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Effects of bacterial endotoxin on regulation of the heart, a sensory-CNS-motor nerve circuit and neuromuscular junctions: Crustacean model

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ABSTRACT

Eatable crustaceans are susceptible to bacterial septicemia from injury or a compromised immune defense, which can possibly have detrimental effects in mammals that consume them. Since many crustaceans (i.e., crabs, lobsters and crayfish) are used for animal food and human consumption, it is of interest to understand the effects potential bacterial infections can have on their health as well as ours, including effects on cardiovascular and neuromuscular activities. The Red Swamp crayfish (*Procambarus clarkii*) was used as a model crustacean to investigate the effect of direct exposure to isolated endotoxin lipopolysaccharide (LPS) and the associated peptidoglycans from gram-negative bacteria (*Serratia marcescens*). *S. marcescens* is a common strain identified to cause septicemia in mammals and is prevalently found in nature. LPS injection into the hemolymph of crayfish revealed acute changes in heart rate and effects on survival. Direct LPS exposure on an in situ sensory-CNS-motor circuit produces a decrease in recruiting of the motor nerve at 500 μg/ml but has no significant effect at 100 μg/ml. At the isolated neuromuscular junction, the direct action of the LPS endotoxin (500 μg/ml) enhances evoked synaptic transmission, while not altering facilitation. Also, the amplitude and the frequency of spontaneous vesicle fusion events was not altered by LPS exposure. However, the resting membrane potential of the muscle transiently hyperpolarizes. These direct actions on tissues appear to be independent of innate immune responses and suggest the LPS targets on these tissues have a role in excitability of cellular function. {242 words}.

1. Introduction

Systemic exposure to lipopolysaccharides (LPS) from gram-negative bacteria triggers a complex immune response in humans and other animals. LPS binds to Toll-like receptor 4 (TLR4) known as the CD14/TLR4/MD2 receptor complex (Yoshida et al., 1996; Steiner, 2004), which is highly conserved from arthropods to primates (Levin and Malik, 2017). These receptors are located on various cells, such as neurons, skeletal muscle, and cardiac muscle. Surprisingly little is known about the direct action of LPS on cells through the CD14/TLR4/MD2 receptor complex, or on other targets, to result in cellular changes independent of responses caused by the secondary immune response, such as secretion of cytokines. There are several putative receptors to initiate an innate immunity in arthropods (e.g., insects and crustaceans), and their expression profile in various tissues is not fully known. The targeted action of pathogens and subsequent gene regulation reveals many downstream effects in expression profiles (Beutler, 2004; Lai and Aboobaker, 2017). It is likely that the pathogen's receptors (e.g., Toll, Imd and JAK-STAT) are similar among species along with some species' unique forms (Coscia et al., 2011; Loker et al., 2004). However, the effects may vary greatly depending on the species and tissue investigated (Loker et al., 2004). It is of interest to examine the kinetics in the response of various tissues and to learn more on the *non-transcriptional* processes and their effects. Since commercially obtained LPS is likely a mixture of associated peptidoglycans (Cooper et al., 2019; Kaneko et al., 2004; Lai and Aboobaker, 2017), there is a greater potential for broader effects and possible binding to multiple receptor subtypes than for highly purified LPS forms, but the effects reported from preparations exposed to LPS and associated peptidoglycans still represent the effects an animal or tissue would be exposed to from a given strain of gram-negative bacteria.

Many crustacean organisms can be used as models to evaluate the effects of bacterial-derived endotoxins and polysaccharides on systemic and tissue-specific functions, such as effects on cardiac and neural physiology. In particular, crustaceans are susceptible to bacterial septicaemia by injury or through a leaky gastrointestinal track. A strain of

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bacteria (i.e., *Vibrio parahaemolyticus* and *Vibrio alginolyticus*) that has acquired a plasmid can produce protein complexes (eg, PirAvp and PirBvp), which make ionic pores in the cells of the gastrointestinal tract leading to rapid death of the host (Lee et al., 2015; Theethakaew et al., 2017). This issue is causing a large economic loss to the shrimp industry worldwide. Since many crustaceans (i.e., crabs, crayfish) are used for animal food and human consumption, it is of interest to know more about the effects various potential bacterial infections can have on their health. Here, we use the Red Swamp crayfish (*Procambarus clarkii*) as a model crustacean to study since it is an eatable crustacean, and there is substantial technical information on the neurobiology and physiology to draw on for assessing and comparing cardiac, neuronal, and skeletal muscle function (Wiese, 2002; Asakura, 2011).

In freshwater streams, lakes, and swamps there are local differences in strains of bacteria to which the Red Swamp crayfish is exposed. This crayfish species occurs in a wide geographical range, as it has been introduced in Asia as a food source and is an aggressive invading species found from northern Mexico to the Great Lakes of the United States and Canada, as well as throughout Southern Europe to the Scandinavian Peninsula (Crandall, 2010; Invasive Species Compendium, 2018; Nagy et al., 2015). Considering *Serratia marcescens* is known to play a role in septicemia in humans and other animals (Mahlen, 2011; Olexy et al., 1979; Piening et al., 2017; Villalon et al., 2018), we focused the responses of the exposure to LPS from this strain to build a foundation of experimental protocols for further examining other strains and combinations of LPS forms from multiple bacteria. The concentrations examined in previous studies vary depending on the species of animal or animal tissue examined. It appears rodents can withstand very high concentrations of LPS systemically as compared to humans (Fink, 2014). It was determined that the optimal lymphocyte-activating concentration of peptidoglycan on mouse lymphocytes was in the range of 400–1000 micrograms/ml (Dziarski, 1987). The concentration to kill half the number of mice exposed to LPS is 1–25 mg/kg, which is noted to be in the range of 1000-fold to 10,000-fold greater than the dose of LPS to cause severe illness in humans (Dziarski, 1987; Taveira da Silva et al., 1993). Crayfish, specifically *Procambarus clarkii*, live in freshwater where there are local differences in various forms of bacteria. Their diets consist of dead and live animal tissue, plants, and detritus in the soil where there are many forms of LPS and combinations of LPS types, which can be studied to examine the effect on this species. In this study, the focus was on *S. marcescens* as a foundational study to compare to other reports in insects and crustaceans. Considering *Procambarus clarkii* is an edible crustacean and farmed commercially in Asia and Europe as a food source (Crandall, 2010; CABI, 2017), it is of interest to know more about their health and the effects of potential bacterial infections. This study utilized concentrations of 500 μg/ml and higher for comparative purposes to those used previously in investigation with Blue Crab (*Callinectes sapidus*) and the insect, *Drosophila melanogaster* (Anyagaligbo et al., 2019; Cooper et al., 2019; Istas et al., 2019; Stanback et al., 2019)

The immune response in the whole animal is complex, so in order to address the effects of pathogens, specific tissues can be isolated or cultured cells can be used. Isolated systems can help to address the direct actions of LPS from the secondary immune response. Isolated neuromuscular junctions (NMJs) offer convenient preparations to investigate the effects on synaptic transmission in the occurrence of spontaneous quantal responses and evoked postsynaptic junction potentials. However, to address the effects on neural circuits, some preparations offer advantages to keep the CNS or segments of it intact without compounding humoral responses. Larval *Drosophila* have served an advantage in this regard (Cooper et al., 2019; Istas et al., 2019). Crustaceans offer similar advantages. Even with an isolated nervous system the varied non-neuronal cell types present can have a compounding effect on the actions of LPS secondarily impacting neurons. In the central nervous system of rodents, LPS increases release of cytokines from microglia, which then act on neurons. This leads to an increase in intracellular Ca^{2+} and can induce apoptosis (Calvo-Rodríguez et al., 2017; Liu et al., 2014; Cui et al., 2018). However, in other studies with rat hippocampal CA1 neurons, LPS appears to suppress NMDA receptor-mediated excitatory postsynaptic currents via blocking Ca^{2+} entry and is assumed to be a rationale for the amnesic action of bacterial sepsis (Jo et al., 2001). In the insect *Drosophila*, direct application of LPS dampens induced sensory-CNS-motor circuits. The decreased neural activity may account for the reduced sensory responsiveness in intact animals that have consumed LPS in the diet (Istas et al., 2019). It has yet to be established if the effect is directly on neurons or through supportive cells, such as microglia in mammals, which then may release substances as an immune response to alter neuronal function. Using the NMJ preparation provides a reductionist approach to investigate direct pre- and post-synaptic effects of LPS for gaining additional insights into actions within neural circuits, as well as effects on synaptic transmission at NMJs. It is likely the insects and crustaceans are conserved in ligand-receptor and signaling cascades involved with pathogenic responses. Since LPS induced a depressing effect of synaptic responses on the larval Drosophila NMJs, it is of interest to determine if similar effects are observed in a cousin among arthropods.

One previous study conducted on synaptic transmission with LPS exposure of *S. marcescens* for an abdominal muscle in the crayfish revealed an enhancement in the amplitude of the excitatory junction potentials (EJPs) (Parnas et al., 1971). This earlier study did not distinguish the type of abdominal muscle nor if the spontaneous quantal responses changed in amplitude. Most likely the synaptic events recorded were from a fast motor unit due to the size of the single EJP reported (2 to 10 mV). The abdominal muscles are polyneuronally innervated, so it is not clear if additional units were recruited to give rise to larger EJPs in this earlier study. In this current study, to address the effect of LPS on synaptic transmission, the walking leg opener muscle was used, which is innervated by a single excitatory motor neuron. The size of the single quantal events was measured to determine if receptivity in the postsynaptic target changed. In addition, the opener NMJ has low output motor nerve terminals (Atwood and Cooper, 1996; Mykles et al., 2002). The synaptic responses show a good degree of short-term facilitation and are fatigue resistant (Crider and Cooper, 2000; Sparks and Cooper, 2004). Thus, by measuring facilitation, one can better allude to the mechanism of LPS affecting Ca^{2+} handling and the responses on synaptic transmission.

To assess the effects of LPS on the whole animal, LPS was injected into the hemolymph while the heart rate was monitored in freely moving animals. Crayfish have a neurogenic heart, so heart rate measures in crayfish serve as a monitor of stress and even neural function. The heart rate can be altered by providing a sensory stimulus such as a tap on the dorsal carapace or the abdomen (Li et al., 2000; Listerman et al., 2000; Schapker et al., 2002; Shuranova et al., 2006). A response to the sensory stimulus generally results in a slight pause between heart beats and then a speeding up of the overall rate (Shuranova et al., 2006). The responses can be followed after an injection at various times. To develop a clearer understanding on the influence of LPS on neural circuity, a sensory-CNS-motor nerve circuit was assessed in crayfish (Strawn et al., 2000; Bierbower and Cooper, 2013). The sensory input to the CNS-ventral nerve cord involves a cholinergic transmission (i.e., acetylcholine as a transmitter). Lastly, the excitatory NMJ of the opener muscle in the walking leg was utilized to address pre- and post-synaptic effects at these glutamatergic synapses.

2. Methods

2.1. Animals

Experiments were performed using Red Swamp Crayfish (*Procambarus clarkii*). They were obtained from a distribution center in Atlanta, GA, USA and delivered to, and bought from, a local

supermarket in Lexington, KY, USA. Throughout the study, midsized crayfish measuring 6–10 cm in body length and 12.5–25 g in body weight were used. Each animal was housed in individual standardized plastic containers with weekly exchanged dry fish food and aerated water (20–21 °C).

2.2. Heart rate activity

The electrocardiograms (ECG) were obtained by standardized procedures (Listerman et al., 2000; Wycoff et al., 2018), which is demonstrated in video format (Bierbower and Cooper, 2009). The preparation of the recording wires consisted of insulated stainless steel wires (diameter 0.005 in./0.008 in. with coating; A-M Systems, Carlsburg, WA, USA). The insulation was burned off the wire ends with a flame to provide a good connection of the bare wire with the recording device. To obtain an optimal HR measure, two holes were formed on the dorsal carapace directly over the heart using a fine-point scalpel. The insulated steel wires were placed into the carapace, spanning the heart to facilitate an accurate impedance measure (UFI, model 2991; Listerman et al., 2000). The impedance detector, which measures the dynamic resistance between the two wires, was linked to a PowerLab/4SP interface (AD Instruments) and calibrated with the PowerLab Chart software version 5.5.6 (AD Instruments, Australia). Zeroing of the impedance apparatus was monitored carefully during any acquisition. The acquisition rate was set on 10 kHz. The calculation of the HR was accomplished by direct counts of each beat over various intervals and transformed into beats per minute (BPM) as described in the results section. Two different estimated concentrations (500 and 5000 μg/ml) of circulating LPS within the hemolymph were examined for the effect on heart rate and survival. Animals were wired for recording three days prior to measures. Heart rate measures were obtained prior to injection of LPS or sham saline and after injection at 5 min, 15 min, and 12 h. These times were selected for assessing the relative acute and long-term effects of the injections. The amount of hemolymph was estimated based on animal's weight assuming that about 30% of an animal's weight is hemolymph (Gleeson and Zubkoff, 1977; Guirguis and Wilkens, 1995). The total volume was established for the appropriate volume of stock LPS to be injected. LPS was diluted in physiological crayfish saline (see below).

2.3. Abdominal neural circuit physiology

All animals were sacrificed in less than five seconds by rapid decapitation and splitting the cerebral ganglion followed by removal of the abdomen. As detailed in Strawn et al. (2000) and Bierbower and Cooper (2010) the ventral nerve cord (VNC) was cut between T5 and A1. The abdomen was separated from the thorax, and pinned ventral side up in a Sylgard coated dish. The nerve roots of the VNC, segmental ganglia, and superficial flexor muscles were exposed and bathed in physiological saline solution. Neural activity recordings were obtained from the 2nd or 3rd abdominal segment. Extracellular recordings of nerve activity in the 3rd motor nerve root to the superficial flexor muscle were obtained by a suction electrode with a differential setting. One led was placed in the suction electrode and the other led wrapped around the outside of the suction electrode. Step by step details in video format of these procedures is provided in Baierlein et al. (2011). All data were recorded by a computer via PowerLab/4 s A/D converter (ADInstruments).

For stimulation of the cuticle, a stiff-bristled paintbrush was mounted on a micromanipulator to control pressure and movement. The brush was positioned along the lateral side of the recording segment and moved an approximate distance of 2.54 cm in a forward and then backward motion. Neural circuit analysis used direct counts of the evoked spike frequency recordings. The brushing motion also resulted in the baseline of the signal waving in some preparations. The baseline did not fluctuate as long as the cuticle was lightly brushed and the abdomen was well-mounted in the Slygard dish. Frequency counts were performed by directly counting visually for any recording with an unstable baseline and by using computer software (Chart Software). A 30 s time period prior to bush stimulation and a 30 s time period during the stimulation were used for analysis of spike frequency. This was repeated 5 times and the average percent change in frequency was the index used for the effect of stimulating the circuit. The average activity prior to stimulation and the average activity during the stimulation were also used to assess the effect of exogenously applied compounds on the activity without and with stimulation in addition to the percent change in activity due to stimulation. Counts were then used to obtain an average value for each condition on every individual trial. These individual values were then used to obtain percent changes in the number of spikes.

2.4. Neuromuscular junction

Details of the dissection and electrophysiological recordings of the opener neuromuscular junction of the walking legs are described in Cooper and Cooper (2009). The short term facilitation (STF) was induced by providing a train of 25 stimuli at 40 Hz at ten second intervals to the excitatory nerve. Intracellular excitatory junction potentials (EJPs) recordings were performed by standard procedures (Sparks and Cooper, 2004). Analysis of response used the amplitudes of the 15th and 25th EJPs of the short-term facilitation train of pulses as determined by procedures previously described (Crider and Cooper, 2000). Even though the EJPs amplitudes reach a plateau around the 15th stimulus of the stimulation train at 40 Hz, the responses are not saturated, because either an exposure of 5-HT or higher stimulation frequency to 60 Hz further enhances the EJPs amplitudes (Sparks and Cooper, 2004). So, to better assess if the responses within the plateaued region in the train increased with LPS, it was important to measure the 25th EJP response as well as the 15th. Amplitudes were measured prior to exposure of LPS and after 10 min of exposure. To test if LPS was directly altering the frequency of spontaneous vesicle fusion events (miniature excitatory junction potentials; mEJPs), the number of occurrences were measured during exposure between 8 and 10 min. Amplitudes of the mEJPs were directly measured by manually placing cursors from base of the event prior to the rise and at the peak of the event. Electrical signals were recorded on-line to a PowerLab/4 s interface (ADInstruments, Australia) and calibrated with the Powerlab Chart software version 7.

2.5. Chemicals

Commercial LPS from *Serratia marcescens* (*S. m.*) was dissolved in physiological saline the day of experimentation. This LPS may also contain some associated peptidoglycans from *S. m.* The crayfish saline used is a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2{\cdot}2H_2O$, 2.45 $MgCl_2{\cdot}6H_2O$, and 5 HEPES adjusted to pH 7.4). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Concentrations of LPS used are listed with the results of the various experimental paradigms. A 500 μg/ml was used in all experiments. A concentration of 100 μg/ml was also used on the sensory-CNS-motor circuit. However, since no effect was noted on the CNS circuit at 100 μg/ml, only 500 μg/ml was used at the NMJs. The injected LPS to provide 500 μg/ml did not kill the crayfish in the whole animal studies, so a 10× increase in concentration to 5000 μg/ml was used, which did result in death after 24 h.

2.6. Statistical analysis

An ANOVA was also used to compare differences among treatments with LPS dosage. This analysis was performed with SigmaStat software. P of ≤ 0.05 is considered statistically significant. Normality Test (Shapiro-Wilk) and Equal Variance Test (Brown-Forsythe) were

performed by the software. All Pairwise Multiple Comparison Procedures used the post analysis with a Bonferroni *t*-test. Paired *t*-test and Sign test were also used for statistical analysis. Averaged data is expressed as a mean (± SEM).

2.7. Reproducibility in analysis of data sets

Analysis of the data occurring over time may result in biases even with automated software due to not fully knowing what to measure as a signal as compared to background noise or spurious deflections in a trace being measured. Different individuals analyzed some of the same data sets independent of each other in determining the HR and frequency of spikes in the nerve root recording and measures of synaptic responses at the NMJs. The degree of reproducibility in analysis on a data set was compared among individuals. Discrepancies were discussed among the individuals conducting the analysis. The independent confirmation of our data analysis and collections is important to show that recordings of HR and other measures are true changes in physiological processes, and thus are not biases or from human errors in the analysis.

3. Results

3.1. LPS effect on heart rate

The injection of 100 µl of LPS stock to produce an estimated concentration of 5000 µg/ml drastically altered the deflection amplitudes, as the amplitude of the impedance signals decreased (Fig. 1A); however, the HR increased as compared to saline control injection. The approximate circulating 500 µg/ml LPS group of crayfish did produce an increase in HR after injection; however, five out of the six decreased in HR lower than the initial rate after 12 h (Fig. 1B; $P < .05$, ANOVA). This was not the case for the saline control injected crayfish, which did not show any significant changes over all (Fig. 1B). The normalization of the data to a percent change to baseline prior to injections at 5 min, 15 min and 12 h show the same similar trends for saline and low concentration of LPS injection as compared to the sustained higher percentage for the high injected dose of LPS (Fig. 1; C1,D1). The HR also remained elevated after 15 min of injection and even 12 h later (Fig. 1 C,D; $P < .05$, ANOVA). Nevertheless, all six of the crayfish injected to obtain a circulating level of 5000 µg/ml were dead after 24 h. The six saline control injected crayfish and the six 500 µg/ml (estimated circulation level) injected crayfish were all alive a week after injections. The wires for the impedance measures were placed on the anterior and posterior regions of the heart. Thus, the deflections in the current between the two wires picked up only the heart and fluid (i.e. hemolymph) movements between the wires and did not measure movement of the body wall muscles. When the amplitude of signal dropped into the baseline, this represented the time point when the animal died and showed no behavioral responses. The traces are clear in Fig. 1, and it was shown that the impedance technique is far superior to the ECG measures made with a P-15 amplifier (Listerman et al., 2000).

3.2. LPS action on sensory-CNS-motor circuit activity

Inducing activity in a sensory-CNS-motor circuit by brushing on the ipsilateral side in which the recording is occurring on the 3rd root is reliable to induce increase neural activity (Fig. 2A, B). The brushing location and degree of force on the cuticle before and during LPS exposure is consistent, but it does vary between preparations which produces the various levels of activity among preparations (Fig. 3A1, A2). Thus, percent changes in the activity before and after exposures are measured within each preparation to normalize differences in the number of recorded spikes.

The exposure of the circuit to a 100 µg/ml did not produce any significant alterations in the activity profile (Fig. 3A1, A2); however, at 500 µg/ml the activity was reduced right after exchanging the bathing media and continued to decrease 5, 10 and 20 min during the LPS exposure (Fig. 3B1, B2). The activity continued to remain depressed even after exchanging the bathing media to saline untainted by LPS (Fig. 3B1, B2). The average (+/− SEM) number of spikes occurring in each condition reveals the overall trends. The percent differences for exposure to 100 µg/ml (Fig. 3A3) and 500 µg/ml (Figure 3 B3). Decreases in activity only occur for the 500 µg/ml ($P < .05$, ANOVA) and the suppressed activity remained even after 10 min from flushing out the LPS on the preparation.

3.3. LPS action at neuromuscular junctions

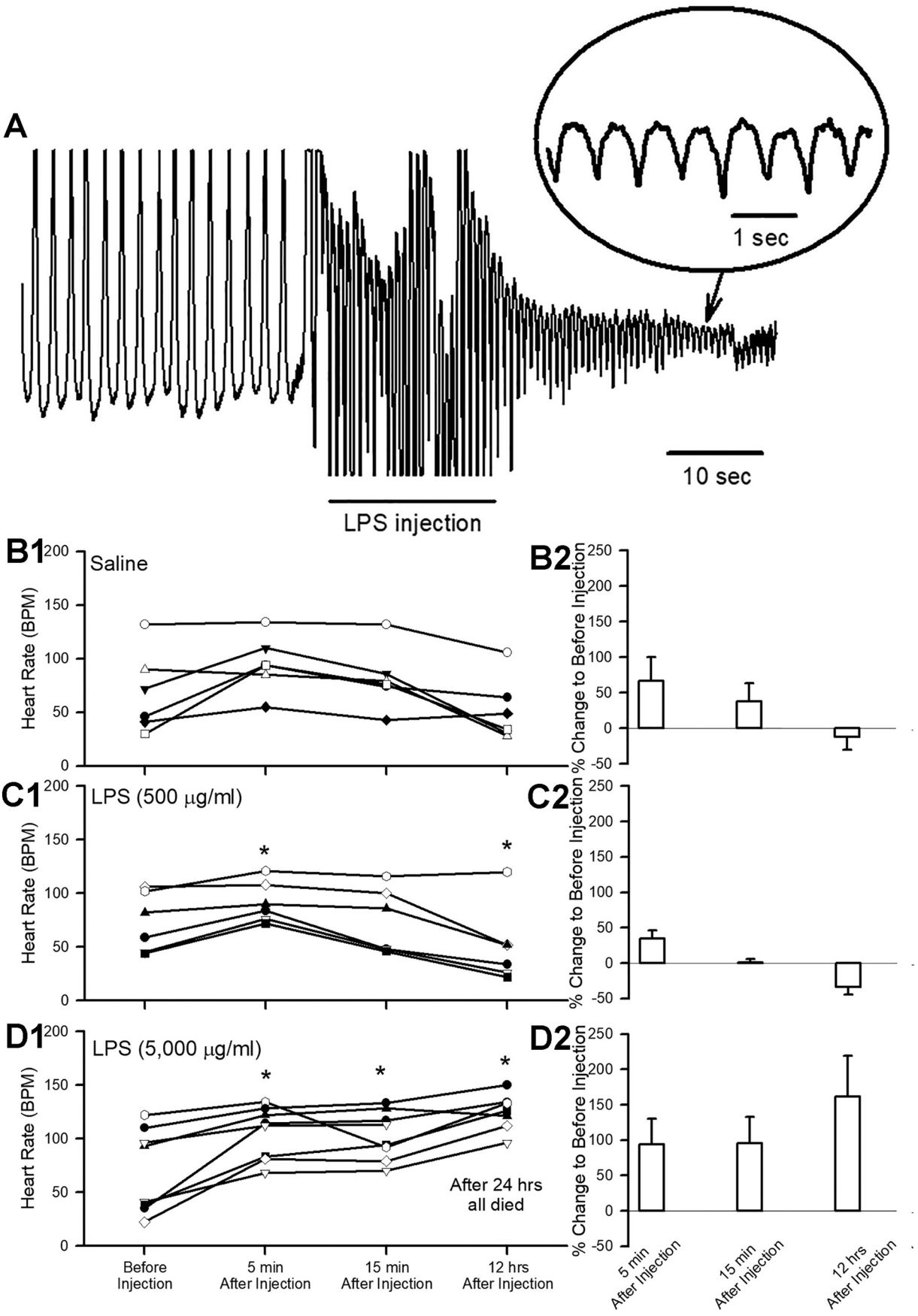
The 25 stimuli delivered at 40 Hz induces EJPs that facilitate in amplitude, and by the 25th EJP, the amplitudes have plateaued (Fig. 4A). In order to observe the variation among preparations, the amplitudes of the 15th and 25th EJP within the train of events is shown before and during LPS exposure (Fig. 4B1, B2). The results are shown in two parts in order to avoid overlapping points. The exposure to LPS increased EJP amplitudes for both the 15th and 25th EJPs after 10 min of exposure ($P < .05$, Paired *t*-test). However, the facilitation index did not show significant differences (Fig. 4C1, C2). To normalize the differences among preparations, percent differences from the initial values to exposure of LPS for the 15th and 25th EJP amplitudes (Fig. 4D) as well as the facilitation index differences (Fig. 4E) from saline to LPS exposure were measured.

3.4. Membrane potential and spontaneous events

With exposure of 500 µg/ml LPS, the membrane potential rapidly hyperpolarized and slowly regained the membrane potential during the exposure (Fig. 5). The hyperpolarization was consistently observed among the preparations ($P < .05$, Sign test). The average amount of hyperpolarization was 1.4 mV (0.36 +/− SEM; $N = 6$). There was no significant effect in the frequency of quantal events from saline to during LPS exposure (Fig. 6; N = 6 preparations; ANOVA within preparation comparison). Only in one of six preparations did the amplitude of the spontaneous quantal events show a significant decrease during LPS exposure (Fig. 7; ANOVA within preparation comparison: Saline, LPS and wash out $N > 40$ for each variable). Overall, there was no consistent effect on the amplitude of the spontaneous events within the 10 min exposure of LPS when comparing a percent change from saline to LPS all six preparations. Also, no statistical effects occurred after removing the LPS media and bathing for 10 min in saline wash (Fig. 7; ANOVA, within preparation comparisons).

3.5. Reproducibility in data quantification

As a measure to verify reproducibility and consistency in data quantification, we used participants at different institutions to independently asses the analysis in the recordings. These individuals were recruited and guided as how to count the number of spikes in the 3rd root recordings, heart rate analysis, and the amplitudes in the EJPs in subsets of data randomly chosen. As a means to standardize the instructions and to ensure the general understanding in how to perform the analysis, video recordings were made of the analysis of sample data sets and provided to the participants. As an example, two independent analysis are shown for indexing the motor nerve activity when evoking the sensory-CNS-motor circuit (Fig. 8A). As expected, the number of spikes counted in the individual trials within the preparations mentioned above shows there is some variation between participants making the counts. However, upon averaging the five trials within a given condition, it was showed that there was no significant differences present between participants (Fig. 8B). A similar blind control for measures in HR also revealed no participant data quantification differences (Fig. 8C).



(caption on next page)

Fig. 1. Effects of LPS on heart rate in whole animals. (A) Representative trace of the recordings obtained of the heart rate before and after injection of LPS (estimated circulating concentration of 5000 μg/ml). Note the decrease in the amplitude of the signal but increase rate after the injection. (B1) The heart rates of six crayfish injected with saline (100 μl) to serve as a control for the stress of injection and handling. Values are of before, 5 and 15 min as well as 12 h after injection. (B2) The percent change in the heart rates are compare to prior the injection ($P < .05$, ANOVA). The heart rates for 500 μg/ml (C1) and 5000 μg/ml (D1) levels are shown along with the corresponding percent changes for 500 μg/ml (C2) and 5000 μg/ml (D2) levels ($P < .05$, ANOVA).

4. Discussion

This study demonstrated that injected LPS and associated peptidoglycans from *S.m.* into the hemolymph had an effect of increasing HR after 5 min and 12 h later for an estimated circulating level of 500 μg/ml, whereas 5000 μg/ml increased HR at 5 min and remained elevated after 15 min and even after 12 h. However, within 24 h, the crayfish died due to the high level of LPS exposure. The high dosage of 5000 μg/ml rapidly depressed the rate of the hemolymph pumped from the heart, as the amplitude of the impedance measures decreased drastically. In semi-intact abdomens, a mechanosensory-CNS-motor neural circuit was depressed within ten minutes of exposure to LPS at 500 μg/ml but not for 100 μg/ml. Evoked EJPs at the NMJ increased in amplitude within ten minutes of exposure to LPS at 500 μg/ml; however, facilitation of the EJPs as measured with the ratio of the 25th /15th EJPs within a response train did not indicate a significant effect. There was no significant effect on the amplitude of the postsynaptic quantal responses after ten minutes of LPS exposure. Interestingly, the resting membrane potential transiently hyperpolarized in a rapid fashion. Lastly, there was no observable effect on the frequency of spontaneous vesicular fusion events during the time LPS was influencing the resting membrane potential.

Our initial assumption was that systemic injections of LPS would result in clotting through the innate immunity response (Lorenzon et al., 1999) and that the circulating level would decrease very rapidly and perhaps before there was a chance to have much of a response on the neural control of the heart or the heart itself. To determine the mechanism of altering the amplitude of the signals, the force in the hemolymph ejection from the heart or the force of heart contraction needed to be measured directly. Despite the rapid drop in the amplitude of the signal, the rate increased, suggesting that the neural circuit was active enough to drive the heart rate. The potential of nitric oxide (NO) having an influence on the heart is feasible since NO was shown to regulate heart rate in *Daphnia magna* (Bownik et al., 2015) and is known to be present in shrimp. However, in shrimp there was no significant difference in NO synthase mRNA expression after stimulation with LPS either by different concentrations or time course (Wu et al., 2013.).

Given the depression of the neural circuit in the abdomen, one might expect a decrease in the neural circuit driving the heart rate. In larval *Drosophila melanogaster*, the heart tube is not innervated, and when exposed to 500 μg/ml of *S.m.* the HR rapidly increased and then decreased (Anyagaligbo et al., 2019). However, LPS from *Pseudomonas aeruginosa* had no consistent effect on HR at 1 μg/ml and 100 μg/ml, but at 500 μg/ml the HR continued to decrease over time even after 10 min of flushing 3 times with untainted saline. The larval heart of a blowfly (*Phaenicia sericata*) was unaltered by LPS exposure. Thus, LPS produces species dependent effects on cardiac function in insects and likely among other crustaceans as compared to the Red Swamp Crayfish (Anyagaligbo et al., 2019; Lorenzon et al., 1999).

In intact neural circuits, it is difficult to know the precise mechanism of action of LPS, as effects could be on neurons or other cell types (i.e., microglia) that are not directly activated by a stimulus but that influence the overall activity of the circuit. In a sensory-CNS-motor circuit of larval *Drosophila melanogaster*, LPS of *S.m.* also depressed induced activity (Istas et al., 2019) similar to the effects we report on the crayfish circuit. Similar to the *Drosophila* CNS circuit, the action of LPS was very rapid in depressing the evoked sensory-CNS-motor nerve. This suggests that either LPS is gaining quick access to the central neurons to have an effect or a response is relayed to alter the circuit. The sensory input is cholinergic in both the larval *Drosophila* and the crayfish, but it is not known if the action of LPS is potentially on the pre- or post-

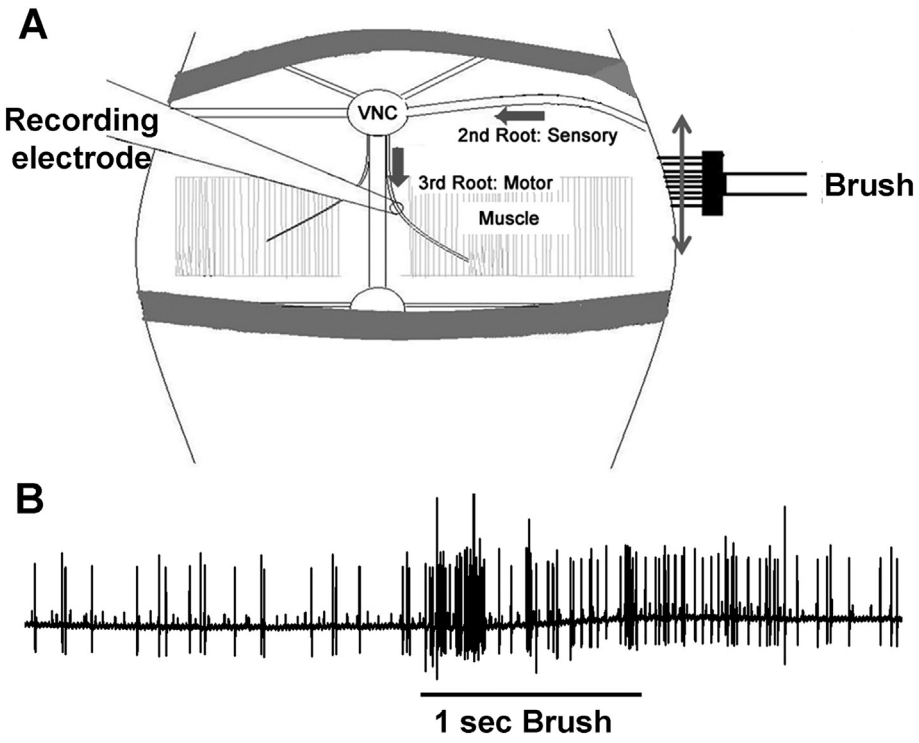


Fig. 2. Inducing sensory-CNS-motor nerve activity in crayfish. (A) Stimulating sensory activity, by a second brushing motion on the lateral cuticle, produces enhanced extracellular spike activity. (B) Recording the 3rd nerve root, which innervates the superficial flexor muscles with a suction electrode, readily responds to the ipsilateral sensory stimulation.

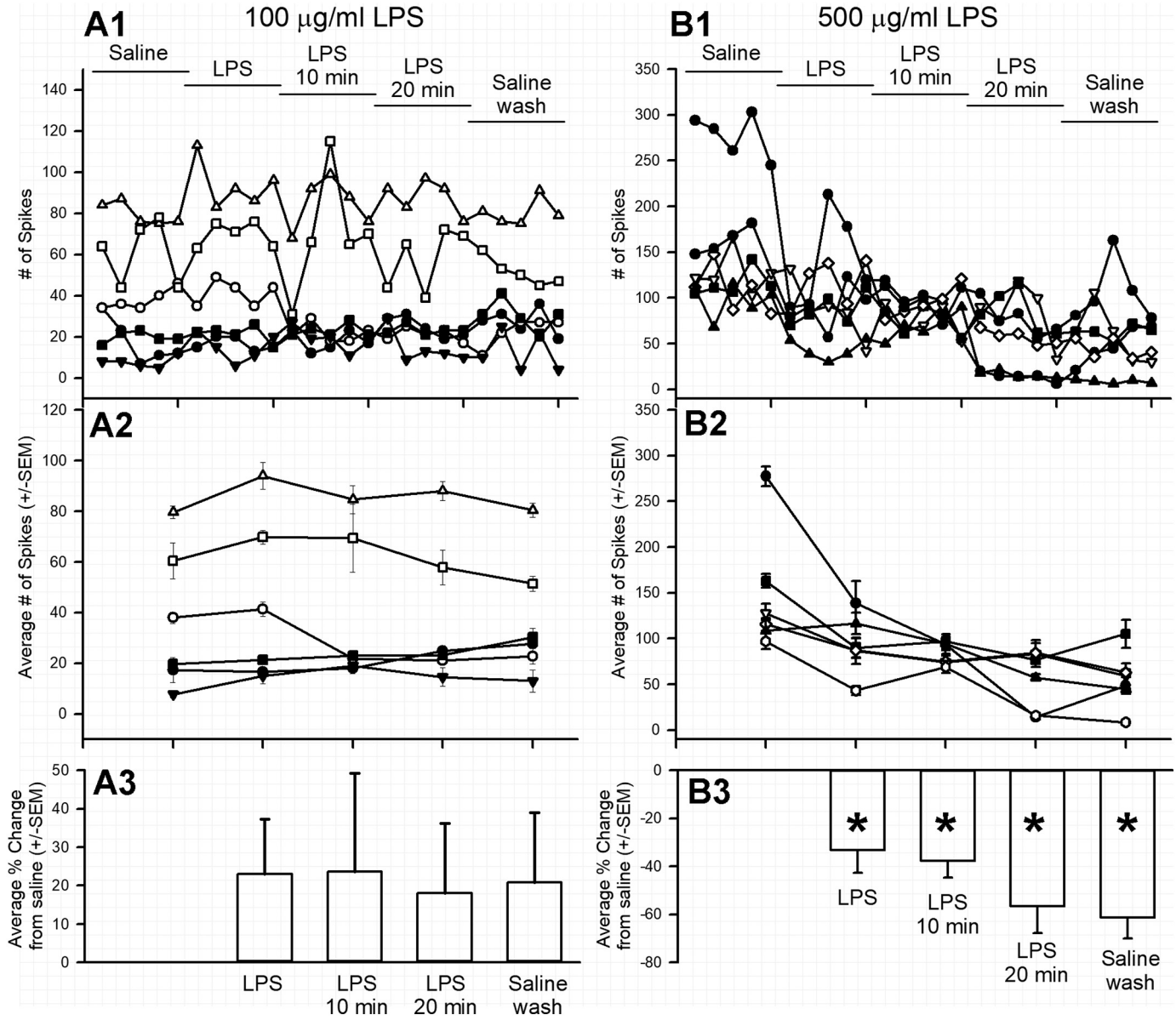


Fig. 3. Extracellular spike activity during sensory stimulation during exposure to LPS. 100 and 500 µg/ml exposure to the neural circuit while providing sensory stimulation resulted in depressed neural activity only for the 500 µg/ml (left and right columns respectively). The number of spikes recorded for each preparation within a minute are shown in the top panels (A1, B1) with the average responses for each condition over 10 min the middle panels (A2, B2) and percent changes to the activity prior to LPS exposure. The 500 µg/ml resulted in depressed activity 10 min after flushing of the preparation with saline not containing LPS ($P < .05$, ANOVA). The lines represent individual preparations and different symbols are depicted to separated out the various preparations.

synaptic targets of the sensory neurons within the CNS or on interneurons or motor neurons directly. Since no alteration in evoking sensory activity and conduction was noted for the muscle receptor organ (MRO) in crayfish or primary sensory neurons of a chordotonal organ in a crab leg at the same concentration 500 µg/ml (Stanback et al., 2019), it appears LPS is not altering the mechano-transduction process or conduction along the sensory neurons. However, at the nicotinic cholinergic synaptic preparation of the frog NMJ, LPS was shown to have a presynaptic action, as the size of the quantal responses are not altered but evoked EJPs are reduced (Person, 1979a,b; McNabb et al., 2019).

The sensory input induced by mechanical stimulation of the lateral cuticle on the crayfish abdomen used in this study likely involves multiple types of sensory neurons and interneurons that drive the motor neurons measured in the 3rd motor root (Strawn et al., 2000). However, the sensory neurons driving the third root to the superficial muscles account for the evoked activity when rubbing on the cuticle (Strawn et al., 2000; Bierbower and Cooper, 2013). It is likely the motor neurons are coupled with the lateral giant (LG) axons, as there are motor neurons coupled with the LG in the third root which project to the deep flexor muscles for the rapid tail flips (Furshpan and Potter, 1959). It has been established that the LG does contain synaptic vesicles along with gap junctions at synapses onto the fast flexor motor neurons (Leitch et al., 1992). Thus, it appears feasible to suggest a similar type of input on the slow flexor motor neurons. The driven circuit is not limited by electrical gap junctions, as heptanol does not completely block the sensory to 3rd motor root (Bierbower and Cooper, 2013).

The enhancement of evoked EJPs in the opener muscle is likely due to a presynaptic action in promoting vesicular fusion (Sparks and Cooper, 2004). Since the membrane potential rapidly hyperpolarizes but is only transit over the ten minutes and evoked EJPs are still enhanced in amplitude, the effect is not due to an increased driving

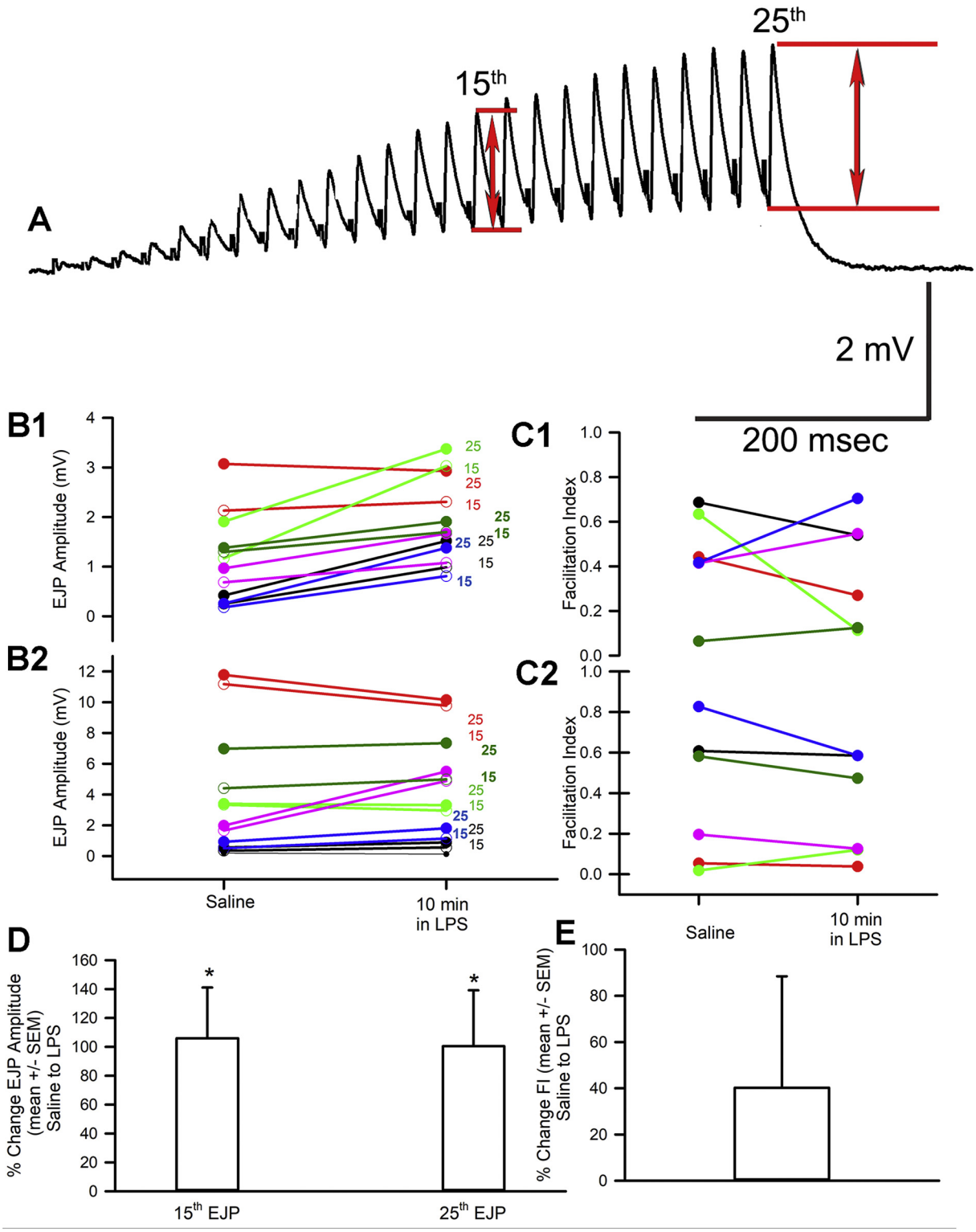


Fig. 4. The effect of LPS exposure on the amplitude of the excitatory junction potentials (EJPs) measured in the opener muscle of the crayfish first walking leg. (A) Stimulation at 40 Hz of the opener excitatory motor nerve induces an EJP train of responses which facilitate. (B1, B2) The amplitudes of the 15th and 25th EJPs within a train increase with exposure to LPS (500 μg/ml); (C1, C2) however, the LPS exposure did not significantly further enhance facilitation. (D) The average percent increase in the 15th and 25th EJP amplitudes from saline to LPS exposure are shown ($P < .05$, Paired *t*-test). (E) No significant effect on the facilitation index were noted by exposure to LPS.

gradient of the ionotropic glutamate receptors. In addition, the range in the amplitudes in the spontaneous events is not increased. Thus, the effect is likely due to increased responsiveness to presynaptic Ca^{2+} influx or even enhanced influx of Ca^{2+} with depolarization of the motor nerve terminal. Since there is no increase in the frequency of spontaneous events, the possibility of a Ca^{2+} leak into the presynaptic nerve terminal or a leak from internal stores of Ca^{2+} is unlikely. However, it is hard to conceive of a mechanism where the rise in presynaptic Ca^{2+}

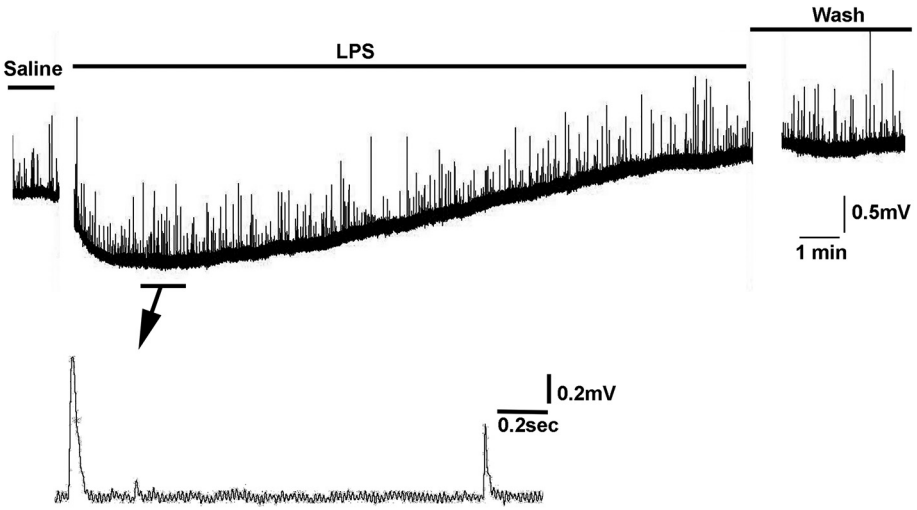


Fig. 5. Membrane potential and spontaneous quantal responses measured in the opener muscle of the crayfish before and during LPS exposure. A representative intracellular muscle recording depicting the effects of switching the bathing media to one containing 500 μg/ml LPS. The membrane shows a transit hyperpolarization. The miniature EJPs (mEJPs) or quantal responses are present before and during LPS exposure. Note the enlarge section of the trace to illustrate the quantal responses and varied sizes measured. Three quantal responses are shown.

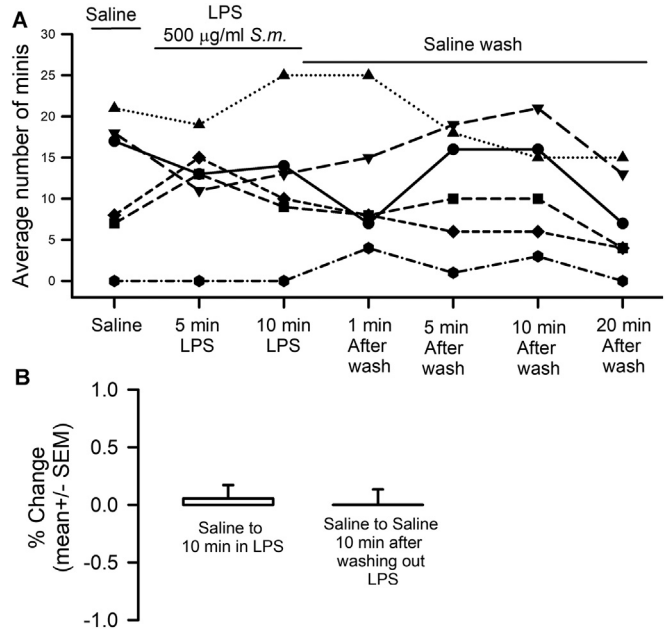


Fig. 6. The effect of the LPS on the occurrence of mEJPs. (A) There is no significant effect on the number of mEJPs by exposure to LPS (500 μg/ml) over a ten minutes. Following 20 min after flushing away LPS the number of mEJPs is not significantly altered. (B) No differences in the percent change from saline to LPS exposure was noted among grouping the preparations.

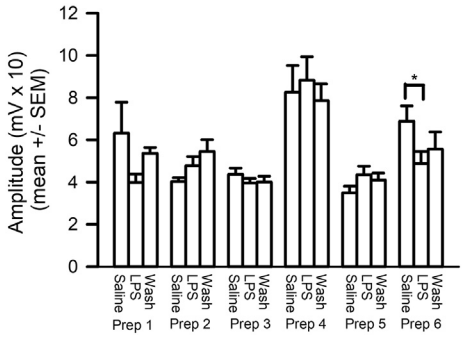


Fig. 7. The amplitude of the spontaneous quantal events with exposure to LPS. The amplitude of the mEJPs among six preparations only showed a significant decrease in preparation 6 during LPS exposure ($P < .05$ ANOVA within a preparation, $N > 40$ sample in each data set). In grouping all six preparations there is no significant difference in the amplitude of the mEJPs induced by LPS.

promoted evoked release and where there is no change in facilitation. Therefore, a more logical explanation is that vesicle docking is enhanced without a rise in the Ca^{2+} within the presynaptic terminal or that the postsynaptic receptivity is changed. Given that the amplitude of the spontaneous quantal events did not show a significant increase, it is more practical to assume a mechanism by which the number of vesicles docked were enhanced and if the same amount of Ca^{2+} entered then an increase in the amplitude of the evoked EJP would occur. This is somewhat reminiscent in the proposed action of serotonin at the crayfish NMJ, where a single evoked response is increased in amplitude after exposure to serotonin (100 nM) by an IP3 induced second messenger cascade to promote vesicle docking (Dropic et al., 2005; Logsdon et al., 2006).

The larval *Drosophila melanogaster* NMJs are similar in pharmacological profile to those of the crayfish NMJ in which both are a quisqualate subtype and are ionotropic (Shinozaki and Shibuya, 1974; Shinozaki and Ishida, 1981; Lee et al., 2009; see review Titlow and Cooper, 2018). However, LPS from *S.m.* appears to block the glutamate receptors on the postsynaptic muscle in *D. melanogaster* at 500 μg/ml but not at 100 μg/ml (Cooper et al., 2019; Istas et al., 2019).

The hyperpolarization of the skeletal muscle in the crayfish limb with exposure to LPS is interesting, as it has not been reported to occur in mammalian skeletal or cardiac muscles. In addition, this phenomenon was not reported to occur in frog skeletal muscle (Person, 1979a,b) nor in the only previous report examining the action of LPS on crustacean NMJs (Parnas et al., 1971). However, in two recent reports it was shown the hyperpolarization response in body wall muscle of larval *Drosophila melanogaster* is robust with exposure to *S.m.* at 500 μg/ml (Cooper et al., 2019; Istas et al., 2019). The mechanisms to cause the hyperpolarization have not yet been determined, but it appears it is not due to an alteration in the input resistance, Ca^{2+} influx, TEA sensitive K^+ channel, Ca^{2+}-activated K^+ channel, or nitric oxide synthase induction in muscle fibers (Cooper et al., 2019; Istas et al., 2019). The mechanisms behind this hyperpolarization in body wall muscle in *D. melanogaster* are likely similar to those of the crayfish, but this remains to be determined. To aid in determining the mechanism of the synaptic enhancement at the NMJ by LPS, we are currently using Ca^{2+} sensitive indicators in the motor nerve terminals of the crayfish and ones expressed in the motor neurons of the larval *D. melanogaster*.

The redundant analysis by participants in determining the frequency of extracellular signals (i.e., spikes) in the same data set was beneficial specifically to show that human error is not resulting in significant results. The added procedural explanations by making a movie recording of analysis in the way it was being performed at one

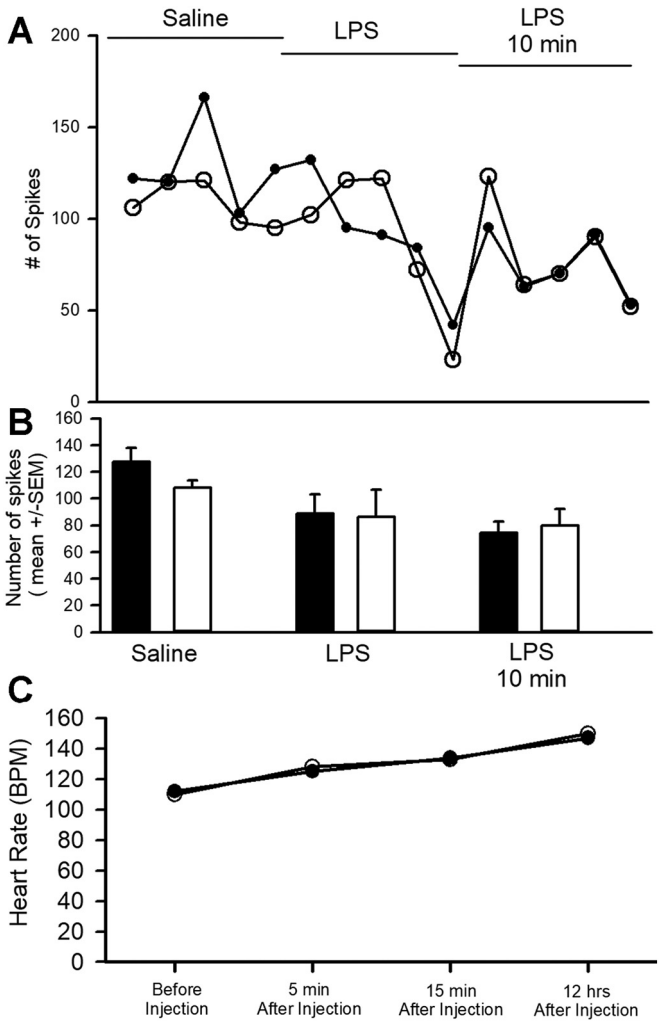


Fig. 8. Reproducibility in data analysis. Analysis of the same data set was performed for determining the number of spikes in the motor nerve by evoking a response in sensory-CNS- motor nerve circuit (A) and the mean (+/− SEM) of the spikes in varying experimental conditions (B). (C) The analysis of the heart rate was determined independently for a given data set. Open and filled symbols and bars represent analysis by two different participants.

location and sharing it with participants at another location appeared to be beneficial to ensure confidence in data quantification and interpretation. Knowing that the general analysis procedure is documented, preferably by video, provides assurance that unconsciously biased analysis can be negated. As shown from experience and this study, the number of spikes in the nerve recording is more challenging for reproducibility as compared to the counting the number of beats in the traces of the heart rate. This difficulty led to not surprising results of variability in analysis in the number of spikes. The main reason for this is because each participant has to vary the threshold in the signal to noise ratio throughout the recording as it appears more base line movement in the trace occurs during the brushing of the lateral cuticle movement. Importantly, for the analysis in these data sets no differences were obtained for the average values in the responses between participants in the analysis. As mentioned in a recent report, in which the same data sets were analyzed and compared, the increase demand for publications to also submit data sets is not advantageous for examining reproducibility of analysis without explicit instructions in the procedures (Stanback et al., 2019). Documentation in video format can potentially be provided with individual archived data files (Stanback et al., 2019). Computer algorithms can be generated, tested, and retested with various sets of data to also test for reliability (Lancaster et al., 2007); however, we have found that the automated software provided with the data acquisition Chart software would not be reliable with a baseline which waves even with sine wave averaging to follow the baseline. The standard deviation of the baseline for a detection of a spike would have to be constantly changed to detect the spikes with a fluctuating baseline. We had better reliability in some cases counting by eye with direct observations.

Preliminary experiments using agonist and antagonist to ATP activated K^+ channels ($K_{(ATP)}$) did not induce hyperpolarization nor block the action of LPS in hyperpolarizing the muscle. So, subsequent investigations proceed to determine this unique action of LPS and associated peptidoglycans. In addition, it is of interest to learn what extent the various bacterial forms of LPS have on the physiology in multiple animal models.

5. Conclusions

It is important to understand the direct actions of bacterial endotoxins, such as for LPS, as well as systemic effects induced by endotoxins (i.e. cytokines). By knowing the initial rapid effects, one may be able to target them to promote or reduce secondary responses in order to provide better treatment depending on the induced effect. The rapid hyperpolarization of skeletal muscle observed in crayfish and *Drosophila* has yet to be reported in mammalian systems. Perhaps, this type of response does not occur in mammals. The increase in evoked synaptic EJPs without altering the facilitation is intriguing and hints at other mechanisms involved in synaptic vesicle docking rather than altering Ca^{2+} influx within the presynaptic terminals as proposed previously. The systemic injections of LPS and effect on the heart rate for crayfish informs us that they are robust in dealing with a level of 500 μg/ml of LPS. This is important to know as this is a common eatable species of crayfish and maybe be infected with high titers of endotoxins without showing changes in behavior or survival. This study also indicates that multiple systems need to be investigated as to the effects of LPS, as the in-situ sensory-CNS-motor preparation suggested that sensory input maybe be depressed but that synaptic function at the NMJs is enhanced. The effects shown herein are contrary to the effects reported previously in the rate of spontaneous vesicle fusion events at the NMJ, emphasizing that repeating experiments by others is sometimes necessary. Lastly, species differences in the effects of LPS on synaptic transmission indicates different mechanisms of action by the same strain of LPS on similar types of glutamatergic synapses. Future investigations are warranted to determine the reason for these differences as both crayfish and larval *Drosophila* NMJs are used as models in the basis of investigating process in synaptic transmission.

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Data references

Data set are provided here

Cooper, R. (2019, May 27). Crayfish NMJ heart CNS-effects of LPS 2019. osf.io/w5c9h *as well as a video format on how to analyze the data sets.*

https://www.youtube.com/watch?v=uBjkwbCKrWs
https://www.youtube.com/watch?v=eWm7m_vBfDw
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Declaration of Competing Interest

There is no conflict of interest.

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