SYSTEMIC INFLAMMATION INCREASES EXPRESSION OF INTERLEUKIN-1 RECEPTORS ON NEURONS AND ASTROCYTES, BUT NOT MICROGLIA, IN THE CERVICAL SPINAL CORD

by

KELLY ROYSTER

A THESIS

Presented to the Department of Human Physiology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2018

An Abstract of the Thesis of

Kelly Royster for the degree of Bachelor of Arts
in the Department of Human Physiology to be taken June 2018

Title:
Systemic inflammation increases expression of Interleukin-1 Receptors on neurons and astrocytes, but not microglia, in the cervical spinal cord

Approved: _____

Adrianne G. Huxtable

Inflammation is a component of all diseases, whereby key pro-inflammatory signaling molecules and receptors increase systemically and in the central nervous system (CNS). Systemic inflammation activates astrocytes and microglial cells in many regions of the CNS, which alter neuronal function and lead to behavioral changes. Our work has shown systemic inflammation impairs respiratory function through activation of the pro-inflammatory interleukin-1 receptors (IL-1RI) in the cervical spinal cord, but the roles of different cell types are unknown. To better understand how activation of IL-1RIs undermines breathing, we first need to determine how systemic inflammation changes IL-1RI expression and what CNS cell types express IL-1RIs. Based on the expression of IL-1RIs in other CNS regions, we hypothesized systemic inflammation would increase IL-1RI expression on identified neurons, astrocytes and microglia in the cervical spinal cord. Using immunohistochemistry to fluorescently label cell types and IL-1RIs, we first determined the optimal concentration of the IL-1RI antibody (1:250) by a dilution series (n=5) in the hippocampus since previous work demonstrate IL-1RI expression in the hippocampus after systemic inflammation. Preliminary data suggest systemic inflammation increases IL-1RI expression on neurons (labeled by NeuN, 1:500, n=8), astrocytes (labeled by GFAP, 1:500, n=3), and endothelial cells (labeled by RECA-1, 1:250, n=2), but not microglia (labeled by Iba1, 1:1000, n=4). These results suggest neurons, astrocytes, and endothelial cells are likely the key cells undermining respiratory function after systemic inflammation. Understanding the mechanisms by which systemic inflammation undermines respiratory function may lead to targeted therapeutic interventions to promote breathing.

Acknowledgements

I would like to express my sincerest gratitude to each of the members of my thesis committee: Dr. Adrianne Huxtable, Austin Hocker, and Dr. Rebecca Lindner. I feel privileged to have had the opportunity to have worked with these mentors, and to have been a part of the Huxtable laboratory. Thank you to Dr. Adrianne Huxtable for sharing your time and knowledge, and always pushing me to improve as a writer and researcher. Thank you to Austin Hocker for the many hours you spent aiding me with completing this project, helping me learning more about the field, and guiding me to be a better researcher. Thank you to Dr. Rebecca Lindner for your support and enthusiasm. Thank you all for the time and effort you have given to develop and finish this project, and to create this document.

I would like to extend my gratitude to the Academic & Thesis Coordinator Miriam Alexis Jordan for assisting me throughout the process of completing this project, and for creating the template for this document along with CHC Librarian Miriam Rigby. I would also like to thank the rest of the Huxtable laboratory for their support. Finally, I would like to thank my family and friends, especially my parents, for their unwavering support.

Table of Contents

List of Figures

Introduction

Background

Breathing is a vital function for life by maintaining CO_2, H^+, and O_2 concentrations, and is controlled by the central nervous system (CNS). However systemic inflammation can undermine one aspect of the control of breathing, respiratory neuroplasticity (Huxtable *et al.*, 2013). Respiratory neuroplasticity is an important component of breathing, whereby a change in motor output (e.g. increased depth of breathing) based on prior experiences, helps maintain homeostasis during internal and external changes (Mitchell & Johnson, 2003). The loss of respiratory neuroplasticity endangers adaptability of the respiratory system to maintain homeostasis, especially in response to disease or injury. Thus, systemic inflammation due to disease or injury may impact the control of breathing.

The brain was once believed to be immune privileged, where inflammatory signals from the periphery would not impact the CNS (Medawar, 1948); however, we now know pro-inflammatory signaling molecules (such as cytokines) pass from the periphery to the CNS, causing neuroinflammation (Faggioni *et al.*, 1995). The CNS is also alerted to peripheral inflammation by activation of afferent (traveling from the periphery to the CNS) vagal nerve fibers, thus promoting cytokine release in the CNS (Watkins *et al.,* 1995). Pro-inflammatory cytokines abolish a model of respiratory neuroplasticity (Fuller *et al.*, 2000; Devinney *et al.*, 2015); respiratory neuroplasticity is mediated by phrenic motor neurons (signal transmitting cells that innervate the diaphragm, the main inspiratory muscle) (Fuller *et al.*, 2001; Devinney *et al.*, 2015; Dale *et al.*, 2017) . Pro-inflammatory cytokines in the CNS also activate microglia,

which are the resident immune cells of the CNS (Parkhurst & Gan, 2010), but compose only a small percentage (5-15%) of CNS cells (Lawson *et al.*, 1992) compared to astrocytes which comprise 20-40% of non-neuronal CNS cells (Verkhrasky & Butt, 2013). Astrocytes are CNS cells with immune and metabolic functions, actively interact with neurons and blood vessels (as illustrated by Figure 1). Astrocyte and microglial activation increase or decrease the activity of neurons by secreting cytokines and other signaling molecules during inflammation (Araque *et al.,* 1999).

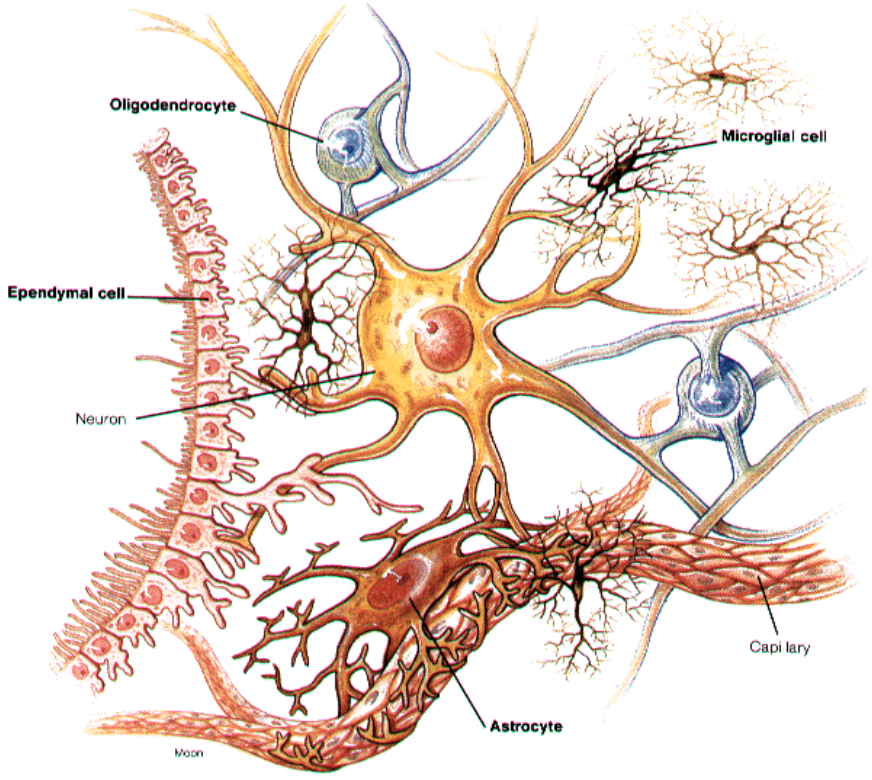


Figure 1. Diagram of the heterogeneous cellular makeup of the CNS. Astrocytes have projections interacting with both the blood vessels (endothelial cells) and with neurons. Microglia are located throughout the CNS and interact with many cell types such as neurons and astrocytes. *The neuron – What happened to Chris*. N.d. Accessed Feb. 8, 2018.[http://whathappenedtochris.weebly.com/uploads/1/3/7/6/13761436/973220257.gif]

Peripheral inflammation ultimately likely causes the abolishment of respiratory neuroplasticity by way of cytokine signaling as well as vagal afferents (Wang *et al.*,

2002). However, the cellular mechanisms by which inflammation undermines respiratory neuroplasticity remain poorly understood.

Previous work has demonstrated systemic inflammation increases gene expression for cytokines (Huxtable *et al.*, 2013), specifically Interleukin-1 beta (IL-1β) in the cervical spinal cord, suggesting IL-1β signaling is an important mechanistic step in impairing neuroplasticity. Further, recent work has demonstrated activation of the receptor for IL-1β, the Interleukin-1 Receptor I (IL-1RI), in the cervical spinal cord is a necessary mechanistic step for impairing neuroplasticity (Hocker and Huxtable, 2018). However, we do not know if expression of IL-1RI increases in the cervical spinal cord after systemic inflammation, or the identity of the CNS cells expressing IL-1RIs. Knowing the identity of cells able to respond to systemic inflammation will allow for better determination of the IL-1RI signaling pathway by which inflammation impairs the control of breathing. Since inflammation increases IL-1β mRNA in the cervical spinal cord, we hypothesize a concomitant increase in IL-1RI expression in the cervical spinal cord following systemic inflammation. Previous work has also demonstrated increased IL-1RI expression on neurons and astrocytes after inflammation in the rodent dorsal cervical spinal cord (Holló *et al.*, 2017) and hippocampus (another area of the brain where neuroplasticity occurs) (Lynch & Lynch, 2002). Thus, we hypothesize systemic inflammation increases expression of IL-RI on astrocytes, microglia, and neurons, ultimately leading to the abolishment of respiratory neuroplasticity.

Goals

The main goal of these studies is to determine how systemic inflammation alters IL-1RI expression, and the location of IL-1RIs on distinct CNS cells (microglia, astrocytes, and neurons) in the cervical spinal cord.

Clinical Relevance

Neuroplasticity (a general term for plasticity occurring throughout the CNS, including respiratory-related regions) is being tested to temporarily restore some motor function to patients who suffered from spinal cord injuries (incomplete spinal cord injuries) (Trumbower *et al.*, 2012; Hayes *et al.*, 2014). Ankle flexion degree and strength (Trumbower *et al.*, 2012; Hayes *et al.*, 2014), as well as walking speed and distance (Hayes *et al.*, 2014) improved in spinal cord injury patients using a similar model to that used to evoke respiratory plasticity. Additionally, high cervical spinal cord injuries cause impaired breathing due to damaged neuronal connections to the muscles they innervate, and therapeutic neuroplasticity interventions are being developed to restore vital ventilatory motor functions in these patients (Dale-Nagle *et al.*, 2010). At present, it hypothesized inducing respiratory neuroplasticity will strengthen neuronal connections to aid in improving ventilatory functioning following a spinal cord injury (Vinit *et al.*, 2009), but it is not known how neuroinflammation impacts therapeutic applications of neuroplasticity. Since significant neuroinflammation occurs after spinal cord injuries (Popovich *et al.*, 1997), this neuroinflammation will likely impair or abolish neuroplasticity, decreasing therapeutic efficacy of neuroplasticity to restore function (Huxtable *et al.*, 2013). In order to understand how inflammation may impact therapeutic uses of plasticity for spinal cord injury patients,

we first need to understand the cellular mechanisms by which inflammation abolishes respiratory neuroplasticity. The techniques for inducing and abolishing neuroplasticity in respiratory-related regions likely induce and abolish neuroplasticity in other areas of the CNS by similar mechanisms. By knowing the cell types IL-1RI are expressed on, and thus the cells able to play a role in the key mechanistic step of abolishing respiratory neuroplasticity, future investigations can address how inflammation can impair neuroplasticity in non-respiratory related regions.

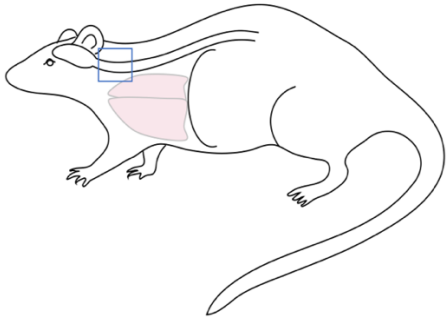
Methods

Tissue Harvesting and Sectioning

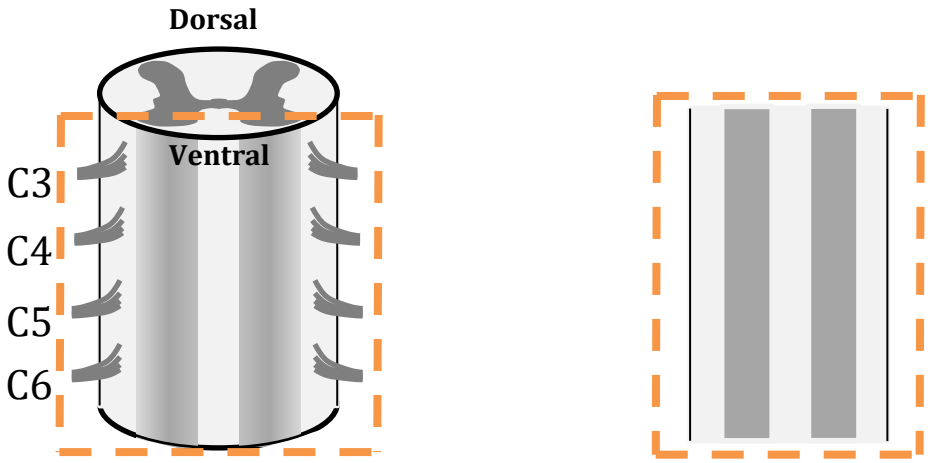
All animal use and experiments were approved by the Institutional Animal Care and Use Committee at the University of Oregon and conform to the policies outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Sprague Dawley rats (3-4 months) were housed under standard conditions, with food and water ad libitum and a 12 h light/dark cycle. Male rats were given intraperitoneal injections (into the abdominal cavity) of saline (control, 24 hrs) to identify the non-specific effects of injections and environmental factors, or lipopolysaccharide (100 μg/kg LPS, 24 hrs) a component of gram negative bacterial cell membrane to induce systemic inflammation prior to tissue harvesting. Female rats were given a single intraperitoneal injection of LPS (300 μg/kg, 4.5 hrs) or one intraperitoneal injection of LPS (500 μg/kg) per day for 6 days as indicated. Peripheral injections of LPS have been previously shown to induce systemic inflammation (Schnydrig *et al.,* 2007), and abolish respiratory neuroplasticity (Huxtable *et al.*, 2013). Rats were anesthetized and transcardially perfused by insertion of a needle through the left ventricle into the aorta. The right atrium was cut, the cardiovascular system flushed of blood cells and plasma with phosphate buffered saline (PBS), followed by 4% paraformaldehyde in PBS to fix the tissue and prevent cellular degradation. Harvested tissues were immersion fixed (4% paraformaldehyde in PBS) for a minimum of 24 hours or up to 13 months. Once fixed, the dura (membrane surrounding the CNS) was removed and cervical spinal cords and cerebrum including hippocampi and cortex tissue (outer layer of the cerebrum) were

isolated (cervical spinal cord and hippocampus location indicated by Figure 2). Cervical spinal cords (segments C3-C6, where phrenic motor neurons are located) were sectioned (40µm slices) on a vibrating microtome (Leica, VT1200S). The cerebral tissue was sectioned medial to lateral on a vibrating microtome. Sections were stored in PBS at 4°C or antifreeze at -20°C, as specified in the results.

A.



B.



C.

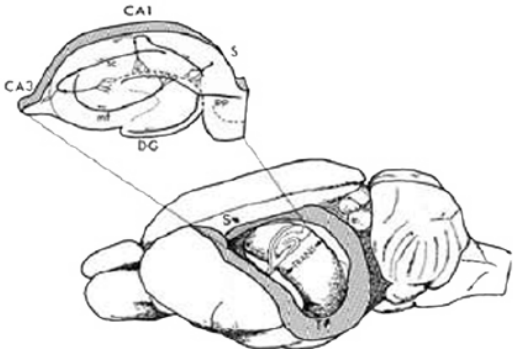


Figure 2. Schematic of the rat brain, highlighting the direction of sectioning of cervical spinal cord and hippocampal tissue used for immunohistochemistry. The cervical spinal cord is located in the neck region of the rat CNS (indicated by the blue box), just caudal to the brain and contains phrenic motor neurons (A). An isolated depiction of the cervical spinal cord segments C3-C6 (B) containing phrenic motor neurons, astrocytes, and microglia. Cerebrum of a rat CNS demonstrating the location of the hippocampus (C)((Brotons-Mas *et al.*, 2006)). Insert illustrates a medial section of the hippocampus as was used for immunohistochemistry (C).

Immunohistochemistry

Immunohistochemistry (IHC) is a technique used to identify the location of proteins of interest in tissue using targeted antibodies (Figure 3). Here, IHC was used to identify the location of IL-1RI proteins on free-floating, cervical spinal cords or cerebral tissues from LPS and saline-treated animals, by determining co-localization of IL-1RI and cell specific proteins.

In IHC, antibodies are Y-shaped proteins that can bind to receptors (such as the IL-1RI), or other types of proteins located on or within a cell to enable visualization of the protein localization. The IL-1RI primary antibody was commercially developed by injecting the IL-1RI protein of interest into a host animal (goat), whereby the host's immune system produces antibodies against the IL-1RI protein. The antibodies are then collected from the blood of the host animal. The secondary antibody used to visualize IL-RI was developed to bind to specific proteins found only on an antibody produced by a specific animal (goat, rabbit, or mouse), and a colored fluorophore (fluorescent tag) was attached to secondary antibodies facilitating visualization of the IL-

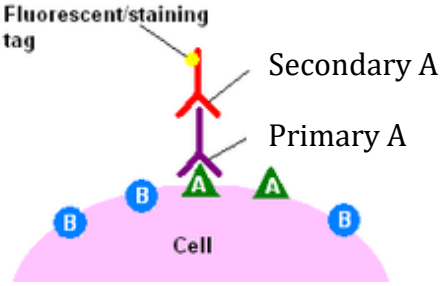


Figure 3. Schematic of immunohistochemical staining. Primary antibody "A" binds to the target A molecule, while a secondary antibody bonds to the primary antibody and has a fluorescent tag. N.d. Date accessed Feb. 8, 2018 [https://upload.wikimedia.org/wikipedia/commons/thumb/3/37/Immunohistochemicalstaining2.PNG/300px-Immunohistochemicalstaining2.PNG]

1RI primary-secondary antibody complex. The host species for primary antibodies must be different from the species of the tissue containing the target protein or the secondary antibody would bind to the tissue and the primary antibody. All other primary antibodies used in this project were commercially developed similarly, using host species other than rats.

Primary antibodies used for this project include: Anti-IL-1RI (Goat, 1:250; R&D Systems AF771), Anti-Iba1 (Rabbit, 1:1000; Wako 019-19741), Anti-GFAP (Rabbit, 1:500; EMD Millipore Corporation AB5804), Anti-NeuN (Mouse, 1:500; EMD Millipore Corporation MAB377), and Anti-RECA-1 (Mouse, 1:250; Abcam, ab9774). Anti-IL-1RI binds IL-1RI and was used to determine the location of IL-1RI expression. Anti-Iba1 antibodies identify microglia by binding to the calcium-binding adaptor molecule 1 (Iba1) localized to the cytoplasm of only microglia (Imai *et al.*, 1996). Anti-GFAP antibodies identify astrocytes by binding to the glial fibrillary acidic protein (GFAP) located on intermediate filaments within astrocytes (Eng, 1985). Anti-NeuN antibodies identify neurons by binding the Hexaribonucleotide Binding Protein-3 (NeuN) localized mainly to the nucleus of neurons (Mullen *et al.,* 1992). Anti-RECA-1 antibodies identify endothelial cells by binding to the rat endothelial cell antigens (RECA) expressed by all rat endothelial cells (Majoor *et al.,* 1992). Astrocyte and microglia primary antibodies were fluorescently labeled with Alexa Fluor Donkey-anti-rabbit 647 (1:2000 Iba1 and 1:1000 GFAP; Thermo Fischer) secondary antibody, neuron and endothelial cell primary antibodies with Alexa Fluor Donkey-anti-mouse 488 (1:1000; Thermo Fischer), and IL-1RI primary antibody with Alexa Fluor Donkey-anti-goat 555 (1:1000; Thermo Fischer).

For IHC, tissue sections were washed with PBS (for 5 minutes on a shaker, three times) to remove debris. In experiments with antigen retrieval (as indicated in the results section), tissue sections were heated at 80°C (25-30 min) in sodium citrate buffer (10mM, pH 6.0) and washed again with PBS. Antigen retrieval encompasses a variety of techniques used to promote primary antibody binding to the target protein, the technique used here breaks protein cross-links which may form during PFA fixation, covering antigens, such as IL-1RI, and inhibiting primary binding. All sections were then permeabilized (0.3% Triton, EMD Millipore Corporation) to permit intracellular primary antibody penetration and access to intracellular proteins, while non-specific binding sites were blocked (1% bovine serum albumin, Research Products International Corporation; 1 hr, room temperature on a shaker). Primary antibodies (up to three antibodies from different host species, described above) were incubated with the tissue for 16 hours at room temperature. Tissues were then washed with PBS (for 5 minutes on a shaker, three times) to remove unbound primary antibodies. Secondary antibodies were incubated with the tissue (2 hrs, room temperature on a shaker) to bind to the primary antibodies already bound to the target protein and fluorescently label target protein location. After tissues were incubated with secondary antibodies, exposure to light was minimized to prevent bleaching the fluorescent tags. Lastly, tissue sections were washed to remove any unbound secondary antibodies and mounted on charged slides with anti-fade solution (40µL of ProLong Gold anti-fade reagent; Invitrogen). Tissue sections incubated without primary antibodies were used as negative controls to ensure secondary antibodies were not interacting with more than the primary antibody target (no-primary control). Tissue sections were also incubated without secondary

antibodies to ensure primary antibodies and tissue were not auto-fluorescing (no-secondary control).

Confocal Microscopy

The locations of proteins of interest were visualized using a laser scanning confocal microscope (Olympus FLUOVIEW FV1000). A laser scanning confocal microscope utilizes a combination of lenses, mirrors, and lasers (of wavelengths 488, 546, and 647nm), which excite the conjugated fluorophores (of wavelengths 488, 555, and 647nm) to emit a particle of light detected and recorded by the imaging software (Olympus FLUOVIEW FV1000 software Version 2.1.c), while the background and out-of-focus light is filtered out (Figure 4). In contrast, other types of fluorescent microscopes (such as epiflourescent microscopes) allow both in-focus and out-of-focus light to be captured, decreasing the ability to determine co-localization between IL-1RI and the cellular identity proteins. It would not be possible to clearly determine if the fluorescence from the IL-1RI and the cellular identity proteins were from the same depth of the tissue. Co-localization is determined by examining the fluorescence from multiple secondary antibodies in a single tissue slice. To determine if IL-1RIs are expressed on neurons, astrocytes, or microglia the IL-1RI

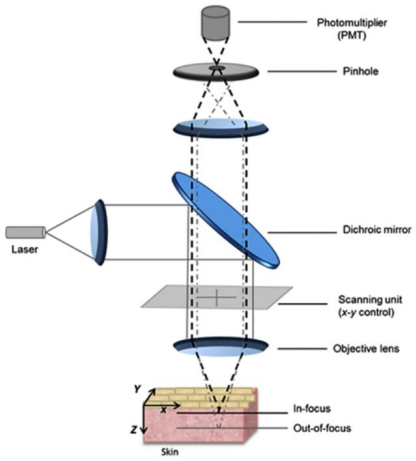


Figure 4. Diagram of a confocal microscope. The microscope has a pinhole allowing only a specific section of in-focus light to be detected. *Confocal Laser Microscopy-Principles and Applications in Medicine*, Biology, and the Food Sciences. Accessed Feb. 8, 2018

and GFAP, Iba1, or NeuN primary antibodies (and their corresponding secondary antibodies) are incubated on the same tissue section. Then, using the confocal microscope the fluorophores conjugated to the secondary antibodies are excited and the emissions recorded. The recorded fluorescence can be viewed as just NeuN, GFAP, Iba1, or RECA-1 secondary antibody fluorescence, just IL-1RI secondary antibody fluorescence, or viewed as an "overlay" image where both NeuN, GFAP, Iba1 or RECA-1 and IL-1RI secondary antibody fluorescence is visible. In areas where co-localization occurs, there will be a visible overlap in fluorescence. The brightness and contrast of the digital images were optimized for each set of images (in order to be compared, tissues must been incubated with antibodies in the same experiment, and images be taken on the same day) to minimize background fluorescence (background fluorescence determined using control images) using Fiji ImageJ (Schindelin *et al.*, 2012). Using these methods, we determined IL-1RI location and relative expression on distinct cell types (astrocytes, microglia, neurons, and blood vessels) within the hippocampus and cervical spinal cord based on co-localization of IL-1RI and the cellular identity proteins. Qualitative analysis of relative IL-1RI fluorescence was used to determine an increase in IL-1RI expression between tissues from saline and LPS treatment groups.

Results

Identification of Primary Antibody Dilutions

Multiple dilutions for NeuN, Iba1, GFAP, RECA-1, and IL-1RI antibodies were assessed to determine the optimal antibody concentration for target visualization while minimizing non-specific antibody binding, and to determine the necessity of antigen retrieval. Dilution experiments were completed on positive control tissue from CNS locations where the protein was known to be expressed. A dilution was selected if it allowed distinct cellular visualization and low levels of background fluorescence. Cervical spinal cord tissue sections were incubated with NeuN primary antibody dilutions of 1:100, 1:500, 1:1000, and 1:2000 (Figure 5). The 1:500 NeuN primary antibody dilution was optimal based on limited background staining and clear neuronal visualization. The more concentrated NeuN primary antibody dilution (1:100) displayed high non-specific background fluorescence, while lower concentrations of the NeuN primary antibody caused fluorescence not easily distinguishable from the non-specific background fluorescence. For Iba1, cervical spinal cord tissue sections were incubated with 1:100, 1:500, 1:1000, 1:2000 dilutions. The optimal Iba1 primary antibody dilution was 1:1000 based on limited background staining and clear cellular visualization of microglia and their corresponding projections. Based on the laboratory's previous unpublished experiments using the GFAP primary antibody in the central nervous system, the 1:500 dilution was selected. For the RECA-1 primary antibody, cervical spinal cord sections and cortex sections (where RECA-1 is known to be expressed on endothelial cells) were incubated with 1:50, 1:250, 1:750, and 1:1000 dilutions. The

optimal RECA-1 primary antibody dilution was determined to be (1:250) based on high non-specific background fluorescence at more concentrated dilutions and no clear visualization of endothelial cells at lower concentrations.

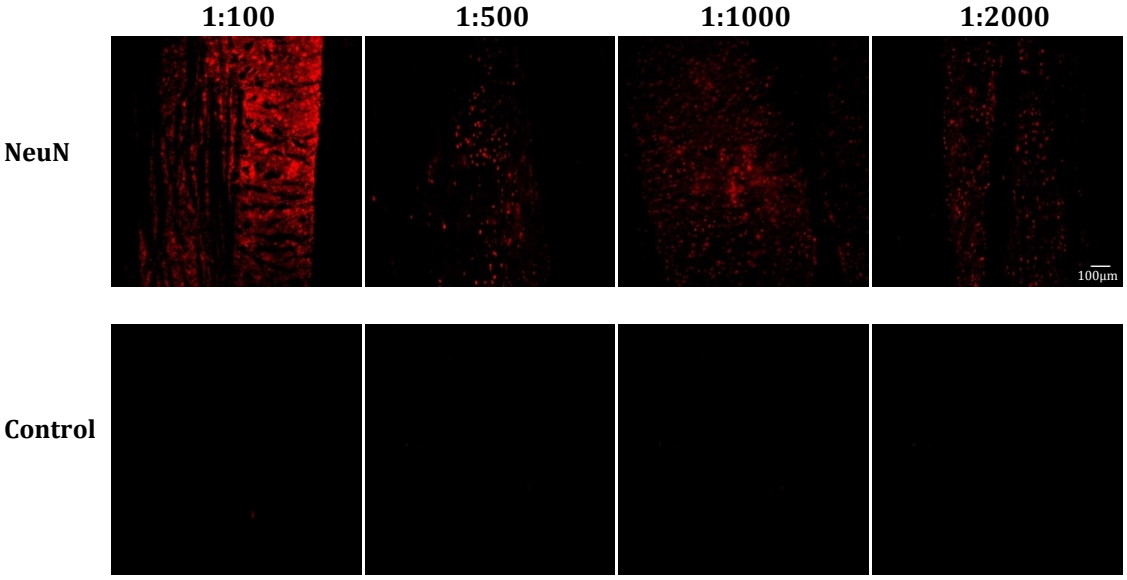


Figure 5. A representative dilution for the NeuN primary antibody dilution demonstrated 1:500 was the optimal dilution based the cellular visualization of the neurons and lowest non-specific background staining. NeuN dilution series in coronal sections of the cervical spinal cord of a saline treated male rat (immersion fixed for 5 months in PFA, stored in antifreeze for 7 months after sectioning). NeuN dilutions used were 1:100, 1:500, 1:1000, and 1:2000. Lower panels contain no-NeuN primary control images (images of tissue incubated with secondary antibody and no primary antibody to control for secondary antibody binding other than to the NeuN primary antibody) identically processed as the images in the top row.

Hippocampal tissues, where IL-1RIs are known to be expressed on neurons and astrocytes (Ravizza & Vezzani, 2006), was incubated with 1:50, 1:100, 1:250, and 1:500 dilutions of the IL-RI primary antibody to determine the optimal dilution in a location of known expression. The 1:250 primary antibody dilution was determined optimal as there was clear visualization of astrocytes and limited background fluorescence (astrocytic IL-1RI primary antibody localization confirmed by co-localization with GFAP primary antibody, data not shown). Higher concentrations

demonstrated high non-specific fluorescence, while lower antibody concentrations demonstrated fewer astrocytic projections (Figure. 8).

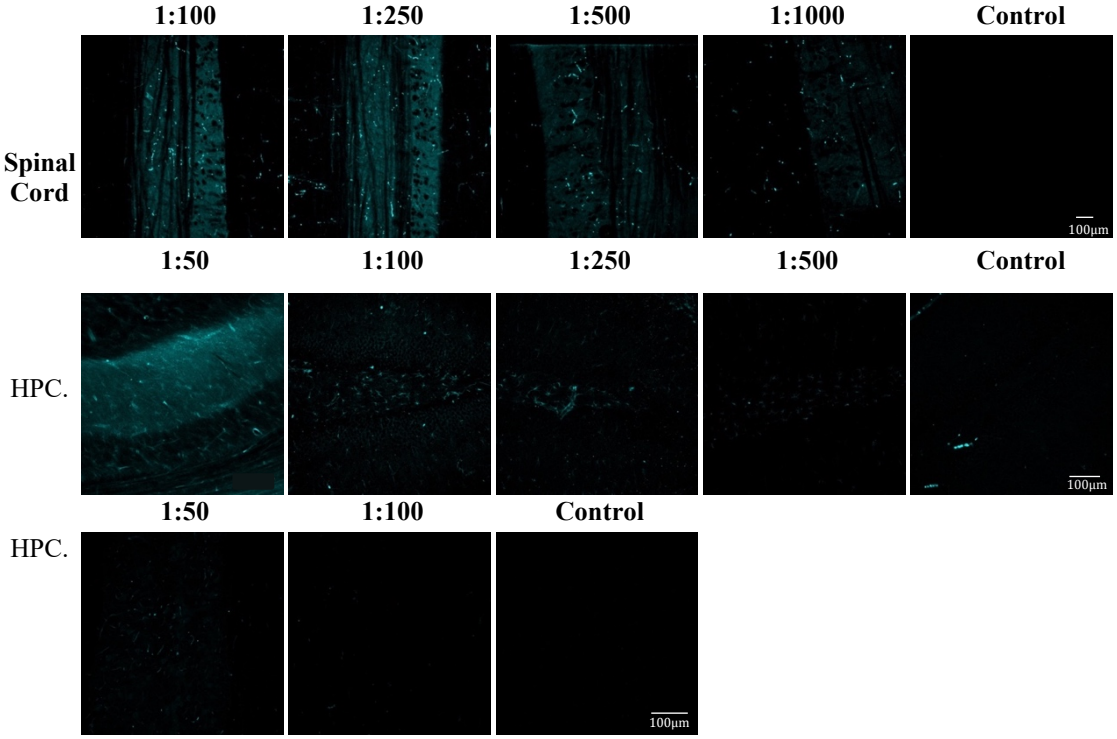


Figure 6. The 1:250 IL-RI dilution was appropriate to identify presumptive blood vessels in the cervical spinal cord, while minimizing non-specific binding, the 1:250 and 1:100 dilutions provided similar visualization of presumptive blood vessels, but in the 1:250 dilution presumptive blood vessels are more easily distinguished from the background and have less non-specific staining (e.g. background staining is lower). Representative confocal images (10x magnification, immersion fixed for 5 months, stored in antifreeze for 7 months) demonstrate a dilution series of IL-1RI (blue) staining in the cervical spinal cord (n=3). Subsequent IL-1RI dilution series experiment in the hippocampus (HPC. LPS treated male, immersion fixed for 20 months, stored in antifreeze for 24 hours) demonstrated 1:250 was sufficient to visualize IL-1RI fluorescence on astrocytes (middle row). However, a third dilution series experiment, also in the hippocampus (female LPS treated 6 times, immersion fixed for 16 days, stored in antifreeze for 6 days), indicated that even 1:50 was too dilute to clearly visualize IL-1RI florescence. All control images are no-IL-1RI primary antibody controls, to verify that the IL-1RI secondary antibody is not binding non-specifically to the tissue or to the other primary antibodies.

While one experiment demonstrated IL-1RI on astrocytes in the hippocampus with an optimal antibody dilution of 1:250 (Figure 9, middle row) images were not

taken with settings optimized for each dilution, but rather all at the same settings. In order to accurately determine optimal dilution, images must be taken with settings that are optimal for each individual antibody dilution, so a subsequent dilution series experiment in the hippocampus was necessary. In the subsequent hippocampal dilution series with the IL-1RI primary antibody, staining was not co-localized with astrocyte primary antibody fluorescence and the 1:250 dilution was insufficient for visualizing IL-1RI primary antibody fluorescence (Figure 9, bottom row).

A series of troubleshooting experiments were conducted to determine the cause of the inconsistency in IL-1RI primary antibody localization. It was hypothesized an experimental error (such as a pipetting error), physical interaction between the two tissues blocking the IL-1RI binding sites during antibody incubation, antifreeze physically blocking the IL-1RI binding sites preventing, or prolonged storage in PFA causing protein crosslinks to form occurred which interfered with the IL-1RI primary antibody binding to the IL-1RIs. However, the troubleshooting experiments demonstrated the inconsistency was not caused by incubating two tissue sections per well; there was no appreciable difference in NeuN or IL-1RI secondary antibody fluorescence when comparing a tissue section incubated in a well by itself to a tissue section from the same animal incubated two tissue sections in one well. The inconsistency was not caused by lingering antifreeze, as a lack of IL-1RI fluorescence localized to astrocytes was observed in a spinal cord and hippocampal tissue sectioned and stored immediately in PBS in the fridge rather than in antifreeze. Prolonged storage in PFA was not the cause of IL-1RI fluorescence inconsistency, as no IL-1RI fluorescence was visualized on astrocytes in hippocampal sections from an LPS-treated

animal immersion perfused in PFA for 24 hrs. Fixing the tissue with PFA for over 24 hours was also determined to not be the cause of the inconsistency as an antigen retrieval technique (eliminates protein crosslinks) did not illustrate IL-1RI fluorescence localized to astrocytes in the hippocampus. However, the antigen retrieval technique did appear to increase IL-1RI fluorescence in the cervical spinal cord. Additionally, the lack of hippocampal IL-1RI fluorescence on astrocytes was not caused by an unknown experimenter inconsistency as the dilution series was replicated by another member of the Huxtable laboratory and also found no IL-1RI florescence localized to astrocytes in the hippocampus. Thus, in total, our treatments did not provide evidence suggesting IL-1RI presence on astrocytes in the hippocampus. These data also suggest 1:250 is the optimal dilution of the IL-1RI primary antibody in the cervical spinal cord.

Interleukin-1 Receptor expression increases on neurons and astrocytes after systemic inflammation

To determine if systemic inflammation induced by LPS increased IL-1RI expression in the cervical spinal cord, the IL-1RI fluorescence (utilizing the antigen retrieval technique) was visually compared between control saline treated animals and LPS treated animals. Qualitatively, IL-1RI expression appears increased in the LPS treated animals (Figures 7-9, top rows) compared to the saline treated animals (Figures 7-9, bottom rows). A qualitative increase in IL-1RI fluorescence in the LPS-treated animals suggests increased IL-1RI expression in the cervical spinal cord of animals after systemic inflammation.

To determine the cellular location of IL-1RIs, LPS and saline-treated ventral cervical spinal cord tissues were triple-labeled with either NeuN, GFAP, and IL-1RI primary antibodies or NeuN, Iba1, and IL-1RI primary antibodies. Then, co-labeling of IL-1RI fluorescence with fluorescence from cell specific markers was analyzed to determine the location of IL-1RI on specific cell types. In the cervical spinal cord sections of LPS-treated animals, IL-1RI fluorescence co-localized with NeuN fluorescence (Figure 7, top row white circles), indicating IL-1RI are expressed on neurons. The overlay images illustrate IL-1RI fluorescence co-localizing with the GFAP fluorescence (Figure 8, white circles), indicating IL-1RI are expressed on astrocytes. IL-1RI fluorescence did not co-label with Iba1 fluorescence in LPS or saline treated animals (Figure 9, top row white circles), indicating IL-1RIs are not expressed on microglia. The qualitative results indicate IL-1RI expression increases with systemic

inflammation and is present on neurons and astrocytes, but not microglia, in the cervical spinal cord.

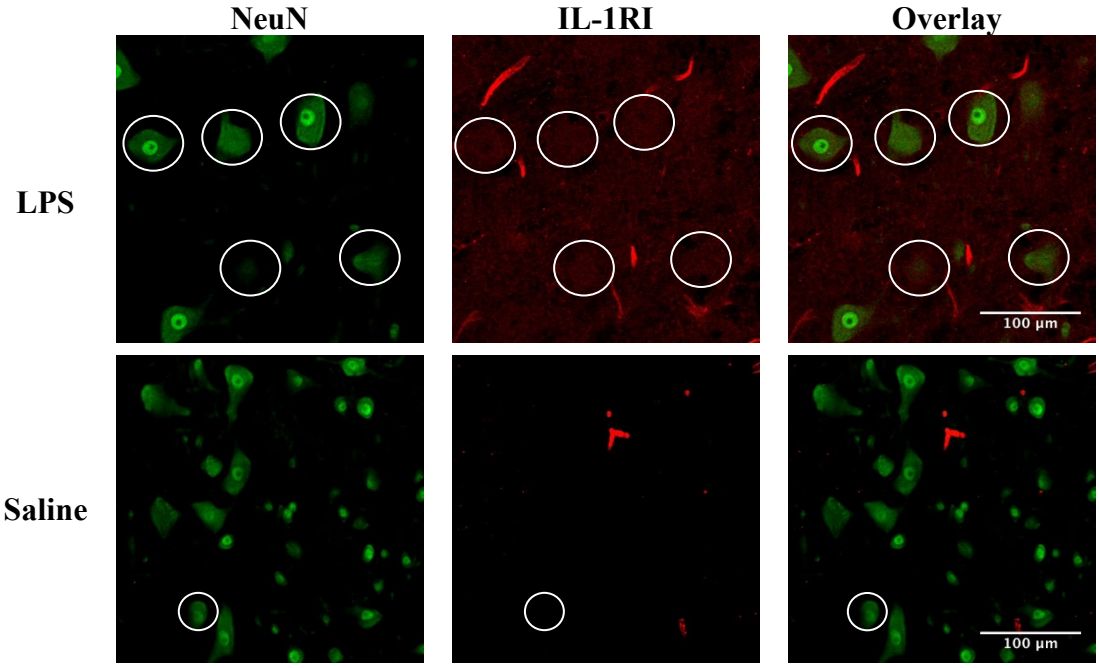


Figure 7. IL-1RI expression is present on neurons, and neuronal IL-1RI expression increases after systemic inflammation compared to saline treated. Representative confocal images (20x magnification, LPS treated stored in antifreeze for 13 months, saline treated in antifreeze for 6 days) illustrate IL-1RI (red) staining in the cervical spinal cord colocalized with neurons (NeuN, green) after systemic inflammation (n=8). As illustrated by the white circles, IL-1RIs are expressed on most neurons after systemic inflammation (LPS), and not expressed on neurons without systemic inflammation (saline).

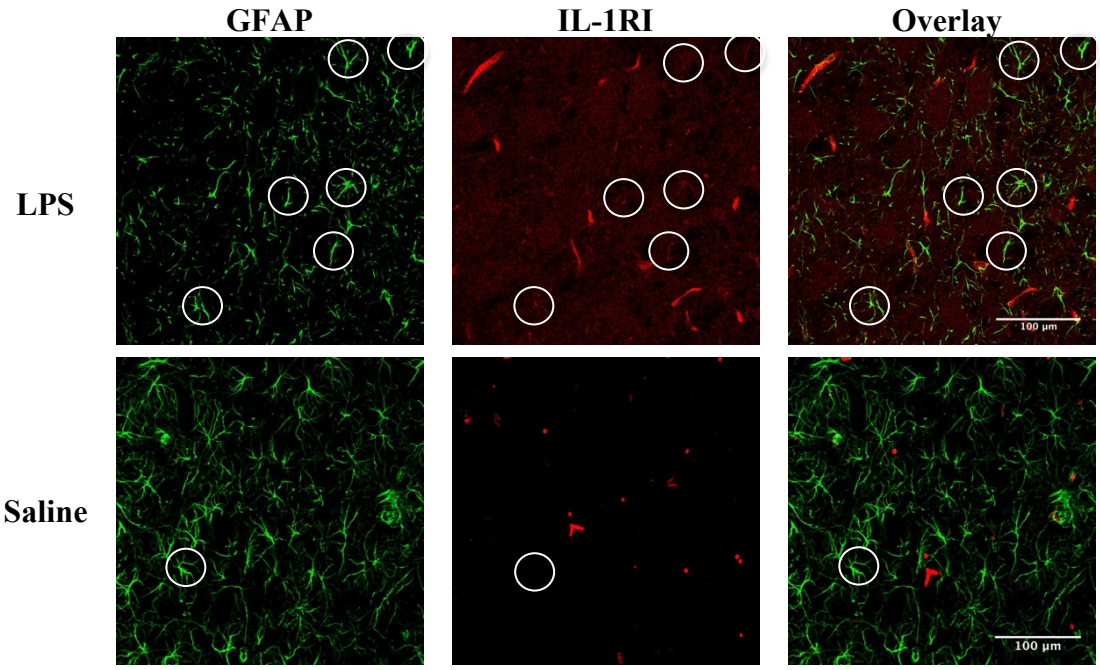


Figure 8. IL-1RI expression is present on astrocytes, and astrocytic IL-1RI expression increases after systemic inflammation compared to saline treated. Representative confocal images (20x magnification, LPS treated stored in antifreeze for 13 months, saline treated stored in antifreeze for 6 days) illustrate IL-1RI (red) staining in the cervical spinal cord colocalized with astrocytes (GFAP, green) after systemic inflammation (n=3). As illustrated by the white circles, IL-1RIs are expressed on some astrocytes after systemic inflammation (LPS), and not evident on astrocytes from saline treated (e.g. those without systemic inflammation).

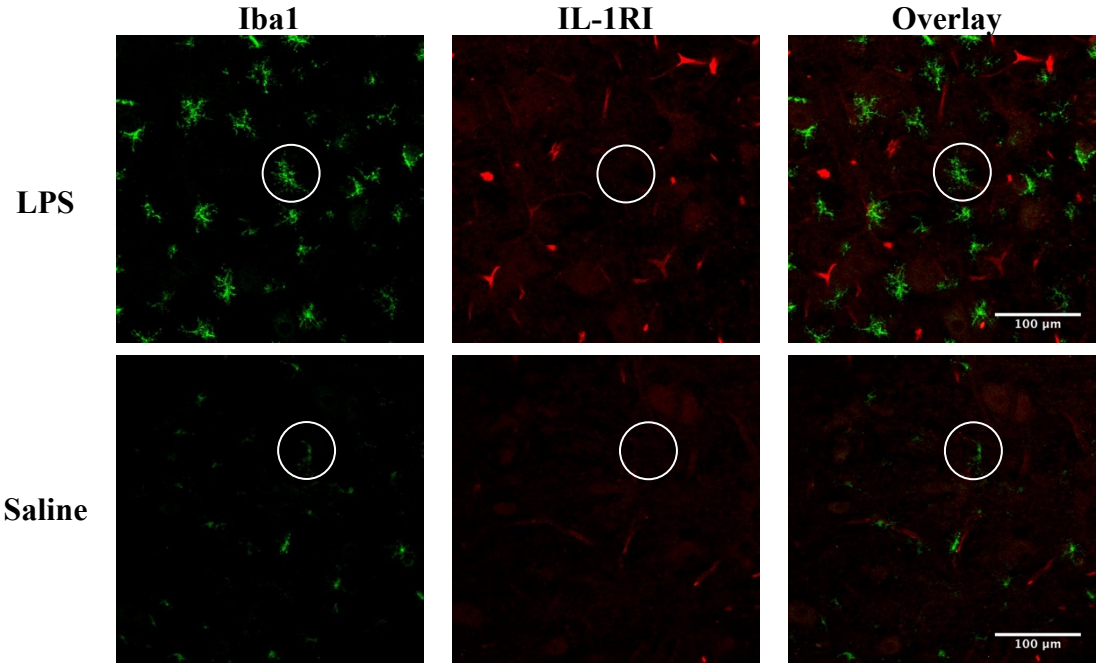


Figure 9. IL-1RI expression increases after systemic inflammation compared to saline treated, but IL-1RI is not present on microglia. Representative confocal images (20x magnification, LPS treated stored in antifreeze for 12 months, saline treated stored in antifreeze for 11 months) illustrate minimal IL-1RI (red) staining present without systemic inflammation (saline), and increased IL-1RI staining with systemic inflammation (LPS) compared to the saline treatment. IL-1RI staining in the cervical spinal cord did not colocalize with microglia (Iba1, green) after systemic inflammation (n=4).

Interleukin-1 Receptors are expressed on endothelial cells after systemic inflammation

Since IL-RI antibody fluorescence was visualized in locations that did not co-localize with any of the three specific cell types, it was hypothesized that IL-1RI are expressed on endothelial cells in the cervical spinal cord. To determine if IL-1RIs are expressed on endothelial cells after inflammation, LPS-treated cervical spinal cord tissue was incubated with RECA-1 and IL-1RI primary antibodies (utilizing the antigen retrieval technique). Preliminary data suggests IL-1RI expression is localized to some endothelial cells in the cervical spinal cord after systemic inflammation, as IL-1RI secondary fluorescence co-localizes with RECA-1 secondary fluorescence (Figure. 10).

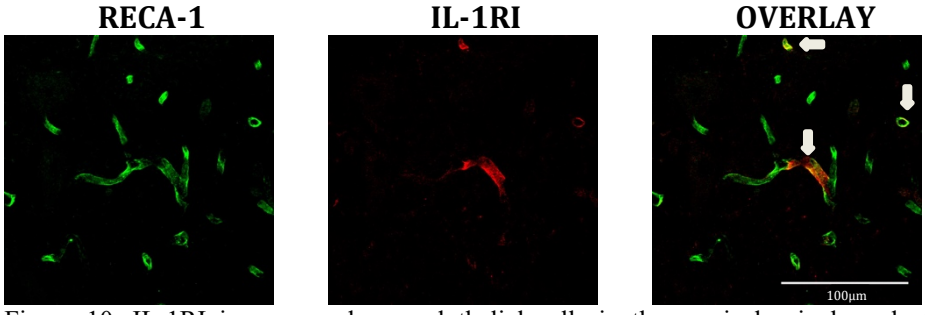


Figure 10. IL-1RI is expressed on endothelial cells in the cervical spinal cord after systemic inflammation. Representative confocal images (60x magnification, 25 minute antigen retrieval, stored in antifreeze for 3 months) demonstrate IL-1RI (red) staining colocalizing with endothelial cells (RECA-1, green) in the cervical spinal cord after systemic inflammation (n=2). As illustrated by the white arrows, IL-1RIs are expressed on endothelial cells.

Discussion

The purpose of this study was to identify the effects of systemic inflammation on expression of IL-1RI in the cervical spinal cord and determine which cells express IL-1RI in the cervical spinal cord in order to determine the cell types able to participate in the IL-1RI mediated inflammatory signaling cascade. The results suggest IL-1RI expression is increased in the cervical spinal cord after inflammation, and IL-1RIs are expressed on neurons, astrocytes, and endothelial cells in the cervical spinal cord. First, the optimal primary antibodies dilutions were determined and, due to an inconsistency in IL-1RI positive control results, troubleshooting experiments were completed to elucidate the cause of the inconsistencies and determine the optimal IL-1RI primary antibody dilution. These troubleshooting experiments confirmed a lack of astrocytic IL-1RI expression in the hippocampus, contrary to previously published results, was not due to an experimental error, such as antifreeze not being properly washed off, unknown experimenter specific errors, or fixation in PFA causing protein crosslinks. It was then concluded that the IL-1RI localization observed in the initial hippocampus dilution experiment was not reproducible, and in this particular strain of rat, astrocytes in the hippocampus likely do not express IL-1RIs. Second, triple label experiments were conducted on cervical spinal cord tissue sections from control and LPS treated animals. IL-1RI secondary antibody fluorescence on tissue from the LPS treated animals was greater than the control tissue IL-1RI secondary fluorescence. Co-localization between GFAP and IL-1RI secondary antibody fluorescence was visualized indicating IL-1RI are expressed on astrocytes in the cervical spinal cord. Similarly, RECA-1 and NeuN secondary fluorescence both co-localized with IL-1RI secondary fluoresces. It was

concluded that IL-1RI expression increased in the cervical spinal cord after systemic inflammation, and IL-1RI expressed was localized to neurons, astrocytes and endothelial cells.

As expected, expression of IL-1RI in the cervical spinal cord increased after systemic inflammation. Increased expression of IL-1RI after systemic inflammation was expected since IL-1β mRNA in the rat cervical spinal cord was shown to increase after systemic inflammation (Huxtable *et al.*, 2013). However, it was previously not known if abolishment of neuroplasticity after systemic inflammation was caused by increased synthesis of IL-1β and activation of IL-1RIs expressed on select cells of the CNS, or if the expression of IL-1RIs also increased after systemic inflammation. During inflammation not only does the relative amount of IL-1β protein likely increase, but the present study indicates the expression of the IL-1RIs also increases. Increased IL-1RI expression supports the proposed mechanism that inflammation allows neurons and astrocytes to become more responsive to further inflammatory signals. As demonstrated here, systemic inflammation causes an upregulation of IL-1RIs on neurons and astrocytes, potentially allowing more IL-1β to bind and propagate the inflammatory signal. With more receptors present on the neurons and astrocytes, it is possible for more IL-1β to bind and both propagate inflammation, as well as induce behavioral alterations in neurons, potentially leading to the abolishment of neuroplasticity.

Here, IL-1RI was shown to be expressed on astrocytes and neurons in the cervical spinal cord, but surprisingly not on microglia, the main immune cells of the CNS. Other studies have shown IL-1RI mRNA expression in microglia (from mouse cortex glial culture) is much lower than in astrocytes (Krasnow *et al.*, 2017). These data

support microglia not expressing IL-1RIs in the cervical spinal cord. When astrocytes are treated with IL-1β, they undergo significant increases in pro-inflammatory gene expression (Krasnow *et al.*, 2017), while microglia only slightly increase inflammatory gene expression when treated with IL-1β (Krasnow *et al.*, 2017). Illustrating that microglia are likely not key cells responsible for IL-1RI signaling. Additionally, microglia can become activated by the release of signaling molecules from the astrocytes which have been treated with IL-1β (Krasnow *et al.*, 2017). The findings from the present study demonstrate microglia do not express IL-1RI in the cervical spinal cord, and astrocytes do express IL-1RI, suggesting astrocytes may play a role in activating other CNS cells and propagating systemic inflammation following astrocytic IL-1RI activation.

As IL-1RI fluorescence appeared to be localized to presumptive blood vessels, follow-up experiments were conducted to determine if the IL-1RI fluorescence was localized to identified endothelial cells of blood vessels. The results suggest IL-1RIs are expressed on some endothelial cells in the cervical spinal cord after inflammation. Recent studies have indicated IL-1RI receptor activation on endothelial cells is responsible for mediating the central response to systemic inflammation (Krasnow *et al.*, 2017). IL-1RI mRNA studies indicate that in addition to IL-1RI expression present on endothelial cells after systemic inflammation, there is a basal level of IL-1RI expression on endothelial cells in the control murine CNS (Krasnow *et al.*, 2017). Endothelial cells make up the blood brain barrier between the CNS and the blood stream, suggesting that those cells would be able to respond to circulating pro-inflammatory cytokines.

The IL-1RI visualized in locations other than co-labeling with neurons, astrocytes, microglia, or endothelial cells could be a soluble form of the IL-1RI receptor located in the extracellular matrix (Jacobs *et al.*, 1991). In mice, soluble IL-1RI are released from immune cells to reduce the toxicity of excessive amounts of IL-1RI activation (Jacobs *et al.*, 1991)(Verschueren *et al.*, 1999). Soluble IL-1RIs are able to bind free pro-inflammatory IL-1 molecules and reduce the amount of IL-1β able to bind to IL-1RIs located on the cell membranes. Less IL-1RI activation could mitigate the inflammatory response and would be an important inflammation inhibitor.

Inflammation abolishes respiratory neuroplasticity and involve key respiratory-related neurons of the cervical spinal cord, phrenic motor neurons (Huxtable *et al.,* 2013; Huxtable *et al*., 2015; Hocker & Huxtable, 2017). Since the neuroplasticity of respiratory-related neurons is decreased with inflammation, and with spinal applications of IL-1β (Hocker & Huxtable, 2018), IL-1β appears increase output from respiratory-related neurons. Levels of IL-1β circulating in the blood increase with systemic inflammation (Kakizaki *et al.*, 1999), and may induce central inflammation by activating the vagus nerve and causing the release of pro-inflammatory cytokines (including IL-1β) in the CNS, which could bind to neurons or astrocytes (Watkins *et al.*, 1995; Goehler *et al.*, 2000). Since IL-1RI is expressed on neurons in the cervical spinal cord, IL-1β may be able to act directly on phrenic motor neurons to abolish neuroplasticity. IL-1β may also be able to indirectly affect neurons through an astrocyte-mediated signaling cascade, similarly to how IL-1β can indirectly activate microglia via astrocyte activation (Krasnow *et al.*, 2017). Circulating of systemic levels of IL-1β may also induce peripheral inflammation by binding to the endothelial cells,

which make up the blood brain barrier and where IL-1RI has been demonstrated in this study to be expressed, to propagate the peripheral inflammatory signal to the central nervous system. Astrocytes are potentially able to propagate an IL-1β-mediated pro-inflammatory response from the endothelial cells (Krasnow *et al.*, 2017), to phrenic motor neurons to ultimately abolish respiratory plasticity. Astrocytes, which have projections wrapping around endothelial cells within the CNS (as illustrated by Figure 1), could be activated by IL-1β and propagate the inflammatory signal to the neurons and other CNS cells, like microglia. The results from this study indicate neurons, astrocytes, and endothelial cells can bind the pro-inflammatory singling molecule IL-1β, but do not indicate the mechanism by which IL-1RI activation abolishes respiratory neuroplasticity.

Knowing IL-1RI expression is upregulated after systemic inflammation and IL-1RIs are located on neurons, astrocytes, and endothelial cells in the cervical spinal cord, provides the field with a greater knowledge about the potential mechanism for the impairment of respiratory neuroplasticity. Future research can investigate the role of astrocytes, neurons, and endothelial cells may play in abolishing respiratory neuroplasticity. When utilizing neuroplasticity as a therapy for patients suffering from cervical spinal cord injuries, a better understanding the mechanisms by which neuroplasticity is undermined will aid in developing maximally effective therapies. In developing therapies relying on respiratory neuroplasticity, it is key to know not only does systemic inflammation increase IL-1β and IL-1RI expression, but also the cellular location of the IL-1RIs in order to mitigate the effects of inflammation on respiratory neuroplasticity.

Limitations

There are number of potential expansions on the study, but these limitations do not diminish the results. One potential limitation or area for expansion is NeuN binds to a protein present in all neurons, and as such it cannot be determined if IL-1RIs are expressed on phrenic motor neurons rather than interneurons or non-phrenic motor neurons. Determining the neuron type would expand the study since IL-1RIs fluorescence was not present on all neurons in the cervical spinal cord. Another potential limitation was GFAP does not label all astrocytic projections (Mudannayake *et al.*, 2016), meaning some of IL-1RI staining in regions not co-localized with GFAP may represent unlabeled astrocytic projections or another population of astrocytes not expressing GFAP. Additionally, labeling all astrocytic projections would allow for better determination of IL-1RI expression of astrocyte foot projections, which interact with endothelial cells. Another limitation is there was IL-1RI fluorescence which did not appear localized to neurons, microglia, astrocytes, or endothelial cells. NeuN labels the cell bodies of the neurons, not axons (neuronal projections), so it was not possible to determine if the IL-1RI expression seen in the cervical spinal cord not localized to a specific cell type was expressed on unlabeled neuronal axons, astrocytic projections, or if it was present in the extracellular matrix. An astrocytic marker present on the projections could be used, such as Aldehyde Dehydrogenase 1 Family Member A1 (ALDH1A1)(Mudannayake *et al.*, 2016), which would allow for clear determination of IL-1RI fluorescence localized to astrocyte projections. A different neuronal label (such as TAU or NFP) could be used to label the axons of the neurons, which would allow for clear determination of IL-1RI fluorescence localized to axons. A label for proteins

located the extracellular matrix (such as anti-collagen I) could also be used to determine if IL-1RI is expressed there. A final limitation is immunohistochemistry was utilized as a qualitative determination of IL-1RI changes, not a quantitative assessment. IHC was not used to make a quantitative assessment of IL-1RI expression since quantification of fluorescence levels is complicated by an arbitrary determination of signal to noise, which may impact the concluded results. Bias may also be introduced when deciding which fluorescence levels are background and which are not, if it is decided by the experimenter rather than a blinded third party viewer. IHC was also not used to make a quantitative assessment since it is easier to get a false positive result when mathematically determining significant increases or decreases in fluorescence, than when they are determined visually; differences in fluorescence may be deemed mathematically significant when there is not a clear visual difference.

Future directions

In the future, phrenic motor neurons should be identified in order to determine if the IL-1RI expression in the cervical spinal cord is localized specifically to phrenic motor neurons. Phrenic motor neurons could be labeled using a technique called "back-labeling". Cholera toxin B subunit is injected into the intrapleural space, where it is taken up by the phrenic motor neuron axons and transported to the neuron cell bodies within the cervical spinal cord (Dale *et al.*, 2017). Cholera toxin presence in phrenic neurons can then be detected using IHC (Dale *et al.*, 2017).

Another experiment which could be completed to confirm IL-1RI is expressed on astrocytes and motor neurons would be to do cell isolation. The astrocytes could be isolated from a chemically or mechanically digested, heterogeneous representative

sample of cervical spinal cord tissue using a number of techniques. One way to isolate astrocytes would be to insert a green fluorescent protein (GFP) into an astrocyte specific DNA region of the animal, harvest the cervical spinal cord tissue, and use a machine called a fluorescently-activated cell sorter to isolate the fluorescently labeled astrocytes from the digested heterogeneous cell mixture (Foo, 2013). In the same way, GFP neurons could be isolated. Once the astrocytes or neurons have been isolated, the homogenous mixture could be further digested and analyzed to determine IL-1RI protein expression. Analysis methods such as an enzyme-linked immunosorbent assay (ELISA) would utilize an IL-1RI primary antibody to bind the IL-1RIs from the digested astrocytes to a surface, a secondary antibody is then bound to the IL-1RI primary antibody for visible detection. Unlike with immunohistochemistry, the fluorescence from an ELISA can be more accurately quantified. Relative increase in IL-1RI receptor expression on these cell types after induced systemic inflammation could be quantifiably determined using the cell isolation methods. The cell isolation experiment could also determine if IL-1RIs are more highly expressed on neurons or astrocytes.

Glossary

1. Antibody: A protein synthesized by the immune system to target foreign substances, particularly in the blood. Used in IHC to bind target proteins for visualization.
2. Cardiovascular or circulatory system: Organ system consisting of the heart, blood, blood vessels, lymph, and lymphatic tissues. Cardiovascular system is used to transport oxygen and nutrients throughout the body.
3. Central Nervous System: Organ system consisting of the spinal cord and brain. Responsible for control of the body, higher reasoning, and other organ systems.
4. Cervical spinal cord: The section of the spinal cord located along the neck which controls breathing.
5. Coronal sectioning: Slicing along the coronal plane. This plane divides the body in half front to back.
6. Cytokines: Signaling molecules secreted by cells.
7. Cytoplasm: All parts of the cell within the cell membrane, except for the nucleus.
8. Dorsal: Towards the backside/rear of the body.
9. Endotoxin: A toxin released from a bacterial cell.
10. Heterogeneous: Consisting of different elements/constituents.
11. Hippocampus: Area of the brain that is part of the limbic system. It is involved in learning and memory.
12. Homeostasis: The regulation of the body, keeping its internal conditions near specific dynamic equilibrium values.
13. Homogenous: Consisting of similar elements/constituents.
14. Intracellular: Within the cell.
15. Intercellular: Between cells.
16. Intermediate Filaments: Protein involved in maintaining cell shape and movement.
17. Lipopolysaccharide: Endotoxin found on the cell membrane of Gram-negative bacteria (such as E. coli).
18. Microtome: Machine with a sharp blade used to cut sections of tissue into thin sections for microscopic visualization, without damaging the tissue by exposure to dehydration, extreme temperatures, or harsh chemicals as are used in other sectioning methods.
19. Medial: Closer to the midline.
20. Periphery: The aspect of the body not including the CNS.
21. Proteins: Large biological molecules with a variety of functions
22. Quantitative: Measuring by amount, or quantity.
23. Qualitative: Measuring by a characteristic (e.g. size or appearance) rather than quantity.
24. Respiratory system: Organ system including the lungs, as well as the oral and nasal respiratory tracts. Responsible for gas exchange mediating homeostatic levels of oxygen, carbon dioxide, and hydrogen ions.

25. Sagittal sections: Slicing along the sagittal plane. This plane divides the body in half left to right.
26. Systemic Inflammation: Inflammation spread throughout the body.
27. Transcardial: Through the heart.
28. Ventral: Towards the front of the body.

Bibliography

Al-Ali SY & Al-Hussain SM (1996). An ultrastructural study of the phagocytic activity of astrocytes in adult rat brain. *J Anat* **188,** 257–262.

American Society of Biological Chemists., Rockefeller Institute for Medical Research. & American Society for Biochemistry and Molecular Biology. (n.d.). *The Journal of biological chemistry.* American Society for Biochemistry and Molecular Biology. Available at: http://www.jbc.org/content/262/7/2941.short [Accessed April 5, 2018].

Araque A, Parpura V, Sanzgiri RP & Haydon PG (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci* **22,** 208–215.

Baker-Herman TL, Mitchell GS, Muir GD, Wilkerson JER, Hoffman MS, Vinit S & Mitchell GS (2002). Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. *J Neurosci* **22,** 6239–6246.

Berlowitz DJ, Brown DJ, Campbell DA & Pierce RJ (2005). A Longitudinal Evaluation of Sleep and Breathing in the First Year After Cervical Spinal Cord Injury. *Arch Phys Med Rehabil* **86,** 1193–1199.

Dale EA, Fields DP, Devinney MJ & Mitchell GS (2017). Phrenic motor neuron TrkB expression is necessary for acute intermittent hypoxia-induced phrenic long-term facilitation. *Exp Neurol* **287,** 130–136.

Colonna M & Butovsky O (2017). Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annu Rev Immunol* **35,** 441–468.

Dale-Nagle EA, Hoffman MS, MacFarlane PM, Satriotomo I, Lovett-Barr MR, Vinit S & Mitchell GS (2010). Spinal plasticity following intermittent hypoxia: implications for spinal injury. *Ann N Y Acad Sci* **1198,** 252–259.

Dapson R (2007). Macromolecular changes caused by formalin fixation and antigen retrieval. *Biotech Histochem* **82,** 133–140.

Eng LF (1985). Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. *J Neuroimmunol* **8,** 203–214.

Faggioni R, Benigni F & Ghezzi P (1995). Proinflammatory cytokines as pathogenetic mediators in the central nervous system: brain-periphery connections. *Neuroimmunomodulation* **2,** 2–15.

Foo LC (2013). Purification of astrocytes from transgenic rodents by fluorescence-activated cell sorting. *Cold Spring Harb Protoc* **2013,** 551–560.

Friedman WJ (2001). Cytokines Regulate Expression of the Type 1 Interleukin-1 Receptor in Rat Hippocampal Neurons and Glia. *Exp Neurol* **168,** 23–31.

Fuller DD, Zabka AG, Baker TL & Mitchell GS (2001). Selected Contribution: Phrenic long-term facilitation requires 5-HT receptor activation during but not following episodic hypoxia. *J Appl Physiol* **90,** 2001–2006.

Fuller D., Bach K., Baker T., Kinkead R & Mitchell G. (2000). Long term facilitation of phrenic motor output. *Respir Physiol* **121,** 135–146.

Giri JG, Wells J, Dower SK, McCall CE, Guzman RN, Slack J, Bird TA, Shanebeck K, Grabstein KH & Sims JE (1994). Elevated levels of shed type II IL-1 receptor in sepsis. Potential role for type II receptor in regulation of IL-1 responses. *J Immunol* **153,** 5802–5809.

Goehler LE, Gaykema RPA, Hansen MK, Anderson K, Maier SF & Watkins LR (2000). Vagal immune-to-brain communication: a visceral chemosensory pathway. *Auton Neurosci* **85,** 49–59.

Hayes HB, Jayaraman A, Herrmann M, Mitchell GS, Rymer WZ & Trumbower RD (2014). Daily intermittent hypoxia enhances walking after chronic spinal cord injury: a randomized trial. *Neurology* **82,** 104–113.

Hocker AD & Huxtable AG (2018). IL-1 receptor activation undermines respiratory motor plasticity after systemic inflammation. *J Appl Physiol*japplphysiol.01051.2017.

Hocker AD, Stokes JA, Powell FL & Huxtable AG (2017). The impact of inflammation on respiratory plasticity. *Exp Neurol* **287,** 243–253.

Holló K, Ducza L, Hegyi Z, Dócs K, Hegedűs K, Bakk E, Papp I, Kis G, Mészár Z, Bardóczi Z & Antal M (2017). Interleukin-1 receptor type 1 is overexpressed in neurons but not in glial cells within the rat superficial spinal dorsal horn in complete Freund adjuvant-induced inflammatory pain. *J Neuroinflammation* **14,** 125.

Huxtable AG, Smith SMC, Vinit S, Watters JJ & Mitchell GS (2013). Systemic LPS induces spinal inflammatory gene expression and impairs phrenic long-term facilitation following acute intermittent hypoxia. *J Appl Physiol* **114,** 879–887.

Huxtable AG, Vinit S, Windelborn JA, Crader SM, Guenther CH, Watters JJ & Mitchell GS (2011). Systemic inflammation impairs respiratory chemoreflexes and plasticity. *Respir Physiol Neurobiol* **178,** 482–489.

Imai Y, Ibata I, Ito D, Ohsawa K & Kohsaka S (1996). A Novel Geneiba1in the Major Histocompatibility Complex Class III Region Encoding an EF Hand Protein

Expressed in a Monocytic Lineage. *Biochem Biophys Res Commun* **224,** 855–862.

Jacobs CA, Baker PE, Roux ER, Picha KS, Toivola B, Waugh S & Kennedy MK (1991). Experimental autoimmune encephalomyelitis is exacerbated by IL-1 alpha and suppressed by soluble IL-1 receptor. *J Immunol* **146,** 2983–2989.

Jafri A, Belkadi A, Zaidi SIA, Getsy P, Wilson CG & Martin RJ (2013). Lung inflammation induces IL-1β expression in hypoglossal neurons in rat brainstem. *Respir Physiol Neurobiol* **188,** 21–28.

Kakizaki Y, Watanobe H, Kohsaka A & Suda T (1999). Temporal profiles of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in the plasma and hypothalamic paraventricular nucleus after intravenous or intraperitoneal administration of lipopolysaccharide in the rat: estimation by push-pull perfusion. *Endocr J* **46,** 487–496.

Krasnow SM, Knoll JG, Verghese SC, Levasseur PR & Marks DL (2017). Amplification and propagation of interleukin-1β signaling by murine brain endothelial and glial cells. *J Neuroinflammation* **14,** 133.

Lawson LJ, Perry VH & Gordon S (1992). Turnover of resident microglia in the normal adult mouse brain. *Neuroscience* **48,** 405–415.

Liddelow SA et al. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541,** 481–487.

Ling L, Fuller DD, Bach KB, Kinkead R, Olson EB & Mitchell GS (2001). Chronic intermittent hypoxia elicits serotonin-dependent plasticity in the central neural control of breathing. *J Neurosci* **21,** 5381–5388.

Lopez-Castejon G & Brough D (2011). Understanding the mechanism of IL-1β secretion. *Cytokine Growth Factor Rev* **22,** 189–195.

Lynch AM & Lynch MA (2002). The age-related increase in IL-1 type I receptor in rat hippocampus is coupled with an increase in caspase-3 activation. *Eur J Neurosci* **15,** 1779–1788.

Majoor G, Duijvestijn AM, Van Goor H, Klatter F, Majoor GD, Bussel V, Van PJC & Vriesman B (1992). Antibodies defining rat endothelial cells: RECA-1, a pan-endothelial cell- specific monoclonal antibody Antibodi es Defining Rat Endothel ial Cells: RECA-1, APan-End othelial Cell-Sp ecific Monoclon al Antibody. Available at: https://www.researchgate.net/publication/21571617 [Accessed May 18, 2018].

MEDAWAR PB (1948). Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol* **29,** 58–69.

Menachem-Zidon O Ben, Avital A, Ben-Menahem Y, Goshen I, Kreisel T, Shmueli EM, Segal M, Hur B & Yirmiya R (2010). Astrocytes support hippocampal-dependent memory and long-term 3 potentiation via interleukin-1 signaling 4. *BRAIN, Behav Immun*; DOI: 10.1016/j.bbi.2010.11.007.

Mitchell GS, Baker TL, Nanda SA, Fuller DD, Zabka AG, Hodgeman BA, Bavis RW, Mack KJ & Olson EB (2001). Invited Review: Intermittent hypoxia and respiratory plasticity. *J Appl Physiol* **90,** 2466–2475.

Mitchell GS & Johnson SM (2003). Invited Review: Neuroplasticity in respiratory motor control. *J Appl Physiol* **94,** 358–374.

Mosser C-A, Baptista S, Arnoux I & Audinat E (2017). Microglia in CNS development: Shaping the brain for the future. *Prog Neurobiol* **149–150,** 1–20.

Mudannayake JM, Mouravlev A, Fong DM & Young D (2016). Transcriptional activity of novel ALDH1L1 promoters in the rat brain following AAV vector-mediated gene transfer. *Mol Ther - Methods Clin Dev*; DOI: 10.1038/MTM.2016.75.

Mullen, R.J., Buck, C.R. and Smith, A.M., 1992. NeuN, a neuronal specific nuclear protein in vertebrates. *Development*, *116*(1), pp.201-211.

The neuron – What happened to Chris. N.d. Accessed Feb. 8, 2018. [http://whathappenedtochris.weebly.com/uploads/1/3/7/6/13761436/973220257.gif]

Olson EB, Bohne CJ, Dwinell MR, Podolsky A, Vidruk EH, Fuller DD, Powell FL & Mitchel GS (2001). Ventilatory long-term facilitation in unanesthetized rats. *J Appl Physiol* **91,** 709–716.

Parkhurst CN & Gan W-B (2010). Microglia dynamics and function in the CNS. *Curr Opin Neurobiol* **20,** 595–600.

Perregaux DG & Gabel CA (1998). Post-translational processing of murine IL-1: evidence that ATP-induced release of IL-1 alpha and IL-1 beta occurs via a similar mechanism. *J Immunol* **160,** 2469–2477.

Popovich PG, Wei P & Stokes BT (1997). Cellular inflammatory response after spinal cord injury in sprague-dawley and lewis rats. *J Comp Neurol* **377,** 443–464.

Ravizza T & Vezzani A (2006). Status epilepticus induces time-dependent neuronal and astrocytic expression of interleukin-1 receptor type I in the rat limbic system. *Neuroscience* **137,** 301–308.

Röhl C, Lucius R & Sievers J (2007). The effect of activated microglia on astrogliosis parameters in astrocyte cultures. *Brain Res* **1129,** 43–52.

Rossetti, Fábia Cristina, Lívia Vieira Depieri, and Maria Vitória Lopes Badra Bentley. "Confocal laser scanning microscopy as a tool for the investigation of skin drug delivery systems and diagnosis of skin disorders." *Confocal Laser Microscopy-Principles and Applications in Medicine, Biology, and the Food Sciences.* InTech, 2013. Accessed on Feb. 8, 2018

Schematic of immunohistochemistry N.d. Date accessed Feb. 8, 2018 [https://upload.wikimedia.org/wikipedia/commons/thumb/3/37/Immunohistochemicalstaining2.PNG/300px-Immunohistochemicalstaining2.PNG]

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P & Cardona A (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9,** 676–682.

Schnydrig S, Korner L, Landweer S, Ernst B, Walker G, Otten U & Kunz D (2007). Peripheral lipopolysaccharide administration transiently affects expression of brain-derived neurotrophic factor, corticotropin and proopiomelanocortin in mouse brain. *Neurosci Lett* **429,** 69–73.

Svenson M, Hansen MB, Heegaard P, Abell K & Bendtzen K (1993). Specific binding of interleukin 1 (IL-1)β and IL-1 receptor antagonist (IL-1ra) to human serum. High-affinity binding of IL-1ra to soluble IL-1 receptor type I. *Cytokine* **5,** 427–435.

Trumbower RD, Jayaraman A, Mitchell GS & Rymer WZ (2012). Exposure to Acute Intermittent Hypoxia Augments Somatic Motor Function in Humans With Incomplete Spinal Cord Injury. *Neurorehabil Neural Repair* **26,** 163–172.

Verkhrasky A & Butt A (2013). Neuroglia: Definition, Classification, Evolution, Numbers, Development. In *Glial Physiology and Pathophysiology*, pp. 73–104. John Wiley & Sons, Ltd, Chichester, UK. Available at: http://doi.wiley.com/10.1002/9781118402061.ch3 [Accessed April 18, 2018].

Verschueren M, Dinarello CA, Van Der Mihai JWM, Netea G, Kullberg BJ, Boerman OC, Netea MG, Verschueren I & Van Der Meer JWM (2018). α Release of Constitutive IL-1 Soluble Murine IL-1 Receptor Type I Induces Soluble Murine IL-1 Receptor Type I Induces Release of Constitutive IL-1␣ 1. *J Immunol* **162,** 4876–4881.

Vinit S, Lovett-Barr MR & Mitchell GS (2009). Intermittent hypoxia induces functional recovery following cervical spinal injury. *Respir Physiol Neurobiol* **169,** 210–217.

Wang X, Wang B-R, Duan X-L, Zhang P, Ding Y-Q, Jia Y, Jiao X-Y & Ju G (2002). Strong Expression of Interleukin-1 Receptor Type I in the Rat Carotid Body. *J Histochem Cytochem* **50,** 1677–1684.

Watkins LR, Goehler LE, Relton JK, Tartaglia N, Silbert L, Martin D & Maier SF (1995). Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. *Neurosci Lett* **183,** 27–31.

Werman A, Werman-Venkert R, White R, Lee J-K, Werman B, Krelin Y, Voronov E, Dinarello CA & Apte RN (2004). The precursor form of IL-1alpha is an intracrine proinflammatory activator of transcription. *Proc Natl Acad Sci U S A* **101,** 2434–2439.