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TLR2/4 Antagonism Blocks Increased Hippocampal IL-1 β and Associated Contextual Long-Term Memory Deficits in a Rodent Model of Multiple Sclerosis

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**TLR2/4 Antagonism Blocks Increased Hippocampal IL-1 β and Associated
Contextual Long-Term Memory Deficits in a Rodent Model of Multiple
Sclerosis**

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Abstract

Approximately half of individuals suffering from multiple sclerosis (MS) express cognitive deficits in the form of memory impairments. Recent research in rats attributes these impairments to pro-inflammatory cytokine induced inflammation in the hippocampus. Toll-like receptors 2 and 4 (TLR2/4) have been implicated as possible therapeutic targets for their probable role in propagating the inflammatory response of MS. Using experimental autoimmune encephalomyelitis (EAE) as a model, we investigated the effects of a TLR2/4 antagonist, (+)-naltrexone, on hippocampal IL-1 β mRNA expression and related cognitive deficits. Treatment with (+)-naltrexone successfully blocked the increased IL-1 β levels and prevented contextual long-term memory impairments induced by EAE. These findings provide the first evidence supporting TLR2/4 antagonism as an effective mechanism against EAE related memory deficits.

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Introduction

Multiple sclerosis (MS) is a chronic disease with serious health, economic, and social implications for those who suffer from it. The world health organization estimates that more than two million individuals worldwide are affected by this debilitating disease (Browne et al., 2014). MS is characterized by inflammation, demyelination and axonal degradation within the central nervous system (CNS). The severity and symptoms can vary by individual and range from neuropathic pain and motor disturbances to social and cognitive impairments (Brassington & Marsh, 1998). Symptoms generally develop within 20-40 years of life and persist with gradual worsening for the remainder of one's lifetime. The relapsing-remitting form of MS, which affects approximately 85% of patients, commonly develops into a secondary progressive form due to the evolving nature of the disease (Compston & Coles, 2008).

The pathophysiology of MS is characterized as an autoimmune disorder. An initial antigen, which remains unknown in current research, triggers infiltration of antigen reactive T lymphocytes across the blood brain barrier and into the CNS. The invasion triggers chemoattractant cytokines to recruit acute inflammatory cells such as macrophages, monocytes, and microglia. These cells form plaques that develop lesions on the brain and spinal cord (Wingerchuk, Lucchinetti, & Noseworthy, 2001). The recruited immune cells produce a highly inflammatory environment that is thought to be involved in demyelination and neurodegeneration (Frischer et al., 2009). Though the exact cause is unknown, it is generally believed that the disease is triggered by environmental factors in genetically predisposed individuals

(Hewagama & Richardson, 2009). In order to study the mechanisms and methods of treatment for MS, animal models of the disease have been developed. The most commonly used model, experimental autoimmune encephalomyelitis (EAE), provides an opportunity to gain critical insight to the mechanisms and pathologies of MS.

EAE is effective in modeling the four key physiopathologies seen in MS including demyelination, axonal degradation, inflammation, and gliosis (Constantinescu et al., 2011). Several successful drug treatments have been developed using this model (Constantinescu, Farooqi, O'Brien, & Gran, 2011). To induce EAE, a myelin-associated antigen is peripherally injected into the animal. In some strains, an adjuvant of mycobacterium is injected with the antigen in order to further sensitize the immune system. The MOG variant of EAE, wherein myelin oligodendrocyte glycoprotein (MOG) acts as the antigen, is often used as a model because it produces inflammation, demyelination and brain lesions similar to those seen in MS (Wingerchuk et al., 2001). In Dark Agouti rats, the full MOG protein (MOG1-125) with incomplete Freund's adjuvant (IFA) can be used to induce the relapsing-remitting form of EAE.

The normal dosage of MOG used to induce EAE in rats for MS research has proven very effective in answering some questions. However, the severe motor symptoms produced by the normal dose limit the ability to perform certain behavioral tests. A low-dose MOG model allows more behavioral testing of subjects including contextual fear conditioning to measure memory.

The cognitive deficits seen in MS include learning, memory, attention, processing speed, and visuospatial disabilities. These impairments are, for many, the greatest contributor to the reduced quality of life associated with MS (Patti, 2009). Long-term and working memory impairments are typically compromised with an overwhelming 40-65% of patients displaying deficits (Chiaravalloti & DeLuca, 2008). Cognitive impairments can be present in any form or stage of MS, but typically are more common later in the disease progression (Chiaravalloti & DeLuca, 2008). Recent research suggests that problems in initial contextual encoding results in impaired retrieval of long-term memories (Thornton, Raz, & Tucke, 2002) and MRI studies show atrophy of the CA1 region of the hippocampus suggesting a hippocampal basis (Mandolesi, Grasselli, Musumeci, & Centonze, 2010). Fear conditioning is a behavioral test developed to investigate learning and memory and is often used as a measure of cognition in rats.

Classical fear conditioning is a form of associative learning and testing that can be used to measure memory. In contextual fear conditioning, an unconditioned stimulus, generally an aversive stimulus such as a foot shock, is paired with a conditioned stimulus, which in this case is a specific context. Upon presentation of the conditioned stimulus alone, the subject will display a fear behavior if it remembers the context and associates it with the foot shock. The freezing behavior in rats is a defensive mechanism that is commonly used and widely accepted as a measure of conditioned fear (Curzon, Rustay, & Browman, 2009). This paradigm relies on encoding in which the subject forms a representation of the context and the association between the context and the aversive stimuli. Both memories must

then be consolidated if they are to form long-term memories. The context is thus involved in retrieval of the association.

There is a significant amount of literature on the importance of the hippocampus in spatial representation. Consistent with this, is the finding that the hippocampus is involved in encoding the contextual representation in fear conditioning and spatial context retrieval (Anagnostaras, Gale, & Fanselow, 2001; Maren, Phan, & Liberzon, 2013). Thus, freezing behavior 24 hours or more after conditioning can be used as a measurement of hippocampal-dependent long-term memory formation. Fear conditioning deficits have been found in EAE animals along with deficits in other hippocampal-dependent memory tasks (Acharjee et al., 2013; Di Filippo et al., 2016; Mandolesi et al., 2010) suggesting EAE is a useful model to study MS-related cognitive deficits.

Though there has been disagreement regarding the mechanisms underlying cognitive deficits in MS and EAE, there is supporting EAE research attributing it to inflammation in the CNS (Acharjee et al., 2013; Mandolesi et al., 2010). This research suggests that inflammation, as a result of microglia activation, causes synaptic degeneration, spine loss and neurodegeneration and this correlates to hippocampal-dependent memory deficits (Centonze et al., 2009; Mandolesi et al., 2010). One recent study attributes cognitive deficits to changes in hippocampal synaptic plasticity as a result of inflammation, including interleukin-1 β (IL-1 β) cytokine expression (Nisticò et al., 2013). Additionally, there is much evidence to support the complicated role that the pro-inflammatory cytokine, IL-1 β , plays in contextual long-term memory. While a basal level of the cytokine is critical for hippocampal-

dependent memory formation, studies reveal that supra-physiological levels impair synaptic function (Lynch, 2010) as well as long-term memory (Spulber et al., 2009). In one study, inflammation in the CNS decreased synaptic plasticity and was accompanied by increased levels of IL-1 β in the hippocampus (Di Filippo et al., 2013). Both contextual fear conditioning and consolidation have been shown to be impaired following IL-1 β injection in the hippocampus (Gonzalez, Schiöth, Lasaga, & Scimonelli, 2009; Machado, González, Schiöth, Lasaga, & Scimonelli, 2010; Pugh, Fleshner, Watkins, Maier, & Rudy, 2001), and sustained overexpression of IL-1 β in the hippocampus leads to contextual memory deficits (Hein et al., 2010). Lastly, and most importantly, IL-1 β mRNA expression in the hippocampus has also been correlated with cognitive impairments in fear conditioning in mice with EAE (Acharjee et al., 2013).

In this study we hypothesized that, in MS and EAE, cellular damage due to inflammation and myelin breakdown releases damage-associated molecular patterns (DAMPs) that initiate an inflammatory response and it is this inflammation in the hippocampus that leads to cognitive deficits, specifically through IL-1 β as the primary signaling molecule. Toll-like receptors (TLRs), expressed primarily by microglia (Bsibsi, Ravid, Gveric, Noort, & M, 2002), recognize pathogen-associated molecular patterns (PAMPs) as well as DAMPs and function to activate acute and adaptive immune responses (Yang et al., 2010). Recognition of PAMPs allows the cell to initiate a response against invading pathogens while DAMP recognition, which preferentially occurs by TLR2 and TLR4 receptors, initiates the immune system against endogenous damage/danger signals (Schaefer, 2014). Although

DAMPs have a physiological importance for signaling tissue damage to the body, there is evidence that they may be involved in inflammatory and autoimmune pathologies (Piccinini & Midwood, 2010). The activation of TLRs by DAMPs in MS can create chronic inflammation by triggering a positive feedback loop of pro-inflammatory cytokines such as IL-1 β (Piccinini & Midwood, 2010). DAMPs including high mobility group box chromosomal protein 1 (HMGB1) and fibrinogen activate TLR2 and/or TLR4, and are present at elevated levels in active lesions of MS patients (Adams, Schachtrup, Davalos, Tsigelny, & Akassoglou, 2007; Andersson et al., 2008). In one study, MS patients also exhibited elevated TLR2/4 mRNA expression in the brain (Bsibsi et al., 2002). Given these findings, the possible implication of these receptors in MS pathophysiology encourages novel therapeutic development. By specifically targeting blockade of DAMP signaling at TLR2/4, it could be possible to combat TLR2/4 associated inflammation without affecting the host cell's protective capabilities.

(+)-Naltrexone [(+)-NTX] functions as a highly selective TLR2/4 receptor antagonist and has most recently been demonstrated to be effective at reversing neuropathic pain (Ellis et al., 2014; Hutchinson et al., 2008). The drug contains only the positive enantiomer and thus doesn't disrupt signaling through classical opioid receptors, as these only bind the negative enantiomer. Currently, (+)-NTX is moving toward FDA Investigational New Drug (IND) status and is slated to begin human trials in the future. Moreover, the drug can be taken orally and is permeable to the blood brain barrier. The combination of these factors makes (+)-NTX a clinically-relevant compound that may be effective for the treatment of various

diseases/disorders associated with inflammation. In this study, we explore whether (+)-NTX can block EAE-induced deficits in contextual fear conditioning and the associated increases in hippocampal IL-1 β in male Dark Agouti rats. We predict that by blocking TLR2/4, (+)-NTX will prevent the increased expression of the pro-inflammatory cytokine IL-1 β in the hippocampus and thereby prevent contextual long-term memory deficits associated with EAE.

Materials and Methods

Subjects

Subjects were male Dark Agouti rats (225-250g; Envigo) between 9-10 weeks old upon arrival (n=48). Subjects were housed two per cage on a 12 hour light/dark cycle (lights on at 0700 hour). The animals received access to water and food *ad libitum*. Experiments were conducted between 0800 and 1600 hour. The procedures followed for this experiment were in accordance with protocols approved by the University of Colorado Boulder Institutional Animal Care and Use Committee.

MOG Administration

Upon arrival, the rats were randomly assigned to either the MOG or saline group. The MOG assigned rats received a 4 μ g injection of MOG1-125 (VU Medical Center, Netherlands, gifted by Dr. Anne-Marie Van Dam) in a vehicle consisting of sodium acetate and IFA (Sigma; St. Louis, MO). The injection was given intradermally at the base of the tail and the syringe left in place for 3 minutes to

avoid leakage from the injection site. The rats in the saline group received saline injections following the same procedure. All animals were between 10-12 weeks old upon receiving the injections.

Motor Scoring

All rats were motor scored daily to assess the severity of their EAE symptoms. The motor score quantified physical paralysis and 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.

(+)-Naltrexone Administration

The rats were counterbalanced based on motor scores into the (+)-naltrexone [(+)-NTX] and saline treatment groups. (+)-NTX (NIH, gifted by Dr. Kenner Rice) administration to the (+)-NTX animals began 2 weeks post MOG administration. The animals received subcutaneous (+)-NTX injections three times daily (0900, 1200, and 1500 hour) for 2 weeks during behavioral testing. The volume administered was 1mL/kg. The saline animals received three times daily injections of saline at the same volume.

Contextual Fear Conditioning and Testing

Behavioral conditioning and testing were conducted using methods identical to our previously published methods (Barrientos, Hein, Frank, Watkins, & Maier, 2012). On fear conditioning and testing days, the morning injections of saline and (+)-NTX were withheld until after conditioning/testing. Contextual fear conditioning of the subjects occurred 3 weeks post MOG administration. Two rats were taken from their home cages and placed in conditioning apparatuses. Each apparatus consisted of a clear plastic box (26 cm [L] X 21 cm [W] X 24 cm [H]) inside a larger sound-attenuating chamber (ice chest). The top of the plastic box consisted of wire mesh and the floor was constructed from wire rods that connected to the shock generator and scrambler (Coulbourn Instruments). Mounted inside each ice chest were a speaker, a fan, one red light bulb and one white light bulb. After the rats had spent two minutes in the cage, a 15 second tone (76 dB) was sounded followed by a 2 second footshock (1.5 mA). Directly after receiving the shock, the rats were administered their morning injections and returned to their home cages. Animals with a motor score of 3 or higher at the time of conditioning were excluded from the study to avoid the confound of sensory/motor impairment.

The contextual fear test occurred 72 hours following conditioning. Rats were placed in the same context (apparatuses) in which they were conditioned and observed for freezing behavior. Freezing scores were recorded for each rat every 10-second period for 6 minutes. Each score was either 0 (active) or 1 (inactive except for breathing, here defined as “freezing”). Directly after testing, the rats received their morning injections and were returned to their home cages.

Tissue Preparation

Four weeks post MOG administration, the rats were transcardially perfused with saline while under sodium pentobarbital anesthesia. Animals continued to receive their normal (+)-NTX injections until the time of dissection. The hippocampus was harvested and stored at -80 °C.

Real Time PCR: IL-1 β

Real time PCR was performed on hippocampal tissue that was collected as described above.

RNA Extraction

Tissue samples were homogenized on ice with 800 μ L of trizol (Thermo Fischer Scientific; Waltham, MA) and allowed to sit at room temperature for 5 minutes. To separate the organic and aqueous layers, 160 μ L of chloroform (Sigma; St. Louis, MO) was added to the samples before vortexing for 15 seconds and sitting at room temperature for 3 minutes. The samples were spun in a 5810R Centrifuge (Eppendorf; Hamburg, Germany) at 12,000 g at 4°C for 15 minutes. The aqueous phase was separated from the organic phase and added in a 1:1 ratio to 100% 2-propanol where the samples incubated at room temperature for 10 minutes. The samples were then centrifuged again at the same temperature and speed for 10 minutes. The supernatant was decanted from each sample, 1 mL of 75% ethanol was added and the samples were spun in the centrifuge at 7,500 g for 5 minutes. The samples were decanted for a second time and the prior step was repeated. After the

two cycles, the supernatant was decanted once more and the pellet was allowed to air dry. The pellet was then resuspended in 40 μ L of nuclease free water (Bio-Rad; Hercules, CA). The concentration and purity of each RNA sample was measured using a NanoDrop One (Thermo Fischer Scientific; Waltham, MA).

cDNA Synthesis

The volume of sample containing 3 μ g of RNA was added to PCR grade water for a total of 10 μ L as calculated from the previously determined RNA concentrations. The samples were then incubated in an iCycler (Bio-Rad; Hercules, CA) at 65°C for 5 minutes in Mastermix 1 consisting of 50% random primers (Thermo Fischer Scientific; Waltham, MA) and 50% dNTP mix (Thermo Fischer Scientific; Waltham, MA). The samples received a quick chill on ice before a second incubation at 25°C for 2 minutes in Mastermix 2. Mastermix 2 consisted of 66% 5X first strand buffer (Thermo Fischer Scientific; Waltham, MA) and 33% 0.1M DTT (Thermo Fischer Scientific; Waltham, MA). Immediately after the incubation, 1 μ L of Superscript II Reverse Transcriptase (Thermo Fischer Scientific; Waltham, MA) was added to each sample. The samples were then incubated at 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes, and then maintained at 4°C.

RT-PCR

Samples were analyzed in duplicate on 96-well PCR plates (Bio-Rad; Hercules, CA) in a Real Time System Thermocycler C100Touch (Bio-Rad; Hercules, CA). Each well contained 13 μ L SYBR mix (Qiagen; Hilden, Germany), 1 μ L forward

primer, 1 μ L reverse primer, 10 μ L nuclease free water, and 1 μ L sample cDNA. Primer sequences (GenBank, National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) listed in Table 1 were validated previously (Grace et al., 2016). The plate was centrifuged at 10,000 rpm at 21-23°C for 1 minute. The BioRadIQ5 program was used to run the real-time polymerase chain reaction at temperatures 95°C, 94°C, 57°C, 72°C, and 95°C. The level of IL-1 β mRNA expression were quantified using the $\Delta\Delta C_T$ 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 and behavioral data were done with a 2-way ANOVA and Tukey's multiple comparison post-hoc test. For all tests, statistical significance was set to $\alpha=0.05$.

Results

(+)-NTX blocks impairments in long-term contextual memory associated with EAE

To investigate the effects of EAE and treatment with (+)-NTX on long-term contextual memory, rats were treated with (+)-NTX or saline and tested for contextual fear conditioning (Figure 1). Animals with EAE exhibited contextual long-term memory impairments; however, when treated with (+)-NTX, these

impairments were blocked. A two-way ANOVA (EAE x treatment) revealed a significant interaction between EAE and treatment group ($F_{1,24} = 8.991, p < 0.01$). Tukey's multiple comparisons post-hoc test confirmed that EAE rats showed a significant decrease in freezing compared to rats without EAE ($p < 0.01$), indicating a substantial deficit in long-term contextual memory. Post-hoc analysis showed that (+)-NTX treatment blocked the long-term contextual memory deficits in EAE animals ($p < 0.05$). There were no significant differences between any of the other groups.

(+)-NTX blocks the increased IL-1 β mRNA expression in the hippocampus associated with EAE

Because EAE deficits in contextual long-term memory are known to be dependent on increased IL-1 β in the hippocampus, we assessed the interaction of EAE and (+)-NTX on hippocampal IL-1 β mRNA (Figure 2). Hippocampal IL-1 β mRNA expression was increased in rats with EAE and treatment with (+)-NTX blocked this increase. Results were analyzed via a two-way ANOVA (EAE x treatment). There was no significant interaction between EAE and treatment group. There were significant main effects of EAE ($F_{1,35} = 12.72, p < 0.01$) and (+)-NTX treatment ($F_{1,35} = 4.737, p < 0.05$). A post hoc analysis using Tukey's multiple comparisons test revealed that EAE animals had significantly increased IL-1 β levels compared to animals without EAE ($p < 0.01$), and furthermore that administration of (+)-NTX prevented the elevated level of IL-1 β mRNA expression induced by EAE ($p < 0.05$). There were no significant differences between any of the other groups.

Discussion

This study investigated the effectiveness of the TLR2/4 antagonist, (+)-NTX, for preventing contextual long-term memory deficits. The research was based on prior evidence implicating neuroinflammation in cognitive dysfunction and the finding that inflammatory cytokines can disrupt synaptic transmission in the hippocampus (Mandolesi, Grasselli, Musumeci, & Centonze, 2010). Consistent with MS and EAE literatures, EAE produced a deficit in contextual long-term memory associated with elevated levels of IL-1 β . Daily injections of (+)-NTX blocked the increase in IL-1 β expression and thus prevented these memory deficits.

The contextual fear-conditioning test was used to investigate the hippocampal-dependent aspect of long-term memory because of the significant focus on it in the literature. The success of the low-dose MOG model in creating inhibited long-term memory symptoms provides encouragement for its use in other studies that are limited by the normal-dose effects. Preliminary research showed that short-term memory was not impaired by EAE, thus showing that inhibition of contextual long-term memory supported the hypothesized mechanism (data not shown). The elevated IL-1 β mRNA expression seen in this study matches research detailing increased hippocampal IL-1 β concentration in EAE (Di Filippo et al., 2013). These results were expected, as previous research has shown that elevated hippocampal IL-1 β impairs consolidation of hippocampal-based memories and prevents long-term potentiation in the hippocampus (Bellinger, Madamba, & Siggins, 1993; Pugh et al., 2001).

While most currently available therapeutics for MS focus on preventing the infiltration of immune cells into the CNS, this target is ineffective in preventing long-term progression of the disease (Gao & Tsirka, 2011). We decided instead to target TLR2/4, which could prevent memory deficits related to both acute and chronic inflammation. This decision was based on the known role of TLR2/4 activation by MS-related DAMPs and their role in self-propagating inflammation (Piccinini & Midwood, 2010). Our findings suggest that (+)-NTX competitively inhibits the binding of DAMPs to TLR2/4 and thus prevents receptor activation. As a result of the inhibited cascade, IL-1 β is maintained at normal physiological levels. Presumably, the positive feedback loop of pro-inflammatory cytokines is never initiated in the hippocampus and therefore, neither is the propagation of inflammation. These findings are significant for both therapeutic and mechanistic reasons. The effectiveness of (+)-NTX in preventing these cognitive deficits provides further evidence for neuroinflammation as a significant symptom-causing pathology in MS and EAE. It also provides support for DAMP activation of hippocampal TLR2/4 as a significant aspect of EAE-related inflammation.

TLR2/4 are expressed by microglia, dendritic cells, T cells, monocytes, astrocytes, oligodendrocytes, macrophages, endothelial cells and fibroblasts (Bsibsi et al., 2002))(Singh & Jiang, 2004)(Bsibsi et al., 2002; Jang, Park, Won, Yun, & Kim, 2012; MacLeod & Wetzler, 2007; Parker, Whyte, Vogel, Dower, & Sabroe, 2004; Singh & Jiang, 2004; Uronen-Hansson et al., 2004) and thus IL-1 β release could be mediated by any one, or a combination, of these cells. Since blocking TLR2/4 on any cell will result in the inhibition of the same pathway (presumably IL-1 β release and

inflammation), (+)-NTX would likely be effective in preventing inflammation initiated by any of the possible mediator cells. Due to the fact that TLR2/4 are primarily expressed on microglial cells (Bsibsi, Ravid, Gveric, Noort, & M, 2002), it is likely that they are the main source of IL-1 β and resulting inflammation.

Microglia, the resident immune cells of the CNS, have been a significant focus in neuro-degenerative disease research due to their involvement in CNS inflammation and release of cytotoxic factors. Microglia can exist in a resting or activated state. In the resting state, the cells extend their processes in order to monitor the environment of the CNS. When PAMPs or DAMPs are recognized by TLRs, a cascade of morphological and biomolecular changes occur in the microglial cell converting it to the activated state. The role of activated microglia is complex because it exhibits a dual nature of pro and anti-inflammation (Goldmann & Prinz, 2013). Its activation states have been generalized into two categories, M1 and M2, depending on what signals they have been activated by. M1 state is characterized by the release of pro-inflammatory cytokines and cytotoxic factors, which promotes tissue injury and likely causes the negative MS symptoms associated with inflammation. This process is critical to the immune response in the healthy CNS but becomes a problem in MS when DAMPS of endogenous ligands activate the microglial receptors. IL-1 β has been found in high-levels in both MS and EAE and has been shown to shift microglial towards the M1 phenotype (Goldmann & Prinz, 2013). IL-1 β is then produced by activated microglia and propagates the inflammatory signal by binding to other microglia and initiating the release of more pro-inflammatory cytokines (Kaushik, Thounaojam, Kumawat, Gupta, & Basu, 2013).

Ultimately, the initial microglial activation primes a self-perpetuating cycle of over activation and inflammation via pro-inflammatory cytokines. When activated in the M1 state, microglia can also function as antigen presenting cells to potentiate the chronic inflammatory response (Aloisi, Ria, & Adorini, 2000), and mediate T cell activation and differentiation (Becher, Bechmann, & Greter, 2006). It is notable that microglia activation occurs early in the EAE disease progression (Ponomarev, Shriver, Maresz, & Dittel, 2005) and remains activated into the chronic phase (Rasmussen et al., 2007) suggesting that the cells function as both initiators and propagators of the inflammation. The high expression of TLR2/4 receptors and their implicated roles in MS/EAE pathology suggests microglia acts as a mediating cell in the TLR2/4 inflammation pathway.

There is some controversy about developing anti-inflammatory drugs to treat MS. This discussion is based on the idea that inflammation has both promotive and regulatory effects on the disease progression (Ziehn, Avedisian, Tiwari-Woodruff, & Voskuhl, 2010). The concern is that using nonspecific inhibition to block inflammation can prevent the beneficial aspects that the body needs for healing. The chronic inflammation and resulting symptomology of MS suggests an obvious dysregulation in the body's normal inflammatory responses. Our focus on specifically targeting receptors 2 and 4 in the TLR family was to best limit the pathology causing aspect of inflammation while sparing the beneficial side. It is possible for (+)-NTX to produce unwanted immuno-suppressant side effects, but as for all therapeutics, the drug is considered effective if the benefit outweighs the cost. In terms of the relapsing-remitting form EAE, hippocampal IL-1 β and associated

cognitive deficits remain increased during remission (Di Filippo et al., 2016) suggesting a potential role of (+)-NTX as a therapeutic even in periods of remission.

The generality of the TLR2/4 antagonism to treat inflammation suggests that it could be an effective treatment for other cognitive, motor, and pain symptoms. For example, (+)-NTX has been shown to reverse neuropathic pain in rodent models (Ellis et al., 2014; Hutchinson et al., 2008) and in the context of EAE, (+)-NTX reversed neuropathic pain associated with elevated IL-1 β in the spinal cord (unpublished observations). Affective symptoms such as depression and anxiety are also thought to be related to IL-1 β -associated inflammation (Di Filippo et al., 2016; Gentile et al., 2015) and thus could benefit from (+)-NTX or other TLR2/4 antagonist treatments.

This study focused on long-term memory dysfunction due to its high prevalence in MS populations (Chiaravalloti & DeLuca, 2008). It is important to note that this represents just one aspect of memory impairments seen in MS. For example, deficits in working and episodic memory are also often exhibited (Bobholz et al., 2006; Rao et al., 1993). The effectiveness of (+)-NTX preventing contextual long-term memory impairments cannot be extended for all forms of memory as elevated IL-1 β levels exert detrimental effects on hippocampal-dependent but not on hippocampal-independent memory (Huang & Sheng, 2010). There is research, however, implicating elevated hippocampal IL-1 β levels in working memory deficits (Matsumoto, Yoshida, Watanabe, & Yamamoto, 2001). To test the effect of (+)-NTX on working memory, a radial arm maze test could be used (Dudchenko, 2004).

Investigating the effect of (+)-NTX on working memory in future studies would provide a greater insight into the range of its treatment options.

It is important to note that EAE is a limited model of MS. In EAE, the initial antigen causing the autoimmune response is known (MOG in this case). In MS, however, the antigen is not known and thus its determination as an effective model is by its symptomology. Some models of MS require artificial activation of the innate immune system using bacterial antigens. The EAE model in DA rats, however, removes this bias by only using IFA ('t Hart, Gran, & Weissert, 2011). It could be argued that TLR2/4 antagonism blocks the formation of anti-MOG antibodies and thus the development of EAE altogether. It is unlikely that this explains our findings as (+)-NTX administration occurred after the formation of anti-MOG antibodies. Additionally, the animals still developed motor impairments indicative of EAE, which is unlikely to occur if the anti-MOG antibodies were not formed initially. There has been some evidence of therapeutics developed in EAE models failing to translate to human clinical trials ('t Hart, Gran, & Weissert, 2011). One explanation for this disconnect comes from differences in the adaptive and acute immune systems of rodents and humans (Mestas & Hughes, 2004). TLRs and their resulting pathways, however, are highly conserved between the species (Mestas & Hughes, 2004) suggesting a more successful translation to human clinical trials. Significantly, all FDA approved immunotherapeutics for treating MS have shown efficacy in EAE and three therapeutics approved for MS treatment, glatiramer acetate, natalizumab, and mitoxantrone, were developed using EAE models (Steinman & Zamvil, 2006). Despite its limitations, EAE is a beneficial model and the success of (+)-NTX in

treating contextual long-term memory deficits in EAE suggests (+)-NTX may also be successful in treating cognitive deficits in MS.

In terms of future research, the next step is to investigate the effect of (+)-NTX directly on microglial activation in the hippocampus. Immunohistochemistry techniques can be used to visualize microglial activation and pro-inflammatory cytokine protein expression including IL-1 β . This will further elucidate the pathway involved and verify microglia as a mediating cell in the mechanism. It will also be important to determine if (+)-NTX is effective in treating other EAE-related cognitive symptoms, as well as other symptoms not related to cognition including anhedonia, reduced social interaction, and neuropathic pain.

This research provides the first evidence of a TLR2/4 antagonist functioning as an effective inhibitor of cognitive deficits and demonstrates the effectiveness of a systemic TLR2/4 antagonist at reducing inflammation in the hippocampus. As a significant number of individuals with MS are affected by long-term memory dysfunction, these findings could lead to an increased quality of life for many sufferers of the disease.

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References

- Acharjee, S., Nayani, N., Tsutsui, M., Hill, M. N., Ousman, S. S., & Pittman, Q. J. (2013). Altered cognitive-emotional behavior in early experimental autoimmune encephalitis--cytokine and hormonal correlates. *Brain, Behavior, and Immunity, 33*, 164–172. <https://doi.org/10.1016/j.bbi.2013.07.003>
- Adams, R. A., Schachtrup, C., Davalos, D., Tsigelny, I., & Akassoglou, K. (2007). Fibrinogen signal transduction as a mediator and therapeutic target in inflammation: lessons from multiple sclerosis. *Current Medicinal Chemistry, 14*(27), 2925–2936.
- Anagnostaras, S. G., Gale, G. D., & Fanselow, M. S. (2001). Hippocampus and contextual fear conditioning: recent controversies and advances. *Hippocampus, 11*(1), 8–17. [https://doi.org/10.1002/1098-1063\(2001\)11:1<8::AID-HIPO1015>3.0.CO;2-7](https://doi.org/10.1002/1098-1063(2001)11:1<8::AID-HIPO1015>3.0.CO;2-7)
- Andersson, A., Covacu, R., Sunnemark, D., I Danilov, A., Dal Bianco, A., Khademi, M., ... Harris, R. (2008). Pivotal Advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis. *Journal of Leukocyte Biology, 84*, 1248–1255. <https://doi.org/10.1189/jlb.1207844>
- Barrientos, R. M., Hein, A. M., Frank, M. G., Watkins, L. R., & Maier, S. F. (2012). Intracisternal interleukin-1 receptor antagonist (IL-1RA) prevents post-operative cognitive decline and neuroinflammatory response in aged rats. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 32*(42), 14641–14648. <https://doi.org/10.1523/JNEUROSCI.2173-12.2012>

- Bellinger, F. P., Madamba, S., & Siggins, G. R. (1993). Interleukin 1 beta inhibits synaptic strength and long-term potentiation in the rat CA1 hippocampus. *Brain Research*, 628(1-2), 227-234.
- Brassington, J. C., & Marsh, N. V. (1998). Neuropsychological Aspects of Multiple Sclerosis. *Neuropsychology Review*, 8(2), 43-77.
<https://doi.org/10.1023/A:1025621700003>
- Browne, P., Chandraratna, D., Angood, C., Tremlett, H., Baker, C., Taylor, B. V., & Thompson, A. J. (2014). Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. *Neurology*, 83(11), 1022-1024.
<https://doi.org/10.1212/WNL.0000000000000768>
- Bsibsi, M., Ravid, R., Gveric, D., Noort, V., & M, J. (2002). Broad Expression of Toll-Like Receptors in the Human Central Nervous System. *Journal of Neuropathology & Experimental Neurology*, 61(11), 1013-1021.
<https://doi.org/10.1093/jnen/61.11.1013>
- Centonze, D., Muzio, L., Rossi, S., Cavasinni, F., De Chiara, V., Bergami, A., ... Martino, G. (2009). Inflammation Triggers Synaptic Alteration and Degeneration in Experimental Autoimmune Encephalomyelitis. *Journal of Neuroscience*, 29(11), 3442-3452. <https://doi.org/10.1523/JNEUROSCI.5804-08.2009>
- Chiaravalloti, N. D., & DeLuca, J. (2008). Cognitive impairment in multiple sclerosis. *The Lancet Neurology*, 7(12), 1139-1151. [https://doi.org/10.1016/S1474-4422\(08\)70259-X](https://doi.org/10.1016/S1474-4422(08)70259-X)
- Cid, C., García-Villanueva, M., Salinas, M., & Alcázar, A. (2007). Detection of anti-heat shock protein 90 β (Hsp90 β) antibodies in cerebrospinal fluid. *Journal of*

- Immunological Methods*, 318(1-2), 153-157.
<https://doi.org/10.1016/j.jim.2006.09.017>
- Compston, A., & Coles, A. (2008). Multiple sclerosis, 372, 16.
- Constantinescu, C. S., Farooqi, N., O'Brien, K., & Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British Journal of Pharmacology*, 164(4), 1079-1106.
<https://doi.org/10.1111/j.1476-5381.2011.01302.x>
- Curzon, P., Rustay, N. R., & Browman, K. E. (2009). Cued and Contextual Fear Conditioning for Rodents. In J. J. Buccafusco (Ed.), *Methods of Behavior Analysis in Neuroscience* (2nd ed.). Boca Raton (FL): CRC Press/Taylor & Francis. Retrieved from <http://www.ncbi.nlm.nih.gov/books/NBK5223/>
- Di Filippo, M., Chiasserini, D., Gardoni, F., Viviani, B., Tozzi, A., Giampà, C., ... Calabresi, P. (2013). Effects of central and peripheral inflammation on hippocampal synaptic plasticity. *Neurobiology of Disease*, 52, 229-236.
<https://doi.org/10.1016/j.nbd.2012.12.009>
- Di Filippo, M., de Iure, A., Giampà, C., Chiasserini, D., Tozzi, A., Orvietani, P. L., ... Calabresi, P. (2016). Persistent activation of microglia and NADPH oxidase drive hippocampal dysfunction in experimental multiple sclerosis. *Scientific Reports*, 6, 20926. <https://doi.org/10.1038/srep20926>
- Ellis, A., Wieseler, J., Favret, J., Johnson, K. W., Rice, K. C., Maier, S. F., ... Watkins, L. R. (2014). Systemic Administration of Propentofylline, Ibudilast, and (+)-Naltrexone Each Reverses Mechanical Allodynia in a Novel Rat Model of

- Central Neuropathic Pain. *The Journal of Pain*, 15(4), 407–421.
<https://doi.org/10.1016/j.jpain.2013.12.007>
- Frischer, J. M., Bramow, S., Dal-Bianco, A., Lucchinetti, C. F., Rauschka, H., Schmidbauer, M., ... Lassmann, H. (2009). The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain*, 132(5), 1175–1189. <https://doi.org/10.1093/brain/awp070>
- Gonzalez, P. V., Schiöth, H. B., Lasaga, M., & Scimonelli, T. N. (2009). Memory impairment induced by IL-1beta is reversed by alpha-MSH through central melanocortin-4 receptors. *Brain, Behavior, and Immunity*, 23(6), 817–822. <https://doi.org/10.1016/j.bbi.2009.03.001>
- Grace, P. M., Strand, K. A., Galer, E. L., Urban, D. J., Wang, X., Baratta, M. V., ... Watkins, L. R. (2016). Morphine paradoxically prolongs neuropathic pain in rats by amplifying spinal NLRP3 inflammasome activation. *Proceedings of the National Academy of Sciences of the United States of America*, 113(24), E3441–E3450. <https://doi.org/10.1073/pnas.1602070113>
- Hein, A. M., Stasko, M. R., Matousek, S. B., Scott-McKean, J. J., Maier, S. F., Olschowka, J. A., ... O'Banion, M. K. (2010). Sustained hippocampal IL-1beta overexpression impairs contextual and spatial memory in transgenic mice. *Brain, Behavior, and Immunity*, 24(2), 243–253. <https://doi.org/10.1016/j.bbi.2009.10.002>
- Hewagama, A., & Richardson, B. (2009). The genetics and epigenetics of autoimmune diseases. *Journal of Autoimmunity*, 33(1), 3. <https://doi.org/10.1016/j.jaut.2009.03.007>

- Hutchinson, M. R., Zhang, Y., Brown, K., Coats, B. D., Shridhar, M., Sholar, P. W., ... Watkins, L. R. (2008). Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *The European Journal of Neuroscience*, 28(1), 20–29.
<https://doi.org/10.1111/j.1460-9568.2008.06321.x>
- Jang, S., Park, J.-S., Won, Y.-H., Yun, S.-J., & Kim, S.-J. (2012). The Expression of Toll-Like Receptors (TLRs) in Cultured Human Skin Fibroblast is Modulated by Histamine. *Chonnam Medical Journal*, 48(1), 7–14.
<https://doi.org/10.4068/cmj.2012.48.1.7>
- Lynch, M. A. (2010). Age-Related Neuroinflammatory Changes Negatively Impact on Neuronal Function. *Frontiers in Aging Neuroscience*, 1.
<https://doi.org/10.3389/neuro.24.006.2009>
- Machado, I., González, P., Schiöth, H. B., Lasaga, M., & Scimonelli, T. N. (2010). α -Melanocyte-stimulating hormone (α -MSH) reverses impairment of memory reconsolidation induced by interleukin-1 beta (IL-1 beta) hippocampal infusions. *Peptides*, 31(11), 2141–2144.
<https://doi.org/10.1016/j.peptides.2010.07.018>
- MacLeod, H., & Wetzler, L. M. (2007). T Cell Activation by TLRs: A Role for TLRs in the Adaptive Immune Response. *Sci. STKE*, 2007(402), pe48-pe48.
<https://doi.org/10.1126/stke.4022007pe48>
- Mandolesi, G., Grasselli, G., Musumeci, G., & Centonze, D. (2010). Cognitive deficits in experimental autoimmune encephalomyelitis: neuroinflammation and

- synaptic degeneration. *Neurological Sciences*, 31(S2), 255–259.
<https://doi.org/10.1007/s10072-010-0369-3>
- Maren, S., Phan, K. L., & Liberzon, I. (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. *Nature Reviews Neuroscience*, 14(6), 417–428. <https://doi.org/10.1038/nrn3492>
- Mestas, J., & Hughes, C. C. W. (2004). Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology*, 172(5), 2731–2738. <https://doi.org/10.4049/jimmunol.172.5.2731>
- Nisticò, R., Mango, D., Mandolesi, G., Piccinin, S., Berretta, N., Pignatelli, M., ... Centonze, D. (2013). Inflammation Subverts Hippocampal Synaptic Plasticity in Experimental Multiple Sclerosis. *PLOS ONE*, 8(1), e54666.
<https://doi.org/10.1371/journal.pone.0054666>
- Parker, L. C., Whyte, M. K. B., Vogel, S. N., Dower, S. K., & Sabroe, I. (2004). Toll-Like Receptor (TLR)2 and TLR4 Agonists Regulate CCR Expression in Human Monocytic Cells. *The Journal of Immunology*, 172(8), 4977–4986.
<https://doi.org/10.4049/jimmunol.172.8.4977>
- Patti, F. (2009). Cognitive impairment in multiple sclerosis. *Multiple Sclerosis Journal*, 15(1), 2–8. <https://doi.org/10.1177/1352458508096684>
- Piccinini, A. M., & Midwood, K. S. (2010). DAMPening Inflammation by Modulating TLR Signalling [Research article]. <https://doi.org/10.1155/2010/672395>
- Rachal Pugh, C., Fleshner, M., Watkins, L. R., Maier, S. F., & Rudy, J. W. (2001). The immune system and memory consolidation: a role for the cytokine IL-1beta. *Neuroscience and Biobehavioral Reviews*, 25(1), 29–41.

- Schaefer, L. (2014). Complexity of Danger: The Diverse Nature of Damage-associated Molecular Patterns. *Journal of Biological Chemistry*, 289(51), 35237–35245.
<https://doi.org/10.1074/jbc.R114.619304>
- Singh, A. K., & Jiang, Y. (2004). How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology*, 201(1–3), 197–207.
<https://doi.org/10.1016/j.tox.2004.04.015>
- Spulber, S., Mateos, L., Oprica, M., Cedazo-Minguez, A., Bartfai, T., Winblad, B., & Schultzberg, M. (2009). Impaired long term memory consolidation in transgenic mice overexpressing the human soluble form of IL-1ra in the brain. *Journal of Neuroimmunology*, 208(1–2), 46–53.
<https://doi.org/10.1016/j.jneuroim.2009.01.010>
- Thornton, A. E., Raz, N., & Tucke, K. A. (2002). Memory in multiple sclerosis: contextual encoding deficits. *Journal of the International Neuropsychological Society: JINS*, 8(3), 395–409.
- Uronen-Hansson, H., Allen, J., Osman, M., Squires, G., Klein, N., & Callard, R. E. (2004). Toll-like receptor 2 (TLR2) and TLR4 are present inside human dendritic cells, associated with microtubules and the Golgi apparatus but are not detectable on the cell surface: integrity of microtubules is required for interleukin-12 production in response to internalized bacteria. *Immunology*, 111(2), 173–178. <https://doi.org/10.1111/j.0019-2805.2003.01803.x>
- Wingerchuk, D. M., Lucchinetti, C. F., & Noseworthy, J. H. (2001). Multiple Sclerosis: Current Pathophysiological Concepts. *Laboratory Investigation*, 81(3), 263–281. <https://doi.org/10.1038/labinvest.3780235>

Yang, H., Hreggvidsdottir, H. S., Palmblad, K., Wang, H., Ochani, M., Li, J., ... Tracey, K. J. (2010). A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(26), 11942–11947. <https://doi.org/10.1073/pnas.1003893107>

Figures/Tables Legends

Figure 1. Percent time freezing during a 6-minute contextual fear test. MOG administration resulted in significantly less freezing relative to saline controls, * $p < 0.01$. Animals that were treated with MOG and (+)-naltrexone froze significantly more relative to animals treated with MOG and saline, # $p < 0.05$. Data presented as mean \pm SEM. Group sizes: saline x saline (n=8), MOG x saline (n=6), saline x (+)-NTX (n=8), MOG x (+)-NTX (n=6).

Figure 2. Hippocampal IL-1 β mRNA levels relative to GAPDH housekeeping gene. MOG administration resulted in significantly elevated hippocampal IL-1 β mRNA relative to saline controls, * $p < 0.01$. Animals that were treated with MOG and (+)-naltrexone had significantly lower hippocampal IL-1 β mRNA levels relative to those treated with MOG and saline, # $p < 0.05$. Data presented as mean \pm SEM. Group sizes: saline x saline (n=7), MOG x saline (n=10), saline x (+)-NTX (n=8), MOG x (+)-NTX (n=10).

Figures/Tables

Figure 1.

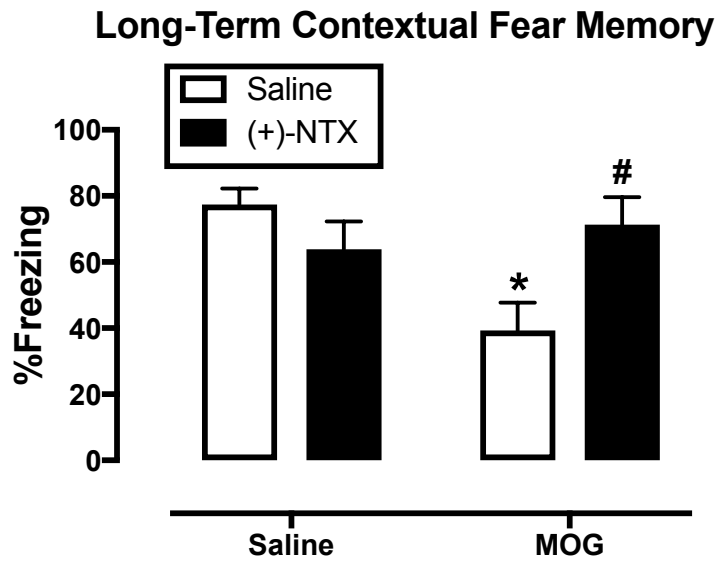


Figure 2.

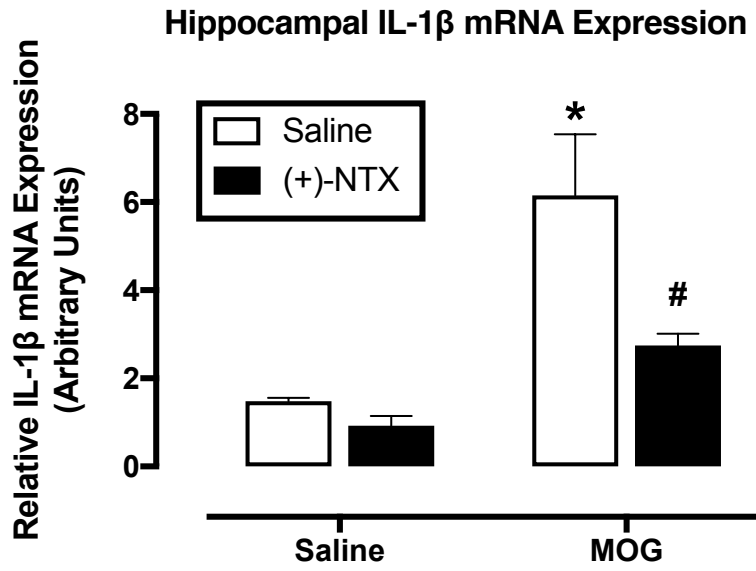


Table 1. PCR Primers

Gene	Primer Sequence (5'-3')
GAPDH	F: AGGGACAATCTCACACAGG R: GACTCAACCTTCCTCTCCA
IL-1β	F: GAAGTCAAGACCAAAGTGG R: TGAAGTCAACTATGTCCCG