Toll-like 2 and 4 Receptor Antagonism Reverses Neuropathic Pain and Associated Spinal Inflammation in Male and Female Dark Agouti Rats in a Model of Multiple Sclerosis

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Toll-like 2 and 4 Receptor Antagonism Reverses Neuropathic Pain and Associated Spinal Inflammation in Male and Female Dark Agouti Rats in a Model of Multiple Sclerosis

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Abstract:

More than 92% of patients with multiple sclerosis (MS) report frequent and disabling neuropathic pain. In MS, neuropathic pain develops after demyelination, neuroinflammation, and damage to axons in the central nervous system. Although several treatments for MS-related neuropathic pain exist, many patients’ symptoms are refractory to current treatments. Recent research has provided evidence that toll like receptors 2 and 4 (TLR2/TLR4) are implicated in propagating the inflammatory response, raising the potential of TLR2/TLR4 importance in inflammatory conditions such as MS. Moreover, previous research in our lab has shown that TLR2/TLR4 antagonists are effective at reversing neuropathic pain in various rodent models involving peripheral nerve injury. In this study we thus investigated the effects of the non-opioid TLR2/TLR4 antagonist, (+)-naltrexone ((+)-NTX), on mechanical allodynia and transcription levels of spinal TLR2/TLR4-related inflammatory markers (i.e., TLR2/TLR4, nod-like receptor protein 3 (NLRP3) inflammasome, interleukin-1β (IL-1β), Tumor Necrosis Factor (TNF), and NF-kappa-B inhibitor alpha (IκBα)), as well as the Th17 cell signalling molecule, Interleukin-17 (IL-17), using experimental autoimmune encephalomyelitis (EAE), a model of MS and its associated central neuropathic pain. Male and female Dark Agouti rats were induced with EAE and 14 days later began 14 days consecutive treatment with subcutaneous (+)-NTX or saline. (+)-NTX treatment successfully reversed neuropathic pain in both male and female rats compared to the rats receiving saline treatment. Moreover, (+)-naltrexone treatment resulted in significantly lower inflammatory mRNA markers (i.e., TLR2/TLR4, NLRP3, IL-1β, TNF, IκBα, and IL-17) in the spinal cord, relative to the saline-treated animals. XT-203, another TLR2/TLR4 antagonist, reproduced behaviour results, and also reversed mechanical allodynia in male rats. Lastly, administration of intrathecal interleukin-1 receptor antagonist (IL-1ra) on day 15 and 29 post EAE induction demonstrated that ongoing spinal IL-1β signalling is necessary for EAE-induced mechanical allodynia, both early and late in disease development. Collectively, our findings provide the first evidence supporting TLR2/TLR4 and intrathecal IL-1β antagonism as effective interventions against EAE related chronic neuropathic pain in both males and/or females and suggests decreased spinal IL-1β and other related inflammatory signals may be important mechanisms by which (+)-naltrexone exerts its therapeutic effects.
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Introduction:

Multiple Sclerosis (MS) is a debilitating, life-long chronic inflammatory neurological disease of the central nervous system (CNS) with a high prevalence in North American and Europe (Haines et al., 2011), affecting almost one million people in the United States (Wallin et al., 2019) and 2.5 million people globally (Duffy et al., 2018a; Huang et al., 2017). MS is one of the most commonly acquired neurologic disorders in the world (Browne et al., 2014), generally affecting young adults ages 20-40, and is characterized by axonal damage, demyelination, and lesion formation in the CNS, which lead to severe physical, cognitive, and neurological defects (Ghasemi et al., 2017). The classic symptoms of MS involve motor disturbances, such as the loss of motor function (paralysis), muscle tremors, and spasticity. In addition, MS patients experience neuropathic pain, visual impairments, and cognitive disturbances, including impaired social interaction, memory deficits, and the inability to feel pleasure (i.e., anhedonia) (Haussleiter et al., 2009). These symptoms can result in a reduced quality of life.

The pathophysiology of MS is still unknown, but it is widely believed to be an autoimmune disease, in which the immune system directly participates in the destruction of myelin and neurons (Wu & Alvarez, 2011). An initial unidentified antigen triggers infiltration of myelin-specific autoreactive CD4+ T cells, specifically T helper cells 1 and 17 (Th1 and Th17) across the blood brain barrier and into the CNS. The invasion triggers the release of chemokines and neurotoxic chemicals to recruit inflammatory immune cells, such as macrophages, monocytes, and microglia (Frischer et al., 2009; Koning et al., 2007). These cells produce a highly inflammatory environment that promotes the formation of axonal and neuronal lesions in the CNS, which are thought to be involved in the main symptoms associated with MS (Dhabhar, 2014). T regulatory cells are a specific subpopulation of T cells that act to suppress the immune system, maintain self-tolerance, and avoid autoimmune diseases (Goverman, 2011). These cells have been observed to be suppressed during MS pathology. Likewise, the etiology of the disease remains elusive, although research has indicated environmental, genetic, and epigenetic factors can determine the risk for developing MS. Moreover, certain environmental factors can increase the risk for genetically vulnerable individuals to develop the disease (Canto & Oksenberg, 2018; Hewagama & Richardson, 2009; Muñoz-Culla et al., 2013). To investigate the pathogenesis of MS and potential therapeutic targets, numerous animal models have been developed, of which the animal model
experimental autoimmune encephalomyelitis (EAE) is the most utilized (Procaccini et al., 2015).

EAE is a complex model in which the interaction between the immune system and central nervous system leads to the approximation of the key characteristics of MS: inflammation, demyelination and axonal, and gliosis, or the nonspecific reactive change of glial cells in response to damage to the central nervous system (CNS) (Constantinescu et al., 2011; Pekny & Pekna, 2016). EAE is induced through an intradermal injection of a myelin-associated antigen, myelin oligodendrocyte glycoprotein (MOG). The introduction of MOG results in multiple immunopathological and neuropathological mechanisms similar to MS, like T cells infiltrating the CNS, immune cells attacking myelin, formation of brain and spinal cord lesions, and the production of inflammatory cytokines. The common dose of MOG that produces typical EAE-related disease severity and associated motor symptoms varies between laboratories (i.e., 16 ug in our lab), and these standard dosing regimens have answered many questions about the causation and progression of the disease. In addition, they have allowed several drugs that are currently used to treat MS and its symptoms to be tested and validated (Bjelobaba et al., 2018; Constantinescu et al., 2011; Glatigny & Bettelli, 2018; Kornek et al., 2000). However, this standard dose of MOG also results in severe motor symptoms as the disease progresses, such as full hind paralysis and partial upper limb paralysis. The dose specifically used for the behavioural experiments here (i.e., 4 ug) was chosen to avoid such extreme EAE expression and impairments, as motor dysfunction could confound the behavioural testing used to measure pain.

Chronic central neuropathic pain is a one of the most common and troubling symptoms of MS, with more than 75% of patients reporting experiencing persistent pain over the course of the disease (Drulovic et al., 2015). Individuals suffering from neuropathic pain suffer from different forms of pain, including paroxysmal pain (shooting electric shock-like sensations), paraesthesia (the abnormal sensations, such as tingling, prickling a “pins-and-needles” type sensations), dysesthetic pain (burning sensations in legs and arms), and evoked pain, like hyperalgesia (increased sensitivity to painful stimuli) and allodynia (perception of innocuous or non-painful stimuli as painful) (Murphy et al., 2017). Although several treatments for MS-related neuropathic pain exist many patients’ symptoms are refractory to current treatments (Khan & Smith, 2014; Murphy et al., 2017; Solaro et al., 2013). Studies reveal that many patients report insufficient relief of pain with currently available treatments which include antidepressants, opioid analgesics, anticonvulsants, and cannabinoid drugs (Duffy et
al., 2018b; Fornasari, 2014; Khan & Smith, 2014; Murphy et al., 2017). Untreated pain adversely affects the quality of life for individuals suffering from MS chronic pain and can result in to depression, sleep disturbances, insomnia, fatigue, and decreased mental, social, and physical functioning (Brola et al., 2014; Haythornthwaite & Benrud-Larson, 2000; O’Connor et al., 2008).

An evolving body of work reveals that animals expressing EAE show hypersensitivity and allodynia, similar to that of MS (Mallucci et al., 2015; Svendsen et al., 2004). EAE has thus been established as a useful and reliable model to study neuropathic pain in MS (Olechowski et al., 2009). As a result of inflammation, demyelination and cell damage in the CNS, both MS patients and EAE subjects are associated with elevated levels of pro-inflammatory cytokines in the CNS, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-17 (IL-17), tumor necrosis factor (TNF), and interferon-γ (IFN-γ) (Komiyama et al., 2006; Milligan & Watkins, 2009; Schoeniger-Skinner et al., 2007; Vitkovic et al., 2001) Elevated levels of pro-inflammatory cytokines and chemokines have been causally linked to both the expression of motor impairments, as well as secondary symptoms, like neuropathic pain (Khaibullin et al., 2017; Khan & Smith, 2014; Palle et al., 2017; K. Wang et al., 2018).

The cells in the CNS express a variety of different receptors that can recognize a wide range of foreign pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs), which are released into the extracellular space under conditions of activation, cellular stress, or tissue damage (Álvarez & Vasquez, 2017). When these receptors are activated, they activate an innate and adaptive immune cascade in response to invading pathogens (PAMPs) or endogenous damage/danger (DAMPs). Among these pathogen recognizing receptors (PRRs) is the family of toll-like receptors, which are broadly expressed throughout the CNS (Bsibsi et al., 2002), providing a key molecular link between tissue injury and inflammation. In this study, we hypothesized that in MS and EAE, cellular damage, myelin breakdown, and lesion formation due to chronic inflammation releases damage-associated molecular patterns (DAMPs) that initiate and activate the Toll-like receptors 2/4/ (TLR2/TLR4) pathway, amplifying the proinflammatory response in the CNS (Kielian, 2006; Piccinini & Midwood, 2010). Although DAMPs have physiological importance for signalling tissue damage to the body and to initiate healing, there is accumulating evidence that they are involved in the promotion of chronic inflammatory states and autoimmune diseases. High levels of DAMPs have been recorded and found in lesions in
the CNS in MS and EAE, such as high mobility group box chromosomal protein 1 (HMGB1), fibrinogen, heat shock protein 70 (like HSP70), etc. (Andersson et al., 2008; Hernández-Pedro et al., 2016; Mansilla et al., 2012; Miranda Acuña et al., 2017). These DAMPs are all known to be endogenous ligands for TLR2 and TLR4, two extracellular TLRs that are constitutively expressed by both microglia and astrocytes (Miranda-Hernandez & Baxter, 2013; Nicotra et al., 2012; Yu et al., 2010). The activation of TLR2/TLR4 by DAMPs in MS initiates chronic neuroinflammation through activation of MAPK signalling pathways, nod-like receptor protein 3 (NLRP3) inflammasome, NF-κB transcription factor and caspase-1, all resulting in the amplification of inflammatory mediators (CHU et al., 2006; Drexler & Foxwell, 2010; Fernandes et al., 2014; Inoue & Shinohara, 2013). This finally results in an excitatory positive feedback loop of proinflammatory cytokines, promoting a chronic inflamed state. TLR2/TLR4 have emerged as important targets for treatment of MS and EAE symptoms to suppress proinflammatory cytokines (Gooshe et al., 2014; Miranda-Hernandez & Baxter, 2013; Racke & Drew, 2009).

One of the biological effects of cytokines and chemokines produced due to TLR activation is the generation of pain, nod-like receptor protein 3 (NLRP3) inflammasome-dependent release of IL-1β, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent release of TNF are considered two of the key players (Inoue & Shinohara, 2013; Keane et al., 2018; Vincenzi et al., 2013). Both molecules are expressed in higher amounts in the dorsal root ganglion (DRGs) and spinal cord lesions of patients with MS and animals with EAE. (Rodrigues et al., 2016). In addition, elevated levels of IL-1β have been observed in other animal models of neuropathic pain (Burm et al., 2016; Lin & Edelson, 2017). IL-1β can act on the IL-1 receptors on the microglia and macrophage surface, activating a positive feedback mechanism accelerating the transcription and translation of IL-1β (Friedman, 2001; Loram et al., 2009). Overexpressed levels of IL-1β can act as potent hyperalgesic agents, and high levels of IL-1β and TNF are thought to exert their pain-inducing effects through their neuroexcitatory effects on neurons (Bustamante et al., 2011; Ren & Torres, 2009a). Furthermore, it has been previously shown that TLR4 antagonism dampens the inflammatory signals and reverses neuropathic pain in rodent models (X. Wang et al., 2016). Given the findings of the involvement of TLR pathways in MS and neuropathic pain (Gooshe et al., 2014; Racke & Drew, 2009), it is likely it could be possible to block the plethora of neurotoxic molecules and associated chronic inflammation in MS and reduce associated motor symptoms and neuropathic pain.
The idea to target pro-inflammatory cytokines is not novel to MS research. Etanercept, a TNF receptor antagonist, was investigated, raising a possibility that it has unexpected side effects, such as demyelination (Pfueller et al., 2008). The trials were discontinued due to the exacerbation of the disease. The molecular diversity of the pro-inflammatory molecules might have compensated for the loss of one specific cytokine. We anticipate that by blocking the inflammatory response more upstream (TLR2/TLR4 receptors) versus downstream (cytokine receptors) (Nish & Medzhitov, 2011), we can block a more general inflammatory response by blocking multiple cytokines and chemokines.

(+)-Naltrexone [(+)-NTX] is a highly selective TLR2/TLR4 receptor antagonist. (+)-NTX contains only the positive enantiomer and, therefore, does not interfere with the classical mu opioid receptors, as does (-)-naltrexone (Selfridge et al., 2015). (+)-Naltrexone is permeable to the blood brain barrier, and, therefore, active with systemic administration. In addition, most immunomodulatory approaches require complicated or painful routes of administration, such as intrathecal injections, but (+)-NTX is orally available (Brightbill & Modlin, 2000; Liaunardy-Jopeace & Gay, 2014). In this study, we are investigating the therapeutic efficacy of the TLR2/TLR4 antagonists (+)-NTX and XT-203 for treating both classical motor symptoms, as well as chronic neuropathic pain and inflammation associated with EAE in male and female Dark Agouti rats. We predict by antagonizing TLR2/TLR4 systemically, (+)-NTX will prevent the elevated levels of proinflammatory cytokines and chemokines, decreasing the inflammation state in the CNS, and thereby preventing impaired motor dysfunction, hyperalgesia, and allodynia associated with EAE.
Materials and Methods

Subjects

Subjects were pathogen free, male and female Dark Agouti Rats (225-280g; Envigo), 10-12 weeks old. Subjects were housed two per standard Plexiglas cage with lights set to 12-hour light/dark cycle with lights on at 07:00 and off at 19:00 and temperature at 23 °C ± 3°C, with standard rodent chow and water available ab libitum. Rats were given an acclimation time period of one week upon arrival before the start of any experiment. All procedures were approved by the University of Colorado Boulder Institutional Animal Care and Use Committee (IACUC) and followed the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals.

MOG Administration and EAE Induction

Rats were randomly assigned to the MOG or saline group. A low-dose relapsing-remitting EAE was induced with 4 µg rat myelin oligodendrocyte glycoprotein (MOG1-125) (VU Medical Center, Netherlands, gifted by Dr. Anne-Marie Van Dam), in a vehicle containing 1:1 sodium acetate (pH=3.0) and incomplete Freund’s adjuvant (IFA) (Sigma; St. Louis, MO). Saline or MOG was administered intradermally at the base of the tail. The needle remained in place inside the base of the tail for three minutes to avoid outflow from the injection site. MOG doses were reduced to 4 µg compared to our standard dosing regimen of 16 µg to decrease the severity of paralysis that might confound testing for mechanical allodynia.

Motor Score and Body Weight

All rats were monitored daily for weight changes and motor score symptoms to assess the severity of EAE progression. The motor scores were scored on a scale from 0 to 7 based on the degree of physical paralysis. Scoring was based on the following designations: 0 = no signs of paralysis, 1 = partial tail paralysis, 2 = full tail paralysis, 3 = hind limb weakness, 4 = partial hind limb paralysis, 5 = full hind limb paralysis; and 6 = partial upper limb paralysis. Rats that reached a score of 6 were euthanized if paralysis exceeded one day. Euthanized rats received a score of 7.
Drugs

Endotoxin-free (+)-NTX was kindly gifted by Dr. Kenner Rice (National Institutes of Health, Rockville, MD, USA). IL-1 receptor antagonist was gifted by NIH Biological Response Modifier Program (Rockville, MD, USA). XT-203 was gifted by Xalud Therapeutics. All drugs were in a saline vehicle.

Toll Like Receptor 2/Toll-Like Receptor 4 Antagonist Drug Administration

Treatment groups receiving either (+)-naltrexone [(+)-NTX] or XT-203 and respective control groups receiving saline were counterbalanced for approximately equivalent motor scores. Drug administration began on day 15 post-EAE induction. The animals received three subcutaneous injections of TLR2/TLR4 antagonist or saline daily, administered at 09:00, 12:00, and 15:00 hr for two weeks during behavioural testing. The (+)-NTX dose used was 6 mg/kg 3x/day (dose determined in this study), and for XT-203 the dose used was 15 mg/kg 3x/day (determined by BV2 culture studies looking for LPS-stimulated NO release (Xalud unpublished studies)).

Interleukin-1 Receptor Antagonist Administration – Acute Intrathecal Injections

Animals were lightly anesthetized with 3% isoflurane. The lumbar region was shaved and cleaned. An 18-gauge guide needle, with the hub removed, was inserted into the L5/6 intervertebral space. A PE-10 catheter was inserted via the guide needle, premarked such that the proximal end of the PE-10 tubing rested over the L4-L6 lumbar spinal cord. All intrathecal drugs were administered over 20 seconds (1 ul of IL-1ra followed by 2 ul of sterile saline flush), with a 30 s delay before removing the catheter and guide needle. Each animal was anesthetized for a maximum of 5 min, and none incurred observable neurological damage from the procedure.

Manual Von Frey Testing for Mechanical Allodynia

Rats were habituated to the testing apparatus for four consecutive days before testing for an hour each day. The von Frey test was performed on the plantar surface of both the left and the right hind paw. A logarithmic series of ten calibrated Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL, USA) were sequentially applied, from low to high intensity.
threshold to both hind paws in random order. Each filament was applied for eight seconds with constant pressure to determine the stimulus intensity threshold stiffness required to provoke a paw withdrawal reaction. Log stiffness of the hairs was determined by \( \log_{10}(\text{milligrams} \times 10) \) and the range of filaments used in this experiment was 0.4 - 15g. The stimulus intensity threshold that elicited a withdrawal response was used to compute the 50% paw withdrawal threshold, using a maximum-likelihood fit method to fit a Gaussian integral psychometric function (Harvey, 1986), which normalizes the data for parametric analysis (Loram et al., 2009). The behavioural testing was performed blind with respect to drug administration.

**Tissue Preparation for mRNA quantification**

Tissue was collected on day 30 post-MOG administration. Rats were transcardially perfused with ice cold 0.9% saline solution while under sodium pentobarbital anaesthesia. Animals continued to receive the normal timed injections until the time of dissection. Tissue was extracted and stored at -80°C.

**Processing of Tissue for Real Time PCR (RT-PCR)**

- **mRNA Extraction**

  Tissue samples were homogenized on ice with 800 µL of trizol (Thermo Fischer Scientific; Waltham, MA) and were incubated at room temperature for 10 minutes and then spun in a 5810R Centrifuge (Eppendorf; Hamburg, Germany) at 4°C and 11,900 x g (rcf) for 10 minutes. The supernatants were transferred to new tubes and the pellet was discarded. To separate the aqueous and organic layers, 160 µL of chloroform (Sigma; St.Louis, MO) was added to the samples before vortexing for 2 minutes and sitting at room temperature for 3 minutes. The samples were then centrifuged at 4°C and 11,900 x g (rcf) for 15 minutes. The aqueous layer was separated from the organic phase and added to 400 µL of 100% 2-propanol (Sigma; St. Louis, MO). The samples were vortexed and incubated at room temperature for 10 minutes before they were centrifuged again at 4°C and 11,900 x g (rcf) for 10 minutes. The supernatant was decanted from each sample, and 1 mL of 75% ethanol was added to each sample before they were centrifuged again at 4°C and 7,500 x g (rcf) for 5 minutes. The samples were decanted again and the ethanol wash was repeated. The tubes were inverted and, once the pellet dried and turned clear, the samples were resuspended in 12 µL in nuclease-free water (Bio-Rad; Hercules, CA). The mRNA concentration and purity for each sample was
measured using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific; Waltham, MA). Samples were stored at -80°C.

- **cDNA Synthesis**

  3 µg of mRNA from each sample was added to nuclease-free water (Bio-Rad; Hercules, CA) in a total volume of 10 µL. 2 µL of Mastermix 1 (Thermo Fischer Scientific; Waltham, MA), consisting of 50% dNTP mix and 50% random hexamer primers, was added to the samples before they were incubated in an iCycler (Bio-Rad; Hercules, CA) at 65°C for 5 minutes. The samples were quickly cooled down on ice for two minutes before a second incubation in the iCycler at 25°C for two minutes in Mastermix 2 (Thermo Fischer Scientific; Waltham, MA). Mastermix 2 consisted of 33% 0.1 M DTT and 66% 5X first strand buffer. After the incubation, 1 µL of Superscript II Reverse Transcriptase (Thermo Fischer Scientific; Waltham, MA) was added to each sample before they were incubated in the iCycle for 10 minutes at 25°C, 50 minutes at 42°C, 15 minutes at 70°C, and then held at 4°C until retrieval. Samples are stored in -20°C

- **Real Time Polymerase Chain Reaction (RT-PCR)**

  Each sample contained 13 µL SYBR mix (Qiagen; Hilden, Germany), 1 µL of cDNA sample, 1 µL forward primer and 10 µL nuclease free water. The plate was centrifuged at 10,000 rpm at 21-23°C for 1 minute. Primer sequences were obtained from the GenBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Primers were generated to span an intron to eliminate genomic interference. The BioRadiQ5 program was used to run the real-time polymerase chain reaction in a sequential series of temperatures (95°C, 94°C, 57°C, 72°C, and then 95°C). The levels of IL-1β, TLR2, TLR4, NLRP3, TNF, IxBα and IL-17 mRNA expression were quantified using the ΔΔCT method relative to the housekeeping gene GAPDH. There were no group differences in GAPDH mRNA expression levels.

**Statistical Analysis**

Statistical analyses were conducted using GraphPad Prism v.7.0d software. Analysis of PCR data was done with a one-way ANOVA. Behavioural von Frey data was analysed with repeated measures 2-way ANOVA and Bonferroni’s multiple comparison post-hoc test. For all tests, statistical significance was set to α=0.05.
Primer Specifications

Table 1: PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’- 3’)</th>
</tr>
</thead>
</table>
| GAPDH  | **F:** AGGGACAATCTCACACAGG  
          **R:** GACTCAACCTTCCTCTCCCA |
| IL-1β  | **F:** GAAGTCAAGACAAAGTGG   
          **R:** TGAAGTCAACTATGTCCCG |
| TLR 2  | **F:** TGGAGGTCTCCAGGTCAAATC  
          **R:** ACAGAGATGCTGCGAGAAT |
| TLR 4  | **F:** TCCCCGTGACTAGGACTTC  
          **R:** CACACCTGGATAAATCCAGC |
| NLRP3  | **F:** AGAAGCTGGGGTGGTGAATT  
          **R:** GTTGTCTAACTCCAGATCC |
| TNF    | **F:** CAAGGAGGAGAAGTTCCCA  
          **R:** TTGGTGGTGGCTACGACG |
| IκBα   | **F:** CACCAACTCAAAGGCCCACA  
          **R:** GCTCTGAGCGTGGACTCA |
| IL-17  | **F:** TCCATCCATGCTGCCTGATGC  
          **R:** ACTCTGAGCCCGCAATGAGGA |

Results:

1. **(+)NTX treatment reverses neuropathic pain associated with EAE in male rats**

Von Frey testing was used to investigate the mechanical allodynia in both male and female rats. In this test, monofilaments with different diameters are applied perpendicular to the surface of the hind paw for eight seconds, delivering a constant pre-determined force. Nocifensive behaviour, which includes paw withdrawal, licking, or shaking during application of the stimulus, is recorded for every monofilament and force for both hind paws. As you apply different forces you can determine threshold in which 50% of the time the animal withdraws its paw. (Deuis et al., 2017; Olechowski et al., 2009). To test the effects of EAE and treatment with (+)-NTX on allodynia, rats were treated with (+)-NTX or saline on day 14 post-MOG. Animals with EAE expressed lower threshold of pain; however, when treated with (+) NTX,
allodynia was reversed in both hind paws in male rats (Figure 1A and 1B). Intergroup differences were analysed using a two-way ANOVA and revealed a significant effect of treatment ($F_{2,13} = 10.55, p = 0.0019$), effect of time ($F_{8,104} = 39.11, p < 0.0001$), and a significant interaction between treatment and time ($F_{16,104} = 6.607, p < 0.0001$) in the left hind paw and a significant effect of treatment ($F_{2,13} = 12.94, p = 0.0008$), effect of time ($F_{8,104} = 36.41, p < 0.0001$), and a significant interaction between treatment and time ($F_{16,104} = 5.33, p < 0.0001$) in the right hind paw. Bonferroni’s multiple comparison post hoc test revealed significant differences in between saline and both (+)-NTX treatment groups (6 mg/kg vs 12 mg/kg) after three days of treatment on days 22 ($p_{6mg/kg} < 0.0001$, $p_{12mg/kg} = 0.0016$), 25, and 28 (all $p$ values for both doses $< 0.0001$) post-MOG in the right hind paw. In addition, significant difference between 6 mg/kg (+)-NTX and saline-treated animals were noted on day 17 ($p = 0.0184$), 22, 25 and 28 (all $p < 0.0001$) in the left hind paw. Likewise, this was seen for the 12 mg/kg (+)-NTX dose on day 22 ($p = 0.0022$), 25 ($p = 0.0003$), and 28 ($p < 0.0001$) in the left hind paw. There was no significant difference between the (+)-NTX doses, therefore we have chosen to use the lower dose (6 mg/kg) for the entire study moving forward.

### 2. (+) NTX treatment has no effect on motor disturbances associated with EAE in males

To investigate the effect of (+)-NTX on motor dysfunction, paralysis level of the rats’ tail and hind paws were assessed daily for males (Figure 1C). A two-way ANOVA revealed a significant effect of time ($F_{16,384} = 4.89, p < 0.0001$). However, it revealed that there was no significant effect of treatment ($F_{2,24} = 2.290, p = 0.1229$). Bonferroni’s post hoc test highlighted that the low dose MOG resulted in motor deficits, but that neither the 6mg/kg or 12 mg/kg (+)-NTX dose stopped the progression of the motor disturbances.
Figure 1: 6 mg/kg/day (+)-NTX treatment reverses the mechanical allodynia in male Dark Agouti rats associated with low-dose EAE progression and is equivalent to 12 mg/kg/day.

Figure 1: Dose Effect in Male Rats on Motor Scores and Allodynia. (A, B) Von Frey thresholds were assessed. 6 mg/kg (+)-NTX treatment significantly attenuated allodynia associated with EAE in both the left hind paw on days 17 (p = 0.0184), 22, 25 and 28 (p < 0.0001) post-MOG and the right hind paw on days 22, 25 and 28 (p < 0.0001) post-MOG relative to saline controls. 12 mg/kg (+)-NTX treatment significantly relieved mechanical allodynia on days 22 (p= 0.0022), 25 (p = 0.0003) and 28 (p < 0.0001) post-MOG in the left hind paw, and on days 22 (p = 0.0016), 25 and 28 (p < 0.0001) post-MOG relative to saline controls. No significant difference was observed between the 6 mg/kg vs. 12 mg/kg (+) NTX doses. (C) Motor scores were taken daily. There was no significant difference in motor scores between treatment and the saline control group (p = 0.1229) or between the 6 mg/kg vs. 12 mg/kg (+) NTX dose. Data presented as mean ± SEM. Group sizes: MOG-saline (n=5), MOG - 6 mg/kg (+)-NTX (n=6), MOG – 12 mg/kg (+)-NTX (n=5).* indicates significant difference between saline and 6 mg/kg (+)-NTX treatment in males. # indicates significant differences between saline and 12 mg/kg (+)-NTX. ^ indicates a significant difference between 6 mg/kg (+)-NTX treatment and 12 mg/kg (+)-NTX treatment.
3. (+)-NTX treatment reverses alldynia in female Dark Agouti EAE rats

Sex and gender can have a crucial impact on the desired therapeutic effect and efficacy (Anderson, 2008), therefore it is important to ensure that (+)-NTX is a viable drug in treating alldynia in both male and females (Figure 2). Both male and female EAE rats developed mechanical alldynia, and when treated with (+) NTX, alldynia was reversed in both hind paws (Figure 2A and 2B). A repeated measures two-way ANOVA revealed a significant effect of sex (F_{3,20}= 15.91, p <0.0001), significant effect of time (F_{8,160} = 43.71, p < 0.0001) and a significant interaction between the variables (F_{24,160} = 7.143, p < 0.0001) in the left hind paw. The same significant effects were for the right paw (effect of sex (F_{3,20} = 20.10, p <0.0001), significant effect of time (F_{8,160} = 42.04, p < 0.0001), and interaction (F_{24,169} = 6.459, p < 0.0001). Bonferroni’s multiple comparison post hoc test revealed significant differences in males treated with saline versus males treated with (+)-NTX on days 17 (p=0.0184), 22, 25, and 28 (p < 0.0001) post-MOG in the left hind paw. The same significant effects were seen on days 17 (p = 0.0153), 22, 25, and 28 (p < 0.0001) post-MOG in the right hind paw in males. Likewise, there was a significant difference between female EAE rats treated with (+)-NTX and saline on days 17 (p = 0.0013), 25, and 28 (p < 0.0001) post-MOG in the left hind paw, and days 22 (p = 0.0106), 25 (p = 0.0032), and 28 (p <0.0001) post-MOG for the right hind paw.

4. (+) NTX treatment has no effect on motor disturbances associated with EAE in female Dark Agouti rats

To investigate the effect of (+)-NTX on motor dysfunction in females compared to males, paralysis level of the rat’s tail and hind paws were assessed daily (Figure 2C). A two-way ANOVA revealed a significant effect of time (F_{16,384} = 4.898, p < 0.0001) indicating a progression of disease and motor deficits. However, there was no significant effect of sex (F_{2, 24} = 2.290, p = 0.1229). Bonferroni’s post hoc test stresses that (+) NTX does not influence or significantly affect motor symptoms for both males and females.
Figure 2: (+)-NTX treatment reverses allodynia associated with EAE in both male and female Dark Agouti rats.

**Figure 2:** Effect of (+) NTX on allodynia in female and male rats. Von Frey thresholds were assessed for both male and female Dark Agouti rats. (+)-NTX treatment significantly attenuated allodynia associated with EAE in male rats on days 17 (p=0.0184), day 22, 25, and 28 (p < 0.0001) post-MOG in both hind paws (Figure 2A) and on days 17 (p=0.153), 22, 25 and 28 (p < 0.0001) post-MOG in the right hind paw (Figure 2B). (+) NTX treatment reversed mechanical allodynia in female EAE rats on days 17 (p=0.0013), 25 and 28 (p < 0.0001) post-MOG in the left hind paw (Figure 2A) and only on days 22 (p=0.0106), 25 (p=0.0032) and 28 (p<0.0001) post-MOG in the right hind paw (Figure 2B). (C) Data presented as mean ± SEM. Group Sizes: Male-Saline (n=9), Male- (+)-NTX (n = 10), Female-Saline (n=8) and Female- (+)-NTX (n= 9). * indicates a significant difference between saline and (+)-NTX treated male rats. # indicates a significant difference between saline and (+)-NTX treated female rats.
5. Interleukin 1 receptor enduringly mediates central neuropathic pain in the rat EAE model of MS

To test the importance of pro-inflammatory cytokine IL-1β signalling in the propagation of neuropathic pain in EAE in the earlier stages and later states of disease propagation, 1 µL of IL-1 receptor antagonist (IL-1ra) (dose determined in (Grace et al., 2016) was intrathecally injected before von Frey testing, and pain was assessed for three hours in EAE rats (Figure 3A-D). IL-1ra treatment attenuated the allodynia associated with EAE on day 15 and 29 post-MOG. A two-way ANOVA revealed a significant effect of treatment (F$_{1,13}$ = 12.96, p = 0.0032), effect of time (F$_{6,78}$ = 169.9, p < 0.0001), and a significant interaction between treatment and time (F$_{6,78}$ = 22.6, p < 0.0001) in the left hind paw (Figure 1C) and a significant effect of treatment (F$_{1,13}$ = 13.83, p = 0.0026), effect of time (F$_{6,78}$ = 128.9, p < 0.0001), and a significant interaction between treatment and time (F$_{6,78}$ = 19.33, p < 0.0001) in the right hind paw (Figure 3B) on day 15 post-MOG. In addition, another two-way ANOVA analysis showed a significant effect of treatment (F$_{1,12}$ = 16.51, p = 0.0016), effect of time (F$_{7,84}$ = 132.9, p < 0.0001), and a significant interaction between treatment and time (F$_{7,84}$ = 12.64, p < 0.0001) in the left hind paw (Figure 3C) and a significant effect of treatment (F$_{1,12}$ = 24.48, p = 0.0003), effect of time (F$_{7,84}$ = 179.5 p < 0.0001), and a significant interaction between treatment and time (F$_{7,84}$ = 15.72, p < 0.0001) in the right hind paw (Figure 3D) on day 29 post-MOG. Bonferroni’s multiple comparison post hoc test revealed a significant difference between saline and IL-1ra treatment groups in allodynia in male rats.

6. (+) NTX treatment blocks TLR2, TLR4, IL-1β, NLRP3, IκBα, TNF and IL-17 mRNA expression in dorsal lumbar spinal cord tissue induced by EAE

EAE central neuropathic pain is known to be associated with increased transcription of inflammatory markers, and TLR2/TLR4 are critical players in the proinflammatory cytokine and chemokine cascade (Nicotra et al., 2012; Ren & Torres, 2009a; Sommer et al., 2017) and, therefore, we assessed the effect of (+) NTX on mRNA expression levels of inflammatory mediators in the dorsal lumbar region of the spinal cord. Results were analysed with a one-way ANOVA revealing a significant effect of treatment for TLR2 (F$_{2,16}$ = 13.38, p = 0.0004), TLR4 (F$_{2,16}$ = 18.54, p < 0.0001), IL-1β (F$_{2,16}$ = 8.918, p = 0.0025), NLRP3 (F$_{2,12}$ = 12.26, p = 0.0013), IκBα (F$_{2,16}$ = 8.918, p < 0.0001), TNF (F$_{2,16}$ = 8.650, p = 0.0028), and IL-17 (F$_{2,16}$ = 6.117, p = 0.0106). A post hoc analysis using Bonferroni’s multiple comparison test revealed that MOG-treated rats have significantly elevated levels of TLR2 (Fig. 4A, p = 0.0003), TLR4

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(Fig. 4B, p < 0.0001), IL-1β (Fig. 4C, p = 0.0022), NLRP3 (Fig. 4D, p = 0.0011), IκBα (Fig. 4E, p < 0.0001), TNF (Fig. 4F, p = 0.0023), and IL-17 (p = 0.0094) vs. naïve controls and that (+)-NTX significantly reversed these effects, except for IL-17 (TLR2 (Fig. 4A, p = 0.0291), TLR4 (Fig. 4B, p = 0.0123), IL-1β (Fig. 4C, p = 0.0254), NLRP3 (Fig. 4D, p = 0.0208), IκBα (Fig. 4E, p = 0.0010), and TNF (Fig. 4F, p = 0.0247) compared to saline controls. Although there was no significant difference in IL-17 mRNA expression between saline and (+)-NTX-treated subjects, this analysis reached very close to significance (p = 0.0504).

**Figure 3:** EAE associated central neuropathic pain is dependent on IL-1β signalling within the spinal cord in both early and late stages of disease progression.

**Figure 3:** Effect of IL-1ra on allodynia. Von Frey thresholds were assessed for three hours after IL-1ra intrathecal administration. (A, B) Interleukin 1 Antagonism significantly attenuated allodynia associated with EAE in both the left (p=0.0032) and the right hind paw (p = 0.0026) relative to saline controls on day 15 post-MOG. (C,D) IL-1ra treatment significantly attenuated allodynia associated with EAE in both the left (p=0.0016) and the right hind paw (p = 0.0003) relative to saline controls on day 29 post-MOG. Data presented as mean ± SEM. All group sizes n=7. * indicates significant difference between saline and IL-1ra treated rats.
Figure 4: (+) NTX treatment reverses elevated TLR2, TLR4, IL-1β, NLRP3, IκBα, TNF and IL-17 mRNA expression in dorsal lumbar spinal cord tissue induced by EAE

Figure 4: Effect of (+)-NTX on inflammation. Quantitative real-time PCR analysis of TLR2 (A), TLR4 (B), IL-1β (C), NLRP3 (D), IκBα (E), TNF (F) and IL-17 (G) mRNA levels in the dorsal lumbar region of the spinal cord tissue (L4-L6) relative to GADPH housekeeping gene 30 days post-MOG. MOG administration resulted in significantly increased expression of TLR2 (Fig. 4A, p = 0.0003), TLR4 (Fig. 4B, p < 0.0001), IL-1β (Fig. 4C, p = 0.0022), NLRP3 (Fig. 4D, p = 0.0011), IκBα (Fig. 4E, p < 0.0001), TNF (Fig. 4F, p = 0.0023) and IL-17 (p = 0.0094) mRNA relative to animals who did not have EAE. Animals that were treated with MOG and (+) NTX had significantly lower TLR2 (p = 0.0291), TLR4 (p = 0.0123), IL-1β (p = 0.0254), NLRP3 (p = 0.0208), IκBα (p = 0.0010), TNF (p = 0.0247) mRNA relative to saline treated animals. There was no significant difference in IL-17 mRNA expression (p = 0.0504) between (+)-NTX and saline treated animals. Data is represented as ± SEM. Group sizes: naïve (n=8), MOG-saline (n=4), MOG-(+)-NTX (n=7). * indicates the effect of MOG vs. naive animals. # sign indicates the effect of (+) NTX vs. saline as determined by post hoc analysis.
7. XT-203, another TLR2/TLR4 antagonist, produces the similar effects as (+)-NTX in reversing allodynia

Another TLR2/TLR4 antagonist was used with the same proposed mechanism to attempt to replicate the reversal of mechanical allodynia as seen with (+)-NTX treatment. Dark Agouti male rats underwent the same treatment regimen as (+)-NTX, receiving three subcutaneous injections a day of 15 mg/kg XT-203. Animals with EAE expressed lower pain thresholds, but XT-203 treatment attenuated the allodynia in both left and right hind paws (Figure 5A and 5B). A repeated measures two-way ANOVA revealed a significant effect of time (F_{15,80} = 57.52, p < 0.0001), significant effect of groups (F_{3,16} = 35.94, p < 0.0001), and a significant interaction of the variables (F_{15,80} = 10.67, p < 0.0001) for the left hind paw (Figure 5A). There was also a significant effect of time (F_{15,80} = 67.42, p < 0.0001), significant effect of groups (F_{3,16} = 37.22, p < 0.0001), and a significant interaction of the variables (F_{15,80} = 12.79, p < 0.0001) for the right hind paw (Figure 5B). Bonferroni’s multiple comparison post hoc test indicated a significant difference between XT-203 and saline treatment groups on pain thresholds in both the hind paws on days 22 (p_{left} < 0.0001, p_{right} = 0.0003) and 28 post-MOG (p_{left} = 0.0007, p_{right} = 0.0003).

8. XT-203 treatment has no effect on motor dysfunction associated with EAE

To investigate the effect of XT-203 on motor dysfunction, paralysis level of the rats tail and hind paws were assessed daily for males (Figure 5C). A two-way ANOVA revealed a significant effect of time (F_{18,268} = 1.987, p = 0.0105). There was, however, no significant effect of XT-203 treatment on motor scores (F_{3,16} = 2.111, p = 0.1391). Bonferroni’s multiple comparison post hoc test highlighted that XT-203 did not affect motor deficits and paralysis levels in EAE rats (p > 0.9999).
Figure 5: XT-203 treatment reverses the mechanical allodynia in male Dark Agouti Rats associated with low-dose EAE progression.

**Figure 5: XT-203 effect on Motor Scores and Allodynia.** (A, B) Von Frey pain thresholds were assessed. XT-203 treatment significantly diminished allodynia associated with EAE on day 22 and 28 post-MOG in both the left (p<sub>day 22</sub> < 0.0001, p<sub>day 28</sub> = 0.0003) and the right hind paw (p<sub>day 22</sub> = 0.0007, p<sub>day 28</sub> = 0.0003) relative to saline treated EAE rats. (C) Motor scores were assessed daily. There was no significant difference in motor scores between XT-203 treatment and the saline control group (p > 0.9999). Data presented as mean ± SEM. Group sizes: Saline–Saline (n=5), Saline – XT-203 (n=5), MOG – Saline (n=5) and MOG- XT-203 (n=5). * indicates a significant difference between saline vs. XT-203 treated male rats.
Discussion:

Neuropathic pain is a devastating symptom of MS, leaving many patients with a lower quality of life. Existing therapies, such as various anticonvulsants, antidepressants, opioids, and cannabinoids, have limited efficacy to treat neuropathic pain in MS (Murphy et al., 2017; Nielsen et al., 2018; Solaro et al., 2013). The mechanism through which neuropathic pain is generated in MS remains unclear, but has been shown to involve pro-inflammatory cytokines and related phenomena. Identification of specific key molecules and cells that mediate neuropathic pain will facilitate the development of novel therapies and may provide better understanding of chronic neuropathic pain, and MS pathology. In this study, we investigated the effect of subcutaneously administered TLR2/TLR4 antagonist, (+)-naltrexone, on classical motor disturbances, neuropathic pain and neuroinflammation. Our research was based on evidence implicating a role of neuroinflammation in neuropathic pain and that chemical mediators, such as cytokines and chemokines, released during the inflammatory response can stimulate and sensitize nociceptors (Ellis & Bennett, 2013; Schomberg et al., 2012; Sommer et al., 2017).

Consistent with MS and EAE literature, EAE subjects produced an allodynic effect in both male and female subjects associated with high levels of inflammatory mediators IL-1β, TNF, IL-17 NLRP3, and IκBα (Komiyama et al., 2006; Mallucci et al., 2015; Vincenzi et al., 2013). The success of low-dose MOG (4 μg) in creating neuropathic pain and indication of neuroinflammation provides encouragement for its use in other studies that could be limited by paralysis symptoms. The systemic administration of a pharmacological TLR2/TLR4 antagonist, (+)-NTX (Figure 1 and 2), and XT-203 (Figure 5) reversed alldynia associated with EAE, indicating that TLR2 and 4 mediate central neuropathic pain in the rat EAE model of multiple sclerosis. Using two drugs with the same proposed mechanisms to reproduce the same results further strengthens the idea that TLR2/TLR4 antagonism can be used to treat neuropathic pain associated with EAE. Moreover, it is important to note that (+)-NTX also has therapeutic effects in female rats (Figure 2). Neuropathic pain is more prevalent in female MS patients and, in addition, sex can influence analgesic efficacy (Campesi et al., 2013; Marck et
Therefore, it is important to study sex differences associated with pain in MS and treatment. Moreover, it is important to note that both TLR2/TLR4 antagonists [(+)-NTX and XT-203] treatment had no effects on the classical paralysis symptoms observed in EAE, indicating that TLR2/TLR4 receptors are not necessary in the ongoing maintenance of motor disturbances. This was unexpected, as previous experiments have investigated TLR4 knockouts and NLRP3 inhibitors, have successfully attenuated motor disturbances (Coll et al., 2015; Imani et al., 2018; Khan et al., 2018; Khan & Smith, 2014). This could, however, be explained by the fact that the (+)-NTX dose might be too weak to diminish motor deficits, as the dose 6 mg/kg 3x/day was chosen based on allodynic effect. However, it is important to recognize that the experimental groups had mild, if any, motor impairments at points across the time course due to the low-dose EAE model. To rule out a floor effect, (+)-NTX treatment has been tested against a more robust level of paralysis with the standard 16 µg dose (data not shown).

Regardless of the MOG dose, (+) NTX had no significant effect on motor deficits. A (+)-NTX or XT-203 dose effect study for motor scores where we can explore higher doses and their effect on motor scores to investigate if TLR2/TLR4 antagonism could still be a suitable treatment option to treat already established motor deficits.

In addition to behavioural testing, our study revealed the importance of pro-inflammatory cytokine IL-1β signalling in the spinal cord in the propagation of neuropathic pain in EAE. Allodynia was reversed in animals treated intrathecally with an interleukin 1 receptor antagonist (IL-1ra), mechanistically indicating that spinal II-1β controls EAE-induced mechanical allodynia in both early and late stages of disease development, and that it is a critical player in CNS neuropathic pain generation (Figure 3). Likewise, in this study we measured inflammatory markers in the dorsal lumbar region of the spinal cord to identify differentially expressed cytokines and chemokines that may mediate neuroinflammation during EAE (Fig. 4). Consistent with other studies, this experiment revealed that mRNA transcription of a variety of inflammatory molecules are induced during EAE, and (+)-NTX treatment blocked the heightened levels of mRNA in the dorsal lumbar region of the spinal cord of TLR2, TLR4, IL-1β, NLRP3, IκBα, and TNF. (+)-NTX did not significantly block IL-17
upregulation (p = 0.0504), but was very close to statistical significance. This research suggests that decreased spinal cord IL-1β and other related inflammatory signals are important mechanisms by which (+) NTX exerts its therapeutic effects in reversing neuropathic pain.

Current available therapies mainly focus on preventing the infiltration of a variety of peripheral immune cells into the CNS, however, these therapies only delay MS symptoms and are not effective at stopping disease progression (Dhib-Jalbut, 2007; Gao & Tsirka, 2011; Koning et al., 2007, 2009). Instead of preventing intrusion of innate and adaptive immune cells into the CNS, our experiment focused on competitively antagonizing TLR2/TLR4 as a therapeutic intervention to neuropathic pain. Our findings suggest that (+)-NTX competitively binds to TLR2/TLR4, inhibiting the binding of endogenous DAMPs, which results in the inhibition of the transcription of pro-inflammatory molecules and inflammation. Moreover, our study also provides support for DAMP binding to spinal cord TLR2/TLR4 as a major aspect of EAE-related inflammation and indicates TLR2/TLR4 as critical drivers of neuropathic pain and inflammation. Presumably, the positive feedback loop of inflammatory mediators is halted in the spinal cord, and, therefore, also the propagation of inflammation. Our findings are significant for mechanistic and therapeutic reasons. Not only have we shown the effectiveness of (+) NTX in preventing EAE-induced neuropathic pain, but we have provided further evidence that neuroinflammation plays a critical role in symptom-causing pathology in EAE. Likewise, it provides support that DAMP activation of TLR2/TLR4 pathway in the lumbar region of the spinal cord is a significant contributor to EAE-related inflammation.

TLR2/TLR4 are expressed on microglia, astrocytes, oligodendrocytes, dendritic cells, T lymphocytes, monocytes, macrophages, neutrophils, fibroblasts, and endothelial cells (Bsibsi et al., 2002; Hemmi & Akira, 2005; Jang et al., 2012; MacLeod & Wetzler, 2007; Oliveira et al., 2015; Parker et al., 2004; Shover & Hochstetler, 2005; Uronen-Hansson et al., 2004) and the release of inflammatory cytokines and chemokines can be mediated by any one, or any combination, of these cells. Systemic blockade of these cells would result in the inhibition of pro-inflammatory molecule release, presumably IL-1β and TNF, and, therefore, limit neuroinflammation. TLRs are primarily expressed by, and most abundantly on, microglial cells (Bsibsi et al., 2002); therefore, microglia could possibly be the main contributors to neuroinflammation and, therefore, disease and symptom progression.
The neurological synapses in the CNS are encapsulated by glia cells, outnumbering neurons, and constituting of 70% of total cell population in the CNS (Gosselin et al., 2010; Jha et al., 2012). Lately, there has been a significant increase in research focusing on glial cells due to their involvement in CNS inflammation, release of inflammatory molecules, and their potential role in autoimmune, neurodegenerative, and demyelination disorders (Popescu & Lucchinetti, 2016; Sriram, 2011; Trudler et al., 2010).

Microglia cells are the resident macrophages in the CNS that are critically involved in both the initiation and maintenance of neuroinflammation as well as in immune responses (Luo et al., 2017; Pöllmann & Feneberg, 2008). Microglia, like most immune cells, exist in many different states, distinguished into two categories: the active state and resting state. In resting state, microglia monitor for tissue homeostasis and surveillance the local milieu for potential tissue damage or pathogen through its surface receptors. Microglia activation is heterogeneous, and when PAMPs or DAMPs associate with its microglia receptor, signals initiate a cascade of pathways and biomolecular changes, converting it to its active state. Microglia activation is a complicated topic as they serve a dual function of both pro-inflammatory and anti-inflammatory states (Goldmann & Prinz, 2013; Mittelbronn, 2014). Activated microglia reprogram into either the “classic” M1 phenotype or the “alternative” M2 phenotype, depending on the type of activation signals received (Goldmann & Prinz, 2013; Lively & Schlichter, 2018; Raivich, 2005). While M2 microglia are often involved in tissue repair, inflammation dampening, and mostly involved in protective functions, the M1 phenotype is characterized by the release of proinflammatory cytokines and cytotoxic proteins, promoting an inflammatory state, tissue damage, and cytotoxic responses (Dhabhar, 2014; Goldmann & Prinz, 2013; Orihuela et al., 2016).

Although these functions are critical to maintain a normal level of health in the CNS, DAMPs of endogenous ligands cause microglia to enter a pro-inflammatory activated state during MS (Chu et al., 2018; Goldmann & Prinz, 2013; Tang & Le, 2016), potentially resulting in the adverse secondary symptoms observed in MS pathology. Elevated levels of IL-1β and TNF have been observed in the CNS of both EAE rats and MS patients (Kemanetzoglou & Andreadou, 2017; Ren & Torres, 2009b; Rizzo et al., 2018), and these two cytokines have been invariably associated with shifting microglia activation response to the cytotoxic M1 phenotype (Goldmann & Prinz, 2013; Schmitz & Chew, 2008). Once IL-1β and TNF stimulate microglia to become M1 microglia, more cytokines and chemokines are released, propagating the production of inflammatory factors and neurotoxic proteins through positive feedback.
mechanisms (Orihuela et al., 2016; Schönrock et al., 1998). This often results in a self-perpetuating cycle of promoting an extreme inflammatory state. The initial activation of microglia primes a vicious exaggerated cycle of overactivation, inflammation, and production of pro-inflammatory factors. Moreover, M1 microglia also have the ability to become antigen-presenting cells and engage with T cells through multiple surface receptors to activate T cells, which can lead to the enhancement of chronic inflammatory responses (Aloisi et al., 2000; Miranda-Hernandez & Baxter, 2013; Schetters et al., 2017; Strachan-Whaley et al., 2014).

Microglia activation has been observed to occur early in EAE and MS disease progression and remain in an enduring active state in later stages of EAE and MS disease progression (Bjelobaba et al., 2018; Duffy et al., 2016; Ponomarev et al., 2005), giving us reason to believe that microglia play a crucial role in both initiating and promoting chronic neuroinflammation in EAE and MS. Moreover, research has highlighted that glial activation is necessary to produce enhanced pain and is involved in hyperalgesia and allodynic responses (L R Watkins et al., 1997). In addition to releasing cytokines and chemokines to trigger inflammation, activated microglia also release substances, such as reactive oxygen species, nitric oxide, excitatory amino acids, and substance P, that excite and activate pain-responsive neurons in the spinal cord (L R Watkins & Maier, 2000), which can dramatically alter pain processing and nociceptive transmission. Due to the high number of TLR2/TLR4 receptors expressed on microglia and their observed involvement in MS and EAE pathology (Luo et al., 2017; Sriram, 2011), we believe they play a pivotal role in the propagation of immune-induced exaggerated neuropathic pain during disease progression (Correale & Farez, 2015; Duffy et al., 2016; Olechowski et al., 2009; Linda R Watkins et al., 2007).

Current work aims to further explore the involvement of microglia in the propagation of inflammation in the spinal cord by utilizing immunohistochemistry staining for CD11B marker on microglia. Comparing the densities of CD11B between experimental groups will provide insight on whether microglia are activated in the low dose EAE model and verify that microglia are our main contributors to inflammation. It will also reveal if systemic TLR2/TLR4 antagonist administration reduced the microglia activation. Likewise, (+)-NTX treatment is also used to investigate other adverse secondary symptoms associated with EAE and MS, like anhedonia, or the diminished ability to feel pressure using saccharin preference and social interaction testing.

Alternatively, we could explore the concept of “designer receptor exclusively activated by designer drugs” (DREADDs), which is a collection of chemogenetically-engineered G
protein-coupled receptors (GPCR) that offer novel advantages for scientific research. The recombinant GPCR cannot be activated by any endogenous ligand, thereby allowing investigators to control its spatial activation when transfected into specific cells through viral vectors. The activation is controlled by inert synthetic ligands (Roth, 2016; Smith et al., 2016; Urban & Roth, 2015). This allows the target cell to be turned “on” or “off”. It would be interesting to investigate microglia silencing and its effect on neuroinflammation and neuropathic pain in EAE rats. One issue that could arise is that the DREADDs need to be introduced through viral vectors, which could alter EAE expression and pathogenesis. To avoid this, the introduction of DREADDs would have to be done before MOG induction to ensure the virus components do not affect the disease progression.

TLR2/TLR4 antagonism significantly lowered molecular indicators of inflammation, and the generality to treat inflammation suggests that it could be an effective treatment for other negative symptoms associated with inflammation. Cognitive and affective symptoms accompanying MS, such as memory loss, learning deficits, depression, anxiety, and anhedonia have also been linked to increased levels of IL-1β and the associated inflammation in the CNS (Bent et al., 2018; Di Filippo et al., 2018; Mori et al., 2014; Rossi et al., 2017; Warabi, 2007). This suggests that TLR2/TLR4 antagonism can be a novel therapeutic target to treat multiple adverse symptoms.

Due to the complex and unknown etiology of MS, it is hard to develop an animal model that will exactly replicate the pathology and symptoms of MS, and, therefore, it is important to note that EAE is a limited animal model. It could be argued that the pharmacological blockade of TLR2/TLR4 could block the formation of anti-MOG antibodies and, thus, affecting the development of EAE; however, in our study, treatment begins after two weeks of disease progression, suggesting this is not the case here. Additionally, animals developed motor impairments and pain deficits indicative of EAE, and it would be unlikely for this to occur if anti-MOG antibodies were not produced. Although EAE has been used to develop current immunomodulatory therapies for MS, there have been cases where successful drugs in EAE studies failed to translate to human clinical trials (Hart et al., 2011; Martin, 1997). This could be explained due to the distinct differences between rodent and human adaptive and innate immune systems (Becker, 2016; Mestas & Hughes, 2004). TLRs and their signalling pathways, however, are highly conserved between species, and a limited number of differences have been noted between rats and humans (Brennan & Gilmore, 2018; Mestas & Hughes, 2004). Therefore, we have more reason to believe that, despite the limitations of the EAE animal
model, that TLR2/TLR4 antagonism and its effects on allodynia in EAE can be used to infer that it may be a successful treatment for chronic pain in MS patients.

There is also a great deal of controversy surrounding the use of immunosuppressive drugs to treat MS. Immunosuppressive drugs can affect the body’s natural way of healing, and inflammation is a part of the body’s natural response to infection and tissue damage, and has been crucial for healing (Filippi & Rocca, 2003; Kanwar, 2005; Martino et al., 2002). However, under normal circumstances, inflammation is tightly regulated and controlled. During EAE and MS, there is an obvious dysregulation of the normal inflammatory response (Constantinescu et al., 2011; Zorzella-Pezavento et al., 2013) resulting in a detrimental overamplified inflammatory response. TLR2 and TLR4 are both upregulated in EAE and MS (Drexler & Foxwell, 2010). Therefore, by pharmacological blockade of only two of the ten known TLRs, we are targeting the main contributors of inflammation while, ensuring the rest of the immune system can contribute beneficially. Like any treatment or medication, (+)-NTX might produce undesired immunosuppressive side effects, however, the drug will be an effective treatment option if the benefits outweigh the downsides.

In terms of future research, a secondary behavioural pain test could be conducted to also measure affective pain. Von Frey only measures mechanical allodynia, which does not incorporate the emotional components of pain, which are as important, if not more important, to treat patients. There is some controversy surrounding the validity of a withdrawal reflex as an preclinical surrogate of human neuropathic pain (Harte et al., 2016). A behavioural test like conflict avoidance incorporates the emotional and cognitive components of pain by letting the rat voluntarily choose to complete a task that will deliver a noxious stimulus in order to receive a reward. Due to the conflict between motivation and aversion, this test may be more indicative of the behavioural complexity associated with chronic pain (Vierck & Yezierski, 2015).

Additionally, it would be important to use western blot analysis to look at protein levels of the inflammatory markers IL-1β, TNF, IL-17, NLRP3, and IκBα. Although we already analyzed the mRNA transcripts, it is important to recognize that not all mRNA transcripts get translated into protein. It is possible that disease state increases the degradation of mRNA transcripts in cells, and, therefore, it is not guaranteed that mRNA elevated transcripts level directly correlate with protein concentrations. Moreover, it is important to determine if anti-MOG antibodies were produced in our low-dose EAE model, to establish the validity of the low dose model and to ensure a MOG effect has taken place.
Collectively, our findings provide the first evidence supporting TLR2 and TLR4 and intrathecal IL-1β antagonism as effective interventions against EAE-related chronic neuropathic pain in both male and females. Our research suggests that reductions of EAE-induced IL-1β and other inflammatory signals are important mechanisms by which (+)-NTX exerts its therapeutic effects and the effectiveness of a systemic TLR2/TLR4 antagonist at attenuating inflammation in the lumbar region of the spinal cord.

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