Xanthine Oxidase Inhibitors and Their Role in Neuropathic Pain

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Xanthine oxidase inhibitors and their role in neuropathic pain

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Abstract

Chronic pain is a debilitating condition that affects millions of people. There exists a need to develop effective pain therapeutics that replace current addictive or non-effective treatments. The purpose of this research was to explore the potential therapeutic value of xanthine oxidase (XO) inhibitors for persistent chronic pain. XO inhibitors are the standard treatment for gout, an inflammatory disease characterized by inflammation and pain in the joints. Evidence of heightened XDH levels in mice post injury hinted towards a potential therapeutic role for XO inhibitors. Febuxostat, an XO inhibitor, was administered orally to assess the role in neuropathic pain using a standard mouse model of neuropathic pain, the spared nerve injury (SNI) model, and Von Frey filaments to measure mechanical pain. Results showed no change in mechanical pain thresholds in mice treated with febuxostat compared with vehicle. RT-PCR was also used to assess the levels of XDH in mice after injury to confirm elevation. Results showed no significant elevation in injured versus naïve animals. XO inhibitors administered orally did not have a significant effect on pain thresholds. Further research is needed to ensure febuxostat delivery and mechanism of action at the pain site.
Dedication

I dedicate this work to Parimal Rana for always being my biggest supporter.
Acknowledgments

I would like to acknowledge my Principle Investigator and mentor, Dr. Isaac Chiu. His scientific inspiration and continued support throughout this work has been critical to my success. I have grown immeasurable amounts in science and personally from his mentorship and am truly grateful.

I also want to acknowledge the entire Chiu Lab for their unwavering encouragement and friendship. My time in the lab was fun filled and happy because I could share it with them. Their guidance in science and life has made this work possible. I would like to especially thank Nicole Yang for answering my relentless questions and for offering help along they way. She answered my questions usually before I even knew I had them.

Lastly, I would like to acknowledge my friends and family for being my moral support and believing in me.
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Chapter I.
Introduction

Chronic pain affects 25.5 million adults in the United States and causes major economic and health burdens. Chronic pain occurs when nociceptive neurons are constantly activated. Current treatments for chronic pain are often non-effective, non-specific, and cause major negative side effects. Opioids are widely prescribed for chronic pain due to their analgesic effectiveness, but off target effects cause major side effects including constipation, nausea, wooziness, and addiction. Addiction to opioids is an increasing economic and health burden that has resulted in four times the number of opioid overdoses since 1999 (CDC, 2016). Chronic pain is a symptom of many common diseases including gout, cancer, diabetes, and nerve injury. The cellular and molecular mechanisms of the development and persistence of chronic pain are not widely understood. There is an obvious need for more effective and targeted pain therapeutics that eliminate the negative side effects that plague analgesic medicine.

Chronic Pain and Limitations of its Treatment

Chronic pain is a debilitating symptom of many conditions including gout, cancer, diabetes, and nerve injury. According to a recent study conducted by the National Center for Health Statistics Centers for Disease Control (CDC) and Prevention, 11.2% of 34,525 surveyed adults, reported having pain every day, indicating an estimated 25.5 million people in the United States suffer from chronic pain (Nahin, 2015).

Chronic pain not only causes discomfort, but can also lead to disability. In the same study, participants were given a supplemental survey to assess the extent chronic
pain causes disability. Results showed that patients experiencing chronic pain were bed-disabled 15.26 days in the previous 12 months compared with 1.46 days of those without pain. Participants reporting pain also had more difficulty walking and sought out medical care more often than those reporting no pain (Nahin, 2015). Chronic pain, if not treated, can lead to additional symptoms. These can include depression, anxiety, frequent falls, sleep disorders, and appetite changes (American Geriatrics Society Panel on the Pharmacological Management of Persistent Pain in Older Persons, 2009). Due to debilitating symptoms caused by chronic pain, physicians were urged in 1999 to increase their treatment of pain symptoms.

From 1999, prescriptions of opioids for chronic pain dramatically increased. Currently, opioids are the most prescribed pharmaceutical for pain and have shown consistent efficacy in reducing pain in patients experiencing chronic pain (Furlan, 2011). However, opioids are associated with many major side effects, including risk of overdose and death. In a 2013 survey, 96% of patients taking opioids for chronic pain experienced at least one side effect (Gregorian, Gasik, Kwong, Voeller, & Kavanagh, 2010). Side effects include constipation, nausea, vomiting, itching, drowsiness (Gregorian et al., 2010), loss of motivation (Moore et al., 2013), addiction, and death. Addiction leading to abuse of opioids has been positively correlated with the number of opioid prescriptions (Edlund et al., 2013). Moreover, the CDC reported 165,000 deaths due to opioid overdose from 1999-2014 (Dowell, Haegerich, & Chou, 2016) and these deaths are also positively correlated with the increase in prescriptions for opioids (Bohnert et al., 2011; CDC, 2011).
Developed tolerance to opioids may contribute to addiction and abuse. Oddly enough, chronic opioid use for the treatment of neuropathic pain can cause allodynia, or increased sensitivity to normally innocuous stimuli (Ballantyne & Mao, 2003). This may be due to the development of tolerance to opioids, which leads to decreased analgesic effects. Mechanisms of sensitization and tolerance are not fully known.

Because opioids are highly abused, the economic burden is also high. Costs due to abuse of prescription opioids include criminal justice, medical complications, abuse treatment, and productivity loss which encompasses incarceration, unemployment, and death. Together these costs total to be 53.4 billion dollars in 2006 (Hansen, Oster, Edelsberg, Woody, & Sullivan, 2011).

These profound side effects, namely the increase in abuse of and death due to opioids, have made the need for more targeted and safe chronic pain treatments abundantly clear. The CDC released a detailed report of recommendations for prescriptions of opioids, aiming to reduce the death seen from opioid abuse and addiction (Dowell et al., 2016). Addiction to opioids and barbiturates is an increasingly common occurrence and is causing national concern for the need for more effective and targeted pain therapeutics.

Alternate non-opioid therapeutics for treatments for chronic pain are not without major drawbacks. Treatments include non-pharmacologic and pharmacologic options. Non-pharmacologic treatments include cognitive behavioral therapy (CBT) and exercise therapy. CBT uses a physiological based intervention that focuses on developing coping strategies to target health problems. CBT is commonly used for mental disorders, but more recently is being applied to chronic pain management. Similarly, exercise therapy
uses physical intervention aimed to reduce pain at injury sites. CBT and exercise therapy have both been shown to have improvements in pain, although the extent to which pain symptoms are mitigated are ambiguous (McBeth et al., 2012). Some groups show significant improvement while other show little or no improvement. In all cases, however improvements due to CBT or exercise therapy were less than opioid therapy.

Pharmacologic intervention with acetaminophen, NSAIDs, COX-2 inhibitors, and anticonvulsants can have a significant improvement on pain symptoms, although chronic use of these drugs have major side effects (Tremont-Lukats, Megeff, & Backonja, 2000). Chronic use of acetaminophen is associated with liver failure, while NSAIDs and COX-2 inhibitors can lead to gastrointestinal bleeding, renal disorders, and increased cardiovascular risks (American Geriatrics Society Panel on the Pharmacological Management of Persistent Pain in Older Persons, 2009). Anticonvulsants can lead to dizziness and fatigue which can lead to a decreased quality of life for chronic use patients (Siniscalchi, Gallelli, Russo, & De Sarro, 2013). The lack of therapeutic options without major side effects raises the need for more effective treatments for chronic pain.

Xanthine Dehydrogenase and Xanthine Oxidase

Xanthine dehydrogenase (XDH) and xanthine oxidase (XO) are two interconvertible forms of an enzyme, xanthine oxidoreductase (XOR), that are involved with metabolic breakdown of purines. XO and XDH catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid (Figure 1), which is dissolved in the blood or excreted in the urine (Pacher, 2006). Elevated uric acid levels are associated with gout, a chronic inflammatory arthritis, characterized by chronic pain and disability at the joints. The first line of treatment for gout is XO inhibitors; allopurinol and febuxostat being the most
prescribed (Gray & Walters-Smith, 2011; Petrie et al., 2013). XO inhibitors exhibit non-competitive binding to inactivate actions of XOR, both XO and XDH. Allopurinol is a purine analogue that will bind to the molybdenum cofactor, thereby reducing it through self-oxidation to oxypurinol, an inhibitory metabolite. Reduction of molybdenum cofactor can lead to production of reactive oxygen species (ROS). Allopurinol, having purine structure, can also inhibit purine nucleoside phosphorylase (PNP) and orotidine-5’-monophosphate decarboxylase (OMPDC) which are needed for purine metabolism and pyrimidine synthesis respectively. Without these, RNA & DNA synthesis can be affected (Shirakura et al., 2016). Alternatively, febuxostat is not a purine analogue and binds at the molybdenum pterin center, which is the XO & XDH active site, blocking their actions (Hille, Nishino, & Bittner, 2011).

Allopurinol and febuxostat have minor side effects for patients. Allopurinol dosages must be modified for patients with renal disease or potential renal problems, as allopurinol can cause increased renal toxicity. Side effects of allopurinol may be due to the aforementioned off target inhibitions of PNP and OMPDC and production of ROS. As will be discussed later, ROS have been shown to increase pain. Research shows that febuxostat does not cause renal complications and is as effective as allopurinol in lowering serum urate levels and reducing acute flare-ups of gout (Becker et al., 2005; Frampton, 2015). For these reasons, the experiments in the following studies will only be using febuxostat to avoid any confounding effects of allopurinol.

The use of XO inhibitors as a treatment for gout targets hyperuricemia, the major cause of joint pain in gout patients. Hyperuricemia is abnormally high serum urate levels leading to the abnormal deposition of monosodium urate (MSU) crystals in the joints.
These MSU deposits cause inflammation and tophus resulting in debilitating pain. XO inhibitors are effective in lowering urate serum levels to less than 6mg/dL and reducing inflammation and painful symptoms associated with gout (Neogi, 2011).

Figure 1. Diagram of the purine metabolism pathway.

*Focused in on xanthine dehydrogenase (XDH) and xanthine oxidase (XO) which catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid at the end of purine catabolism. Modified from Pacher, et al. (2006) Pharm. Reviews.*
XDH and Neuropathic Pain

The therapeutic value of XO inhibitors for reduction of gout symptoms, including pain and inflammation, suggests their benefit for reducing chronic pain separate from gout.

XDH upregulation leads to deposition of MSU. MSU are 168 daltons and are recognized by toll-like receptors (TLRs), located on leukocytes (including macrophages) and dendritic cells. MSU recognition by TLRs causes the body to produce an immune response. Macrophages will phagocytize MSU, stimulating the NLR-related protein 3 (NLRP3) inflammasome (Martinon, Mayor, & Tschopp, 2009). Activation of NLRP3 will cause the release of caspase 1, which will produce Interleukin-1β (IL-1β). IL-1β is a proinflammatory cytokine produced during an innate immune response that is recognized by Interleukin 1-receptor (IL-1R). Recognition causes the release of proinflammatory cytokines and chemokines, which recruit leukocytes, thereby creating an inflammatory loop propagated by positive-feedback (Figure 2).

IL-1β is especially an especially important mediator during an inflammatory response, whose classic signs are pain, redness, and swelling. Activation of the NLRP3 inflammasome and production of IL-1β are associated with a number of inflammatory diseases in addition to gout, including type 2 diabetes mellitus and fibrosing disorders (Masters, Simon, Aksentijevich, & Kastner, 2009; Ren & Torres, 2009; Yang, Shin, & Jo, 2012).
In gout, an autoinflammatory disease, the NLRP3 inflammasome is activated leading to an inflammatory loop. MSU are recognized by TLRs on leukocytes, phagocytosed, and active the NLRP3 inflammasome. This leads to the release of caspase-1 and ultimately IL-1β, which is then recognized by IL-1R and release proinflammatory cytokines and chemokines. These proinflammatory factors then recruit leukocytes which propagate this positive feedback inflammatory loop. Modified from Yang, et al (2012) International Neurology Journal.

It is well known that injection (intraplantar or intraperitoneal) of IL-1β can cause pain and overproduction of IL-1β at the injury site and spinal cord is associated with neuropathic pain (Martinon et al., 2009). It is also known that IL-1β can act directly on nociceptors to increase excitability and promote action potentials and is mediated through p38 MAP kinase pathways; nociceptors therefore can act as IL-1β sensors (Binshtok et al., 2008). Uric acid level increase in gout is also correlated with an increase in levels of
IL-1β. XO inhibitors therefore may reduce levels of IL-1β, thereby reducing painful symptoms of chronic pain.

Additionally, XOR generates reactive oxygen species (ROS). XDH and XO act as reducing agents to transfer electrons to NAD+ to yield NADH or to molecular (O2) to yield superoxide (O2-) respectively, causing oxidative stress. Imbalance of NAD+/NADH will cause reductive stress that will reduce glutathione reductase, leading to elevated oxidized glutathione. This oxidized glutathione cannot donate electrons to the ROS H2O2 to neutralize its damaging effects (Wu, Jin, Zheng, & Yan, 2016). ROS levels are also known to increase during environmental stress. ROS can cause damage to proteins, lipids, DNA and lead to cell injury and death. Increased ROS levels are often correlated with chronic neuropathic pain, inflammation and ischemic injury, the latter two eventually leading to painful symptoms (Gwak, Hassler, & Hulsebosch, 2013; Yowtak et al., 2011). The mechanisms, by which ROS leads to neuropathic pain, however are not clear.

A potential mechanism by which ROS can lead to pain relates to ROS activation of the inflammasome. ROS can activate the NLRP3 inflammasome through association with thioredoxin-interacting protein (TXNIP). Endoplasmic reticulum (ER) stress is also dependent on ROS to activate the inflammasome (Yang et al., 2012). As previously mentioned, activation of the inflammasome releases IL-1β, which can directly depolarize nociceptive neurons causing pain. This may hint towards a potential mechanism by which XO inhibitors can reduce pain. Febuxostat has been seen to reduce ROS in renal and myocardial models of ischemia-reperfusion (Tsuda et al., 2012; Wang et al., 2015).
Recently our lab has shown increased $XDH$ gene expression in neurons after injury. We used Nav1.8-Cre/Td-tomato mice in order to select for Nav1.8 expressing neurons. Nav1.8 neurons are a subset of nociceptive neurons. Spared nerve injury (SNI) surgery was performed in mice to induce injury of neurons. 24 hours post surgery, dorsal root ganglia (DRG) neurons innervating the injured paw (ipsilateral) and corresponding DRGs in the uninjured paw (contralateral) were collected. Neurons were sorted by fluorescence-activated cell sorting (FACS) and selected for Nav1.8-cre/Td/tomato expressing cells (Figure 3). Using whole population transcriptional profiling (Chiu et al., 2014), after sciatic nerve injury, $XDH$ was highly expressed in dorsal root ganglia neurons that innervate the injured hind paw. The contralateral DRG neurons and naïve mice showed no increase in $XDH$ expression (Figure 4) (Chiu et al. 2014, unpublished).

Our findings of increased $XDH$ transcript levels post nerve injury, complement studies that show XO activity increase in circulation in other traumas including thoracoabdominal surgery, intestinal, hind limb, myocardial & renal ischemia-reperfusion, skins burns, and liver transplant (Pacher, 2006; Tsuda et al., 2012; Wang et al., 2015). XO increase leads may lead to increased ROS, which causes oxidative damage in the aforementioned traumas. Therefore inhibition of XO with febuxostat, would reduce ROS to prevent further tissue damage. Unsurprisingly, febuxostat has been shown to protect the kidneys from ischemic-reperfusion damage (Tsuda et al., 2012) and pretreatment with febuxostat has decreased myocardial ischemia-reperfusion injury (Wang et al., 2015). We hypothesize that increased $XDH$ post nerve damage may be causing further damage by way of ROS leading to the activation of the inflamasome, release of IL-1β and the persistence of neuropathic pain. Inhibition of XO therefore
could protect against chronic pain. The therapeutic value of XO inhibitors on pain in gout patients combined with the increase of XDH seen in neuropathic pain mouse models suggests their benefit for reducing pain in patients with chronic neuropathic pain.

Figure 3. Selecting for Nociceptive Neurons.

Figure 4. Fold change and transcript levels for XDH expression

(A) Differential volcano plot analysis. Comparison showing expression differences of spared nerve injury (SNI) contralateral dorsal root ganglia (DRG) neurons versus SNI ipsilateral DRGs. (B) XDH gene is highly expressed post SNI in ipsilateral DRGs compared with contralateral DRGs. Chiu, et al. (2014) Unpublished data.
Chapter II.

Methods and Materials

The following section describes the methods and materials used throughout this research. In brief, a neuropathic pain mouse model called spared nerve injury (SNI) was used to assess the therapeutic value of the XO inhibitor, febuxostat, in reducing pain. Von Frey filaments were utilized to measure mechanical pain sensation. RT-PCR was used to measure levels of XDH in mouse DRGs and spinal cord.

Spared Nerve Injury: A Model of Neuropathic Pain

The purpose of this research was to assess the therapeutic value of XO inhibitor febuxostat in reducing pain. Using the spared nerve injury (SNI) model, neuropathic pain was induced in C57BL/6J mice. SNI is a well-established model of neuropathic pain and previously described (Decostern, I. and Woolf, C. 2000; Richner, Bjerrum, Nykjaer, & Vaegter, 2011). The SNI model targets the sciatic nerve at the bifurcation of the three branches: tibial, peroneal, and sural. To ensure proper anesthesia, reflexes were tested by pinching the tail and hind paws looking for unresponsiveness. Prior to surgery, sterile saline was injected subcutaneously between the shoulder blades to prevent dehydration and eye ointment was applied to prevent eye damage. The surgical area (right hind leg) was shaved from below the knee to the hip and the mouse was secured in position with tape. The shaved surgical area was then disinfected three times alternating betadine and ethanol, starting in the center and making circular motions moving towards the edge of the surgical area.
Using sterile scissors, a small 1 cm cut was made longitudinally proximal to the knee and the skin was opened using blunt dissection. After locating the clearly visible blood vessel that is close to the femur, the muscle layer was separated using blunt dissection to reveal the sciatic nerve immediately below the muscle. Using a stereomicroscope (Leica, Germany) and #2 forceps (Roboz), the muscle is carefully moved to visualize the sciatic nerve. Posterior to the bifurcation, the tibial and peroneal branches were ligated with a Silk, 3-0, non-absorbable suture (Johnson & Johnson) by making a tight surgical knot, taking care to spare the sural branch. Silk sutures are used for the ligation because a good handling suture material is needed to provide easy manipulation around the tibial and peroneal branches and avoiding contact with the sural nerve. Silk sutures also provide good knot security, which is needed for the ligation. The tibial and peroneal nerves were then grasped below the ligation using #5 forceps (Roboz) and cut using Vannas spring scissors (Roboz), thereby removing a piece of the two nerves. The muscle layer was then closed and the skin sutured closed using 4-0 Ethicon Vicryl (polyglactin 910) sutures (Johnson & Johnson).

Post surgery, animals were placed in a clean recovery cage and eye ointment is reapplied. A heating pad was placed under part of the cage to maintain proper body temperature. Proper recovery was ensured for all mice before returning them to a clean home cage.

SNI surgery caused hypersensitivity in the lateral of the innervated paw. Figure 5 shows the innervation of the sciatic nerve in the footpad and DRGs at L4-L6 (Decostern, I. and Woolf, C. 2000). This innervation is crucial to understand when measuring hypersensitivity developed from the spared sural nerve.
Figure 5. Sciatic nerve innervation in the rodent hind paw.

(A) Innervation of sciatic nerve into L4-L6 dorsal root ganglia (DRG) and branching of peroneal, tibial, and sural, nerves. (B) Innervations of each sciatic branch into the rodent paw, with sural innervating the lateral most part, peroneal innervating the middle, and the saphenous or tibial innervating the medial part. Shows both the top (left) and bottom (right) foot. Modified from Decosterd and Woolf (2000) Pain.

Von Frey Assay: Measure of Mechanical Pain

The Von Frey assay measures mechanical nociception in the mouse hind paw using the Von Frey apparatus and Von Frey filaments. The Von Frey apparatus (Plastic Concepts) has a mesh wire bottom, allowing access to the bottom of the mouse hind paws. Opaque chambers on top of the mesh keep the mice isolated from one another and reduce outside distractions. Von Frey filaments (Bioseb, France) are nylon filaments that have differing weights (g) and apply a constant force as they bend.

Prior to the Von Frey test, mice were habituated to the experimental room and Von Frey apparatus twice for one hour on different days. On measurement days, mice were acclimatized to the apparatus for at least 15 minutes prior to beginning
measurements. Calibrated Von Frey filaments (.02g-1.4g) were applied perpendicularly to the plantar region of the foot, careful to contact the lateral area of the hind paw, as this is where the sural nerve (the spared nerve) innervates (Figure 5). The up-down Von Frey method was used beginning measurements with the 0.14g filament that is in the middle of the series of filaments. Nocifensive responses, defined as brisk withdrawal, paw flinching, licking, or biting, to filaments were recorded. In the case of a response, the next highest filament in the series is used and in the case of no response, the next lowest filament in the series is used. After a change in direction of filaments, four additional filaments are tested. Each time point is recorded twice, waiting 20 minutes in between, and the thresholds generated are averaged. To generate the thresholds, the algorithm determined by Chaplan et al. (1994), was used. Calibration of the filaments using a scale (Mettler Toledo) to determine exact weight is important to generate accurate thresholds.

*Per Os* Treatment of Febuxostat

Drinking Water Treatment

Febuxostat was dissolved in DMSO and the final concentration of the DMSO in water was 5%. The drinking water of 6-week-old, C57BL/6J mice (Jackson Laboratories) was either treated with febuxostat (Sigma) 5mg/kg/day in 5% DMSO (n=8) or vehicle only (5% DMSO) (n=8) starting on day -1, prior to surgery. Dosage was estimated based on starting body weights (averaged by cage) and average daily water consumption of C57BL/6J mice described previously (Bachmanov, Reed, Beauchamp, & Tordoff, 2002). Prior to water treatment, mice were twice habituated to the Von Frey apparatus and three baseline Von Frey measurements were taken on different days. On
day 0, mice underwent spared nerve injury (SNI) surgery. Von Frey measurements were taken on both the ipsilateral and contralateral hind paws on days 1, 7, 14, 21, and 28 (Figure 6A). Water consumption was measured weekly and average consumption per mouse was estimated using the average body weight per cage. Throughout the study, behavior and water consumption was measured by a blinded researcher.

A second similar experiment was conducted with an increased febuxostat dose of 10mg/kg/day in 10% DMSO or vehicle in the drinking water (Figure 6B).

End points included unanticipated distress, extreme lethargy, tissue damage, or more than 20% loss of body weight.

Oral Gavage Administration

Oral gavage administration is a widely used administration technique that better ensures accurate dosing in mice (Turner, Brabb, Pekow, & Vasbinder, 2011). 8-10 week old, C57BL/6J mice (bred in house) were given either 15mg/kg febuxostat in 1% carboxymethyl cellulose (n=8) or vehicle (n=9) by oral gavage (Roboz, 20g, 1.5”, 2.25mm) daily for 28 days starting at Day -4. Carboxymethyl cellulose was chosen in this experiment, due to febuxostat’s insolubility in water and the potential for toxicity from DMSO (Galvao et al., 2014). Carboxymethyl cellulose has been shown to be safe in rodents and is commonly used as an emulsifier and suspending agent in pharmaceuticals (Gad, Cassidy, Aubert, Spainhour, & Robbe, 2006; Kamel, Ali, Jahangir, Shah, & El-Gendy, 2008). Baseline Von Frey habituations and baselines were recorded prior to treatment. Von Frey measurements were taken at days -1 (prior to surgery), 1, 7, 11, 14, 21, & 28. SNI surgery was performed on all mice at day 0 (Figure 6C). Body weights were taken prior to baseline, and days -4, 7, 14, & 21. Blood was collected from the
submandibular vein prior to treatment and on days -1, 7, 14, & 21. Blood was allowed to clot for at least 30 minutes and then centrifuged at 2000g for 10 minutes. Serum was collected and stored at -80°C.

Figure 6. Experimental timeline outlining Von Frey measurement, treatment start, and SNI surgery.

(A) 5mg/kg/day febuxostat in 5% DMSO or vehicle treatment administered in the drinking water. Dose determined by cage average body weights. (B) 10mg/kg/day febuxostat in 10% DMSO or vehicle treatment administered in the drinking water. Dose determined by cage average body weights. (C) 15mg/kg/day febuxostat in 1% carboxymethyl cellulose or vehicle treatment administered via oral gavage. Dose determined by individual body weight.
6-9 week old, C57BL/6J mice (bred in house) underwent SNI surgery (n=10). Contralateral and ipsilateral dorsal root ganglia (DRGs) and spinal cord were collected 24 hours post surgery (n=5) or 24 hours post surgery (n=5). DRGs and spinal cord from naïve mice (n=5) were also collected as a control group.

DRG & Spinal Cord Dissection

Only DRGs at L4-L6 innervate the sciatic nerve (Figure 5) and were the ones of interest in this project (Decostern, I. and Woolf, C. 2000). A stereomicroscope (Leica, Germany) was used to trace the ipsilateral and contralateral sciatic nerves to the corresponding L4-L6 spinal nerves which lead to the L4-L6 DRGs in the dorsal root that emerges from the spinal cord. Careful consideration was taken to collect only L4-L6 DRGs and differentiate between ipsilateral and contralateral sides. The corresponding spinal cord was also collected and the contralateral and ipsilateral sided were separated. DRGs and spinal cord samples were collected into trizol (Life Technologies) on ice and pooled to yeild 3 biological replicates per group. Samples were homogenized using plastic, autoclaved manual homoegnizers. Samples were stored at -80°C until RNA extraction.

RNA Extraction

Samples were thawed on ice and chloroform was added at a 1:5 chloroform:trizol ratio. Samples were shaken vigourously and left to settle for 2 minutes at room temperature. Samples were then spun down for 15 minutes at 12,000G and kept at 4°C to
minimize RNA degredation. The spun sample will form three layers: the aqueous phase (RNA), the middle layer (lipid), and bottom layer (protein). The RNA aqueous layer is removed and added to a new eppendorf tube careful to not include any lipid layer. 70% ethanol is then added at a 1:1 ratio and mixed gently. Sample is then applied to the RNeasy Mini Kit column (Qiagen) and spun at 8000g for 15 seconds to capture the RNA. On-column DNase digestion was done according to the RNeasy Mini Kit handbook to remove all DNA from samples. RNA extraction steps from the RNeasy Mini Kit handbook were then followed until RNA elution. RNA was then stored on ice to minimize degradation and concentrations were measured with Nanodrop, A260/280 ratio is 2, A260/230 is 2.0-2.2. Elution buffer was used as the blank. RNA was stored at -80°C until cDNA synthesis.

cDNA Synthesis

RNA was then synthesized into cDNA. RNA template was created using RNA concentrations as determined from the Nanodrop. The amount of RNA (ng) needed was calculated from the most dilute sample, 6.3ng/uL. RNA was then combined with ddH₂O to yield a total volume of 15uL. 4ul iScript reaction mix (5x), 1uL iScript reverse transcriptase (Biorad), and 15uL RNA template were combined in a PCR tube. PCR protocol (Table 1) was performed using a ProFlex PCR system (ThermoFisher Scientific).
Table 1. PCR protocol for cDNA synthesis

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (˚C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Priming</td>
<td>25</td>
<td>5 min</td>
</tr>
<tr>
<td>2: Reverse Transcription</td>
<td>42</td>
<td>30 min</td>
</tr>
<tr>
<td>3: RT inactivation</td>
<td>85</td>
<td>5 min</td>
</tr>
<tr>
<td>4: Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

RT-PCR

5uL Power SYBR Green PCR Master Mix (5x) (Life Technologies), 0.5uL forward primer, 0.5uL reverse primer, 1.5uL cDNA template, 2.5uL ddH₂O was mixed. Samples were plated in technical replicates (n=2) in a 96-well plate. Primer sequences can be found in Table 2. Plate was sealed with MicroAmp Clear Adhesive Film (Applied Biosystems) careful to press down securely on edges and corners of the plate. StepOne™ Real-Time PCR System (ThermoFisher Scientific) was used and PCR cycle was used according to Table 3.

Table 2. XDH primer sequences.

<table>
<thead>
<tr>
<th>Forward</th>
<th>ATGACGAGGACAACGCTAGAT</th>
</tr>
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<tbody>
<tr>
<td>Reverse</td>
<td>TCATCCTTGGAGATCATCACGCT</td>
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</tbody>
</table>

Table 3. RT-PCR Protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (˚C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>10 min</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
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<tr>
<td></td>
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<tr>
<td>3</td>
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<td>15 s</td>
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<tr>
<td></td>
<td>60</td>
<td>1 min</td>
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<td></td>
<td>95</td>
<td>15 s</td>
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Chapter III
Results

The purpose of this research was to determine if a xanthine oxidase inhibitor could provide therapeutic pain relief in the context of neuropathic pain. Prior to testing any treatments, the spared nerve injury model (SNI) of neuropathic pain needed to be established and measurable in the lab in untreated mice. Once this model was established, it could be used to measure the therapeutic value for febuxostat in post injury pain treatments. Different routes of drug administration and vehicles were used following the literature to ensure intended delivery and dose in order to study its effect. The results are outlined in this section.

Establishment of the Spared Nerve Injury Model

Prior to treatment, the spared nerve injury (SNI) mouse model needed to be established in the lab. Although well established in the pain field and widely used as a model of neuropathic pain, implementation of a new technique in the lab comes with environmental and technical differences. To be confident in testing therapeutic value of an unknown compound, first a measurable increase in pain post surgery in naïve mice was important to establish.

Assessment of Two Von Frey Methods

Pain thresholds were measured with Von Frey filaments. Two methods of Von Frey methods are commonly used: the repeated mechanical stimuli and the up-down
method. The repeated mechanical stimuli stimulates the mouse hind paw with ascending filaments 10 times per filament until the response frequency is below 50%. The threshold can therefore be only numbers of the filaments. The up-down method required less sustained contact with the mouse and yields a more accurate and specific threshold, which can be a number in between filament sizes. Conducting behavior with the repeated measure and up-down methods yielded different results, with the up-down method being more precise (data not shown). The up-down method was then chosen as the continued mechanical pain measurement.

Mice Showed Increased Sensitivity Post SNI surgery

Mice (n=8) having undergone spared nerve injury (SNI) surgery clearly showed an increase in mechanical pain on the plantar ipsilateral hind paw. This increase in pain was measured with the up-down Von Frey assay and seen as a drop in response threshold in the ipsilateral hind paw as compared to the contralateral hind paw in the same mice (Figure 7). Habituation, baseline measurements, and post surgery measurements were performed in identical environments and conditions. Three baseline mechanical pain thresholds were measured prior to surgery and averaged for each mouse to yield one baseline value. Mechanical pain thresholds were measured on days 3, 7 and 10 post surgery to determine the development of neuropathic pain following SNI. Two-way ANOVA with Bonferoni multiple comparison test was used to determine the significance of thresholds comparing the ipsilateral and contralateral paws of the same mice. The establishment of the baseline neuropathic pain was a critical component to moving forward with testing the compound.
Figure 7. Mice have increased mechanical pain sensitivity after SNI surgery.

Response thresholds (in grams) after stimulation of the lateral plantar surface of the mouse hind paw with Von Frey filaments using the up-down method. The ipsilateral paw (n=8) showed a significant decrease in threshold post SNI surgery compared with the contralateral paw (n=8). Statistics: Two-way ANOVA with Bonferroni’s multiple comparison test.

Testing the Therapeutic Value of Febuxostat

Febuxostat treatment in drinking water did not reduce pain after SNI (Figure 8). Mice were group housed (n=4) and given either febuxostat in 5% DMSO or vehicle only in their autoclaved drinking water. 0.02mg/mL of febuxostat in water was used to yield an intended dose of 5mg/kg/day, which was based on an average body weight of 24g and daily water consumption of 6mL per mouse. The solution was thoroughly mixed to yield a homogenous and clear solution. Water was checked daily.

Large, white precipitates were found in treatment cages on day 3 post treatment. Water was changed and water consumption was measured when precipitates were seen.
on day 3. Although the researcher was blinded to groups previously, the presence of precipitates in 2 out of 4 cages hinted at the groups. This information may have contributed to the slightly higher threshold that is seen in the febuxostat ipsilateral versus the vehicle ipsilateral paw (Figure 8). Precipitates in the treatment group water were seen throughout the course of the treatment. These precipitates may have impeded the ability for mice to drink water throughout the day, although water was checked daily and changed at first site of precipitates. Figure 9 shows the average water consumption for both the treatment and vehicle groups for the entire experiment. Mice were group housed (n=4) with each cage receiving only one treatment, 0.02mg/mL of febuxostat in 5% DMSO in drinking water or 5% DMSO in drinking water. Water consumption for each cage was measured every day starting on day 3. Average water consumption per day per mouse was calculated. The mice in the treatment group drank less water per day compared to the mice in the vehicle group. Average water consumption was lower in the treatment group (4.78mL/mouse/day) compared with the vehicle group (6.9mL/mouse/day). Mice with lower water consumption, in the febuxostat treated group, did not produce any signs of dehydration or any other clinical signs. Due to lower than anticipated water intake in the treatment group, the intended febuxostat dose of 5mg/kg/day based on a 6mL water intake per day (Bachmanov et al., 2002) was inaccurate. Based on the average body weights per cage and the average water consumption per day per cage, the actual febuxostat dose was 4.147mg/kg/day.
Figure 8. Febuxostat treatment in drinking water at 5mg/kg/day did not reduce pain after injury.

Response thresholds (in grams) after stimulation of the lateral plantar surface of the mouse hind paw with Von Frey filaments using the up-down method. No significant differences in response thresholds were seen between the febuxostat treated (n=8) and vehicle treated (n=8) mice either on the ipsilateral or contralateral hind paw. All mice underwent SNI surgery on day 0. Statistics: Two-way ANOVA with Bonferroni’s multiple comparison test.
Figure 9. Water consumption differed between treatment and vehicle group.

Mice were group housed (n=4) with each cage receiving only one treatment, 0.02mg/mL of febuxostat in 5% DMSO in drinking water or 5% DMSO in drinking water. Water consumption for each cage was measured every day starting on day 3. Average water consumption per day per mouse was calculated. The mice in the treatment group drank less water per day compared to the mice in the vehicle group.

The lower dose urged us to increase the dose to 10mg/kg/day. With febuxostat being soluble in DMSO 1mg/1mL, the increase to 10mg also required an increase in DMSO to 10%. Many researchers have administered 10% DMSO orally with no toxicity or clinical signs seen. 10 days after beginning treatment, all mice presented clinical signs of dehydration (hunched, skin tenting, lethargic). Water measurements showed mice had very low daily consumption, likely due to 10% DMSO vehicle, as all mice exhibited these signs. The study was ended early. Figure 10 shows the inconclusive data from day 7 Von Frey measurement.
Figure 10. Von Frey thresholds from inconclusive experiment.

Response thresholds (in grams) after stimulation of the lateral plantar surface of the mouse hind paw with Von Frey filaments using the up-down method. The ipsilateral paw (n=8) showed a significant decrease in threshold post SNI surgery compared with the contralateral paw (n=8). Statistics: Two-way ANOVA with Bonferoni’s multiple comparison test.

Drinking water treatment, especially in group-haired animals does not yield exact treatment doses. The uncertainty of dose amounts and the toxicity of the DMSO vehicle pushed the experiments towards a more exact treatment model. The next experiment used daily oral gavage dosing with doses calculated from individual body weights. To ensure maximum effect, 15mg/kg/day was chosen as it has been used previously (Shankpal, Tadke, Hotwani, Chitnis, & Kokani, 2015). Due tofebuxostat’s insolubility in water, 1% carboxymethyl cellulose was used as the vehicle. Febuxostat makes a uniform suspension in carboxymethyl cellulose. To ensure a homogenous mixture, the compound
was inverted gently prior to dosing. Vortexing or vigorous mixing could cause foaming, which can impede accurate measurement of doses. Treatment was started four days prior to SNI and Von Frey measurements were made as outlined in Figure 6C. No significant change in mechanical pain threshold was seen in febuxostat treated versus vehicle treated mice (Figure 11). A comparison of body weights across the course of the experiment yielded no significant change in body weights in the febuxostat treated group compared to vehicle treated group (Figure 12).

Figure 11. Oral gavage treatment of febuxostat did not decrease pain after injury

Response thresholds (in grams) after stimulation of the lateral plantar surface of the mouse hind paw with Von Frey filaments using the up-down method. Mice were treated via daily oral gavage with either 15mg/kg febuxostat in 1% carboxymethyl cellulose or vehicle. No significant differences in response thresholds were seen between the febuxostat treated (n=8) and vehicle treated (n=9) mice either on the ipsilateral or contralateral hind paw. All mice underwent SNI surgery on day 0. Statistics: Two-way ANOVA with Bonferroni’s multiple comparison test.
Figure 12. No significant difference in body weights was seen between febuxostat and vehicle treated mice.

*Statistics: Two-way ANOVA with Sidak's multiple comparison test.*

RT-PCR Yields No Increase in XDH Levels Post Injury

Contrary to previous results (Figure 4), no increase in XDH expression was seen after injury. In fact, relative fold change expression showed a down regulation in mice that had undergone SNI surgery, 24 hour and 48 hour post surgery in both contralateral and ipsilateral DRGs compared to naïve mice (Figure 13). Fold change expression was calculated $2^{-\Delta\Delta CT}$. CT mean was averaged for all sample types (biological triplicates and technical replicated). $\Delta CT = CT(XDH) - CT(GAPDH)$ with XDH as our gene of interest and GAPDH as our housekeeping gene. $\Delta\Delta CT = \Delta CT(\text{Test}) - \Delta CT(\text{Naïve})$. XDH expression at 24 and 48 hours post SNI surgery had decreased expression in both contralateral and ipsilateral DRGs compared to naïve animals. This was a striking difference from previous results (Figure 4) that showed a clear increase in XDH.
transcript levels 24 hours post SNI surgery compared to naïve animals. The results from RT-PCR data may be confounded due to non-neuronal cells in the samples. These non-neuronal cells could include satellite cells, meninges from the spinal cord, or connective tissue. The samples from whole population transcriptional profiling were specifically sorted using FACS, selecting for neurons, specifically Nav1.8 expressing neurons. In the more recent samples, DRGs were taken as a whole, which can include the aforementioned non-neuronal cells, but also different subsets of neurons, not only the Nav1.8 subset. If XDH expression is only increased in a specific neuronal subset, relative expression may not be apparent in whole DRGs.
Figure 13. XDH expression was down regulated post injury compared to naïve mice

Fold change expression was calculated $2^{\Delta \Delta CT}$. CT mean was averaged for all sample types (biological triplicates and technical replicated). $\Delta CT = CT(XDH) - CT(GAPDH)$ with XDH as our gene of interest and GAPDH as our housekeeping gene. $\Delta \Delta CT = \Delta CT(\text{Test}) - \Delta CT(\text{Naïve})$. XDH expression at 24 and 48 hours post SNI surgery had decreased expression in both contralateral and ipsilateral DRGs compared to naïve animals.
Chapter IV
Discussion

Febuxostat has been a highly effective and clinically relevant treatment of gout, relieving symptoms of hyperuricemia and pain, due to its ability to inhibit xanthine oxidase (XO). The mechanisms of action for its therapeutic action, however, are not clearly understood. It was hypothesized that febuxostat through inhibition of XO would inhibit downstream effects that lead to pain, including ROS and IL-1β production. The experiments done in this study looked at the PO treatment of febuxostat in mice post spared nerve injury and showed no change in mechanical pain response compared to vehicle treatment.

PO treatment of febuxostat may have been a major limitation in these experiments. Chosen for clinical relevance, we cannot be sure that PO administered febuxostat entered the blood stream from the gastrointestinal tract to target the intended neuronal cells. Submandibular blood samples were taken, spun, and serum was stored at -80°C on days -1, 7, 14, and 21 throughout the experiment (Figure 6C). Future work could include testing the serum samples for febuxostat levels to determine if febuxostat was in circulation. Another dose verification could include testing XO activity in the neurons (Atlante, Valenti, Gagliardi, & Passarella, 2000). If XO inhibition is not seen in our cells of interest, it would support the lack of mechanical pain sensitivity changes seen.

Due to off target effects of current pain relieving pharmaceuticals, not many drugs are effective in treating neuropathic pain and cause long term suffering. About 60% of
neuropathic pain is localized to one area (Mick et al., 2011), so broad acting therapeutics may not be as effective. The neuropathic pain model, spared nerve injury, used in these studies is more similar to localized neuropathic pain that is most experienced. The need for a more targeted therapeutic still exists, and in addition to an effective molecule, an effective dosing route is also of high importance.

XO is found ubiquitously in the body, in the liver, gut, kidneys, heart, brain, and plasma, meaning that when administered febuxostat will non-selectively inhibit XO in the body. Due to the localized site of pain in this neuropathic pain model, it could be beneficial to administer treatment closer to where inhibition of XO would be most therapeutic. High levels of XDH were seen in injured Nav1.8 DRG neurons versus uninjured neurons (Figure 4). Intrathecal injection, into the L5-L6 subarachnoid space in the vertebrae (Fairbanks, 2003), would target these DRGs at L4-L6 that specifically innervate the sciatic nerve which is the location of injury in the spared nerve injury (Decostern, I. and Woolf, C. 2000). Intrathecal injection is a minimally invasive and non-damaging procedure in mice.

Some caveats with intrathecal injections do exist. The maximum administered volume that can be safely injected into the mouse is only 5uL. This administration method has a high learning curve; 6 hours per week for 2-6 months is needed to achieve 95-100% success rate (Fairbanks, 2003), assuming previous mouse experience. There is a lack of post-injection verifiability; after injection, researchers cannot verify that injections actually entered the intended subarachnoid space. Researchers use a lateral tail flick as a sign of successful injection as they enter the vertebral space, but the flick is not a guarantee of proper injection, as the placement of the needle can move after the tail
flick is seen. In addition, although this method has been used without anesthesia (Fairbanks, 2003), more recent and standard protocol is to use light, short lasting anesthesia when injecting (Njoo, Heinl, & Kuner, 2014). This is due to the intricacies of the procedure. The use of anesthesia with the intended daily dosing of febuxostat may confound measured pain results in the experiments. Generally it is best to avoid using any anesthetics or analgesics in pain research. Despite these caveats, intrathecal or other direct and targeted dose administration would prove worthwhile. Direct XO inhibition may lead to more potent effects in the area of interest and potentially decrease pain in the intended area.

Previous work showing high XDH gene expression in Nav1.8 expressing neurons post SNI (Figure 4) is compelling evidence that points towards a significant role of XO in neuropathic pain. In the experiment, DRGs were dissected from the L4-L6 region that represents the cell bodies whose axons travel to the injured sciatic nerve in SNI. These DRGs were then sorted using FACs selecting for Nav1.8 marked neurons made possible by using the Nav1.8 Cre+/Tdtomato+ mouse line. XDH gene transcript levels in DRGs showed a marked increase 24 hours post injury compared to contralateral DRGs in the same mice and also compared to naïve mice with no injury (Figure 4). This increase suggests a role of XO in pain and injury. However, in more recent RT-PCR experiments, XDH expression in DRGs did not show an increase 24 or 48 hours after injury compared to naïve mice or the contralateral DRGs (Figure 13). Differences between the two studies are essentially important to assess the conflicting results.

The former study, specifically analyzed XDH levels in Nav1.8 neurons, a subset of neurons involved in pain transmission, while more recent experiments did not
specifically select for Nav1.8 expressing neurons. Nav1.8 is a voltage dependent sodium channel encoded by the SCN10A gene and is expressed in 85% of nociceptive neurons (Stirling et al., 2005). Additionally, 75% of all Nav1.8 expressing cells are DRG neurons, with 25% being mechanoreceptors (Shields et al., 2012). Nav1.8 plays an important role in nociception. It has a slow rate of inactivation, which allows higher frequency firing and longer action potentials. These channels also recover quickly from inactivation, allowing them to fire again quickly. Genetic mutations in Nav1.8 channels are found in patients experiencing painful neuropathies and lead to decreased firing thresholds and increased firing frequencies (Han, Huang, & Waxman, 2016). The intrinsic properties of Nav1.8 channels and the gain of function genetic mutations seen in patients with neuropathy suggest a major role for these channels in the mechanisms of neuropathic pain.

A major role for Nav1.8 in pain transmission could be an explanation for why no $XDH$ increase was seen in the recent RT-PCR experiments (Figure 13). DRG samples may have included Nav1.8 negative neurons or non-neuronal cells including satellite cells, meninges from the spinal cord, or connective tissue that also do not express Nav1.8. $XDH$ upregulation after injury could be cell-type specific, only increasing in Nav1.8 expressing cells and therefore the contributing Nav1.8 negative cells may have masked any increase that would be seen in Nav1.8 positive cells. Increased $XDH$ levels in Nav1.8 expressing neurons after injury should be confirmed with further experiments. It should be of note that circulating XO activity has been shown to correlate with increased $XDH$ transcript expression (Dupont et al., 1992), but further experiments should be conducted to verify XO activity after neuronal injury as well.
Verification of $XDH$ and XO activity increase would indicate grounds for further exploration of ROS in order to identify a mechanism if pain decrease is also seen. Research shows XO has been shown to be a large contributor to ROS acting as a reducing agent to transfer electrons from $O_2$ to yield $O_2^-$. It can be hypothesized that XO increase leads to increased ROS, which causes oxidative damage. Therefore inhibition of XO with febuxostat, would reduce ROS to prevent further tissue damage. Unsurprisingly, febuxostat has been shown to protect the kidneys from ischemia-reperfusion damage (Tsuda et al., 2012). Increased $XDH$ post nerve damage therefore may be causing further damage by way of ROS leading to the activation of the inflammasome, release of IL-1β and the persistence of neuropathic pain. Inhibition of XO therefore could protect against chronic pain.

If after further experiments, XDH levels are indeed not increased in the spared nerve injury model, a change in model may be an important next step. It has been shown that allopurinol, an XO inhibitor, increases nerve and vascular function in diabetic rats (Inkster, Cotter, & Cameron, 2007). Researchers used a standard streptozotocin intraperitoneal injection to induce diabetes and verified disease state by measuring hyperglycemia in the blood and glycosuria in the urine. These findings showed benefits of allopurinol on nerve conduction, vasodilation/blood flow, and mechanical hypoalgesia (Inkster et al., 2007). It is well known that diabetes is associated with neuropathy and intense pain that is debilitating for patients. Diabetes in mice induces hypoalgesia, both mechanical and thermal, which can be measured in the hind paw using the Hargreaves test and Von Frey filaments.
ROS have also been shown to be elevated in diabetes due to hyperglycemia causing an increase in glycolysis, which creates an NADH/NAD+ imbalance. Imbalance of NAD+/NADH will cause reductive stress that will reduce glutathione reductase, leading to elevated oxidized glutathione. This oxidized glutathione cannot donate electrons to the ROS $\text{H}_2\text{O}_2$ and therefore $\text{H}_2\text{O}_2$ will accumulate. In diabetes, ROS can cause insulin resistance, insulin deficiency, and cell death (Wu et al., 2016). As discussed previously ROS are also a large contributor to neuropathic pain. Accumulation of ROS in diabetics may be a contributor to the neuropathy and pain experienced. These data suggest the therapeutic benefit of administering febuxostat in diabetic neuropathy in a streptozotocin induced diabetic mouse model to access its role in pain development.
References


