LANCL2: A POTENTIAL TARGET FOR THE TREATMENT OF NEUROPATHIC PAIN

by

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B.S. Biology, Middle Georgia State University, 2017

A Thesis Submitted to the Faculty
of Mercer University School of Medicine
Department of Biomedical Sciences
in Partial Fulfillment of the
Requirements for the Degree

MASTER OF SCIENCE IN BIOMEDICAL SCIENCES

Macon, Georgia

June 2020

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ACKNOWLEDGEMENTS

I would like to express my greatest appreciation to my advisor and principal investigator, Dr. Han-Rong Weng, for his guidance and support. His knowledge and understanding provided an excellent graduate school experience for me. I would also like to thank my thesis committee members for their support: Dr. Kristen Horner and Dr. Jaehwa Choi.

Many other people have contributed to my work on this project and my growth as a scientist. For that, I would like to thank Lingwei Kong, Dr. Karpinski, Shirley Powell, and Troy Kendrick. I would also like to express my thanks to my fellow graduate student Elizabeth Shoaga and undergraduate student Takahiro Nakamura.

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ABSTRACT

LANCL2: A POTENTIAL TARGET FOR THE TREATMENT OF NEUROPATHIC PAIN
By: DAVID JAMES CHRISTY
Under the direction of DR. HAN-RONG WENG, Ph.D, M.D.

Dysfunctional pain signaling is a hindrance to a normally functioning organism. Neuropathic pain is caused by damage or dysfunction of the nervous system, such as a pinched nerve, diabetic neuropathy, cancer, and chemotherapy treatments. Current drugs used for treatment of neuropathic pain are either not safe or effective. Discerning signaling molecules regulating neuropathic pain in animal models can offer molecular targets for the development of novel analgesics.

Neuroinflammation in the spinal dorsal horn is a crucial mechanism underlying the genesis of neuropathic pain. Given that LANCL2, a membrane associated protein, is engaged in regulation of inflammation, the purpose of this study was to determine whether LANCL2 has pro- or antinociceptive effects in the spinal dorsal horn. We analyzed levels of pro-inflammatory cytokines as well as LANCL2 to determine the role of spinal LANCL2 in the genesis of neuropathic pain.

Neuropathic rats induced by partial sciatic nerve ligation were used. Specifically, we found that protein expression of LANCL2 in the spinal dorsal horn was reduced in neuropathic rats 10-days after the nerve injury in comparison with sham operated animals. This was accompanied with activation of microglia and astrocytes, increased levels of ERK, TNF-α, and IL-1ß at the same site. Meanwhile, the spinal dorsal horn of neuropathic rats had lower levels of abscisic acid, an endogenous ligand of LANCL2. Furthermore, intrathecal application of abscisic acid suppressed mechanical and thermal hypersensitivity in neuropathic rats. Immunostaining experiments demonstrated that LANCL2 is expressed in neurons but not astrocytes; the data from microglial staining was inconclusive. Our study suggests that activation of LANCL2 signaling pathway in the spinal cord is a powerful approach to conquer neuropathic pain.

CHAPTER 1

INTRODUCTION

An Overview of Pain

Nociception is defined as the process in the central nervous system of encoding harmful stimuli (Smith 2018). This stimulus can be thermal (activating free nerve endings), mechanical (activating stretch receptors), or chemical (activating thermoreceptors). First order neurons, with their nerve endings in the skin, muscle, joints, bone, and viscera, are the cells which receive the stimulus and initiates transmission to the brain. The neuron synapses onto a second order neuron in the spinal dorsal horn of the ipsilateral side. The axon of the second order neuron crosses to the contralateral side of the spinal cord and ascends to the thalamus, where it synapses on the third order neurons. These neurons send that signal to the cerebral cortex where we can perceive pain and respond to the pain. Descending pathways exist that are modulatory in nature, acting on various neurons in the spinal dorsal horn to enhance or inhibit nociceptive signaling. The focus of the current experiments is within the spinal dorsal horn, where the integration of the signal from the primary afferent neurons along with crosstalk from glial cells can alter the signaling pathway.

Nociception is an essential function of a healthy organism. The ability to detect noxious stimuli is important for reacting and preventing further injury. This is typified in acute pain, where a pain stimulus indicates damage to tissue. The timeframe is short term, typically lessening as the tissue heals. Chronic pain is regularly occurring or persistent pain over several months and affects 11 – 19% of the adult population (Breivik et al. 2006; Nahin 2015; Riskowski 2014). A subset of chronic pain is neuropathic pain, caused by a lesion or disease of the somatosensory nervous system. Some common causes of neuropathic pain include diabetic neuropathy, postherpetic neuralgia caused by shingles, pinched nerves, cancer, and chemotherapy treatments.

What happens to the tissue affected by neuropathic pain? Sensitization of the peripheral and central nervous systems causes two prevalent conditions termed allodynia and hyperalgesia. Allodynia is described as a painful response to a normally non-painful stimulus (International 2017). Hyperalgesia is an increased response to a normally painful stimulus (2017). These conditions are useful, testable parameters used for these experiments.

Inflammation of the Nervous System

Injury in the peripheral nerve leads to the activation of glial cells and the subsequent release of pro-inflammatory cytokines. Within the spinal dorsal horn are two types of glial cells: microglia and astrocytes. These glial cells are activated

in the neuropathic state; their specific cell markers and inflammatory proteins are an index of neuroinflammation.

Microglia are the resident immune cells of the central nervous system, akin to macrophages in their function. Microglia exhibit rapid activation in response to small changes in the central nervous system. The number of microglia in the spinal dorsal horn is dramatically increased after peripheral nerve injury (Gilmore 1975; Gilmore and Skinner 1979); this process is termed microgliosis. These cells are activated by ATP, colony-stimulating factor-1 (CSF-1), chemokines, and proteases that are released by injured or activated neurons. In turn, they release several factors including brain-derived neurotrophic factor (Ulmann et al. 2008), TNF-α (Kawasaki et al. 2008), and IL-1ß (Kawasaki et al. 2008; Yan et al. 2018; Yan and Weng 2013). These glial mediators can enhance excitatory synaptic transmission and suppress inhibitory synaptic transmission, thereby potentiating the neuropathic state.

Astrocytes perform many functions, such as neuromodulator recycling, regulation of extracellular ion concentration, and modulation of synaptic transmission. Astrocytes have direct neuronal connections via gap junctions; they are activated by persistent synaptic transmission, and proinflammatory cytokines. In turn, astrocytes release glutamate, ATP, and several chemokines. There is consensus that astrocyte-mediated neuroinflammation is crucial for the

maintenance of chronic pain (Ji et al. 2018), and so the role of astrocytes in the spinal dorsal horn and their potential link to this project cannot be undervalued.

Neuroinflammation plays a key role in neuropathic pain through the upregulation of several key factors, most notably the pro-inflammatory cytokines TNF-α and IL-1ß (Hung, Lim, and Doshi 2017). Protein kinases also play an important role, and extracellular signal-regulated kinase 1 and 2 (henceforth collectively notated as ERK in this paper) play a dynamic role in early and late phase neuropathic pain. Neuronal phosphorylated ERK (p-ERK) is immediately present for several hours, followed by microglial p-ERK present during early phase nerve injury and slowly decreases, and finally astrocytic p-ERK is upregulated in late phase nerve injury (Zhuang et al. 2005). These data suggest that p-ERK plays a significant role in the initiation and maintenance of neuropathic pain.

Pain Management

Pain management for neuropathic pain is unfortunately limited, which can leave patients in a difficult state. Diagnosis of neuropathic pain is problematic as identifying the source is often not obvious. To help identify neuropathic pain, several clinical tests have been developed such as Neuropathic Pain Scale (Galer and Jensen 1997), Neuropathic Pain Questionnaire (Krause and Backonja 2003), Neuropathic Pain Symptom Inventory (Bouhassira et al. 2004), and Pain Quality Assessment Scale (Jensen et al. 2006) to name just a few. None of these tests are identical, though there are some similarities in pain description between most:

“tingling”, “burning”, and “shooting” seem to be the three best descriptors for neuropathic pain and are included in virtually all of these tests (Gilron, Baron, and Jensen 2015). Once it is determined a patient suffers from neuropathic pain and a treatment plan has been formulated, frequent checkups are needed to rule out other treatable underlying medical conditions.

Based on the advice from several associations and societies, a recommendation guide (based on the Grading of Recommendations Assessment, Development, and Evaluation system) has been compiled (Guyatt et al. 2008). Strongly recommended are tricyclic antidepressants, gabapentin, pregabalin, and serotonin-norepinephrine reuptake inhibitor antidepressants (Cavalli et al. 2019). Gabapentin and pregabalin have similar structures to GABA. However, they do not bind to GABA receptors, but competitively inhibit Ca^{2+} voltage-dependent channels in the presynaptic nerve terminal, lowering Ca^{2+} influx, and inhibit the release of several neurotransmitters (2019). Weakly recommended are lidocaine patches, high-concentration capsaicin patches, opioids, botulinum toxin A, and combinations of strongly recommended agents (2019). Lidocaine patches lower spontaneous ectopic nerve discharge by blocking voltage-gated sodium channels (2019). Clinical trials of opioid use for managing neuropathic pain show positive results, but the misuse and potentially fatal effects put this class of drugs near the bottom of the recommended list (Finnerup et al. 2015).

Lanthionine Synthetase C-Like 2 Protein

The lanthionine synthetase C-like (LANCL) proteins are homologs of bacterial lanthionine synthetase C, a cyclase involved in the production of lanthionine rings in lantibiotics (Bauer et al. 2000). Lantibiotics are not produced in animals, so the LANCL protein family must possess a different role. Three LANCL proteins are encoded in the human genome: LANCL1 is located on chromosome 2, LANCL2 is located on chromosome 7, and LANCL3 is located on the X chromosome (Katoh and Katoh 2003; Mayer, Bauer, and Prohaska 2001a). LANCL1 has been shown to possess an array of functions. It was shown to possess antioxidant functions in the nervous system based on LANCL1 knockout mice (Huang et al. 2014). Little is currently known about the role of LANCL3. LANCL2 is a cytoplasmic membrane bound protein attached by myristoylation of an N-terminal glycine (Landlinger, Salzer, and Prohaska 2006). LANCL2 has varied roles depending on cell type. In human granulocytes the LANCL2 signaling pathway is pro-inflammatory. LANCL2 is coupled to a G_i G-protein which sequentially activates adenylate cyclase, generating cAMP; this abundance of cAMP stimulates CD38, which converts NAD^+ to cyclic ADP-ribose and ADP-ribose thereby increasing Ca^{2+} entry and calcium-induced calcium-release (Sturla et al. 2009). A similar pathway is activated in human monocytes (Magnone et al. 2009). A study performed in human hepatocytes indicates an anti-inflammatory pathway; LANCL2 activation leads to the activation of pro-survival AKT/protein kinase B (Zeng, Van Der Donk, and Chen 2014). These data indicate that LANCL2 activation

and the resulting pathway, whether pro- or anti-inflammatory, may be cell-type specific. LANCL2 mRNA was measured in a variety of tissues and shown to be present in the spinal cord (Mayer, Pongratz, and Prohaska 2001b) though exact protein quantification has not been analyzed.

Abscisic acid (ABA) is the naturally occurring ligand of LANCL2 in mammals. First identified in plants, this hormone is involved in varied plant processes including seed maturation, seed dormancy, root growth, leaf senescence, and flowering (Gao et al. 2007). ABA was first shown to bind LANCL2 in human granulocytes and rat insulinoma cells (Sturla et al. 2009). Further studies have shown that ABA is produced and released by granulocytes (Bruzzone et al. 2007) and monocytes (Magnone et al. 2009) reacting to environmental stimuli. In mouse models of diabetes, inflammatory bowel disease, and atherosclerosis, oral ABA dosing has shown therapeutic effectiveness (Guri, Hontecillas, and Bassaganya-Riera 2010a; Guri et al. 2007; Guri et al. 2010b). Transport of ABA into cells has been documented through two related anion transporters: anion exchanger 1 (AE1) in human erythrocytes and anion exchanger 2 (AE2) in the chronic myeloid leukemia cell line K562 (Vigliarolo et al. 2015; Vigliarolo et al. 2016). The transport of ABA in the cells of the central nervous system likely occurs through AE2 as it is the most widely distributed transporter of the anion exchanger family (Alper et al. 1999; Alper et al. 1997).

Preliminary data gathered from a previous student, Dylan Maixner, indicates that LANCL2 may play a role in neuropathic pain regulation (Figure 1). Naïve rats were treated over two days with LANCL2 siRNA and control siRNA morning and night, and on the third morning behavior tests were performed. Afterwards the spinal cord was collected for protein analysis. Accordingly, in the LANCL2 siRNA group, the behavior data indicates a paw withdrawal threshold decrease similar to pSNL data. The western blot data confirms knockdown of LANCL2, as well as an increase of pro-inflammatory markers. The results presented represent the mean of each group and the error bars indicate the standard error of the mean.

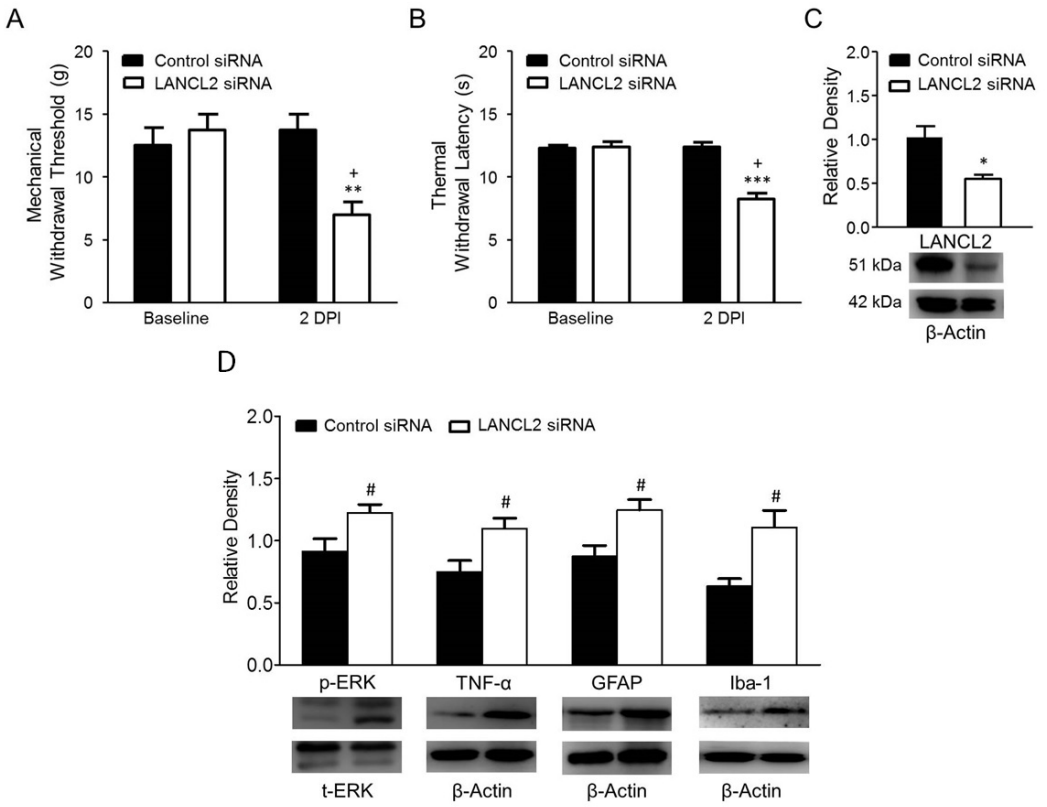

Figure 1. Intrathecal LANCL2 siRNA and control siRNA administration to naïve rats. Mechanical and thermal behavior indicates LANCL2 knockdown mimics neuropathic state (A and B). Western blot analysis confirms LANCL2 knockdown and increased production of pro-inflammatory cytokines in LANCL2 siRNA group (C and D). Statistical significance between LANCL2 siRNA and control siRNA groups are labeled with a + in A and B, labeled with * in C, and labeled with # in D. Statistical significance between two days post injection and baseline are labeled with * in A and B. One symbol: $p < 0.05$; two symbols: $p < 0.005$; three symbols: $p < 0.001$.

Specific molecular targets offer a more precise treatment of neuropathic pain than the previously listed treatments. The present study aims to identify a protein that can be manipulated to alleviate neuropathic pain. Specifically, it is hypothesized that LANCL2 is downregulated in a rat model of neuropathic pain

and that activation of LANCL2 will stimulate anti-inflammatory signaling in the spinal dorsal horn and thereby alleviate the symptoms of neuropathic pain. The specific aims to test the hypothesis are: 1) determine what role LANCL2 plays in neuropathic pain, whether it is pro- or anti-inflammatory, and 2) determine the cellular mechanisms by which LANCL2 acts in the spinal dorsal horn.

CHAPTER 2

METHODOLOGY

All experiments for this study took place in Mercer University School of Medicine's laboratories and animal care facility. The animals used for this study were Sprague-Dawley rats obtained by Charles Rivers Laboratories (Raleigh, NC) at 7 to 8 weeks of age and fed Teklad Global 2014 (Envigo; Indianapolis, IN). Rats were maintained on a 12-hour light/dark cycle and offered food and water ad libitum. The rats were housed 3 to 4 per cage for standard experiments and one per cage for intrathecal cannulations to limit the risk of social grooming which could lead to the removal of the tubing. Before any experiments took place, rats were allowed three days to acclimate to the new environment.

Neuropathic Pain Model and Behavior

Many animal models are used for the study of neuropathic pain, including chronic constriction injury (Bennett and Xie 1988), spared nerve injury (Decosterd and Woolf 2000), and complete Freund's adjuvant injection to the sciatic nerve (Eliav et al. 1999), to name a few. The partial sciatic nerve ligation (pSNL) was determined to be the most reliable, minimally invasive, and cost-effective technique to consistently produce both thermal hyperalgesia and

mechanical allodynia along the short timespan (10 days) of the experiment (Seltzer, Dubner, and Shir 1990).

Animals were first anesthetized with 3% aerosolized isoflurane (Covetrus; Portland, ME) and continued throughout the surgery by affixing the outlet tube over the mouth and nose. The left outer thigh was shaved and sanitized by swabbing with 10% povidone iodine solution then 70% isopropanol, twice over. A skin incision was made along the length of the thigh to expose the biceps femoris. To expose the sciatic nerve, an incision was made parallel with the muscle fibers to minimize tissue damage and inflammation. The wound was carefully expanded using a pair of curved scissors until it penetrated the space below the muscle. Once the sciatic nerve was visualized, the same curved scissors were used to separate the sheath by placing the tip along either side of the length of the nerve and spread to cut the sheath in a controlled fashion. Once the nerve was free from its sheath, a length of 3-0 silk suture (KeeboMed; Mount Prospect, IL) was passed under the nerve and tied twice to constrict the nerve to roughly half its original diameter. The nerve was then tucked back into the space below the muscle and the wound was closed using 9mm stainless steel AutoClips (Mikron Precision Inc.; Gardena, CA). The sham control for this surgery involved a complete reproduction of the pSNL up to tying the sciatic nerve. After the nerve was free of the sheath, the wound was closed with skin staples.

When performed correctly, the pSNL imitates neuropathic pain through the development of mechanical allodynia and thermal hyperalgesia. Behavior tests for each mode of sensory tests were used to quantify the effectiveness of the surgery. All rats were allowed at least three days of recovery from pSNL/sham surgeries before behavior experiments were performed. All rats in this study underwent either mechanical or thermal testing at set points throughout the experiments. Behavior data was taken before surgeries (pain model and catheter placement), after abscisic acid administration, and before spinal cord removal to ensure the pSNL/sham surgeries were performed correctly.

To measure mechanical allodynia, rats were loosely restrained in a plexiglass box atop a wire mesh platform. A 30-minute acclimatization period was allowed to reduce stress for the animals and eliminate potential errors in measurements. A series of von Frey monofilaments (DanMic Global; Campbell, CA), ranging from 2g to 15g bending force, was used to apply pressure to the mid plantar area of the left hind paw. Each monofilament was applied in ascending order five times for a maximum of one second. The pain threshold was determined as the filament in which the rat exhibited a painful paw withdrawal to greater than 50% of applications, and this threshold was later averaged across all animals in the same group. A five-minute rest period was taken between each application to eliminate any possible residual pain from the previous application that could skew the data.

To measure thermal hyperalgesia, rats were loosely restrained in a plexiglass box atop a preheated glass plate (32°C). A 30-minute acclimatization period was allowed to reduce stress for the animals and eliminate potential errors in measurements. A radiant heat beam (IITC Life Sciences; Woodland Hills, CA) was directed at the midplantar area of the left hind paw. The heat source was adjustable through a range of temperatures, so a series of tests were performed that determined the optimal power output that allowed the pSNL and sham rats to show a significant difference in latency of paw withdrawals. The withdrawal latency was defined as the period from the onset of radiant heat to paw withdrawal. Three readings were taken for each animal and averaged to determine a final individual time, and these times were later averaged across all animals in the same group. A 20-second cutoff was used to avoid damage to the animal. A five-minute rest period was taken between each application to eliminate any possible residual pain from the previous application that could skew the data.

Protein Measurements of LANCL2 and Inflammatory Molecular Markers

The comparison of protein levels between the pSNL and sham models was an important aspect to correlate with the behavior test results; increased levels of inflammatory markers accompany the reduced thresholds seen from pSNL rats. Assuming these inflammatory markers follow suit, then measuring the levels of LANCL2 in pSNL and sham rats would help determine whether it has a pro- or anti-

inflammatory effect. The technique utilized for measuring protein levels was the western blot.

At 10 days post-surgery, rats were placed under continuous isoflurane anesthesia and the lumbar enlargement of the spinal cord was removed. The left dorsal quarter was taken and either immediately homogenized or placed in -80°C and homogenized the next day. Protein content was determined, and volumes were adjusted to load 30 μg protein per well. Either a 10% or 13% SDS-PAGE gel was prepared depending upon the molecular weight of the target protein. Gels were run at a constant 0.01 amps until the loading buffer ran out of the gel. The gels were set to transfer to polyvinylidene membranes (Bio-Rad; Hercules, CA) overnight in a 4°C cold room. Membranes were cut to the molecular weight of target proteins and incubated for two hours with 5% non-fat milk in 1x phosphate buffered saline/tween (PBST). After washing three times, 10 minutes each with 1x PBST, membranes were incubated with primary antibodies as specified (Table 1) for two hours. The wash cycle was repeated and then incubated with secondary antibodies as specified in table 1 for one hour. A final wash cycle was performed, then membranes were developed using Pierce Enhance Chemiluminescence Western Blotting Substrate (Thermo Scientific; Rockford, IL), imaged using Bio-Rad ChemiDoc (Bio-Rad; Hercules, CA), and analyzed using Bio-Rad Image Lab software and ImageJ.

1° Antibody	1° Antibody Concentration	2° Antibody	2° Antibody Concentration
Mouse anti-GFAP (Cell Signaling Technology; Danvers, MA)	(1 : 3000)	Goat anti-Mouse-IgG, horseradish peroxidase conjugated (Thermo Fisher Scientific; Waltham, MA)	(1 : 3000)
Mouse anti-TNF-α (Proteintech; Rosemont, IL)	(1 : 500)		(1 : 250)
Mouse anti-GAPDH (Proteintech; Rosemont, IL)	(1 : 10000)		(1 : 5000)
Mouse anti-IL-1ß (Santa Cruz Biotechnology; Dallas, TX)	(1 : 100)		(1 : 200)
Mouse anti-ERK 1/2 (Santa Cruz Biotechnology; Dallas, TX)	(1 : 100)		(1 : 1000)
Rabbit anti-CD11b (Abcam; Cambridge, United Kingdom)	(1 : 250)	Goat anti-Rabbit-IgG, horseradish peroxidase conjugated (Thermo Fisher Scientific; Waltham, MA)	(1 : 1000)
Rabbit anti-p-p44/42 MAPK (Cell Signaling Technology; Danvers, MA)	(1 : 250)		(1 : 1000)
Rabbit anti-LANCL2 (MyBioSource; San Diego, CA)	(1 : 250)		(1 : 500)

Table 1. Antibodies used for western blot analysis. All antibodies were diluted to working concentration with 1x PBST.

Spinal Administration of Abscisic Acid

LANCL2 protein levels were expected to vary between the pSNL and sham models. The next goal was to determine whether application of ABA could rescue the neuropathic pain state and reverse the pain threshold of the pSNL rats.

To study this in real-time intrathecal catheters were placed in naïve rats and ABA was administered immediately before a behavior test. A 30 cm length of

sterilized PE-10 tubing was inserted into the intrathecal space from the intervertebral space between L5 and L6 vertebrae. Approximately 4 cm of tubing was inserted. The muscles were sutured together, as well as the tubing to provide some resistance from being pulled out. The other end of the tubing was tunneled under the skin by an 18-gauge needle, exited at the base of the neck, and secured to a skin staple at the same area. The tubing was flushed with 20 µL saline and closed with a soldering iron until the day of behavior testing. Rats were allowed five days to recover before randomly assigned either pSNL or sham surgeries. Ten days after pSNL/sham surgeries, each group was randomly separated into ABA + vehicle and vehicle only groups. The ABA + vehicle groups were administered 15 µg ABA in 10 µL vehicle (2% ethanol in saline) followed by a 20 µL saline flush; the concentration is equal to 5.67 millimolar ABA. The only other study to date in which ABA was injected into the central nervous system used a maximum dose of 10 µg in an intraventricular injection (Soti et al. 2019). It was thought to deliver a high enough dose to the L4 and L5 spinal sections, a larger amount of ABA should be injected to counteract the possibility of the dose dissipating along the length of the spinal cord, and so 15 µg was decided as the dosage in this experiment. The vehicle groups were given only 10 µL vehicle followed by a 20 µL saline flush. Behavior data was collected at the following time points: 15 minutes post injection (mpi), 30 mpi, 60 mpi, 90 mpi, 120 mpi, and 180 mpi.

Abscisic Acid Content

The role of LANCL2 in the regulation of neuropathic pain was the primary focus of this study. It has been shown in earlier studies that regulation of certain proteins, like glycogen synthase kinase beta and adenosine monophosphate-activated protein kinase, has a dramatic effect on the production or termination of neuropathic pain (Maixner et al. 2016; Weng, Gao, and Maixner 2014). The natural ligand of LANCL2, abscisic acid, is not a protein but is produced and released by mammalian cells. Regulation of ABA levels could have an effect on neuropathic pain, and as such the levels of ABA were quantified using an ABA ELISA kit (MyBioSource; MBS 2000214).

In short, 10-day post-surgery pSNL and sham rats were euthanized, and the left spinal dorsal horn of the lumbar enlargement was collected. All of the following steps were performed according to the manufacturer's protocol. Fresh tissue was homogenized with ice-cold 80% methyl alcohol and shaken on an orbital platform shaker for 24 hours in a 4°C cold room. The supernatant was collected, additional alcohol added to the pellet, and shaken for one hour in a 4°C cold room. The total supernatant was collected and evaporated on a rotary evaporator. Petroleum ether was added to the liquid and mixed. After the liquid became layered, the top layer of petroleum ether was removed by pipetting, then the bottom methyl alcohol layer was collected and used immediately. Positive controls of 100, 33.33, 11.11, 3.7, and 1.23 ng/mL ABA were used as well as a

negative control of the diluent used to create the dilutions of positive control. 50 µL of each control and samples were added to the wells of a 96 well plate. 50 µL Detection Reagent A was added, gently shaken by hand, covered with plate sealer, and set in an incubator for one hour at 37° C. The solution was aspirated and washed with 350 µL of 1x Wash Solution four times. 100 µL of Detection Reagent B was added to each well, covered, and incubated for 30 minutes at 37° C. Aspiration and wash process was repeated five times. 90 µL of Substrate Solution was added to each well, covered, and incubated for 15 minutes away from light. 50 µL of Stop Solution was added and immediately run on a microplate reader at 450nm.

Immunofluorescent Labeling of LANCL2

An immunofluorescent co-localization study was used to determine the cell type in which LANCL2 was present. Ten-day post-surgery sham rats were anesthetized with urethane (Fisher Scientific; Pittsburgh, PA) at 1.2 g/kg body weight and a transcardial perfusion was performed to fix the whole animal. First, blood was removed by flushing with 300 mL heparinized saline followed by perfusion with 300 mL of the fixative solution (0.1% picric acid and 4% paraformaldehyde in 0.1 M PBS). The spinal section was then removed and placed in the same fixative solution overnight in a 4° C cold room. The section was then transferred to a 30% sucrose solution in a 4° C cold room for three days. Sections were then embedded in OCT medium and cut on a freezing microtome to 30 µm

thickness. The sections were processed free floating. Sections were washed three times, 10 minutes each with 0.1 M PBS then blocked with 10% NGS and 0.3% Triton X-100 in 0.1 M PBS for two hours. Wash cycle was repeated, then sections were incubated with primary antibody Rabbit anti-LANCL2 (1:100 dilution (Bioss; Woburn, MA)) for two hours at room temperature, followed by overnight incubation at 4° C. Sections were washed and then incubated with the secondary antibody Texas Red anti-rabbit-IgG (1:500 dilution (Vector Laboratories; Burlingame, CA)) for two hours. Further processes were performed in low light conditions to minimize photobleaching of fluorescent dyes. The wash cycle was repeated, then sections were incubated with one of three primary antibodies: Mouse anti-GFAP (1:1000 dilution (Cell Signaling Technology; Danvers, MA)), Mouse anti-NeuN (1:200 dilution (Cell Signaling Technology; Danvers, MA)), or Mouse anti-Iba1 (1:25 dilution (Santa Cruz Biotechnology; Dallas, TX)), for two hours at room temperature followed by overnight incubation at 4° C. Sections were washed then incubated with the secondary antibody Fluorescein anti-Mouse-IgG (1:500 dilution (Vector Laboratories; Burlingame, CA)) for two hours. Sections were washed, placed on slides to dry, and mounted with Vectashield Antifade Mounting Medium (Vector Laboratories; Burlingame, CA). Images were taken on Zeiss Axio Imager Z1 Upright Trinocular Fluorescence Microscope (Zeiss; Oberkochen, Germany). Images were taken at 10x magnification with CY3/TRITC and CY5/FITC fluorescent cubes, and the program associated with the microscope automatically overlaid the images.

Data Analysis

All data are presented as the mean ± standard error of the mean. One-way and two-way analysis of variance (ANOVA) with repeated measures were used respectively to detect statistical significance in behavioral data over different time points in the same group and behavioral data between groups over different time points. Minimal statistical significance was determined by a p-value < 0.05. The Bonferroni post-hoc test was used to determine sources of differences. Paired t-tests were used to make comparisons between groups for western blot and ELISA data.

CHAPTER 3

RESULTS

Protein Content of LANCL2 and Inflammatory Molecular Markers

To test the hypothesis that LANCL2 expression mediates anti-inflammatory reactions in neuropathic pain, the protein content of LANCL2 was assessed. In addition, expression of markers of inflammation such as TNF-α and IL-1ß were also determined.

Western blot analysis indicates increased levels of all inflammatory markers in the pSNL model (Figure 2). Phosphorylated-ERK was compared to total-ERK, while all other proteins were normalized to GAPDH. All inflammatory markers showed a statistical increase in pSNL compared to sham while LANCL2 showed a statistical decrease in pSNL compared to sham. The results were presented as the mean of four rats per group and the error bars indicate the standard error of the mean.

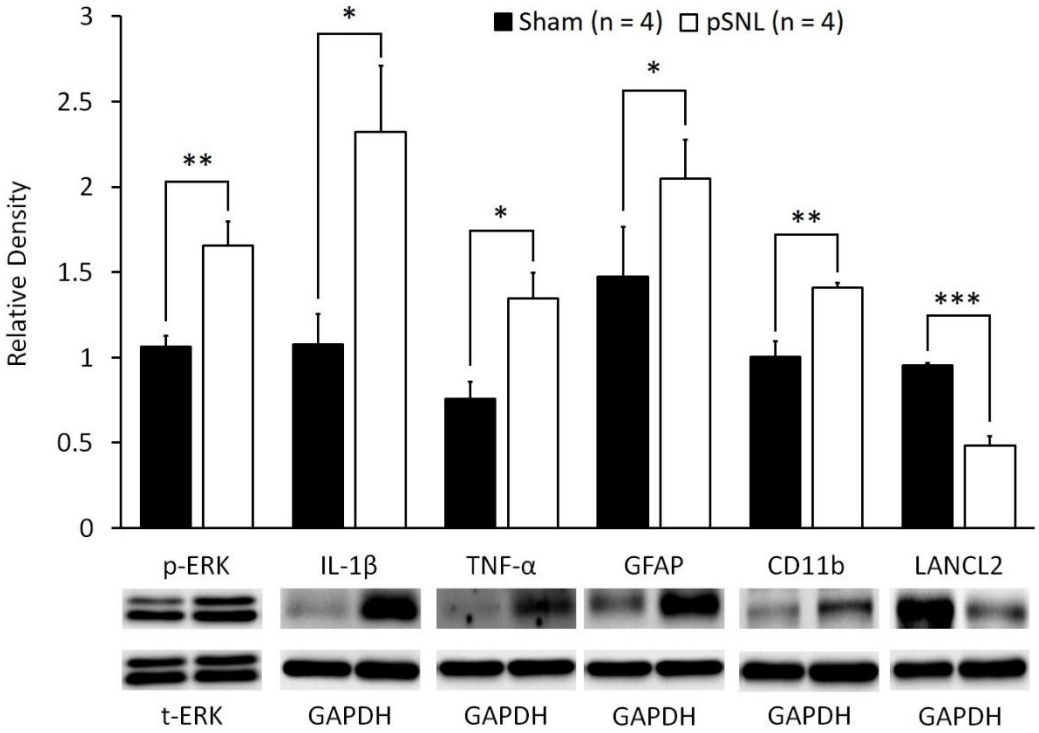

Figure 2. Protein levels of inflammatory markers and LANCL2 at 10-days post-surgery. Rats in the pSNL group displayed increased levels of pro-inflammatory markers and decreased levels of LANCL2. Statistical significance between pSNL and sham for each protein is labeled with *. One symbol: $p < 0.05$; two symbols: $p < 0.005$; three symbols: $p < 0.001$.

Effects of Spinal Administration of Abscisic Acid on Pain Behavior

Abscisic acid's role in the activation of LANCL2 in the nervous system is not documented. The activating pathway can be either pro- or anti-inflammatory in other cell types of the body. Accordingly, administration of ABA into pSNL and sham rats would indicate what role ABA and LANCL2 play in the spinal dorsal horn.

Behavior data indicates that ABA administration has an acute inhibitory effect on both mechanical allodynia (Figure 3) and thermal hyperalgesia (Figure 4)

in pSNL rats. The maximum effective timeframe occurred from 30 minutes post injection to 90 minutes post injection, with some residual effect occurring up to 180 minutes post injection. During the 30 to 90-minute timeframe in both mechanical and thermal tests, the pSNL + 15 µg ABA groups showed a statistically significant difference compared to the baseline values (marked with *) and a statistically significant difference compared to the same timepoint as the pSNL + vehicle group (marked with +). The results presented represent the mean of each group and the error bars indicate the standard error of the mean.

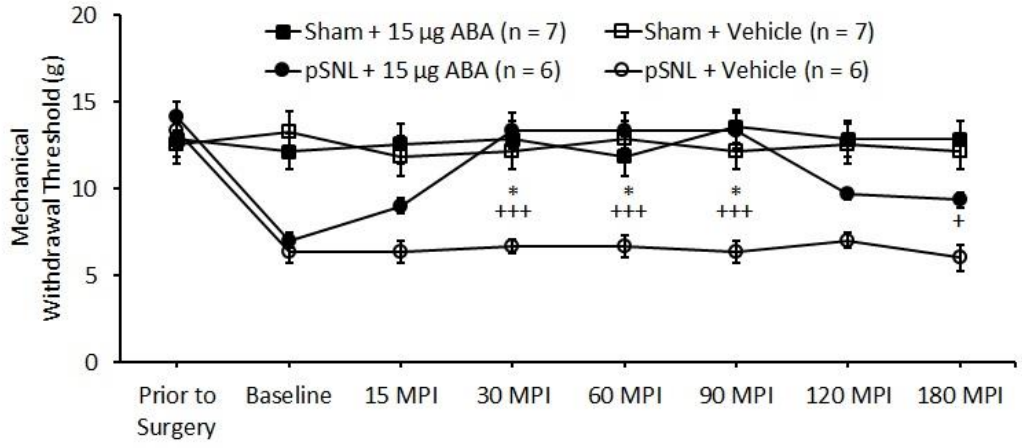

Figure 3. Mechanical behavior tests following ABA administration. Mechanical behavior tests on 10-day post-surgery rats indicates ABA administration provided an acute antinociceptive effect on pSNL rats, with maximum effect occurring from 30 minutes post injection to 90 minutes post injection. Some residual effect continued to occur up to 180 minutes post injection. Statistical significance between different time points in the pSNL + 15 µg ABA group compared to baseline is labeled with *. Statistical significance between each pSNL + 15 µg ABA and pSNL + vehicle time point is labeled with +. One symbol: $p < 0.05$; two symbols: $p < 0.005$; three symbols: $p < 0.001$.

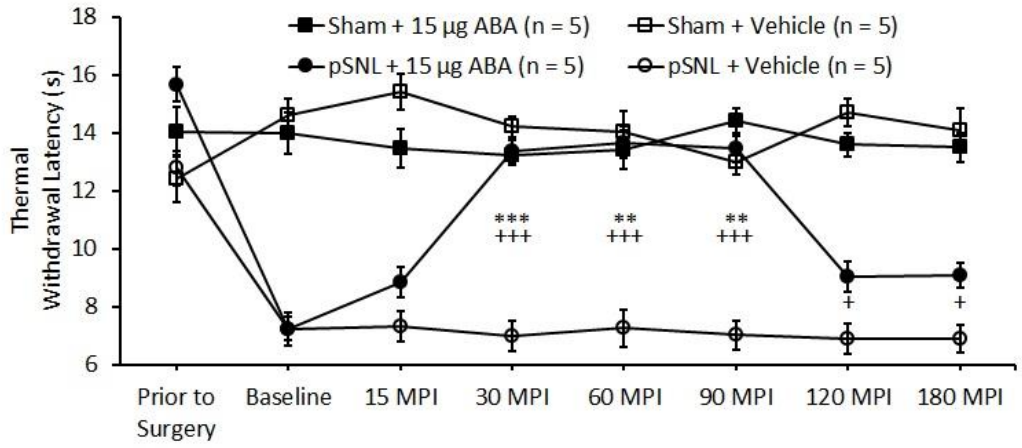

Figure 4. Thermal behavior tests following ABA administration. Thermal behavior tests on 10-day post-surgery rats indicates ABA administration provided an acute antinociceptive effect on pSNL rats, with maximum effect occurring from 30 minutes post injection to 90 minutes post injection. Some residual effect continued to occur up to 180 minutes post injection. Statistical significance between different time points in the pSNL + 15 µg ABA group compared to baseline is labeled with *. Statistical significance between each pSNL + 15 µg ABA and pSNL + vehicle time point is labeled with +. One symbol: $p < 0.05$; two symbols: $p < 0.005$; three symbols: $p < 0.001$.

Evaluation of Abscisic Acid Content in the Spinal Cord

Abscisic acid is an endogenous chemical produced by animals, though levels in the nervous system between pSNL and sham rats have not been elucidated. ABA is not a protein, but an ELISA kit has been produced with antibodies targeted at ABA. By means of this ELISA kit, levels of ABA in the spinal dorsal horn were collected (Figure 5). A standard curve was constructed according to the manufacturer's specifications, and the optical density measurements of the samples were plotted along the curve to determine the concentration of ABA. This data was then used to determine the total amount of ABA in each sample based on the concentration, volume, and weight of each sample. Abscisic acid showed a

statistically significant decrease in pSNL compared to sham rats. The results presented represent the mean of five rats per group and include the standard error of the mean.

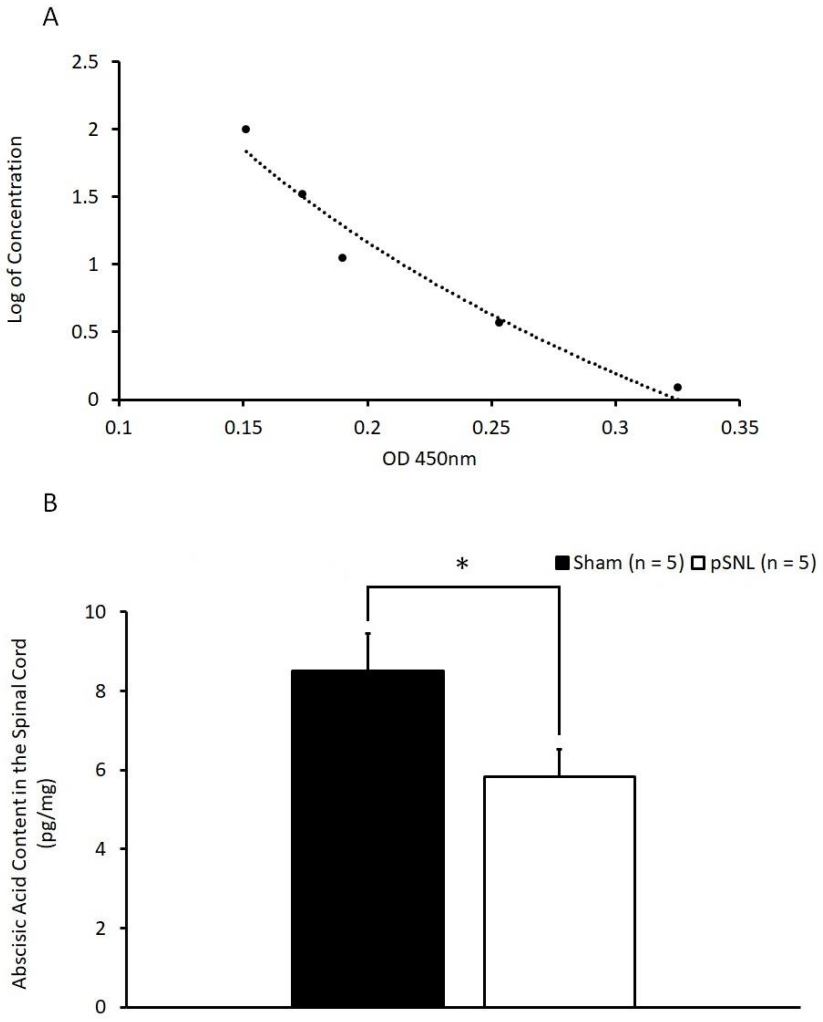

Figure 5. Abscisic acid content in the spinal dorsal horn. A standard curve was first constructed using the known quantities of the positive controls (A). This curve was then used to determine the ABA concentration of each sample using their optical density measurements. The total amount of ABA (B) in each sample was determined based on the concentration, volume of lysate, and the weight of spinal cord. ABA displays a statistically significant decrease in pSNL rats. Statistical significance between pSNL and sham is labeled with *. One symbol: $p < 0.05$.

Cellular Localization of LANCL2

Little is known of LANCL2 expression in the nervous system. It is for this reason that determining cellular expression through co-localization is important in understanding the protein content displayed by the western blot data. Sequential antibody staining of spinal cord slices was demonstrated using fluorescent microscopy (Figure 6). These data reveal that LANCL2 seems to be solely expressed by neurons in the spinal cord.

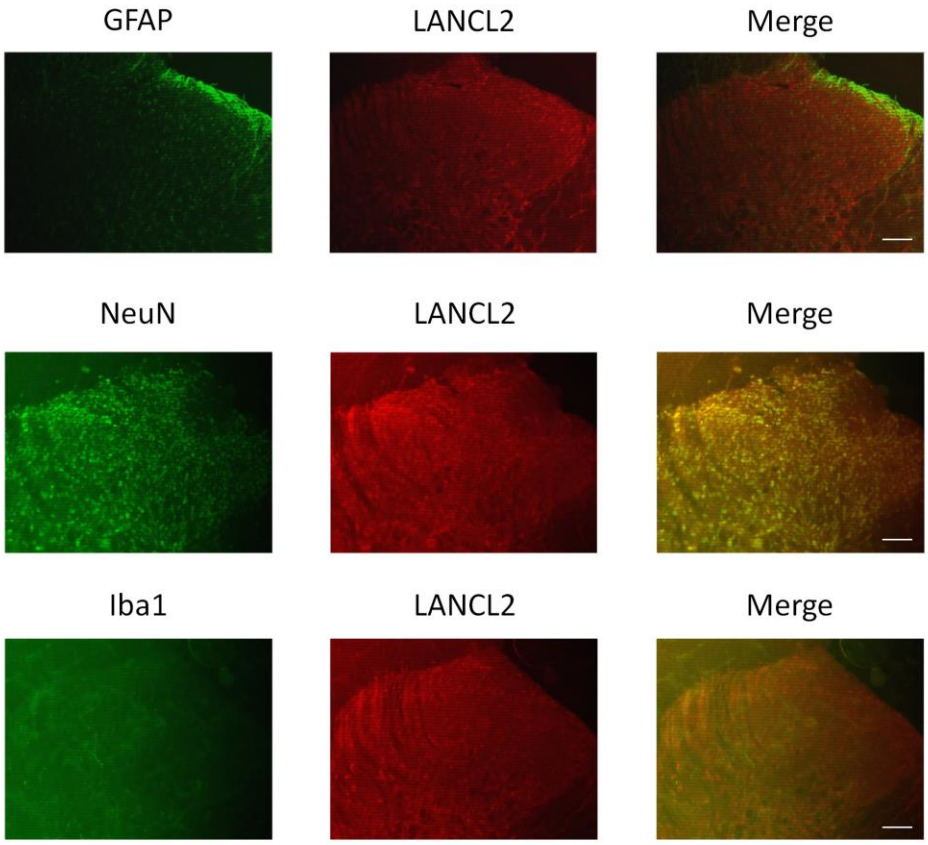

Figure 6. Co-staining LANCL2 with GFAP, NeuN, and Iba1. The only visible co-staining from these 10-day post-surgery spinal dorsal horn slices from sham rats is with NeuN, indicating LANCL2 is expressed in neurons in the spinal dorsal horn. Scale bar = 30 µm. *Images in this document are presented in color.*

CHAPTER 4

DISCUSSION

The purpose of the present study was to identify whether LANCL2 plays a role in either reduction or escalation of neuropathic pain. The basis of this work lies in the pSNL animal model and our ability to measure behavior, measure protein levels through western blot analysis, deliver treatment to the correct spinal cord section, and visualize the protein of interest.

The preliminary data obtained by Dylan Maixner indicates the LANCL2 siRNA produces a neuropathic like state, significantly decreasing the withdrawal threshold of both mechanical and thermal behavior tests compared to the scrambled control siRNA. Western blot analysis of LANCL2 confirmed the efficacy of the LANCL2 siRNA. Western blot of p-ERK, TNF-α, GFAP, and Iba1 further exemplified the LANCL2 siRNA data to be comparable to previous pSNL western blot data. Though this knockdown experiment may typify neuropathic behavior similar to pSNL/sham experiments, ultimately it does not exactly equate. The first course of action was to evaluate LANCL2 levels in our rat models. Western blot analysis of 10-day post-surgery pSNL and sham rats indicated that LANCL2 levels were decreased in the neuropathic rats; this data lines up with the preliminary

data provided by Dylan. This data definitively displays that LANCL2 is downregulated in our pain model.

The western blot data is only significant if it can be shown that LANCL2 plays a role in regulating neuropathic pain in the spinal dorsal horn. To assess the impact of LANCL2 activation, intrathecal administration of ABA was performed on 10-day post-surgery rats. In both mechanical and thermal behavior tests, the pSNL + ABA groups were comparable to our sham groups during the 30 to 90-minute post injection timeframe. As ABA only binds to LANCL2, this data indicates that LANCL2 has a transitory anti-inflammatory effect in the spinal dorsal horn. Interestingly, the sham + ABA groups show no effect in terms of increasing their withdrawal threshold.

The ability of ABA to return withdrawal thresholds to control levels led us to question whether ABA is downregulated in our pain model as well? According to ELISA data obtained from five samples each of pSNL and sham, ABA shows statistically decreased levels in pSNL compared to sham rats. As ABA is not a protein and we absorb little of it through a normal diet, the enzymes responsible for production of it must be downregulated or inhibited in some manner in the pSNL model. Unfortunately, the biosynthetic pathway in animals is not well established.

Not all proteins are expressed in every cell type, and so determining which cell type in the spinal dorsal horn expresses LANCL2 is an important step in

understanding its role. Due to data from previous studies showing LANCL2 expression in cells of the immune system, it was thought that microglia would express LANCL2 in the nervous system. According to the data obtained, LANCL2 is definitively not expressed in astrocytes, but is expressed in neurons. Unfortunately, the antibodies for microglia did not stain properly and as such, the exact location of LANCL2 in the spinal dorsal horn is still in question. It is possible that the additional fixation time masked the epitopes for the antibody to bind.

CHAPTER 5

CONCLUSIONS

In summary, the present data displays that LANCL2 is decreased in the animal neuropathic model and suggests that LANCL2 activation within the spinal dorsal horn ameliorates neuropathic pain. The preliminary data provided by Dylan Maixner indicates that LANCL2 knockdown alone is enough to mimic neuropathic pain. It is also found that the administration of ABA increases the animals pain tolerance. To the author's knowledge, this study represents the first time ABA has been quantified in the spinal cord and shown that a quantifiable difference exists between our control and neuropathic pain models. Our findings suggest that activation of the LANCL2 signaling pathway in the spinal dorsal horn is an effective approach to ameliorate neuropathic pain.

CHAPTER 6

FUTURE DIRECTIONS

Although this study provides insight into the anti-inflammatory role of LANCL2 in the spinal dorsal horn, it is imperative that we examine pending questions and future directions. Additional projects are needed to establish a complete understanding of the LANCL2 signaling pathway in pain regulation.

The data from the immunohistochemical study is incomplete and answering this unknown would be the necessary first step in understanding cellular localization of LANCL2. It is clear from the staining that astrocytes do not express LANCL2 while neurons do, but whether microglia play a role is unclear. Obtaining a microglia specific antibody, whether Iba1 or CD11b, from another company would be advised, or performing an antigen retrieval on the tissue to unmask the antigen epitopes.

The behavior data from ABA administration revealed the acute timeframe within which the anti-inflammatory effect peaks. It would be interesting to perform a topical ABA administration. This would be done through direct application of ABA to exposed spinal cord of a pSNL rat for an hour and western blot of spinal dorsal horn tissue to analyze the previously used inflammatory markers and LANCL2.

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