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The effects of bacterial endotoxin LPS on synaptic transmission at the neuromuscular junction

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Abstract

The direct action of bacterial lipopolysaccharides (LPS) endotoxin was shown to enhance synaptic transmission and hyperpolarize the membrane potential at low doses, but block glutamatergic receptors and decrease observable spontaneous events at a high dosage. The dosage effects are LPS type specific. The hyperpolarization is not due to voltage-gated potassium channels or to activation of nitric oxide synthase (NOS). The effects are induced directly by LPS, independent of an immune response.

Keywords: Immunology, Neuroscience, Physiology, Zoology

1. Introduction

Gram-negative bacterial septicaemia infects humans as well as other animals. The immunological response to bacterial infection will activate various cascades of defence cytokines and antibody formation [1]. Two common bacterial strains that cause septicaemia in mammals are *Pseudomonas aeruginosa* (*P. a.*) and *Serratia marcescens* (*S. m.*) [2, 3, 4, 5]. The induced cytokines and defence response to the surface antigens on bacteria account for some of the immune response, but

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lipopolysaccharides (LPS), which are secreted from the bacteria, can have direct effects on cells, in addition to stimulating an immune response. When bacteria are lysed either by the body's own defence or by therapeutic treatments, LPS can surge and trigger a reaction independent of the immune response. The direct action of LPS and the immune induced response are complex. The mechanisms responsible for the neurological effects induced by septicaemia, including mental confusion and in some cases coma, are still debated [6, 7, 8].

Thus, it is important to tease apart the direct actions of LPS from the immune response. Isolated tissue preparations offer the ability to assess the effect of LPS directly without the compounding variables found within a host. The exposure of frog cholinergic neuromuscular junctions (NMJs) to LPS revealed an increase in the occurrence of spontaneous quantal responses but reduced the evoked amplitude of the excitatory postsynaptic junction potentials (EJPs) at doses as low as 10 μg/ml of lyophilized LPS from *Salmonella typhimurium*. LPS was proposed to cause a Ca^{2+} leak, leading to an increase in spontaneous quantal responses, but block the voltage-gated calcium channels, leading to a reduced evoked response [9]. There has yet to be direct confirmation that Ca^{2+} leaks in the presynaptic nerve terminal following LPS exposure or that the voltage-gated channels are directly blocked. It is unlikely that the nicotinic postsynaptic receptors were blocked since an increase in frequency of spontaneous EJPs (mEJPs) was observed. In contrast, the only study of glutamatergic synapses at a NMJ was performed on crayfish, which revealed an increase in the amplitude of the EJPs after exposure to LPS (*S. m.*; 2 μg/ml) [10].

However, an increase in spontaneous quantal events was also found in this preparation [10]. The crayfish NMJ is different from the frog NMJ in that the evoked responses in the muscle fibres are graded and non-spiking, so the enhanced EJP amplitudes are due to an increase in synchronized vesicular fusion. The increase in spontaneous events and the enhanced evoked responses suggest increased Ca^{2+} loading induced by LPS. Previous studies of frog NMJ did not directly measure Ca^{2+} levels within the presynaptic nerve terminal and did not address whether the rise in Ca^{2+} was due to internal release of Ca^{2+} from the ER or from an extracellular source.

The NMJs of crayfish and larval *Drosophila melanogaster* are similar in their pharmacological profile [11, 12]. Even though the neurotransmitter at the larval NMJ is glutamate, the genetic tools available in *Drosophila* have provided a detailed understanding of synaptic homeostasis and the biochemistry involved in synaptic function as a model for most chemical synapses [11]. The purpose of this study was to determine the direct effects of LPS on glutamatergic synaptic transmission.

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2. Results

Stimulating a segmental nerve in the 3rd instar at 0.5 Hz evokes prominent EJPs in muscle 6 (Fig. 1). Alterations in amplitude of synaptic responses occurred during exposure to LPS from *S. m.* and *P. a.* in a dose-dependent manner. Treatment with LPS from *S. m.* at 100 μg/ml produced varied responses in EJP amplitudes (Fig. 2A). Whereas treatment with LPS from *P. a.* significantly increased EJP amplitude ($P = 0.02$, paired T-test) among preparations despite a collective average percent decrease due to a few of the 16 preparations having a pronounce decrease in amplitude (Fig. 2C). The frequency of spontaneous quantal events did not systematically increase for either LPS exposure as was reported for glutamatergic crayfish [10] and cholinergic frog [9] NMJs. In fact, there was a significant decrease in the frequency of spontaneous events after 10 min of exposure to LPS for both *S. m.* and *P. a* (Fig. 2B and D; Wilcoxon Signed Rank Test, $P < 0.005$).

2.1. Changes in EJP amplitude is LPS dose-dependent

The rate of change in the amplitude of the evoked EJPs and mEJPs, as well as the resting membrane potential, is rapid upon exposure to LPS from *S. m.* and *P. a* (Figs 3 and 4). However, LPS from *S. m.* depressed synaptic transmission to a greater

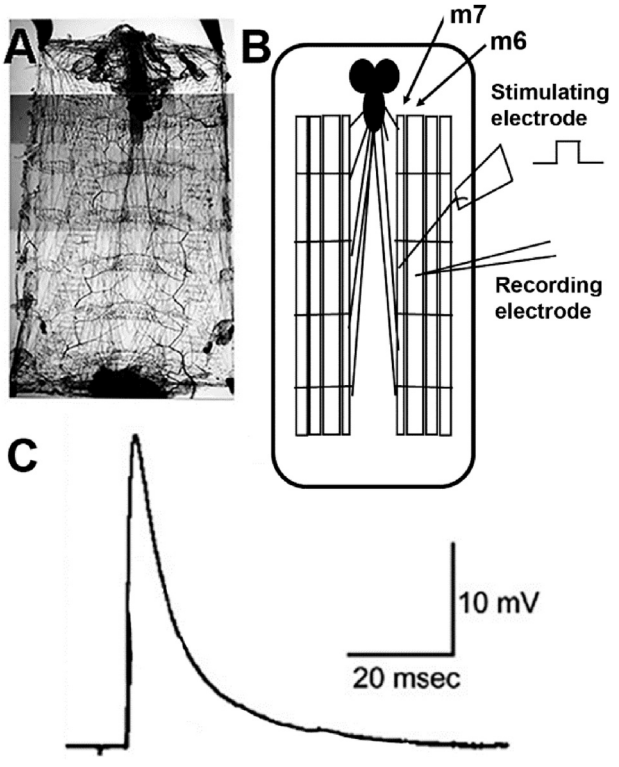

Fig. 1. (A) 3rd instar larvae were filleted open and a (B) segmental nerve was stimulated while (C) EJPs and mEJPs were recorded from m6 muscle fibres.

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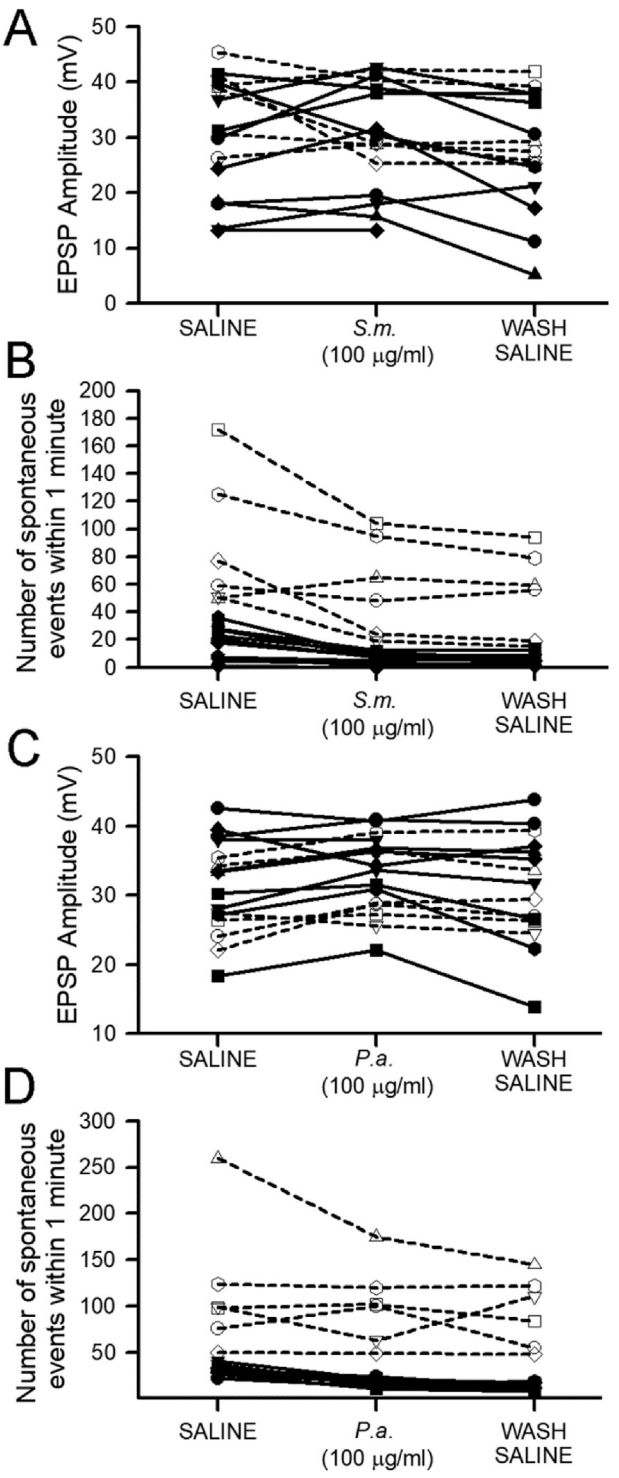

Fig. 2. Comparison of LPS at 100 μg/ml from *S. m.* and *P. a.* The EJP amplitudes did not have a significant change for exposure to *S. m.* (A). The EJP amplitudes significantly increased for *P.a.* exposure (B; $P = 0.02$, paired T-test). There is significant decrease in the frequency of spontaneous events after 10 min of exposure to LPS for both *S. m.* and *P. a.* (Fig. B and Fig. D; Wilcoxon Signed Rank Test, $P < 0.005$).

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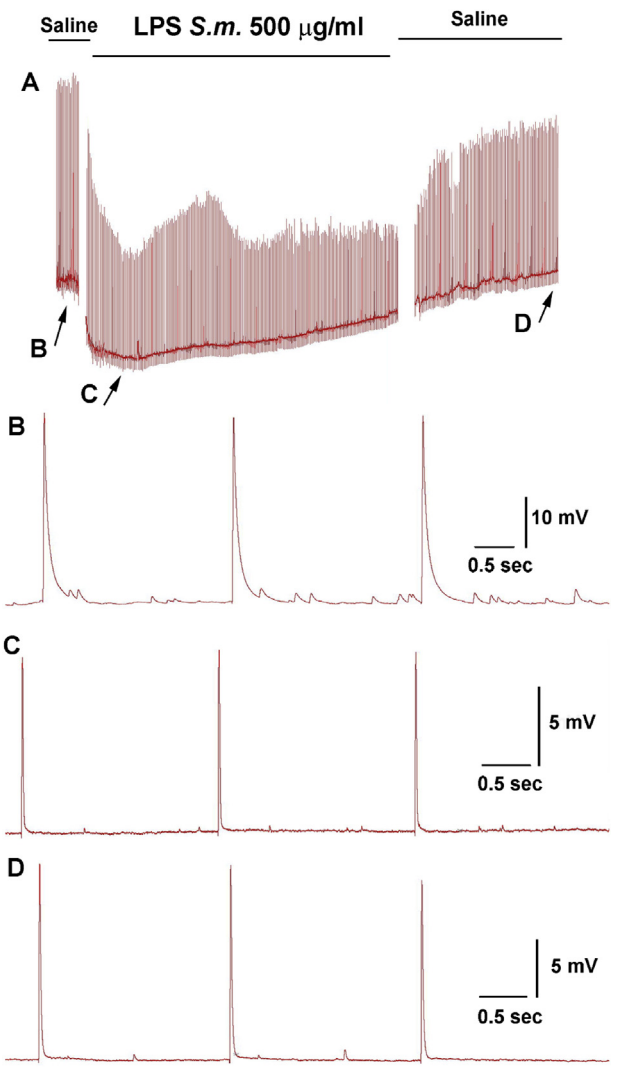

Fig. 3. Representative trace for exposure to LPS from *S.m.* at 500 µg/ml and enlarged segments to highlight the changes in amplitudes of the EJPs.

extent at 500 µg/ml ($P < 0.001$, paired T-test; Fig. 5A2) than LPS from *P. a.* at 500 µg/ml (Fig. 5B2). The LPS from either *S. m.* or *P. a.* rapidly hyperpolarized the membrane potential ($P < 0.001$, paired T-test; Fig. 5A2 and B2).

The decrease in the amplitudes of the EJP is obviously not due to low frequency synaptic depression, which is known to occur at these NMJs [13], since upon removal the of the LPS the amplitude of the responses partially recovers (Figs. 3C, D and 4C, D).

It was possible to quantify the rate of spontaneous mEJPs during exposure to 100 µg/ml LPS since the amplitudes did not decrease. However, since the amplitudes decreased during exposure to 500 µg/ml LPS (*S. m.*), they could not be reliably determined and compared to the exposure of LPS for *P. a.* at 500 µg/ml (Figs. 3C

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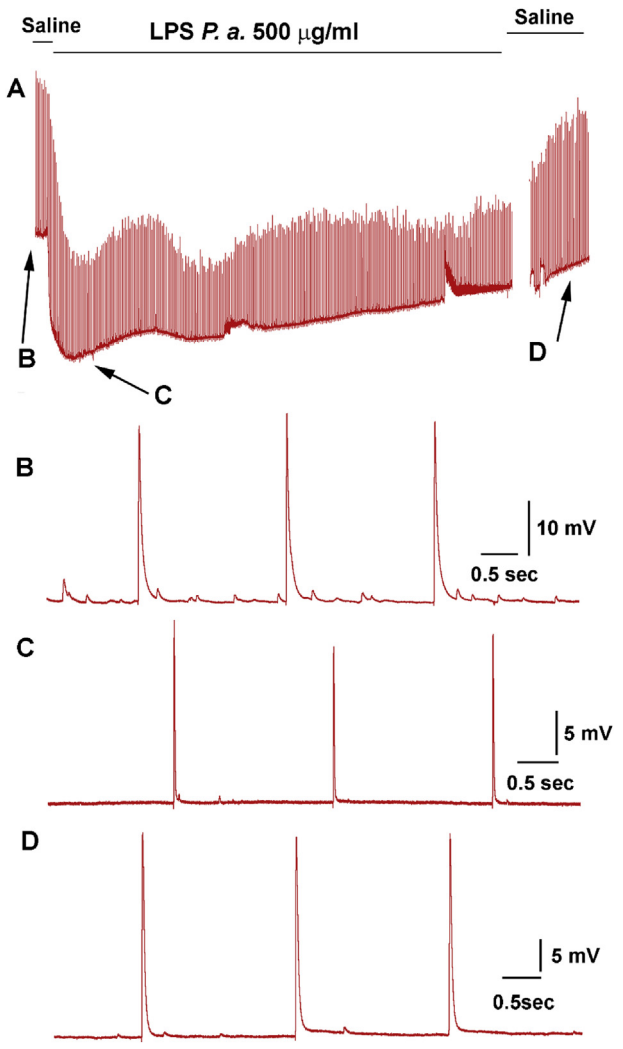

Fig. 4. Representative trace for exposure to LPS from *P.a.* at 500 μg/ml and enlarged segments to highlight the changes in amplitudes of the EJPs.

and 4C). Within the 10-minute exposure to LPS of *P. a.*, one preparation appeared to display a short burst of mEJPs. In general, the frequency and amplitude of mEJPs decreased with the reduction of the EJPs amplitude. The mEJPs were not detectable in most of the preparations at 500 μg/ml since the size became so small and were lost in the background noise. Thus, we did not report on the amplitudes and frequency since it was obvious, they are gradually being lost in the background noise of the recording with exposure to LPS. It is interesting the mEJPs and EJPs gradually reappear upon removing the LPS. Contrary to mEJPS, however, when evoked EJPs drop to zero amplitude, it is appropriate to report this value, as it is known when they should occur.

Increasing the concentration of *P. a.* LPS to 750 μg/ml produced an effect similar to the treatment with 500 μg/ml of LPS from *S. m.*: it decreased the amplitude of EJPs

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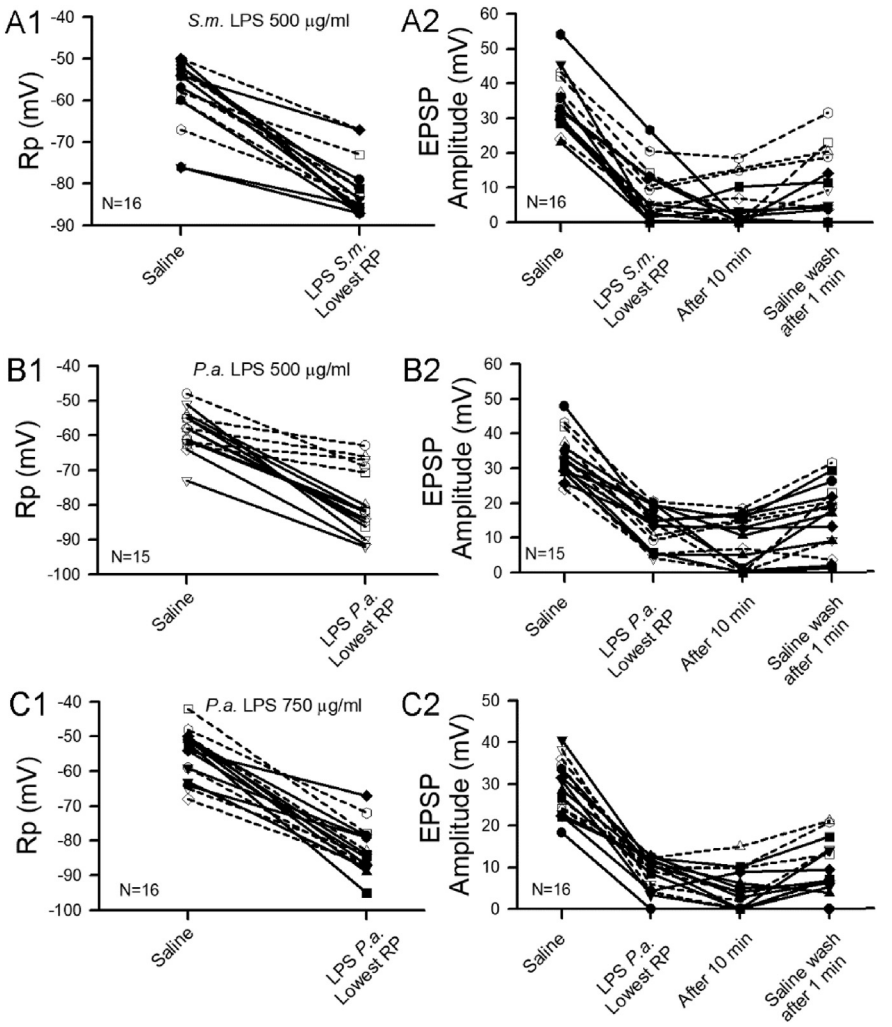

Fig. 5. Composite effects on the amplitude of EJPs and membrane potential during and after treatment with 500 µg/ml *S. m.* and *P. a.* and 750 µg/ml LPS from *P. a.* LPS from either *S. m.* or *P. a.* depressed the EJP amplitudes with 500 µg/ml (A2, B2) and *P. a.* for 750 µg/ml (C2) ($P < 0.001$, paired T-test). The LPS from either *S. m.* or *P. a.* rapidly hyperpolarized the membrane potential at the ($P < 0.001$, paired T-test).

and mEJPs, and produced a pronounced hyperpolarization of the membrane potential (Fig. 5). EJPs quickly increased in amplitude after removal of the LPS, and addition of a saline-alone solution in the trials with *S. m.* at 500 µg/ml and with the *P. a.* at 750 µg/ml. However, repeated flushing of the preparation with fresh saline after exposure to LPS did not fully restore the amplitudes of evoked EJPs. The membrane potential also approached basal levels and the observable occurrences of mEJPs returned. The reoccurrences of mEJPs is likely due to an increased amplitude, as they decreased gradually in amplitude upon exposure to LPS and gradually increase in amplitude after removal of LPS.

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2.2. Membrane hyperpolarization

Even though the values of the resting membrane potentials vary among preparations, the effect of LPS still results in a hyperpolarized response. LPS exposure to either *S. m.* 500 μg/ml, or *P. a*. at 500 or 750 μg/ml, resulted in a more negative membrane potential (Fig. 5A1, B1 and C1; $P < 0.001$, paired T-test). These muscle cells were not voltage clamped in order to measure the effect on the membrane potential and the varying degree in the effect on the EJP amplitude as would occur in intact animals.

Despite the hyperpolarization of the membrane induced by LPS from *S. m.* at 500 μg/ml or *P. a.* at 500 or 750 μg/ml, the EJPs became smaller and continued to vary in amplitude (Fig. 5A2, B2 and C2; $P < 0.001$, paired T-test).

2.3. Potential mechanisms behind muscle hyperpolarization

The mechanism that induces hyperpolarization is not as readily explained. If LPS induced calcium-activated potassium conductance, it would have to be specific to the muscle fiber and not the presynaptic nerve terminal; otherwise, an increase in the evoked EJP amplitude and an increase in the frequency of mEJPs would be expected to occur with any increased Ca^{2+} loading in the nerve terminal. It is possible that LPS acts more prominent on the postsynaptic muscle by blocking glutamate receptors and hyperpolarization via some ionic mechanism such as a calcium-activated potassium or chloride conductance, which may even occur simultaneously.

To examine if LPS induced a Ca^{2+} influx, a Ca^{2+}-free saline was added for 2 minutes prior to exposing the preparation to a Ca^{2+}-free saline containing LPS (*S. m.*, 500 μg/ml). The amplitude of the evoked EJPs rapidly dampened in Ca^{2+}-free saline; however, LPS still resulted in a substantial hyperpolarization (Fig. 6A). It was previously demonstrated that TEA (20 mM) blocks the calcium-activated potassium channel in larval *Drosophila* muscle [14]. TEA (20 mM) did not block LPS induction of hyperpolarization (Fig. 6B) and did not prevent the reduction in the amplitude of the evoked EJPs during LPS exposure. Since LPS has been shown to activate nitric oxide synthase (NOS) and induce hyperpolarization in the membrane potential of rodent cardiac muscle, NOS induction was examined. L-NAME inhibits NOS within 20−30 minutes in birds, rodents and human tissue [15, 16, 17, 18] and treatment of *Drosophila* with L-NAME aided the immune response to parasitoids [19]. Thus, the exposed NMJs were incubated with L-NAME (1 mM) and TEA (20 mM) for 20 min prior to treatment with saline containing L-NAME + TEA + LPS (*S.m.*, 500 μg/ml), which still produced substantial hyperpolarization. To note the change under these conditions only spontaneous quantal vesicular activity and the resting potential is measured (Fig. 6C). This same trend, in response to the pharmacological treatments, is observed in six out of six preparations ($P < 0.05$; sign test).

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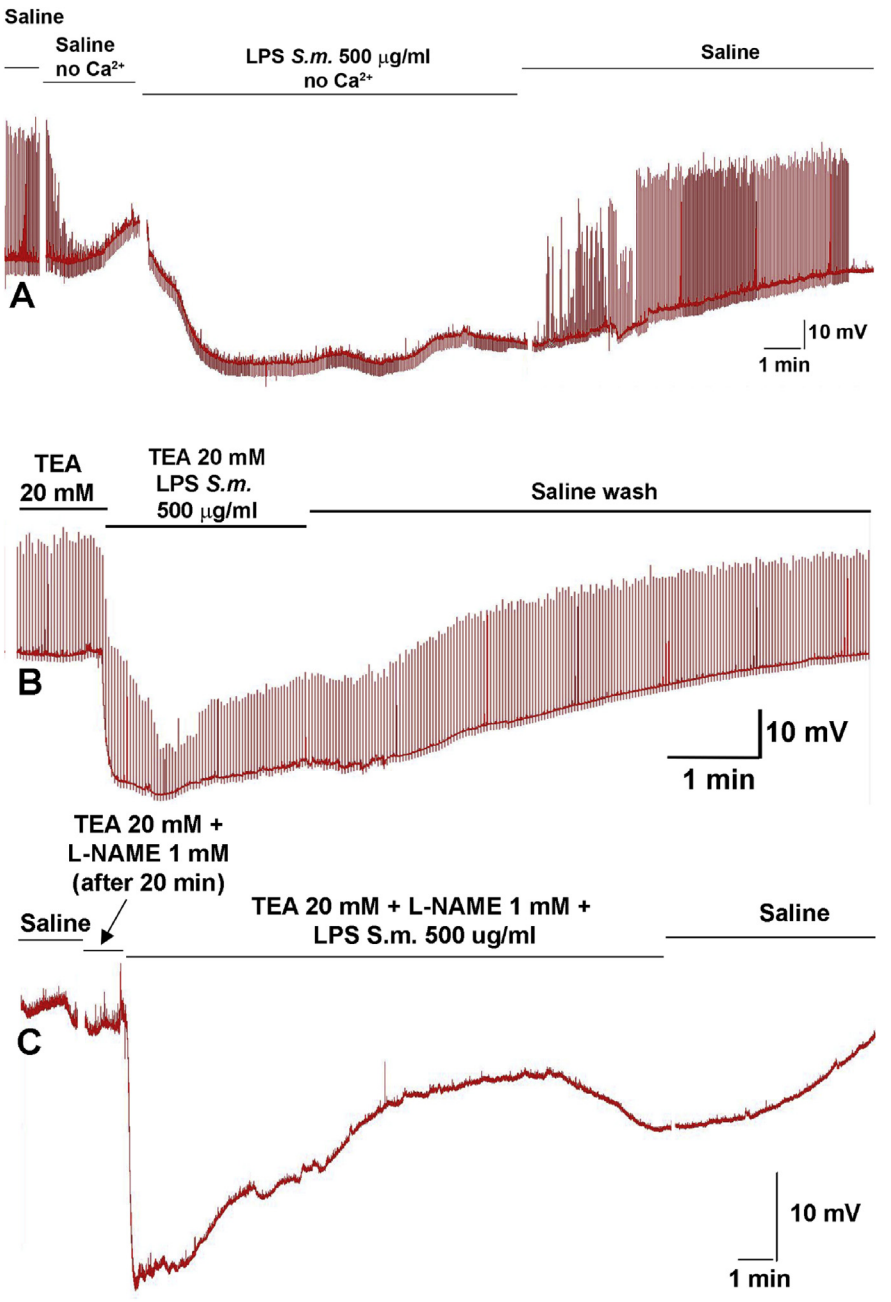

Fig. 6. Examining the mechanisms behind the hyperpolarization of the muscle membrane by LPS. (A) In calcium-free saline, LPS induced hyperpolarization. (B) In blocking the *Drosophila* calcium-activated potassium channel with TEA, the muscle was hyperpolarized upon exposure to LPS and TEA. (C) After nitric oxide production was blocked with a 20-minute incubation with L-NAME and TEA, the muscle membrane continued to be hyperpolarized upon exposure to TEA + L-NAME + LPS. To test this effect only spontaneous quantal vesicular activity and the resting potential is shown.

2.4. Membrane resistance

To examine if the hyperpolarization was due to an alteration in the input resistance of the muscle fibre, changes in voltage responses to 0.2 nA current injection was

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evaluated. Since the membrane potential showed rapid changes during LPS exposure, a range of current steps was not feasible in order to obtain an I-V curve to calculate membrane input resistance at given times of LPS exposure. The alternating pulses of + and − current injections at 0.2 nA prior to LPS and during LPS revealed no differences (N = 6; not significant) in the voltage amplitude as shown in a representative trail (Fig. 7).

3. Discussion

The significance of these findings is that we have shown that LPS decrease the responsiveness of the glutamatergic receptors, potentially by blocking them. Using the genetically amenable *Drosophila* model, this mechanism can be further studied to determine how this occurs and if the binding is competitive or non-competitive. LPS activates the innate immune response by binding to Toll-like receptor 4 (TLR4) known as the CD14/TLR4/MD2 receptor complex in mammals [20]. The TLR4 receptors are highly conserved from primates to insects [21]. These receptors are known to be located on cells and can act directly on cellular function without any influence from a secondary immune response. In *Drosophila melanogaster,* where the Toll receptor was discovered [22, 23], it does not appear LPS mediates its response through the Toll receptor complex [24]. Rather, in *Drosophila,* the Immune deficiency (Imd) signaling pathway, activated by the peptidoglycan proteins, is the main cellular cascade stimulated by LPS [25, 26]. However, the expression profiles for peptidoglycan receptors in the brain of insects has yet to be fully identified, nor the effects on the physiology of organs, or the effects on synaptic transmission in the central nervous system or at the NMJs.

Both *P. a.* and *S. m.* are known to play a role in septicaemia in humans and other mammals [27, 28, 29, 30] but the direct action of LPS, from these bacterial strains, on neural

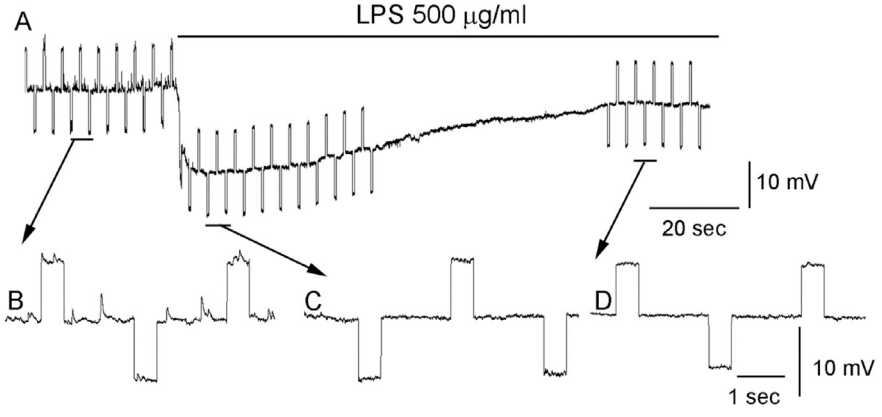

Fig. 7. Input resistance of the muscle fibre. With alternating positive and negative current injections (0.2 nA) before and during LPS exposure revealed no differences in the voltage amplitudes.

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tissue has not been fully investigated. It is known that LPS from *S. m.* is more potent than from *P. a.* in inducing an immune response in mammals [31, 32, 33].

The slight increase in the EJP amplitude with acute exposure to LPS at 100 μg/ml but a large decrease at 500 μg/ml maybe well be due to varying affinities to receptors which mediate different cellular response or even off target effects from the proposed IMD receptors responsibly for mediating the response to gram negative bacterial LPS [25, 26]. It is known that low concentration of some compounds, such as endogenous ouabain, can be stimulatory to the Na-K ATP dependent pump where as high concentrations are inhibitory [34]. Possibly this may also be occurring with LPS induced responses.

Septic myocardial dysfunction in mammals by LPS is thought to occur due to sarcoplasmic leaks leading to altered contractile properties [35]. Given that LPS increases the number of spontaneous events at the crayfish and frog NMJs [9, 10], a similar cellular response may explain the increase in the number of spontaneous events in those preparations; however, this does not appear to be the case in larval *Drosophila* NMJs. Imaging the nerve terminals with Ca^{2+} indicators, such as with using a genetic variant expressing GCaMP6 in *Drosophila*, would address this effect and other forms of Ca^{2+} indicators could be used for motor neurons in the frog and crayfish preparations.

Given that NMJs are very plastic in regulating efficacy of synaptic transmission throughout develop and with various activity profiles, it would not be surprising with a systemic bacterial challenge that a synaptic homeostasis would attempt to occur, particularly for the fast developing larval *Drosophila* [36, 37, 38, 39, 40]. However, if LPS is blocking glutamate receptors, and still present systemically, even the newly expressed receptors would be susceptible. If glutamate receptors are compromised, there would likely be an attempt to enhance synaptic transmission from the muscle to the motor neuron [39, 40]. In addition, it is yet unknown the full extent to which LPS activates cytokine-like factors in *Drosophila* which could impede homeostatic regulatory processes in attempting to maintain synaptic function.

Mechanistic explanation of the transit hyperpolarization remains unanswered. Since the muscle input resistance is not altered during the hyperpolarization, the increasing negative potential is not likely due to leaky membrane channels remaining open for an ion. The equilibrium potential for Cl^- in larval *Drosophila* body wall muscles is more depolarized than the resting membrane potential [41]. So, if Cl^- channels opened, we would expect depolarization, also the same for Na^+ and Ca^{2+}. It is a possibility that leak channels of Na^+ are temporally closing with LPS exposure and the membrane is driven to E_k equilibrium potential. With the injected alternating current pulses, no change was measurable in the input resistance of the membrane, thus perhaps an ionic pump is active. Although it would seem unlikely, if the Na-K ATPase pumps were transitory hyperactivated this could explain the phenomenon in the large hyperpolarization phase of the LPS response.

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The findings of this study may be applicable to treating septicaemia LPS-induced neurological effects in mammals [42, 43]. In addition, the hyperpolarization of skeletal muscle in *Drosophila* may explain the hyperexcitability with enhanced driving gradients or enhanced excitability by preventing the inactivation of voltage-gated ion channels at rest and lowering the threshold. The effect may even result in the inhibition of synaptic transmission. A revitalization of the investigation of the direct effects of LPS on cellular function is on the horizon.

4. Methods

4.1. Larval *Drosophila melanogaster*

The Canton S (CS) strain of flies was used in all experiments. This strain has been isogenic in the lab for several years and was originally obtained from the Bloomington *Drosophila* Stock Centre. Flies were raised on standard cornmeal-agar-dextrose-yeast medium in vials kept at room temperature (22−23 °C) with a 12-hour light/dark cycle.

4.2. Electrophysiology in 3rd instar larvae

Fly saline haemolymph-like 3 (HL3) [44] was used (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) and pH at 7.1 [46]. The EJPs and spontaneous mEJPs were collected and analysed with LabChart 7.0 (ADInstruments, USA) as previously detailed [39, 45].

LPS was dissolved in saline prior to use and was readily exchanged over the dissected preparations during the recording of evoked EJPs and mEJPs. Exposure to LPS for 10 minutes was used followed by changing the bathing saline 3 or 4 times with fresh saline not containing LPS. The total volume of the chamber is only 1 ml which is fully exchanged when switching the media. Two forms of LPS were used (*S. m.* and *P. a.*). LPS and the chemicals used for saline were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acute 10-minute direct exposure of in situ NMJs to saline containing LPS (*S. m.*) at 100 or 500 μg/ml was determined in preliminary experiments to bracket a range from minor to prominent effects on synaptic transmission. The LD50 in rodents for LPS from *S. m.* is 650 μg/ml (*10*) (6×10^6 CFU-colony-forming units, [46]). Thus, we chose to use a relatively high concentration for *D. melanogaster* since they are likely exposed to gram-negative bacterial strains in their native environment.

4.3. Statistical analysis

SigmaPlot (version 13.0) was used for graphing and statistical analysis. The electrophysiological analysis is presented as raw values and percent change from control

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(saline) or from LPS exposure to washout. There is considerable variation among baseline EJP frequency from preparation to preparation. Thus, raw values as well as percent changes are used. The non-parametric sign test for trends was used for testing significance ($P \leq 0.05$).

Declarations

Author contribution statement

Robin L. Cooper: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Micaiah McNabb: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jeremy Nadolski: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study has been deposited at osf.io/psv9m (Cooper, Robin. 2018. "LPS -NMJ-Drosophila Data Bank." OSF. September 26).

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