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Bowei Deng April 12, 2022

The Effect of Systemic Lipopolysaccharide Treatment on Locomotor and Pain Behavior of Spinal Cord Injured Mice

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Abstract

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Importance: Approximately 296,000 persons currently live with a spinal cord injury (SCI) in the United States. Neuropathic pain, which can be expressed as an aggravated, sharp, stabbing pain around the level of SCI, is an important consequence of SCI. The mechanisms underlying neuropathic pain and other behavioral complications after SCI are poorly understood, although such an understanding is essential to discovering novel therapeutic interventions.

Objectives: This study examined the effect of systemic inflammation on chronic pain responses, hind-limb locomotor function, and inflammation-induced neuronal and cellular plasticity accompanying pain after SCI.

Methods: Liposaccharide (LPS) was intraperitoneally injected into mice to induce inflammation. A contusion SCI was created at the thoracic (T) 10 level with an Infinite Horizon impactor. The Basso Mouse Scale (BMS) was used to examine hind-limb locomotor function after SCI. Hind-paw mechanical hypersensitivity was assessed by the von Frey test, while the tail-flick test was used to measure thermal sensitivity. Respiratory rates (RR) were monitored at weekly time points, and the two-chamber conditioned place aversion (CPA) paradigm was used to assess affective pain in response to mechanical stimulation of the trunk. Spinal expression levels of pERK were measured at acute and chronic time points after LPS treatment.

Results: LPS pre-treatment in naïve mice produced mechanical and thermal hypersensitivity, and a short-lasting reduction in resting RRs. Although LPS had no effect on mechanical pain in SCI mice, it impaired the recovery of locomotor function at later time points. LPS had no effect on spinal pERK levels at the acute time point, although pERK2 was decreased at 35 days post-treatment.

Discussion and conclusion: The results suggest that whereas LPS-induced inflammation has a pain-producing effect in naïve subjects after SCI, this effect is diminished due to the more robust effect of SCI. Interestingly, the mechanisms underlying LPS-induced pain appear to be independent of pERK signaling in the spinal cord. Also, LPS impaired locomotion, while having no effect on pain after SCI, suggests that LPS pre-treatment differentially affects locomotor and pain systems after SCI. Additional studies are needed to fully elucidate the impact of LPS and systemic inflammation on pain behaviors after SCI.

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1.0 Introduction

1.1 Overview

The central nervous system (CNS) comprises the brain and spinal cord. The spinal cord serves as a signaling conduit between the brain and the body or periphery (outside the CNS). However, it is well-established that the spinal cord is not a passive conduit of neural impulses. Instead, it is dynamic and enables a wide range of modification and integration of sensorimotor activity and modulation by descending input. Because the spinal cord is the initial central site of integration and modulation of incoming sensory information, it is an essential location for various types of plasticity, including pain processing.

Pain is a physiological response to injury, which can be categorized as adaptive or maladaptive (Woolf CJ, 2010). Nociceptive pain is caused by activating primary afferent nociceptors located in the peripheral nervous system in somatic (skin, muscle, or bone) or visceral (body organs) tissue. Nociceptive pain represents the sensation associated with the detection of potentially tissue-damaging noxious stimuli and is adaptive or protective in nature. Adaptive pain also includes inflammatory pain, which is an increase in sensitivity due to an inflammatory response. Neuropathic pain, which results from injury to the somatosensory nervous system, is an example of a maladaptive pain system (Costigan M et al., 2009; Woolf CJ and Mannion RJ, 1999). Neuropathic pain typically involves abnormal functioning of the nervous system and is therefore not protective.

1.2 Spinal Cord Injury

Spinal Cord Injury (SCI) is a debilitating neurological condition that results in variable degrees of deficits in locomotor and sensory functions (Kraus JF et al., 1975). According to the

National Spinal Cord Injury Statistical Center 2021 report, the annual incidence of SCI is approximately 54 cases per one million people in the United States, which equals about 17,900 new SCI cases each year (NSCISC). Approximately 296,000 persons (range of 252,000 -373,000 persons) currently live with SCI in the United States. SCI is an important pathology to study as it causes many devastating health challenges during the acute and chronic stages post-injury with substantial socioeconomic implications for patients and their caregivers (Alizadeh A et al., 2019). The effects of SCI include the impairment of locomotor functions, numbness, weakness, cardio-respiratory dysfunctions, and pain (Anderson KD, 2004; Backonja MM and Serra J, 2004; Backonja MM and Serra J, 2004; Felix ER et al., 2007; Nepomuceno C et al., 1979; Siddall PJ and Loeser JD, 2001). The pain experienced after SCI may be classified as either neuropathic or nociceptive. "Neuropathic pain following SCI is caused by damage to or dysfunction of the nervous system, while nociceptive pain is caused by damage to non-neural tissue either musculoskeletal due to bone, joint, muscle trauma or mechanical instability" (Hagen EM and Rekand T, 2015). Neuropathic pain is a prevalent and clinically relevant effect of SCI (Turner JA et al., 2001).

1.3 Neuropathic Pain after SCI

Injury-induced neuropathic pain causes severe miscommunication within the central nervous system. However, the mechanism underlying neuropathic pain remains unclear (Shiao R and Lee-Kubli CA, 2018). Chronic neuropathic pain after SCI, often described as a sharp, stabbing pain located around the level of injury, is incredibly disturbing to the wounded (Felix ER,Cruz-Almeida Y and Widerstrom-Noga EG, 2007). Eliminating pain or regaining normal sensation is ranked as one of the highest priorities among SCI patients (Anderson KD, 2004). After SCI,

chronic neuropathic pain is met with severe challenges that have limited therapeutic interventions for its control and alleviation. Thus far, amitriptyline, gabapentin, and pregabalin have been reported to be the most effective in relieving pain (Ahn SH et al., 2003; Rintala DH et al., 2007; Siddall PJ et al., 2006; Tai Q et al., 2002; Vranken JH et al., 2008). However, the efficacy was based on limited patients' responses that have individual variations. Moreover, side effects were not thoroughly studied (Hagen EM and Rekand T, 2015). While the neural mechanisms that underlie neuropathic pain after SCI are still poorly understood, research studies agree that events in the periphery (outside the CNS) and within the CNS are involved, e.g. (Bedi SS et al., 2010; Carlton SM et al., 2009; Christensen MD and Hulsebosch CE, 1997; Crown ED et al., 2006; Garraway SM et al., 2014; Hulsebosch CE et al., 2009; Yezierski RP et al., 2004).

Because research on the mechanisms underlying neuropathic pain after SCI had primarily focused on plasticity within the CNS, typically the lesioned spinal cord, the contribution of peripheral mechanisms remains vague and understudied. The majority of what is known about the peripheral mechanisms comes from work done by ET Walters, who identified nociceptors as potential drivers of neuropathic pain after SCI (Bedi SS,Yang Q,Crook RJ,Du J,Wu Z,Fishman HM,Grill RJ,Carlton SM and Walters ET, 2010). In those studies, the authors showed that small-diameter neurons in the dorsal root ganglia (DRG) sensitive to capsaicin and presumed to be nociceptors exhibit spontaneous firing after SCI. Furthermore, this chronic spontaneous activity was seen from 3 days to 8 months after SCI and strongly correlated to behavioral measures of pain, i.e., mechanical and thermal hypersensitivity (Bedi SS et al., 2010). These results were critical in demonstrating that plasticity occurring in DRG neurons may underlie chronic neuropathic pain after SCI. Additional work in support of the idea that peripheral nociceptor activity contributes to pain after SCI comes from work by Garraway and colleagues, which showed that expression of

pain after SCI is exacerbated by nociceptive inputs such as tail shock (Garraway SM,Woller SA,Huie JR,Hartman JJ,Hook MA,Miranda RC,Huang YJ,Ferguson AR and Grau JW, 2014) or complete Freunds' adjuvant (cFA) (Martin KK et al., 2019). CFA is known to increase the excitability of Aδ and C nociceptors (Xiao W-H and Bennett Gary J, 2007). Hence, these studies support the notion that peripheral nociceptor activity is critical to developing and expressing pain hypersensitivity after SCI.

That primary afferent hyperexcitability contributes to chronic pain is not surprising. The transition from acute to chronic pain (a process known as chronification) in inflammatory and non-SCI chronic pain involves hyperalgesic priming - the process of sensitizing a peripheral nociceptor after which an acute stimulus can trigger long-lasting hypersensitivity of the nociceptor to other stimuli (Dina OA et al., 2008; Ferrari LF et al., 2010). Hyperalgesic priming can trigger exaggerated responses in nociceptors to inflammatory cytokines and normally subthreshold noxious inputs (Kandasamy R and Price TJ, 2015; Reichling DB and Levine JD, 2009). Furthermore, cytokines, which are increased in the spinal cord and periphery after SCI, can initiate hyperalgesic priming (Gonçalves dos Santos G et al., 2020). Altogether, these studies suggest that primary afferent hyperexcitability and hyperalgesic priming are likely to contribute peripherally to the enhanced pain states after SCI.

1.4 Systemic Inflammation after SCI

Inflammation is generally triggered by infections, injuries, or foreign substances. Inflammation is marked by an increase in immune and inflammatory cells and numerous pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), interleukin (IL)-1β, and -6, which are implicated in the devastating effects of SCI (Garraway SM et al., 2014; Murakami T et

al., 2013; Wang XJ et al., 2005). It was recently reported that trauma, including SCI, induces multiple organ dysfunction (Sun X et al., 2016), in part, by promoting an early systemic inflammation (Anthony DC and Couch Y, 2014; Sauerbeck AD et al., 2015). Most of what is known about the inflammatory response initiated by SCI focuses on the events that occur centrally, typically at or near the lesion epicenter. Much light has been shed on glial cell migration and proliferation response, the release of inflammatory cytokines, and subsequent apoptotic cascades. However, the systemic inflammatory response also produces increases in inflammatory cell phenotypes (Gris D et al., 2008; Pillay J et al., 2007) and a plethora of inflammatory markers in peripheral tissue after SCI (Gris D,Hamilton EF and Weaver LC, 2008; Parvin S et al., 2021; Sun X,Jones ZB,Chen XM,Zhou L,So KF and Ren Y, 2016).

1.5 Lipopolysaccharide:

Lipopolysaccharide (LPS), the major component of gram-negative bacterial cell walls, induces a persistent systemic inflammatory response that often leads to sickness behavior and eventual death. However, low doses of LPS induce several characteristic symptoms of systemic inflammation that do not result in fatality (Frank-Cannon TC et al., 2008). LPS exerts its actions by engaging the toll-like receptor (TLR) 4, which activates transcription factor Nuclear Factor Kappa B (NF-κB) signaling, and subsequently increases the expression of IL-1β and TNFα. LPS acting through TLR4 leads to sensitization of primary sensory neurons in the lumbosacral dorsal root ganglion (Wu Y et al., 2019) and trigeminal ganglia (Diogenes A et al., 2011). TLR4 signaling, coupled with NFκB and inflammatory cytokines, contributes to deficits in locomotor recovery, spinal cord edema, and apoptosis after SCI (Ni H et al., 2015). Hence, SCI-induced systemic inflammation is comparable to LPS-induced inflammation.

The elevation of inflammatory cytokines, TNF-α, IL-1β, and IL-6, is significant starting two hours after LPS administration and can last at least 72 hours (Meneses G et al., 2018). LPS models inflammation-induced pain sensitization, and several prior studies have shown that LPS can induce pain when administered to humans (Benson S et al., 2012; Wegner A et al., 2015) and rodents (Inceoglu B et al., 2006; Woller SA et al., 2016). Therefore, LPS can serve as a reliable method to uniformly induce peripheral inflammation in mice to investigate the effect of inflammation on chronic neuropathic pain after SCI. Moreover, LPS-induced inflammation can be used as a positive control in determining whether, like LPS, SCI-induced pain is driven by systemic inflammation that produces primary afferent hyperactivity.

2.0 Hypothesis

The aforementioned studies establish the need to elucidate the neural mechanisms that underlie pain after SCI, with an emphasis on peripheral mechanisms. The overarching goal of this thesis project is to investigate the contribution systemic inflammation makes to functional outcomes after SCI, particularly pain hypersensitivity and locomotor function. I hypothesize that pre-treatment with LPS will induce peripheral inflammation and worsen functional outcomes after SCI. To test my hypothesis, I undertook the following three experiments:

Experiment 1 investigated the effect of LPS pre-treatment on pain behaviors and hind-paw locomotion at acute and chronic time points in adult uninjured (naïve) and spinal cord contused mice. Experiment 2 examined the influence of LPS treatment on thermal sensitivity in adult naïve mice at acute time points. Experiment 3 tested the effect of LPS treatment on the phosphorylated Extracellular Signal-Regulated Kinase (pERK) expression level in the lumbar spinal cord in adult naïve mice at acute and chronic time points.

3.0 Methods

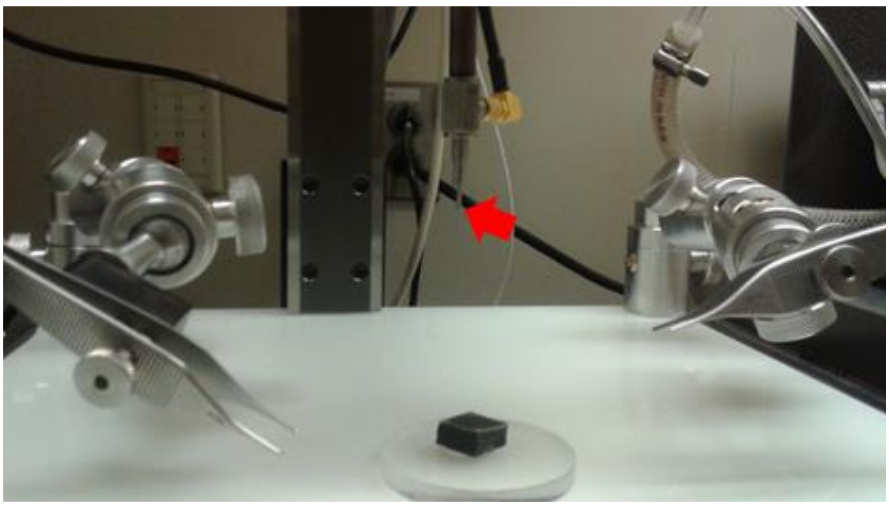
3.1 Animals

Experiments were performed in female and male BALB/C wild-type mice, which were bred in our animal colony or purchased from Jackson Laboratory. Mice were approximately 3-4 months old at the time of surgery or injection and weighed 20-22 grams (females) and 24-26 grams (males). They were housed in standard cages in a vivarium on a 12:12-hour light-dark cycle and fed standard rodent diets *ad libitum*. Experimental procedures were approved by the Animal Care and Use Committee (IACUC) of Emory University and conformed to national standards for the care and use of experimental animals and the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

3.2 Spinal Cord Injury

According to the National SCI Database (NSCID) and National Shriners SCI Database (NSSCID), for the 83.1% of total SCI incidences reported from 2005, 31.5% were from automobile crashes, 25.3% were from falls, 10.4% were from gunshot wounds, followed by 6.8% from motorcycle crashes, 4.7% from accidents, and 4.3% from medical complications (Chen Y et al., 2013). Hence, the common origin of an SCI is from a brutal impact around the spine. To mimic the common and clinically-relevant SCI, I utilized the contusion injury, in which the spinal cord is bruised. The contusion was done at the lower thoracic (T) 10/11 level. This type of complete SCI has been reported in mice to impair locomotor and bladder functions and contribute to the development of neuropathic pain (David BT et al., 2014; Matyas JJ et al., 2017). The mice were deeply anesthetized with 5% isoflurane with oxygen for induction. A hind-paw pinch test and corneal reflex were assessed before the surgery to assess the depth of anesthesia. Once mice were

in the complete-anesthetic state, isoflurane was adjusted to 3-4%. Under sterile conditions, a dorsal skin incision followed by T9 to T12 dorsal laminectomy exposed the point of contusion (T10 or T11). The Infinite Horizon Impactor (Precision Systems and Instrumentation, Lexington, KY, USA), shown in Apparatus 1, was used to contuse the spinal cord's dorsal surface at a force of 70 kilodynes (~0.5mm displacement). The incision was sutured, and skin stapled. The wound area was treated with triple antibiotic ointment (bacitracin-neomycin-polymyxin B) topically.



Apparatus 1. An image of Infinite Horizon Impactor from Precision Systems and Instrumentation, Lexington, KY, with the red arrow pointing at the impactor that creates the bruise on T10 of the anesthetic mice. The accompanying software was installed on a desktop to monitor the force of contusion (~70 kilodynes).

The SCI mice were given meloxicam (5 mg/kg, subcutaneously [SC]) and lactated Ringer's solution (0.5 mL, intraperitoneally) immediately after surgery and left to recover on a heated pad. The mice were also administered 0.9% sterile saline daily (0.5 mL) for the first 48 hours after surgery to maintain hydration. Subsequent administration of saline was given as needed. In addition, mice received the antibiotic Baytril (2.5 mg/kg, SC) immediately after surgery and daily each morning up to 7 days post-operation (DPO) to minimize the risk of urinary tract or bladder infection in SCI animals. Experimenters manually expressed SCI mice bladders twice daily for the

duration of the experiments. Mice were assessed for impairment of locomotor function at 1 DPO using the Basso Mouse Scale (BMS) (Basso DM et al., 2006) to ensure the effectiveness of the injury. SCI mice were only included in the study if they recorded BMS scores of 0 or 1 at 1 DPO.

3.3 Administration of LPS

All the SCI mice received a single intraperitoneal injection of LPS 4 hours before SCI surgery, 5 hours before the behavioral assessments (*note behavioral tests started 1 hour after SCI*), and all the naïve mice received LPS intraperitoneally 5 hours before the behavioral assessments. Lipopolysaccharide was purified from Escherichia coli O111:B4 (Sigma-Aldrich #L2630). It was dissolved in the sterile solution to the final concertation of 100µg/mL. Each mouse received 0.5µg LPS/1g Mouse mass, ~10-13µg of LPS, or an equivalent volume of sterile saline to serve as the vehicle control. The injection of LPS was staggered to ensure all behavioral assessments were perfectly timed. For instance, our setup allows us to record two mice simultaneously for respiratory rate, and each recording lasts 30 minutes. Therefore, LPS injected naïve animals were grouped into pairs to receive LPS in 30-minutes intervals so that each group could start the test at approximately 5 hours post-injection.

3.4 Behavior Room Acclimation

Mice were transported from the animal facilities to the behavioral suite in accordance with the IACUC procedure and with minimal stress to them. They were sufficiently acclimated to the behavioral suite and testing apparatuses for at least 3 days (not necessarily consecutive) prior to testing. During the acclimation period and on testing days, the behavioral suite was maintained at

~25°C. In addition, on testing days, mice were allowed to acclimate to the behavioral room 15 minutes before being transferred to the behavioral apparatuses.

3.5 Basso Mouse Scale

Starting from 1 day-post-operation (DPO), all subjects were assessed for hind-limb locomotor function using the Basso Mouse Scale (BMS) open field test (Basso DM,Fisher LC,Anderson AJ,Jakeman LB,McTigue DM and Popovich PG, 2006). Only SCI mice with 1 DPO BMS score lower than 2 (slight or no ankle movement) are included in the study. Subsequently, BMS was conducted on 1, 7, 14, 21, and 28 DPO to monitor the recovery of locomotor function after SCI and LPS treatment over time.

3.6 von Frey test

I conducted the von Frey (VF) test of hind paw mechanical hypersensitivity (Chaplan SR et al., 1994) on 7, 14, 21, and 28 DPO. Before the assessment started, the mice underwent three 90-minutes acclimations to the von Frey mesh (Apparatus 2). Baseline measurements were taken 2-3 days before surgery. Before each von Frey test, mice were acclimated to the von Frey mesh for 15-20 minutes.

A set of von Frey filaments (NC12775-99, North Coast Medical, Inc., Morgan Hill, CA, USA) were used in this study, starting with filament evaluator size 3.22 that was calibrated to give a specific force (target force 0.16 grams). The filaments were administered from below the mesh platform for 3-5 seconds to test each animal's sensitivity to mechanical stimulation of the hind paw, using the Up-down method described by Chaplan et al. (Chaplan SR,Bach FW,Pogrel JW,Chung JM and Yaksh TL, 1994). Right and left paw withdrawal thresholds were averaged to determine

overall mechanical sensitivity. A reduction in von Frey withdrawal threshold values from their baseline levels corresponded to enhanced mechanical hypersensitivity.

Apparatus 2. Diagonal bottom-up view of the von Frey chambers on a mesh wire table top. Mice were placed in individual chambers with opaque acrylic barriers. This configuration limited possible interferences during the test. Mice were allowed to acclimate and calm before the test started.

3.7 Tail-Flick test

The second reflexive pain assessment used in this study is the tail-flick (TF) test to measure thermal sensitivity. The test was done at baseline (one day before LPS) and 4, 24, and 48 hours post-LPS or saline treatment in naïve mice. A restraining tube (Model 84-IITC life science, Woodland Hills, CA, USA) was used, and each mouse was acclimated to the semi-restraining tube for 3 consecutive days. Each day, the mice spent 2 hours in the tube. The setup of the tail-flick Analgesia meter (IITC life science, Woodland Hills, CA, USA) is shown in apparatus 3. Each mouse was acclimated to the restraining tube for 10 minutes before being placed on the testing apparatus for 5 minutes. A radiant heat stimulus was administered to the tail (approximately 1.5 inches from its tip) three times, with an interstimulus interval of 2 minutes. The latency to withdraw

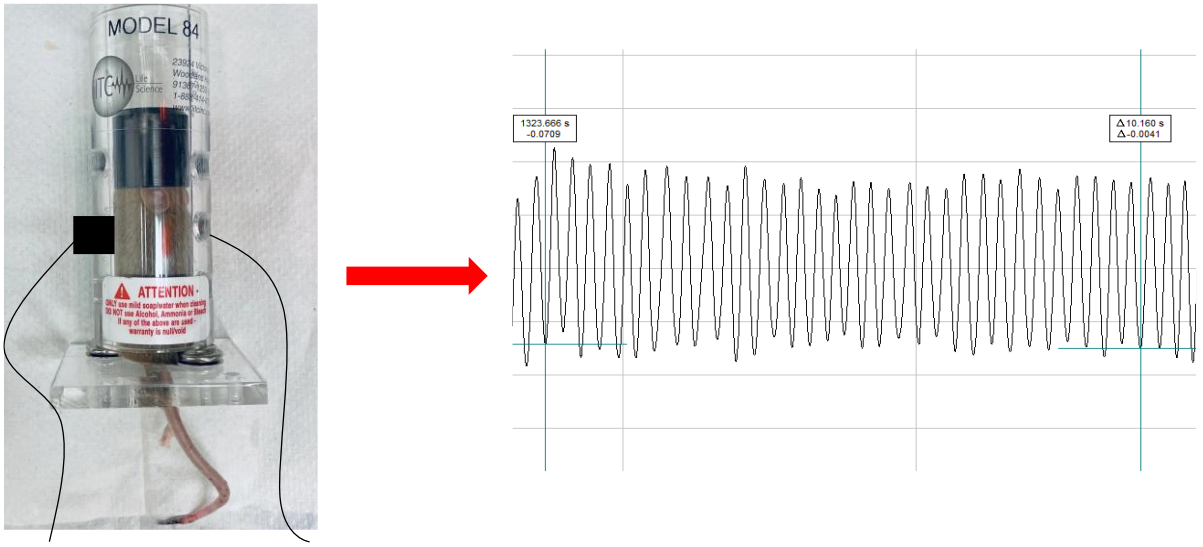
from the noxious stimulation was recorded for each stimulus. A cut-off of 8 sec was pre-determined to prevent inadvertent tissue damage. The average of all three latencies was used for data analysis. The temperature at which a response was elicited was also recorded, ranging from 25 – 31°C.

Apparatus 3. The Tail-Flick Analgesia Meter with a mouse in the semi-restraining tube, ready to be tested. The red arrow points at the focus of light that shines at 1.5 inches from the tip of the tail. After each trial, the withdrawal latency and temperature are shown on the screen in green.

3.8 Respiratory Rate

The Garraway lab had previously shown that SCI increases respiratory rates (RR), and an increase in RR might represent a measurable index of chronic pain states in adult rats (Noble DJ et al., 2019). Here, I utilized non-contact biosensor technology to assess baseline RR before treatment, at 5 hours, and 1, 7, 14, 21, and 28 days after LPS or saline injections. These assessments were done in both naïve and SCI mice. Subjects were loosely restrained in the recording tube (Model 84-IITC life science, Woodland Hills, CA, USA) (Apparatus 4) for 30 minutes, during

which RRs were continuously recorded. Prior to recording, each mouse was acclimated to the restraint tube for 1 hour on 3 consecutive days. Two non-contact biosensors (Plessey Semiconductors) were placed on the side and the bottom of the tube to non-invasively track breathing as previously described (Noble DJ et al., 2017). When attached to the external wall of the restraint tubes, the sensors could accurately record movement behaviors, including respiration.



Apparatus 4. The Semi-Restraining Tube from IITC life science, Woodland Hills, CA, USA. A mouse in the semi-restraining tube was ready for RR recording (left image). The black square represents the non-contact biosensors placed on the left and the bottom of the tube. The breaths of the mice were converted into sinusoidal waves. Steady breathing for 10 seconds was extracted in frequency (Hz) and converted to breaths per minute (BPM) (right image).

The mice spent 1 hour in the tube to prepare for the 30-minutes RR recording. The recorded data were analyzed using Clampfit analysis software (Molecular Devices, San Jose, CA). In Clampfit, the raw signals were analyzed and filtered, power spectral analyses were performed, and threshold-based detection of individual breaths was determined. Specifically, four 10-second epochs (early, middle, and late segments) were analyzed from raw recordings collected throughout the recording period, and dominant spectral peak RRs for each segment were averaged to determine a final value for resting RR. The frequency was then converted to breaths per minute

(bpm) [frequency (Hz) X 60]. The average RR for mice is 250-350 bpm. A significantly increased RR can serve as another approach to measure the effect of inflammation on neuropathic pain.

3.9 Two Chamber Conditioned Place Aversion (CPA) Paradigm

To provide a validated assessment of at-level affective pain after SCI, I used a modified light-dark chamber CPA paradigm similar to previous studies (Bagdas D et al., 2016; Hummel M et al., 2008; Refsgaard LK et al., 2016; Wu Z et al., 2017; Yang Q et al., 2014). The two-chamber conditioned place aversion (CPA) paradigm (Apparatus 5A) made in the Garraway lab was used to assess affective pain responses following mechanical stimulation of the trunk between four to five weeks post-operation (Ideally the week after the last RR recording, starting from 33 DPO). This behavioral assessment is done over 5 days.

On day 1 of 5 days (33 DPO), mice were placed in a custom-built apparatus consisting of a light and a dark chamber separated by a partition that allows them to move freely in-between as they prefer. The number of transitions and time spent in each chamber were recorded as the pre-test measurements by video recordings. At the end of day 1, the trunk fur on both sides of the mouse was removed with an electric shaver while the mouse was deeply anesthetized. On days 2 to 4 of 5 days (34 to 36 DPO), mice received daily 30-minute conditioning. Each CPA box contained a small window permitting the entry of a small histology brush (Camel hair #4, Ted Pella, Inc., Redding, CA, USA) for manual stimulation. In their preferred chambers, animals were administered brush stimulation (once/minute for 15 minutes) using the Camel Hairbrush, delivered a distance of ~3 cm across the trunk in the caudal-to-rostral direction at a speed of ~1 cm/s while they were semi-restrained (Apparatus 5B). Stimulation of the trunk at 1 cm/sec was based on an earlier study that showed an apparent allodynic response at stimulation speeds of 0.3-3 cm/sec (Noble DJ,Martin KK,Parvin S and Garraway SM, 2019). Mice remained unstimulated for the

other 15 minutes in the non-preferred chamber. The non-contact biosensors were used during days 2 to 4 to monitor RRs during the conditioning. On day 5 (37 DPO), each mouse again was allowed free access to explore both chambers for a 30-minute post-conditioning test with no stimuli present. The number of transitions between chambers and cumulative time spent in each chamber on day-5 were recorded as post-test and compared to day 1 (pre-test) measurements to indicate relative place aversion. This test can be referred to in the following sections as the 5-day paradigm (5DP).

A. **B.**

Apparatus 5. (A) Ariel view of CPA Paradigm. A mouse was moving freely in the CPA paradigm on days 1 and 5. Video recordings were taken to extract the number of transitions and chamber preference (time spent in the preferred chamber). **(B)** Side view of CPA Paradigm. A mouse is semi-restrained in the preferred chamber of the CPA, preparing to receive mechanical stimulation with the #4 camel brush. The red arrow points at the entry used to apply the trunk stimulation. RR was taken during the entire 30 minutes, 15 minutes of once per minute stimulation and 15 minutes of non-stimulation.

3.10 Lumber Spinal Cord Collection and Western Blot

At the end of the behavioral assessments, naïve mice were deeply anesthetized with isoflurane, and 1 cm of lumbar spinal cord encompassing L2–L5 was rapidly extracted and flash-frozen in liquid nitrogen for subsequent cellular assay. The entire cord removal procedure was completed within 2 minutes to ensure the integrity of the cord. Protein was extracted from the lumbar spinal cord tissue using modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM

NaCl, 1% NP-40, 1 mM EDTA) with proteases and phosphatases inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), and 2 mM phenylmethylsulfonyl fluoride.

Western blotting was used for quantification of the protein pERK in the lumbar cord. Proteins were extracted from the flash-frozen lumbar cords (1 cm centered lumbar 3). The tissue was immediately sunk. Protein concentration was quantified using the bicinchoninic acid protein assay (Thermofisher Scientific, Waltham, MA). Protein samples were diluted in Laemmli sample buffer at known total protein concentrations (4 μg/μL)and stored at -80 °C. Western blots were performed to quantify the expression of spinal pERK, which is upregulated in the spinal cord of mice that are implicated in the inflammatory (Xu Q et al., 2008) and neuropathic (Crown ED,Ye Z,Johnson KM,Xu GY,McAdoo DJ and Hulsebosch CE, 2006) pain states.

Equal amounts (40 μg) of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), the blots for non-phosphorylated ERK proteins were blocked for 1 h in 5% blotting grade milk (BioRad, Hercules, CA, USA) in tris-buffered saline and Tween-20 (TBST), whereas blots for pERK1/2 were blocked in 5% bovine serum albumin in TBST. After blocking, the blots were incubated overnight at 4 °C in primary antibody diluted in blocking solution as follows:

ERK1/2 (1:2000; #06-182-Millipore, Temecula, CA, USA); pERK 1/2 (1:500, #4370-Cell Signaling Technology, Danvers, MA, USA); and β-tubulin (1:1000; #05-661-Millipore, Temecula, CA, USA) served as control. The following day, blots were washed in TBST (3 x 10 min) at room temperature and then incubated in horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:5,000; #31460 or 31430, respectively; Pierce, Rockford, IL) for 1 hour at room temperature. Following another 3 x 10 min washes using TBST, the blots were

developed with standard enhanced chemiluminescence and imaged with Azure Biosystems c400 Western Blot Imaging System. Ratios of the integrated densitometry of each protein of interest to the loading control (β-tubulin) and pERK to ERK ratio were calculated with AlphaView Software by ProteinSimple, normalized to controls, and averaged for animals within each group (Parvin S,Williams C,Jarrett S and Garraway S, 2021).

4.0 Blinding of Experiments and Statistical Analysis

To minimize differences between treatment groups, mice were randomly assigned into different groups, LPS or Saline injection. To reduce bias in experimental assessment, the injection of LPS was performed by another lab technician such that group allocation was concealed during the data collection process. The assignments were revealed to me only after individual data sets were thoroughly analyzed. It is unachievable to mask SCI mice from naïve mice while examining locomotor functions. Hence, two experimenters simultaneously assessed the BMS of SCI and naïve animals for 2 minutes to collectively provide the most accurate score.

All statistical analyses and correlations were calculated by GraphPad Prism v9 (GraphPad Software, La Jolla, CA, USA). Behavioral data were analyzed using repeated measures (RM) analysis of variance (ANOVA) with timepoint as the within-subjects factor (one-way ANOVA) and treatment/injury group as the between-subjects factor (two-way ANOVA). For individual data points, a paired two-sample for means t-test was performed for comparison within the same group. A two-sample assuming equal variances t-test was performed for comparison treated group with the same number of mice. A two-sample assuming unequal variances t-test was performed for comparison in-between treated groups with a different number of mice. An *a priori* alpha value of

0.05 or below is considered significant. In the figures, * ($p < .05$), ** ($p < .01$), *** ($p < .001$) and **** and ^^^^ ($p < .0001$) show significance compared between groups and data points.

5.0 Experimental Design

For experiment 1, 25 animals were used - 16 naïve and 9 SCI mice. The 16 naïve mice were treated intraperitoneally with saline (n=8) or LPS (n=8) 5 hours before behavioral assessments were undertaken. The 9 mice that received an SCI were treated with Saline (n=4) or LPS (n=5) 4 hours before SCI. Subsequently, the mice underwent behavioral assessment of hind-paw locomotion with the Basso Mouse Scale, pain sensitivity using the von Frey test and CPA paradigm, and continuous monitoring of respiratory rates.

For experiment 2, 12 naïve animals were used. The naïve mice were treated intraperitoneally with saline (n=6) or LPS (n=6) 5 hours before the behavioral assessment. Then, these mice underwent tail-flick tests for thermal sensitivity assessment.

For experiment 3, 28 naïve animals were used. The mice were separated into chronic (n=16) and acute (n=12) groups. The 16 chronic naïve mice were treated intraperitoneally with saline (n=8) or LPS (n=8), and the 12 acute naïve mice were treated intraperitoneally with saline (n=6) or LPS (n=6), both 5 hours before behavioral assessments were undertaken. Animals were sacrificed (chronic = 37 DPO, acute = 2 DPO) with lumbar spinal cords collected to perform western blots in detecting pERK expression.

6.0 Results

6.1 Experiment 1

6.1.1 Basso Mouse Scale (BMS)

A two-way ANOVA was performed to analyze the effect of DPO and saline/LPS injection on the BMS score of naïve mice (**Figure 1**), which revealed that there was not a statistically significant interaction between the effects of DPO and saline/LPS injection ($F_{(4, 56)}$ = 1.0, p = .392). Simple main effects analysis showed that DPO did not have a statistically significant effect on the BMS score of naïve mice ($F_{(4, 56)}$ = 1.7, p = .168), and injections did not have a statistically significant effect on the BMS score of naïve mice ($F_{(1, 14)}$ = 0.8, p = .390).

An indication of a successful spinal cord contusion is the paralysis of the lower body beyond the point of impact, thoracic level 10. Therefore, I expect to observe a BMS score of zero in SCI mice. A mixed-effects analysis was performed to analyze the effect of DPO and saline/LPS injection on the BMS score of SCI mice (**Figure 2**) because one SCI-saline mouse was excluded from the study after 14 DPO. The analyses revealed that there was a statistically significant interaction between days post-operation (time) and treatment groups ($F_{(4, 26)}$ = 5.7, p = .002). Overall, both LPS and Saline mice had significant improvement in locomotor function at later timepoints compared to day 1 scores. However, when comparisons were made between groups over time, there was a significant difference ($F_{(4, 26)}$ = 5.7, p = .002). Specifically, although there were no differences between LPS and Saline treated groups at 1, 7, and 14 DPO; at 21 ($p < .05$) and 28 ($p < .01$) DPO, the saline treated subjects had a better recovery than LPS treated mice. No difference was observed in the degree of recovery between the LPS and saline SCI mice on day 7 and day 14 after surgery. Nevertheless, after day 14, the saline-injected SCI mice continued to recover with an increase in BMS score, while the LPS-injected SCI group's BMS remained stagnant. The BMS score of saline-injected SCI mice (n=3) was significantly higher than the LPS-injected mice (n=5) on day 21 (t = 3.2, $p < .05$) and day 28 (t = 3.8, $p < .01$) post-surgery, which suggests LPS to attenuate locomotor recovery after spinal cord injury (**Figure 2**).

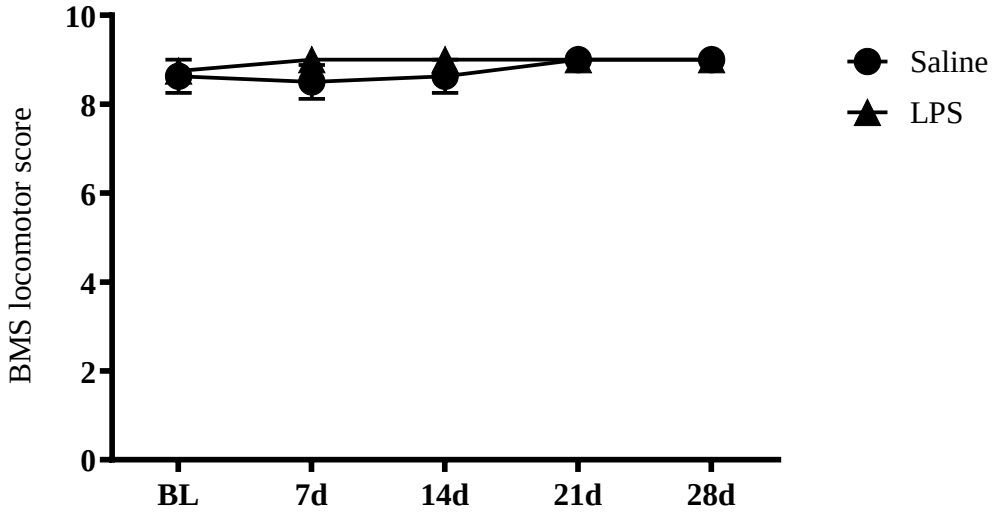


Figure 1. Line graph of BMS locomotor score for Saline/LPS treated naïve mice. Y-axis shows the average BMS locomotor score of the left and right paw. There is no difference between the two groups throughout the testing period. Overall, LPS does not alter locomotion in naïve mice.

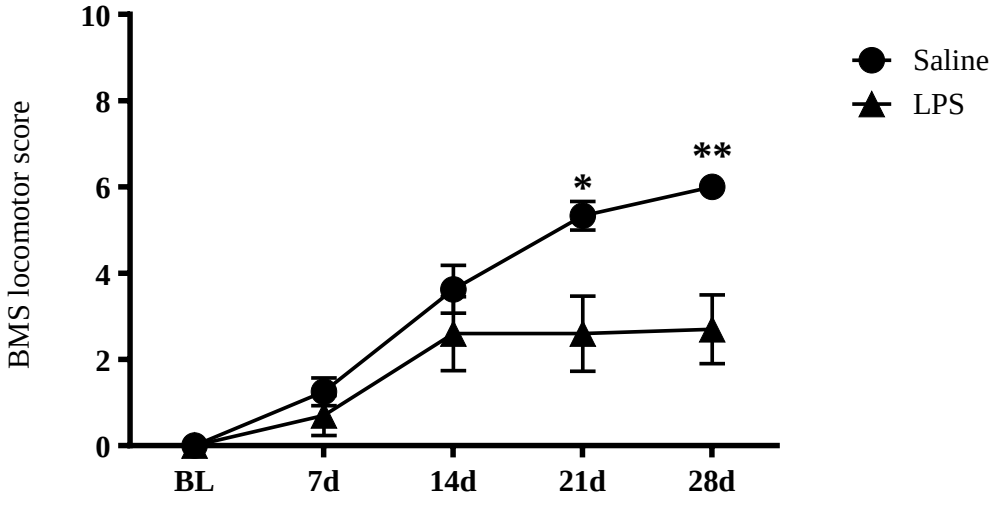


Figure 2. Line graph depicting BMS locomotors score for Saline/LPS treated SCI mice. LPS injected SCI mice (n=5) showed impaired recovery of locomotion compared to Saline treated mice. This effect emerged at 21 days ($p < .05$) and persisted to 28 days ($p < .01$), compared to saline. These results demonstrate a negative effect of LPS on locomotor recovery after SCI.

6.1.2 von Frey (VF)

A two-way ANOVA was performed to analyze the effect of saline or LPS treatment on the withdrawal threshold to mechanical stimulation with von Frey filaments in naïve mice. As shown in **Figure 3**, there were no differences in the withdrawal thresholds between the two groups over time ($F_{(4, 56)} = 1.5$, $p = .206$). While there were no overall differences between the two groups, a simple t-test showed that, on day 7, the LPS treated mice showed a reduction in their withdrawal threshold compared to the saline treated counterparts mice ($t_{(14)} = 2.8$, $p = .015$) and also to their baseline responses ($t_{(7)} = 4.4$, $p = .003$) (**Figure 4**).

I also evaluated the effect of saline and LPS treatments on mechanical sensitivity in mice with an SCI (**Figure 4**). The ANOVA results revealed no significant interaction between days post-operation and saline/LPS treatments ($F_{(4, 26)} = 0.7$, $p = .578$). In both groups, the withdrawal threshold was significantly changed over time ($F_{(4,26)} = 53$, $p < .0001$). Specifically, in both saline and LPS treated SCI groups, mechanical hypersensitivity was observed at 7, 14, 21, and 28 DPO compared to baseline. However, LPS did not produce any additional effects compared to the saline treatment (**Figure 4**).

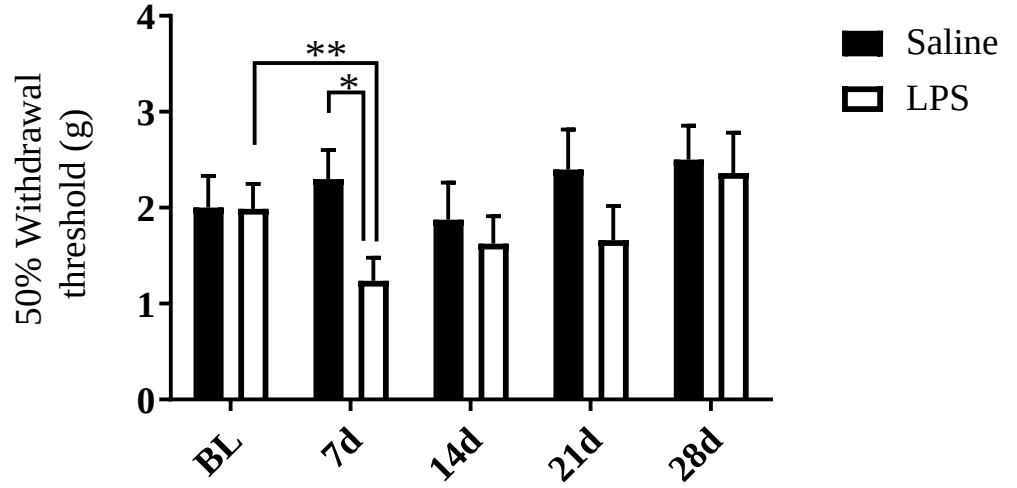


Figure 3. Bar graph of von Frey withdrawal threshold for Saline/LPS treated naïve mice. Y-axis shows the average 50% withdrawal threshold of the left and right paw. The 50% withdrawal threshold for LPS injected naïve mice had decreased on day 7 compared to the baseline ($p < .01$) and to the saline group $p < .05$).

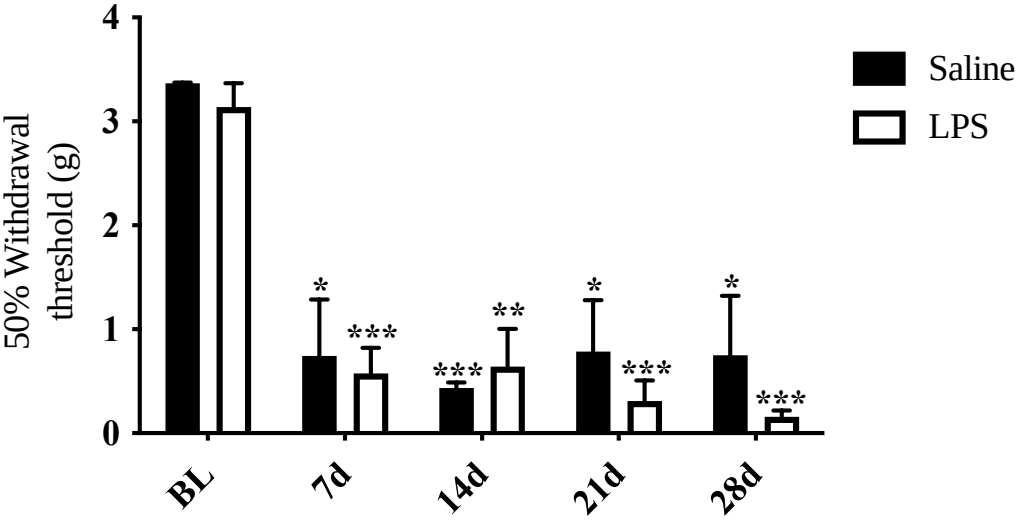


Figure 4. Bar graph of von Frey withdrawal threshold for Saline/LPS injected SCI mice. Y-axis shows the average 50% withdrawal threshold of the left and right paw. Both the LPS and saline-injected SCI mice had significantly decreased 50% withdrawal threshold at all time points after SCI, compared to their baseline thresholds. [*, $p < .05$; **, $p < .01$, ***, $p < .001$).

6.1.3 Respiratory Rates (RRs) measurement: at weekly timepoints and during truncal stimulation

I evaluated the effect of LPS treatment on resting RRs in naïve and SCI mice. In naïve mice (**Figure 5**), a two-way ANOVA revealed that there was a statistically significant interaction between days post-operation and saline/LPS treatment ($F_{(6, 84)} = 7.9$, $p < .0001$). The multiple comparative analyses showed that at 5 hours, RRs were significantly reduced in the LPS treated subjects compared to all other time points (p values ranging from $p < .05$ to $p < .0001$). Furthermore, a follow-up analysis comparing the two groups showed that at 5 hours, there was also a significant difference in resting RRs between the LPS and saline treated groups (^^^^, $p < .0001$).

When RRs were compared in SCI mice, I found no difference in treatment over time between the LPS and saline groups ($F_{(6, 36)} = 1.1$, $p = .365$). Because there was an apparent decrease in RRs in the LPS treated groups at 5 hours after treatment compared to baseline (as was seen in the naïve mice), a paired t-test was performed to assess the effect on LPS. The results confirmed that RRs were significantly reduced in LPS-treated mice shortly after SCI compared to their baseline ($t_{(3)} = 4.5$, $p = .021$). A similar effect was seen at 14 DPO, in that the RRs were reduced compared to baseline ($t_{(4)} = 8.3$, $p = .001$). No other effect was observed. These results are shown in **Figure 6**.

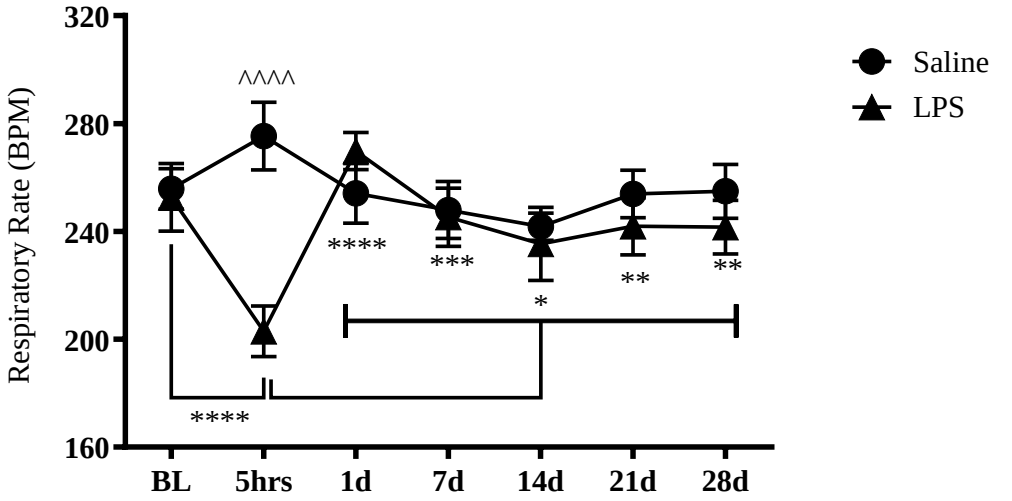


Figure 5. Line graph of weekly RR for Saline/LPS treated naïve mice. Y-axis shows the respiratory rate in breath per minute. The RR of LPS mice was decreased at 5 hours compared to baseline (****; $p < .0001$), although there was a return to baseline subsequently. [*RRs at 5 hours was reduced compared to all other timepoints*.] At 5 hours, average RR of LPS-treated mice was also different to saline treated at the same time (^^^^, $p < .0001$).

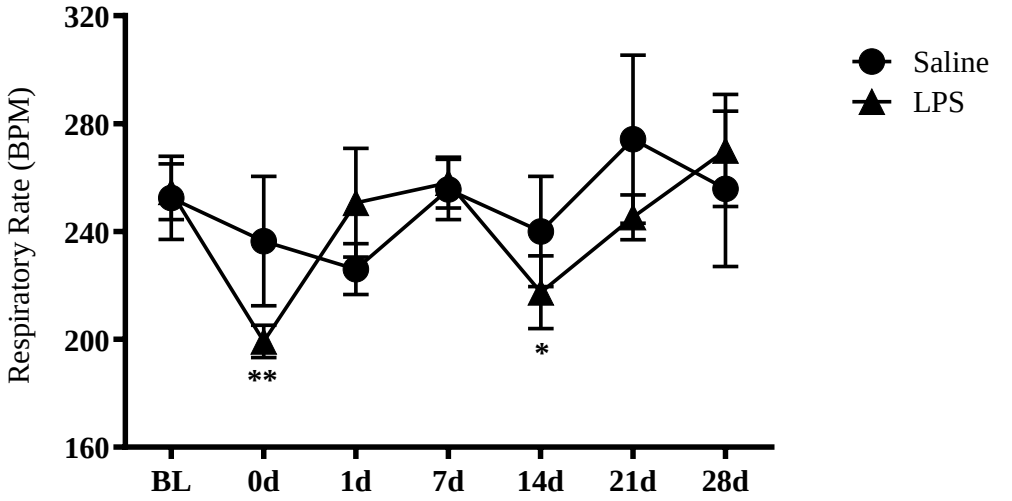


Figure 6. Weekly RRs for Saline/LPS injected SCI mice. The RR of LPS injected SCI mice (n=5) decreased on the day of surgery ($p < .01$) and 14 DPO ($p < .05$) compared to its baseline. There was no change in RRs in the saline treated mice, over the testing period.

Each mouse was stimulated in the preferred chamber (dark) and non-stimulated in the non-preferred chamber (light) for the CPA paradigm as previously discussed. To associate changes in RRs with aversive truncal stimulation, I assessed RRs during the time spent in each chamber. Two way-ANOVA analyses showed that there were no differences in RRs in neither naïve (**Figure 7A**) nor SCI (**Figure 7B**) mice regardless of treatment.

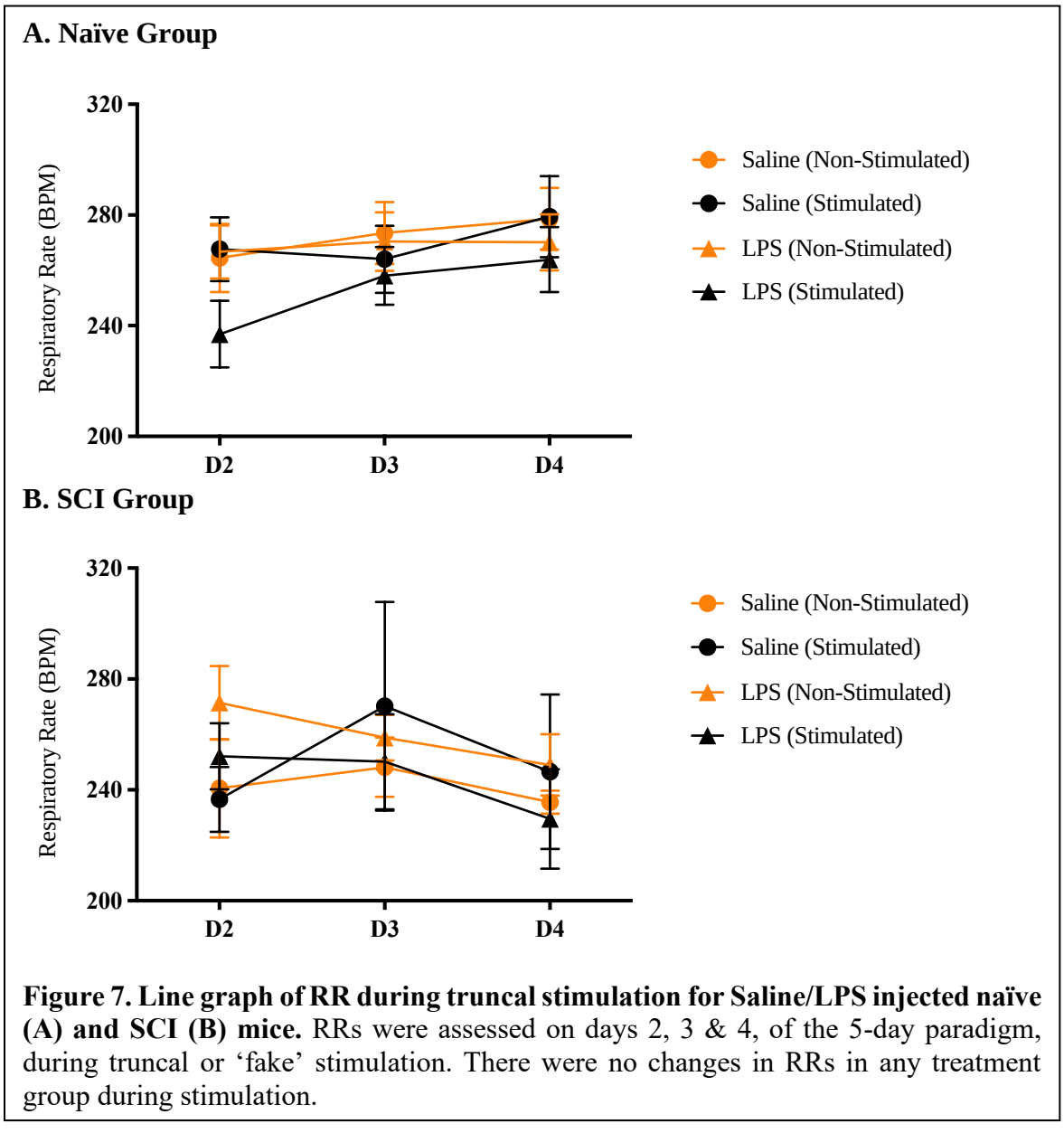


Figure 7. Line graph of RR during truncal stimulation for Saline/LPS injected naïve (A) and SCI (B) mice. RRs were assessed on days 2, 3 & 4, of the 5-day paradigm, during truncal or 'fake' stimulation. There were no changes in RRs in any treatment group during stimulation.

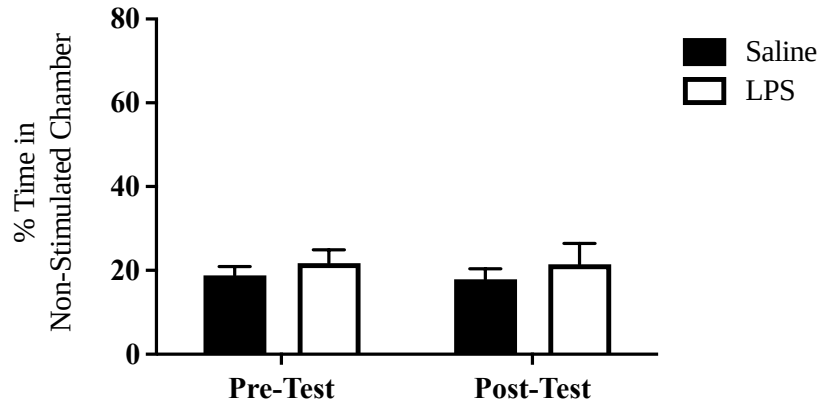
6.1.4 Five-day Paradigm, Chamber Preference (5DP, CP)

It is clear from the chamber preference results of both naïve and SCI groups that mice innately stay longer in the dark chamber (stimulated chamber). However, when before (Day 1) and after (Day 5) comparisons were made, there were no significant differences in time spent in unstimulated chambers (indicative of aversive pain) in the naïve or SCI groups, regardless of treatment (**Figure 8 A&B**, respectively). Nonetheless, there was a modest trend toward increased time spent in the non-stimulated chamber for the LPS injected SCI mice after trunk stimulation.

6.1.5 Five-day Paradigm, Chamber Transitions (5DP, CT)

I also compared the effect of LPS treatment on chamber transitions – when the mouse moves more than half of its body from one chamber to the other. For naïve mice, there were no differences in transition between LPS and saline treated mice (**Figure 9A**). Interestingly in the SCI treated mice, LPS treatment causes a reduction in transitions in the post-stimulation period (day 5), compared to pre-stimulation (day 1), during the post-stimulation period ($t_{(4)} = 3.6$, $p = .023$) (**Figure 9B**). This finding agrees with the BMS results, which showed that LPS treatment impaired the recovery of locomotor function compared to saline treatment. Additional observations worth noting are that there was no change in chamber transitions in saline-injected SCI mice, and the number of transitions in SCI mice was much reduced compared to naïve (~ 22 transitions versus 70 transitions), an observation that aligns with SCI-induced impairment of locomotion.

A. Naïve Group



B. SCI group

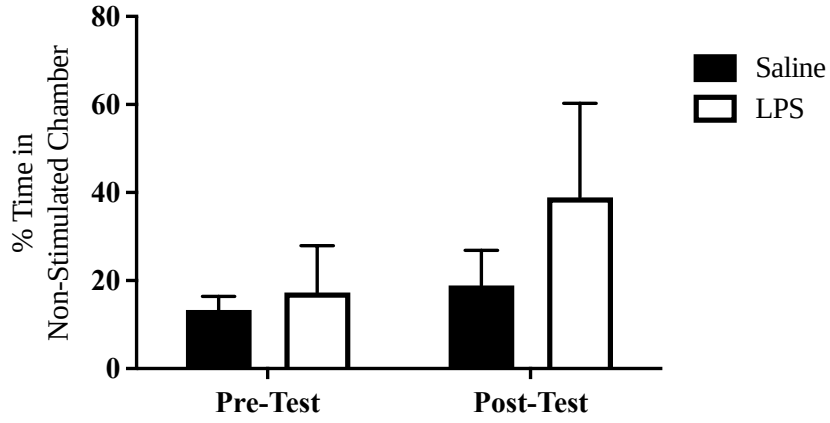
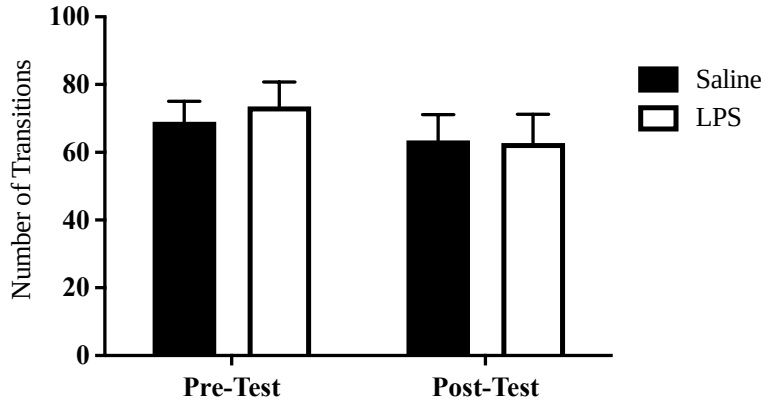


Figure 8. **Bar graph showing the result of the 5-day paradigm for chamber preference following stimulation in Naïve (A) and SCI (B) mice**. **A.** LPS treatment did not increase the mice' time spent in the unstimulated chamber following truncal stimulation, compared to the pre-test preference. **B.** As with naïve mice, there was no significant difference in chamber preference in LPS treated mice compared to before treatment or the saline treated groups. However, although no effect of LPS or stimulation was observed, LPS did cause a modest increase (*not significant*) in preference for the non-stimulated (light) chamber during the post stimulation period.

A. Naïve Group



B. SCI Group

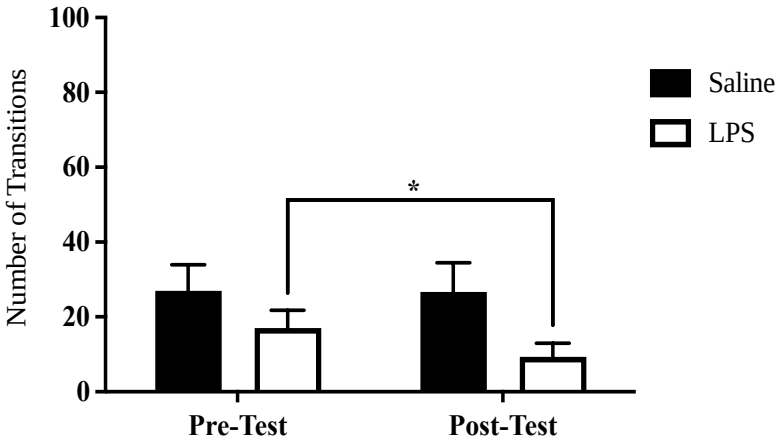


Figure 9. **Effect of LPS on transitions between chambers in Naïve (A)** and **SCI (B)**. **A.** There were no differences in pre- and post-stimulation transitions in Saline and LPS naïve mice. **B.** In the SCI subjects, LPS treatment caused a significant reduction in number of transitions after trunk stimulation compared to pre-stimulation ($p < .05$). There was no difference in saline treated subjects. Overall, SCI subjects (**B**) transitioned less than naïve animals (**A**), an indication of reduced locomotion caused by SCI.

6.2 Results from Experiment 2

6.2.1 Tail-Flick Test

The VF results for the naïve mice suggest a temporary, 7-day impact on hind-paw mechanical hypersensitivity when treated with LPS. To explore the onset of LPS' effects and test allodynia from a different perspective, 12 mice were incorporated into experiment 2, assessing the impact of LPS on the thermal sensitivity of mice. A two-way ANOVA was performed to analyze the effect of DPO and saline/LPS injection on withdrawal latency of naïve mice **(Figure 10)**, which revealed that there was a statistically significant interaction for treatment over time ($F_{(3, 30)} = 8.5$, $p = .0003$). LPS treated mice had a significant reduction in their withdrawal latencies at all time points compared to baseline. A significant group effect was observed ($F_{(1, 10)} = 15$, $p = .003$), and Šídák's multiple comparisons test showed a significant difference in withdrawal at all post-treatment timepoints latencies between the saline and LPS treatment groups. Together with the von Frey data, these results show that LPS alone can temporarily increase mechanical and thermal sensitivity in naïve mice.

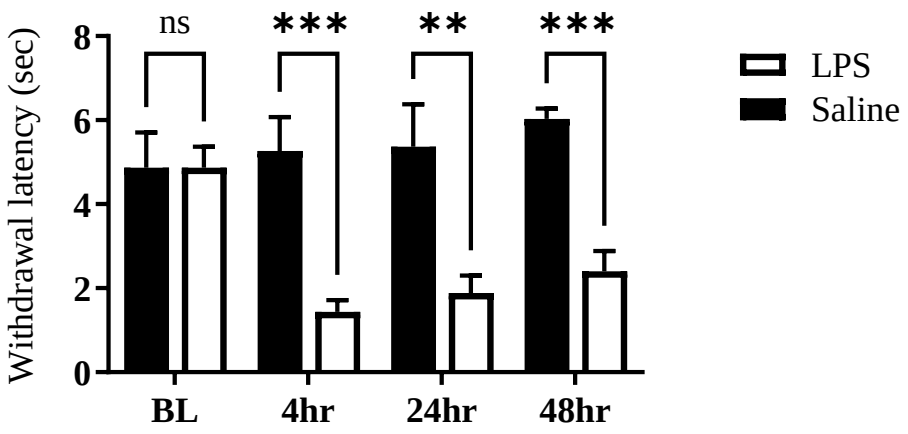


Figure 10. Bar graph of Tail Flick Test for Saline and LPS treated Naïve mice. The thermal withdrawal latency (sec) is significantly reduced in LPS treated mice compared to Saline-injected mice at all post-treatment times (**, p< 0.01 and ***, p < .001). Importantly, LPS treated mice have significant thermal sensitivity at all times compared to baseline ($F_{(3, 20)} = 12.8$, $P < .0001$).

6.3 Results from Experiment 3

6.3.1 Western Blot for phosphor-ERK (p-ERK)

To access nociceptive plasticity occurrence in the spinal cord, I performed western blots to quantify changes in activated ERK (pERK) expression in the lumbar spinal cord. Densitometry analyses with Alphaview software (ProteinSimple) were performed. pERK44 and pERK42 ($pERK_{1/2}$) were compared to total ERK44 and ERK42 ($ERK_{1/2}$) levels. There was no difference in $pERK_1$ and $pERK_2$ levels in LPS treated mice compared to saline at the acute time point [($t_{(8)}$ = 0.7, p = .495), ($t_{(8)}$ = 0.5, p = .653), n=6 each, (**Figure 11A)**]. At the chronic time point, no change was found for $pERK_1$ expression in saline-injected (n=8) and LPS-injected naïve mice (n=8). However, a decrease in $pERK_2$ expression was observed in LPS-injected naïve mice compared to the saline-injected naïve mice [($t_{(7)}$ = 1.9, p = .046), n=8 each, (**Figure 11B**)].

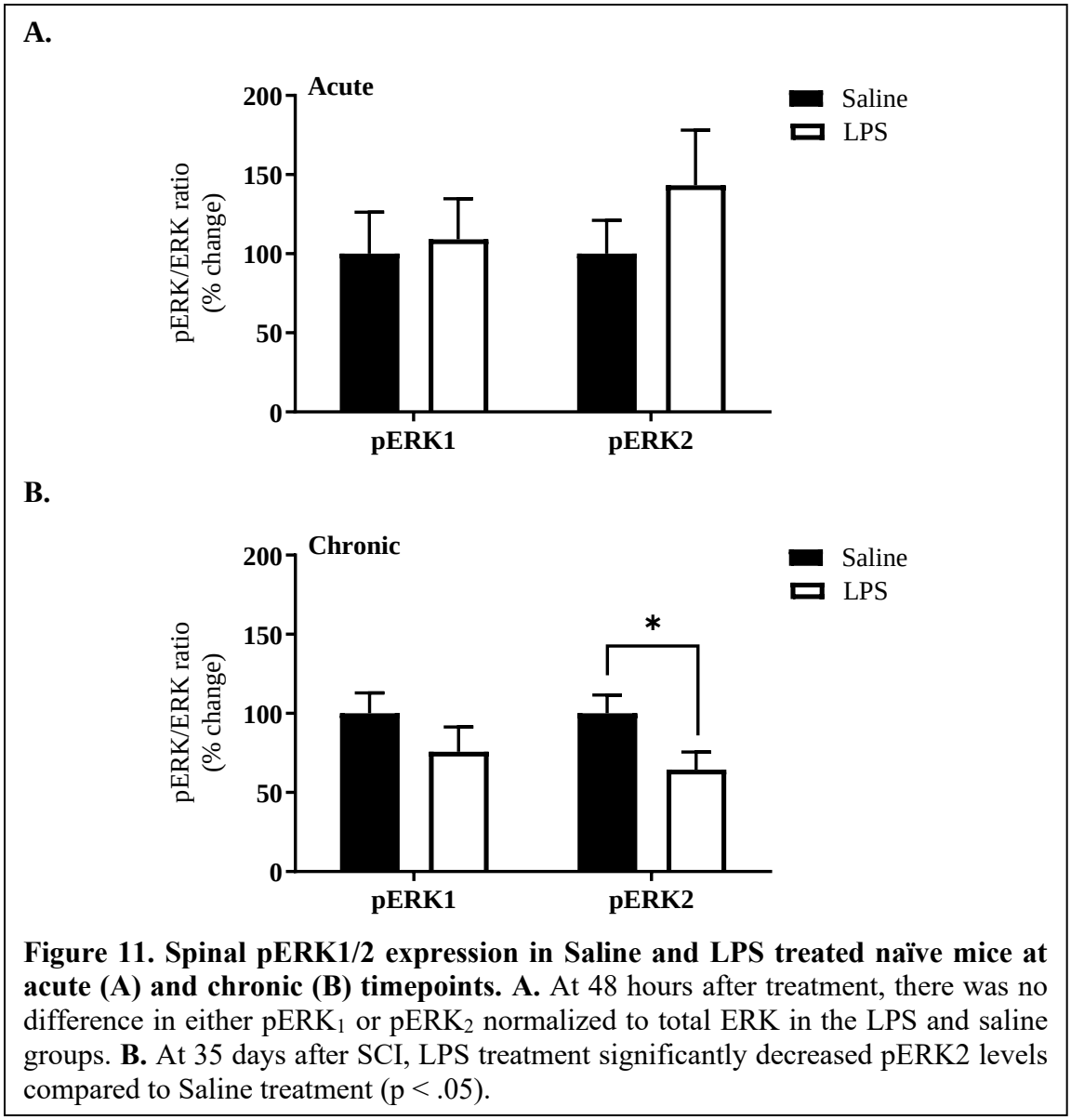


Figure 11. Spinal pERK1/2 expression in Saline and LPS treated naïve mice at acute (A) and chronic (B) timepoints. A. At 48 hours after treatment, there was no difference in either $pERK_1$ or $pERK_2$ normalized to total ERK in the LPS and saline groups. **B.** At 35 days after SCI, LPS treatment significantly decreased pERK2 levels compared to Saline treatment ($p < .05$).

7.0 Discussion

The overarching goal of my thesis project was to evaluate the impact LPS, a model of systemic inflammation, has on pain responses and locomotor function in naïve and SCI mice. There are four key findings. First, LPS caused a short-lasting mechanical hypersensitivity in naïve mice. Second, LPS produced robust thermal hypersensitivity in naïve mice. Third, LPS failed to exacerbate mechanical hypersensitivity after SCI, although SCI caused significant mechanical pain.

Fourth, LPS attenuated the recovery of hind-limb locomotion after SCI. The observation that LPS pre-treatment in naïve mice induces mechanical hypersensitivity 7 days post-injection and thermal hypersensitivity from 4 hours to 48 hours post-injection is important. Specifically, the temporary increase in mechanical and thermal sensitivity suggested that LPS alone induces an acute pain state in adult mice. Unlike the effect seen in naïve mice, it was surprising that LPS was less effective in SCI subjects. Because SCI alone produces a significant mechanical hypersensitivity response, as previously shown (Garraway SM et al., 2014; Martin KK,Parvin S and Garraway SM, 2019), the mechanical threshold has likely reached its minimum, preventing additional decreases by LPS treatment. If this is the case, it would also suggest that LPS does not worsen the systemic effect caused by SCI, particularly as it relates to pain states. However, it should be noted that LPS worsened locomotor outcomes after SCI and decreased chamber transitions. Thus, I can postulate that LPS effects on pain responses and locomotor functions are mediated by different neural mechanisms. Unfortunately, I was not able to fully elucidate the underlying mechanisms of LPS functions in this study.

The initial physiological damage to the musculoskeletal structure and neural damage to the spinal cord had made SCI a traumatic pathology to acquire. The secondary injuries following an SCI, such as systemic inflammation, apoptosis of injured tissue, loss of myelin, and glial scar formation, made a recovery from SCI a journey demanding great effort and exertion (Crowley ST et al., 2019). It has been reported in a previous study that mildly injured SCI animals were capable of full recovery of locomotor function 5 weeks after the surgery, and continued recovery of animals with moderate and severe SCI was observed starting 3 hours to 3 weeks post-surgery (Kakuta Y et al., 2019). Similar results from BMS scores of SCI animals were found in this study as the saline group continued to recover in locomotor function 4 weeks post-surgery. As briefly mentioned in

the LPS section, its mechanism of inflammation induction is initiated from interactions with the TLR4, which leads to intracellular reactions and signaling cascades that incorporate transcription factor NF-κB, and then starts the secretion of inflammatory chemokines and cytokines like Interleukin (IL)-1β, 6, and Tumor Necrotic Factor-alpha (TNF-α) (Heinbockel L et al., 2018). Inflammation after SCI has also been shown to be mediated by IL-1β, IL-6, and TNF-α. Extensive research on these inflammatory cytokines has been done, and scientists found them to be significantly upregulated within hours after the spinal trauma (Parvin S,Williams C,Jarrett S and Garraway S, 2021), which leads to massive infiltration of immune cells such as microglia, PDMs, and neutrophils. These immune cells continue to produce inflammatory mediator that leads to an augmentation of the inflammation response and the apoptosis of neurons (Hellenbrand DJ et al., 2021; Zhang N et al., 2012). In alignment with the general hypothesis, the attenuation in recovery for the LPS group starting from 14 DPO had indicated the LPS effect alone in locomotor recovery, suggesting that the inflammation before SCI will worsen secondary injuries that impairs recovery.

Changes in behavior to thermal and mechanical stimuli were commonly used as a parameter to access inflammatory allodynia in rodents. It has been shown that mice possess hypersensitivity to temperature and pressure 24 hours after LPS injection (Hsieh C-T et al., 2018). Comparing the von Frey result from naïve and SCI mice in this study, it is clear that the inflammation induced by LPS caused a temporary increase in mechanical sensitivity in the naïve group at 7 DPO compared to baseline ($n=8$, $p < .01$). This effect was not readily observed in the SCI result. However, comparing the 50% withdrawal threshold for the LPS injected SCI group on 14, 21, and 28 DPO, the mice had a continuously decreasing withdrawal threshold, although non-statistically significant, compared to the baseline that went from 14 DPO ($t_{(4)} = 6.8$, $p = .003$) to 21 DPO ($t_{(4)} = 11$, $p = .0004$) and 28 DPO ($t_{(4)} = 15$, $p = .0001$). This uninterrupted increase in

mechanical hypersensitivity was not observed in the saline-injected SCI mice, suggesting that LPS may exert a synergistic effect with SCI that further exacerbates pain response after the injury.

Whereas LPS shows profound effects in von Frey sensitivity at 7 days and thermal hypersensitivity up to 48 hours, its effects on SCI were less clear. It is worth noting that the von Frey results of the LPS injected SCI animal presented are statistically underpowered. In fact, it is with caution that I conclude many of the findings in this study because a limited number of subjects were used. Additional studies with an increased number of subjects are needed to fully elucidate the effects of LPS on pain responses, especially after SCI.

LPS as a reliable inducer of inflammation have been studied in many contexts, including its effect on respiration. Huxtable et al. had demonstrated that LPS injection alone would increase baseline breathing frequency in rats (Huxtable AG et al., 2011). In addition, previous studies showed that nociceptive stimuli could activate the sympathetic nervous system to increase respiratory rate (Noble DJ,Martin KK,Parvin S and Garraway SM, 2019; Santuzzi CH et al., 2013). However, my results failed to support their findings. For both naïve and SCI groups, I observed a decrease in weekly resting RR 5 hours after LPS injection (naïve n=8, SCI n=5, $p < .01$).

Although not significant, a modest increase in 5DP RR was found in LPS injected naïve group while the mice were experiencing trunk stimulation on D3 compared to D2. Despite that the results had projected in the opposite direction than expected, the decrease in resting RR reflected the acute effects of LPS. Moreover, a change in RR is complex and intertwined with many factors. As reported by the Journal of American Association for Laboratory Animal Science, the RR of mice can be altered by handling, restraints, earmarking, tail vein bleeding, nail clipping, and retroorbital bleeding (Raşid O et al., 2012).

The expression of pERK in the lumbar spinal cord was examined in this study because it is induced within minutes after noxious stimuli or tissue damage. Due to its dynamic activity and ease of handling, pERK has been identified as an excellent marker for inflammatory pain states (Gao Y-J and Ji R-R, 2009). Unfortunately, my results were unsuccessful in showing a consistently drastic increase in pERK expression in both acute (2 DPO) and chronic (35 DPO) timepoint after LPS injection. Contrarily, the $pERK_2$ expression in LPS injected naïve mice at 35 DPO was lower than in the Saline injected group ($n=8$, $p < .05$). Nevertheless, one aspect of experimental design might have contributed to these conflicting results. In prior similar studies, pERK expression was assessed in the dorsal spinal cord only after SCI and noxious shock or hind paw inflammation (Garraway SM,Woller SA,Huie JR,Hartman JJ,Hook MA,Miranda RC,Huang YJ,Ferguson AR and Grau JW, 2014; Xu Q,Garraway SM,Weyerbacher AR,Shin SJ and Inturrisi CE, 2008). This approach was taken to concentrate primarily on pain-processing neurons in the dorsal horn. In this study, the entire lumbar spinal cord (dorsal and ventral) was used. I believe the inclusion of the ventral portion diluted any effects that might be occurring in the dorsal horn. In fact, there was a trend towards an increase in $pERK_2$ in the spinal cord of LPS treated naïve mice at the acute time point. This trend aligns with increased $pERK_2$ in the spinal cord following peripheral inflammation (Xu Q,Garraway SM,Weyerbacher AR,Shin SJ and Inturrisi CE, 2008).

This study has demonstrated that LPS treatment alone in naïve mice causes pain response, while this response was not observed in mice with SCI. LPS attenuates the recovery of locomotion after SCI and acutely increases the thermal sensitivity in naïve mice. The weekly RR results for LPS injected naïve and SCI mice, von Frey results for LPS injected naïve mice, and tail-flick results for LPS injected naïve mice altogether suggest that the LPS effect lasts around seven days. Future experiments should be designed considering this timeline. A von Frey test arranged on 3,

5, and 7 DPO may better elucidate LPS's acute effects on hind-paw mechanical sensitivity, and the tail-flick test can be arranged on the exact dates, serving as collaborative evidence. To better understand the causality embedded in data, more SCI mice need to be included in the study to increase the statistical power of the results.

It had been proposed in other studies that inflammation-induced after SCI may have beneficial effects for recovery (Hayakawa K et al., 2014). LPS can facilitate M2 activation in microglia that vascularize around the injured site to improve tissue rearrangements and functional recuperation. All subjects in this study were injected with LPS/Saline 4 hours before the surgery or 5 hours before the behavioral test. To investigate the potential curative effect of LPS injection, a new group of animals can be tested with LPS injection after SCI surgery. Furthermore, to explore changes in neural activity and cellular plasticity after LPS treatment, electrophysiological and histological studies on dorsal root ganglion could be integrated into the current research.

In conclusion, it is essential to note that despite the limitations of this study, there are several strengths. Importantly, I showed that SCI pathophysiology is worsened by systemic inflammation. Furthermore, the results of this study strengthen the notion that the neural mechanisms that underlie neuropathic pain after SCI are indeed complex and might be influenced by both peripheral and central processes.

8.0 References

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