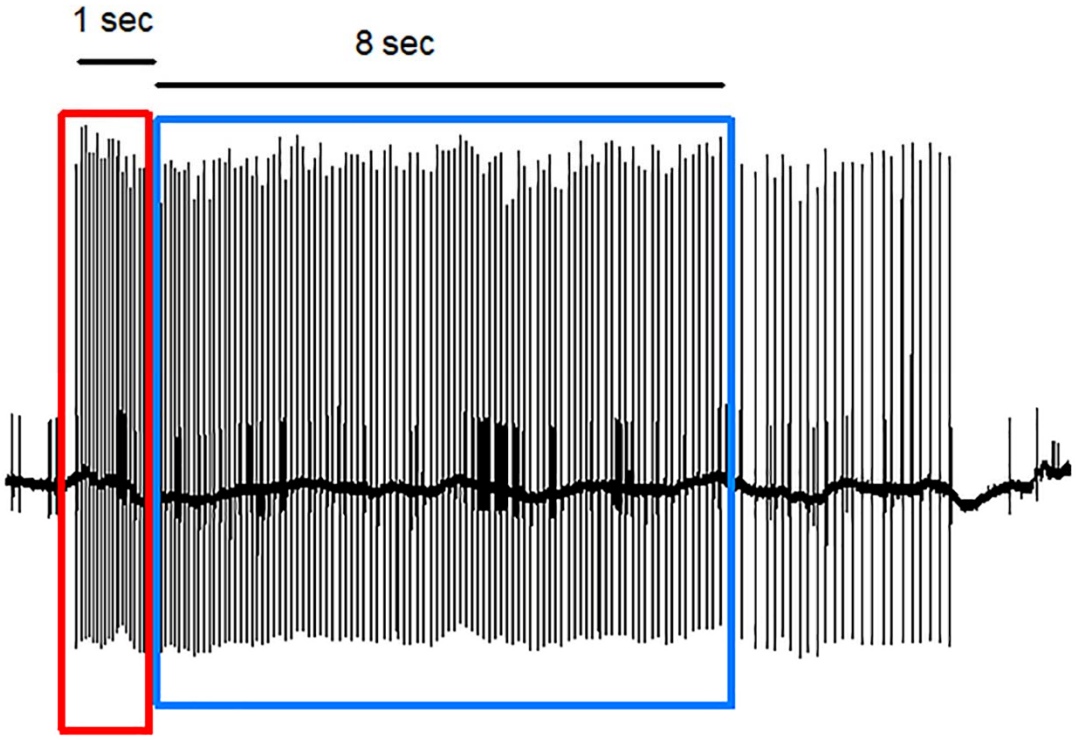

Figure 1: A representative trace of neural activity obtained from a crayfish MRO preparation. The extracellular recorded action potentials (i.e. spikes) from the nerves are shown during the 1 s displacement and static held position. An analysis of the number of spikes within the first second and the following 8 s was used for quantification of the neural activity.

Results

Following are the results from exposing both the crab PD organ and the crayfish MRO to LPS from *S. m.*, *P. a.*, and a combination of the two, as well as control trials with saline only. Activity from rapidly adapting neurons, which are active during the first 1 s of movement, and slowly adapting neurons, which are active during the 8 s held stretch, were distinguished.

Crab PD organ

The frequency of spikes measured in the dynamic movement of 1 s and in the static 8 s recordings of the PD nerve did not show a significant trend in alteration when exposed to the LPS from *S. m.* (Figure 2). The individual measures from each of the three movement and hold trials in saline and during the LPS exposure, after incubating in LPS for 20 min, of the 6 preparations is shown (Figure 2 A1, B1). The average of the three trials in saline and after exposure to LPS illustrated that 5 out of the 6 preparations showed a downward trend for the 1 s dynamic movements, although some changes were very slight (Figure 2 A2; $N = 7$, $p < 0.05$ non-parametric sign test). The averaged changes for the static measures varied, with 3 showing a downward trend and 3 showing an increasing trend with LPS exposure (Figure 2 B2). An average percent change for all 6 preparations for the dynamic activity showed a decrease (Figure 2 A3); however, the average percent change for the activity during the static hold before and during LPS exposure did not have a general trend (Figure 2 B3).

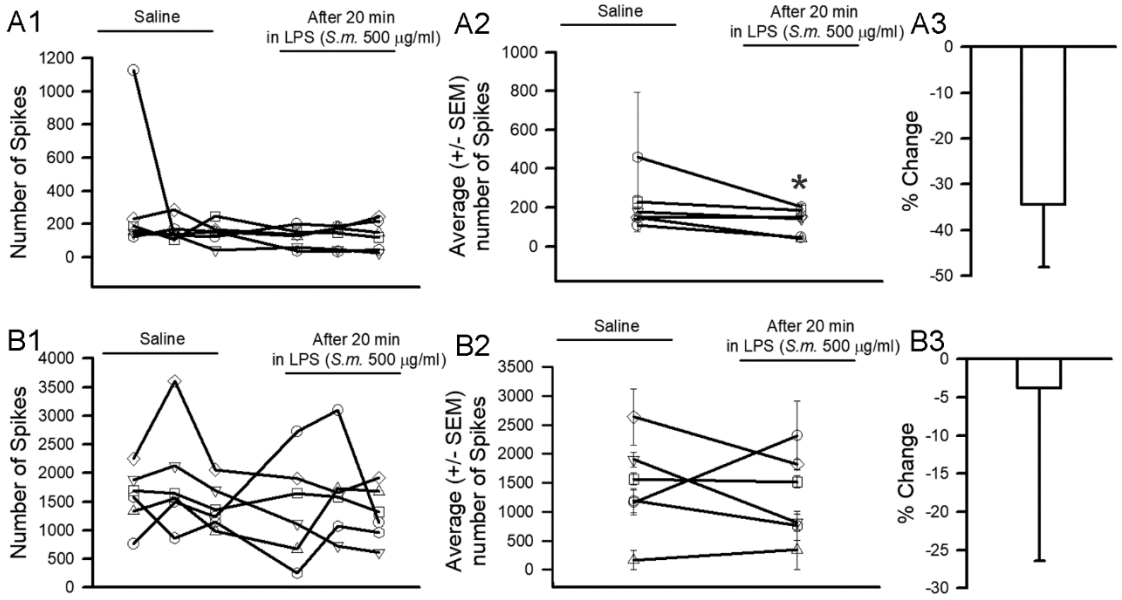

Figure 2: Activity of the PD organ before and during exposure to LPS from *Serratia marcescens (S. m.)*. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The dynamic displacement was significant N = 7, $p < 0.05$ non-parametric sign test. The effect of 500 µg/ml for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials and the average of the three displacement trials after 20 min exposure to LPS. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to LPS.

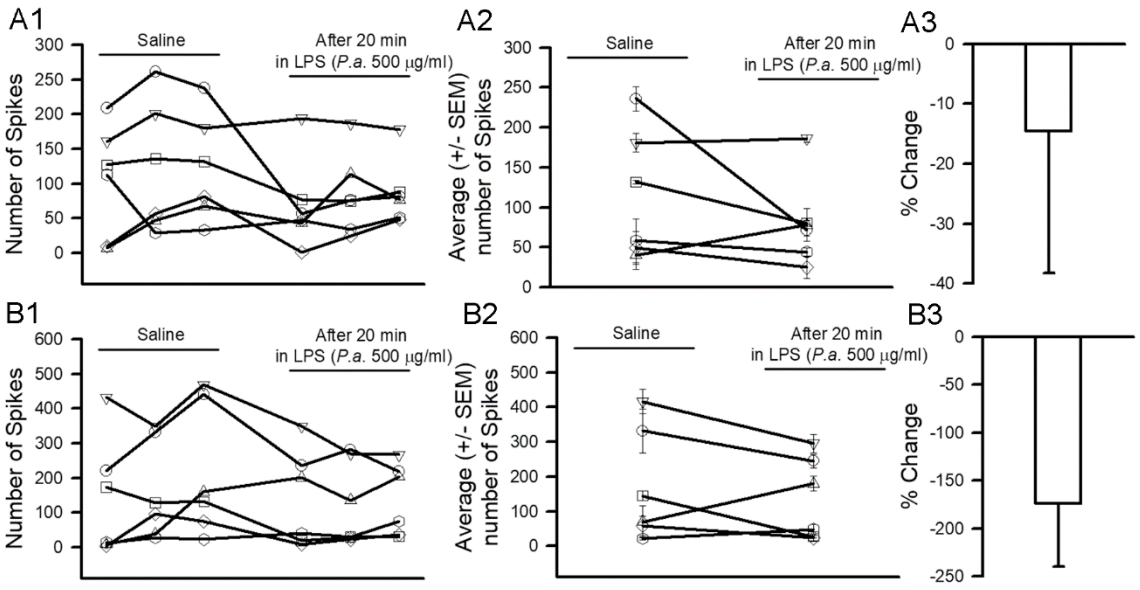

Figure 3: Activity of the PD organ before and during exposure to LPS from *Pseudomonas aeruginosa (P. a.)*. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The effect of 500 µg/ml for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials and the average of the three displacement trials after 20 min exposure to LPS. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to LPS.

The same analysis that was performed for before and during exposure to *S. m.* was done for exposure to *P. a.* No significant trends in neural activity occurred for the dynamic or static positional changes in the PD organ (Figure 3).

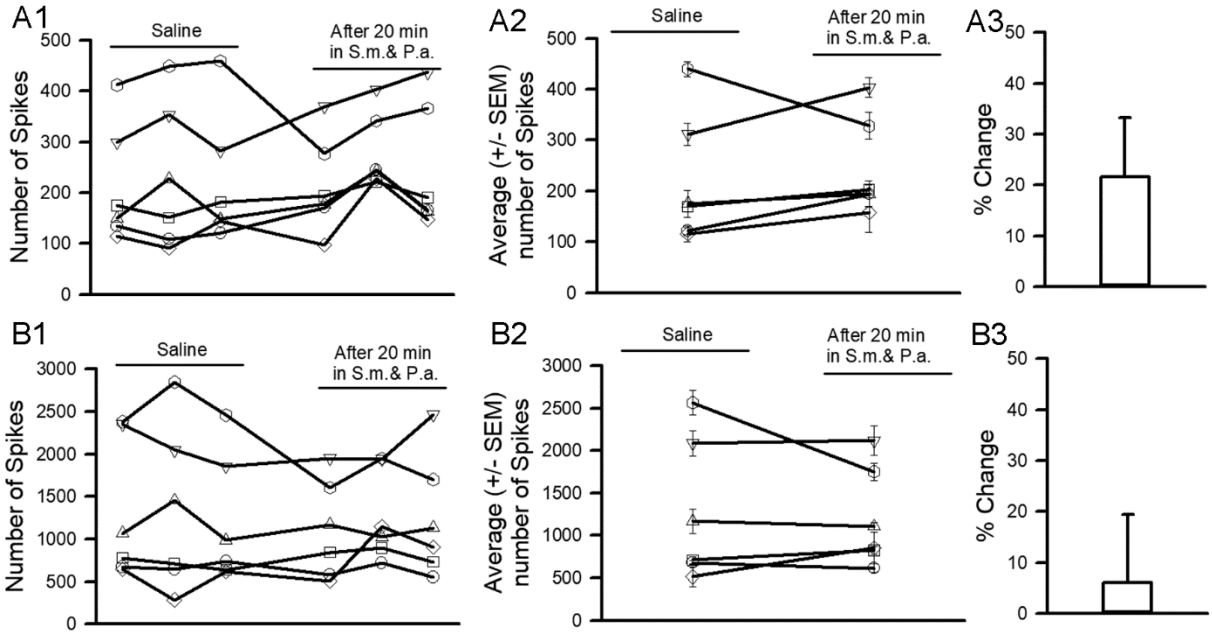

Figure 4: Activity of the PD organ before and during exposure to LPS from both *Serratia marcescens (S. m.)* and *Pseudomonas aeruginosa (P. a.)*. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The effect of 500 µg/ml for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials and the average of the three displacement trials after 20 min exposure to LPS. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to LPS.

When considering the effect of changing the bathing media in the experimental chamber and controlling for the 20 min period of incubation time in LPS, control experiments were performed where the bathing media was exchanged for only saline without LPS and held also for 20 min. No significant changes in neural activity occurred for the saline to saline control experiments (Figure 5). In considering potential effects of a combination in the two forms of LPS, they were mixed in saline and then exposed to the PD organ. With the individual LPS strains, no statistically significant trend occurred for the dynamic or static displacements (Figure 4). However, 5 of the 6 preparations now showed an increase in the frequency of activity for the

dynamic 1 s movement (Figure 4 A2) during LPS exposure and an average percent increase for the combined 6 preparations (Figure 4 A3).

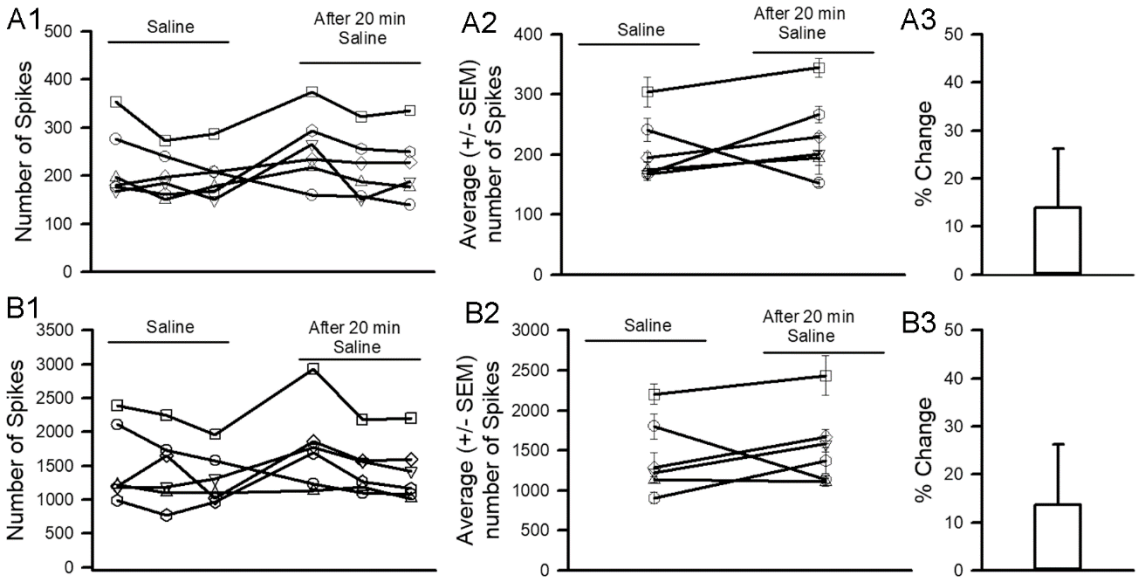

Figure 5: Activity of the PD organ before and after exchanging the bathing media for saline as control experiments for changing the bathing media and duration of incubation time for the LPS exposures. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The effect of saline exchanges for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials and the average of the three displacement trials after 20 min after a saline exchange. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to saline.

Crayfish MRO

The same series of experiments were also conducted for 6 crayfish MRO preparations as for the 6 crab PD organs. The frequency of spikes measured in the dynamic movement of 1 s and in the 8 s recordings of the MRO nerve did not show a significant trend in alteration when exposed to the LPS from *S. m.* (Figure 6), *P. a*. (Figure 7), or a combined LPS exposure (Figure 8). The saline-to-saline exchange which served as experimental controls also indicated no significant alteration in neural activity (Figure 9).

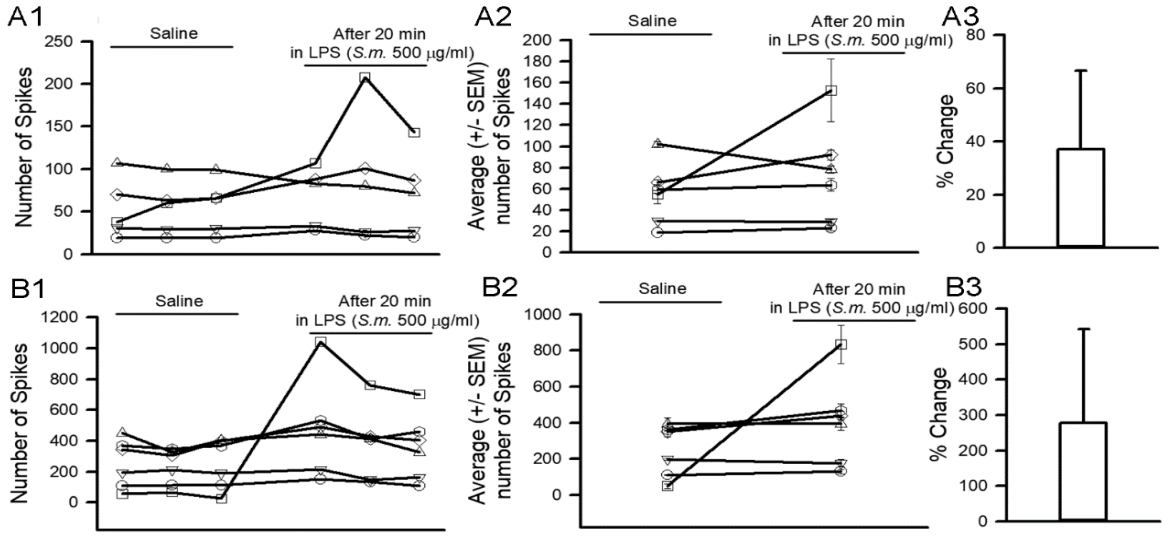

Figure 6: Activity of the MRO organ before and during exposure to LPS from *Serratia marcescens (S. m.)*. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The effect of 500 µg/ml for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials to the average of the three displacement trials after 20 min exposure to LPS. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to LPS.

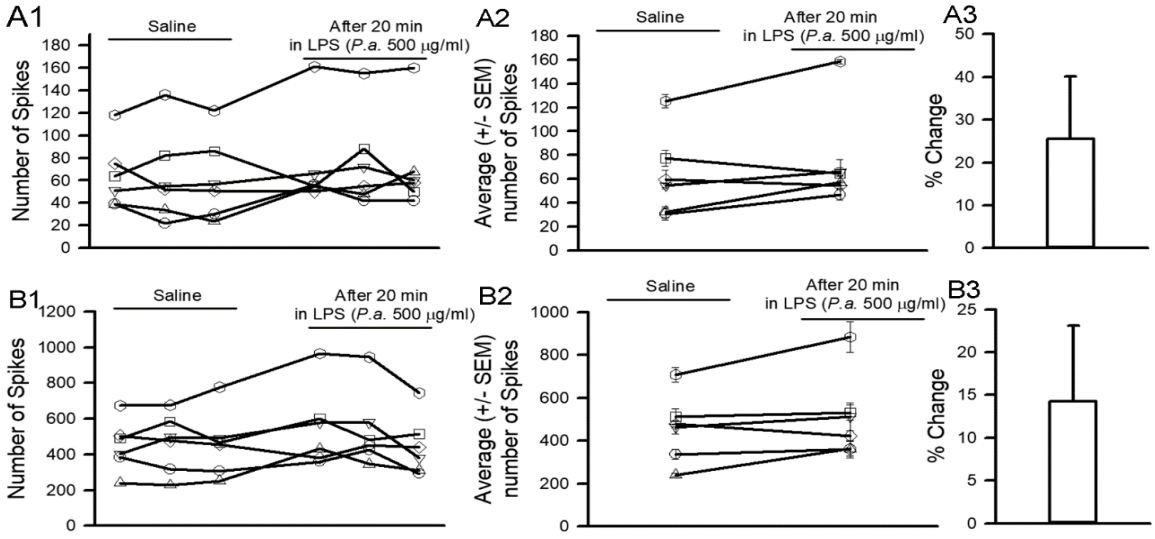

Figure 7: Activity of the MRO before and during exposure to LPS from *Pseudomonas aeruginosa (P. a.)*. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The effect of 500 µg/ml for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials to the average of the three displacement trials after 20 min exposure to LPS. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to LPS.

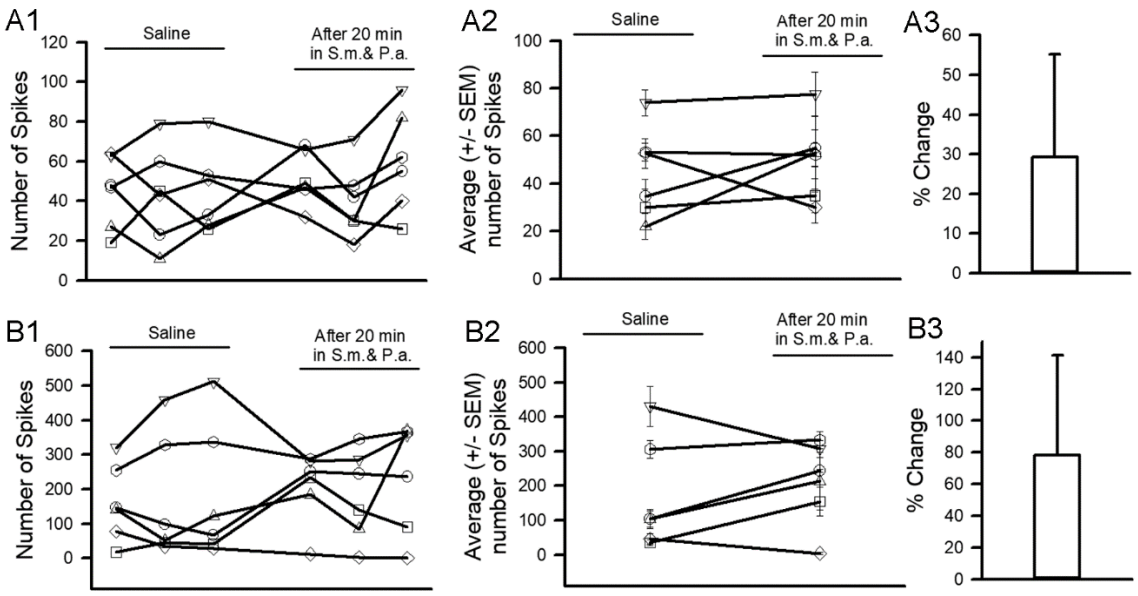

Figure 8: Activity of the MRO before and during exposure to LPS from both *Serratia marcescens (S. m.)* and *Pseudomonas aeruginosa (P. a.)*. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The effect of 500 µg/ml for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials to the average of the three displacement trials after 20 min exposure to LPS. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to LPS.

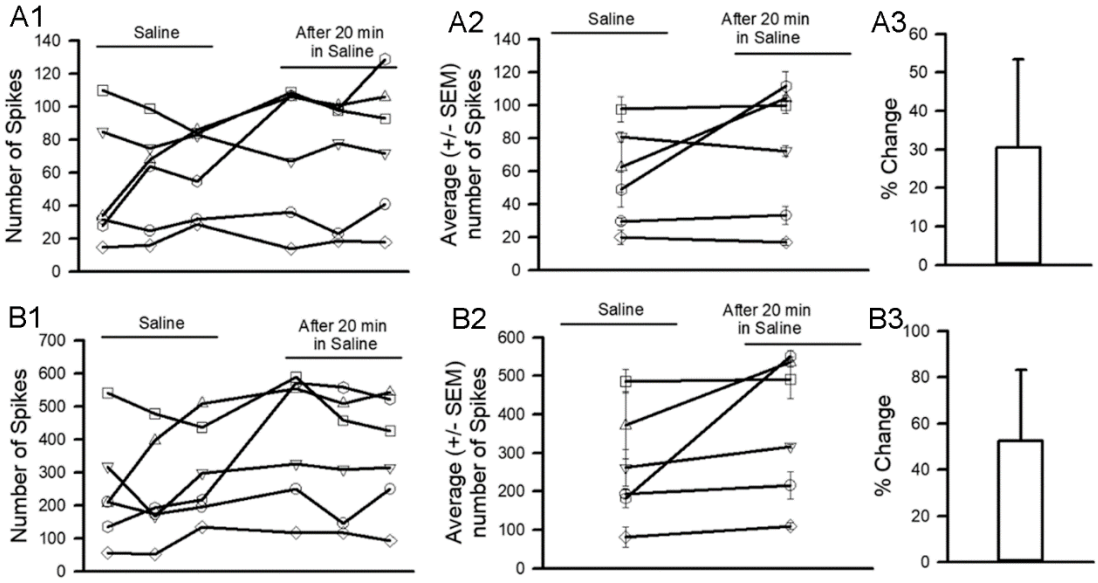

Figure 9: Activity of the MRO before and after exchanging the bathing media for saline as control experiments for changing the bathing media and duration of incubation time for the LPS exposures. The activity of the initial displacement within 1 s (A1) and the activity for static responses for 8 s (B1) after the initial first second of recording. The effect of saline exchanges for six preparations are shown. (A2, B2) The average (+/-SEM) of the three saline trials and the average three displacement trials after 20 min after a saline exchange. (A3, B3) The overall percent changes of the averaged (+/-SEM) values for activity in saline to saline.

Reproducibility in analysis of data sets

Analysis of the number of spikes (extracellular signals) with various participants in a classroom setting or in a data set provided to an individual to analyze may result in biases even with the use of automated software, due to differences in analysis procedures. In these sets of experiments, spurious electrical activity can be obtained in extracellular recordings when the recording electrode is moved or touched while displacing the joints of the crab leg or crayfish abdomen in the recording chamber. The degree of reproducibility in analysis of a data set was compared with different individuals who did not know the experimental conditions or if the preparations were from the crab PD organ or the crayfish MRO.

The same data file was used for analysis among three groups. Group 1 in the data set knew the experimental preparation was from the MRO and conducted the experiments (data from group 1 are the red lines, Figure 10 A1, B1). From visual observations, this group knew that the electrical activity could be picked up from the muscle or cuticle touching the recording electrode. When providing the same data set to group 2 or group 3, different measures in the frequency of activity were obtained, but the files were blind to the participants. While using the same software for analysis, these differences occurred.

In determining the number of spikes in the 1 s or 8 s measures of activity, a threshold discriminator was used to detect the deflections. The number of deflections were then collected for the dynamic movements and for the static held position. A sine wave fitting curve in the automated analysis of the software (Chart 7

version, ADI) was used as the baseline sometimes slowly changes with the movements of the preparations. The standard deviation of the mean from the baseline can be varied to capture or not capture small signals in the raw traces, meaning the original trace containing both baseline noise and spikes of nerve activity. The small signals are often noise, but they may be mixed with activity from the nerve that produces a smaller amplitude spike, perhaps due to a less tight seal with the recording electrode, or slippage of the nerve from the electrode. The crab PD organ also is innervated by many small neurons, and some of this activity shows up on the trace as very small signals that may be mixed in with the baseline noise. The important aspect in analyzing these traces is to be consistent throughout with what amplitude a person considers noise versus a spike from nerve firing. This is illustrated in Figure 10C with the blue ellipses capturing noise in the baseline. Notice the blue arrow in Figure 10C indicating the small circles that show which deflections are being counted. The green ellipse depicts a region in the baseline where the noise is small and is not being captured as a deflection with the set standard deviation for this analysis. Note the arrows for groups 1, 2 and 3 in Figure 10A2 and 10B2. Due to the detecting of different amplitudes of deflections in the noise within the trace, the general levels above the baseline shown will result in different measures in the number of spikes.

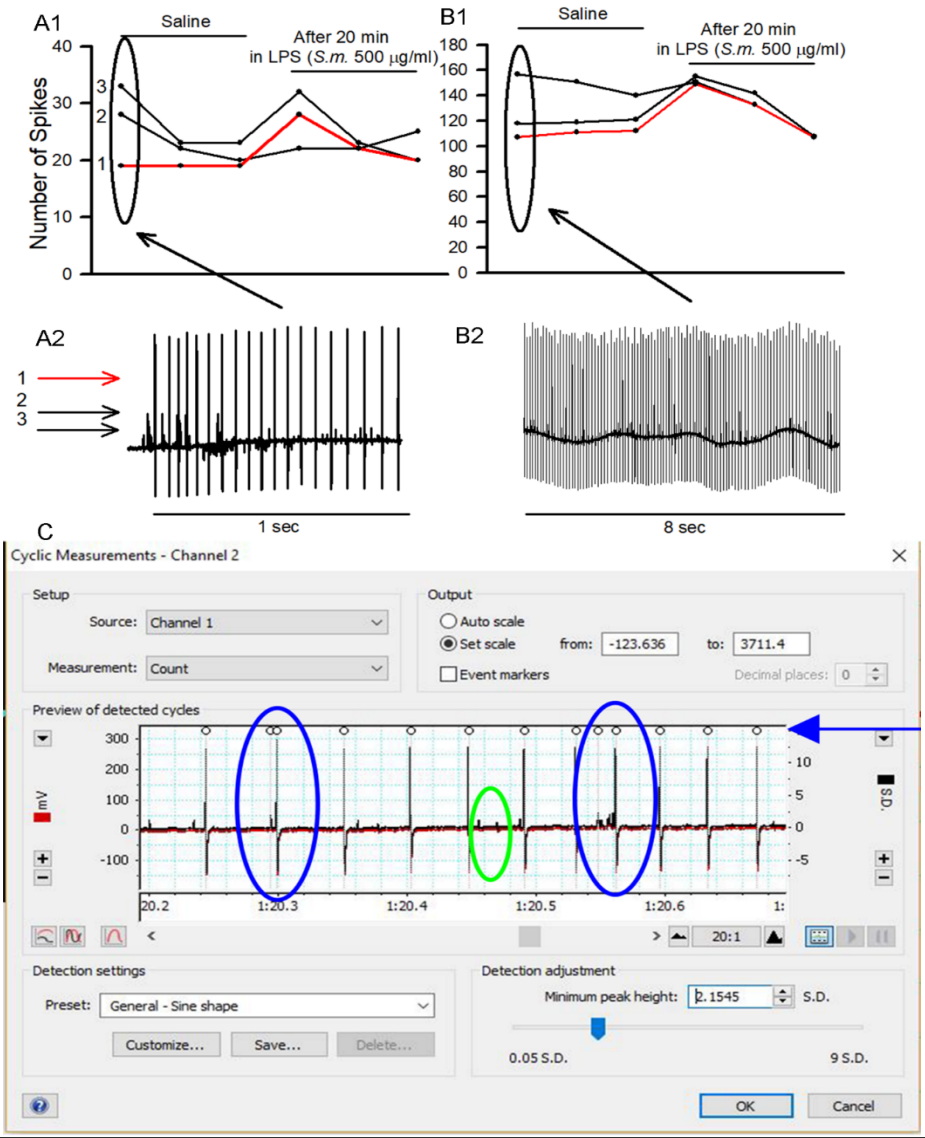

Figure 10: Reproducibility in analysis of a data set. The top panel shows the analysis for the number of spikes measured during the 1 s displacements (A1) and 8 s static positions (B1) for the three trials in saline and three trials in LPS after incubation for 20 minutes. The raw traces for the first trial of the 1 s movement (A2) and the first trial of the 8 s of the static position (B2) are shown. The analysis by the three different groups of participants are labelled as 1, 2, and 3. Group number 1 conducted the experiments for the data set and received extensive training in the measures for extracellular recordings in these preparations. Groups 2 and 3 were given the data sets for analysis with little training and only a brief exposure to the experimental preparations. (C) The window within the software where the analysis indicates which deflections are being collected as spikes. The sine wave fitting curve is used for the curve fitting to the raw trace to follow baseline movements. The standard deviation of the mean from the baseline can be varied to capture or not capture small signals in the raw traces. The blue arrow points to the small circles within the analysis window indicating which deflections are being measured. The blue ellipses show extra measures being obtained by capturing noise in the baseline. The green ellipse depicts a region in the baseline where the noise is small and is not being captured as a deflection.

Discussion

In this study of the direct effects of LPS on primary sensory neurons, it was shown that the endotoxin did not demonstrate clear, direct effects on the crab PD organ or the crayfish MRO proprioceptor. Only the PD organ before and during exposure to LPS from *S. m.* showed a trend for the initial first second of recording in the dynamic displacement with a non-parametric sign test. This may mean that the rapidly adapting neurons are more sensitive to *S. m.* than the slowly adapting neurons. However, decrease in the activity was very slight, demonstrating that stretch-activated and voltage-gated channels work normally without any significant change in sensory transduction. There was a large degree of variability among preparations when exposed to the two different forms of LPS. Comparisons with control experiments of saline-to-saline exchanges indicated as much variation in the changes in activity as exposure to LPS. Even though 5 out of 6 preparations showed some of the activity for dynamic movements or the static positions (i.e., MRO for the 8 s of holding the joint static during the combined exposure to *S. m.* and *P.a.*), several of the changes were not very large and not considered significant by a sign test among preparations. It was noted that, as the movement was performed on the MRO, the muscles seemed to tighten and contract on their own after the addition of LPS. As Parnas et al. (1971) discovered, the motor neuron innervating the crayfish abdominal muscle demonstrated increases in evoked synaptic response from the endotoxin LPS released from *S.m.* Since the primary sensory neurons are embedded in muscle fibers, the effects may in part be contributed by the innervating motor neurons on the fibers. Despite this observed phenomenon, a consistent increase in MRO activity did not occur.

The electrophysiological recordings of the primary sensory nerves in the crab PD organ are devoid of any effect on synaptic transmission within the sensory neuron by LPS. If there were direct effects, they would likely be on the stretch activated ion channels in the sensory endings, on input resistance of the membrane through leak channels, or on the ionic channels responsible for electrical conduction. The action of LPS on the motor nerve terminals needs to be addressed in the context of the anatomical differences of the crayfish MRO and the crab PD organ. As mentioned, Parnas et al. (1971) demonstrated an increase in evoked synaptic responses by LPS from *S.m.* when the motor nerve was stimulated in an abdominal phasic muscle of the crayfish. They also reported an increase in spontaneous quantal events in muscle fibers. We have not found any reports on the action of LPS on synaptic transmission on neuromuscular junctions of crabs or direct effects on primary sensory neurons of any crustacean species.

The muscle fibers of the MRO of the crayfish may increase their stiffness by the activity of the motor neurons. The motor neurons are contained within the nerve in which the sensory neurons are being monitored. Even though the motor neurons are not being electrically stimulated by the recording electrode, the nerve terminals innervating the muscle may alter the spontaneous occurrences of individual synaptic vesicles. The resultant effect could be graded from mild to more intense local contraction of the muscle. The innervation of the MRO is complex, as there is not only innervation by excitatory glutamatergic motor neurons but also inhibitory GABA-ergic motor neurons (Kuffler, 1954; Rydqvist et al. 2007). The increased force of the muscle fibers would be relayed to the sensory endings. Thus, the resting position of the MRO prior to displacement may show an increase in firing frequency of the sensory neurons. If the muscle is stiffer due to an increase in tension in the fibers from the motor neurons, it may show an increase in firing during the passive movements. The complexity of the MRO's innervation may require a more complex and rigorous experimental setup. One approach to resolve this possibility would be to record the force of the MRO fibers with a force transducer while passively stretching them over a

displacement range, as well as recording intracellularly in the MRO muscle fibers to measure the potential change in the spontaneous occurrence of quantal synaptic events. However, these MRO muscle fibers are very small, single fibers that are easily damaged when removed, and the intracellular recordings of these small muscle fibers were beyond the scope of this project.

As for the PD organ in the crab leg, these sensory neurons are embedded directly into an elastic strand spanning the joint without muscle fibers markedly influencing the activity of the sensory neurons while the joint is being displaced. Since the induced displacement starts in the most flexed state, is physically moved to the extended state within 1 s, and held for at least 10 s before physically being moved back to the flexed state, any stiffness of the muscle fibers attached to the same apodeme as the PD organ would have minor impact. No spontaneous contraction was observed when releasing the joint from the extended state, like what occurs in the MRO, and it was still required for the joint to be physically moved by the experimenter to the flexed state prior to the next displacement trial.

LPS did not directly impact the firing frequency of the primary sensory neurons in the PD organ, indicating that the stretch-activated and voltage-gated ion channels in the axons continue to function without significant change. It can also be inferred that LPS does not impact leak channels in the sensory neurons to change the threshold of inducing action potentials. Similarly, the sensory system in the crayfish MRO did not demonstrate any significant change in firing frequency. To control variability in exchanging the saline bath with a bath containing LPS, the saline bath was replaced with new saline as a control for the mechanical disturbances of the sensory organs. Similarly, no significant changes in the frequency of the spikes were noted for the control runs.

Only two forms of LPS were investigated in this study. Given that there are many different forms and concentrations of gram-negative bacteria in the marine and freshwater environments in which these animals live, further studies examining these other forms and concentrations of LPS may reveal effects on the types of sensory neurons used in this study (Colwell et al., 1975; Scott and Thune, 1986; Madetoja and Jussila, 1996; Popović et al., 2014; Loureiro et al., 2015). Even within bodies of marine or freshwater, there are local differences in the potential exposure of various forms of bacteria, considering the ecological range and what they are consuming in their diets. This could mean that greater affects may possibly be seen with increased concentrations of the LPS or with different strains of gram-negative bacteria. *Callinectes sapidus* (commonly referred to as the Blue Crab) are commercially harvested from Chesapeake Bay in the east coast of the United States throughout the gulf coast of Texas and Mexico (Johnson, 2015). The freshwater *Procambarus clarkii* (commonly referred as the Red Swamp Crayfish) is found in a wide geographical range as it has been introduced in Asia as a food source and is an aggressive invading species. As such, it is found in northern Mexico and the Great Lakes of the United States and Canada, as well as throughout Southern Europe to the Scandinavian Peninsula (Crandall, 2010; Johnson, 2015; CABI, 2017).

Given that these species are edible and farmed commercially, it is of interest to know more about the effects that various potential bacterial infections can have on their health. It is also of importance to examine combinations of various types of LPS from different species of bacteria, as it is unlikely that a condition of septicemia would result from a single bacterial species in these animals, just as it is uncommon in humans in most cases (Sancho et al., 2012; Bouza et al., 2013). It is known that some shrimp are exposed to bacteria *Vibrio parahaemolyticus* and *Vibrio alginolyticus* which produces protein complexes that make ionic pores in the cells of the gastrointestinal tract. This leads to bacterial entry into the body and death of the shrimp, causing large financial losses for shrimp farmers

(Lee et al., 2015; Theethakaew et al., 2017; De Schryver et al., 2014). It is noted that the infected shrimp have an increase in activity of Toll-like receptors and the presence of lysozyme in the hemolymph (Hong et al., 2016).

There may be synergistic effects of LPS action on targeted tissue, or even competing effects if LPS of different bacteria is binding to the same receptors or proteins. LPS is a primary trigger of the innate immune response by activating cells containing Toll-like receptor 4 (TLR4) known as the CD14/TLR4/MD2 receptor complex (da Silva Correia et al., 2001; Park and Lee, 2013). Further studies with LPS from different bacteria and in varying concentrations could potentially demonstrate these competing or synergistic effects.

The duplicate analysis by different participants in a student laboratory revealed differences in determining the frequency of extracellular signals (i.e., spikes) in the same data set. Such independent analysis is important to consider, as one individual may be unconsciously biased while analyzing signals from perceived noise in the recordings. The analysis of the spikes may vary depending on the threshold one uses to measure the signals from the noise. This is not usually a concern for the crayfish MRO preparation, as one or two spikes are detected since only the dynamic and static position sensitive neurons are present. However, if the signals are small due to a low seal with the nerve and the suction electrode, then the signal-to-noise ratio could be small. The PD organ can be more challenging for reproducibility in analysis as there are many more sensory neurons associated with the nerve, each giving a different amplitude spike. The small axons produce small field potentials, so they can fall within the background noise, making it difficult for different people to set a standard threshold for analysis if not specifically shown how. In making this point, data sets for the crayfish MRO were used. One set was provided to students without explicit details in the way the analysis was performed for determining which amplitude spike to record.

Submitting data for accessibility with peer-reviewed publications is a growing trend required of various journals. However, this is not advantageous for examining reproducibility of analysis without explicit instructions. Videos are being developed for data analysis in various files in this study to see if this improves reliability in the analysis. The inclusion of videos or textual packets detailing analysis methods may be a worthwhile approach in the future for anyone placing data files in a repository associated with publications. Additionally, for software in which thresholds in analyses of the signals can be reported, the cut-off limits, or standard deviation from the baseline, should be listed. However, this value can change for each data file and within each file, so this becomes a large undertaking to present for a publication with many data sets. Documentation can potentially be listed with archived data files individually.

In this study we examined the direct effects of LPS on tissues apart from the immune response which would occur within intact organisms. There is still much to be addressed on the direct effects of LPS on the nervous system in all animals, including humans. Over a million people within the United States are hospitalized with septicemia every year, with death occurring in some cases (Anderson et al., 2014; CDC Statistics, 2017). By addressing the direct actions of LPS apart from the immune response, future medical treatments and pharmaceuticals can be developed to better treat such illnesses, in addition to improving issues in aquaculture used for human and animal food supply.

Endnotes

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 the LPS endotoxin could influence proprioception and sensory function. This course project is part of a new trend in teaching science to undergraduates (Linn

et al. 2015). Course-based undergraduate research experiences (CUREs) are relatively new and an approach being adopted by science educators in high schools and colleges (Bakshi et al. 2016).

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