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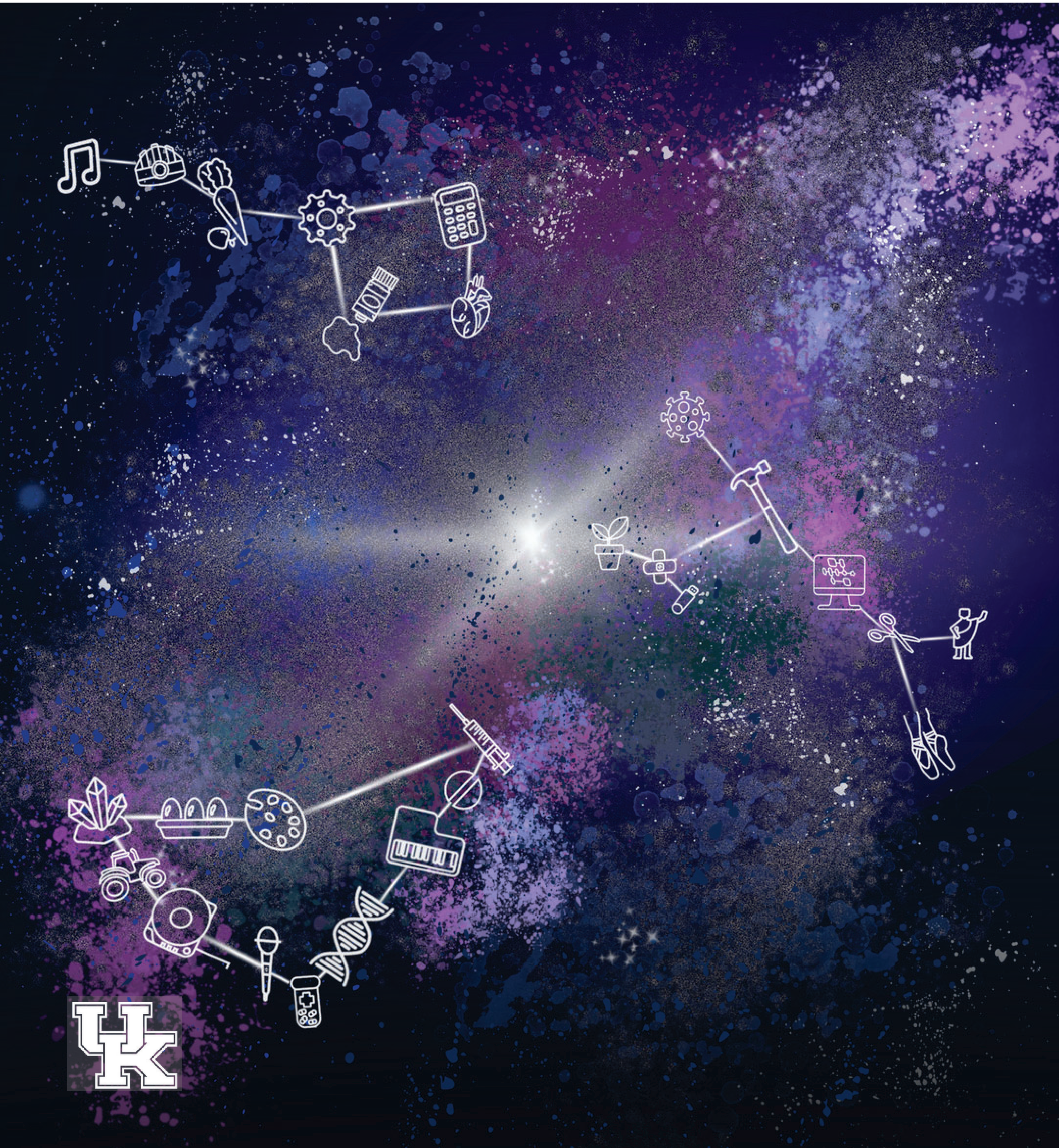

The Effect of Bacterial Toxin Lipopolysaccharide on Neuronal Circuit Function

Rebekah McIntosh

Rebekah McIntosh started at the University of Kentucky in the fall of 2022 as a Neuroscience major and graduated in May 2026. Freshman year, she was part of STEMcats, where she conducted research on the acute physiological effects of zinc and published her findings in the Journal of Pharmacology and Toxicology. She has continued to do research in Dr. Robin Cooper's lab for the past three years, focusing on the effects of LPS on synaptic transmission, and she has presented this research at several meetings.

Abstract

Septicemia due to Gram-negative bacteria is a life-threatening condition affecting 1.7 million adults in America, and the 9th leading cause of death in Kentucky. Gram-negative bacteria contain structural components known as lipopolysaccharides (LPS). However, there is limited research on the mechanistic actions of LPS on neurons and synaptic properties. The leech model preparation offers a distinct advantage for studying the electrophysiological properties of isolated, identifiable neurons. Thus, this study analyzed the acute effects of LPS insertion on Retzius (Rz) cells within (1) an isolated ganglion with intact neural circuitry and glia and (2) an isolated culture separate from the synaptic connections and glia. In the preparation with neural circuitry intact, acute LPS exposure at 250 µg/mL resulted in a significant increase in spontaneous and evoked action potentials, while 500 µg/mL caused varied effects. The isolated cell culture proved problematic, as changing of the medium while maintaining intracellular recording was difficult. Technical procedures need to be better developed for investigating the isolated cells in culture.

Key Words: lipopolysaccharides; neurobiology; neurophysiology

The Effect of Bacterial Toxin Lipopolysaccharide on Neuronal Circuit Function

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INTRODUCTION

Septicemia in animals due to Gram-negative bacteria is life-threatening. Approximately 1.7 million adults in America develop sepsis annually, and, of the adults in this group, around 350,000 adults died during their hospitalization in 2022.[1] In Kentucky alone, septicemia is the 9th leading cause of death.[1] Gram-negative bacteria are particularly dangerous because the associated lipopolysaccharides (LPS) induce the host's immune response, thus increasing circulating cytokines.[2] High levels of cytokines generate abnormal neural and cardiac function, becoming harmful to the host.[3-7] In mammals, the immune response can lead to breakdown of the blood-brain barrier and sepsis-associated encephalopathy.[8,9] The encephalopathy leads to altered function in neural circuitry, likely due to actions on synaptic function. In the case of sepsis-associated encephalopathy, there are many local factors and blood-borne cytokines impacting neuronal function. However, the actions of LPS itself on neurons and synaptic properties in the absence of an immune response have not been well-studied. This is primarily because it is difficult to examine neurons in isolation from other neurons (and their releasing factors) or cell types (e.g., glia, microglia). It is important to investigate how LPS initially acts on neurons, such as effects on various ion channels, pumps, exchangers, and overall excitability of the cell; in turn, these effects can alter membrane potential and synaptic function, thus affecting whole animal physiology.

Neuron excitability and voltage-gated ion channel function depend on the electrical-chemical potential of the membrane. K2P channels (i.e., two-pore-domain K^+ channel) are primarily responsible for K^+ efflux, thus driving the membrane potential towards the equilibrium potential for potassium ions (E_K)[10,11]; NALCN (i.e., sodium leak channels), on the other hand, are primarily responsible for Na^+ influx, thus driving the membrane potential in the opposite direction, towards the Na^+ ion equilibrium potential (E_{Na}).[12,13] It has recently been implied that purified, commercially obtained LPS from Serratia marcescens can rapidly activate K2P channels and, with delay, activate NALCN on the body wall muscles of larval *Drosophila melanogaster*.[14] However, the glutamatergic synaptic communication at the neuromuscular junction (NMJ) in larval *D. melanogaster* is rapidly depressed, as LPS exposure blocks glutamate receptors despite hyperpolarization of the muscle.[15,16] In contrast, at the crayfish glutamatergic NMJ, exposure to an equivalent concentration and form of LPS enhances synaptic transmission with only slight hyperpolarization of the targeted muscle fibers. The primary mechanistic effects appear to be presynaptic, resulting in enhanced probability of evoked vesicular fusion.[17,18]

In both crayfish[19] and larval *D. melanogaster*[20], LPS exposure over a few minutes depressed an evoked sensory-CNS-motor neuron circuit; however, the larval *D. melanogaster* neural circuit was initially enhanced, then depressed with exposure. The effects in both model preparations could be partially reversed by extensive flushing with saline not containing LPS. It was not established where in the neural circuitry the activity was altered; this could implicate the evoking of sensory neurons, sensory neuron synaptic output on postsynaptic interneurons, interneuron or motor neuron recruitment, direct motor neuron activation, or even impacts on electrical conduction along the neuron. Since activity of the motor roots was recorded, the decreased responses of the neural circuit were not due to depression at the NMJ in the *D. melanogaster* model. The sensory input in both larval *D. melanogaster* and crayfish is cholinergic, so it is possible that cholinergic receptors could have been blocked in a similar manner to the glutamatergic receptors at the larval *D. melanogaster* NMJ; however, in a separate study, LPS exposure at the amphibian (i.e. frog) cholinergic NMJ was not blocked.[21,22] The evoked responses were depressed and reversible with LPS wash-out, while spontaneous quantal events were still present. Thus, it appears LPS affected recruitment of the motor neuron, electrical conduction, and/or presynaptic function, though this has yet to be fully confirmed for the frog motor unit.

With an intact CNS, brain slices, or even mammalian neurons in culture, it is difficult to isolate individual neurons from other cell types for investigation of LPS direct action. The activity of the neural circuitry in a rodent brain slice was depressed by LPS exposure at equivalent concentration and subtype to that used in studies on larval *D. melanogaster*, crayfish, and frog preparations. However, in the rodent model, it is not known if the depressed responses were due to direct effects on the neuron, microglia function, or the actions of other neurons.[21,22] This general response has also been demonstrated previously with other forms of LPS.[23-25] In mammals, LPS binds to TLR4 receptors, which are known to be on microglia, astrocytes, oligodendrocytes, and neurons.[26,27] It was even demonstrated that LPS (1 μg/mL, of *Escherichia coli*) had effects on hippocampal neurons in culture independent of glia cells, since an increase in cytosolic [Ca^{2+}] was noted in the neurons but not the surrounding glia. The potential that glial glutamate release causes increased neuronal Ca^{2+} concentration was addressed by blocking the glutamate receptors; however, this did not completely abolish the raised [Ca^{2+}] in the neurons[28], suggesting that the mechanism of action may be both pre- and postsynaptic.

Comparative studies in the past, particularly with invertebrates, have helped develop understanding of response variation and mechanisms of action observed with LPS exposure, as noted by the 2011 Nobel Prize in Physiology or Medicine awarded to Hoffmann and Beutler. It is thus reasonable to continue pursuing comparative studies into bacterial toxins and screening of pharmacological interventions.

The freshwater leech model is advantageous because it has identifiable primary neurons and very characteristic action potential shapes in electrophysiological recordings, allowing for the categorization of each distinct cell type (sensory neuron, motor neuron, or interneuron).[29,30] Neurons of the leech model have been well studied over the years at the single cell level, particularly the Retzius (Rz) neurons.[30,31]. Rz neurons are a large pair of serotonergic, unipolar neurons with known characteristic action potentials and size large enough to ease identification and electrode insertion.[31] Thus, this investigation examined the acute, direct effects of LPS from *S. marcescens* on the electrophysiological responses of Rz cells under two main conditions: (1) within an intact, isolated ganglion and (2) when in culture (i.e., isolated from these surrounding structures).

This study is significant because most of the therapeutic emphasis in treating sepsis-associated encephalopathy in mammals is on dampening the actions of the immune response and not on the direct, neuronal effects of LPS toxin. The more one understands these effects, the more preventive and palliative health care measures can be addressed, such as the complications of the LPS bolus released when high antibiotic doses cause bacterial lysis.

METHODS & MATERIALS

Animals

Leeches (*Hirudo medicinalis*) were bought from Leeches U.S.A. L.T.D. (300 Shames Drive, Westbury, NY, USA11590) and maintained in aquaria with water, as stated for animal care by the supplier. They were stored for no longer than 2 months.

Dissection

A detailed dissection procedure is described in text and video format in Titlow et al.[30] In brief, the leech was stretched and pinned in a longitudinal manner to a large silicone elastomer-lined dish. A longitudinal cut was made to expose the ventral blood sinus (Figure 1A). The blood sinus was cut along the length of the ventral nerve cord without damaging the cord itself (Figure 1B). The ventral nerve cord was removed and placed into a silicone elastomer-lined dish with saline. Leech Ringer's solution consists of the following: 115.3 mM NaCl, 1.8 mM $CaCl_2$, 4.0 mM KCl, and 10 mM Tris/maleic acid or HEPES. The pH was adjusted to 7.4.

The LPS compound used for assessment was *S. marcescens* (product number L6136; Sigma-Aldrich, St. Louis, MO, USA), in solution at concentrations of 250 and 500 µg/mL for comparison with prior studies.[16]

The individual ganglion was cut away and pinned to clearly identify the Rz cells (Figure 1C). Notice the two large Rz cells in the center of the ganglion. The recording set-up consisted of a dissecting microscope (100X) and a fiber-optic lamp projected on a mirror to visualize the neurons (Figure 1D).

Culturing single Rz Neurons

To remove neurons from the ganglia, the isolated ganglion was pinned down with the ventral side up in a clean dish containing saline. With fine scissors, the glial capsule was nicked at the side, and one end of the scissors blade was placed under the capsule to cut across the glial capsule (Figures 2A and 2B). The cell bodies of the neurons looked like grapes on a vine pushing out of the ganglion capsule (Figure 2C). The scissors were used to cut the group of neurons away from the bottom of the ganglion capsule, and the cluster of neurons was pushed to one side of the capsule. The cluster of cell bodies was then placed into a culture dish, which was pre-coated with fetal calf serum (FCS) mixed with saline to avoid the cells sticking to the plastic surface (Figure 2D and 2E).

A pipette attached to a syringe was used to pull saline into and out of the glass capillary tube over the cluster, resulting in the neurons gently loosening from each other. Then, the largest neurons—the cell bodies of the Rz cells—were identified and extracted from the group by sucking them into the capillary. The capillary pipette tip was heat-polished so that it was slightly larger than the cell body diameter. For Rz cells in an adult leech, the diameter is approximately 50 µm. The aspirated cells were discharged into another dish containing saline, which was not pre-coated with FCS (Figure 2D). The cells stuck to the plastic microwell plate (Figure 2E). Intracellular electrophysiological techniques were used to record membrane potential and to inject current pulses to evoke action potentials. The saline medium was reduced in volume to cover just the cells (~2 mL). The saline containing LPS was added very gently along the well wall to allow continued recording from the cells.

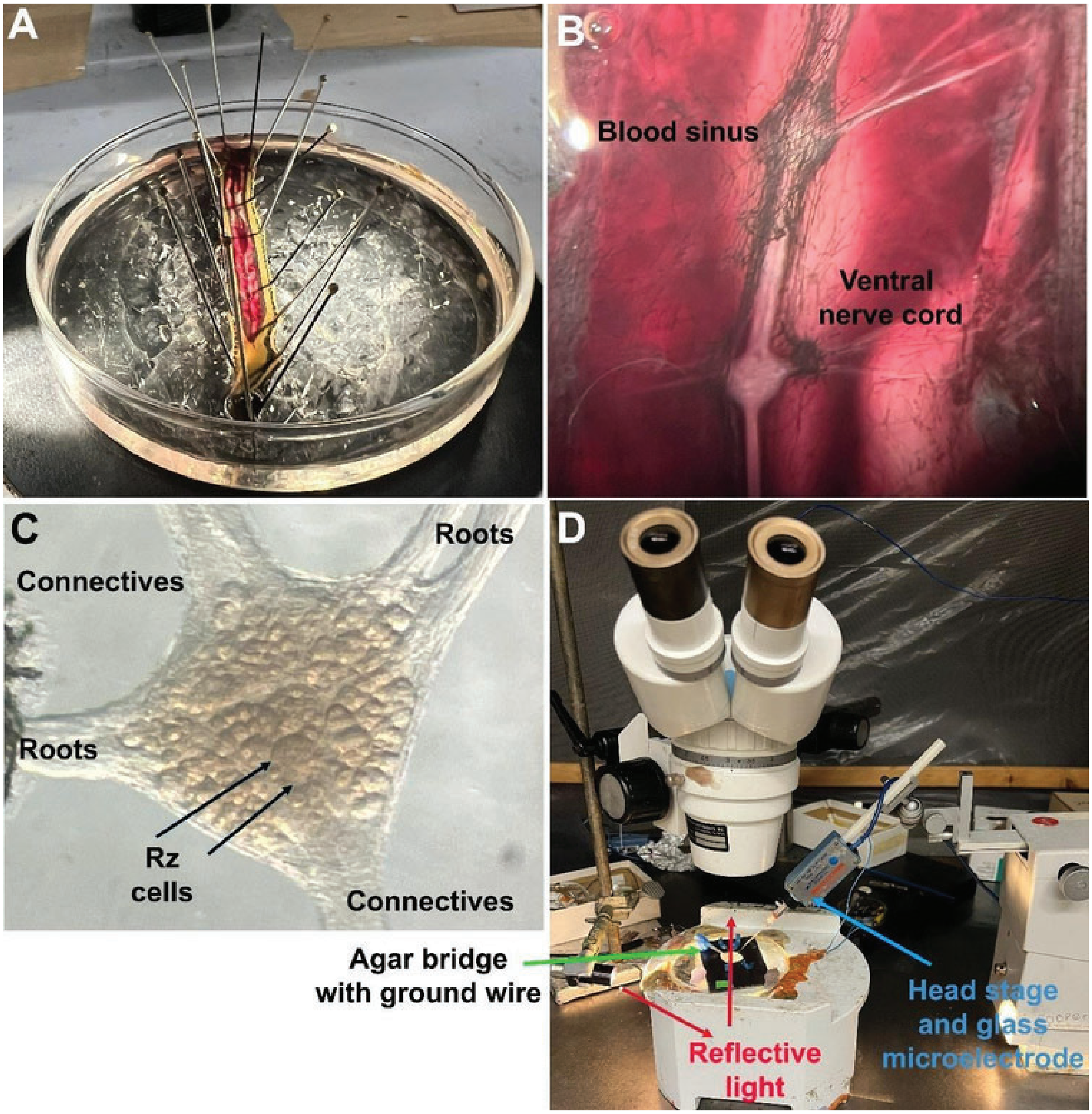

Figure 1: Preparation for electrical recordings of Retzius cells (Rz) within an isolated ganglion of a leech. (A) Ventral side of the stretched and pinned leech. A longitudinal cut is made to expose the blood sinus for the length of the animal. (B) To isolate the ventral nerve cord, the blood sinus is cut along the length of the ventral nerve cord. The two Rz neurons are even visible within the ganglion as whitish dots. (C) A ganglion is transected and removed from the ventral nerve cord, whereupon it is pinned by the connectives that lead to other ganglia. The ganglion was also pinned by the roots, which contain nerves to and from the lateral sides of the ganglion. (D) The recording dish is placed on a platform in which a mirror can reflect the transmitted light through the ganglion to enhance visualization of the individual cell bodies of the neurons. A 1% agar bridge made with leech saline was placed within a plastic pipette tip for ground wire placement. The tip of the plastic pipette was placed into the bathing saline on the dish. The intracellular glass microelectrode was attached to the amplifier head stage. The microelectrode was positioned with the use of a manipulator. A 100X dissecting microscope was used for visualization of the Rz neurons for placement of the microelectrode.

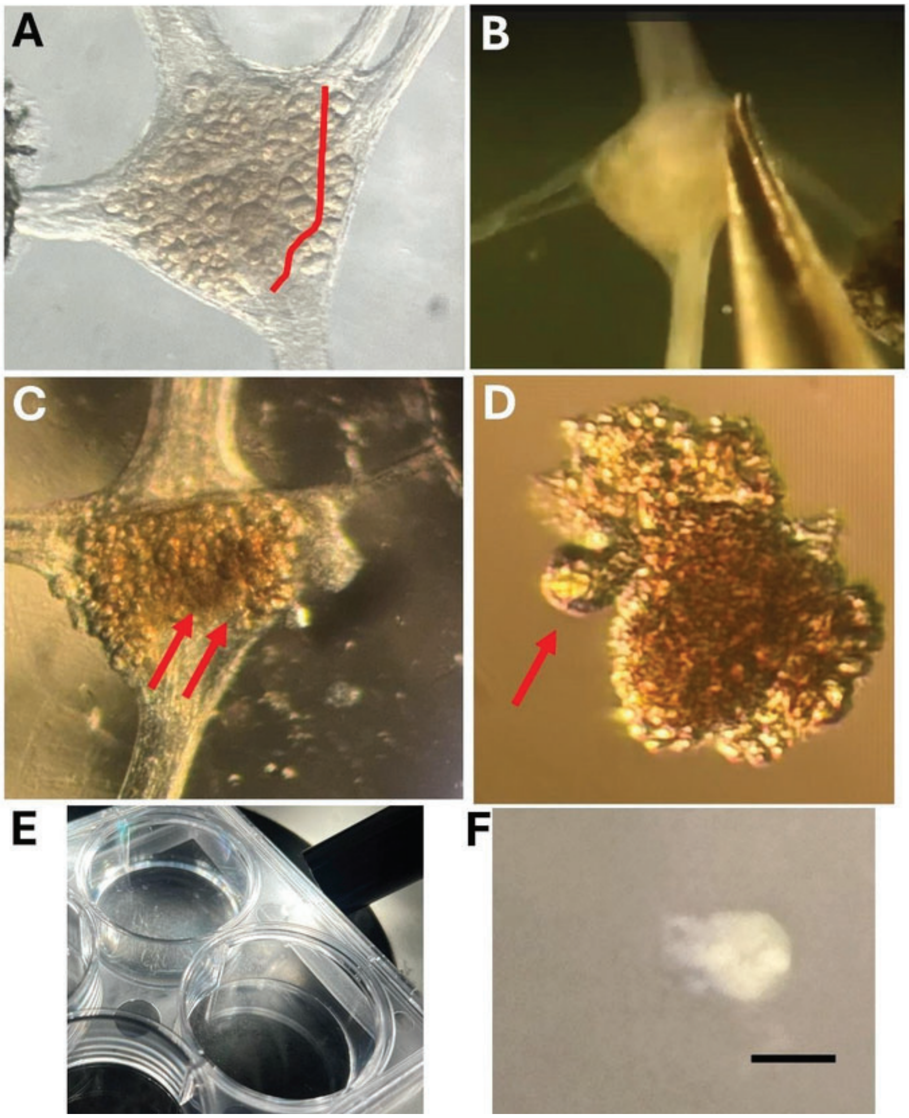

Figure 2: Preparation for electrical recordings of isolated Retzius cells (Rz) in cell culture. (A) Ventral side of the stretched and pinned leech ganglion. The path for cutting open the glia capsule was determined and depicted in red. (B) The ganglion glial capsule was cut along one side with fine scissors. (C) A group of cell bodies, including the large Rz cell bodies, was removed from the glial capsule by cutting underneath the cluster of cells and was placed into a chamber of the culture microwell dish pre-coated with fetal calf serum. The Rz cell bodies are indicated by red arrows. (D) The cluster of cell bodies was aspirated in and out of a glass capillary tube until the cell bodies of the Rz cells separated from the cluster. (E) The single Rz cells were then transferred from the microwell with FCS to one containing only saline so the cell bodies would stick to the plastic bottom. (F) The electrical properties of the isolated neurons were recorded. Scale bar = 50 μm.

Electrophysiology

To monitor the transmembrane potentials of the cells, a sharp intracellular electrode (30 to 40 M resistance) filled with 3M KCl was used. An Axoclamp 2B (Molecular Devices, Sunnyvale, CA, USA) amplifier and 1 X LU head stage were used to record electrical signals. Data were collected using a PowerLab/4sp (ADInstruments, Colorado Springs, CO, USA), and analyzed with LabChart 7.0 (ADInstruments, Colorado Springs, CO, USA) which was recorded on a computer at a 20 kHz sampling rate along with the use of a NPI GMbH filter (type EPMS07 DPA 2F, from Adam and List Associate, LTD., 1100 Shames Drive, Westbury, NY 11590, USA) set at 3.0 kHz with no high-pass filtering.

The resting membrane potential and spontaneous action potentials were recorded. In some cases, short pulses (50 to a 200 msec) of positive current (1-5 nA) into the cell were used to evoke action potentials.

Analysis

Raw membrane potential values were obtained for each preparation. The number of spontaneous action potentials and evoked action potentials were counted to obtain an index of the LPS exposure effects. The activity was binned into one-minute periods.

Paired t-tests were used to compare changes in the number of action potentials before and during exposure to LPS. Percent changes in membrane potential were determined from the initial values in normal saline to that containing LPS as a means of normalizing preparations with varying action potential frequency; these were determined across one minute of 250 ug/mL exposure and two minutes of 500 ug/mL exposure in the following manner: by finding the absolute difference (initial - experimental), dividing that number by the initial value, and then multiplying by 100.

RESULTS

Two methodological approaches were used to assess the effects of LPS on neuronal function. The first was to retain as much neuronal circuitry as possible from an isolated ganglion with intact glia packets while recording from a Rz neuron. The second approach was to record from isolated Rz neurons placed in a culture dish free of synaptic contacts and packet glia. The electrical activity of the Rz neuron while residing inside the ganglion presented synaptic activity. The subthreshold and action-potential-initiating activity levels are shown in Figure 3. Sub-threshold synaptic activity can vary in amplitude based on the synaptic strength and dendritic location. Thus, some synaptic activity is difficult to quantify based on the signal-to-noise ratio of the baseline. Action potentials were induced by delivering current pulses to depolarize the neuron above threshold. For quantification of LPS effects on electrical activity and excitability of the neuron, only initiated action potentials that were evoked by current injection and spontaneous suprathreshold synaptic responses were counted. The small synaptic graded potentials (marked below with arrows) were not included in analysis (Figure 3).

The effect of LPS exposure on Rz cell activity while residing in the ganglion was assessed for two concentrations. A representative trace of the electrical activity before, during, and after (i.e., during wash-out) exposure to 250 µg/m LPS is shown in Figure 4A. In Figure 4A, the current injections are shown in a repetitive fashion (1/2 Hz), along with the spontaneous activity. The effects of current injections and spontaneous activity are highlighted in the enlarged traces from before (Figure 4B), during (Figure 4C), and after (via saline rinse; Figure 4D) LPS exposure. In some cases, changing the bathing media can produce electrical artifacts, particularly when the media is removed below the agar plug during the bath exchange, as illustrated in the traces covered by the shaded blue (Figure 4A). Thus, the electrical activity cannot be measured during medium exhange in all cases, but is possible again as soon as the recording is stable.

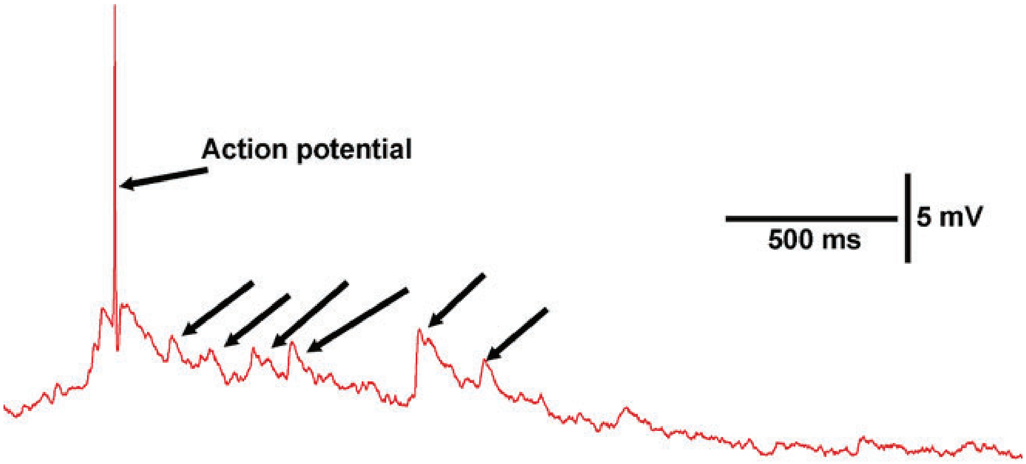

Figure 3: Synaptic activity on a Rz neuron recorded while residing within a ganglion. When the neuron is depolarized above a threshold, an action potential is initiated. There is a substantial amount of varied synaptic activity when a Rz cell is recorded in an acutely isolated intact ganglion. The pronounced synaptic activity is indicated by the arrows in the baseline. Minor synaptic activity (present as small deflections) is difficult to determine from the baseline.

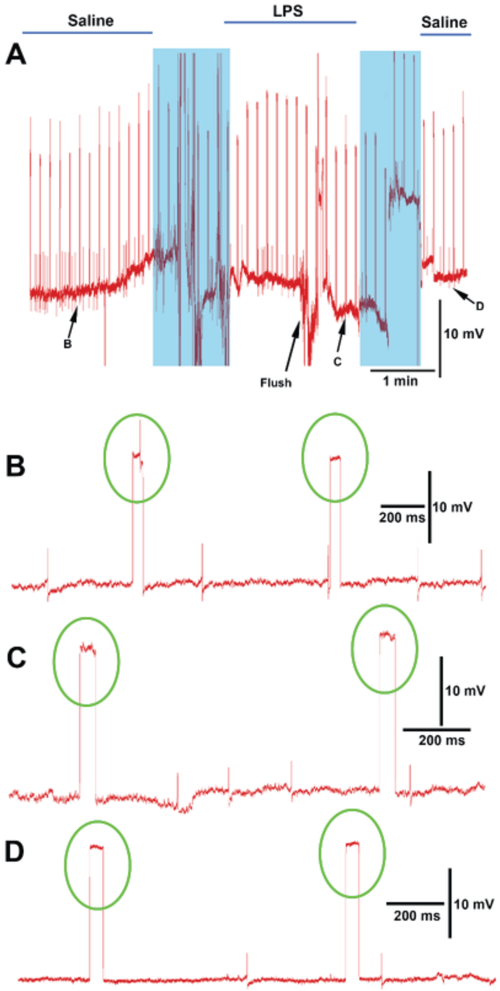

Figure 4: Exposure to LPS at 250 µg/mL while recording in a Rz neuron within a ganglion. (A) The firing of action potentials in saline and during exposure of LPS (250 µg/mL) over time. Current pulses of 2 nA of 100 ms duration were applied every 10 seconds. Note the letters indicating the enlarged regions shown in B through D. (B) An enlargement of A while the preparation is bathed in saline. (C) An enlargement of A directly after exchange of the bathing medium to one containing LPS. (D) An enlargement of A directly after exchange of the bathing medium to fresh saline without LPS. Note that the green circles indicate the current pulses. The shaded blue indicates the changing of the bath to the next solution, which resulted in some electrical artifacts. During the LPS exposure, the bath was flushed around to ensure exposure of the ganglion to LPS.

A similar paradigm was used to examine the effects of 500 µg/mL LPS (Figure 5A). In Figure 5A, the current injections are shown in a repetitive fashion (1/2 Hz), along with the spontaneous activity. The effects of current injections and spontaneous activity are highlighted in the enlarged traces shown before (Figure 5B), during (Figure 5C), and after (via saline rinse; Figure 5D) LPS exposure. In this case, the changing of the bathing medium did not produce large electrical artifacts, while the electrical activity was greatly enhanced by exposure to LPS.

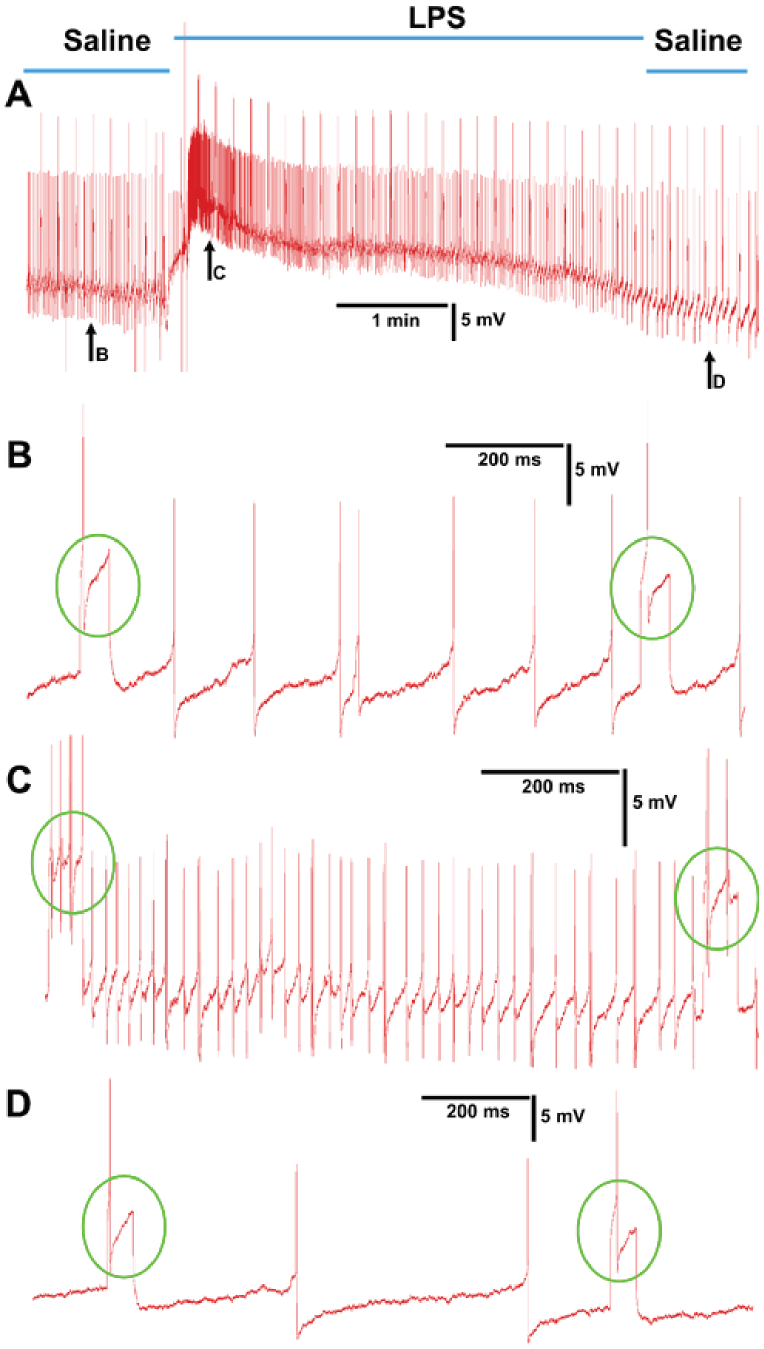

Figure 5: Exposure to LPS while recording in a Rz neuron within a ganglion. (A) The firing of action potentials in saline and during exposure to LPS (500 µg/mL) over time. Current pulses of 2 nA of 100 ms duration were applied every 10 seconds. Note the letters indicating the enlarged regions shown in B through D. (B) An enlargement of A while the preparation is bathed in saline. (C) An enlargement of A directly after exchange of the bathing medium to one containing LPS. (D) An enlargement of A directly after exchange of the bathing medium to fresh saline without LPS. Note that the green circles indicate the current pulses.

Quantification of the excitability in the Rz cells, as measured by the number of action potentials within one-minute periods, revealed that the 250 µg/mL produced a reduction in the number of action potentials for 6 of the 7 preparations (Figure 6A). One preparation was highly excited upon removal of the media containing LPS, as shown in the trace in red (Figure 6A). The activity before LPS exposure was monitored for one minute. The exposure to 500 µg/mL LPS produced more variability in activity than 250 µg/mL, with some preparations having an enhanced activity while others had depressed activity (Figure 6B). Since maintenance of the intracellular recordings was most stable during the first three minutes of LPS exposure, the average activity in this period was used for comparison of both the 250 and 500 µg/mL LPS exposures. The loss of the intracellular recording occurred more often when exposed to the 500 µg/mL LPS. The five minutes prior to bathing medium exchange to fresh saline without LPS were maintained for timing purposes. For preparations in which the recording was lost, a dotted line was used to represent the period prior to the saline rinse. The percent change in activity from the averaged response in saline prior to LPS exposure and the averaged activity in the first three minutes of LPS exposure were used for quantification (Figure 6C). A percent change measure allowed a normalization among preparations with varied initial activity. The activity was significantly reduced for the 250 µg/mL, but not for the 500 µg/mL LPS exposures (Figure 6C; $p<0.05$; paired T-test). There was no significant difference in percent change of the activity between the 250 and the 500 µg/mL LPS ($p>0.05$; t-test) due to the high variability for preparations exposed to 500 µg/mL LPS.

Isolating individual Rz cells for culture has proven to be very challenging while maintaining the integrity of cells. Moving the culturing dish to a recording platform resulted in the cells being pulled off the plastic surface; therefore, maintaining the microwell plate on the recording stage when aspirating the cell bodies in the culture well with the FCS was beneficial. This allowed transfer of the cells to the microwell lacking FCS without cell damage prior to electrical recording. The microscope was mounted on a boom stand so the microscope could be moved over the various wells of the dish. This approach prevented the dislodging of the neurons after placing them in the microwell dish. The exchange of the saline with LPS while recording from the neurons with an intracellular electrode also proved to be difficult. Adding saline containing LPS along the wall of the microwell resolved some of the problems; however, it was still difficult to maintain intracellular recording while removing the saline containing LPS and adding fresh saline. Thus, three well-maintained recordings were obtained as preliminary results regarding LPS effects on resting membrane potential and evoked action potentials. In all three recordings, the membrane potential hyperpolarized with LPS addition (500 µg/mL). The average hyperpolarization of the neurons was 12 mV (+/- 2.4 SEM, N=3). In all three recordings, the ability to evoke an action potential with current injections was dampened. In two of the three recordings, the membrane potential was maintained during removal of the saline containing LPS with two flushes of approximately half the volume in the microwell. In one preparation, recording of the evoked action potential responses was able to be recovered with the saline flush (Figure 7). For the other of the two neurons, the ability to evoke an action potential was not successful even with a range of current injections from 1 nA to 5 nA.

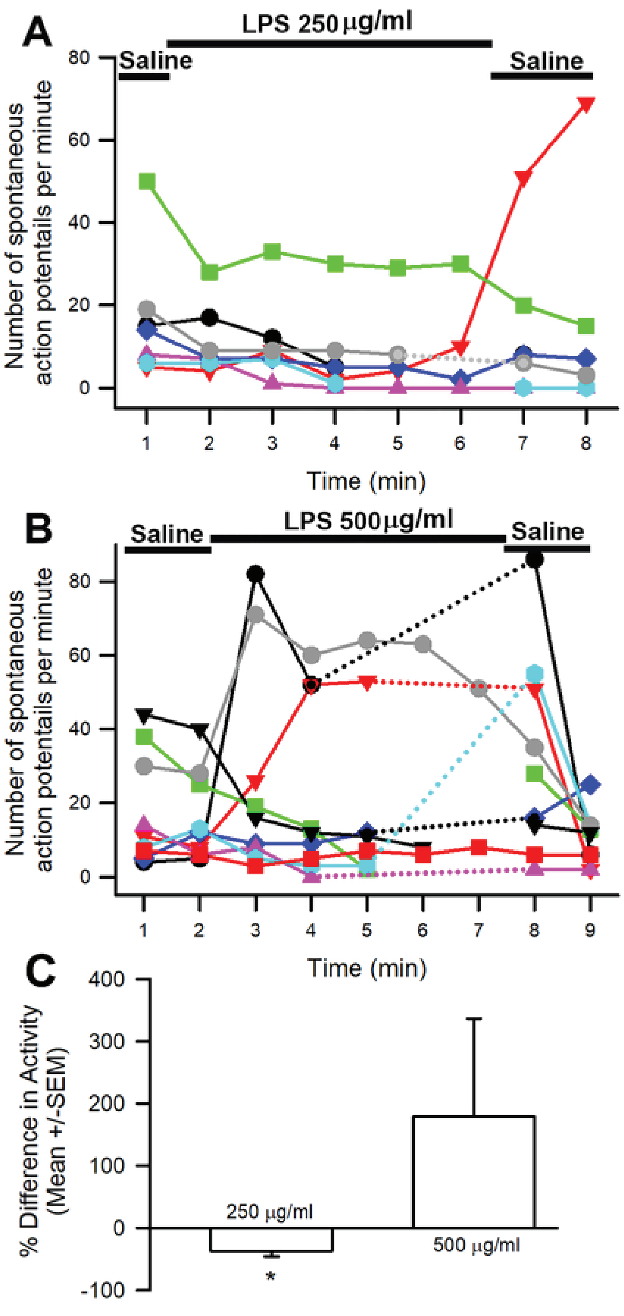

Figure 6: The acute effects of exposure to LPS on ganglia while measuring activity in Rz neurons. (A) Exposure to LPS at 250 µg/mL (N=7). (B) Exposure to LPS at 500 µg/mL (N=9). The activity was measured within 1-minute bins. The initial saline exposure for the preparations in A was one minute prior to LPS exposure. For the traces shown in B, the initial saline exposure was maintained for two minutes. Dotted lines indicate time in which the intracellular recording was lost, but the timing was maintained for the exposure prior to exchanging the bathing media with saline not containing LPS. (C) The percent change in the average activity in saline to the average activity within the first three minutes of exposure to LPS. There was a significant decrease in activity for exposure to 250 µg/mL LPS (Paired T-test; $p<0.05$), while there was no consistent trend for the 500 µg/mL. There was no significant difference between 250 and 500 µg/mL exposure (T-test; $p>0.05$).

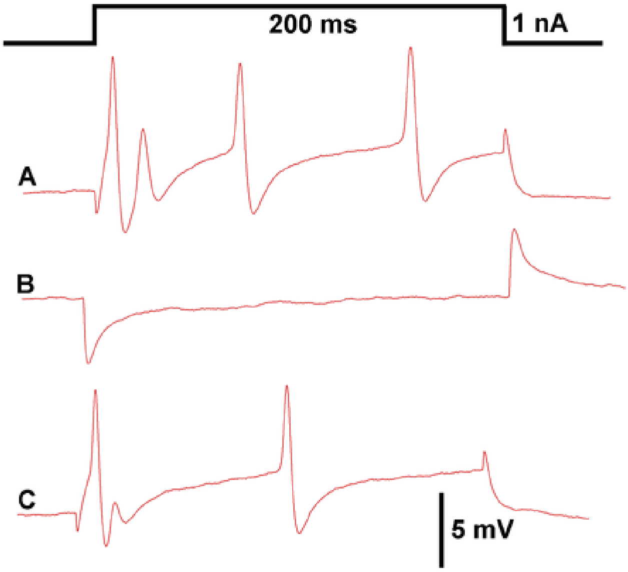

Figure 7: Evoked responses with current injection in cultured isolated Rz neurons. (A) The response with saline alone. (B) The response after 250 µL of LPS (500 µg/mL) was added to the well containing the neuron (2 mL of saline). Note that the evoked responses stopped. (C) After removal of 1 mL saline and replacement with 1 mL of saline twice (to flush out the LPS), the evoked responses returned. Current injection was 1 nA for 200 msec, as shown at the top of the figure.

DISCUSSION

In this investigation, it was demonstrated that acute exposure to LPS within a minute resulted in altered frequencies of spontaneous and evoked APs in Rz cells within a segmental ganglion. The response was dose-dependent, as only the 250 µg/mL showed a significant effect, while 500 µg/mL caused varied responses on preparations. Exposure of isolated Rz neurons in culture was difficult to obtain while maintaining intracellular recordings. The preliminary results indicate that the resting membrane hyperpolarizes and the evoked responses are dampened.

Rz neurons within the ganglion generally decreased in excitability with 250 µg/mL exposure. Some preparations for the 500 µg/mL also decreased in excitability, while other preparations showed pronounced increases in activity. Perhaps, there is a threshold around 500 µg/mL for increasing activity as compared to decreasing activity. Examining a higher concentration of LPS may resolve this possibility. However, 500 µg/mL is likely higher than what these neurons would be exposed to naturally within an intact leech. The LPS concentration in the leech sinus blood has not been determined. LPS within the gastrointestinal tract may even be degraded before transportation to the leech's own blood supply and sinuses, which surround the ventral nerve cord. For humans in severe sepsis, 500 pg/mL of LPS is a lethal concentration.[32] However, it is known that in rodents, 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.[33,34] It remains to be determined what a lethal dose would be for the blood-consuming leech (*Hirudo medicinalis*).

It should be noted that leeches may be unique in their immunity to exposure of LPS, as it has been demonstrated that they can produce antimicrobial peptides, such as Hirunipins, that can suppress pro-inflammatory cytokine expression in LPS-stimulated macrophages.[35] This immunity may have arisen to allow storage of whole blood for long periods of time within the leech gastrointestinal tract.[35,36] It is still not known whether the LPS-initiated immune response occurs via TLR4-like receptors, which are immune receptors that can detect LPS, or via the IMD receptor complex for leeches, which is known as the receptor complex for LPS for insects.[37-39] Mammals use a protein complex known as CD14/TLR4/MD2 complex.[40,41] However, the TLR4 protein is noted to be conserved from insects to mammals.[42-44] Few studies have examined the rapid (i.e., in a few seconds) effects of LPS and direct effects on ion channels as compared to slower responses in the synthesis and release of cytokines by immune cells.

It was demonstrated using larval *D. melanogaster* and crayfish muscle that LPS (500 µg/mL) from *S. marcescens* rapidly hyperpolarizes the membrane potential. *D. melanogaster* muscle hyperpolarizes rapidly and, within a minute, starts to depolarize, resulting in muscle contractions. On the other hand, the crayfish muscle did not present with as much hyperpolarization or muscle contractions at 500 µg/mL.[14,45,46] The mechanisms of action for the larval *D. melanogaster* indicates an activation of K2P channels and NALCN (for K^+ and Na^+, respectively), but this has yet to be established fully for the crayfish preparations. As for the leech Rz neuron, there was no consistent hyperpolarization or depolarization of the membrane potential noted when the Rz cell was monitored within the ganglion.

In isolation, there was difficulty in maintaining a stable baseline, even in saline, and even more so while changing the saline out for one containing LPS. The preliminary evidence of three neurons would indicate a hyperpolarization and reduced excitability of the neurons. However, considering the recordings in Rz ganglia and the frequent loss of intracellular recording with 500 µg/mL LPS exposure, it would be interesting to investigate whether muscles exist in inter-ganglionic connectives to synchronise movement of the ventral nerve cord with body contractions. It appears that leeches may not contain muscles within the connectives of the ventral nerve cord.[47,48] The muscles within the stockings (blood vessel walls), which are pinned to hold the ganglion in place, may contract and result in movements, thus dislodging the microelectrode. When the recording was lost, with a slight repositioning of the microelectrode and re-poking of the neuron, a similar membrane potential and activity could be obtained.

Substantial variation was observed in Rz neuron responses to LPS, whether monitored within the intact ganglion or in isolated cell culture. The potential presence of endogenous LPS within the commercially bought leeches prior to acquisition could not be assessed; similarly, it was not possible to control for the potential that exogenous LPS present on the leech's skin contaminated the dissection process. It is also possible that prior LPS exposure from various strains might have affected the response to subsequent exposures, independent of immune response, though this response would, in turn, produce additional variables that must be considered.

Larval *D. melanogaster* body wall muscles demonstrated similar direct responses to LPS, as the membrane potential hyperpolarized upon acute exposure and thorough wash-out[49]; however, prolonged LPS exposure prompted depolarization without wash-out success, suggesting that the induced influx of Na^+ and Ca^{2+} might have damaged the cell's integrity[14]. The preliminary results reported herein for isolated, cultured Rz cells indicate LPS-induced excitability reduction. Indeed, even after thorough flushing of LPS from the bathing medium, one preparation failed to yield an evoked response at all. More investigations are therefore necessary to determine the mechanism behind reduced evoked action potentials in neurons after LPS exposure.

A similar phenomenon was observed in the sensory-CNS-motor neuron activity profile of the crayfish with exposure to LPS of the same strain and concentrations. This produced an increase, followed by a decrease in activity.[19] It does not appear that the activity increased to a level to produce synaptic depression, whereas, in previous studies, the neural circuit was exposed to serotonin, which resulted in much larger increases in activity for a more prolonged time.[50] However, at the crayfish NMJ, evoked activity was enhanced for more prolonged periods (i.e., longer than 10 minutes).[17-19,45] On the other hand, a hippocampal slice preparation only demonstrated a rapid depression in evoked activity.[21] Thus, the effects of LPS on neural circuitry and NMJs are complex and not fully predictable, as the effects are preparation-dependent. Since it appears LPS can promote K^+ flux through K2P channels and Na^+ through NALCN[14], the differential effects on cells within a circuit and on single cells likely depends on the K2P and NALCN subtypes and expression levels.

It is not known what subtypes of K2P and NALCN are expressed in Rz neurons—or, for that matter, any leech neurons—as it does not appear this has been specifically investigated to our knowledge. However, attempts are being made to look at expression profiles unique to subtypes of leech neurons.[51] Some neurons of *Hirudo medicinalis* have been well studied electrophysiologically, such as the sensory neurons N, T, and P. These neurons have differential expressions of ion channels, which give rise to the characteristic subtypes of action potential shapes.[52] In addition, they maintain their characteristic action potential shapes in culture with a minimal saline bathing medium.[53] Thus, the potential differential effects of LPS on various neuronal subtypes with varying ion channel distributions and membrane properties could be addressed. Isolated neurons of the leech can also be readily paired in culture to form specific types of synapses (i.e. electrical, chemical, and uni- or bidirectional).[54-56] Thus, the effect of LPS on synaptic function in well-identified, isolated neural subtypes without glia or contaminating cell types releasing cytokines could be addressed. By understanding the direct effects of LPS on various neuron types in various organisms, healthcare providers will better understand the potential consequences of exposure on neuronal function in mammals.

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