Neuroscience Research 170 (2021) 59–65

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Bacterial endotoxin lipopolysaccharide enhances synaptic transmission at low-output glutamatergic synapses

Abigail Greenhalgh, Oscar Istas, Robin L. Cooper*

Department of Biology, Center for Muscle Biology, University of Kentucky, Lexington, KY, 40506-0225, USA

ARTICLE INFO

Article history:
Received 15 May 2020
Received in revised form 14 August 2020
Accepted 26 August 2020
Available online 26 September 2020

Keywords:
Lipopolysaccharide
Endotoxin
Serratia
Synapse
Glutamate receptor
Glutamatergic

ABSTRACT

The endotoxin lipopolysaccharides (LPS), secreted from gram-negative bacteria, has direct effects on synaptic transmission independent of systemic secondary cytokine responses. High concentration of LPS (500 μg/mL) from *Serratia marcescens* increased synaptic efficacy at glutamatergic low-output synapses more than for high-output synapses. Over an hour of exposure was not toxic to the preparation and continued to enhance synaptic transmission. A small but significant rapid hyperpolarization of the post-synaptic cells occurred, in addition to a slower enhancement of in the amplitude of evoked excitatory junction potentials. LPS may promote reserve pool vesicles to the readily releasable pool for low-output synapses. The action of LPS at the glutamatergic synapses of the crayfish neuromuscular junction is unique in promoting synaptic transmission as compared to other glutamatergic synapses in *Drosophila* and mammals, where synaptic transmission is depressed.

1. Introduction

The cascade in the immune response induced by gram-negative bacterial strains, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens*, is initiated in part by the bacterial secretion of endotoxins in the form of lipopolysaccharides (LPS). When circulating in the blood stream, in the case of sepsis, a series of immunological responses, such as the release of proinflammatory cytokines (TNF-α, IL-1, or IL-6) into the circulation (Costamagna et al., 2015), can occur. Treating bacterial sepsis with antibiotics results in lysing the bacterial load which can release a surge of LPS; thus, adding to an enhanced response by cytokines (D'Elia et al., 2013; Cavaillon, 2018).

To block the initial step induced by bacteria, a better understanding of the action of LPS and its receptor mediated responses is needed. In addition, understanding the direct action of LPS in various animal and tissue models provides a more complete view of the potential mechanism induced by LPS so that targeted therapeutic approaches can possibly be used to block downstream responses.

The action of LPS on the central nervous system in mammalian models, such as hippocampal slices, is complex due to the mixed cell types present. The proposed mechanism of cytokines, such as TNF-α, have direct effects on neurons and glia, but the direct action of LPS on neurons, independently from glial cells, are difficult to address in intact and CNS slice preparations. It has been reported that hippocampal neurons in culture did show a response to LPS (Olson and Miller, 2004). In this report it was demonstrated that LPS (1 μg/mL, Escherichia coli) increases neuronal cytosolic Ca^{2+} and promotes cell death in older cultures. Surprisingly, direct action of LPS on glutamatergic synapses at the crayfish neuromuscular junction (NMJ) enhances synaptic transmission (Saelinger et al., 2019; Ballinger-Boone et al., 2020). The action of LPS at these NMJs appears to be presynaptic in promoting synaptic transmission. However, these NMJs previously examined contain low output synapses (Cooper et al., 1995) and only acute exposure (10 min responses) to LPS were examined. One earlier report examined what appears to be a higher output NMJs in the crayfish abdomen; however, the muscle was not identified, and it appears only one observation was reported (Parnas et al., 1971). One can record the responses from a single excitatory high output motor neuron in an abdominal muscle, depending on the stimulation and recording arrangement. Whereas the opener muscle in the walking leg of crayfish possesses substantially lower output synapses and the entire muscle is innervated by a single excitatory motor neuron. Both preparations were used in the study herein. Thus, direct action of LPS on synaptic transmission of high- and low-output glutamatergic synapses are addressed in these two models.

Muscles in crustaceans are generally innervated by either a phasic or a tonic motor neuron, although some single fibers can be innervated by both phasic and tonic excitatory motor neurons. Through selective stimulation of one or the other motor neuron,

* Corresponding author: 675 Rose St., Dept. of Biology, Univ. of Kentucky, Lexington, KY, 40506-0225, USA.
E-mail address: RLCOOP1@uky.edu (R.L. Cooper).

https://doi.org/10.1016/j.neures.2020.08.008
0168-0102/

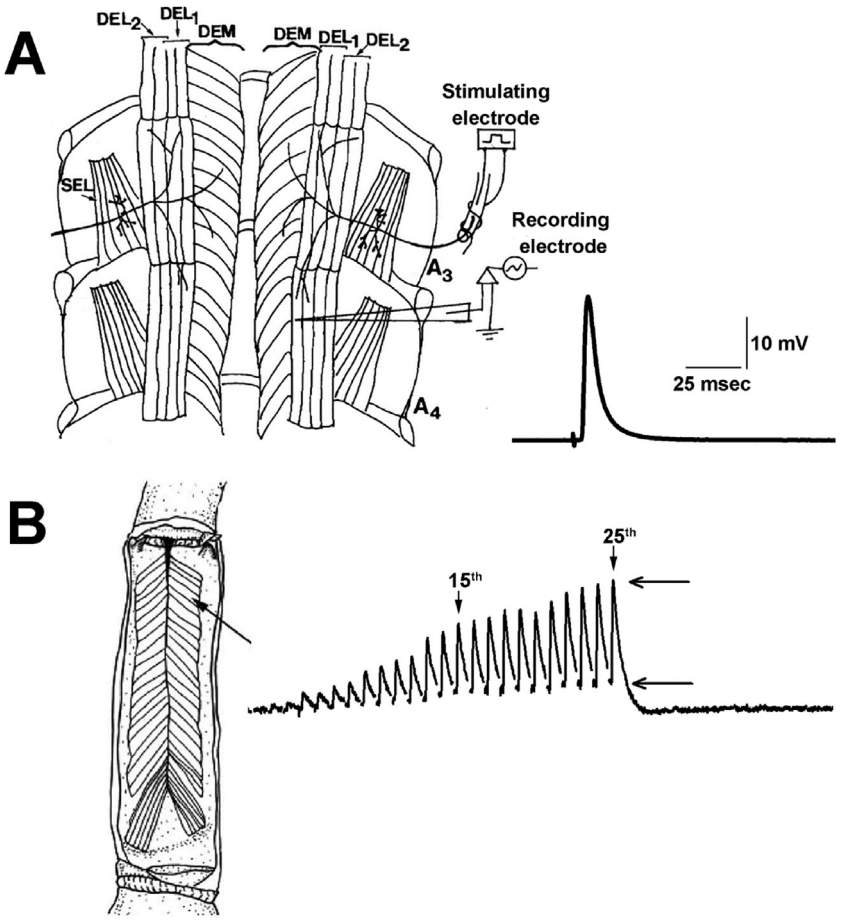

Fig. 1. Anatomical and physiological measures of a high- and low-output preparation. (A) The muscles on the dorsal aspect of the abdomen are shown and labelled. The deep extensor lateral 1 (DEL1), deep extensor lateral 2 (DEL2), and deep extensor medial (DEM) muscles are fast phenotype muscles. The superficial extensor lateral muscle (SEL) is shown to the lateral edge. The arrangements for stimulating the segmental nerves within A3 and recording responses from DEL1 muscles in A4 are illustrated. The recording in DEL1 of the next caudal segment to the one being stimulated is innervated by only a single phasic neuron within the nerve. A typical response is shown. (B) The opener muscle in the 1st walking leg is innervated by a single excitatory motor neuron. The distal muscle bundles provide facilitated responses with a train of stimuli. A response to a 40 Hz train of 25 stimuli is shown. The amplitudes of the 15th and 25th responses were used as a measure (as shown between the two arrows).

physiological differences in the EJPs can be measured. Normally phasic motor neurons are silent, but when active, they can drive rapid twitching of muscle fibers and evoke EJPs on the order of 10–40 mV. The phasic response depresses rapidly with 5–10-Hz trains of stimulation. Tonic motor neurons, however, have a higher intrinsic activity. They give rise to smaller EJPs that can be facilitated in the presence of a high stimulation frequency (10–50 Hz) (Atwood, 1973,1976; Cooper et al., 1998). The tonic muscle contraction is often slower and graded when compared to the phasic response. Structurally, the presynaptic phasic and tonic terminals are different. The synaptic structures also vary between the two types of terminals with tonic terminals having more synapses per length of the nerve terminals and more reserve pool vesicles than the phasic terminals (Atwood and Cooper, 1995, 1996a,b; Bradacs et al., 1997; Cooper et al., 1998; Johnstone et al., 2008).

Crayfish are readily maintained at 21 °C and dissected preparations are stable in minimal physiological saline at 21 °C for several hours for prolonged studies. Considering that the amplitude of the spontaneous quantal EJPs does not increase while the evoked EJPs do when exposed to LPS (Saelinger et al., 2019), it would appear LPS has a presynaptic action in promoting an enhanced evoked vesicular fusion. Because the lower output synapses have a larger leeway in enhancing synaptic efficacy as compared to the phasic higher output synapses, we tested the hypothesis that the tonic lower output synapses will show a more pronounced effect in enhancing the evoked EJP when exposed to LPS.

Reports state that LPS at high concentrations are toxic to cells, but the reports do not differentiate if it is the direct effect of LPS or the secondary effect of the cytokine storm on the tissues (Loppnow et al., 1990; Wassenaar and Zimmermann, 2018). Recently, the direct effect of a high concentration of LPS for 20 min showed a pronounced hyperpolarization followed by a depolarization of the skeletal muscle and reduction in synaptic transmission at the larval *Drosophila* NMJ (Cooper et al., 2019; Istas et al., 2020). After the prolonged exposure, the membrane potential and synaptic responses did not recover after removing the LPS and thoroughly flushing the preparation with saline free of LPS (Istas et al., 2020). Thus, in this present study, an examination of long-term exposure over an hour with high concentration of LPS (500 μg/mL) while stimulating the low output NMJs was performed to determine if such exposure was toxic to the crayfish NMJ preparation. In addition, a comparison in responses to LPS at low- and high-output synapses was made to help elucidate potential mechanism of action in altering transmission.

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 a local supermarket in Lexing-

ton, KY, USA, where they were purchased. Throughout the study, midsized crayfish measuring 6–10 cm in body length were used. Each animal was housed in individual standardized plastic containers with weekly exchanged dry fish food and aerated water (20–21 °C). It was observed that the crayfish had ectoparasites (branchiobdellids) in their gill chambers, but these crayfish were the only ones available for the study (Cooper, 1998). Crayfish are obtained from the wild for most all experimental studies worldwide in which they are used.

2.2. Dissection and electrophysiology

2.2.1. Abdominal muscle preparation

To make intracellular muscle recordings, the ventral surface of the deep extensor was exposed by removing the ventral side of the abdomen after cutting along the length of the abdomen approximately at the midline on each side. The dorsal half was pinned down on a Sylgard coated dish and residual flexor muscle removed. This allowed for excellent visual identification of the deep extensor muscles (DEL1, DEL2, and DEM) and the superficial lateral extensor muscle (SLE) (Fig. 1). Details of the dissection and anatomical descriptions are provided in earlier reports (Cooper et al., 1998; Griffis et al., 2000; Sohn et al., 2000 and in video formats Leksrisawat et al., 2010 and Baierlein et al., 2011). The DEL1 muscle fibers are of a phasic muscle phenotype (Mykles et al., 2002). Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mmol/L: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2{\cdot}2H_2O$; 2.45 $MgCl_2{\cdot}6H_2O$; 10 glucose; 0.5 HEPES adjusted to pH 7.4).

Intracellular recordings were taken from DEL1 muscle fibers of the fourth abdominal segment. These fibers were innervated in part by a descending branch of the motor neuron that innervates the DEL1 muscle of the third segment (Parnas and Atwood, 1966; Lnenicka and Atwood, 1989; Mercier and Atwood, 1989; Sohn et al., 2000). This allowed recordings from fibers innervated by a single identified motor neuron (Cooper et al., 1998; Griffis et al., 2000). Suction electrodes made from plastic tips were used to stimulate the cut nerves (details of making the suction electrodes is provided in Baierlein et al., 2011). The nerve was stimulated with supramaximal pulses using a suction electrode and Grass S-88 stimulator to elicit EJPs. The stimulation paradigm consisted of providing stimulation at 0.5 Hz (Fig. 2A1). An average of 5 responses in each of the conditions was used for measuring the amplitude of the EJP. Responses were recorded with an AxoClamp 2B (Axon Instruments, USA), converted with a PowerLab, 4SP (ADInstruments, USA), and analyzed with LabChart 7.0 (ADInstruments, Colorado Springs, CO, USA) which were recorded on a computer at a 10 or 20 kHz sampling rate.

2.2.2. Opener muscle preparation

The opener muscle for studying synaptic transmission has been historically reviewed (Cooper and Cooper, 2009). The use of the most distal muscle fibers in the preparation provides a reference for consistency among preparations (Cooper et al., 1995). The tonic phenotype of the innervation of these fibers is low output and fatigue resistant but has pronounced facilitation (Crider and Cooper, 1999, 2000). The most distal muscle fibers on the opener muscle of the first and second walking legs are of a tonic muscle phenotype (Mykles et al., 2002). The dissection to expose and selectively stimulate the excitatory motor neuron of the opener muscle is described in textual and video format (Cooper and Cooper, 2009). In brief, the excitatory neuron was isolated from the inhibitor neuron and stimulated in the meropodite segment. The stimulation paradigm consisted of providing a train of 25 pulses at 40 Hz with 10 s or 100 s between trains (Fig. 2B1). An average of 2 trains in each of the conditions was used for measuring the amplitude of the 15th and 25th EJP. The rationale for using 25th pulses in a train is that by the 25th pulse, a plateau in the amplitude of the EJP was usually reached for the opener NMJs when at room temperature (Wu and Cooper, 2012a). The periods in time chosen for obtaining the EJP amplitudes in illustrated in Fig. 2B1 as #1, #2, #3 and #4 demarked by arrows. This provided measures in saline, after 5 min of LPS exposure and after 55 min as well as after 5 min after removing the LPS by flushing the saline bath with fresh saline. LPS exposure was obtained by exchanging the untainted saline with LPS containing saline three times while maintaining intracellular recording. The same paradigm was used as a control with only changing saline with saline not containing LPS. A suction electrode was used to stimulate the excitatory nerve. The saline used and the recording

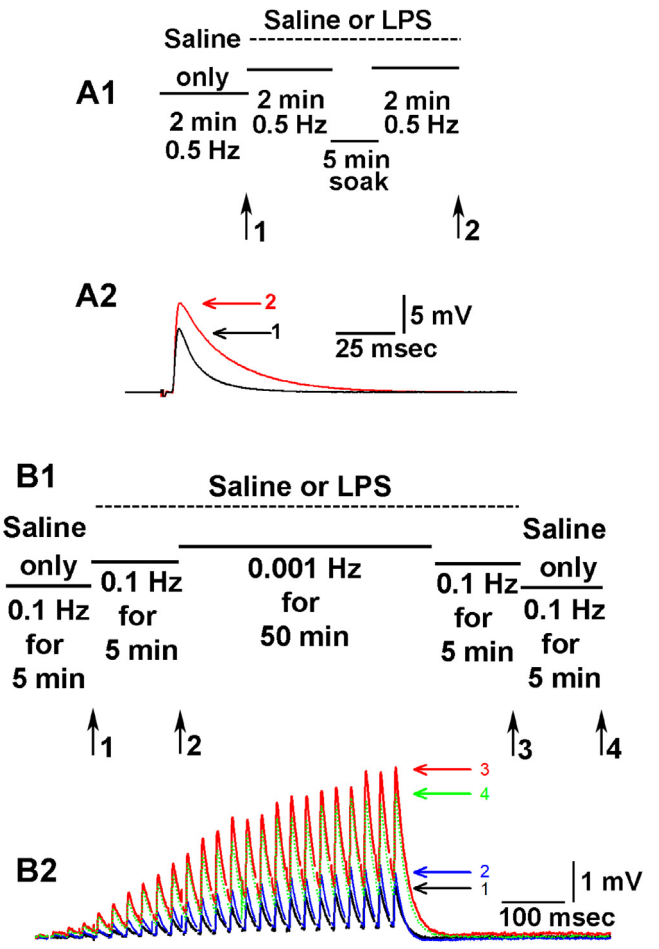

Fig. 2. The stimulation paradigm for the abdominal and opener preparations along with typical synaptic responses before and during exposure to LPS. (A1) The paradigm used to stimulate the high-output neuron to the DEL1 muscle is illustrated. A 0.5 Hz stimulation rate for 50 pulses in saline followed by 50 min more while exposed to LPS. A 5 min incubation time in LPS was then followed with resuming a 0.5 Hz stimulation. The measures used were in saline prior to LPS exposure, and after the 5 min LPS exposures after 2 min at 0.5 Hz stimulation. The arrows at 1 and 2 illustrate the time the measures used and are shown in the synaptic responses for the saline (1) and during the LPS (2) exposure (A2). (B1) The paradigm used to stimulate the low-output neuron to the opener muscle is illustrated. Stimulation trains at 40 Hz for 25 pulses were given every 10 s for 5 min in saline followed by another 5 min after exposure to LPS. This was followed by the trains given every 100 s for 50 min, and then train intervals of 10 s for 5 more minutes. The preparations were then rinsed with fresh saline and continued to be stimulated with trains every 10 s. The measures of the15th and 25th EJP within the trains occurred where the arrows are shown (1, 2, 3 and 4). (B2) The synaptic responses for a single preparation are shown before LPS exposure and after 5 min as well as after 55 more minutes in the stimulation paradigm. The dotted line is the response after 5 min being back in a saline after flushing away the LPS.

of electrical responses were the same as mentioned above for the abdominal preparation.

2.2.3. 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. marcescens*. A concentration of 500 μg/mL of LPS was used to compare to earlier studies used at the crayfish and *Drosophila melanogaster* NMJs. All chemicals for the saline and LPS were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3. Results

3.1. High output terminals

The high output NMJs of the DEL2 show depression within two minutes upon repetitive stimulation at 20 Hz (Cooper et al., 1998) but little depression at a 0.5 Hz stimulation frequency for 10 min; thus, this low frequency of stimulations was used for these studies. The stimulation paradigm used for the DEL1 NMJ provided time to obtain an average amplitude of the EJP prior to exposure of LPS. Since the amplitude of the EJP did not rapidly increase with the exposure, the stimulation at 0.5 Hz was continued for 2 more minutes, followed by a period of incubation to LPS for 5 min, then 2 more minutes of stimulation at 0.5 Hz was performed. At the end of the last stimulation, an average amplitude of the EJP was obtained (Fig. 2A1 and A2).

The low output of the NMJs on the distal fibers of the walking leg opener muscle showed marked facilitation with a train of stimuli. The 15th and 25th facilitated EJP responses were used for indexing the effect of LPS. Note the first few stimuli in the train barely induced a measurable EJP response (Fig. 2B2). Since the NMJs of the opener muscle did not show rapid depression with prolonged stimulation, a prolonged time of exposure and stimulation was used to examine the consequences of long term LPS exposure at a high concentration. During the incubation with LPS, a lower occurrence in the stimulation train (i.e., 50 min with 100 s between stimulation trains) was used followed by a stimulus train every 10 s for 5 min to compare to the initial 5 min of LPS exposure as the same stimulation frequency and train interval (Fig. 2B1 and B2).

To know if the EJP responses were depressing or enhancing over the time frames used in the different paradigms, saline-only exposures were used with the same stimulation paradigms. The bathing solutions were exchanged at the same increments of time as when the LPS containing saline would have been introduced (Fig. 2A1, B1).

The effect of LPS on the membrane potential was slight (~5 mV), but there was some drift in the membrane potential when changing the bathing media and with maintaining intracellular recordings of the preparations over time. The saline to saline changes helped to account for experimental variability and effects on the membrane potential over time to compare to the effects of LPS exposure. To illustrate the rapid effect on the membrane potential on the opener muscle, an enlarged view of the trace upon switching the media is shown in Fig. 3A1. A raw trace of one train during the saline exposure is illustrated to note that the EJPs are small and need to be facilitated to note their presence. Here, only the last 20 EJPs of the 25 stimulations are obvious. To illustrate the prolonged effect on the membrane potential a trace over the period is shown (Fig. 3B). The middle section of the 50 min period is removed. Note the hyperpolarization continues during the LPS exposure and upon removal of the LPS the membrane depolarizes slightly. However, the membrane potential is still more negative than during the initial saline exposure prior to LPS treatment. This trend was consistent in all six

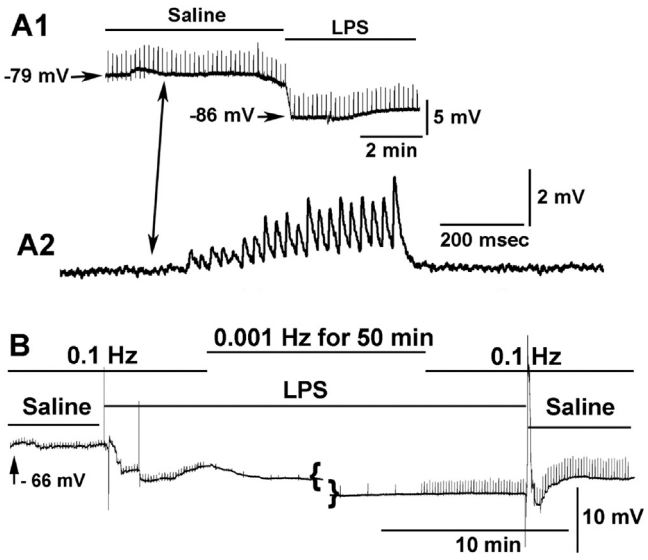

Fig. 3. The membrane potential of the muscles hyperpolarizes when exposed to LPS. (A1) A trace illustrating the response for an opener muscle before and during exposure to LPS. (A2) One of the responses to a stimulation train is shown. Each deflection shown in A1 is a response train. However, only the last 20 or the 25 stimuli resulted in a measurable EJP. (B) The membrane potential for the opener muscle preparation is shown over the experimental paradigm. There is a rapid hyperpolarization upon exposure to LPS and continues over time. When removing the LPS there is a slight depolarization, but the potential is still more negative than the initial starting potential.

preparations and is shown in the percent changes in the membrane potentials among the preparations (Fig. 4).

Exchanging the saline to LPS produced hyperpolarizing responses in the DEL1 muscles within preparations ($p < 0.05$, paired Student's *t*-test, $N = 6$, Fig. 4A). The saline-to-saline exchanges were not significant for the DEL1 muscle as well as for the opener muscle (Fig. 4).

The opener preparations did not show significant differences in the membrane potentials for the control saline to saline exchanges for all the four sampling points (1, 2, 3 and 4 shown in Fig. 2B1) (Friedman repeated measures analysis of variance on ranks, all pairwise multiple comparison procedures with a Bonferroni *t*-test). However, the LPS treatment resulted in hyperpolarized membrane potentials from saline to 5 min after exposure to LPS (period 1 to period 2, see Fig. 2B1), also saline to LPS exposure after the 55 min of exposure (period 1 to period 2) and saline to the saline wash out (period 1 to period 4) (Fig. 4A) in the last comparisons. These were all compared by Friedman repeated measures analysis of variance on ranks with an all pairwise multiple comparison procedures with a Bonferroni *t*-test ($p < 0.05$, $N = 6$). The other time periods for the raw membrane potentials of 2, 3 and 4 were not significant to each other.

A percent change in the membrane potentials were used, as this normalized the differences among preparations in the various raw membrane potentials (Figs. 3 and 4). A percent change within each preparation was calculated and the mean (±SEM) of the percent changes are shown in Fig. 4A for the membrane potentials. A percent change within each preparation was calculated and the mean (±SEM) of the percent changes are shown in Fig. 4B. To compare the same percent changes, such as period 1–2, the values are compared for the saline-to-saline change to the saline-to-LPS change. These equivalent periods for comparisons in the percent changes in the membrane potentials for the saline controls to LPS paradigm were significantly different by *t*-test (1–2, 2–3 as well as 1–4 in Fig. 2B1) ($p < 0.05$, data was not normally distributed, so a Mann-Whitney Rank Sum Test was performed, *t*-test, $N = 6$). The percent change

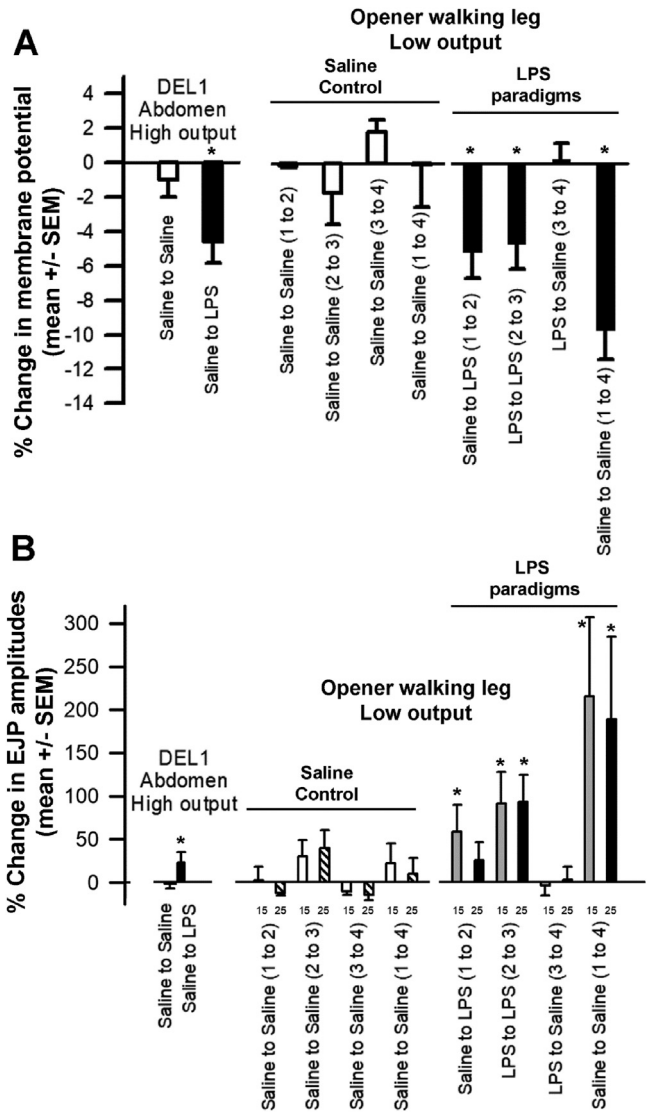

Fig. 4. The changes in the membrane and EJP amplitudes for the high- and low-output neurons for saline to saline (sham control) as compared to saline to LPS (experimental group). (A) The DEL1 phasic like muscles significantly hyperpolarized with exposure to LPS as compared to saline and between saline to saline controls of exchanging the media. The opener muscle also showed significant hyperpolarizing within preparations for LPS exposure but not for saline control paradigm. The percent change for the periods 1 to 2 and 3 to 4 as well as 1 to 4 showed significant differences for the same period of comparison for the saline to saline controls except for the percent changes from 3 to 4 periods (see Fig. 2B1). (B)The high-output neurons had a larger amplitude in the EJP by within preparation comparisons; however, the average percent increase when exposed to LPS as compared to the saline only controls was not significantly different. The low-output opener NMJ for saline to saline did trend to drift to larger responses in the first 5 min (1 to 2) and after the 55 min of stimulation when compared to just after the 5 min of exchanging the bathing solution (2 to 3), but within preparation comparisons did not reveal significant differences, nor did the percent changes among the different periods of time (see Fig. 2B1). The raw amplitudes in the EJPs among the different periods, 2, 3 and 4 were significantly different from the initial saline. The percent change in the amplitudes of the 15th and 25th EJP responses between the periods only showed differences for periods 1 to 2, and 2 to 3 as well as 1 to 4 but not the periods of 3 to 4 (B; see Fig. 2B1 for the defined periods).

of 3–4, which is the effect of wash out of LPS did not show any significant difference from saline controls in the percent changes, although the raw membrane potentials were different within the LPS paradigm from the initial saline to the periods at 3 and 4, as mentioned above.

The within experiments of LPS exposure showed a difference in the amplitudes for the 15th and 25th EJP amplitudes from saline to after 5 min of exposure to LPS (period 1 to period 2; see Fig. 2B1), also saline-to-LPS exposure after the 55 min of exposure (period 1 to period 2) and the saline-to-saline wash out (period 1 to period 4) (Fig. 4A and B) in the last comparisons. These were all compared by Friedman repeated measures analysis of variance on ranks with an all pairwise multiple comparison procedures with a Bonferroni *t*-test ($p < 0.05$, $N = 6$). The other time periods for the raw amplitudes of 2, 3 and 4 were not significant to each other.

The percent change in the amplitudes of the 15th and 25th EJP responses in the trains were used as this normalized the differences among preparations in the various raw amplitudes (Fig. 4). A percent change within each preparation was calculated and the mean (±SEM) of the percent changes are shown in Fig. 4B. The percent changes in the 15th and 25th EJPs were compared to the saline controls for the percent changes in saline controls. To compare the same percent changes, such as period 1–2, the values are compared for the saline-to-saline change to the saline-to-LPS change. These equivalent periods for comparisons in the percent changes in the amplitudes for the saline controls to LPS paradigm were significantly different by *t*-test (1–2, 2–3 as well as 1–4 in Fig. 2B1) ($p <$ 0.05, data was not normally distributed, so a Mann-Whitney Rank Sum Test was performed, *t*-test, $N = 6$). The percent change of 3–4, which is the effect of wash out of LPS, did not show any significant difference from saline controls in the percent changes; although, the raw amplitudes were different within the LPS paradigm from the initial saline to the periods of 3 or 4, as mentioned above.

4. Discussion

The effect of LPS on the high output phasic type of NMJs did show a significant increase when compared as a before and after effect; however, when compared to controls of saline only exchanges, there was not a significant difference. The low output tonic-like NMJs showed a substantial increase in the synaptic responses over time due to exposure of LPS within preparations and when compared to saline only controls. The muscle fibers of the opener muscle showed a hyperpolarization when compared to saline controls. The long term exposure in LPS at a high concentration of an hour did not result in the loss of membrane integrity or a decrement to the amplitude of the EJP. The opposite occurred, in that the membrane remained hyperpolarized and EJP amplitudes remained larger than saline controls over the same period of experimental time. The comparison of the initial values of membrane potential and the amplitudes of the EJPs before the LPS and after wash out of the LPS were in a healthy range indicating that the LPS exposure was not damaging to the crayfish NMJ as it is for the larval *Drosophila* NMJs (Istas, et al., 2020).

The mechanisms of action to increase synaptic transmission is assumed to be presynaptic, since an earlier study had demonstrated no effect on the amplitude of individual quantal responses on crayfish muscle (Saelinger et al., 2019). One mechanistic possibility is an increase in Ca^{2+} within the presynaptic motor nerve terminal. However, no increase in the frequency of quantal responses were observed in an earlier study of crayfish NMJs (Saelinger et al., 2019) as well as for larval *Drosophila* NMJs (Cooper et al., 2019; Istas et al., 2019) when exposed to LPS. One early study which appears to have measured response from a single preparation of an unidentified abdominal muscle of the crayfish showed an increase in the frequencies of quantal events with LPS (Parnas et al., 1971). Thus, the effect on the opener muscle may be to promote evoked Ca^{2+} channels to stay open or to open at a lower threshold if any resting inactivation of voltage gated Na^{+} channels within the motor neuron may be removed with potential hyperpolarization. This would lead to altering the evoked amplitude and duration of the action potential in the neurons. Since the muscle hyperpolarizes in both larval

Drosophila (Istas et al., 2019; Cooper et al., 2019) and opener muscle in crayfish, it may also be feasible that the motor nerve terminal hyperpolarizes. Thus, if inactivation of voltage-gated ion channels occurs at normal resting membrane potential, then removing the inactivation would lower the threshold of activation. This remains to be examined with intracellular recording of the axons of the motor neurons. Imaging the nerve terminals with Ca^{2+} indicators would address if there is an increase in the evoked Ca^{2+} load. In addition, the small (~10 mV) hyperpolarization increases the driving gradient for the inotropic glutamate receptors at these neuromuscular junctions (Titlow and Cooper, 2018) can contribute in part to the increased amplitude of the EJP. To account for the larger increase in the amplitude for the EJPs of the tonic NMJs, there is likely a contribution of recruiting a larger reserve pool of vesicles around the synapses of the tonic NMJs than for the synapses of phasic NMJs (Bradacs et al., 1997; Johnstone et al., 2008). This would increase the probability of vesicle fusion if more vesicles were docked for release upon Ca^{2+} entry in the presynaptic terminal. A larger increase in the amplitude of EJPs also occurs in the presence of serotonin for tonic NMJs than for phasic NMJs of in crustaceans. This is probably due to the synaptic structural differences and population of the vesicle pools among the readily releasable pool and reserve pools between these types of terminals (Cooper et al., 2003; Logsdon et al., 2006; Wu and Cooper, 2012a, b,2013). LPS also behaves similarly to serotonin in enhancing evoked EJPs but it was shown recently that a high concentration of LPS does not appear to diminish the effects of serotonin when given concurrently. This would suggest that LPS might not be competing for the same receptors as serotonin or the affinity for the receptors to LPS and serotonin are substantially different (Bernard et al., 2020a).

There has yet to be a mechanistic explanation to account for the LPS induced hyperpolarization observed in crayfish opener muscle or in larval *Drosophila* muscle (Ballinger-Boone et al., 2020). As stated in a previous report, the hyperpolarization response is not due to an induction of a calcium-activated potassium conductance, a TEA sensitive potassium channel, or mediated through nitric oxide synthase (NOS) as each of these possibilities have been addressed (Cooper et al., 2019; Ballinger-Boone et al., 2020). In addition, the response is not due to the opening of a chloride channel as the equilibrium potential for Cl^- in larval Drosophila body wall muscles is more depolarized than the resting membrane potential (Rose et al., 2007; Stanley et al., 2019). One potential mechanism which could explain the phenomenon is activation of the Na-K ATP pump as the response occurs rapidly in both crayfish opener muscle as well as larval *Drosophila* muscle (Cooper et al., 2019; Ballinger-Boone et al., 2020).

Receptors for LPS on muscle and neurons in crustacean and insects remains to be thoroughly investigated. The toll-like receptor 4 (TLR4), as in mammals (Yoshida et al., 1996; Steiner, 2004), does not appear to be the sole receptors for the gram-negative LPS induced response in *Drosophila* nor is it likely that the Immune deficiency (Imd) signaling pathway is the key. Peptidoglycan recognition proteins (PGRPs) are known to mediate an immune response in *Drosophila*. The three PGRPs (i.e., PGRP-SA, PGRP-LC, and PGRP-LE), out of the 13 identified in the genome of *Drosophila*, were linked with the immune response (Werner et al., 2000; Leclerc and Reichhart, 2004). PGRP-LC and PGRP-LE have been shown to respond to gram negative bacteria (Gottar et al., 2002; Takehana et al., 2002). Using RNAi constructs of PGRP-LC and PGRP-LE for expression in larval *Drosophila* body wall muscles as well as neurons did not alter the responses to direct exposure of LPS (Ballinger-Boone et al., 2020). Thus, the LPS receptors have yet to be identified to account for the responses at the larval NMJ of *Drosophila* as well as for the crayfish NMJs. There is mounting evidence from neuronal responses in *Drosophila* and mammals that the gram-negative bacterial endotoxin lipopolysaccharide (LPS) binds to TRPA1 receptors (Meseguer et al., 2014; Boonen et al., 2018). Recently, the the TRPA1 receptors were knocked down with RNAi in *Drosophila* muscle and neurons (Bernard et al., 2020b). Thus, suggesting the TRPA1 receptors do not mediate the responses to LPS in Drosophila. The TRPA1 receptors are one form of thermal detectors, but it is unknown if these receptors are expressed in crayfish skeletal muscle.

Future comparative studies examining various cell types and species would be beneficial to determine the direct mechanism of action by LPS which could help in addressing therapeutic actions of various cell types and animal species.

Acknowledgments

Chellgren Endowed Professor fund (RLC) at the University of Kentucky, Lexington, Kentucky, USA.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neures.2020.08.008.

References

Atwood, H.L., 1973. Crustacean motor units. In: Stein, R.B., Pearson, K.G., Smith, R.S., Redford, J.B. (Eds.), Control of Posture and Locomotion. Plenum Press, New York, pp. 87–104.
Atwood, H.L., 1976. Organization and synaptic physiology of crustacean neuromuscular systems. Prog. Neurobiol. 7, 291–391.
Atwood, H.L., Cooper, R.L., 1995. Functional and structural parallels in crustaceans and Drosophila neuromuscular systems. Am. Zool. 35, 556–565.
Atwood, H.L., Cooper, R.L., 1996a. Assessing ultrastructure of crustacean and insect neuromuscular junctions. J. Neurosci. Meth. 69, 51–58.
Atwood, H.L., Cooper, R.L., 1996b. Synaptic diversity and differentia-tion: crustacean neuromuscular junctions. Invert. Neurosci. 1, 291–307.
Baierlein, B., Thurow, A.L., Atwood, H.L., Cooper, R.L., 2011. Membrane potentials, synaptic responses, neuronal circuitry, neuromodulation and muscle histology using the crayfish: Student laboratory exercises. J. Vis. Exp. (JoVE). 47, http://dx.doi.org/10.3791/2325 http://www.jove.com/Details.php?ID=2322.
Ballinger-Boone, C., Anyagaligbo, O., Bernard, J., Bierbower, S.M., Dupont-Versteegden, E.E., Ghoweri, A., Greenhalgh, A., Harrison, D., Istas, O., McNabb, M., Saelinger, C., Stanback, A., Stanback, M., Thibault, O., Cooper, R.L., 2020. The effects of bacterial endotoxin (LPS) on cardiac and synaptic function in various animal models: Larval Drosophila, crayfish, crab, and rodent. Int. J. Zool. Res. 16, 33–62, http://dx.doi.org/10.3923/ijzr.2020.33.62.
Bernard, J., Greenhalgh, A., Istas, O., Marguerite, N.T., Cooper, R.L., 2020a. The effect of bacterial endotoxin LPS on serotonergic modulation of glutamatergic synaptic transmission. Biology 2020 (9), 210, http://dx.doi.org/10.3390/biology9080210.
Bernard, J., Marguerite, N., Inks, M., Cooper, R.L., 2020b. Opposing responses of bacterial endotoxin lipopolysaccharide (LPS) and TRPA1 thermal receptors on synaptic transmission and resting membrane potential. Current Res. Bacteriol. 13 (1), 10–21, http://dx.doi.org/10.3923/crb.2020.10.21.
Boonen, B., Alpizar, Y., Meseguer, V., Talavera, K., 2018. TRP channels as sensors of bacterial endotoxins. Toxins. 10 (8), 326, http://dx.doi.org/10.3390/toxins10080326.
Bradacs, H., Cooper, R.L., Msghina, M., Atwood, H.L., 1997. Differential physiology and morphology of phasic and tonic motor axons in a crayfish limb extensor muscle. J. Exp. Biol. 200, 677–691.
Cavaillon, J.M., 2018. Exotoxins and endotoxins: inducers of inflammatory cytokines. Toxicon. 149, 45–53, http://dx.doi.org/10.1016/j.toxicon.2017.10.016.
Cooper, R.L., 1998. Ectoparasites (branchiobdellids) on egg hatching. International Association of Astacology newsletter' Crayfish News'. 20, 20–21.
Cooper, A.S., Cooper, R.L., 2009. Historical view and demonstration of physiology at the NMJ at the crayfish opener muscle. J. Vis. Exp. (JoVE). 33, http://dx.doi.org/10.3791/1595 http://www.jove.com/index/details.stp?id=1595.
Cooper, R.L., Marin, L., Atwood, H.L., 1995. Synaptic differentiation of a single motor neuron: conjoint definition of transmitter release, presynaptic calcium signals, and ultrastructure. J. Neurosci. 15, 4209–4222.
Cooper, R.L., Warren, W.M., Ashby, H.E., 1998. Activity of phasic motor neurons partially transforms the neuronal and muscle phenotype to a tonic-like state. Muscle Nerve 21, 921–931.
Cooper, R.L., Donmezer, A., Shearer, J., 2003. Intrinsic differences in sensitivity to 5-HT between high- and low-output terminals innervating the same target. Neurosci. Res. 45, 163–172.
Cooper, R.L., McNabb, M., Nadolski, J., 2019. The effects of a bacterial endotoxin LPS on synaptic transmission at the neuromuscular junction. Heliyon 5, e01430 https://www.heliyon.com/article/e01430.

Costamagna, D., Costelli, P., Sampaolesi, M., Penna, F., 2015. Role of inflammation in muscle homeostasis and myogenesis. Mediators Inflamm., 805172, http://dx.doi.org/10.1155/2015/805172.
Crider, M.E., Cooper, R.L., 1999. The importance of the stimulation paradigm in determining facilitation and effects of neuromodulation. Brain Res. 842, 324–331.
Crider, M.E., Cooper, R.L., 2000. Differentially facilitation of high- and low-output nerve terminals from a single motor neuron. J. Applied Physiol. 88, 987–996.
D'Elia, R.V., Harrison, K., Oyston, P.C., Lukaszewski, R.A., Clark, G.C., 2013. Targeting the "cytokine storm" for therapeutic benefit. Clin. Vaccine Immunol. 20 (3), 319–327, http://dx.doi.org/10.1128/CVI.00636-12.
Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D., Royet, J., 2002. The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature 416, 640–644.
Griffis, B., Bonner, P., Cooper, R.L., 2000. Sensitivity of transformed (phasic to tonic) motor neurons to the neuromodulator 5-HT. Comp. Biochem. Physiol. A 127, 495–504.
Istas, O., Greenhalgh, A., Cooper, R.L., 2019. The effects of a bacterial endotoxin on behavior and sensory-CNS-motor circuits in *Drosophila melanogaster*. Insects 10, 115, http://dx.doi.org/10.3390/insects10040115.
Istas, O., Greenhalgh, A., Cooper, R.L., 2020. Repetitive exposure to bacterial endotoxin LPS alters synaptic transmission. J. Pharmacol. Toxicol. 15, 65–72, http://dx.doi.org/10.3923/jpt.2020.65.72.
Johnstone, A.F.M., Kellie, S., Cooper, R.L., 2008. Presynaptic depression in phasic motor nerve terminals and influence of 5-HT on docked vesicles. Open Neurosci. J. 2, 16–23.
Leclerc, V., Reichhart, J.M., 2004. The immune response of Drosophila melanogaster. Immunol. Rev. 198, 59–71.
Leksrisawat, B., Cooper, A.S., Gilberts, A.B., Cooper, R.L., 2010. Response properties of muscle receptor organs in the crayfish abdomen: a student laboratory exercise in proprioception. J. Vis. Exp. (JoVE) 45, http://dx.doi.org/10.3791/2323 http://www.jove.com/index/details.stp?id=2323.
Lnenicka, G.A., Atwood, H.L., 1989. Impulse activity of a crayfish motoneuron regulates its neuromuscular synaptic properties. J. Neurophysiol. 61, 91–96.
Logsdon, S., Johnstone, A.F.M., Viele, K., Cooper, R.L., 2006. The regulation of synaptic vesicles pools within motor nerve terminals during short-term facilitation and neuromodulation. J. Applied Physiol. 100, 662–671.
Loppnow, H., Libby, P., Freudenberg, M., Krauss, J.H., Weckesser, J., Mayer, H., 1990. Cytokine induction by lipopolysaccharide (LPS) corresponds to lethal toxicity and is inhibited by nontoxic Rhodobacter capsulatus LPS. Infect. Immun. 58 (11), 3743–3750.
Mercier, A.J., Atwood, H.L., 1989. Long-term adaptation of a phasic extensor motoneurone in crayfish. J. Exp. Biol. 145, 9–22.
Meseguer, V., Alpizar, Y.A., Luis, E., Tajada, S., Denlinger, B., Fajardo, O., Manenschijn, J.-A., Fernández-Peña, C., Talavera, A., Kichko, T., Navia, B., Sánchez, A., Señarís, R., Reeh, P., Pérez-García, M.T., López-López, J.R., Voets, T., Belmonte, C., Talavera, K., Viana, F., 2014. TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. Nat. Commun. 5, 3125, http://dx.doi.org/10.1038/ncomms4125.
Mykles, D.L., Medler, S.A., Koenders, A., Cooper, R.L., 2002. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. J. Exp. Biol. 205 (4), 513–522.
Olson, J.K., Miller, S.D., 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J. Immunol. 173, 3916–3924.
Parnas, I., Atwood, H.L., 1966. Phasic and tonic neuromuscular systems in the abdominal flexor muscles of the crayfish and rock lobster. Comp. Biochem. Physiol. 18, 701–723.
Parnas, I., Reinhold, R., Fine, J., 1971. Synaptic transmission in the crayfish: increased release of transmitter substance by bacterial endotoxin. Science 171, 1153–1155.
Rose, U., Derst, C., Wanischeck, M., Marinc, C., Walther, C., 2007. Properties and possible function of a hyperpolarisation-activated chloride current in Drosophila. J. Exp. Biol. 210 (14), 2489–2500.
Saelinger, C.M., McNabb, M.C., McNair, R., Bierbower, S., Cooper, R.L., 2019. Effects of bacterial endotoxin on regulation of the heart, a sensory-CNS-motor nerve circuit and neuromuscular junctions: crustacean model. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 237, 110557, http://dx.doi.org/10.1016/j.cbpa.2019.110557.
Sohn, J., Mykles, D.L., Cooper, R.L., 2000. The anatomical, physiological and biochemical characterization of muscles associated with the articulating membrane in the dorsal surface of the crayfish abdomen. J. Exp. Zool. 287, 353–377.
Stanley, C.E., Mauss, A., Borst, A., Cooper, R.L., 2019. The effects of chloride flux on Drosophila heart rate. Meth. Protocols 2 (3), 73, http://dx.doi.org/10.3390/mps2030073.
Steiner, H., 2004. Peptidoglycan recognition proteins: on and off switches for innate immunity. Immunol. Rev. 198, 83–96.
Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., Aigaki, T., Kurata, S., 2002. Overexpression of a pattern-recognition receptor, peptidoglycan recognition protein-LE, activates imd/relish mediated antibacterial defense and the prophenoloxidase cascade in Drosophila larvae. Proc. Natl. Acad. Sci. U.S.A. 99, 13705–13710.
Titlow, J.S., Cooper, R.L., 2018. Glutamatergic synthesis, recycling, and receptor pharmacology at drosophila and crustacean neuromuscular junctions. In: Parrot, S., Denoroy, L. (Eds.), Biochemical Approaches for Glutamatergic Neurotransmission. NEuromethods, vol 130. Humana Press, New York, N.Y, pp. 263–291.
Wassenaar, T.M., Zimmermann, K., 2018. Lipopolysaccharides in food, food supplements, and probiotics: should we be worried? Europ. J. Microbiol. & Immunol. 8 (3), 63–69, http://dx.doi.org/10.1556/1886.2018.00017.
Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., Hultmark, D., 2000. A family of peptidoglycan recognition proteins in the fruit fly Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.A. 97 (25), 13772–13777.
Wu, W.-H., Cooper, R.L., 2012a. The regulation and packaging of synaptic vesicles as related to recruitment within glutamatergic synapses. Neurosci. 225, 185–198.
Wu, W.-H., Cooper, R.L., 2012b. Serotonin and synaptic transmission at invertebrate neuromuscular junctions. Exp. Neurobiol. 21 (3), 101–112.
Wu, W.-H., Cooper, R.L., 2013. Physiological separation of vesicle pools in low- and high-output nerve terminals. Neurosci. Res. 75, 275–282.
Yoshida, H., Kinoshita, K., Ashida, M., 1996. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, Bombyx mori. J. Biol. Chem. 271, 13854–13860.