Pilot Study Exploring the Effect of Targeted COX-2 Inhibition in Macrophages Responding to Neuronal Injury; Promoting Enhanced Axonal Regeneration

Alyssa Brauckmann

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PILOT STUDY EXPLORING THE EFFECT OF TARGETED COX-2 INHIBITION IN MACROPHAGES RESPONDING TO NEURONAL INJURY; PROMOTING ENHANCED AXONAL REGENERATION

A Thesis
Submitted to the Rangos School of Health Sciences

Duquesne University

In partial fulfillment of the requirements for the degree of Master of Science

By
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May 2020
PILOT STUDY EXPLORING THE EFFECT OF TARGETED COX-2 INHIBITION IN MACROPHAGES Responding TO NeURONAL INJURY; PROMOTING ENHANCED AXONAL REGENERATION

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ABSTRACT

PILOT STUDY EXPLORING THE EFFECT OF TARGETED COX-2 INHIBITION IN MACROPHAGES RESPONDING TO NEURONAL INJURY; PROMOTING ENHANCED AXONAL REGENERATION

By
Alyssa L. Brauckmann

May 2020

Dissertation supervised by Dr. John A. Pollock

Celecoxib nanoemulsion (CXB-NE) has been developed as a macrophage targeted analgesics by Dr. Janjic and her team at Duquesne University, (Janjic et al, 2018; Liu et al, 2020; Saleem et al, 2019b; Vasudeva et al, 2014). The CXB-NE nanoemulsion carrying a Nonsteroidal Anti-inflammatory (NSAID) inhibitor of COX-2 activity result in a reduction in PGE2 expression in macrophages. Using CXB-NE in rats that have peripheral nerve injury constricting the sciatic nerve relieves hypersensitivity, a pain-like behavior. The treatment also decreases inflammation associated with this chronic constriction injury (Janjic et al, 2018; Saleem et al, 2019b; Stevens et al, 2019). In this project, we evaluated the potential impact of CXB-NE on neuroregeneration. In CCI, the injury and inflammation damages nerves, leading to axon degeneration distal from the site of injury. While peripheral nerves can regenerate axons, if axonal degeneration
continues to occur due to inflammation, then axonal regeneration cannot proceed. Ultimately, axonal regeneration can be an important part of nerve recovery, which in humans can aid in rehabilitation after injury. Since CXB-NE is able to decrease inflammation, we are exploring the effect that CXB-NE has on axonal regeneration in the injured sciatic nerve. To analyze this, we are using immunohistochemistry and epi-fluorescent microscopy to assess the presence of macrophages, Growth Associated Protein (GAP-43), and growth cones as detected through F-actin. The results show that GAP-43, which naturally increases expression during nerve regenerations is precociously activated days earlier than normal when CXB-NE is present. Furthermore, CXB-NE treatment appears to enhance the production of axon growth cones, indicating that inhibiting COX2 with the nanoemulsion therapy promotes axon regeneration. These observations will be discussed in the context of other studies on additional infiltrating inflammatory cells and changes in gene expression that are evident during nerve injury and when CXB-NE treatment provides reduced inflammation and pain-relief.
DEDICATION

Dedicated to all the people who have supported me throughout my years at

Duquesne University
ACKNOWLEDGEMENT

It is with deep gratitude that I would like to thank the multiple people and organizations that made this work possible. Thank you to Dr. John A. Pollock for all the immeasurable amount of guidance and support he gave throughout the years and thank you for all the editing of preliminary and finally drafts of my posters, manuscripts, papers, resumes, and now my thesis. Thank you to Dr. Jelena M. Janjic for collaborating with me through the years, offering the drug-loaded nanoemulsion that was essential to the project as well as reviewing drafts of my abstracts, posters and now the thesis. A special thank you to the Department of Engineering in the Rangos School of Health Sciences at Duquesne University and Dr. John Viator for providing funding for my summer project as well as funding for multiple essential supplies for this work. Special mention and thanks to Dr. Kimberly Williams and Dr. Bin Yang for being a part of my committee and for providing me guidance on the content for my final thesis.

I would also like to acknowledge several of my peers in the lab whom helped to make this work possible. A large thank you to the PhD candidate, Brooke Deal, who has been a peer role model and huge support throughout the years with her help and guidance in and out of the lab. A special thank you to Dr. Muzamil Saleem, PhD candidate Andrea Stevens, and undergraduates: Laura Reynolds and Charles Patterson.

There are also several grants that deserve recognition for instrumental materials that made my thesis possible. Confocal imaging was supported with grants to Dr. John A. Pollock from the NSF DBI-0400776, NSF DBI-1726368. NIR optical imaging was performed on Pearl® Small Animal Imaging System (Li-COR Biosciences) at Duquesne
University (Supported by Pittsburgh Tissue Engineering Initiative Seed Grant). Dr. Jelena M. Janjic acknowledges support from NIDA award number 1R21DA039621–01, NIBIB award number R21EB023104–02 and AFMSA Award number FA8650-17-2-6836. Dr. John A. Pollock and Dr. Jelena M. Janjic acknowledge support from Pittsburgh Tissue Engineering Initiative Seed Grant. Dr. John A. Pollock also acknowledges the Hunkele Dreaded Disease Award, Samuel and Emma Winters Foundation, the Charles Henry Leach II Fund, the Commonwealth Universal Research Enhancement Award. Dr. John A. Pollock and Dr. Jelena M. Janjic acknowledge support from the Duquesne University Inaugural Provost’s Interdisciplinary Research Consortia Grant, which supports the Chronic Pain Research Consortium.
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LIST OF ABBREVIATIONS

• CCI=Chronic Constriction Injury
• CD68=general macrophage marker
• COX-2=Cyclooxygenase-2
• CXB-NE=Celecoxib in a Nanoemulsion
• DF-NE=Drug-Free Nanoemulsion
• F-actin=Growth cones marker
• GAP-43=Growth Associated Protein
• IHC=Immunohistochemistry
• NE=Nanoemulsion
• NSAID=Non-steroidal anti-inflammatory drug
• PGE-2=Prostaglandins E2
• PBS=Phosphate buffered saline
• NDS=Normal Donkey Serum
• OCT=Optimal cutting temperature
Chapter 1

Background

1.1 Chronic Neuropathic Pain

In the United States, there are currently at least 100 million Americans suffering from chronic pain (ISAP, 2011). One of the common forms of chronic pain is peripheral neuropathy that arises from inflammation after injury to peripheral nerves (Clatworthy et al, 1995). Peripheral neuropathy is associated with neuroinflammation, which is generated by activation and infiltration of inflammatory cells such as macrophages that contain cyclooxygenase-2 (COX-2) (Ji et al, 2016). COX-2 is the key enzyme driving the production of prostaglandins (e.g. PGE-2) that further drives the neuroinflammation and hypersensitivity (Bazan et al, 2001; Ren et al, 2008). To treat neuroinflammatory pain, systemic drugs have been a most common option. However, the key challenge is that neuroinflammation requires to high of a dose to reach at an effective concentration at the site of injury. However, that concentration of drug is also evident throughout the entire body and can damage other organs. This has caused a drive to generate a more targeted treatment approach that is able to deliver drug to where it is needed with a lower, overall body burden. Dr. Janjic’s designed targeted delivery strategy with CXB-NE achieves effective dose at the site of injury without systemic exposure to high concentrations (Janjic et al., 2018).

1.2 Targeted Drug Delivery Engineering

Many major diseases have a need for a more targeted drug delivery system because of
their underlying pathology and the inadequacies of systemic delivery methods. Finding alternative methods with a more targeted approach to delivering drugs is a major part of many biomedical engineering and pharmaceutical research initiatives. These fields have already proposed multiple alternatives that include: microdevices that use cantilevers valves to release drugs when a specific, previously determined threshold is met; synthetic polymer scaffolds that have macropores that allow drug to be released once placed at the area of interest; multi-phasic devices that allow for pulsatile release of a drug; or nanoparticles that allow for short-term release of drug through intravenous injections (Cheong et al, 2018; Rambhia & Ma, 2015). Although all of these possible drug systems have their benefits, our research focuses on the advantages of using of nanomedicines as a drug delivery system. Nanomedicines are a recent, but popular drug delivery system studied because of their ability to give therapeutic agents in a more controlled, targeted manner (Patel & Janjic, 2015; Patra et al, 2018). They offer multiple benefits such as their size that gives them ability to move freely within the body and be more readily ingested by cells, increased biocompatibility through use of natural polymers, and decreased toxicity due to the decreased dosage added to the nanoparticles (Patra et al., 2018). Considering these, using a nanomedicine approach for drug delivery should bypass the problems of systemic delivery, increase the success of drug, and thus making the nanomedicines an ideal system for targeted drug delivery in our research.

1.3 Targeted Drug Delivery via Nanoemulsion

As previously stated, we are using a targeted drug delivery approach that employs a drug-loaded nanoemulsion designed by Dr. Jelena Janjic. The nanoemulsion in this paper
was invented and produced by Dr. Jelena Janjic as described in previous literature (Janjic et al., 2018; Liu et al., 2020; Patel & Janjic, 2015). Dr. Janjic developed celecoxib, a COX-2 inhibitor, loaded nanoemulsion (CXB-NE) that is given at a dose that is 2000x less than the typical oral dosage (Janjic et al., 2018; Liu et al., 2020; Saleem et al., 2019b). CXB-NE is able to be administrated at such a low dose because, after intravenous injection, circulating monocytes in the bloodstream phagocytose the drug and then naturally accumulate at the site of injury (Janjic et al., 2018; Saleem et al., 2019b). These monocytes then infiltrate the injured nerve, differentiating into macrophages. Within the sciatic nerve, the nanoemulsion laden macrophages produce less PGE2, resulting in pain relief and a number of other subtle changes in the neuroinflammatory response (Janjic et al., 2018; Saleem et al., 2019b; Stevens et al., 2019). This direct delivery through the immune system allows for CXB-NE to avoid high concentration in other systems in the body and negates damage to other organs. Another quality of CXB-NE is that it has fluorescent dye within it that allows for visualization through live-imaging and fluorescence imaging after tissue staining (Janjic et al, 2013, 2018; Liu et al., 2020; Patel & Janjic, 2015; Patel et al, 2013; Saleem et al, 2019b). This enables us to track the flow of the nanoemulsion and confirm the success of the mechanism of this method of drug delivery. In past experiments, CXB-NE has been shown to have successful accumulated and successful reduce the presence of macrophages at the site of injury, making it a good candidate for reducing pain with a more targeted approach.
1.4 Chronic Pain in vivo

In order to replicate chronic pain in vivo to continue to experiment with CXB-NE, a chronic pain model needs to be used. One such model is chronic constriction injury (CCI) (Bennett & Xie, 1988) as applied to the right sciatic nerve of a rat (Vasudeva et al, 2014). CCI is a technique that involves tying ligatures loosely around the sciatic nerve. These ligatures will generate neuroinflammation that leads to hypersensitivity for the right hind-paw of the rat (Bennett & Xie, 1988; Clatworthy et al., 1995; Vasudeva et al., 2014). This hypersensitivity is measured by assessing pain-like behavior through the Von Frey mechanical allodynia test (Chaplan et al, 1994; Deuis et al, 2017; Janjic et al., 2018). Because of its ability to replicate peripheral neuropathy and to measure changes in pain-like behavior with CCI, it makes it an ideal model for these studies.

1.5 Immunohistochemical assessment of infiltrating cells and molecular markers indicative of nerve regeneration

Immunohistochemical assessment of tissue sections of the affected sciatic nerve can reveal aspects of the cell biology of the nerve such that different cells and biological markers can be identified. We can use fluorescent antibody staining of tissue from uninjured nerves compared with nerves damaged by CCI and nerves damaged by CCI but treated with COX2 inhibiting nanoemulsion. Specific primary antibodies combined with paired secondary fluorescent probes allow for the simultaneous assessment of the molecular probes by epi-fluorescent microscopy. This assessment allows for the visualization and quantification of specific cells, such as macrophages and target proteins such as GAP-43 and F-Actin, which are involved with nerve regeneration.
Chapter 2

Introduction

Peripheral neuropathy is a form of chronic pain that occurs when an axon is not able to regenerate due to the high levels of neuroinflammation from nerve injury (Martin et al., 2013). Continual inflammation after injury leads to the inability of an axon to recover and begins a process that is known as Wallerian Degeneration. Wallerian Degeneration occurs through the increased activity of inflammatory cells such as non-myelinating Schwann Cells and M1 (pro-inflammatory) macrophages (Gaudet et al, 2011; Huebner et al, 2009). With an increased presence of these cells and the inflammatory mediators that they produce, the nerve cannot recover and continues to degenerate. After weeks of excess neuroinflammation, M1 macrophages eventually shift into M2 (anti-inflammatory) macrophages and begin to decrease inflammation around the site of injury (Saleem et al., 2019b). This decreased inflammation occurs, in part, through a shift in macrophage phenotype and allows for myelinating Schwann Cells to align and increase axon recovery. So, when inflammation decreases, axon recovery agents become activated and allow for regeneration. Based on these observations and the past studies, CXB-NE should be able to increase the presence of axon recovery agents because of its ability to decrease inflammation. However, to our knowledge there are no studies that show axonal regeneration following CXB-NE treatment with targeted delivery by nanoemulsion.

In order to study axonal regeneration enhancement from CXB-NE, markers associated with axonal regeneration need to be visualized and compared across conditions. One such protein is Growth Associated Protein-43 (GAP-43). GAP-43 is a
substrate for protein kinase C, playing a role in neurite formation through interaction with actin remodeling; its expression is increased significantly during nerve regeneration (Benowitz & Routtenberg, 1987, 1997). GAP-43 is seen to be suppressed during Wallerian Degeneration due to excess of pro-inflammatory mediators. However, once this inflammation is decreased, it has been shown to have elevated levels of expression in the membrane of the axon during axonal regeneration (Gaudet et al., 2011; Skene & Willard, 1981). Another marker of interest are growth cones that protrude from the ends of damaged axons at the site of injury and begin to make complete axons that are growing toward their natural targets in the lower limb. These growth cones have an abundance of F-actin that functions both structurally in the growth cone and as a component of the chemo-guidance control of growth. As such, both GAP-43 and F-actin can serve as good markers for axon re-growth. Evidence of growth cones is directly representative of regeneration (Lopez-Verrilli et al, 2013; Montani & Petrinovic, 2014). Based on this, it is hypothesized the enhanced transition from M1 macrophages to M2 macrophages, which is supported by CXB-NE after nerve injury, will allow for a subsequent increase in expression of GAP-43 and an increase in the number of identified growth cones. If CXB-NE is able decrease pain-like hypersensitivity as well as promote axonal regeneration, then treatments like this may represent a therapy that can help the 100 million Americans suffering from chronic pain.
Chapter 3

Materials and Methods

3.1 Ethical Statement

This study was performed in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and the Institutional Animal Care and Use Committee (IACUC) at Duquesne University approved the animal protocol (Protocol 1803-02). All efforts were made to minimize animal suffering and number of subjects used. All surgical procedures were performed under isoflurane anesthesia. Male Sprague-Dawley rats weighing 250-350 grams were used in this study (Hilltop Laboratory Animals, Inc Scottsdale, PA). Rats were maintained on a 12:12 hour light-dark cycle and were given ad libitum access to water and purified chow (D10012G, Research Diets, Inc., New Brunswick, NJ). Purified chow was used because it has shown to reduce non-specific fluorescence caused by plant materials in regular chow; regular chow can interfere with the visualization of the near-infrared fluorescence of the nanoemulsion (Vasudeva et al., 2014).

3.2 Surgery and Behavioral Timeline; Two study Groups

Animals in the study are managed over the course of two to three weeks (Figure 1). There were two main groups with 4 surgical conditions per group for this study. The two groups were assessed behaviorally for 12-days post-surgery and a separate group that was behaviorally assessed for 18-days post-surgery. The 4 surgical conditions in each group were (1) naïve (un-operated), (2) CCI with no treatment, (3) CCI ‘vehicle’ treated with Drug Free nanoemulsion (DF-NE), and (4) CCI treated with Celecoxib loaded
nanoemulsion (CXB-NE). The DF-NE condition is a CCI subject that on day 8 of the behavioral timeline received injection of vehicle-nanoemulsion (DF-NE). The CXB-NE condition is a CCI subject that on day 8 of the behavioral timeline received injection of the drug-loaded nanoemulsion (CXB-NE).

A. Experimental Timeline:

![Timeline Diagram]

B. Chronic Constriction

C. Von Frey Behavior

D. Tail Vein Injections:

E. LICOR Live Imaging:

Figure 1. Summary of surgical timeline and corresponding procedures. (A) The timeline of the procedures that a rat undergoes during a 12-day and 18-day experiment is shown above. Every two days, the rat’s pain-like hypersensitivity was tested via Von Frey filaments (C) starting two days prior to chronic constriction injury surgery (Bennett & Xie, 1988) on the right sciatic nerve (B) and finishing on the day of euthanasia. Eight days after CCI surgery, drug-free nanoemulsion (DF-NE) or celecoxib nanoemulsion (CXB-NE) is injected via tail vein (D). Live imaging of the fluorescent dye in the nanoemulsion is taken before injection, after injection, three days after injection, and nine days after for those in the 18-day experiment (E). At the end of the 12-day experiment and 18-day experiment, the rats are euthanized, and their sciatic nerves were collected for immunohistochemistry. B drawing by Robert Hoggard, C photo by Brooke Deal, D process illustration by John Pollock & Jelena Janjic, E NIRF photo by Alyssa Brauckmann.
3.3 Chronic Constriction Injury as in vivo Chronic Pain Model

The chronic constriction injury method developed by Bennett and Xie (1988) is used to generate neuropathic pain in the sciatic nerve of rats as previously described (Janjic et al., 2018; Saleem et al., 2019b; Vasudeva et al., 2014). Briefly, the surgery involves exposing the right sciatic nerve of the rats by making an incision through the skin and carefully separating the muscles of the mid-thigh region of right hind limb. Four ligatures are tied loosely around the exposed sciatic nerve (Figure 1B) approximately 1 mm apart using 4-0 chromic gut suture (Moore Medical, Connecticut). Care was taken to ensure that the ligatures were tight enough for the pain model, but not so tight as to cut off epineural blood flow (Chen et al, 2020). Once the sutures were optimal around the nerve, the incision site was closed by using 9 mm stainless steel auto clips.

3.4 Mechanical Allodynia Testing

Behavior testing was performed through assessment of mechanical allodynia with the up-down method of applying von Frey filaments to the plantar surface of the hind paw (Figure 1C) (Chaplan et al., 1994; Deuis et al., 2017; Janjic et al., 2018; Saleem et al., 2019b). Before testing begins, the animals were placed on a metal grid that allows access to paws with a Plexiglass cover and acclimated for 30 minutes. After acclimating, testing occurs in which the left and right hind paws of all the subjects were probed to the point where the testing filament bends (Figure 1C), and the rat’s responses were recorded. A documented withdrawal is when the rat removed its paw from the filament via lifting its paw or lifting and flicking its paw. Testing begins with a median filament. If this withdrawal happened, then a next smaller filament is used with a smaller gram force. If
this withdrawal did not happen, then the next bigger filament is used. This process continues until either of the following happen: the rat withdrawals when the smallest filament is used, the rat does not withdrawal when the largest filament is used, or four probes after the first change in withdrawal behavior.

Baseline behavior was established two days prior to surgery and on the day of surgery for all groups. Testing continued every other day starting at day 2 until day 12 for the 12-day group and until day 18 for the 18-day group. The 50% paw withdrawal threshold was calculated using the principle of the median lethal dose (LD50) that determines the threshold in grams that the rat will withdrawal its paw 50% of the time (Chaplan et al., 1994; Janjic et al., 2018; Saleem et al., 2019). Hypersensitivity is evident when a lower threshold force causes paw withdrawal. Hypersensitivity is interpreted as pain-like behavior.

3.5 Rat Tail-Vein Injection of Nanoemulsion and NIRF Live Imaging

On day 8 post-surgery, CCI rats begin to reach a plateau of pain-like behavior similar to that seen in previous studies (Janjic et al., 2018; Saleem et al., 2019; Stevens et al., 2019; Vasudeva et al., 2014). The rats were put under isoflurane anesthesia and live NIRF imaging for background of near-infrared fluorescence prior to injection using a LiCOR Pearl Impulse (LI-COR Biosciences, Lincoln, NE). The procedure described in Saleem et al., 2019a (JoVE) involves warming the tail with water to dilate the veins. Using the lateral veins, the needle was placed in the vein without the syringe. Once blood flows through the needle gauge, the needle is considered to be successfully placed in vein (Saleem et al, 2019a). The syringe is added to the needle gauge and the celecoxib-loaded
nanoemulsion (CXB-NE) and the vehicle nanoemulsion (DF-NE) were injected intravenously in this vein. The DF-NE was used as a vehicle control. The injection for CXB-NE represents a single dose of celecoxib ~0.24 mg/kg. Immediately after injection, the rats were imaged on again LiCOR for the near-infrared fluorescence (NIRF) of the nanoemulsion. A successful injection was indicated by the nanoemulsion being completely cleared from the tail vein except for the possibility of a small spot at the site of injection. All injections were successful for this study. NIRF live imaging was continued throughout the experiment on day 11 and day 17 (for the 18-day experiment only). All of images from the LICOR Imaging system that were to be compared were combined with a single linked look-up tables (LUTs) adjusted for brightness and contrast of the positive images. The same LUT was applied to the negative controls and NIRF background images. The LICOR Peal Impulse software gives two mergeable channels for visualization: a white light channel for a body view and 785 nm excitation for 820 emission, which detects the DiD in the nanoemulsion. The relative fluorescence a region of interest (ROI) was determined by drawing an ROI over the incision site to get the NIRF signal intensity using the LICOR ImageStudioLite Software (version 5.2.5). A comparable background image was also acquired and the relative fluorescence per unit area was calculated.

3.6 Euthanasia and Sciatic Nerve Processing

At the end of the experiment, the rats were euthanized. The right (ipsilateral surgically treated) and left (contralateral control) sciatic nerves were then dissected and stored overnight in 4% paraformaldehyde in 1X phosphate-buffered saline (PBS) at 4°C
for fixation. For each animal, the contralateral, left leg sciatic nerve was used as control for the histology. The next day, the tissues were stored in 30% sucrose overnight. The tissues were then placed in optimal cutting compound (OCT) solution for sectioning and frozen with a bath of isopentane surrounded by dry ice at a temperature of about ~-55 to -60°C. The frozen tissues were cut at -20°C, and then sectioned on the cryostat (MICROM HM550, MI) at 20 µm, mounted on gelatin-coated slides (SouthernBiotech, Birmingham, AL). Slides were stored at -20°C until immunohistochemical processing was carried out.

3.7 Immunohistochemistry and Nanoemulsion NIRF Detection

Tissue sections from each condition underwent equivalent immunohistochemistry staining. Briefly the sections for each condition were warmed for 30 minutes on the slide warmer and then fixed in 4 % paraformaldehyde for 15 minutes. Then, they went through 5 washes for 5 minutes each in 1X PBS, permeabilized with 0.3% Triton 1X PBS for 30 minutes, and blocked with 5 % normal donkey serum (NDS) for one hour. 5% NDS was obtained from NDS stock and 1X PBS solution.

Two immunostains that were performed. The first stain was an immunostain for the presence of macrophages using the primary antibody: mouse monoclonal anti-CD68 1:500 (MCA341R, Bio-Rad) and secondary antibody: Alexa Fluor 555 goat anti mouse (A21127, Invitrogen). The second stain was a double immunostain to reveal the number of growth cones (F-Actin) and relative expression of GAP-43 using primary antibodies: mouse monoclonal anti-F-actin 1:100 (Ab205, Abcam) and rabbit polyclonal anti-GAP-43 1:250 (PA1-16729, Invitrogen). The secondary antibodies used for this stain were Alexa Fluor 555 goat anti-mouse 1:200 (A21426, Invitrogen) and Alexa Fluor 488
donkey anti rabbit 1:200 (A21206, Invitrogen). All of the antibodies were diluted using 5% NDS in 1X PBS. After both primary and secondary washes, the slides were rinsed 5 times with 1X PBS. After the washes following the secondary antibody incubation, Prolong diamond antifade reagent with DAPI (P36965, Thermo Fisher Scientific) was applied to mount the coverslip to the slide. DAPI is a blue-fluorescent DNA stain that exhibits enhanced fluorescence when it is intercalated into A-T rich double stranded DNA. As such it is an efficient nuclear stain. A fourth fluorophore detected in some of these experiments is the DiR near infrared dye, which is a component of the nanoemulsion. It is detected with the 640 nm laser on the Nikon A1r confocal microscope.

3.8 Microscopy and Image Analysis

All stained sections were viewed on Nikon NiU Fluorescence Microscope with Nikon NIS-Elements imaging software. Using the built-in programs on the Nikon NIS-Elements, multiple regions of interest (ROIs) were drawn per field of view. In these ROIs, the number of growth cones or CD-68 positive macrophages were counted. These ROIs and the Nikon NIS-Elements built-in functions also allow for the mean fluorescence of GAP-43 to be calculated with a background ROI for normalization. The number of growth cones, number of CD-68 positive macrophages and the normalized mean fluorescence of GAP-43 for all the ROIs were then used for statistical analysis. For higher magnification images, the Nikon A1 confocal microscope equipped with six excitation solid-state diode lasers (405 nm, 440 nm, 488 nm, 514 nm, 561 nm, and 640 nm) was used with image acquisition and processing with the Nikon NIS-Elements.
software. Additionally, coincidence of DiR and CD68 staining in macrophages was demonstrated by confocal microscopy.

3.9 Statistical Analysis

Statistical analysis was done using GraphPad Prism 6 statistical software. Since the number of animals per condition in this analysis is one (n=1 per condition in each group), statistical analysis was not able to be used for behavior and LiCOR live animal imaging data. However, statistical analysis could be made for the stained sections due to the multiple ROIs demonstrating the consistency of image analysis in determining the relative values for in an individual animal’s infiltration of macrophages, and GAP-43 or F-Actin expression in the nerve. The counts for growth cones and macrophages and the relative mean-fluorescence for GAP-43 were analyzed using a one-way ANOVA with a 95% confidence interval and Tukey’s post-hoc test to make multiple comparisons. The averages and standard error of the mean were calculated and graphed.
Chapter 4

Results

4.1 Pain-like hypersensitivity differs across treatment groups

Pain-like hypersensitivity in rats with neuropathic pain caused by CCI was tested using Von Frey filaments that measure mechanical allodynia. The mechanical allodynia testing allows for the calculation of the withdrawal threshold of the rat’s paw in which the lower the threshold, the more pain-like hypersensitivity that rat has. Baseline behavior was established two days prior to surgery and the day of surgery; during these pre-surgery days every group displays the same thresholds and each sub-category represents an n=1(Figure 2). The corresponding days following surgery show that DF-NE and CCI thresholds are consistently low while naïve remains at baseline threshold throughout the duration of the experiments. The changes are seen in CXB-NE after day 8 when CXB-NE is injected. In the 12-day experiment, after injection of CXB-NE the rat’s withdrawal threshold climbs back to baseline by day 12 (Figure 2a), interpreted as significant pain-relief. In the 18-day experiment, after injection of CXB-NE the rat’s withdrawal threshold partially recovers but did not achieve baseline relief (Figure 2b). The threshold at which this CCI CXB-NE Day 18 rat experiences a withdrawal 50% of the times that it was probed was higher than that of CCI and CCI DF-NE. This means that the CCI CXB-NE Day 18 rat was experiencing pain-relief as compared to the pain that the rats exhibited that received no treatment.
Figure 2. Pain-like hypersensitivity behavior testing results for the 12-Day and 18-Day time-points for the animals used in the immunohistochemistry experiments presented below. Mechanical allodynia of the rats was measured through the Von Frey behavior test for both the individual 12-day animals used in the IHC (A) and individual 18-day animals used in the IHC experiments (B). At day 0, CCI surgery occurs and at day 8, nanoemulsion is injected. For both the 12-day and 18-day experiments Naïve, CCI, DF-NE, and CXB-NE n=1. In both time-points, the rats in the naïve condition demonstrated no hypersensitivity while the CCI and DF-NE rats had continued hypersensitivity after CCI with no relief. In the 12-day experiment, the rat with CXB-NE appears to recover back to baseline with no pain-like hypersensitivity while the rat with CXB-NE in the 18-day experiment had a very slight recovery, but still demonstrated pain-like hypersensitivity. (C). Shown are the individual behavioral responses for the animals experiencing different treatments.
4.2 CXB-NE NIRF signal decreased in injured sciatic nerve in live imaging

The NIRF signal in the nanoemulsion was detected in live rats by imaging the relative fluorescence with a LiCOR Pearl Impulse (LI-COR Biosciences, Lincoln, NE). In previous studies, it was shown that the macrophages that contain phagocytosed nanoemulsion infiltrate the injured sciatic nerve at a significant quantity by day 11 (Janjic et al., 2018; Saleem et al., 2019b; Stevens et al., 2019; Vasudeva et al., 2014). As such live animal images for DF-NE and CXB-NE are taken before injection, after injection (Figure 3A.1, 3B.1), at day 11 (Figure 3A.2, 3B.2), and again at day 17 for the 18-day experiment (Figure 3B.3). After the tail-vein injection, a small circle of the NIRF signal can be detected at the site of injection. At day 11, the NIRF can be detected at the sciatic nerve. The intensity of the NIRF signal in DF-NE CCI rats is higher than the intensity of the CXB-NE’s NIRF signal (Figure 3A.3, 3B.4). This trend is continued to day 17, but the intensity of both DF-NE and CXB-NE are both reduced (Figure 3B.4).
Figure 3. LICOR Live Imaging results for 12-Day and 18-Day time-points. LICOR Live imaging is performed before injection of DF-NE and CXB-NE, after injection (A1 and B1), at day 11 (A2 and B2), and at day 17 with one animal for DF-NE and CXB-NE in both the 12-Day and 18-Day experiment (B3) compared with linked look-up tables. In each graph, the n=1 such that there is only one DF-NE and one CXB-NE. The fluorescence intensity of the NIRF signal at day 11 for the DF-NE condition showed an increased fluorescence as compared to CXB-NE (A3 and B4). The fluorescence intensity of the NIRF signal at day 17 showed that the intensity appears to decrease for both DF-NE and CXB-NE, but DF-NE was still slightly elevated as compared to CXB-NE (B3, B4).
4.3 *The number of macrophages in injured sciatic nerve is decreased with CXB-NE treatment*

The number of macrophages present in all groups was detected using anti-CD68 immunohistochemical staining with nuclei staining through DAPI visualized with a Nikon NiU Fluorescence Microscope and Nikon Elements imaging software (Figure 4A). The average number of macrophages per ROI and its corresponding standard error of the mean was calculated for ~28-80 ROIs per condition and the significance between groups was calculated (Figure 4B). At both day 12 and day 18, there was a significant increase (p-value < 0.0001) in the number of infiltrating macrophages per ROI between Naïve and CCI as well as Naïve and DF-NE. However, for animals in pain, there was no significant difference between CCI and DF-NE at both day 12 and day 18. There was, however, a significant decrease (p-value < 0.0001) in the number of infiltrating macrophages per ROI between DF-NE and CXB-NE at both 12-day and 18-day time-points. The presence of the NIRF signal in the infiltrating macrophages was visualized by confocal microscopy demonstrating the presence of the nanoemulsion DiD (Figure 4C).
Figure 4. Presence of CD68 positive macrophages. All images were taken with Nikon NiU Fluorescence Microscope with Nikon Elements imaging software. Nuclei are highlighted with DAPI. CD68 reveals infiltrating macrophages at the site of injury (A). The scale bar = 100 µm. The images for Naïve, CCI, DF-NE, and CXB-NE tissue conditions in the 12-day experiment (A1, A3, A5, A7) and the 18-day experiment (A2, A4, A6, A8), respectively, were used for analysis. Using multiple fields of view and about ~28-80 ROIs per tissue condition, the number of macrophages in each ROI were calculated. The average number of macrophages per ROI was calculated for each tissue condition and the standard deviation of the mean was also calculated which are represented by the error bars (B). There was a significant increase in the number of macrophages from Naïve and CCI (**** p-value of <0.0001) as well as DF-NE (**** p-value of <0.0001). There was no significant difference between CCI and DF-NE, but there was a significant decrease in the number of macrophages from CCI/DF-NE to CXB-NE (**** p-value of <0.0001) (n=1 animal per condition, ~28-80 ROIs, one-way ANOVA with Tukey’s post hoc test). To show that the macrophages had nanoemulsion in them, the NIRF in the nanoemulsion was visualized using confocal microscopy with the CD68 and DAPI overlay (C1). A zoomed in image of the macrophage in the box in C1 was taken and the CD68 channel (C2), nanoemulsion channel (C3) and nanoemulsion with DAPI (C4) were separated to show that nanoemulsion was present.
4.4 GAP-43 expression increases with time after CCI; GAP-43 increase in expression is accelerated with CXB-NE.

The expression of Growth-Associated Protein 43 in all groups was detected using anti-GAP-43 immunohistochemistry staining and the Nikon NiU Fluorescence Microscope with Nikon Elements imaging software for visualization (Figure 5A). The average GAP-43 mean fluorescence per ROI and its corresponding standard error of the mean was calculated for ~20-50 ROIs per condition and the significance between groups was calculated (Figure 5B). At day 12, there was no significance difference in GAP-43 expression between naïve and DF-NE. However, there is a significant increase (p-value <0.0001) in GAP-43 expression in the DF-NE treated sciatic nerve by day 18. The day 8 injection of CXB-NE led to the early increase in the expression of GAP-43, evident at day 12 instead of day 18. Interestingly, the expression of GAP-43 is significantly reduced (p-value <0.01) in the CXB-NE condition at day 18.
Figure 5. GAP-43 protein expression and F-Actin Growth Cones. All images were taken with Nikon NiU Fluorescence Microscope with Nikon Elements imaging software (Bar = 100 µm). (A) GAP-43 protein expression at the site of injury for Naïve, DF-NE, and CXB-NE 12-day (A1-3) and the 18-day (A4-6). The mean fluorescence of ~20-50 ROIs per tissue condition were taken. The fluorescence of each ROI was normalized to background fluorescence for each field of view and the average mean fluorescence for each tissue condition was taken along with the standard error of the mean (the error bars) (B). There was a significant increase in the mean fluorescence of GAP-43 between Naïve and CXB-NE during the day 12 experiment (**** p-value of <0.0001) that isn’t seen until day 18 for DF-NE conditions (**** p-value of <0.0001) and there is also a significant decrease (p-value <0.01) in GAP-43 expression between Day 12 CXB-NE and Day 18 CXB-NE (n=1 animals per condition, ~20-50 ROIs, one-way ANOVA with Tukey’s post hoc test). Next, the number of growth cones per ~20-50 ROIs per condition were counted. The images for F-Actin were taken on the Nikon A.1 Confocal microscope at the 60X objective at the digital 4X zoom with a 50 µm scale bar (C1). A closer look at the growth cones in the box in image C1 was taken (C2). The average number of growth cones per condition was calculated with the standard deviation of mean (C3). There was a significant increase in the presence of growth cones from Naïve and DF-NE to CXB-NE at both Day 12 and Day 18 experiments (n=1 animal per condition, ~20-50 ROIs, **** p-value <0.0001, one-way ANOVA with Tukey’s post hoc test).
4.5 The number of growth cones in injured sciatic nerve is increased with CXB-NE treatment

The number of growth cones present in all groups was detected using anti-F-Actin immunohistochemistry staining and the Nikon A1r Confocal microscope for visualization (Figure 5C.1, 5C.2). The average number of growth cones per ~20-50 ROIs per condition and the standard error of the mean was calculated to be used to test for significance (Figure 5C.3). There was no significant difference between Naïve and DF-NE conditions in both 12-day and 18-day experiments, but there was a significant increase (p-value < 0.0001) between Naïve/DF-NE and CXB-NE conditions in both 12-day and 18-day experiments.
Chapter 5

Discussion

5.1 Theranostic nanoemulsion is able to relieve pain-like hypersensitivity in the 12-day experiment and decrease inflammation at both day 12 and day 18

We hypothesized that after intravenous injection of low-dose celecoxib in a nanoemulsion, the circulating monocytes in the blood phagocytose the nanoemulsion. These monocytes will then naturally accumulate at the site of injury. Triggered by cytokines produced in the sciatic nerve these infiltrating immune cells differentiate into mature macrophages while carrying CXB-NE. This delivers CXB-NE directly to the site of injury and allows for the inhibition of the COX-2/PGE2 neuroinflammatory pain pathway.

The behavioral results for CCI animals treated with CXB-NE during the 12-day experiment, were similar to those previously reported by our laboratory further indicating the success CXB-NE at decreasing hypersensitivity to be equivalent to the baseline naïve control (Janjic et al., 2018; Saleem et al., 2019b). However, this effect was not evident in the 18-day CXB-NE rat in this study. The rat displayed the same patterns of behavioral hypersensitivity through day 12 in much the same way as the 12-day CXB-NE rat. The animal’s walking gait scrunched its toes, general range of motion were all consistent with expected behaviors. The rat was also able to withstand stiffer Von Frey filaments than the hypersensitive CCI and DF-NE rats, but did not exhibit the same robust recovery evident in the CCI CXB-NE rat studied in the day 12 experiment. All of these explanations make the rat an outlier for this study.
However, even though the threshold of behavior were not the same, the decrease in infiltration of macrophages in both the 12-day and 18-day CXB-NE rats was consistent with what has been previously shown (Saleem et al., 2019b). For both experimental conditions of CXB-NE and DF-NE, we also found that the NIRF signal at day 11 for the 18-day experiment had the same relative intensity as the NIRF signal at day 11 for the 12-day experiment (Figure 3). This shows that CXB-NE was able to be delivered at the same efficiency to the injured nerve. The success of the delivery of CXB-NE to the injured nerve clearly reduced the level of inflammation as measured by the number of infiltrating macrophages (Figure 4). The fact that this particular rat in the 18-day study did not achieve complete pain relief can be explained in at least two possible ways. First, it is important to recognize that we are assessing a unique individual. As has been seen in previous studies (Janjic et al., 2018 [n=9]; Saleem et al., 2019b [n=7]; Stevens et al., 2019 [n=5]), this individual’s behavioral response is in the range of behaviors observed with CCI CXB-NE rats similarly studied. This behavior is, nonetheless, an outlier and needs to be considered with caution. For example, there is a possibility that for the day-18 rat, the four chromic gut ligatures may not have been tied with equivalent tension. A recent study explored the variation of behavior that corresponds to loose, normal and tight ligatures (Chen, et al, 2020). It reports that the compression force of the ligatures will result in differing levels of muscle atrophy in which the tighter the suture, the more atrophy. Given this, it is possible that the rats sutures were not loose enough causing slight muscle atrophy and therefore displaying the slowed recovery.
5.2 Theranostic nanoemulsion is able to increase the presence of markers of axonal regeneration

The neuroinflammation caused by CCI on the right sciatic of rats is known to decrease the ability of axons to regenerate through a process known as Wallerian Degeneration (Gaudet et al., 2011; Huebner & Strittmatter, 2009). The mechanisms of axonal regeneration are repressed when inflammation is high at the site of injury. This is shown through the proposed mechanism of action in which the increase in the presence of pro-inflammatory chemicals and macrophages that inhibit expression of GAP-43 and therefore production of growth cones (Figure 6A). With CXB-NE, inflammation is reduced as evident through the inhibition of the COX-2, the reduction of PGE2, the reduction in infiltrating macrophages, as well as the shift in macrophage phenotype and the influence on Mast Cell degranulation (Saleem et al., 2019b). Here we present evidence and a possible mechanism of how CXB-NE supports axon regeneration with the enhancement of GAP-43 expression earlier than normal as well as an increase in the number of growth cones as detected by F-actin expression (Figure 6B). The immunostain results for GAP-43 show that the presence of CXB-NE enhances GAP-43 expression 6 days before there is a naturally occurring increase in GAP-43 expression as seen in DF-NE conditions. With the decrease in inflammation by CXB-NE, GAP-43 was able to become activated early and may be contributing to the axon recovery days before it appears to come on when celecoxib is not present (Figure 6B). The results from the F-actin stain for growth cones showed that in both 12-day and 18-day experiments, CXB-NE was able to enhance the formation of growth cones within the fasciculated nerve bundle. There are substantially more growth cones that are present in CXB-NE than the
other conditions. With the enhancement of both GAP-43 and growth cones when CXB-NE is present, it is concluded that CXB-NE presence is able to promote and enhance axonal regeneration.
Figure 6. Proposed Mechanism of Action of Chronic Constriction Injury on Sciatic Nerve with and without CXB-NE Injection. Part A represents the mechanisms of action after chronic constriction injury without injection of CXB-NE. Once the sciatic nerve is injured, macrophages become activated to begin cleaning up debris and are predominantly pro-inflammatory macrophages, M1. Activated macrophages activate COX-2 to produce PGE-2. Prostaglandins will increase production Substance P and increase the infiltration of macrophages to the site of injury. Substance P increases nociceptor sensitization that leads to the pain-like hypersensitivity experienced with peripheral neuropathy while the increase in infiltrating macrophages increases neurogenic inflammation. When there is an increase in the pro-inflammatory chemicals and macrophages, axonal regeneration agents are inhibited specifically GAP-43. GAP-43 enhances the development of growth cones, so growth cones development is also inhibited. Overall, chronic constriction injury causes increased pain-like hypersensitivity, increased inflammation, and decreased axonal regeneration.

Part B represents the mechanisms of action after chronic constriction injury with injection of CXB-NE. Once CXB-NE is injected and travels to site of injury through differentiated macrophages, Celecoxib binds reversibly and specifically to COX-2 within the macrophage. Celecoxib binding causes an inhibition of COX-2 and subsequent inhibition of PGÉ-2. Without PGE-2, M1 macrophages switch to M2, anti-inflammatory macrophages, which decreases nociceptor sensitization and inflammation. The decrease inflammation also allows for the activation of axonal regeneration agents such as GAP-43. With GAP-43 expression enhanced, growth cones development is enabled, and the axons are to begin to regenerate.
Chapter 6

Limitations and Future Directions

A major limitation to this study is that this is a pilot study with only one subject per condition. With only having one animal to represent each condition, there is a chance the data is due to variation among the individuals. Also, the CCI CXB-NE Day 18 animal did not exhibit pain relief behavior back to baseline as is the normal response to the nanoemulsion therapy. As such, this animal is considered an outlier. It is interesting that the CXB-NE was able to have an effect on this Day 18 animal as is evident in the decrease in the presence of macrophages. The drug was getting to the site of inflammation and was influencing the density of infiltrating macrophages. So, in order to overcome this limitation, additional animals need to be included in the study. Care will need to be taken to ascertain whether this outlier represents an anomaly or whether this individual represents a subset phenotypic behavior.

In future studies, they should consider exploring the involvement of other important axonal regeneration agents. GAP-43 and growth cones are just two of many important agents that contribute to the regeneration of axons. Specific agents of interest are Schwann cells, Toll-Like Receptors (TRLs), and laminin trimers. Schwan cells are shown to increase axonal regeneration through promoting growth cones, so further
characterization of their response to CXB-NE could help to clarify their role in supporting the presence of growth cones and regeneration. TRLs are receptors on Schwann cells that are used to detect tissue damage. Stevens et al (2019) also showed that Toll Like receptor 2 exhibited differential mRNA expression in the CCI DF-NE and CCI CXB-NE conditions as compared to sham sciatic nerves as assayed by qPCR. Finally, laminin trimers act directly on neurons and Schwann cells to help with maintenance and repair of neurons (Gaudet et al., 2011; Lopez-Verrilli et al., 2013). When laminin expression is inhibited, the axon loses its ability to regenerate completely. By analyzing such agents, we can further confirm that CXB-NE is enhancing axonal regeneration and further understand the mechanism that drives axonal regeneration from CXB-NE injection.

Another future direction that can be taken is a second dosage of CXB-NE. In past studies, it was shown that CXB-NE’s pain relief subsides after 6 days (Saleem et al, 2019). However, there have been no studies to date showing what happens when CXB-NE is injected for a second time. In order for this experiment to happen, the second CXB-NE dosage will need a new NIRF dye to allow for the discrimination of both the original and second dosage of CXB-NE. Through visualizing this, we will be able to determine how additional injections affect hypersensitivity, macrophage recruitment, axonal regeneration, as well as the effect on other systems within the body. Janjic and colleagues have already demonstrated the engineering capability needed to introduce additional NIR dyes into a single nanoemulsion (Patel, Patrick, et al., 2013). This will allow us to further understand the mechanisms of peripheral neuropathy after CXB-NE treatment. It will also allow us to determine if additional injections will continue to support the repair of
axons.

A final future direction that can be considered is developing a software routine that embraces the capabilities built into the Nikon Elements software that allows for measuring relative fluorescence brightness as well as the capability to count objects in a field of view or region of interest. The Nikon Elements software has the tools that can be trained to identify cells in an image acquired on either the conventional or confocal microscopes. The code could be set to determine a threshold for positive staining for the agents of interest, such as coincident DAPI (nuclear) and CD68 (macrophage) cell identity, which can then be automatically counted. That data can be exported to a file for statistical analysis. Once validated, engineering such a code would minimize the hours spent counting the targets of interest and allow for more time with other experiments as well as allowing for analysis that helps to establish the significance of the phenomena.
Chapter 7

Conclusions

Overall, this pilot study provides evidence that the pain-relieving micro-dose of COX2 inhibiting CXB-NE also enhances the potential for axon outgrowth and nerve regeneration. The assessment of behavior and macrophage infiltration provided further confirmation to observations previously reported, that CXB-NE is effective in providing pain-relief and reducing inflammation as evident by the infiltration of CD-68 positive macrophages (Janjic et al., 2018; Saleem et al., 2019; Stevens et al., 2019b). Two key markers of axon growth and regeneration are enhanced by the presence of CXB-NE; GAP-43 and F-actin labeled growth cones. These observations are consistent with observation that CXB-NE promotes the transition of M1 macrophage population in the injured sciatic nerve to M2 phenotype, which promote repair and regeneration of injured tissue (Saleem et al., 2019b). The increase in GAP-43 expression is also an indicator of nerve regeneration, which is evident at day 18 without celecoxib, but appears to be enhanced with precocious expression at day 12 when CXB-NE is present. Finally, detection of F-actin in a morphology consistent with the visualization of growth cones in fasciculated nerve bundles of the sciatic nerve provides compelling evidence for nerve regeneration enhancement by localized COX2 inhibition. This observation is also
consistent with the report by Stevens et al (2019) that showed that the expression of actin mRNA in the injured sciatic nerve is significantly enhanced by CXB-NE. Taken together, these results indicate that CXB-NE is able to promote axonal regeneration after CCI injury.

By confirming that CXB-NE contributes to supporting axon regeneration, we can consider the use of nanoemulsion delivered NSAID that not only provide chronic pain relief but may also be effective in aiding regenerating the damaged nerve leading to eventual axon recovery. Relieving pain and promoting nerve regeneration may provide an important alternative to opioids which treat an aspect of pain but does not contribute to healing. Success could help to diminish the opioid crisis in America and significantly improve the lifestyle of those 100 million Americans that suffer everyday with debilitating pain.
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