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TLR2 and TLR4 cascade involved in the multifaceted symptoms of experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis

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Abstract

Multiple sclerosis is a multifaceted disease involving both neurodegeneration as well as inflammation. While paralysis and other motor impairments are central to the disease, it also induces an array of other secondary symptoms including pain, depression and anxiety as well as cognition changes. Inflammation seems to be an overlapping factor in all of the symptoms of MS. Toll-like receptors (TLR) 2 and 4 have been previously implicated in the neuroinflammation that is central to the symptomology of MS and experimental autoimmune encephalomyelitis (EAE), a rodent model of MS. Antagonism of TLR2 and TLR4 via (+)-naltrexone [(+)-NTX] is thus expected to reverse many symptoms of MS including pain and depression. In this study, MS-related neuropathic pain was assessed using the von Frey test in rats with EAE and it was confirmed that (+)-NTX reversed EAE-induced neuropathic pain. Furthermore, blocking nod-like-receptor protein 3 (NLRP3), a component of TLR2-TLR4-mediated inflammatory cascades, also reverses EAE-induced mechanical allodynia. Juvenile social exploration (JSE) is a common behavioral model for anhedonia/depression/anxiety in rodents, and here we used JSE order to assess the presence of these symptoms in EAE. At this time it is unclear if EAE produces reliable deficits in juvenile social exploration. It is likely that a larger statistical power will reveal significant deficits from EAE in JSE testing, as well as reversal of those deficits upon treatment with (+)-NTX. It is crucial that the secondary symptoms of MS be investigated for the development of more efficacious treatments for this debilitating disease.
Introduction

Multiple sclerosis (MS) is an autoimmune and neurodegenerative disease of the central nervous system characterized by the deterioration of the neuronal myelin sheath (Alastair Compston & Coles, 2008; Minagar, 2014). This deterioration impedes neurons’ ability to send electrical signals, and thereby obstructs their ability to communicate. In the United States alone more than 700,000 people suffer from MS without any cure (Wallin et al., 2019). Additionally, the prevalence of MS has been steadily increasing in the past few decades (Wallin et al., 2019). In 2013, the national bill for the management of MS, including treatments and hospitalizations is estimated to be $4.3 billion (Wallin et al., 2019). It is essential that better and more affordable treatments be created to liberate the emotional, physical, and economic burden of MS.

Myelin lesions can occur at any level of the brain or spinal cord, which leads to the diverse clinical manifestations within MS (Haussleiter, Brüne, & Juckel, 2009; Minagar, 2014). However, the primary symptom of MS is motor impairments including problems with balance, coordination, strength, and sensation (Cattaneo et al., 2002). Other symptoms include cognitive decline, pain, psychiatric disorders, sensory loss, fatigue, urinary dysfunction, sexual dysfunction, among others (Minagar, 2014). In fact, 66.5 percent of patient’s report feeling pain associated with their disease state (Drulovic et al., 2015). Additionally, depression and anxiety each affect at least 20 percent of MS population (Marrie et al., 2015). Furthermore, 29.1 percent of patients reported feeling both pain and depression (Drulovic et al., 2015). Despite these prevalent secondary symptoms established in MS, the mechanisms that control pain, cognitive deficits, and psychological disorders in MS are not well understood. There is evidence that the mechanisms underlying these multifaceted symptoms of MS, including pain, anxiety, and depression share similar inflammatory cascades, and that the type of symptoms that manifest are
dependent on the tissue that is affected. Current treatments of MS are mainly pharmaceutical immunomodulatory agents such as dimethyl fumarate (DMF) and fingolimod (Gold et al., 2012); however, there is no evidence that either of these drugs treat the pain, cognitive, or psychological effects of MS, suggesting a need for better treatments for these symptoms.

It is thought that anhedonia and pain, among other secondary symptoms in MS may be controlled by similar inflammatory cascades (Anisman, Merali, Poulter, & Hayley, 2005). Inflammation can occur as a natural consequence of the mechanics behind the innate immune system; however, when inflammation exceeds normal levels it becomes a problem. A crucial part of our innate immune system includes pattern recognition receptors (PRRs) that respond danger associated molecular patterns (DAMPS), which includes things like ATP, and parts of cells, and pathogen associated patterns (PAMPS), such as bacteria and other pathogens. Typically, PRRs respond by upregulating the transcription of genes involved in inflammatory responses. Toll-like receptors (TLR) are one of many PRRs that typically sense these PAMPs and DAMPs (Takeuchi & Akira, 2010). TLRs are predominately expressed on macrophages, microglia and astrocytes (Kumar, 2019).

TLR2 and TLR4 have received considerable attention as initiators of inflammatory cascades that might play a role in these MS symptoms (Takeuchi & Akira, 2010). Activation of TLR2 and TLR4 leads to increased signaling for pro-inflammatory cytokines at the affected area (Jacobsen, Buisman-Pijlman, Mustafa, Rice, & Hutchinson, 2018). Evidence suggests that some MS symptoms may be the result of overactive TLR2 and TLR4 cascades, specifically activation of the NOD-like-receptor protein 3 (NLRP3) inflammasome and related release of pro-inflammatory cytokines like interleukin-1β (IL-1β) that are associated with MS symptoms (Lin & Edelson, 2017; Prins et al., 2013; Takemiya, Kawakami, & Takeuchi, 2018). NLRP3 has
received attention for its role in a variety of diseases involving inflammation including autoinflammatory syndromes and metabolic/inflammatory disorders thus it is likely that is also plays an imperative role in diseases that include neuroinflammation, such as MS. (Pellegrini, Antonioli, Lopez-Castejon, Blandizzi, & Fornai, 2017)

Here, we use (+)-naltrexone (NTX), a non-opioid, selective TLR2 and TLR4 antagonist, to determine the role of TLR2 and TLR4 in MS-like depression/anhedonia/anxiety and pain-like behavior. While (-)-NTX is a classical opioid receptor antagonist, opioid receptors are strictly stereoselective and do not bind (+)-isomers. In the presence of (+)-NTX, TLR2 and TLR4 are blocked, thereby theoretically preventing the activation of NLRP3 and release of pro-inflammatory cytokines such as IL-1β, which might lead to decreased MS-like symptomology. Additionally, we also assess MS-like pain behavior for the effect of MCC950, a NLRP3 inhibitor which can block the inflammatory cascade at the level of the inflammasome, presumably decreasing downstream levels of IL-1β and other cytokines. At the time of MCC950 treatment, NLRP3 activation is blocked and thereby it is anticipated that EAE-induced mechanical allodynia will be reversed.

In this study, we assessed the effect of the TLR2-TLR4 antagonist (+)-NTX on EAE-induced depression/anxiety as well as neuropathic pain. Experimental autoimmune encephalitis (EAE) is the most common animal model of MS (Kipp, Nyamoya, Hochstrasser, & Amor, 2017). EAE is induced by injecting rat myelin oligodendrocyte glycoprotein (MOG) in the base of the rat’s tail. Similar to MS, EAE induces multifaceted symptoms including motor deficits, pain, mood, and cognitive impairments (Bjelobaba, Begovic-Kupresanin, Pekovic, & Lavrnja, 2018; Khan & Smith, 2014). Upon immunization with MOG, an immune response is initiated which leads to demyelination of nonspecific neurons, similar to the demyelination that occurs in
MS (Bjelobaba et al., 2018; Kipp et al., 2017). EAE mirrors MS in that is relapsing-remitting, involves B cells, white and grey matter demyelination, and inflammation (Baker & Amor, 2014; Bjelobaba et al., 2018). Despite these similarities, there are several drawbacks including questionable CD8+ T-cell involvement and lack of axonal loss as seen in MS (Bjelobaba et al., 2018). In MS, CD8+ T-cells play a significant role in disease pathogenesis and inflammation, thus the EAE model will be ameliorated once the involvement of CD8+ T-cells is established (Lassmann & Bradl, 2017). Despite these drawbacks, using EAE as a model has successfully led to the development of several pharmacotherapies to treat multiple sclerosis, as well as heightened our understanding of the disease (Bjelobaba et al., 2018; Kipp et al., 2017).

To assess depression, anxiety, and anhedonia within EAE, a model of juvenile social exploration (JSE) was used. JSE measures the time an adult rat spends interacting with a novel juvenile rat. A decreased social interaction time from pre-EAE baseline interaction levels is thought to indicate anhedonia, which is the inability to experience pleasure, a common symptom in depressed patients, including those with MS (Hatzigiakoumis, Martinotti, Giannantonio, & Janiri, 2011). To assess neuropathic pain, mechanical allodynia was assessed using the von Frey test, which provides information about whether the subject perceives a stimulus to be innocuous or nociceptive based on the force required to initiate a paw withdrawal response. We predict that juvenile social interaction will be reduced by EAE, indicative of an anhedonic-like response, and that this effect will be reversed by administration of (+)-NTX. Additionally, it is expected that MCC950 will immediately reverse mechanical allodynia in a von Frey model.
2. Methods

2.1. Animals

Male Sprague Dawley rats (300-325 g; Envigo Labs) were used in all experiments. Rodents were housed in pairs in standard Plexiglas cages in temperature (23°C ±3°C)- and light-controlled rooms (12:12/light dark). Standard rodent chow and water were provided *ad libitum*. Rats were given a one-week acclimation period upon arrival before the commencement of any study. All procedures were approved by the University of Colorado Boulder IACUC.

2.2. EAE Induction

Relapsing-remitting EAE (Figure 1) was induced via injection of rat myelin oligodendrocyte glycoprotein (MOG; gifted by Dr. Anne-Marie Van Dam at VU university Medical Center in the Netherlands) in incomplete Freund’s Adjuvant (IFA; Sigma) and sodium acetate vehicle (pH = 3). Initially, a MOG dose effect curve was performed in order to determine the appropriate dose of MOG that would reliably affect JSE in a majority of the rats with the least amount of motor disturbances. This was done to avoid motor disturbances confounding measures of JSE and mechanical allodynia, which require intact movement for reliable interpretation. MOG was administered intradermally at the base of the tail in a volume of 100 µl at 0, 8, 16, and 32 µg doses. The dose effect curve experiment determined that the 16 µg dose was the most appropriate dose for JSE experiments, producing minimal motor impairment (Figure 1) with modest impairment of JSE behavior (Figure 2), whereas a previous experiment determined that 8 µg MOG was most appropriate for neuropathic pain studies (data not shown). Moving forward, 16 µg was used for the JSE and MCC950 von Frey experiments as the same cohort of rats was used for these studies, whereas 8 µg MOG was used for the (+)-NTX von Frey experiment. Rats were briefly anesthetized with isoflurane and given a single intradermal
injection at the base on the tail. The needle remained at the injection site for three minutes in order to prevent leakage of the emulsion.

2.3. Drug preparation and delivery

Upon commencement of (+)-NTX (6 mg/kg) or saline (vehicle) treatment, the drug course continued until time of euthanasia. Time of drug onset varied depending on the experiment: JSE experiments began (+)-NTX treatment on day 21, while von Frey experiments began (+)-NTX treatment on day 15. Subcutaneous injections occurred daily at 9 AM, 12 PM, and 3 PM for a total of 18 mg/kg/day (+)-NTX. (+)-NTX was generously synthesized and gifted by Dr. Kenner Rice at the National Institutes of Health. MCC950 (Sigma) or saline (vehicle) administered via a single intrathecal injection in the volume of 10 µl at doses of either 0.01, 0.1, and 1.0 µg on day 23 post MOG. These doses were selected in order to assess a wide range of potential therapies. Rats were briefly anesthetized with isoflurane (Piramal Critical Care).

2.4. Motor Score and Body Weight

Rats were monitored daily for motor disturbances and body mass changes. Motor disturbances were scaled from 0 to 7 based on the degree of ascending paralysis: 0, no motor symptoms; 1, partial tail paralysis; 2, full tail paralysis; 3, hindlimb weakness; 4, partial hindlimb paralysis; 5, full hindlimb paralysis; 6, partial forelimb paralysis; 7, euthanasia due to disease progression. If a rat lost more than 20 percent of its original body weight, the animal was euthanized. One subject was euthanized due to weight loss and disease progression.
2.5. Juvenile Social Exploration (JSE)

JSE was performed at four time points: baseline (pre-MOG), Day 14, Day 21, and Day 28 after MOG injection. The rats were transported to the procedural room and stored in cages that contained bedding from each rat’s homecage in order to prevent the stress that fresh cage bedding induces. The rats were habituated to the procedural room for 45 minutes prior to the test. Juvenile Sprague Dawley rats (28-32 days old) were then placed in the experimental rat’s cage for 180 seconds. Although rats were reused from multiple tests, each week the adult rat was tested with a novel juvenile. Exploration and interaction (e.g. sniffing, pinning, and allogrooming) initiated by the adult rat was monitored and recorded as total time (s) by two individual blind observers, with their scores averaged. Rats that received MOG were selected for consistently decreased JSE scores versus IFA-treated rats’ scores on days 14 and 21 (i.e. at least 5 percent difference between lowest vehicle-treated rat for both days 14 and 21), and then, based on the severity of decreased JSE, rats were equally divided into (+)-NTX and saline treatment groups to receive their final test on day 28 post MOG administration. Only a subset of rats that received MOG (25-33 percent) reached these criteria to be included in the JSE study.

2.6. Von Frey Testing

The rats were transported to the procedural room and habituated for an hour prior to the testing period, as well as habituated for an hour per day for four consecutive days prior to the trial commencement. Animals were kept individually under large cylinders on a spaced shelving. 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$_{10}$(milligrams x 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 percent paw withdrawal threshold, using a maximum-likelihood fit method to fit a Gaussian integral psychometric function, which normalizes the data for parametric analysis (Harvey, 1986). The behavioral testing was performed blind with respect to drug administration.

**Von Frey (+)-NTX Experiment**

Von Frey was performed at five time points: baseline (pre-MOG), day 7, day 14, day 16, day 22, and day 27. Dosing with (+)-NTX (6 mg/kg) began on day 15 post MOG via daily subcutaneous injections that occurred three times per day, for a total of 18 mg/kg/day. Tissue was taken on day 30 post MOG.

**Von Frey MCC950 Experiment**

Von Frey was performed at six time points: baseline (pre-MOG), day 8, day 15, day 23, and 3 hours and 24 hours post MCC950 treatment. MCC950 was given in 10 µl at doses of either 0.01, 0.1, and 1.0 µg on day 23 post MOG.

2.7. **Statistics**

GraphPad Prism v.8.2.1 software was used for all statistical analyses. Behavioral JSE data, Von Frey Data, and motor score data were normalized to individual pre-MOG baselines and assessed via Two-Way ANOVA. A Sidak’s multiple comparison post-hoc test was run in the event of a statistically significant effect. Statistical significance was set at $\alpha=0.05$. 
3. Results

3.1. MOG dose effect curve

In order to determine the appropriate MOG dose for juvenile social exploration experiments, a MOG dose effect was performed. The groups were: IFA vehicle, 8 µg, 16 µg and 32 µg MOG. These doses were selected based on past experiments with both Sprague Dawley and Dark Agouti rats. Motor scores provide insight into the motor deficits a subject may be experiencing and are described on an ascending scale with 0 being no motor disturbances and 7 being euthanized due to disease progression. It was expected that the 32 µg would exhibit the most motor deficits, and therefore would not be optimal for the experiments, but in order to assess a full range of doses, it was tested. 8 µg was chosen because this dose is twice the typical low dose given for the behavioral analysis within our lab when using EAE-susceptible Dark Agouti rats. As SD rats are less susceptible to EAE development, higher doses of MOG are required to produce EAE symptoms versus more susceptible rats, like Dark Agoutis. 16 µg was selected in order to gauge the middle of two more extreme doses. As expected, the 32 µg group expressed the most severe motor impairments (see Figure 1), followed by the 16 µg and 8 µg with minimal motor impairments. A two-way ANOVA between day and MOG doses revealed a significant effect of day ($F(3, 19) = 2.305; p=0.1094$), a significant effect of MOG dose ($F(22, 418) = 2.053; p= 0.0037$), and a significant effect of interaction ($F(66, 418) = 1.803; p=0.0003$). Sidak’s multiple comparisons post hoc reveals a significant difference between IFA and 32 µg MOG ($p=0.0073$), a significant difference between 8 µg and 32 µg MOG ($p=0.0032$), and a significant difference between 16 µg and 32 µg ($p=0.0032$). There was no difference between the IFA, 8µg, or 16 µg groups. This data confirms that typically increasing the amount of MOG administered increases the likelihood that that subject will experience a more severe disease.
progression. For example, only one subject was euthanized early because of the severity of
disease progression, and this subject received the highest dose of MOG, signifying that this dose
is not suitable for experiments focused on the secondary symptoms of MS.

During JSE, on days 14 and 21, approximately one third of the animals in both the 16 µg
and 32 µg groups demonstrated consistent JSE behavior allowing them to be included in the
experiment, compared to approximately one sixth of the 8 µg group. Accordingly, 16 µg was
selected as the optimal dose due to number of animals impacted in JSE in conjunction with
minimal motor deficits.

Figure 1: Motor deficits were monitored using motor scores, an ascending ranking system where
0 represent no motor deficits and 7 represents euthanized due to disease progression. Motor
scores were taken daily. In this experiment, a MOG dose effect curve was conducted to discern
the most appropriate dosage out of 8, 16 or 32 µg MOG. Analysis revealed a significant effect of
MOG dose, and a significant interaction between MOG dose and time. Post hoc analysis
revealed there were significant differences between 0, 8, and 16 µg doses versus the 32 µg dose,
but no differences were found between IFA, 8 µg, and 16 µg.
3.2. (+)-NTX and Juvenile Social Exploration

JSE was used to model psychological symptoms in MS, such as anxiety, depression, and anhedonia, using EAE in male Sprague Dawley rats. Behavior initiated by the adult rat such as allogrooming, sniffing, and pinning was recorded by time spent performing those behaviors. Adult rats are typically highly motivated to interact with a juvenile rat. Therefore, when less time is spent interacting, it is speculated that the animal is expressing anhedonic behaviors. To test the effects of EAE and (+)-NTX treatment on anhedonia, rats were treated with either (+)-NTX or saline on day 21 post-MOG. In order to accurately assess the impact of MOG and (+)-NTX on JSE deficits, only subjects that demonstrated consistent JSE were included in the treatment phase. Consistent scores were determined by scores on day 14 and day 21 on the JSE experiment. If on both days an EAE subject was below 80 percent of their baseline score, they remained in the experiment for the drug phase. Alternatively, if the subject was only below 80 percent on one of those days, they were excluded since they lacked consistency. Furthermore, animals that received the IFA control were also normalized. If the subject’s score was unreliable between baseline, day 14, and day 21 (i.e. greater than 20 percent variability), they were considered to be unreliable and therefore excluded from the study. Typically, one third of the EAE subjects have consistent deficits. On day 28, after 7 days of treatment with (+)-NTX, JSE was performed a final time. Intergroup differences were analyzed using a two-way ANOVA between MOG and (+)-NTX. The two-way ANOVA revealed a nonsignificant effect of MOG (F(1, 17) = 3.984; p = 0.0622), a nonsignificant effect of (+)-NTX (F(1, 17)=0.6514; p = 0.4308), and a nonsignificant effect of interaction (F(1, 17) =4.115; p = 0.0585). However, it is important to recognize the trend between the interaction of JSE scores, MOG and (+)-NTX. Due to this trend, future experiments will be conducted to increase statistical power, which is predicted to
uncover significant statistical relationships between these variables. Once JSE is confirmed as a reliable model for anheonia/depression/anxiety in EAE, the role of TL2 and TLR4 antagonism will be confirmed as an impactful treatment for these symptoms in multiple sclerosis.

**Figure 2:** JSE was performed at baseline (pre-MOG), day 14, day 21, and day 28 post-MOG. Subjects that showed inconsistencies between day 14 and day 21 were excluded. The data is collapsed to include all MOG doses (8, 16, 32 µg for a n=12) within MOG, but in the future the 16 µg dose will be used. 3x/day treatment with (+)-NTX for a total of 18 mg/kg/day commenced on day 21 and lasted the course of the experiment. Analysis revealed there were no significant differences between groups.

3.3. **Von Frey and (+)-NTX**

Von Frey was used to examine mechanical allodynia induced by EAE in Sprague Dawley rats. In order to assess allodynia, filaments of varying diameters were applied to the rat’s hindpaw, and paw withdrawal behavior was recorded. As different forces of distinctive filaments are applied, the threshold that 50 percent of the time induces paw withdrawal can be determined. In order to assess the effects of EAE and treatment of (+)-NTX for mechanical allodynia, rats
were either treated with daily saline or 6 mg/kg three times per day (+)-NTX (for a total of 18 mg/kg/day) beginning on day 14 post-MOG. Animals with EAE expressed a lower threshold of pain, but after treatment with (+)-NTX, mechanical allodynia was reversed in both hindpaws (Figure 3). No motor deficits were observed via the motor scoring system, and no animals were excluded from test due to disease progression (Figure 4). Intergroup differences were analyzed using a two-way ANOVA and revealed a significant effect of treatment on the left paw (F(3,20) = 76.4; p < 0.0001) and right paw (F(3, 20) = 83.52; p < 0.0001), a significant effect of day on the left paw (F(5,100) = 58.34; p < 0.0001) and right paw (F(5,100) = 68.5; p < 0.0001), and a significant interaction between day and treatment on the left paw (F(15,100) = 7.366; p < 0.0001), and right paw (F(15,100) = 8.361; p < 0.0001). Sidak’s multiple comparisons post hoc reveals a significant difference between IFA and MOG on day 7 (p <0.0001), 14 (p <0.0001), 16 (p <0.0001), 22 (p <0.0001), 27 (p <0.0001), and a significant difference between MOG-saline and MOG-NTX on days 16 (p <0.01), 22 (p <0.0001), 27 (p <0.0001), demonstrating that MOG produces allodynia that is reversed by (+)-NTX treatment (Figure 3). This signifies that TLR2 and TLR4 antagonism may be a beneficial treatment for multiple sclerosis patients experiencing neuropathic pain.
**Figure 3:** Left and right hindpaw von Frey mechanical allodynia testing in male Sprague Dawley rats. Subjects were either injected with MOG to produce EAE or with a control vehicle of IFA. On day 15, subjects received 3x per day subcutaneous injections of (+)-NTX for a total of 18 mg/kg/day or received control subcutaneous injections of saline until animal sacrifice. (+)-NTX reversed mechanical allodynia prompted by EAE.

**Figure 4:** Motor deficits were monitored using motor scores, an ascending ranking system where 0 represent no motor deficits and 7 represents euthanized due to disease progression. Motor scores were taken daily. In this experiment, no motor scores were seen at any time point of the experiment, demonstrating no motor deficits. Analysis confirmed that there were no differences among groups.
3.4. Von Frey and MCC950

Following confirmation that the TLR2 and TLR4 cascade regulates mechanical allodynia, we wanted to better understand what part of this molecular cascade might be responsible for (+)-NTX effects, therefore we moved downstream to the level of the inflammasomes. NLRP3 is one inflammasome under TLR2 and TLR4 control, and thus MCC950, an NLRP3 inhibitor, was administered to determine the role of NLRP3 in EAE-induced mechanical allodynia. The von Frey assay was used to test the effects of EAE and treatment of MCC950 for mechanical allodynia. Rats were treated with MCC950 or saline on day 23 post-MOG, and von Frey was preformed 3 hours post treatment. Animals with EAE expressed a lower threshold for pain, but after MCC950 treatment, allodynia was reversed in both hindpaws at the 3-hour time point but not the 24-hour time point (Figure 4), which is consistent with the pharmacokinetics of MCC950 found in other studies (Coll et al., 2015). Intergroup differences were analyzed using a two-way ANOVA and revealed a significant effect of day on the left paw (F (3, 14) = 4.147; p= 0.0268 and right paw (F (3, 14) = 4.410; p= 0.0221), a significant effect of treatment on the left paw (F (5, 70) = 75.78; p< 0.0001), and right paw (F (5, 70) = 72.02; p< 0.0001) and a significant interaction between the day and treatment on the left paw (F (15, 70) = 2.829; p= 0.0017) and right paw (F (15, 70) = 2.863; p= 0.0015). Sidak’s multiple comparisons post hoc revealed a significant difference between saline versus 0.001 mg (p=0.0009), 0.01 mg (p=.0056), and 0.1 mg MCC950 (p<.0001) on the left paw, and a significant difference between saline versus 0.001 mg (p=0.0038), 0.01 mg (p=.0018), and 0.1 mg MCC950 (p<.0001) on the right paw. While there was no significant difference between doses of MCC950, it is worthy to note that the highest of dose produced the strongest reversal of mechanical allodynia (Figure 5). This data further confirms that the TLR2-TLR4 cascade, including the NLRP3 inflammasome, is
necessary for the neuroinflammation that MS patients experience, and that antagonism along this cascade could be an efficacious treatment for neuroinflammation, and thereby the secondary symptoms of multiple sclerosis.

Figure 5: Left and right hindpaw von Frey mechanical allodynia testing in male Sprague Dawley rats. Subjects were either injected with MOG to produce EAE or with a control vehicle of IFA. On day 23, subjects received a single 10 µL intrathecal injection of MCC950 at either .001, .01, or 0.1 µg doses. All doses of MCC950 significantly reversed mechanical allodynia at the 3 hour time point but not the 24-hour time point.

4. Discussion

MS produces devastating multifaceted symptoms including motor deficits, neuropathic pain and psychological deficits such as depression and anxiety. The current standard treatments of MS such as DMF and fingolimod are immunomodulatory agents, aiming to attenuate a range of immune activity (Gold et al., 2012; Mehling, Kappos, & Derfuss, 2011). However, these treatments do not address secondary symptoms such as neuropathic pain nor depression or anxiety (Feinstein, Magalhaes, Richard, Audet, & Moore, 2014). This leaves a large clinical gap in treatment since so many patients are not receiving complete treatment for their symptoms.

This gap may stem from practitioners failing to distinguish between a patient’s complaints of
fatigue, and overlapping symptoms indicative of depression (Hasselmann et al., 2016). These intersecting symptoms can lead to lack of separation and potential for both unnecessary medication or lack of adequate care (Hasselmann et al., 2016).

In this study, we aimed to better understand the TLR2-TLR4-related mechanisms governing MS-related neuropathic pain and depression/anhedonia/anxiety within EAE. TLR2-TLR4 are activated by DAMPS and PAMPS, eventually causing release of proinflammatory cytokines such as IL-1β. Neuroinflammation is an important aspect of the immune system and promotes regeneration and healing of tissue after exposure to infectious agents (Korn & Kallies, 2017). However, when turned maladaptive, this productive neuroinflammation can become destructive in the form of neuropathic pain and psychological disorders, such as depression/anhedonia/anxiety (Anisman et al., 2005; Sommer, Leinders, & Üçeyler, 2017; Zunszain, Hepgul, & Pariante, 2013). Macrophages, microglia, astrocytes, T-lymphocytes, chemokines and cytokines all play an important role in contributing to neuroinflammation (Sommer et al., 2017; Zunszain et al., 2013). Understanding these mechanisms will guide future treatments that hopefully target secondary symptoms of MS and relieve some of the burden that patients endure. Juvenile social exploration was selected as a behavioral model to measure depression/anhedonia/anxiety induced by EAE while the von Frey test was used in order to evaluate mechanical allodynia produced by EAE. Results revealed no differences between treatment groups in JSE. Nonetheless, we believe there are significant differences between treatment groups and larger statistical power will be incorporated in order to investigate this. We furthermore found that mechanical allodynia was enduringly-reversed by daily (+)-NTX treatment and acutely-reversed by MCC950 3 hours post injection, but not 24 hours post injection. Overall, these behavioral assessments have led us to the conclusion that neuropathic
pain as well as depression/anhedonia/anxiety likely involve neuroinflammation controlled by TLR2-TLR4, and that blocking TLR2-TLR4 mitigates these secondary symptoms present in multiple sclerosis.

Firstly, in order to effectively study anhedonia induced by EAE, an optimal MOG dose had to be chosen. Based on prior experience with MOG within our lab, 8 μg, 16 μg, and 32 μg doses were selected for the MOG dose effect experiment. This quantity was predicted to be a ‘low-dose’ model of MS compared to a standard-dose model that elicits more severe motor impairments. A standard-dose model is used when studying paralysis as well as treatments that impact motor impairments and can be seen in many papers using EAE as an MS model (Balatoni et al., 2007; Matsumoto, Sakuma, Kohyama, & Park, 2007). A standard dose model is better equipped to study paralysis and other motor impairments implicated in EAE than a low-dose model. However, in order to study anhedonia, depression, and anxiety, as well as neuropathic pain, it was postulated that these symptoms will be present even without pervasive motor impairments, which has been found previously (Acharjee et al., 2013). Therefore, it was decided that a low-dose model would be used in order to evoke these secondary symptoms of MS and attempt to avoid severe motor deficit progression, which would serve as confounds.

Additionally, the use of Sprague Dawley rats is not standard in MS research. Most commonly either Dark Agouti or Lewis rats are used because they exhibit a predictable disease course (Bjelobaba et al., 2018). Alternatively, Sprague Dawley rats are less susceptible to EAE, and thereby express more variable disease progression (unpublished observations). However, due to our focus on secondary symptoms, which seem to be present regardless of the progression of motor impairments, Sprague Dawley’s serve as a satisfactory model. As expected, a subset of the subjects that received MOG still expressed motor impairments indicated by a motor score greater
than one: the 8 µg dose had an average motor score of 0, while the 16 µg had an average motor score of 0.235 and the 32 µg dose with the most severe motor deficits with an average of 1.19.

The 8 µg dose produced a reliable JSE deficit in 1/6 of the subjects followed by the 16 µg and 32 µg doses which produced a reliable JSE deficit in 1/3 of the subjects. Thus, the 16 µg dose was chosen because it optimized the amount of subjects with consistent deficits in JSE while limiting the progression of motor impairments.

Social interaction has been a dependable tool in measuring anxiolytic, anxiogenic, anhedonic and depressive behaviors (File & Seth, 2003; Knyazev, Savostyanov, Bocharov, & Rimareva, 2016). In this experiment, juvenile social interaction was used due to its advantages over other social interaction tests including using familiar cage testing conditions or using interaction with adult rats, which limits aggressive behaviors that can misrepresent anxiolytic behaviors (Christianson et al., 2008). It was expected that animals with EAE would express anhedonic behaviors, and therefore spend less time interacting with the juvenile. While the effects of MOG, (+)-NTX, nor the interaction between MOG and (+)-NTX was significant, it is trending towards significance, suggesting there may actually be an effect of MOG as well as (+)-NTX on juvenile social interaction. In order to assess this, an additional experiment will be done to expand the statistical power. With a larger sample size, it is expected that the effect of MOG will become statistically significant, as was found in a previous study (Grace et al., 2017).

Neuroinflammation no doubt has implications in the occurrence of neuropathic pain as well as depression/anhedonia/anxiety, but the mechanisms are complicated and poorly understood. There is very little known about the mechanisms that link neuroinflammation, depressive disorders, and neuropathic pain, however the relationship between the two is well established (Altamura, Buoli, & Pozzoli, 2014; Anisman et al., 2005; Cowen, Sharp, & Lau,
The role of neuroinflammation in depression/anhedonia/anxiety within MS is corroborated by clinical research examining the relationship between patients’ relapse and current psychological standing. They found that patients’ rates of depression and anxiety increased in severity during a period of relapse. Additionally, patients that showed more symptoms had a lower mean time of relapse, perhaps signifying the overlapping control of the neuroinflammation (Rossi et al., 2017). Moreover, MS patients experiencing depression or anxiety are more likely to report feeling pain (Archibald et al., 1994). Various studies demonstrate that neuropathic pain in MS can be modulated by suppression of several inflammatory pathways (Donvito et al., 2018; Giacoppo, Iori, Bramanti, & Mazzon, 2017). Even so, in both animal and clinical research, there are limited results regarding the mechanisms underlying these symptoms, and the mechanism has yet to be elucidated.

In order to better understand the mechanisms underlying depression/anhedonia/anxiety, neuropathic pain and inflammation, key proteins along the respective pathways must be identified. Toll-like receptors (TLR) respond to PAMPS and DAMPS and have long been recognized for their importance in neuroinflammation (Kumar, 2019). Therefore, it was expected that when TLR2 and TLR4 were antagonized using (+)-NTX that EAE-induced mechanical allodynia and EAE-reduced JSE would be reversed. As expected, (+)-NTX significantly reduced the mechanical allodynia induced by the EAE and also appears to reverse the reduced JSE caused by EAE. NLRP3 is an inflammasome downstream from TLR2 and TLR4, that cleaves the inactive pro-interleukin (IL)-1β, into its active IL-1β form, a pro-inflammatory cytokine (Jia et al., 2017). Therefore, it was expected that when NLRP3 was blocked using MCC950 that mechanical allodynia would be reversed. As expected MCC950 significantly reduced the
mechanical allodynia induced by the EAE. While NLRP3 has been associated with EAE disease progression and allodynia, it had not previously been implicated as a spinal cord mediator for EAE-induced mechanical allodynia. Therefore, this study demonstrates that spinal NLRP3 is necessary for mechanical allodynia. Future studies will address the NLRP3 specificity of EAE-reduced JSE, pending statistically significant effects of EAE and (+)-NTX in follow up studies. Implicating TLR2, TLR4 and NLRP3 as part of this inflammatory cascade that controls alldynia provides insights at both the mechanistic and therapeutic levels for multiple sclerosis.

Previous studies demonstrated that IL-1β is involved and expressed in higher levels in the spinal cord and brain of both MS patients and EAE subjects (Burm et al., 2016; Prins et al., 2013). Therefore, in order to better understand the role of IL-1β, a natural next step involves examining the role of IL-1β in both EAE-induced mechanical allodynia and EAE-reduced juvenile social exploration. In fact, our lab previously intrathecally administered the IL-1 receptor antagonist IL-1ra in the context of EAE-induced mechanical allodynia and discovered that mechanical allodynia was reversed by this treatment (data not shown), implicating spinal IL-1β is also necessary for EAE-induced mechanical alldynia. Assuming expanding the statistical power for the (+)-NTX JSE experiment reveals significance, it would be expected that blocking IL-1β within the reward pathway and/or the amygdala would reverse the deficits MOG induces in JSE, further implicating the TLR2 and TLR4 cascade as a potential therapeutic point for the secondary symptoms of multiple sclerosis.

While our JSE data are very promising, it is possible that the need for more subjects in the JSE experiment is a reflection that MOG produces a weak deficit in JSE, as well as of (+)-NTX’s treatment for anhedonic and depressive behaviors. In some studies, depressive-like behaviors were examined prior to the onset of motor impairments (Acharjee et al., 2013). It is
possible that these secondary symptoms are more prominent before motor symptom onset, but it is improbable that these symptoms would disappear with disease progression. Therefore, it is unlikely that timing is a concern for this experiment. Moreover, subjects’ express reversible mechanical allodynia with and without motor symptoms through the course of the experiment, so it is doubtful that the timing of these experiments is confounding. Additionally, while JSE is a common module to measure anhedonia, anxiety, and depression, it is impossible to say with certainty that the animals are behaving in the manner they are due to anhedonia. However, this problem regarding lack of efficacy behind emotional tests is persistent among all tests measuring for affective behaviors.

The next step in this project is to expand the statistical power in the JSE experiment with the prediction that this will reveal statistically significant effects of MOG and (+)-NTX. After this, assuming the trend remains intact, the brains will be processed and we will perform immunohistochemistry to stain for IL-1β, as well as microglia and astrocyte activity. Specifically, we will stain for brain areas along the reward pathway such as the ventral tegmental area and the nucleus accumbens. Furthermore, we will stain for brain areas involved in fear and anxiety such as the amygdala. These areas have been previously implicated in the expression of anxiety or control of social behaviors (Christianson et al., 2010; Felix-Ortiz & Tye, 2014; Smith, Mogavero, Tulimieri, & Veenema, 2017). It is expected that these brain areas will express an increase in activation of microglia, astrocytes, as well as more IL-1β due to over-activation of the TLR2 and TLR4 cascade. However, among treatment with (+)-NTX, it is expected that these levels will return to normal levels, as seen in a control animal.

Overall, these findings provide novel insights into the secondary symptoms of EAE and MS as well as the importance of neuroinflammation for the presence of these symptoms. Prior to
these experiments, juvenile social exploration had not been used in the context of EAE. With time, it is likely that JSE will be confirmed as an adequate behavioral measure in which MOG elicits consistent deficits. TLR2 and TLR4 antagonism is a potentially impactful treatment for the secondary symptoms found in multiple sclerosis.

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