Effects of Post Trauma Morphine On Dorsal Horn Neuron Excitability: Studies using cFOS and RNAscope

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Defense Date: Tuesday, 1 November 2022

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Abstract

Previous research has shown that a 5-day course of morphine enhances nociceptive sensitivity and allodynia when given 10 days after chronic constriction injury (CCI) as measured by the Von Frey test, a test where the hindpaw is poked with force (measured in grams) calibrated filament. This increased sensitivity to touch suggests that post trauma morphine makes second order sensory dorsal horn neurons more excitable. Therefore, it is hypothesized that during morphine enhanced allodynia, dorsal horn neurons will be more excitable to nociceptive stimulus. It was found that morphine enhanced allodynia causes more excitable neurons across a larger spatial range of the spinal cord, both rostral-caudal and dorsal-ventral along the dorsal horn. Mechanisms for the increased excitability of the dorsal horn are proposed and explored. These findings add to a robust literature which has detailed the paradoxical pain amplifying effects of morphine. Further, this study predicts that hyperexcitability of pain and touch pathways may occur as a clinically unintended side effect of morphine when administered to treat ongoing neuropathic pain.

1. Introduction

1.1: Introduction to morphine

Morphine has been one of the most commonly used drugs for its agonistic action on the endogenous opioid system, causing significant pain relief. Unfortunately, morphine has potentially dangerous side effects, including drug tolerance and addiction. The dangers of its use have led to an opioid crisis and demand for safer alternatives for pain management (Coussens et al., 2019; Chisholm-Burns et al., 2019; Volkow et al., 2021). One side effect of morphine is to paradoxically increase pain, as in morphine induced hyperalgesia and morphine-enhanced allodynia.

1.2: The Two-Hit hypothesis

Hyperalgesia is defined as increased pain sensitivity to a stimulus that would normally cause mild pain. Allodynia is defined as pain in the presence of normally non-painful stimuli. Morphine enhanced allodynia is counterproductive to the analgesic effects of morphine and lingers for weeks or months after the drug has worn off. Additionally, glial cells play an active role in neuroinflammatory processes. The Two-Hit hypothesis proposed in Grace 2016 proposes the role of glial cells to play an active role in morphine-induced neuropathic pain. After initial activation of glial cells by a glial activating event such as trauma, age stress, etc., the cells become primed to the next activating event, and the response to this secondary event is stronger and prolonged. It is predicted that opiates act as this secondary glial activating event. These phenomena have been demonstrated in many models across species (Ellis et al., 2016, Grace et al., 2016). Increasing evidence suggests this enhanced nociceptive sensitivity depends

upon activation of innate immune signaling (Grace et al., 2016). However, changes to the excitability of dorsal horn neurons have not been quantified in tissue from animals.

1.3: Pain and nociception

Touch and nociceptive (pain) sensory pathways transmit sensory signals from the periphery to the dorsal horn of the spinal cord, where it is then transmitted to the brain. Previous research shows that morphine enhanced allodynia is caused by neuroinflammation in the spinal cord, but changes to excitability of the relevant second order spinal cord sensory neurons have not been studied. Our prior studies examining the paradoxical effects of morphine-induced allodynia have led us to further examine the effects of morphine on wide dynamic range neurons, which receive both nociceptive and mechanical inputs. Nociceptive inputs derived from the periphery have cell bodies (somas) located in the dorsal root ganglion (DRG), where the axon then continues into laminae I/II to synapse on the secondary pain neuron. Laminae I/II contain nociceptive neurons, as well as laminae IV/V, leading us to study the dorsal-ventral spread of these markers in the dorsal horn.

1.4: Neuronal hyperexcitability

To measure changes in neuronal excitability in the lumbar dorsal horn after post-trauma morphine, we quantified cFOS+ cells in lamina I/II as well as lamina IV/V after thermal challenge in rats with morphine-induced hyperalgesia. This thermal challenge requires a hot water dunk of the neuropathic paw, inducing production of immediate-early genes (namely, cFOS). cFOS is the protein end product of immediate-early gene

expression. An increase in cFOS expression is a marker of excitability and is indicative of active neurons. An increase in total dorsal horn cFOS may suggest that the spread of sensitized neurons extends farther rostral in morphine-treated animals.

1.5: Involvement of NMDA system

To assess possible mechanisms of sensitization we have begun to work out technical steps for assaying calcium permeable excitatory neuronal channel pNR1, specifically in dorsal horn lamina I/II, the layer of the spinal cord sensory pathway that specializes in pain transmission, and lamina IV/V, which respond to both touch and pain. Phosphorylated NMDA Receptor Subunit 1 (pNR1) is of interest because recent literature has observed that treatment with NMDA receptor antagonist MK-801 reverses thermal hyperalgesia and mechanical allodynia in mice with opioid induced hyperalgesia, suggesting that the NMDA system may be responsible for hyperexcitable neurons (Gao et al., 2016). An increased expression of pNR1 on the membrane of neuronal cells in laminae I/II and IV/V of the dorsal horn is predicted to be due to the hyperexcitability that occurs through the NMDA system.

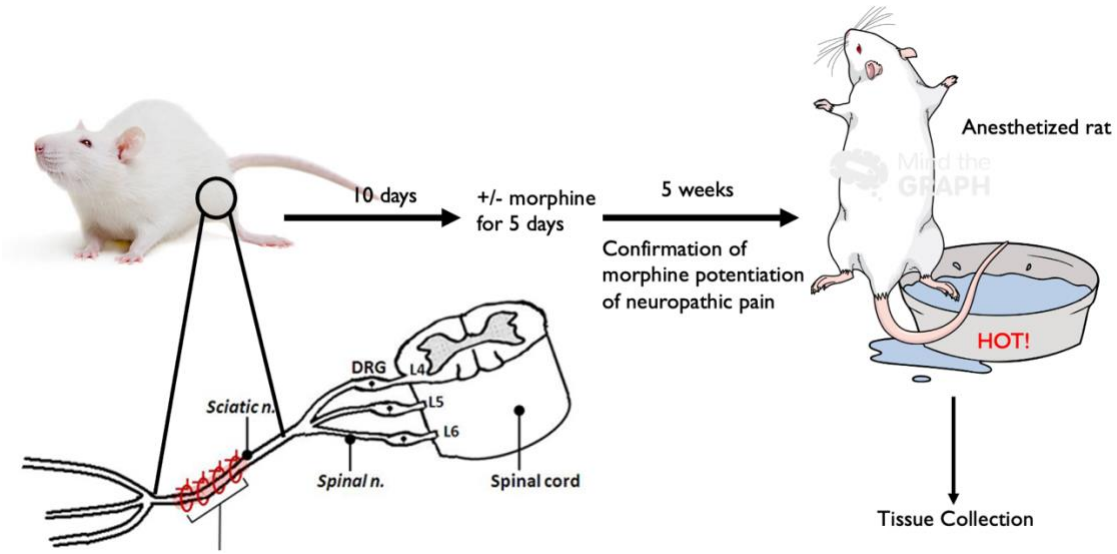
1.6: Role of glial TLR4

Finally, prior literature regarding Toll-like receptor 4 (TLR4) in CCI models has shown that increasing TLR4 levels is necessary for morphine enhanced allodynia (Grace et al, 2016). TLR4 is located in the membranes of both neurons and glial cells, and contains domains which bind both opiates and endogenous inflammatory molecules and this is predicted to potentiate neuropathic pain following morphine cessation. In

total, these findings suggest that the neuroinflammation-dependent enhanced allodynia observed in prior studies leads to more excitable nociceptive neurons of the dorsal horn. Prior research regarding glial TLR4 suggests that activation of these receptors and release of interleukins, ROS, etc. in response to both nerve injury AND opiate binding- results in pain amplification.

2. Methods

Timeline: Rats received a unilateral CCI followed by a 5-day course of morphine or saline beginning on day 10 post CCI. After the morphine cohort showed significantly enhanced allodynia as measured by von Frey test, rats were subjected to a thermal challenge via immersion in the paw in hot water, and tissue was collected 2h later for IHC analysis.



2.1 Subjects: Male Sprague-Dawley rats (290-310 grams) were housed in pairs on a 12-h light/dark cycle. Rats were allowed to acclimate to colony conditions for 7–10 days prior to surgery.

2.2 Chronic Constriction Injury (CCI) Surgery: Neuropathic pain was induced using a light version of the sciatic nerve CCI model. Briefly, rats were put under isoflurane anesthesia, the mid-thigh level of the left hind leg was shaved and the skin treated with Nolvasan. The sciatic nerve was gently isolated, and one chromic gut suture was loosely tied around the nerve. Animals were monitored postoperatively until fully ambulatory before returning them to their home cage. Surgeries were performed aseptically.

2.3 Morphine Administration: Morphine sulphate was made into saline solution at a concentration of 5mg/ml. Twice daily for 5 days morphine (5mg/kg) or vehicle control was administered via subcutaneous injections.

2.4 Thermal Challenge: On day 36 post morphine administration, rats were subjected to a nociceptive thermal challenge based on Tolle et al., 1994, via repeated immersion of the hind paw in a hot water bath while under light anesthesia. First, rats were brought under 2% isoflurane for approximately 5 minutes. This level of anesthesia was chosen to keep the animal just before the surgical plane to minimize the number of sensory neurons shutting down. After the rat was anesthetized, the left leg was then shaved up to the knee, a cast was created with electrical tape tied across the knee joint- preventing the knee and hip from achieving full reflexive flexion while still allowing blood flow. A noxious thermal stimulus was applied by immersing the foot and lower half of the shank of the surgical leg in 52^{o}C water for 10 rounds of 20 seconds with 90 seconds

between rounds. After termination of the last round of stimulation, rats were moved to lighter anesthesia (1% iso) for 2 hours before cardiac perfusion. 1% isoflurane was judged to be the lightest anesthesia stage which prevented the rats from becoming ambulatory.

2.5 Tissue Collection: On day 36 post morphine administration rats were overdosed with sodium pentobarbital, and then transcardially perfused with ice-cold saline. The saline perfusion was followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Lumbar spinal cords were isolated.

2.6 Tissue Processing: Tissues were postfixed by immersion in 4% paraformaldehyde overnight at 4°C, and cryoprotected sequentially in 15%, 20%, and 30% sucrose with 0.1% azide at 4°C. Lumbar sections were then freeze-mounted in OCT and cut at 20µm and mounted onto slides.

2.7 Immunohistochemistry: For fluorescent immunohistochemical analysis, tissue sections were washed in 0.01M phosphate buffered saline (PBS) and blocked for 1 hour with 10% NGS, 0.3% Triton-X in PBS. To label cFos, slides were then incubated for 72 hr at 4°C in 10% normal goat serum (NGS) together with 1:100 Rabbit x cFos (Synaptic Systems, Göttingen, Germany). To label pNR1, a separate slide set was incubated for 24 hr at 4°C in 10% NGS together with 1:500 Mouse x NeuN (Product #MAB377) + 1:500 Rabbit x pNR1 (EMD Milipore, Massachusetts, USA, product #ABN99). Slides were then washed and incubated in Alexafluor488 Goat x Rabbit (GFP, 1:500) (Thermo Fisher, Waltham, MA USA) for cFos or Alexafluor647 Goat x Rabbit (Cy5, 1:500) for two hours at room temperature. To label Iba1, another slide set was incubated for 24 hr at 4°C in 10% NGS together with 1:500 Guinea pig x IBA1 (Synaptic Systems, Göttingen,

Germany, product #234004). Slides were then washed in PBS and coverslipped with ProLong diamond mountant (Thermo Fisher).

2.8 RNAscope + Immunohistochemistry: For fluorescent *in-situ* detection of TLR4 RNA, RNAscope multiplex kit from ACD bio was used. Instructions were followed as per the manufacturer's recommendation but with several changes. Namely, a weaker protease was used to process the tissue so that antigens remained for immunohistochemistry, and rats were perfused with paraformaldehyde rather than used fresh frozen.

2.9 Microscopy: cFOS images were acquired using an Olympus BX61 widefield microscope (Olympus, Center Valley, PA) and Hamamatsu Flash 4.0 camera (Hamamatsu, Hamamatsu, Japan) with cellSens Dimension software (Olympus). All tissue samples were acquired with the same acquisition settings (illumination, exposure time, etc.). For ipsilateral lumbar dorsal horn, images from four to six sections per animal were acquired in mosaic format with a mechanical stage at 20x magnification.

2.10 Image Analysis: Imaris modeling software has been proven to be effective in the analysis of various cellular structures, with its ability to process images based on modeling protocols we have developed. Common features we used in Imaris included image processing, cropping of images, and detection of surfaces and spots within the image. We used thresholds we have discovered to be most effective in studying these specific molecular markers. Imaris allows for cellular structures to be masked and modeled into an image that is capable of anatomical analysis, either manually or automatically. Imaris' manual surfaces setting requires surfaces to be created by hand on each image individually, whereas automatic surfaces utilizes specifically designed

thresholds and filters on all images to control for variation in modeling. The methods described below include analysis using these protocols, as well as results that corroborate with prior research and our current hypothesis that wide dynamic range neurons in the dorsal horn are found to be more active following morphine treatment. Further description of each analysis is described below.

2.11: cFOS

Spinal cord tissue was first processed for IHC of the immediate early gene cFOS. Morphine treatment after CCI makes the nociceptive neurons more excitable to a subsequent nociceptive stimulus so we predict this will increase cFOS in laminae I/II and IV/V of the morphine cohort. To quantify the rostral-caudal spread of neuronal sensitization, tissue sections were collected from throughout the rostral-caudal length of the lumbar enlargement and labeled for cFOS with fluorescently labeled via rabbit x cFOS antibody cFOS+. Cells of each lamina of the dorsal horn were then counted blind.

2.12: pNR1

Spinal cord sections of CCI morphine vs saline rats for pNR1 and NeuN and quantified the expression of pNR1 in laminae I/II neurons. This protocol was similar to the protocol followed for cFOS, but the tissue set analyzed for pNR1 was not given a thermal challenge. Spinal cord tissue was then processed for IHC of pNR1 using Cy5. In order to assay the expression, as well as rostral/caudal and dorsal/ventral spread of pNR1, spinal cord sections were collected from throughout the whole lumbar enlargement, and fluorescently labeled with antibodies as per standard IHC methods.

Sections were imaged on a widefield microscope. To quantify dorsal/ventral spread of neuronal excitability, the ratio of neuronal pNR1 expression in laminae I/II was compared between groups

The original widefield image with TRITC to detect NeuN antigen to mark neuronal cell bodies, and Cy5 was used to model pNR1 expression through fluorescent activity. Laminae I/II were created as a masked surface on the original widefield picture **(Fig. 1A).** The images underwent image processing, which included adjusting the TRITC channel's gamma correction and background subtraction. The Cy5 channel also underwent gamma correction and background subtraction. Then, neuronal cell bodies were automatically modeled using a NeuN surface model to capture neurons within the desired threshold **(Fig. 1B).** pNR1 was modeled using automatic spots on Imaris, including object to object statistics to filter pNR1 within NeuN only **(Fig. 2C).** This process was repeated on 4 rats, with an average of 7 sections each, totaling to 29 images analyzed in this tissue set for both laminae I/II and IV/V.

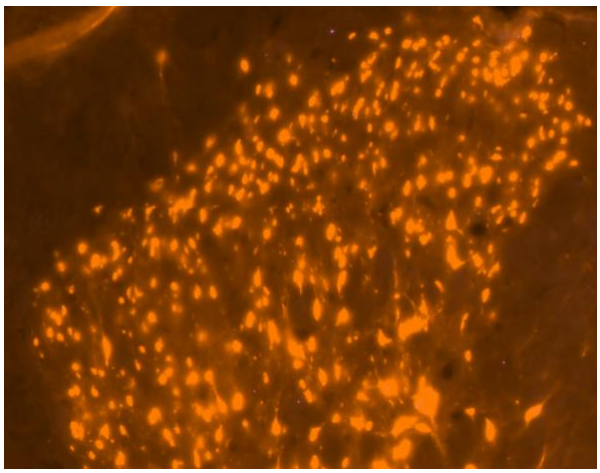


Figure 1A

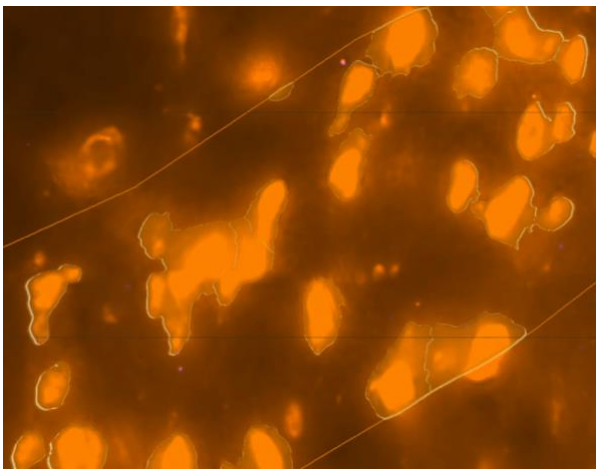


Figure 1B

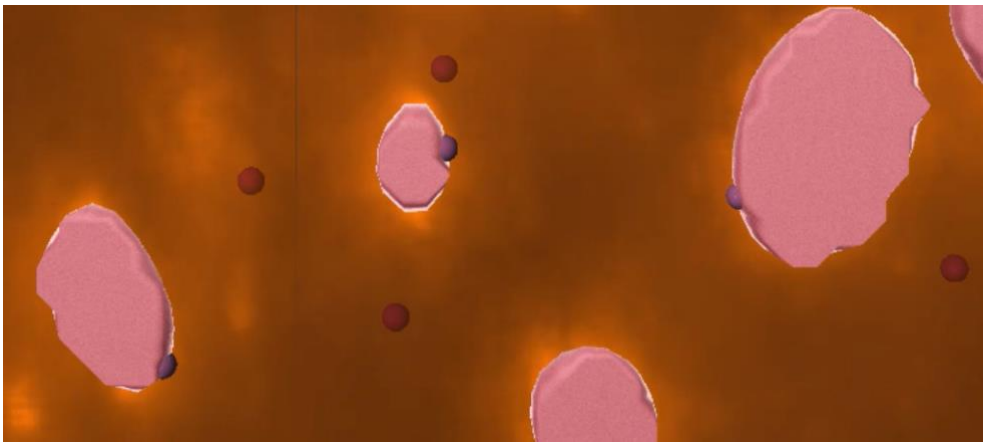


Figure 1C

Figure 1A. Widefield image showing TRITC and Cy5 channels. **Figure 1B.** Image after image processing, cropped for laminae I/II with neuronal cell bodies modeled. **Figure 1C.** Masked image showing neurons (shown in pink), pNR1 located in neurons (purple spots), as well as pNR1 located outside of neurons (red spots).

2.13: TLR4

We predict that locating TLR4 RNA transcripts using RNAscope, as well as IHC to locate microglia and neurons will allow for better visualization and analysis of the location and what kind of cells make TLR4. If results indicate that TLR4+ RNA is increased in CCI, we will then examine the quantification of TLR4 in rats who receive CCI and sham, as well as morphine/saline administration. To measure neuronal TLR4 RNA expression, tissue sections from the middle of the lumbar enlargement were labeled for TLR4, as well as neuronal NeuN and the microglial marker Iba1. Spinal cord tissue was analyzed via RNAscope to examine the distribution and location of TLR4 following CCI and treatment with morphine. NeuN was used to spatially define the neuronal soma within laminae I/II, inside of which the average TLR4 expression within laminae I/II NeuN+ regions was quantified as average TLR4 fluorescence intensity. Following this, we used NeuN antigen to spatially define the neuronal soma within the whole left dorsal horn, inside of which the average TLR4 expression within all NeuN+ regions was quantified as average TLR4 fluorescence intensity. The original RNAscope image is shown below with channels for TRITC, Cy5, and CFP labelled NeuN+, Iba1+ and TLR4, respectively **(see Fig. 2A)**. Images were then processed, where baseline subtraction, background subtraction, and gamma correction were applied to all channels **(Fig. 2B)**. Laminae I/II were created by drawing a surface to capture this section of the dorsal horn and masking the channels within **(Fig. 2C).** By selecting to analyze laminae I/II, the processed TRITC channel was masked **(Fig. 2D).** NeuN was then modeled using automatic surfaces, then smoothing the channel and adding background subtraction in order to analyze groups of neurons that resulted in a higher intensity stain **(Fig. 2E).** The threshold level for NeuN was adjusted by eye. TLR4 modeling was

achieved through creation of automatic spots **(Fig. 2F).** Object to object statistics, as well as a quality filter to analyze only spots found in neurons was set **(Fig. 2H)**. This analysis was performed on 8 rats, with an average of 3 sections per rat, resulting in a total of 24 images analyzed.

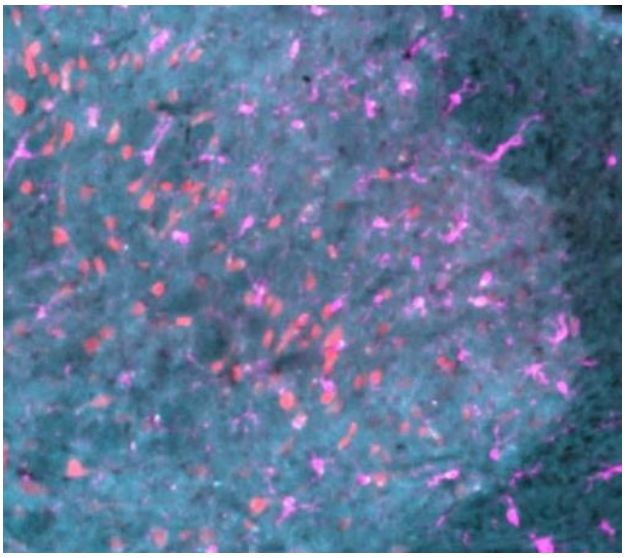


Figure 2A

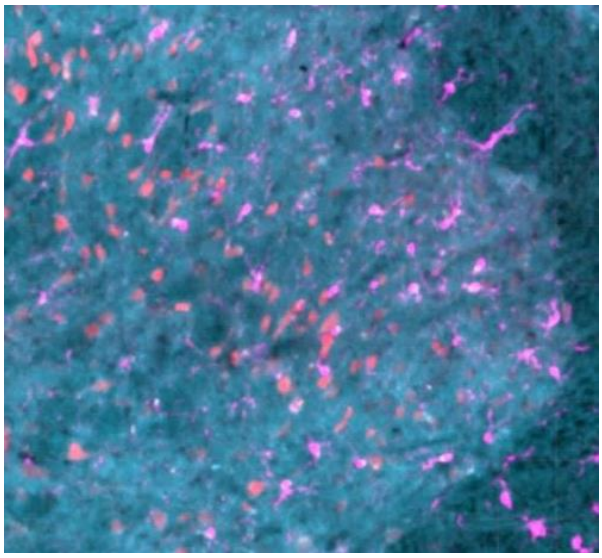


Figure 2B

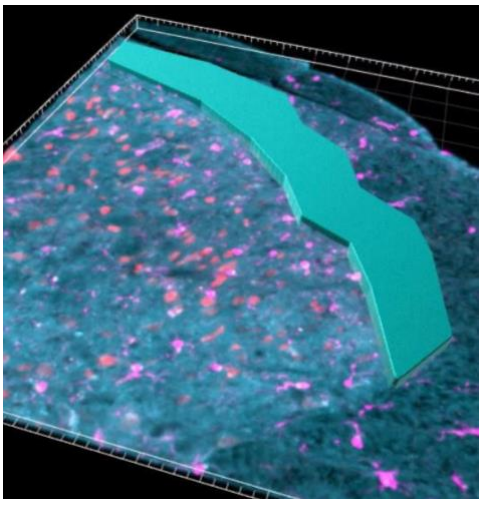


Figure 2C

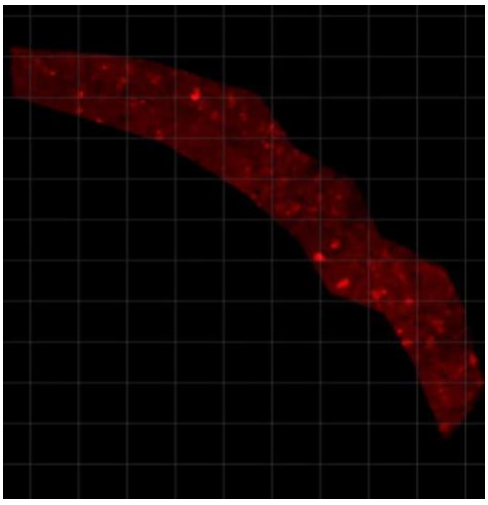
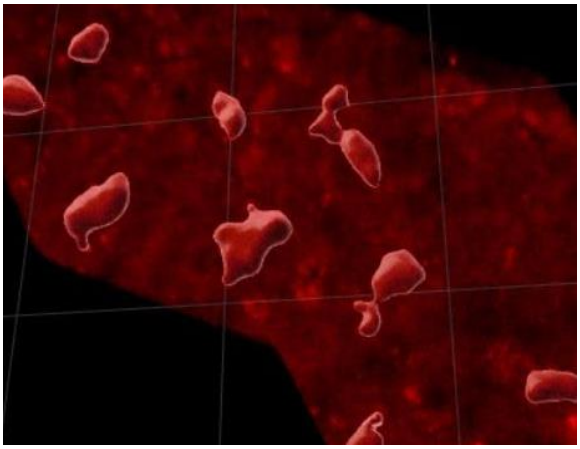


Figure 2D



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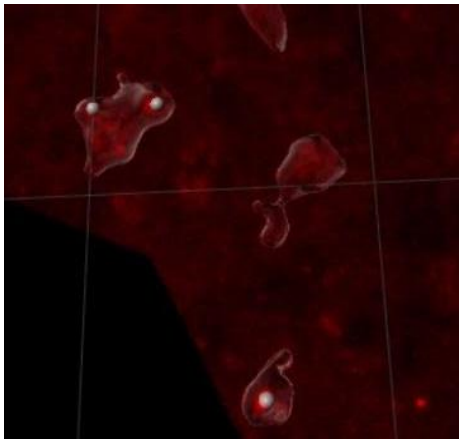

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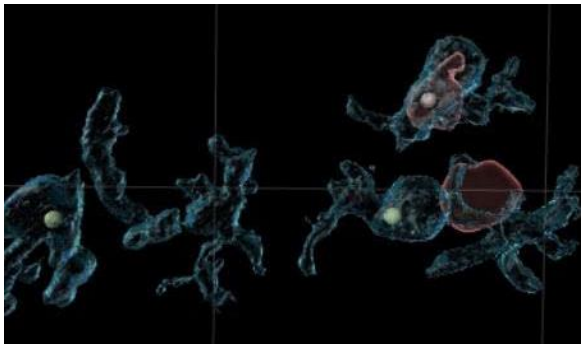

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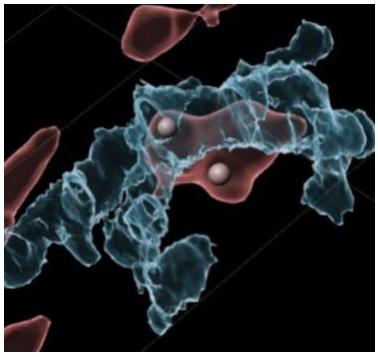

Figure 2H

Figure 2A. Original RNAscope image with TRITC, Cy5, and CFP channels displayed. **Figure 2B.** RNAscope after undergoing image processing. **Figure 2C.** The creation of a laminae I/II mask. **Figure 2D.** Laminae I/II with only the processed TRITC channel displayed. **Figure 2E.** Neurons modeled via automatic surfaces. **Figure 2F.** TLR4 RNA transcripts modeled using automatic spots. **Figure 2G.** Neurons (red) and microglia (blue) with TLR4 transcripts displayed (white spots indicate that it is contained within a neuronal surface). **Figure 2H.** The final model derived from the tissue set.

Results

A 5 day morphine regimen increased the magnitude of CCI-allodynia in the Lewis) rat strain. The increased magnitude of allodynia was replicated with a second cohort, which was used for tissue collection after thermal challenge as discussed in the following section **(Fig. 3)**. These behavior data add to the evidence for morphine-induced enhancement of chronic pain after nerve injury.

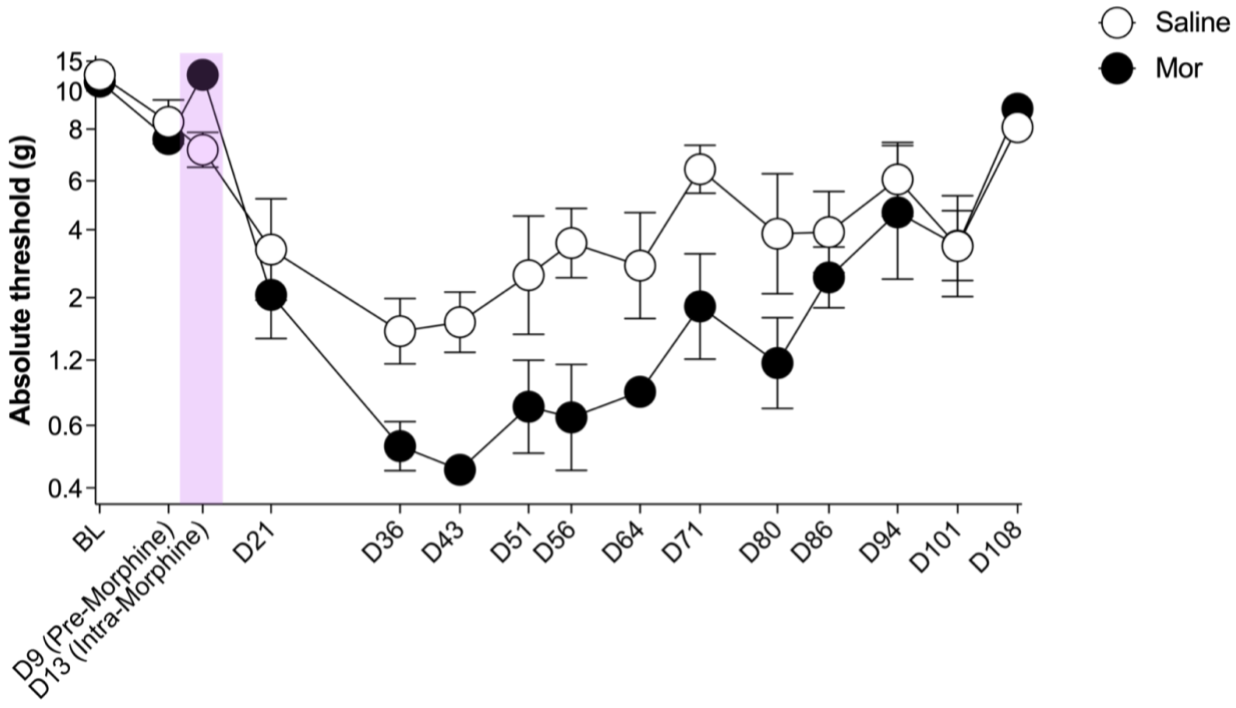

Figure 3. Von Frey time course of morphine induced hyperalgesia. CCI + morphine groups develop greater sensory allodynia than CCI + saline.

Following thermal challenge, cFOS expression was measured in laminae I/II and laminae IV/V in the dorsal horn. A comparison of the morphine and saline cohort showed that cFOS levels rose between just below 3 cFOS+ cells per cm to a maximum

of well over 8 cFOS+ cells per cm in laminae I/II in rats who received morphine, and a minimum of just over .05 cFOS+ cells per cm to a maximum of .15 in laminae IV/V . On the other hand, rats who received saline showed a range of 0.5-7 cFOS+ cells per cm in laminae I/II, and these rats also reached a maximum of 0.5 in laminae IV/V. A two-tail T-test comparing the morphine and saline cohort was repeated for both laminae I/II and laminae IV/V on 14 rats, with an average of 3 sections per rat, resulting in 39 sections of tissue analyzed for both laminae **(Fig. 4A&B, 5A&B)**.

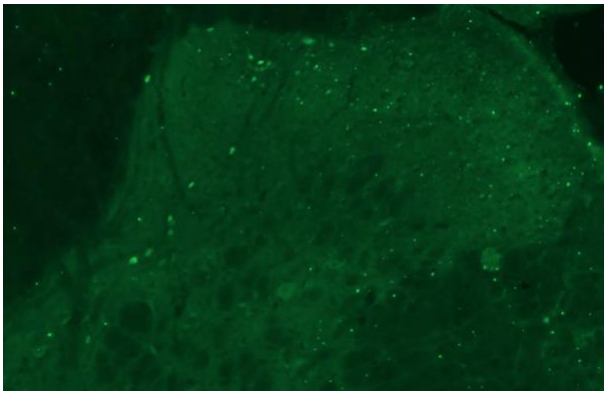

Figure 4A

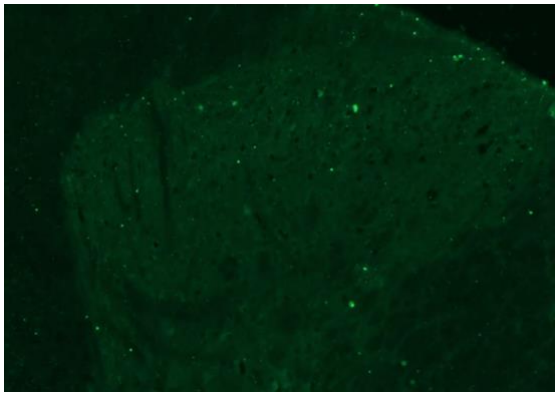

Figure 4B

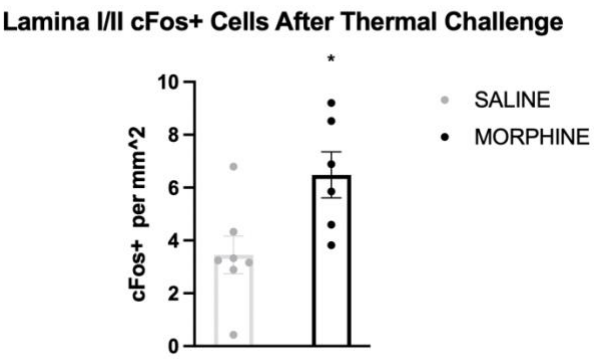

Figure 5A

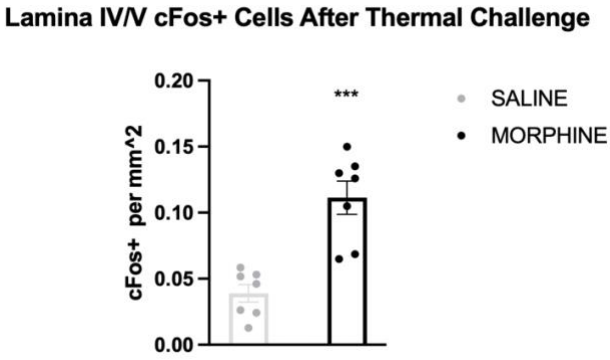

Figure 5B

Figure 4A. cFOS+ Neurons of the Dorsal Horn in a rat that received morphine. **Figure 4B.** cFOS+ Neurons of the Dorsal Horn in a rat that received saline. **Figure 5A.** Each rat was averaged for cFOS+ neurons in each section, and then normalized to the area of laminae I/II. A two-tail T-test between morphine and saline cohorts resulted in a significant, nearly two-fold, increase in the average cFOS+ between the rats who received saline to morphine, which had an average cFOS+ expression of 3 and 6, respectively ($p=.02$) in cFOS+ neurons in laminae I/II in rats who received morphine followed by a thermal challenge. **Figure 5B.** A similar increase in cFOS+ neurons in laminae IV/V ($p=.0003$) was shown in rats who received morphine followed by a thermal challenge

After the modeling protocol for pNR1, we then ran statistical analysis by normalizing neurons and pNR1 within lamina I/II voxels. Average pNR1 expression was taken per animal, and CCI + morphine and CCI + saline groups were compared. When examining pNR1 spots per cm in laminae I/II of rats who received saline, we noticed several rats displaying more pNR1 than those who received morphine. Contradicting our hypothesis, we also had several outliers that showed expression of pNR1 that was higher than the maximum expression in rats who received morphine (removed from this analysis), which confirmed that our protocol likely did not accurately label pNR1 expression.

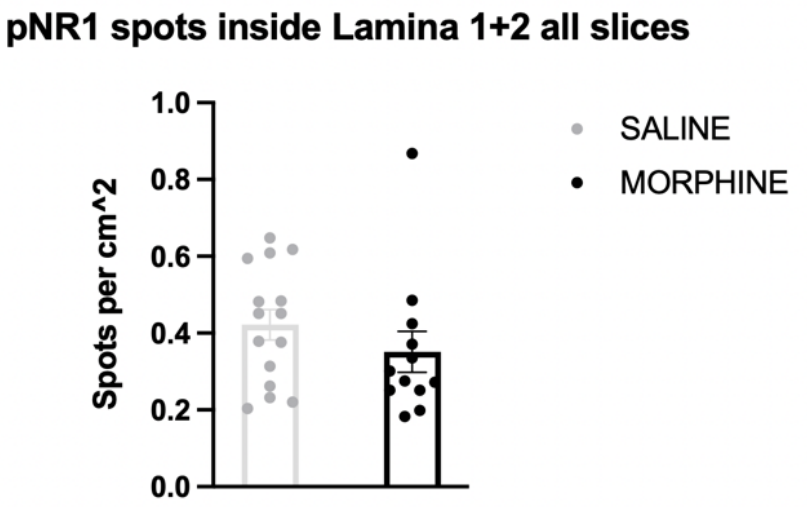

Figure 6

Figure 6. Each rat was averaged for pNR1 in each section, and then normalized to the area of laminae I/II. Results from a two-tail T-test show no significant increase in pNR1 in laminae I/II in rats who received morphine.

After finalizing the model, statistical analysis was run by dividing the models into CCI and naive groups, and normalizing microglia to the dorsal horn. This average was taken for each animal, as well as the average TLR4 RNA transcripts per microglia per rat. With results indicating a highly significant increase in total TLR4 puncta in microglia in the dorsal horn within rats who received CCI, we predict that these rats are recruiting more microglia to the dorsal horn, leading to the increased expression of TLR4. After rats received CCI or no surgery (naive), TLR4 expression was measured in the dorsal horn. A comparison of the CCI and naive cohort showed that TLR4 RNA expression increased to a minimum of approximately 400 and a maximum of approximately 800 TLR4 RNA transcripts in rats who underwent CCI. In contrast, the naïve showed a maximum TLR4 expression of nearly 350 RNA transcripts. The difference between

groups shown in this analysis leads us to believe this is caused by increased microglial expression as well as recruitment of more microglial cells.

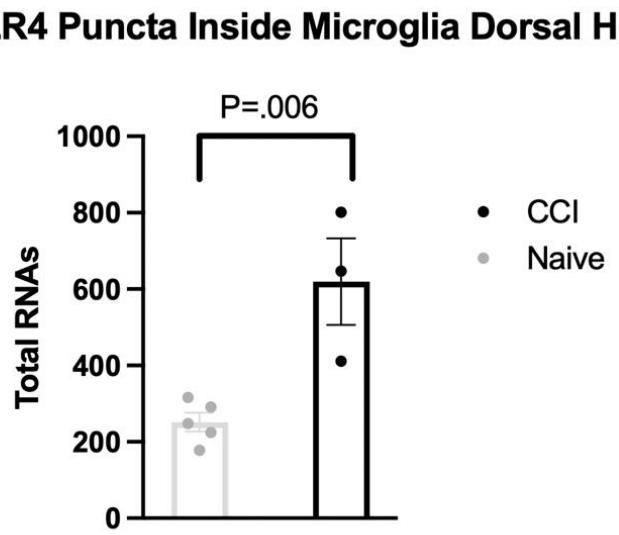

Figure 7

Figure 7. Two-tail T-test of TLR4 total puncta inside of microglia, displaying the average TLR4 puncta per microglia cell. These results show an increased average number (p=.006) of TLR4 transcripts per microglia in rats that received CCI.

Discussion

Our prior research has shown that a 5-day course of morphine beginning 10 days after chronic constriction injury (CCI) of the sciatic nerve causes a brief analgesic effect which is followed by enhanced neuropathic pain in several strains of rats and both sexes. These effects appear to be independent of opioid withdrawal mechanisms, as preventing dependent binding to TLR4, a receptor of the innate immune system, drives activation of NLRP3 inflammasome in the spinal cord, producing an increase in gliosis

and signaling molecules associated with pain (Grace et al., 2016). Administration of a variety of innate immune system antagonists, such as TLR4 and NLRP3 antagonists, prevent the allodynic effects of morphine. Previous research has shown the mechanistic importance of both neuroinflammation and NMDA receptor activation in morphine induced hyperalgesia but has not shown the effects of morphine on the neurons of the spinal cord of living animals. Our results showing increased expression of cFOS in laminae I/II and lamiae IV/V shows the location of neuronal hyperexcitability in the spinal cord. In total, these findings suggest that the neuroinflammation-dependent enhanced allodynia observed in prior studies leads to more excitable neurons of the dorsal horn.

One hypothesis about post-trauma morphine neuronal excitability is that CCI + morphine animals would show a larger rostral caudal and/or dorsal ventral spread of neuronal excitability. This is because of the nature of inflammation, which the morphine enhanced nociceptive response relies upon. Namely, because inflammation can be propagated by soluble factors that diffuse in tissue, we expected to find that CCI + morphine animals not only had an increased number of excitable neurons within a given section of the lumbar enlargement, but that the range of excitable neurons would spread throughout larger distances of the dorsal horn in all dimensions. Based on the data collected regarding immediate early gene cFOS expression, as well as phosphorylated NR1 (pNR1), and TLR4 RNA, our research corroborates and expands on recent literature regarding dorsal horn excitability following chronic neuropathic pain and/or morphine pretreatment. Current literature suggests that hyperalgesia occurs via

inflammatory agitation caused by morphine binding, with the inflammatory mechanism most pronounced during a preexisting inflammatory state, such as an ongoing chronic nerve injury, Fitting the two-hit hypothesis of Grace et al. 2016, and suggesting that when CNS tissue is primed toward a pro-inflammatory state of the tissue (with chronic/ongoing nerve damage etc.), “second hit” of morphine on TLR4 will drive enough immune activation to produce hyperalgesia. One way to test this hypothesis is to administer morphine to chronically inflamed animals as in an aging model and look for subsequent hyperalgesia.

Future research could fully sample the range of excitable neurons in order to definitively say whether this range of neurons is increased with post-trauma morphine. Also, although the tissue collection extended caudally enough past the lumbar enlargement to show a “baseline” level of neuronal excitability that lies beyond the excitable range of neurons, we did not collect rostral enough samples to sample the whole range of neuronal excitability across cohorts. One limitation of this analysis is that not all the animals had the same total length of tissue rostrally and caudally in the frozen tissue blocks, causing the statistical power of the rostral/caudal ends of the tissue to be lower than the center of the enlargement since fewer animals are represented in those groups. Future experiments should collect more rostral regions in order to fully sample the range of excitable neurons and definitively say whether the excitable range of neurons is increased with post-trauma morphine. Another limitation was found in our TLR4 analysis, where TLR4 was compared in rats who has received CCI or naïve rats. Our future studies plan to examine this effect in CCI vs sham rats.

Furthermore, these results indicate the difficulty of detecting pNR1 antibodies in tissue slices, so neuronal excitability changes will be assayed using dendritic spine counts. The increased expression of TLR4 transcripts in the dorsal horn of chronically injured neuropathic rats is in line with data presented in Grace et al., 2016, and further studies using a similar protocol may be used to analyze the location of other markers of inflammation, including NLRP3. We are piloting the methods for NLRP3 RNAscope in CCI vs sham tissue. Currently, this tissue set is being stained and imaged**.** Once the protocol for RNAscope is successful, we will analyze a CCI + morphine vs saline tissue set to assay differences with morphine administration. Finally, to examine the spread of cFOS+ neurons through the dorsal horn, a coordinate system marking the 4 corners of the dorsal horn (dorsolateral, dorsomedial, ventrolateral, ventromedial) will be created to normalize the location of all cFOS+ cells in each image. To quantify the spread of neuronal excitability across the dorsal-ventral axis of the dorsal horn, images of sections from the middle of the lumbar enlargement of each rat will then be divided into laminae I/II, lamina III, and laminae IV/V. The interest in examining the dorsal-ventral spread of cFOS is to analyze the hypothesis of wide-dynamic range pain neurons, located caudally in the dorsal horn, should also express as hyperexcitable in rats who received morphine.

With the data we collected regarding neuronal hyperexcitability, location of inflammatory transcripts, and barriers to overcome when imaging spinal cord tissue, a methods paper will be sent to be reviewed for publication- as the visualization of these markers in animal spinal tissue has been limited in terms of effective protocols prior.

Acknowledgements

Thank you to Dr Linda Watkins and Jayson Ball, as well as other team members, for the support and knowledge they provided during the 2 years I worked in Dr Watkins laboratory. Further gratitude is extended towards Joe Dragavon and the Biofrontiers Microscopy Center for their support and guidance with our imaging protocols, as well as the funding this project received from the NIH RO1 Grant, the Biological Sciences Initiative, and the Undergraduate Research Opportunities Program. Finally, my last appreciation is for my two biggest cheerleaders- Mike and Carin Jean Woodall (my mom and dad).

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