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Morphological Changes in Dorsal Root Ganglia Macrophages Associated with Neuropathic Pain Mechanisms Suggest a Novel Target for Chronic Pain Therapy

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Claremont Mckenna College

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Author Note:

This research was conducted under doctoral candidate Matthew Hunt and the Yaksh laboratory at the University of California, San Diego.

For further information, contact: ekussick21@cmc.edu.

Table of Contents

Glossary

DRG: Dorsal Root Ganglia
SN: Sciatic Nerve
SC: Spinal Cord
BBB: Blood Brain Barrier
CNS: Central Nervous System
PNS: Peripheral Nervous System
IT: Intrathecal
TLR4: Toll-Like Receptor 4
TLR4 activation leads to an intracellular signaling pathway and the release of inflammatory cytokines that are together responsible for activating the immune system
LPS: Lipopolysaccharide
Part of bacterial cell wall that is an inflammatory TLR4 agonist.
WT: Wild Type
Cc2-/-: C-C Chemokine Receptor Type 2
Transgenic mice that lack this gene do not express the CCR2 receptor that is responsible for recruiting circulating macrophages and monocytes to the DRG during an immune response
Iba1: Allograft Inflammatory Factor 1
Microglia antibody that is also present on macrophages
MCH II: Major Histocompatibility Complex, class II
Antibody present on endothelial cells
CX3CR1: Chemokine (CX3C) Receptor, class I
Gene that encodes for the receptor of chemokines (CX3XCR1) and fraktaline ligands that are present on macrophages
NMDA: N-Methyl-D-Aspartate Receptor
GFAP: Glial Fibrillary Acidic Protein
Protein expressed by astrocytes (CNS) and glial cells (PNS)
ATP: Adenosine Triphosphate
An important extracellular signaling molecule that acts as a neurotransmitter in the PNS and the CNS. ATP is released from synaptic terminals and causes post-synaptic currents
NeuN: Neuronal Nuclear Protein
Target for fluorescent staining of neuronal nuclei.
DAPI: 4′,6-Diamidino-2-Phenylindole
Fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA and allows visualization of cell nuclei.
t**-SNE plots:** T-distributed Stochastic Neighbor Embedding
A machine learning algorithm for visualization of high-dimensional data in 2- or 3-dimensional space
3D: Three Dimensional

Abstract

The present study examined morphological changes in the dorsal root ganglia (DRG) following an innate immune stimulus. The importance of the DRG has increasingly become recognized in pain processing as more than just the home of primary afferent cell bodies. All sensory information passes through the DRG via the primary afferents, and on to the spinal cord. The primary afferents synapse with second-order neurons in the spinal cord that ascend towards the brain, where they transmit the pain signal to the limbic forebrain and/or the somatosensory cortex for processing. The DRG is an interesting niche to study at as it lies outside the blood-brain-barrier (BBB) but projects to the CNS. Therefore, neurons in the DRG are exposed to large circulating molecules (such as LPS) that normally would not be able to act on second-order neurons in the spinal cord, due to the BBB, and thus can indirectly act on second-order neurons by sensitizing their primary afferent inputs, which increases primary afferent output into the spinal cord. Of particular interest, the DRG constitutively possesses a significant population of macrophages. Mice injected with lipopolysaccharide (LPS) will lead to an enhanced pain response that has been speculated to result from the activation of innate immune receptors (TLR4 expressed on the macrophage which leads to the release of active factors that sensitize the sensory neuron). To enable study of these DRG macrophages, we employed a novel 3D imaging technique to visualize morphological changes in whole cells, as opposed to previous imaging methods that only captured slices of cells, which greatly improved our ability to depict the dense and complex cellular environment of the DRG. A machine learning network was employed to train 3D stacks of DRG tissue images at different depths and intensities. After running thousands of replicates (epochs), through curated images, the machine learning program became very accurate and enabled us to characterize, in 3D reconstructions, the DRG macrophage population.

This approach enabled several observations. 1) Two distinct sub-populations of macrophages in the DRG were found: non-vascular and peri-vascular macrophages. 2) Spinal delivery of LPS which activated TLR4 receptors was found to increase the size of the individual macrophages, but in contrast to previous studies no the number. 3) This change in macrophage size vs. number was confirmed by the lack of change in the macrophage response in CXC3R (-) mutants which do not have circulating macrophages. The macrophage production of many spiny, amorphic processes were likely "double counted" in previous studies done on tissue slices (as opposed to the 3D, whole-cell modeling employed in these studies). By visualizing whole 3-D reconstructed cells, we were able to differentiate between increases in macrophage number versus increased macrophage volume that manifested. These techniques were validated by comparing our data analysis pipeline with commercially-available software, which yielded similar results. Further application, of our techniques and pipeline will enable the 3D imaging of different cell types and their interactions within the DRG.

Keywords: DRG, chronic pain, peripheral sensitization, central sensitization, macrophages, satellite glial cells, cytokines, chemokines, LPS, 3D image pipeline

Background: Pain as a problem

Chronic pain (>3 months) arises from a variety of disease states such as arthritis, cancer, osteoporosis, back pain, and nerve injury, cumulatively effecting over 126 million people globally. Not only does chronic pain diminish quality of life, it contributes to a loss of substantial economic productivity (Gaskin & Richard, 2012). Chronic pain has also been associated with psychological conditions like depression and anxiety, impacting even more people around the world. Pain therapy, and its efficacy, varies greatly, in part, due to the distinct and disparate mechanisms that underlie different pain states. While many analgesic drugs (e.g. opioids) provide effective short-term pain relief, they are less effective and have undesirable side effects when employed chronically, which is necessary in the treatment of persistent pain (Rosenblum et al., 2008). This decreased efficacy is due to a multitude of reasons including tolerance, dependence and addiction. Understanding pain perception in humans is crucial for the development of analgesics and therapeutic strategies for pain reduction. In this thesis, I will explain the larger pain circuit in general, and then introduce one component of the pain pathway that has been identified as a potentially effective target area for novel chronic pain therapies – the dorsal root ganglion.

Nociception and pain processing

A high intensity stimulus that induces the transmission of a pain signal represents the activation of a series of neuronal linkages. First, primary sensory neurons that respond selectively to high intensity thermal and mechanical stimuli (e.g A∂ nociceptive specific afferents and small unmyelinated C polymodal nociceptors) project to the spinal cord and synapse with second order neurons in the superficial dorsal horn. In these nociceptors, the frequency of discharge covaries directly with the stimulus intensity. The second order neurons,

activated by primary afferents (again with their frequency of discharge covarying directly with stimulus intensity), send their axons contralaterally to ascend in the anterolateral pathway to several regions of the thalamus. Here, third order neurons in lateral thalamic nuclei project to the somatosensory cortex, while third order neurons in the more medial thalamic nuclei project to cortical regions such as the anterior cingulate and inferior insula. Importantly, the somatosensory regions are thought to mediate perception of the location and intensity of the stimulus (so called sensory discriminative components), while the other regions (part of the "old limbic forebrain") are thought to underlie emotion and affective aspects that correspond to sensory perception of the stimulated area.

The shape of the primary afferent

Sensory primary neurons are pseudo unipolar, meaning that two axonal branches project from the neuron's cell body: one longer axon innervating peripheral tissue and the other projecting centrally to synapse in the dorsal horn. During embryogenesis, the cell body buds off from the sensory axon, retaining connection with the axon by a glomerulus that links the cell body to the axon. This glomerulus serves as the route by which the products (e.g. protein, metabolic products, enzymes, etc.) synthesized in the cell body are transported to the axon. Further, the glomerulus can transmit action potentials from the cell body through the proximal branch in passage to the spinal cord, and conversely can receive conducted potentials originating from the proximal or distal terminals (Nascimento et al., 2018). All sensory information detected in the periphery is sent by the innervating afferents, passing though their respective DRGs, to synapse in the dorsal horn. (Lu & Richardson, 1993; Nascimento et al., 2018)

Sensitization and chronic pain

It has been observed that in the setting of tissue injury and inflammation, the relationship between stimulus intensity and pain behavior is altered such that a given stimulus yields a stronger pain response. This enhanced response, referred to as hyperalgesia or allodynia (hyperalgesia: excessive sensitivity to mildly noxious input, and allodynia: low-intensity mechanical stimuli yields a pain response), reflect two mechanisms: peripheral and spinal sensitization.

Peripheral sensitization follows tissue injury and inflammation, when afferent activation and discharge is altered such that the otherwise silent nociceptor begins to display spontaneous activity and an augmented frequency–stimulus intensity response relationship (e.g a left shift in the stimulus-intensity response curve) (Xie et al., 2007). Consequentially, a modest, non-aversive, low-intensity, stimulus may generate an augmented afferent discharge that leads a stimulus to be perceived as painful when it normally would not. This enhanced afferent response is caused by a sensitization of the primary afferent terminal generated by the local release of active factors such as hydrogen ions, kinins, chemokines and cytokines arising from tissue injury, capillary leakage, and the in-migration of inflammatory cells (Grace et al., 2011; Kiguchi et al., 2012; Milligan et al., 2001).

Spinal sensitization occurs when the intense, high-frequency afferent barrage generated by injury and inflammation leads to a sensitization of the dorsal horn second order projection neurons. This sensitization of the second order neurons reflects a number of underlying pain mechanisms. As an example, high frequency afferent input leads to depolarization of the second order neuron that lay deep in the dorsal horn and which are referred to as wide dynamic range neurons. These cells are activated by glutamate released from small afferents displaying high frequency output and this leads to massive depolarization though the activation of the NMDA

receptor. This phenomenon is known as wind up. Further, the increase of NMDA activation leads to increases in intracellular calcium that serve to activate kinases that phosphorylate channels and receptors increasing their activation. Activation of enzymes that release lipids like prostaglandin sensitize local neuronal membranes and then initiate further transcription leading to the upregulation of a variety of pro-excitatory channels and receptors (Ji et al., 2012; Peters et al., 2006; Milligan et al., 2001). In addition, this massive excitation serves to activate non-neuronal cells in the dorsal horn such as astrocytes and microglia which themselves release pro-inflammatory cytokines. The chemical cascades initiated by the injury-induced high frequency input results in increases in the transmittal system (Simeoli et al., 2017; Kiguchi et al., 2012; Ji et al., 2012). Here the enhanced response to an otherwise innocuous stimulus (allodynia or hyperalgesia) is fundamentally driven by central and peripheral sensitization. A third element in this cascade is the role played by the afferent cell bodies in the DRG.

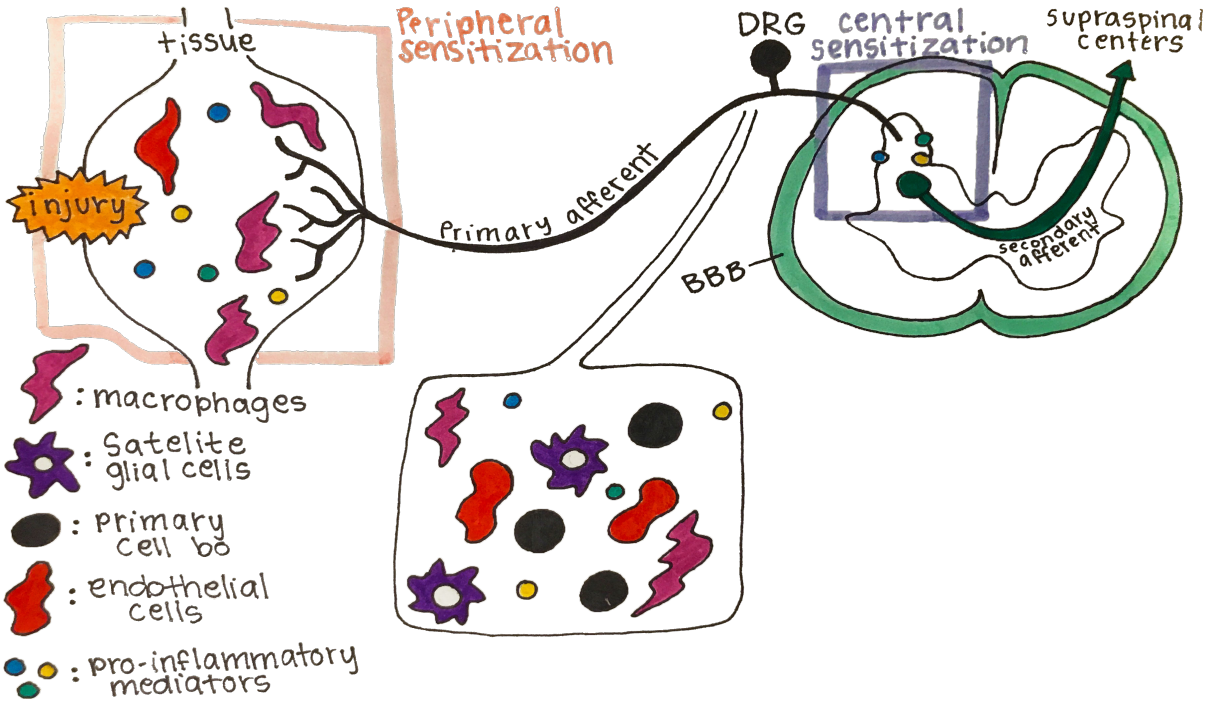

Figure 1: Visual representation of pain processing following tissue injury. As part of the immune response, pro-inflammatory mediators are released by capillaries, endothelial cells, macrophages, ETC. which act on the nociceptive afferent and effectively sensitize the neuron such that any sensory input triggers augmented afferent discharge into the spinal cord. Augmented discharge into the spinal cord depolarizes the secondary afferent and causes the recruitment of immune cells, like astrocytes and microglia, that further sensitize secondary afferents projecting to the brain. All sensory input detected on the periphery is sent through the DRG before reaching the spinal cord. The DRG is home to a complex environment of neuronal somas, SGCs, macrophages, and endothelial cells. The DRG lies outside of BBB but sends projections into the CNS, enabling peripheral input to induce changes in the protected CNS environment.

The Dorsal root ganglion (DRG)

The DRGs are composed of the afferent cell bodies (somas) that provide sensory innervation for each of the spinal segments. These DRGs are located in the foramen formed at the junctures of each pair of vertebral bodies. The DRG is unique in that it lies outside the blood brain barrier (BBB) of the central nervous system (CNS), making it a novel and distinctive neuraxial system to study pain transmission. Of particular interest has been the appreciation that the DRG neuron, when activated by mechanical stimuli (as with a disc avulsion) or the presence of inflammatory products, is able to generate afferent activity that leads to an output to the dorsal horn (Xie et al., 2007; Peters et al., 2006). Thus, consider that one characteristic feature of the primary afferent nociceptors is their uniform silence in the absence of adequate stimulation. However, after tissue and nerve injury, many studies have demonstrated abnormal spontaneous activity of primary sensory neurons originating at the site of injury (Xie et al., 2007; Lu & Richardson, 1993; Milligan et al., 2001). It is appreciated that this spontaneous, or ectopic activity, can originate from the peripheral terminals (as at the site of local nerve injury) and that prolonged activity may also arise from the neuronal cell body in the DRG of the injured axon (Kiguchi et al., 2012; Xie et al., 2007; Peters et al., 2006).

The origin of this ectopic afferent activity in the DRG appears to reflect the roles played by complex elements found within the DRG. The DRG thus provides an interesting collection of effects for pain transmission as it lies outside of the BBB

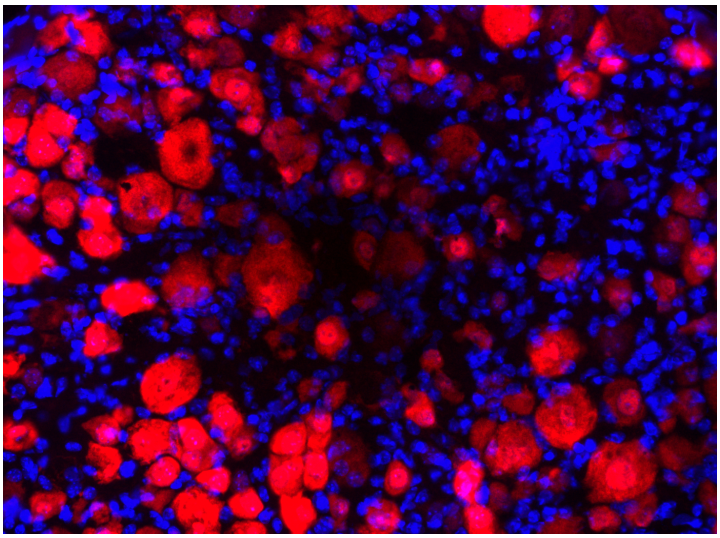

Figure 2: Visualization of the complex environment of the DRG. Neuronal cell bodies are stained for the antigen NeuN (red) that is present in neuronal nuclei. The nuclei of other cells, including glia, vascular cells, and inflammatory cells, are stained with the DNA-binding dye - DAPI (blue).

(in the PNS), such that its vasculature, unlike that in the CNS, does not have tight junctions, exposing the DRG to peripherally circulating substances that could not originally enter the spinal parenchyma and cause central sensitization (Jimenez-Andrade et al., 2008). These DRG components will be reviewed below.

DRG Satellite cells (GFAP +)

It is well known that neural activity enhances the release of trophic factors by regulatory cells surrounding neurons – a process that has implicated the complex DRG environment in the sensitization of primary afferent cell bodies (Kiguchi et al., 2012; Jimenez-Andrade et al., 2008). The DRG is classically thought of as the home of neuronal cell bodies but anatomically they are also comprised of large populations of satellite glial cells (SGCs) and macrophages that also exist in the DRG (Lu & Richardson, 1993, Ji et al., 2012). These SGCs lay in very close proximity to the DRG afferent cell body and are referred to as the "resident astrocytes" of the PNS as they form astrocyte-like gap junctions with somas in the DRG and exhibit astrocyte-like behaviors such as clearing debris, releasing trophic factors, recycling neurotransmitters, and modulating synaptic strength but uniquely, they reside outside of the BBB (Ji et al., 2012). Satellite glial cells in the DRG form neuron-glia units with primary afferent cell bodies via gap junctions such that the distance between the SGC and the neuronal soma is only 20 nm (Pannese, 2013; Ji et al., 2012). Communication through these gap junctions is enhanced in persistent pain conditions (Hu & McLachlan, 2002, Ji et al., 2012) and results in the up-regulation of channels and receptors (e.g. NMDA, ATP, Iba1, TLRs and chemokine receptors) on SGCs that can be activated by substances secreted by DRG somas (Ji et al., 2012; Kiguchi et al., 2012; Pannese, 2013). Many studies have demonstrated bidirectional communication between primary afferent cell bodies in the DRG and glial cells, and this communication has been correlated with changes

in primary afferent spontaneous activity (Nascimento et al., 2018; Hu & McLachlan, 2002; Kiguchi et al., 2012). Data suggests that SGCs respond to spontaneous activity with an elevation of internal calcium concentration that leads to the release of neurotransmitters, causing feedback regulation of neuronal activity. Additionally, SGCs have been observed to activate primary afferents through the release of ATP, which plays an important role in peripheral sensitization (Ji et al., 2012). Trophic factors such as cytokines, chemokines, and growth factors released in the DRG after injury alter the morphology and sensitization of glia-neuron units in the DRG such that SGCs may play a key role in persistent augmented afferent output into the dorsal root of the spinal cord causing central sensitization (Hu & McLachlan, 2002; Pannese, 2013).

DRG Macrophages (Iba1 +)

Macrophages are considered the "resident microglia" cells of the PNS as their phenotype mimics that of microglia in the CNS but they reside outside of the BBB (Ji et al., 2012). Microglia in the CNS have been extensively studied and are known to activate following injury, causing them to up-regulate Iba 1 and MHC-II receptors, change their morphology, and proliferate (Grace et al., 2011; Kiguchi et al., 2012; 68). Microglia activate following both direct damage to the CNS and damage to the peripheral nerve, suggesting that peripheral injury can induce morphological changes in and communication to the spinal cord (Peters et al., 2006). Injury-induced microglia changes are associated with neuropathic pain such that blocking microglia following peripheral nerve injury can prevent central sensitization (Grace et al., 2011; Kiguchi et al., 2012; Ji et al., 2012; Pannese, 2013).

Distinct populations of macrophages exist all throughout the body, cleaning synapses through phagocytosis but behaving slightly differently based on the demands of their environment (Grace et al., 2011; Kiguchi et al., 2012). The supportive role of macrophages in

general is through the consumption and subsequent removal of harmful debris surrounding neurons, preventing Wallerian degeneration (Kiguchi et al., 2012; Lu & Richardson, 1993).

Macrophages present a third cell population present in the DRG that could act on afferent somas and contribute to increased afferent output into the spinal cord such as SGCs and somas themselves. Macrophages makeup 5% of the volume of the DRG such that their potential role in regulating sensory afferent activation through interactions with DRG somas is underappreciated with respect to their number and microglia-like functioning (Lu & Richardson, 1993). Activated macrophages can release several families of active molecules that can act upon primary afferent somas to enhance their excitability. Previous research has noted that DRG cell bodies release vesicles containing microRNA that are thought to recruit macrophages to the DRG following peripheral nerve injury. Altered macrophage-neuron interactions as such has correlated with the extent of hypersensitivity (Simeoli et al., 2017; Kiguchi et al., 2012).

Peripheral nerve damage activates two distinct populations of macrophages: one at the site of injury and one around the afferent cell bodies in the ipsilateral DRG (Yu et al., 2020; Lu & Richardson, 1993; Simeoli et al., 2017). The contribution of altered macrophage functionality in response to nerve injury is not fully understood but research suggests that changes in DRG macrophages that initiate and maintain mechanical hyper sensitivity around afferent cell bodies persist longer than macrophage activation at the site of nerve injury (Yu et al., 2020; Lu & Richardson, 1993; Hu & McLachlan, 2002). Macrophages release pro-inflammatory mediators such as cytokines and chemokines that activate the vascular endothelium and alter nociceptive transduction such that sensory afferents become active (Simeoli et al., 2017; Grace et al., 2011; Hu & McLachlan, 2002).

The actions and particular role of macrophages in neural pain responses has been disputed in existing research but one common finding is the proliferation of macrophages in the DRG following nerve injury (Lu & Richardson, 1993; Simeoli et al., 2017; Hu & McLachlan, 2002; Peters et al., 2006; Pannese, 2013). Like microglia, macrophage proliferation and expansion after nerve damage is hypothesized to enhance their effect on pain transduction. Studies have proposed that an increase in macrophages around the peripheral terminal can be beneficial for axon regeneration (Lu & Richardson, 1993) while an increase in macrophages in the DRG has correlated with afferent sensitization and persistent pain (Lu & Richardson, 1993; (Hu & McLachlan, 2002; Yu et al., 2020). Regardless of their specific effects on afferent transmission, extensive changes in macrophage number, shape, and function following nerve injury prompts an investigation into the extent of neuron-glia-macrophage changes and interactions and their contribution to pain processing.

DRG Vasculature

Because the DRG lies outside of the BBB and encapsulates many blood vessels, circulating products in the blood stream, that would normally be unable to affect central processing, can act on primary afferent cell bodies in the DRG that project to the CNS. If macrophages act on primary afferent cell bodies in the DRG, their previously studied interactions with blood vessels could enable them to act as a conduit for circulating large molecules into the DRG. While inflammatory molecules like LPS usually could not be transmitted across the BBB and into the CNS, uptake of LPS by local macrophages in the DRG or macrophages that migrate into the DRG after injury could sensitize primary afferents by releasing LPS onto cell bodies. Previous research has indicated that macrophages typically lay in very close proximity to the DRG vasculature (Jimenez-Andrade et al., 2008; Lu & Richardson, 1993). Additionally, DRG

blood vessels receive sympathetic innervation from other peripheral tissues that could further cause the sensitization of macrophages through some effect of their interaction.

Macrophages as potential targets for pain therapy

Drugs have been developed that isolate peripheral or DRG macrophages, leaving the CNS environment un-altered due to the protection of the BBB. For example, the FK-binding protein dimerizer, AP20187, selectively kills macrophages in mice by manipulating the CSF1R promoter that is expressed by monocytic cells (Yu et al., 2020). For this reason, to the extent that activated DRG macrophages serve to augment injury-induced activation or become ectopic generators themselves, macrophage targeted interventions present a possible strategy for therapeutic intervention with regard to chronic pain. These DRG macrophages have received relatively little attention in the neuroscience community but appear to play a large role in the regulation of peripheral cells and could therefore pose therapeutic benefits from the perspective of peripheral aid, not impacting the CNS or neurons.

DRG and macrophage imaging

DRGs are densely packed with neuronal soma, fibers, satellite glial cells, macrophages, and blood vessels that lack any specific organization. The DRG also lacks definite anatomic land marks to orient sectioning, making it difficult to know what part of DRG you are sectioning through. Previous studies that have employed typical histochemistry analysis techniques to image DRGs have been limited in their ability to isolate individual macrophages due to their amorphic structure. Because macrophages exist in unique shapes with erratic skinny processes (Nascimento et al., 2018) that can show up as separate from their cell body (e.g. from noise and imaging confines), they can appear in and out of a single plane, distorting accurate representations of morphological nuances and lead to miscounting. Therefore, the analysis of

many individual sections would be required to achieve a meaningful sample using typical histochemistry methods and may still limit the ability to detect whole-cell morphological and contextual information that accurately informs how macrophages change and interact with their environment with regard to changes in the DRG following injury. However, the DRG being relatively small lends itself to study using whole mount methodologies

Methods

Animals

Wild type C57/BI6 and Ccr2-/- functional knock out mice were used in this study. Mice were housed with a maximum of three other mice. Ccr2-/- mice lack the gene that encodes for chemokine receptor that is responsible for recruiting macrophages to the DRG (Yu et al., 2020). All experimentation and animal care dimensions were approved by the Institutional Animal Care and Use Committee of UC San Diego.

Intrathecal drug delivery

Mice were anaesthetized using 2-3% isoflurane until breathing substantially slowed and behavioral responses were inhibited. 50uL of stock Lipopolysaccharide (LPS) solution (2mg/ml stored at -20 degrees Celsius) was diluted to a concentration of 0.2 ug/ul by combining a ratio of 10 ul stock/90uL saline.

Intrathecal (IT) injections were administered using a 25 ul Hamilton syringe that was attached to a 30g needle by polyethylene tubing. Devices were flushed with sterile water before injections. Slowly, 5 ul of LPS solution was injected between the L4-L5 intervertebral-space until the mouse's tail observably twitched. If air bubbles appeared in the tubing, it was recorded and noted as impeded flow.

Tissue collection

Mice were deeply anesthetized with isoflurane and then administered a 0.1 ml dose of intraperitoneal Beuthenasia. Following the effective euthanization of mice with Beuthenaia, they were transcardially perfused using ice cold saline and 4% PFA. For the proceeding 24 hours mice were post-fixed in 4% PFA and then immediately stored in a PBS that included 0.02% sodium azide.

Laminectomy of the vertebral column exposed the spinal cord and DRGs. DRGs corresponding to the sciatic nerve were identified in L3-5. During DRG collection, 0.5-1 cm of the peripheral nerve and the dorsal root surrounding the DRG were left in-tact to prevent damage to any cell body rich portions of the DRGs. Tissue damage can distort precise imaging.

Design and materials for imaging chambers

In order to avoid tissue damage caused by sectioning the organ, imaging chambers were uniquely tailored to analyze whole mounted DRGs on inverted confocal microscope. Due to previous comparisons, we determined SLA printers using 3Dresyn CR UHT as the best DRG imaging chamber. The chambers were perfectly flat and were able to be reused for the longest time: they did not degrade with over 100 uses. SLA chambers also never fell of the slides even when glued with low viscosity cyanoacrylate.

Tissue clearing for immunohistochemistry

A slightly modified version of the iDISCO and fDISCO protocols were employed to clear and stain the DRG tissues. Because DRGs are anatomically small, pretreatment was not always necessary and if avoidable, tissue working time was greatly reduced. DRGs were stained with *Iba1 (wacko, 1:1000), CD-31(,1:50), NeuN (1:300), mCherry(Rockland, 1:1000), and DAPI(1:10,000).* DRGs were always washed in metal plates with transfer baskets and

incubations were carried out in securely sealed Eppendorf tubes of glass vials. DRGs were washed three times for 15 minutes each in PTx.2 and then incubated in permeabilization solution for 2 hours at 37 degrees Celsius on a shake plate. The DRGs were then transferred to blocking solutions, on a shake plate, for two hours at 37 degrees Celsius. Following, DRGs were incubated for 24-72 hours in a 1 degree antibody on a shake plate at 37 degrees Celsius. Combining multiple DRGs in the same clearing process helped save reagents but required larger (1.5 ml) Eppendorf tubes. With individual DRGs, PCR tubes with 200 microliters of solution sufficed. DRGs were then washed five more times in 15 minute durations with PTwH. For the following 24-72 hours, DRGs were incubated on a shake plate in a secondary antibody solution (PTwH/3% serum) at 37 degrees Celsius. Lastly, the DRGs were washed for one to two hours in PTwH + DAPI 1:10,000, and then washed five times in 15 minute durations with PTwH prior to clearing.

THF proved to be a better reagent for tissue clearing compared to menthol but posed methodological challenges as it cannot be stored in plastic vials. Accordingly, DRGs were dehydrated in a series of glass vials containing THF/H2O at the following concentrations: 50%,70%,80%,100%, 100%, Then DCM 33%/THF 66%, 100%DCM, and finally 100% DBE.

When using DBE, some slight shrinkage of the DRG can occur. Volumetric reduction of the DRG can be beneficial in assuring that the working distance of the magnification objective spans the depths of the DRG. Notably, upon DRG submersion in BDE, the structure becomes invisible and can be lost. As a preventative measure, DAPI was added so that a weak UV light could be used (along with UV glasses) to visualize the DRG. BDE can be used to store DRGs but the length that it can store DRGs depends on the temperature in which it is kept.

Imaging

Inverted SP5 confocal microscopes with 10x air, 20x air, and 63x water objectives have a working distance greater than the depth of the DRG. These machines were employed to obtain high resolution images of the DRG that controlled for photo bleaching and loss of fluorescent intensity at deep tissue depths by optimizing settings at the lowest laser signal intensity that effectively produced signal.

10 image analysis pipeline

Traditional imaging methods failed to accurately quantify macrophage activation states and 3D datasets (DRG tissue) were largely limited due to the inaccessibility of advanced imaging software. Despite being extremely expensive and time-consuming (as you must set specific thresholds for signal and noise in each image), Imaris was used for the manual detection of individual macrophages as it is a commercially available and validated cell-labeling method. I acted as a trained and expert observer in the identification of macrophages by isolating signal from noise in DRG images taken from different depths, with different intensities, and under different conditions (Figure 3.a). Emphasis was given to track processes throughout stacks of tissue images to capture macrophage morphology across image planes. These labels were used as a ground truth for training data and were supposed to encompass all of the possible data that the model would encounter while analyzing DRGs.

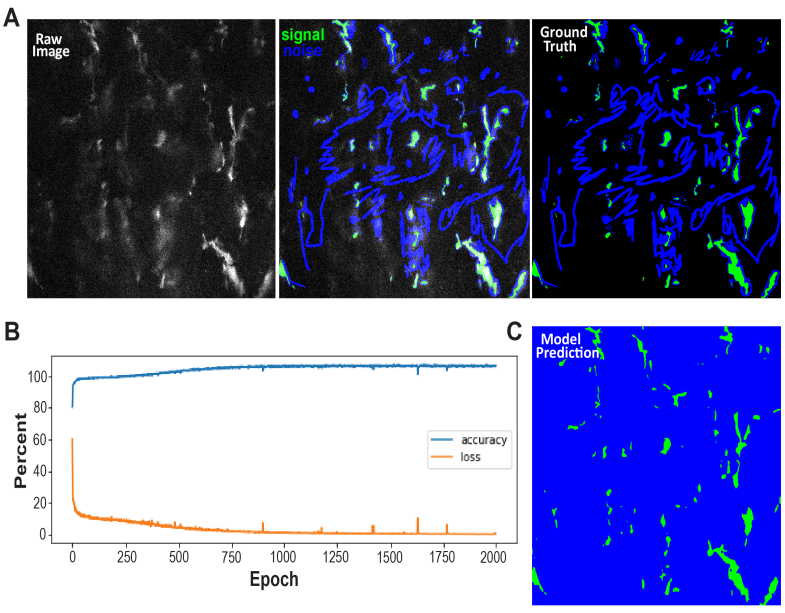

Figure 3: *A.* raw images of DRG tissue at different depths and intensities were analyzed. An expert observer differentiated between signal (green) and noise (blue). The labeled images were used as ground truth for training the model. *B.* accuracy increased and loss reduced after training the model over 2000 epochs. *C.* Model predictions reflected macrophages (green) and background (blue).

The machine learning program was trained to recognize macrophages as the expert observer did, among stacks of 30 images that contained 5-10 labeled macrophages per image. These models were trained for up to 2000 epochs and resulted in highly reproducible and accurate predictions (Figure 3.b). The model takes raw macrophage images as an input and outputs individual macrophage data in binary. This methodology serves to reduce bias in cell labeling as the trained labeler did not know what population of macrophages they were exposed to (e.g. WT, LPS, Ccr2-/-, Ccr2-/- and LPS tissues). Future applications of this model would further reduce human bias that is inherent in the expensive and time-consuming individual labeling of cells through Imaris.

Expanding upon the 3D modeling techniques stated above, we employed a pipeline to segment our raw image-labeled macrophages, and reconstruct the original raw images, ultimately enabling us to view macrophages through their 3D morphological features, staining intensity, and the type of cells that they were in contact with. To accomplish this, we strung together raw a pipeline in the form of a shell script that takes raw images and runs them through the machine learning models, then subsequently segments and analyzes the resulting outputs.

In accordance with our interest in the DRG vasculature and its interactions with macrophages, the pipeline was

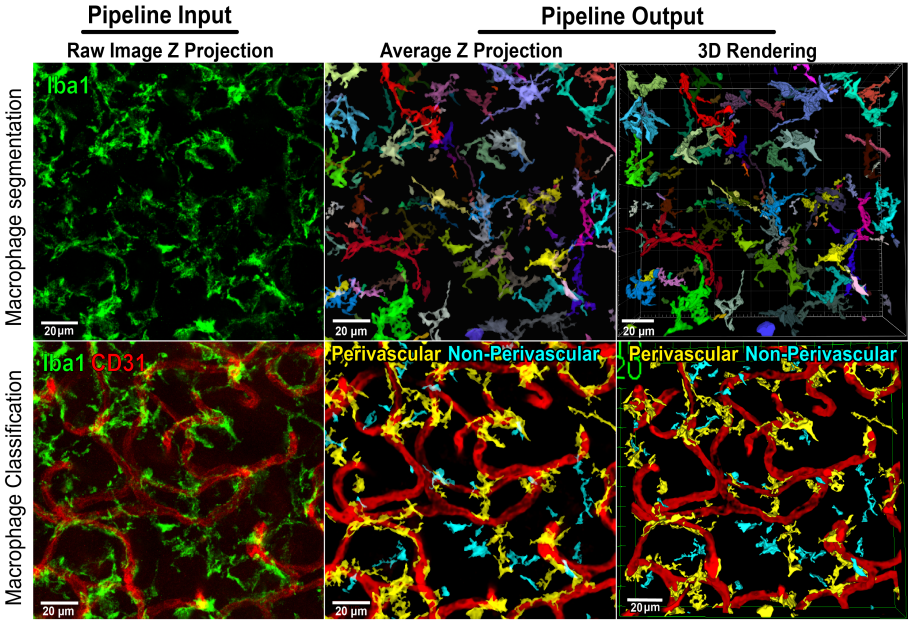

Figure 4: Visual representationof image analysis pipeline. *Top row:* input and output of Iba1 stained DRGs showing the average raw image Z projection and the 3D rendering of randomly colored macrophages. *Bottom row:* input and output of raw Iba1 positive macrophages and CD31 labelled blood vessels. The pipeline output classifies macrophages as perivascular (yellow) or non-perivascular (cyan) based on their proximity to blood vessels.

designed to explicitly identify macrophages and then classify those macrophages as perivascular (in physical contact with a blood vessel), or non-perivascular, based on their contact with blood vessels. The model then quantified macrophage volume, number, and image characteristics (e.g. intensity, depth) based on 3D representations of whole cells. A visual representation of these functions is shown in figure 4.

Results

The goal of the present study was to validate a novel 3D imaging technique that would capture morphological changes that occur in the DRG following IT LPS, maintaining the accurate depiction of cellular complexity through whole-cell identification.

Distinct populations of macrophages in the DRG, the sciatic nerve (SN), and the spinal cord (SC)

The study illustrated in Figure 5 was done as preliminary research generated in the Yaksh laboratory, using standard multiparametric flow cytometric sample processing and data analysis methodologies. Fresh tissues from the mouse DRG, SN, and the SC were each disaggregated and evaluated separately by multiparametric flow cytometry, using antibodies to the macrophage-associated antigens CD45, CD64, CD11b, MHCII, CX3CR1, and F4/80, plus propidium iodide (PI) as a viability marker. Figure 5.a shows that when the analysis is restricted to the CD45-positive/PI-negative cells in each of these three locations, two discrete macrophage subsets are identifiable by virtue of their differing levels of expression of CD64, F4/80, MHCII, and CD11b when the flow data are visualized on 2-dimensional dotplots; all of the macrophages express similarly high levels of Cx3Cr1 (Figure 5.b), the fraktaline ligand and chemokine receptor that specifically defines peripheral monocytic cells (Yu et al., 2020). When t-distributed stochastic

neighbor embedding (t-SNE, a machine learning algorithm for visualization of high-dimensional data a low-dimensional space of two or three dimensions) is applied to the data (Figure 5.c), the presence of two discrete macrophage subsets is confirmed, with one subset (colored black Figure 5.c) being largely limited to the spinal cord (CNS origin), and the other subset consisting of both DRG and sciatic nerve components (both of PNS origin). Figure 5.d (left) shows the different macrophage densities in these three anatomic locations, with the highest density of macrophages being in the spinal cord, and the lowest density being in the sciatic nerve. Finally, when green fluorescent protein (GFP)-tagged Cx3Cr1 can be used to visualize the tissue distribution of macrophages in these three locations (Figure 5.d, right)

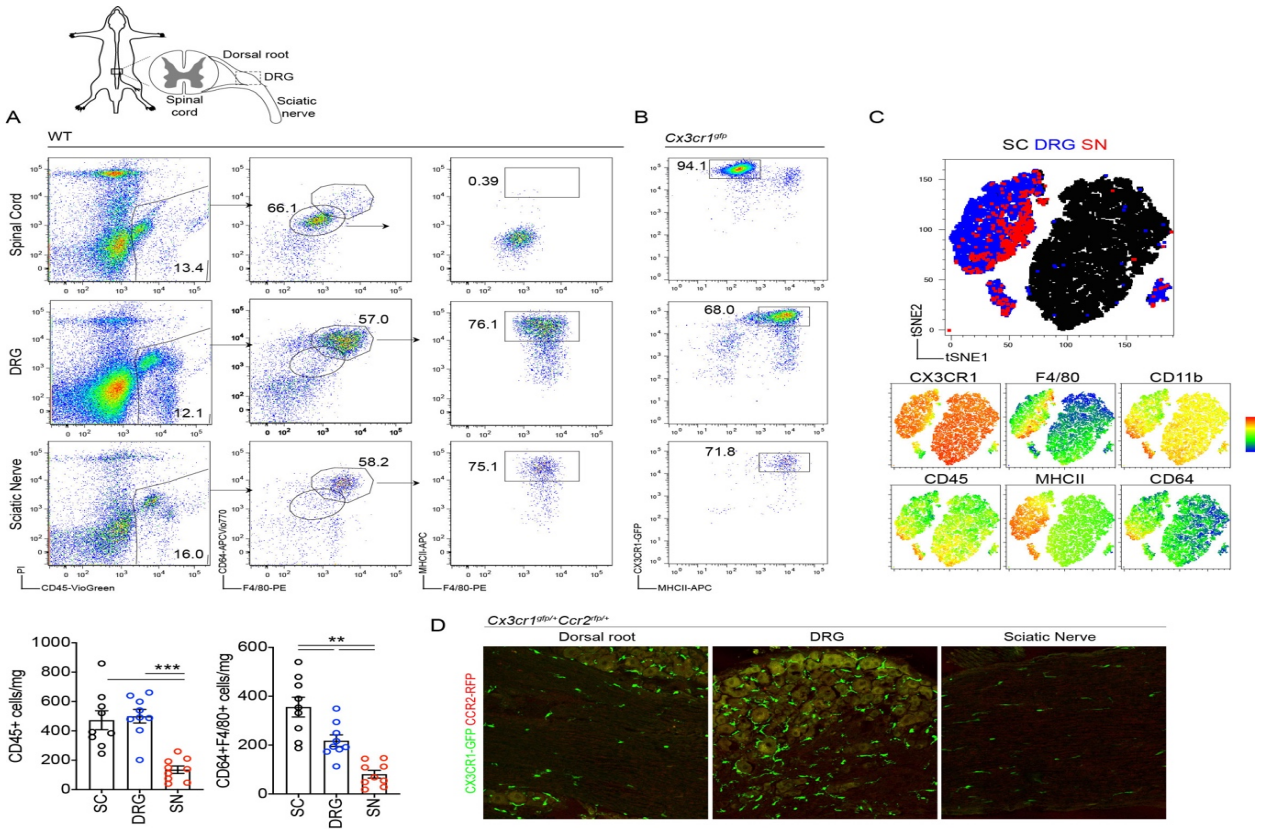

Figure 5: Figure 4: Distinct populations of macrophages reside in the DRG, the sciatic nerve (SN), and the spinal cord (SC). A, B: Flow cytometric evaluation of known macrophage markers in disaggregated samples from these 3 locations. Flow cytometry of WT (a) and Cx3CR1-gfp mouse tissues (b) quantified the presence of these markers and accordingly pictured macrophage populations. C. t-SNE plots show cells with similar characteristics as distinct clusters in Cartesian space, allowing visualization of two distinct macrophage populations. D, left. Graphs illustrate a significant difference in the number of CD45 positive and CD64+F4/80 macrophages present in the DRG, SN, and SC. D, right. Visual representation of Cx3cr1-GFP+CCR2-RFP positive macrophages.

DRG Macrophage activation following intrathecal LPS

Intrathecal LPS administration was used authenticate the functionality of the imaging methods and pipeline. A visual difference of macrophage morphology was observed as a larger volume of Iba1 positive cells were present in the DRG of mice injected with IT LPS compared to those injected with saline (Figure 6.a). Ccr2-/- mice were also injected with IT LPS to assess macrophage infiltration of the DRG after nerve injury. In Ccr2-/- mice, a chemokine receptor that is crucial for the recruitment of macrophages during immune responses in deleted.

Therefore, the observed increase in macrophage size and volume in Ccr2-/- mice following IT LPS indicates that macrophage expansion in the DRG is more likely to occur by local proliferation than by the infiltration of circulating macrophages.

The results of our pipeline indicate that DRG macrophage volume increases after IT LPS in both WT and Ccr2-/- mice (figure 6.c). The data produced by our pipeline replicated Imaris results, confirming the validity of the present imaging methods (Figure 6.b and 6.c). Our pipeline also enabled the accurate counting of macrophages by number in the DRG which showed no increase in the number of macrophages in the DRG after IT LPS (Figure 6.d).

The model was able to identify significant volumetric increases of both non-perivascular macrophages (Figure 6.e) and perivascular macrophages (Figure 6.f) following IT LPS. Significant increases in the volume of non-perivascular macrophages, perivascular macrophages, and all macrophages were observed in the DRGs of mice treated with LPS compared to saline (Figure 6.g, 6.h, and 6.i). This change was larger in perivascular macrophages, suggesting that macrophage-vessel interactions in the DRG have some unique function or role in mediating pain transmission.

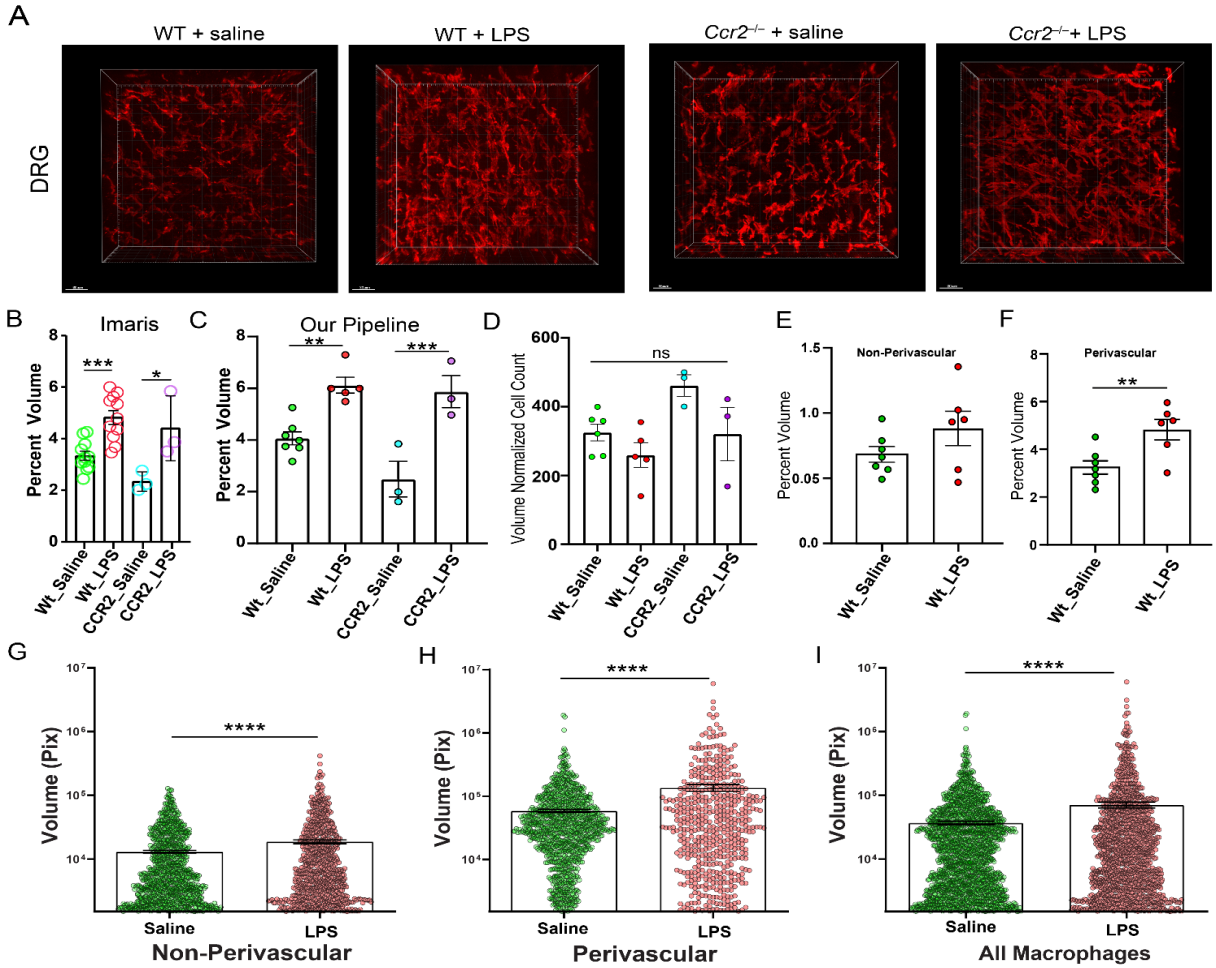

Figure 6: The effect of IT LPS on macrophage activation in the DRG. *A.* Visual representation of macrophages in WT and CCR2-/- mice. *B.* Analysis with Imaris reflected an increase in macrophage volume in following IT LPS in C57/BI6 and CCR2-/- mice. *C.* Analysis with our pipeline replicates data produced by Imaris. *D.* No change was observed in the number of macrophages present in the DRG before and after IT LPS. *E, F, G, H.* significant increases were observed in non-perivascular and perivascular macrophages following IT LPS. *I.* macrophages increase in volume following IT LPS. [**** P<0.0001]

Discussion

Many studies have examined macrophages in the DRG through fluorescence-activated cell sorting (FACS) and flow cytometry (Yu et al., 2020), but these studies have lacked the ability to access complex 3D morphology of macrophage projections in the densely packed DRG environment. In our study, flow cytometry provided important preliminary evidence for the existence of a distinct macrophage population in the DRG, while comparison to the imaging

program Imaris enabled us to develop our own method to quantify labeled agents in the DRG in a pain state, and to assess 3D cell-cell interactions (such as those between blood cells and macrophages). In the present study, we present a novel, efficient, and informative way to process, stain, and image DRGs using high-resolution confocal microscopy and a novel pipeline that enables the analysis of large stacks of high quality 3D images.

The pipeline we created was validated by confirming the significant apparent changes in macrophage morphology, in response to IT LPS (immune mediated pain) that were expected based on previous research that was summarized in the introduction. LPS activated macrophages in tests on WT and CCR2 knockout mice. Further proof of our pipeline was demonstrated as it replicated data produced by Imaris, a commercially available and widely used imaging program (Figure 5.c). While useful, Imaris is expensive, time consuming, and labor intensive. It also reflects more potential bias introduced due to exhaustive involvement by human labelers. Our pipeline reduced bias and let us see the greater complexity of 3D images.

These results demonstrate the complexity of the DRG and macrophage activation after LPS exposure. Our data suggests that previous studies that have employed traditional histology techniques have had the limitations inherent to imaging amorphous cells using slice histology. Because larger macrophages with more projections could pass in and out of sectioned planes (slices) and appear as separate from the cell body they belong to, traditional histologic analysis risks quantifying more macrophages than are actually present.

In contrast with previous research, we observed that distinct populations of macrophages in the DRG do not proliferate following nerve injury, but rather significantly increase in volume. Volumetric increases in DRG macrophages holds true for WT and CCR2-/- mice that lack infiltrating monocytes and macrophages (Figure 6.b). This result indicates that volumetric

macrophage increases occur in pre-existing DRG macrophage populations, and do not reply on external macrophages that infiltrate the DRG.

In contrast to the slow-onset, adaptive immune response that is entirely based on T-lymphocytes, macrophages are part of our innate immune response that uses non-specific defense mechanisms immediately or shortly after the appearance of an antigen in the body. Innate immune signaling, as initiated by the activation of TLR4 receptors by LPS may drive activation of DRG macrophages and cause the release of products into the DRG that excite DRG somas, resulting in ongoing ectopic activity arising from the DRG. While the direct effect of macrophage activation was not examined in this study, it has been suggested that macrophages release more pro-inflammatory mediators when they are activated (as evidenced by their increase in volume) (Xie et al., 2007; Hu & McLachlan, 2002).

The pipeline demonstrated in this study illustrates the power of using machine learning to analyze large stacks of 3D data and visualize macroscopic processes that occur in the body. The same methods and techniques presented here can be applied to future studies investigating the specific cellular mechanisms that underlie behavioral phenomena. Potential basic science topics of interest related to the DRG include the expression and release of mediators from the neuronal soma, cell-cell interactions, and environmental characteristics including relationship to the vasculature. Potential clinical topics of interest related to the role of DRG macrophages in chronic pain include isolating the casual relationship between macrophage morphology and afferent sensitization (e.g. do macrophages cause persistent pain or are they merely a symptom of it?), analyzing of DRG macrophage changes across a variety of different pain states, and evaluating the clinical relevance of macrophage manipulation on persistent pain. Furthering our understanding of the influential components of pain processing should uncover specialized and

novel targets for chronic pain therapy, which represents the fundamental promise this line of research.

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