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Eric H. Mitten
University of Colorado Boulder, ermi1863@colorado.edu

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(+) - Naltrexone Effects on Anhedonia-Like Symptoms in a Low-Dose Model of Experimental Autoimmune Encephalomyelitis (EAE)

By:

Eric Mitten
Department of Psychology and Neuroscience, University of Colorado Boulder

Defense Date: April 1, 2019

Linda Watkins, Department of Psychology and Neuroscience

Defense Committee: Linda Watkins (Department of Psychology and Neuroscience), Heidi Day (Department of Psychology and Neuroscience), Chris Lowry (Integrative Physiology)

Acknowledgements: This research was supported by a grant funded by the National Institute of Health’s Neuroendocrinology, Neuroimmunology, Rhythms, and Sleep Section (NIH/NNRS; RO1 NS097313-01). Sincere gratitude is expressed to Linda Watkins, Andrew Kwilasz, and Anouk Schrama for help throughout the entire process of the creation of this thesis.
Abstract

The demyelinating and inflammatory disorder multiple sclerosis (MS) primarily causes paralysis. However, MS also causes secondary symptoms, including: chronic pain, memory deficits, and depression. Work in our lab has shown that antagonism of toll-like 2/4 receptors with (+)-naltrexone ([+] -NTX) can reverse chronic pain and memory deficits in experimental autoimmune encephalomyelitis (EAE), a rat model of MS. We thus hypothesized that (+)-NTX would also reverse the expression of anhedonia, a symptom of depression defined as an inability to experience pleasure, as measured by saccharin preference testing within EAE. Four separate cohorts of male Dark Agouti and Sprague Dawley rats were tested. Two cohorts of each strain helped characterize saccharin drinking behavior within and outside the context of EAE. Another two cohorts from each strain were utilized to examine (+)-NTX’s effects on EAE-induced anhedonia. A low-dose model of EAE was used to remove the confound of motor paralysis. Subjects in (+)-NTX studies received (+)-NTX or saline three times per day for two weeks fourteen days following EAE induction. Reliable drinking behavior was found in both strains with and without EAE induction. EAE modestly produced motor scores and mechanical allodynia, consistent with normal expression of low-dose EAE symptoms. (+)-NTX had no effect on motor scores. (+)-NTX significantly reversed mechanical allodynia associated with EAE. However, no effect of EAE or (+)-NTX on anhedonia-like symptoms was detected. Despite negative results, continued examination of (+)-NTX is encouraged due to its ability to reverse other secondary symptoms associated with neuroinflammation within EAE.
Introduction

Multiple sclerosis (MS) is a life-long debilitating disease affecting 2.5 million people worldwide (Duffy et al., 2018) which manifests as a multifaceted series of symptoms (Sand, 2015). MS impedes the ability of neurons to communicate effectively through the chronic demyelination of neurites in the central nervous system (CNS). The primary symptom of MS is paralysis. However, MS is associated with a host of additional symptoms, including: neuropathic pain, motor disturbances, cognitive deficits, impaired memory, anxiety, and depression (Duffy et al. 2018; Marck et al., 2017; Rocca et al., 2015; Scherder et al., 2018). Major depressive disorder (MDD) has been reported as moderately prevalent in the MS community, with 25% to 50% of all MS patients developing some form of comorbid MDD (Feinstein et al., 2014; Patten et al., 2017). Comorbid MDD is associated with increased cognitive dysfunction, increased risk for self-harm, decreased treatment adherence, and decreased quality of life in MS patients (Hasselmann et al., 2016). Despite the fact that this comorbidity is well known and characterized, it is undertreated in MS patients (Fiest et al., 2016). Current treatments for MS, such as fingolimod (Cohen et al., 2016; Kappos et al., 2015; Lublin et al., 2016) and dimethyl fumarate (Bomprezzi, 2015; Linker and Haghikia, 2016), solely focus and treat the primary symptom of paralysis. Moreover, current antidepressants have little to no effect in the treatment of depression symptoms within the MS population (Koch et al., 2015). Therefore, novel treatments for MS that address these comorbid symptoms, as well as the primary symptom of paralysis, are necessary for improving the quality of life of MS patients.

Current research indicates that secondary MS symptoms are primarily mediated by immune responses mounted against the myelin sheath exclusively in the CNS (for review see
Yadav et al. 2015). The immune response found in MS is incredibly complex and consists of many molecular markers that are currently being identified. Toll-like receptors 2 and 4 (TLR2/TLR4; for review see Akira and Takeda, 2004) are major components of the innate immune system that have been shown to mediate the immune response in MS. Examination of TLR2/TLR4’s within several contexts has shown that activation of these receptors potentiates the immune response by increasing inflammation through the modulation of cytokine release, such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF) (Becher et al., 2016; Jack et al., 2005; Linker and Lee, 2009; Luo et al., 2017; Milligan and Watkins; 2009; Paré et al., 2017).

TLR2/TLR4s have been shown to exist predominately on microglia in the CNS, but also are present in other resident cells, such as astrocytes, infiltrating macrophages, T cells, oligodendrocytes, fibroblasts, endothelial cells, and B cells (Bsibi et al., 2002), and recognize molecules produced through tissue damage, known as damage associated molecular patterns (DAMPs) (Liu et al., 2014; Vénéreau et al., 2015). Within MS, the source of DAMPs is primarily thought to be from the breakdown of neurites as a result of chronic demyelination. Due to this interaction, several DAMPs have been identified and implicated in the pathology of MS (Hernandez-Pedro et al., 2016; Varhaug et al., 2017). These results have led our lab to hypothesize that TLR2/TLR4 binding by DAMPs produced through tissue damage, such as demyelination, continually exacerbates the immune response and worsens these aforementioned secondary symptoms associated with MS. Therefore, research in our lab is examining the effect of the non-opioid TLR2/TLR4 antagonist (+)-naltrexone ([+]NTX) in a rodent model of MS, termed experimental autoimmune encephalomyelitis (EAE) (for review see Constantinescu et al., 2011). This model has been shown to create an immune response similar
to what would be experienced in human MS patients and has been characterized through primary behavioral symptoms, such as graded levels of paralysis (Blakeley et al., 2015; Kim et al., 2010; Shin et al., 2000), and secondary behavioral symptoms, such as neuropathic pain (Khan and Smith, 2014; Khan et al., 2014), memory loss (Dutra et al., 2013; Kim et al., 2012), cognitive deficits (Mandolesi et al., 2010), and depression-like symptoms (Pollak et al., 2002). Lastly, EAE has also been characterized through tissue effects such as peripheral and central cytokine release and central markers of demyelination (Barclay and Shino, 2017; Liu et al., 2018; Zhang et al., 2018).

In our lab, we utilize a low-dose model of EAE to produce the secondary symptoms of MS without significant expression of motor paralysis symptoms. Further characterization of this model will allow for unconfounded testing of these aforementioned secondary symptoms to better develop new pharmacotherapies to address all symptoms associated with MS. Thus far in our low-dose EAE models, (+)-NTX has been shown to significantly reverse chronic neuropathic pain and memory deficits associated with EAE as measured through von Frey pain testing and contextual fear conditioning, respectively. Due to these results, our lab is currently examining the effect of (+)-NTX on the expression of EAE-induced depression-like symptoms. Specifically, we are assessing a model of EAE-induced anhedonia, a common MDD symptom (Rizvi et al., 2016), through the use of saccharin preference testing. This measure of anhedonia-like symptoms examines the subject’s intake of a sweetened solution compared to a neutral solution (water), inferring that increased intake of the sweetened solution over the neutral solution is an index of motivation to engage in a pleasurable behavior. Therefore, an anhedonia-like response manifests as a decreased preference for the sweetened solution.
compared to the neutral solution. Sweetened solution preference testing, through the use of sucrose, saccharin, and other associated sweeteners, has been well characterized to reliably measure anhedonia-like symptoms in several other paradigms in which depression-like symptoms are expressed (Alkhlaif et al., 2017; Darkazalli et al., 2016; Grimonprez et al., 2015; Remus et al., 2015). Furthermore, this expression of anhedonia-like symptoms through this test is also reliably reversed by antidepressant treatment (Abdel-Tawab et al., 2015; Burstein et al., 2017; Lu et al. 2017). Therefore, we hypothesize that (+)-NTX administration will significantly attenuate the decrease in saccharin preference associated with anhedonia-like symptoms induced by EAE as compared to saline controls. Examining this effect will help elucidate possible mechanisms mediating depression-like symptoms in EAE and determine if TLR2/TLR4 antagonists are a possible intervention for the depression symptoms of MS.

**Methods**

*Subjects and Materials*

Subjects were either male Dark Agouti (DA; Envigo) or Sprague Dawley (SD; Jackson Laboratories) rats. The initial cohort of DA rats (6 rats total; $n = 3$) was utilized to conduct a pilot study. The second cohort of DA rats (18 rats total; $n = 6$) was treated with our standardized protocol. From here, our lab switched to the use of SD rats due to high operating costs of DA rats and to properly control for vehicle effects, as described below. The first cohort of SD rats (8 rats total; $n = 8$) was utilized as a pilot to determine drinking behavior. Finally, the second cohort of SD rats (24 rats total; $n = 6$) was treated with our standardized protocol. Subjects were housed in pairs in clear cages with a filtered top. The colony room was kept at $25^\circ$ C and
ambient humidity was maintained. Subjects were kept on a 12-hour light/dark cycle with lights on at 0700 hours. Subjects were provided with food and water *ad libitum*. Animals entered into the facility and were left untouched for a week. The subsequent week, animals were handled and habituated to behavioral testing conditions. All procedures were conducted in accordance with protocols approved by the University of Colorado Boulder Institutional Animal Care and Use Committee.

*Induction of Experimental Autoimmune Encephalomyelitis (EAE)*

Subjects were randomly assigned to EAE (MOG) or saline/vehicle groups the following week after general handling and habituation. DA rats assigned to the MOG group received a 4 μg injection of recombinant rat myelin oligodendrocyte protein (MOG) 1-125 (VU University Medical Center, Netherlands, gifted by Dr. Anne-Marie Van Dam) in a vehicle consisting of 50% sodium acetate buffer (pH = 3) and 50% incomplete Freund’s adjuvant (IFA; Sigma; St. Louis, MO). SD rats assigned to the MOG group received an 8 μg injection of MOG in the same conditions. These doses are lower than the standard dose utilized in other models. We chose to use these doses to remove the confound of motor paralysis in the expression and measurement of depression-like symptoms through saccharin preference testing.

For all experimental conditions, subjects were anesthetized using isoflurane. MOG injections were administered intradermally at the base of the tail and the syringe was left in place for three minutes to avoid leakage at the injection site. For DA cohorts, the control injection consisted of saline utilizing the same procedure. For SD cohorts, the control injection
consisted of the MOG vehicle (50% sodium acetate buffer; 50% IFA) utilizing the same procedure.

**Motor Scoring**

Motor behavior was scored daily in all rats to assess the severity of their EAE paralysis symptoms. The motor 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, 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. All rats received motor scoring to ensure that paralysis remained below a score of 3 during behavioral testing. Animals exhibiting symptoms at a 3 or above were removed from behavioral testing to remove the confound of motor paralysis.

**Von Frey Testing for Mechanical Allodynia**

Rats were habituated to the testing area for four consecutive days before testing. The von Frey testing apparatus consisted of subjects being placed on a wire rack above the researcher and covered with a large plastic cup to hold the subject in place. The plantar surface of the left and right hind paws of each subject were tested in random order. For the test, a logarithmic series of ten calibrated Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL, USA) were applied consecutively from low to high intensity threshold. The stimulus intensity threshold needed to produce a paw withdrawal response was ascertained by applying each filament for eight seconds with constant pressure. Log stiffness of the hairs was determined by
the following equation: \( \log_{10}(\text{milligrams} \times 10) \). The range of filaments used in this experiment was 0.4 -15g. Testing was performed blind with respect to group assignment. For this study, von Frey testing was conducted in the second cohort (Dark Agouti) and fourth cohort (Sprague Dawley) of subjects. This test was utilized to ensure that the previously validated symptom of mechanical allodynia expressed within our low-dose model of EAE applied to these cohorts. Furthermore, this test was utilized to determine if this effect was reliably reversed with (+)-NTX administration, which has been shown in previous studies within our lab.

\textit{(+)\textbf{-Naltrexone Administration}}

Subjects were equally-assigned to (+)-NTX and saline treatment groups based on the presence of motor symptoms. (+)-NTX (National Institute of Health, gifted by Dr. Kenner Rice) administration began 2 weeks after MOG administration. The animals received subcutaneous (+)-NTX (6 mg/kg) or saline injections three times daily (0900, 1200, and 1500 hours) for 2 weeks during behavioral testing, for a total of 18 mg/kg/day. The volume of each injection administered was 1 mL/kg.

\textit{Saccharin Preference Testing}

Saccharin, as opposed to the commonly utilized sucrose, was chosen for testing of this behavior in an EAE model so as to remove the confound of nutritive value. By using saccharin, we can more confidently discern that changes in drinking behavior can be explained by a change in pleasure associated with the sweetened solution rather than any nutritive value associated with it. To create varying concentrations of saccharin water, a stock mix of 0.1%
saccharin water was created through sonication and diluted accordingly. The primary concentrations of saccharin used were 0% (i.e. regular water), 0.032%, 0.01%, and 0.0032%. For the first cohort of DA rats, 0.1% and 0.001% saccharin were also utilized.

For behavioral testing, subjects were singly housed and given the choice between two water bottles. Weights of the water bottles were measured directly before and after the testing period. The testing period consisted of placing the water bottles in the late afternoon (at about 1400 or 1500 hours) and allowing ad libitum drinking behavior for 24 hours. For all testing periods, the placement of the sweetened water was alternated to control for a position preference. Saccharin water was administered through a Latin square design, in which subjects were randomly assigned to the four concentrations tested (0%, 0.0032%, 0.01%, and 0.032%) for four consecutive testing periods. Therefore, all animals were exposed to each concentration throughout the four testing periods. At the end of the testing period, the weight of the water bottles was measured and subtracted from the original weight to determine water or saccharin intake in grams. Finally, percent preference was determined utilizing the following formula:

$$\frac{\text{Saccharin intake (g)}}{(\text{Saccharin intake [g]} + \text{Water intake [g]})} \times 100$$

Data Analysis

Statistical analyses were conducted utilizing GraphPad Prism (version 8.0.2) software. Motor score, von Frey pain testing, and saccharin preference testing data were analyzed using repeated measures two-way ANOVA with Sidak’s multiple comparisons post hoc test. Data acquired from the pilot study conducted in Sprague Dawley rats utilized a repeated measures one-way ANOVA. For all tests, statistical significance was set to p < 0.05.
Results

Low-Dose model EAE does not produce anhedonia-like symptoms in Dark Agouti rats

A pilot study was conducted initially to determine the optimal parameters for conducting saccharin preference testing within our low-dose EAE model. Additionally, this pilot was run to determine the drinking behavior of DA rats within our low-dose model of EAE. Figure 1 shows the results of this initial pilot study. This test did not include (+)-NTX administration so as to determine solely an effect of EAE. Motor score data for this cohort were not saved due to this being a pilot study. However, all scores remained significantly low (less than 3) so as to allow for the unconfounded testing of drinking behavior. No significant differences were observed between MOG treated rats and saline controls ($F_{1,4} = 0.01434; p = 0.9105$). A significant effect was observed between saccharin concentrations, indicating that as saccharin concentration increased, saccharin preference increased ($F_{1.849,7.396} = 143.0, p < 0.0001$). Finally, no interaction was detected between saccharin concentration and treatment conditions ($F_{4,16} = 1.468; p = 0.2581$). A ceiling effect seemed to emerge at the concentration of 0.032%, indicating subjects were not drinking more saccharin at higher concentrations than this.
Figure 1: Saccharin preference testing results for a Dark Agouti rat pilot study examining the effect of EAE induction on saccharin drinking behavior. Subjects were either induced with EAE utilizing a MOG injection (MOG) or given a saline control injection (saline). No significant difference was detected between the MOG and saline group, indicating that EAE did not significantly decrease saccharin preference. Data are presented as mean ± SEM and analyzed using a repeated measures two-way ANOVA (n = 3 per group; 6 total).

(+)-NTX has no effect on anhedonia-like symptoms of low-dose EAE in Dark Agouti rats

This experiment was conducted to examine the effect of (+)-NTX on expression of anhedonia-like symptoms within low-dose EAE through saccharin preference testing. Figure 2.1 shows the motor score data for MOG-treated DA rats including the administration of (+)-NTX. No significant differences in motor scores were observed between treatment groups (F1,11 = 1.913; p = 0.1941). A significant effect of time was found, indicating that motor scores significantly increased through the duration of the study (F20, 220 = 3.811; p < 0.0001). Finally, no significant interaction was detected between treatment group and time concerning motor scores (F20, 220 = .8425; p = 0.66). Analysis from day sixteen forward was utilized to examine the possible effect of (+)-NTX on the relapse of paralysis symptoms. Analysis did not detect a
significant effect of treatment ($F_{1,11} = 1.491; p = 0.2475$), time ($F_{12,132} = 1.706; p = 0.0721$), or interaction between these two variables ($F_{12,132} = 1.274; p = 0.2413$).

**Figure 2.2** shows the results of presentation of different concentrations of saccharin in a Latin square design to these subjects. Due to the observation of a ceiling effect in the pilot study, we excluded the 0.1% saccharin concentration for this experiment. No significant differences in saccharin preference were observed between treatment groups (MOG/(+)/NTX; MOG/saline; saline/saline) at any concentration ($F_{2,14} = 1.568; p = 0.2429$). As previously shown, a significant effect was found between saccharin concentrations, indicating that as saccharin concentration increased, saccharin preference increased ($F_{2.182,30.55} = 5.596; p = 0.0072$). Finally, no interaction was detected between saccharin concentration and the treatment group ($F_{6,42} = 0.6645; p = 0.6786$).

Von Frey testing was conducted to ensure that symptoms other than anhedonia associated with EAE were still being expressed in this cohort. **Figure 2.3** shows the results from von Frey pain testing in this cohort. Analysis revealed a significant effect of time ($F_{3, 15} = 107.2; p < 0.0001$), significant effect of treatment ($F_{1,5} = 9.564; p = 0.0271$), and a significant interaction between these two variables ($F_{3,15} = 11.61; p = 0.0003$) in the left hind paw. Additionally, analysis revealed a significant effect of time ($F_{1.604,16.04} = 77.55; p < 0.0001$), a significant effect of treatment ($F_{1,10} = 6.028; p = 0.0339$), and a significant interaction between these two variables ($F_{3,30} = 8.188; p = 0.0004$) in the right hind paw. Sidak’s multiple comparisons post hoc test revealed a significant difference between the MOG/saline and MOG/(+)/NTX groups at 21 days ($p = 0.0002$) and 28 days ($p = 0.0004$) post EAE induction in the left hind paw and at 28
days ($p = 0.0419$) post EAE induction in the right hind paw. These results indicate that (+)-NTX significantly reversed EAE induced chronic neuropathic pain at these timepoints.

**Figure 2.1:** Motor score data for the second cohort of Dark Agouti rats. Subjects were either induced with EAE utilizing a MOG injection (MOG) or given a saline control injection (saline). Subjects then either received three times daily subcutaneous injections of (+)-NTX (6 mg/kg) for a total of 18 mg/kg/day ([+]-NTX) or received saline control injections (saline). A significant effect of time was detected, indicating that motor scores increased over time. However, no significant difference was detected between groups, indicating that (+)-NTX had no effect on motor scores. Analysis from day sixteen forward indicated no significant effect of treatment, time, or their interaction, indicating (+)-NTX had no effect on the relapse of paralysis symptoms within EAE. Data are presented as mean ± SEM and analyzed using a repeated measures two-way ANOVA ($n = 6$ per group; 18 total).

**Figure 2.2:** Saccharin preference testing results from the second cohort of Dark Agouti rats. Subjects were either induced with EAE utilizing a MOG injection (MOG) or given a saline control injection (saline).
Subjects then either received three times daily subcutaneous injections of (+)-NTX (6 mg/kg) for a total of 18 mg/kg/day ([+]NTX) or received saline control injections (saline). No significant effect was detected between all four groups, indicating no effect of EAE or (+)-NTX. Data presented as mean ± SEM and analyzed using a repeated measures two-way ANOVA (n = 6 per group; 18 total).

**Figure 2.3:** Left and right hind paw von Frey pain testing results for the second cohort of Dark Agouti rats. Subjects were either induced with EAE utilizing a MOG injection (MOG) or given a saline control injection (saline). Subjects then either received three times daily subcutaneous injections of (+)-NTX (6 mg/kg) for a total of 18 mg/kg/day ([+]NTX) or received saline control injections (saline). Data from the saline/saline group were not included in the von Frey test. A significant effect of time, treatment, and their interaction was detected in both hind paws. Post hoc analyses revealed a significant difference at 21 days post EAE induction in the left hind paw and 28 days post EAE induction in both hind paws, indicating a significant reversal of EAE induced chronic neuropathic pain due to (+)-NTX administration. Data are presented as mean ± SEM. Data were analyzed using a repeated measures two-way ANOVA coupled with Sidak’s multiple comparisons post hoc test (n = 6 per group; 18 total; * p < .05).

**Sprague Dawley rats reliably drink saccharin-sweetened water**

A pilot study in Sprague Dawley rats was conducted to determine their drinking behavior and determine whether or not they would preferentially drink saccharin-sweetened water. **Figure 3** shows the results of SD rat drinking behavior in our Latin square paradigm without EAE induction or (+)-NTX administration. Considering EAE was not induced, no motor scores were recorded for this pilot. A significant effect of saccharin concentration was shown, indicating that saccharin preference increased as saccharin concentration increased (F_{1.943, 15.54} = 18.71; p < 0.0001).
Figure 3: Saccharin preference testing results for the cohort of Sprague Dawley rats utilized for a pilot study to determine drinking behavior. EAE induction and (+)-NTX administration were not conducted in this cohort. A significant effect of concentration was detected, indicating that saccharin preference increased with concentration. Data are presented as mean ± SEM and analyzed using a repeated measures one-way ANOVA ($n = 8$).

(+) NTX has no effect on anhedonia-like symptoms of low-dose EAE in Sprague Dawley rats

This experiment was conducted to examine the effect of (+)-NTX on expression of anhedonia-like symptoms within low-dose EAE through saccharin preference testing in SD rats. Figure 4.1 shows the motor score data for MOG-treated SD rats, including administration of (+)-NTX. No subjects in any conditions exhibited motor symptoms, indicated by scores of 0’s throughout the entire study.

Figure 4.2 shows the results of presentation of different concentrations of saccharin in a Latin square design to these same subjects. No significant differences in saccharin preference were observed between treatment groups (MOG/(+) NTX; MOG/saline; saline/(+) NTX; saline/saline; $F_{3,16} = 0.7953; p = 0.5143$). As in the previous experiments, a significant effect of saccharin concentration was detected, indicating that saccharin preference increased as
saccharin concentration increased \((F_{2.310,36.96} = 14.90; p < 0.0001)\). Finally, no interaction was detected between saccharin concentration and treatment group \((F_{9,48} = 0.7816; p = 0.6341)\).

Von Frey testing was conducted to ensure that symptoms other than anhedonia associated with EAE were still being expressed in this cohort. Figure 4.3 shows the results of von Frey pain testing in this cohort. Analysis revealed a significant effect of time \((F_{5,100} = 58.34; p < 0.0001)\), a significant effect of treatment \((F_{3,20} = 76.14; p < 0.0001)\), and a significant interaction between these two variables \((F_{15,100} = 7.336; p < 0.0001)\) in the left hind paw.

Additionally, analysis revealed a significant effect of time \((F_{5,100} = 68.50; p < 0.0001)\), a significant effect of treatment \((F_{3,20} = 83.52; p < 0.0001)\), and a significant interaction between these two variables \((F_{15,100} = 8.361; p < 0.0001)\) in the right hind paw. Sidak’s multiple comparisons post hoc test revealed significant differences between the IFA/saline and MOG/saline groups at 7 days \((p < 0.0001)\), 14 days \((p < 0.0001)\), 16 days \((p < 0.0001)\), 22 days \((p < 0.0001)\), and 27 days \((p < 0.0001)\) post EAE induction in the left hind paw. This same effect was detected at 7 days \((p < 0.0001)\), 14 days \((p < 0.0001)\), 16 days \((p < 0.0001)\), 22 days \((p < 0.0001)\), and 27 days \((p < 0.0001)\) post EAE induction in the right hind paw. These results indicate that our low-dose EAE model significantly and reliably produced chronic neuropathic pain throughout the duration of the study. Additionally, significant differences between the MOG/saline and MOG/(+)-NTX groups were detected at 16 days \((p = 0.0090)\), 21 days \((p < 0.0001)\), and 28 days \((p < 0.001)\) post EAE induction in the left hind paw. This same effect was detected at 16 days \((p = 0.0023)\), 21 days \((p < 0.0001)\), and 28 days \((p < 0.0001)\) post EAE induction in the right hind paw. These results indicate that (+)-NTX administration significantly reversed EAE induced chronic neuropathic pain at these timepoints.
Figure 4.1: Motor score data for the second cohort of Sprague Dawley rats. Subjects were either induced with EAE utilizing a MOG injection (MOG) or given a vehicle injection of incomplete Freund’s adjuvant (IFA). Subjects then either received three times daily subcutaneous injections of (+)-NTX (6 mg/kg) to a total of 18 mg/kg/day ([+] -NTX) or received saline control injections (saline). None of the subjects expressed motor symptoms throughout the duration of the study. Due to this, no statistical analysis could be conducted. Data are presented as mean ± SEM (n = 6; 24 rats total).

Figure 4.2: Saccharin preference testing results from the second cohort of Sprague Dawley rats. Subjects were either induced with EAE utilizing a MOG injection (MOG) or given a vehicle injection of incomplete Freund’s adjuvant (IFA). Subjects then either received three times daily subcutaneous injections of (+)-NTX (6 mg/kg) to a total of 18 mg/kg/day ([+] -NTX) or received saline control injections (saline). No significant effect was detected between all four groups, indicating no effect of EAE or (+)-NTX. Data presented as mean ± SEM and analyzed using a repeated measures two-way ANOVA (n = 6; 24 rats total).
Figure 4.3: Left and right hind paw von Frey pain testing results for the second cohort of Sprague Dawley rats. Subjects were either induced with EAE utilizing a MOG injection (MOG) or given a vehicle injection of incomplete Freund’s adjuvant (IFA). Subjects then either received three times daily subcutaneous injections of (+)-NTX (6 mg/kg) to a total of 18 mg/kg/day ([+] NTX) or received saline control injections (saline). Analysis revealed a significant effect of time, treatment, and interaction between the two variables in both hind paws. Post hoc analysis revealed significant differences between the IFA/saline and MOG/saline groups at 7 days, 14 days, 16 days, 21 days, and 28 days post EAE induction in both hind paws, indicating a significant expression of EAE induced chronic neuropathic pain at these timepoints. Post hoc analysis also revealed significant differences between the MOG/saline and MOG/(+)NTX groups at 16 days, 21 days, and 28 days post EAE induction in both hind paws, indicating a significant reversal of chronic neuropathic pain expression by (+)-NTX administration at these timepoints. Data are presented as mean ± SEM. Data were analyzed using a repeated measures two-way ANOVA coupled with Sidak’s multiple comparisons post hoc test (n = 6 per group; 24 total). [# indicates comparison between IFA/saline and MOG/saline groups; p < 0.05; * indicates comparison between MOG/saline and MOG/(+)NTX groups; p < 0.05]

Discussion

This study examined whether or not two separate male rat models of MS could produce anhedonia-like symptoms, as measured through saccharin preference testing. Moreover, the effect of TLR2/TLR4 antagonist (+)-NTX on anhedonia-like symptoms associated with EAE was examined. As the results showcase, this low-dose model of EAE did not produce a reliable expression of anhedonia-like symptoms, as measured through a decrease in saccharin preference, nor did we see any effect of (+)-NTX administration on saccharin preference. The small amount of studies examining depression-like behavior in standard-dose EAE find reliable expression of these symptoms in multiple tests, including forced swim, tail suspension, and
social interaction testing (Acharjee et al., 2013; Ayatollahi et al., 2017). However, these results are limited to the pre-symptomatic phase of the disease due to motor paralysis confounding behavioral testing later in the disease progression. Despite this limitation, these results help eliminate the possibility of a lack of depression-like symptoms in standard EAE. Importantly, EAE is not a perfect model of MS in humans (for review of MS experimental models see Ransohoff, 2012). Therefore, there are other possibilities as to why the low-dose model of EAE was not associated with anhedonia-like symptoms in these studies. Firstly, the lack of anhedonia-like symptoms may be a limitation of our low-dose EAE model. Considering this model has not been standardized, it is possible that this model does not cause the same expression of symptoms as a standard dose would. However, this seems unlikely due to the presence of mechanical allodynia in these subjects that is reliably reversed by (+)-NTX administration which is consistent with previous studies within our lab. Secondly, depression-like behaviors associated with EAE may only be present within the pre-symptomatic phase, as shown in the aforementioned studies. Thirdly, another possibility underlying this lack of expression of anhedonia-like symptoms might be the use of saccharin preference testing as compared to sucrose preference testing. Moreover, other depression measures may more sensitively measure anhedonia-like symptoms in the EAE model as compared to saccharin preference.

Despite saccharin preference being a valid measure of anhedonia-like symptoms, no reliable effect was seen within our current study. This might be due to two main factors. First, it might be possible that intake of saccharin by rodents is unreliable in this model of EAE compared to other sweeteners. This suggests that use of saccharin in this study might be
obscuring the results due to a lack of reliable intake, not a lack of depression-like symptoms. One recent study has shown that non-nutritive sweeteners (specifically a saccharin-sucralose mixture) produced weaker preferences than nutritive sweeteners (Sclafani and Ackroff, 2017). Also, it has recently been found that other forms of saccharin may be more preferred by rodents, such as sodium saccharin salt hydrate (Rehn et al., 2018). Therefore, finding a non-nutritive sweetener that will provide optimal intake is necessary to measure anhedonia-like symptoms in disease states in which nutritive value might be a confound. A lack of effect may also be attributable to the utilization of a saccharin preference paradigm as compared to sucrose preference. As aforementioned, sucrose preference testing has been validated as a reliable measure of depression-like symptoms throughout several paradigms. Although, this has been used rarely within EAE. One study found that sucrose preference decreased in the acute phase (six to nine days after induction), while it increased well above control levels in the recovery and chronic phases (out to day 50; Pollak et al., 2000). However, this increase in drinking was concurrent with increases in body weight, creating the possibility that their increased intake throughout the study could be attributed to its nutritive value. Therefore, saccharin was utilized in this experiment to avoid this possible confound. Saccharin preference has been shown to reliably decrease with high sensitivity in depression-like states throughout several studies (Darkazalli et al., 2016; Gogos et al., 2018; Grimonprez et al., 2015; Jaehne and Baune, 2014; Portillo-Salido et al., 2015). Therefore, assuming that saccharin preference testing would generalize to EAE is warranted. Secondly, it might be possible that these effects are only reliably measured in rodents who prefer sweetened solution at baseline. The separation of subjects between high vs. low intake of saccharin at baseline or rodent lines being selectively
bred based on this characteristic is commonly seen (Gahtan et al., 1996; Gosnell and Krahn, 1992; Radke et al., 2015; Regier et al., 2012). Further, it has been shown that sensitivity of saccharin intake at baseline can predict the expression of anhedonic states induced by social defeat (Spierling et al., 2017). This suggests that the expression of anhedonia-like symptoms in EAE might be more likely in high saccharin consumers compared to low saccharin consumers. Therefore, separating subjects by saccharin intake may help reveal a possibly concealed effect.

Based on these observations, there are several future directions for our research. A dose-response study should be conducted for anhedonia-like symptoms within the EAE model below the levels of hind limb motor paralysis. This study will ensure that depression-like symptoms can be expressed in lower dose models of EAE, allowing for reliable measurements without the confound of motor paralysis. Future research should include the use of a non-nutritive sweetener solution possibly more preferred by rodents and include the separation of low vs. high saccharin consumers at baseline. This may help remove the possibility of a concealed saccharin preference effect through lack of reliable intake. Examination of other depression measures, such as the forced swim test, social interaction test, or tail suspension test, utilizing this low-dose model of EAE is also needed. These other types of behavioral testing may more sensitively measure depression-like symptoms within EAE compared to saccharin preference testing. However, careful monitoring of motor paralysis and exclusion of subjects exhibiting confounding symptoms would be imperative in the use of these other measures.

Finally, examination of tissue effects in structures of interest will help support whether or not this low-dose model of EAE is robust enough to produce anhedonia-like symptoms. One set of implicated structures are the dopaminergic mesolimbic and mesostriatal pathways,
containing structures such as the ventral tegmental area (VTA), nucleus accumbens (NAc), caudate nucleus, and putamen (for review Ikemoto, 2007). Dysregulation located in these pathways has been implicated in the expression of depression-like symptoms throughout various paradigms (Argyropoulos and Nutt, 2013; Nestler and Carlezon, 2006). Within MS, it has been shown that patients show significant gray matter atrophy within several of these aforementioned structures as compared to healthy controls (Batista et al., 2012; Nourbakhsh et al., 2016; Zivadinov et al., 2012). One study has also shown that MS patients experiencing chronic pain had significantly decreased co-activation of the NAc and caudate nucleus (Seixas et al., 2016). Within EAE, destruction of dopaminergic neurons with MPTP increased the severity of EAE symptoms (Balkowiec-Iskra et al., 2007) while activation of dopaminergic signaling with a D2/D3 receptor agonist seems to attenuate these same symptoms (Lieberknecht et al., 2017). These results show that these dopaminergic pathways may be dysregulated within MS as well as EAE and may be contributing to the expression of depression-like symptoms.

Another implicated brain structure in the development of depression-like symptoms is the lateral habenula (LHb). It has been shown that dysfunction of the LHb may dysregulate the dopaminergic and serotonergic systems by exhibiting overt inhibitory influences, producing depression-like symptoms (Christensen et al., 2013; Nair et al., 2013; Proulx et al., 2014). However, little to no work has been conducted examining its effects within the context of MS or EAE. The most closely associated study examined the role of the LHb in pain-associated depression in a chronic constriction injury (CCI) model of the sciatic nerve. This study shows that CCI led to over-excitation of the LHb, causing inhibition of the dorsal raphe nucleus (Li et al., 2017).
The primary candidate for dysregulation of these structures within MS and EAE is inflammation within the CNS. Neuroinflammation in structures associated with dopaminergic signaling has been implicated in the expression of depression-like behaviors (Felger and Treadway, 2017; Gentile et al., 2015). Despite a lack of work concerning the LHb within MS, neuroinflammation may be causing overactivation of the LHb in this model, leading to the expression of depression-like symptoms. Therefore, examination of these aforementioned areas for inflammatory markers would allow for a more holistic view of the expression of anhedonia-like symptoms within the EAE model. Additionally, examination of these areas within our low-dose model of EAE specifically may help determine whether or not it can reproduce these depression-like behaviors as seen in the standard dose models. Analysis of these tissue effects might be executed by conducting a dose-response curve of EAE examining microglial activation and other inflammatory markers in these structures of interest.

This study provided one of the first glimpses of the effects of a TLR2/TLR4 antagonist on the expression of anhedonia-like symptoms within a low-dose model of EAE. Despite a lack of positive results, this study helps provide a stepping stone and future directions for continued research. Continued pursuit of this line of research may help create a reliable model for the expression of depression-like symptoms within EAE without the confound of motor paralysis. Furthermore, continued examination of (+)-NTX may help provide a novel therapeutic strategy that provides a holistic coverage of the symptoms associated with MS.
References


