Impacts of *Hymenolepis diminuta* (benign helminth worm) colonization on chronic pain and the central nervous system in Sprague Dawley rats

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Abstract

Over the last century, members of post-industrial societies have experienced a significant depletion of gut microbiota in terms of parasitic "old friends". Consequently, the ability of the remaining microbiota to modulate immune responses has been drastically limited. Reduced immunoregulation causes the immune system to be overactive due to an improper balance between T helper 1 (Th1) and T helper 2 (Th2) immunity. Th1 immunity is used to fight intracellular pathogens, and it is mediated by inflammatory effector T cells. Th2 immunity fights extracellular pathogens through a humoral response that upregulates antibody production. It has been demonstrated that intestinal helminth worms, such as *Hymenolepis diminuta,* are responsible for a shift away from Th1 cell immunity and towards Th2 cell immunity, which promotes an anti-inflammatory phenotype through suppression of inflammatory effector T cells. A helminth-induced anti-inflammatory shift could potentially be used to counteract inflammatory disorders such as chronic neuropathic pain. Neuropathic pain is responsible for an upregulation of pro-inflammatory cytokines and a shift toward Th1 immunity. This study explored the use of helminthic therapy as a treatment for neuropathic pain in the periphery and both neuropathic pain and cognitive dysfunction in the central nervous system through increased immune regulation.

We investigated the effects of *H. diminuta* on neuropathic pain development and cognition in male Sprague Dawley rats following chronic constriction injury of the sciatic nerve. Rats were colonized with *Hymenolepid diminuta* cystercercoids (HDCs; larval stage) prior to CCI surgeries. Von Frey testing measured levels of mechanical allodynia, Pavlovian fear conditioning measured declarative memory, and juvenile social exploration measured levels of anxiety. Inconsistent results from Von Frey and fear conditioning suggest that helminth worm therapy most likely does not improve mechanical allodynia or hippocampal-dependent learning and memory cognition. Neither CCI surgery nor helminth colonization impacted anxiety levels. Additionally, impacts of *H. diminuta* on molecular regulation of cytokine levels in the hippocampus were assessed using qRT-PCR. Hippocampus analysis demonstrated a shift toward an anti-inflammatory cytokine milieu following helminth treatment. These studies indicate that, although helminths did not consistently impact behavior following CCI surgeries, *H. diminuta* therapy is a promising treatment for neuroinflammation in the brain.

Introduction

Chronic pain is a world-wide crisis that impacts more than 50 million people in the United States alone, and it has been connected to anxiety and depression, opioid addiction, and limited mobility (Dahlhamer, 2018)**.** Because neuropathic pain has become an incredibly widespread issue, the neuroimmune causes of chronic pain are of great interest to the research community.

The immune mechanisms that underly the modulation of neuropathic pain are responsible for the amplification of inflammatory signaling molecules. In response to damage to the somatosensory nervous system, non-neuronal cells of the central nervous system, called glial cells, become activated. Glial cell activation triggers an increase of proinflammatory cytokines from the site of injury, and this signal projects to the spinal cord and the brain. This neuroimmune connection causes the development and amplification of pain, which is a phenomenon referred to as hyperalgesia (P. M. Grace, Hutchinson, Maier, & Watkins, 2014).

Additionally, immunoregulatory processes are responsible for modulating inflammation through the use of effector T cells. Regulatory $CD4^+$ T (T_{reg}) cells serve a critical role in preventing overactive T cells from promoting inflammation aberrantly, thereby protecting against autoimmunity and controlling the cellular response to pathogens. These regulatory T cells interact directly with other effector T cells in order to minimize tissue damage. If these cells are not present, T helper 17 (Th17) cells and eosinophils are able to proliferate and promote an inflammatory phenotype (Parham, 2015). Regulatory T cells have also been found to regulate the immune response to neuropathic pain through the alteration of cytokine expression (Lees; Luchting), directly opposing the action of activated glial cells. Activating T_{reg} cells suppresses the cytotoxic T cells and prevents them from releasing proinflammatory cytokines. Because this directly opposes the cytokine release by activated glial cells, T_{reg} activation could potentially be an effective treatment for neuropathic pain.

According to the Center for Disease Control and Prevention, approximately 20% of the United States population is affected by chronic pain (Cragg, 2018), and this may be due, in part, to overactive effector $CD4^+$ T cells that are not being adequately suppressed by T_{reg} cells. The presence of certain beneficial microbiota within the gut microbiome have been linked to increased numbers of regulatory T cells (Hewitson), so it is possible that exposure to these

specific microbiota could be a potential treatment for chronic pain. Though humans coevolved with these microorganisms, humans within post-industrialized countries no longer serve as hosts to many of these microorganisms. It has proposed that the use of antibiotics, clean water, and sterilized medical techniques have caused the gut microbiome of humans living in post-industrialized countries to become a hostile environment for many commensal microorganisms (Bilbo, Wray, Perkins, & Parker, 2011; Ege, 2017; A. H. Liu, 2015; Villeneuve et al., 2018). This decreased microbial diversity correlates with decreased immunoregulatory functions of the host, and the proposed link between these two factors is referred to as the hygiene hypothesis. More specifically, the hygiene hypothesis states that the depletion of microorganisms within the gut microbiome of a human can cause the immune system to be poorly educated, which in turn, can cause the immune system to be overactive in response to inappropriate stimuli. The positive correlation between the presence of microorganisms and improved immunoregulation can be supported on a molecular basis by the promotion of an anti-inflammatory phenotype of animals that host microorganisms.

An example of one of these microorganisms is *Mycobaccterium vaccae* (*M. vaccae*). *M. vaccae* restores the immune balance between T helper 1 (Th1) and T helper 2 (Th2) responses. Th1 responses harness effector Th1 and Th17 cells to fight intracellular pathogens, and Th2 responses use antibody-mediated responses and suppression of inflammatory effector T cells to fight extracellular pathogens. *M. vaccae* modulates immunity of the infected animal by activating Th1-mediated responses and suppressing Th2 responses (Akkoc et al., 2008; Grange, Bottasso, Stanford, & Stanford, 2008; Hernández-Pando et al., 2008; Stanford, Stanford, & Grange, 2004; L. Zhang et al., 2016). This is the natural response of the body to attempt to attack and destroy the pathogen. The Th1 response activates tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and cytokines such as IL-12 and IL-2 (Dlugovitzky, Stanford, & Stanford, 2011; L. Zhang et al., 2016). Over time, infection with *M. vaccae* activates type 1 interferons, which correlates with the activation of T_{reg} cells and the suppression of Th1 responses (Frank, Fonken, Dolzani, et al., 2018; L. Zhang et al., 2016). This switch from primarily Th1 responses to Th2 responses increases levels of IL-4, which causes a positive feedback loop that further enhances the Th2 shift (Chen et al., 2004). This molecular mechanism that drives Th1/Th2 balance is responsible for promoting an overall anti-inflammatory phenotype due to the expression of T_{reg}

cells and anti-inflammatory cytokines, such as IL-10, IL-4, and TGF-β (Frank, Fonken, Watkins, Maier, & Lowry, 2018).

Depending on the microorganism, upregulation of Th1 responses or upregulation of Th2 responses can be utilized to enhance immunoregulation and benefit host immunity, because both of these techniques can be used to restore the Th1/Th2 balance. For instance, parasitic microbes, such as helminth tapeworms, can regulate the immune system by shifting the immunophenotype of their hosts from primarily mediating Th1 responses to facilitating Th2 responses (Villeneuve et al., 2018). Helminth tapeworms, called *Hymenolepis diminuta*, were used in this study because they are able to restore the Th1/Th2 balance of the host organism by promoting T_{reg} activation and effector $CD4^+$ T cell suppression. These factors contribute to promoting an anti-inflammatory phenotype in the host. In addition to promoting immunoregulation, *H. diminuta* also avoid piercing the epithelial wall of the small intestines of their hosts, so they are considered benign helminths (Smyth et al., 2017). These two features make treatment with benign helminth parasites a very attractive research subject in regard to alleviating immunological disorders without causing tissue damage to the host. In the wild, rats are colonized by *H. diminuta,* so this treatment is used to restore the immune functions that rats normally demonstrate (McKenney et al., 2015; Ren et al., 2017). Re-introduction of these helminth worms to the gut microbiome has been linked to both increased numbers of T_{reg} cells as well as increased expression of anti-inflammatory cytokines such as IL-10 (Parfrey et al., 2017). Specific molecules on the surface of *H. diminuta* shed from the surface of the tapeworms, and these molecules serve as foreign particles, called antigens, which elicit an immune response from the host. These antigens are processed and presented by antigen-presenting cells, such as dendritic cells and T cells (Bień et al., 2016; Lightowlers & Rickard, 1988), and this antigen presentation has been linked to suppression of classically activated macrophages (Bień et al., 2016) and polarization of alternatively activated macrophages (Fernando, Reyes, Iannuzzi, Leung, & McKay, 2014; Hewitson, Grainger, & Maizels, 2009). Alternatively activated macrophages are responsible for promoting the differentiation of T_{reg} cells from naïve T cells (Gundra et al., 2014). Polarization of alternatively activated macrophages and activation of T_{reg} cells are responsible for promoting an anti-inflammatory phenotype in the host.

In previous studies, *H. diminuta* have been proven to alleviate neuroinflammation, decrease cognitive disorders (Williamson et al., 2016), and decrease risks of autoimmune diseases (Fairweather & Cihakova, 2009). Additionally, the life cycle of *H. diminuta* offers a benefit that makes it an ideal candidate for the colonization of rats and mice in a laboratory setting. These helminths require a secondary, intermediate host for reproduction (Smyth et al., 2017; M. Zhang, Mathew, & Parker, 2018), which prevents these helminths from transmitting directly from rat to rat or from rat to human unintentionally. Common grain beetles can be used to allow *H. diminuta* eggs to mature into *Hymenolepis diminuta cysticercoids* (HDCs) that can be directly fed to rodents. The HDCs then colonize their hosts and grow into full helminth worms within the small intestines of the rodents. At this point, the helminth worms produce eggs that are secreted in the fecal matter of the rodents; grain beetles eat the eggs, and the life cycle starts again (M. Zhang et al., 2018). The anti-inflammatory properties of *H. diminuta,* along with the benefits of laboratory use, make this species of helminth an ideal candidate for research in neuroinflammation and chronic pain.

This following set of experiments conducted in this lab aimed at answering two questions concerning the effects of the introduction of the benign helminth worm, *H. diminuta*, into the gut microbiome of Sprague Dawley rats. The first goal of this experiment was to determine whether helminthic treatment was able to counteract the negative behavioral effects of a chronic constriction injury (CCI) on the left sciatic nerve of Sprague Dawley rats. A CCI surgery was chosen as a model for chronic pain due to its ability to amplify inflammation and decrease the number of T_{reg} cells at the injury site (Austin, Kim, Perera, & Moalem-Taylor, 2012; Austin et al., 2012; Lees, Duffy, Perera, & Moalem-Taylor, 2015). Inflammation from the CCI surgery is amplified from the site of injury to the spinal cord and the brain. In order to determine the behavioral effects of *H. diminuta* colonization, measures of neuropathic pain (Von Frey test), learning and memory (Pavlovian fear conditioning), and anxiety (juvenile social exploration test) were determined. Von Frey measured relative levels of mechanical allodynia (pain that is generated by a normally innocuous stimulus), Pavlovian contextual fear conditioning measured declarative memory, and juvenile social exploration measured levels of anxiety. Chronic constriction injuries have been shown to increase mechanical allodynia (Austin & Moalem-Taylor, 2010) and decrease declarative memory (Qian et al., 2018) in rodents. This peripheral

nerve injury may also cause decreased social interaction due to the increased levels of pain, which corresponds with increased anxiety (Descalzi et al., 2017).

The second goal of this set of experiments, analysis of the hippocampus, showed a significant difference in the mRNA levels of several cytokines in rats that were colonized by *H. diminuta*. There was an increase of the anti-inflammatory cytokine, IL-10, along with a decrease in the IL-10 receptor. Additionally, there was a decrease in the expression of IL-13. Though this Th2-induced cytokine has been linked to several anti-inflammatory processes, it is also associated with allergen-induced asthma (Corren, 2013). Since treatment with *H. diminuta* causes an increase in immunoregulatory Th2 responses and a reduction in airway inflammation, this result was expected (McKenney et al., 2015). Colonization by *H. diminuta* generated an anti-inflammatory cytokine milieu within the hippocampus of Sprague Dawley rats, which suggests potential therapeutic uses for benign helminthic treatment.

Background: The Hygiene Hypothesis

The original hygiene hypothesis, introduced in 1989, suggested a potential explanation for the epidemiological trend of increased occurrences of allergies and asthma in post-industrial countries (Strachan, 1989). It proposed that improved standards of hygiene could lead to poor immunological defense mechanisms later in life. Additionally, it has been observed that there is a negative correlation between the number of older siblings a person has and the likelihood of having an immune disorder, such as allergies or autoimmunity (Okada, Kuhn, Feillet, & Bach, 2010). Because there have been no clear correlations between socioeconomic status and immunological deficiencies within exclusively industrialized countries, increased hygiene and sanitization may be the factor that is causing immune disorders by transforming the gut microbiome into a hostile environment for the microbial "old friends" that coevolved with humans (Okada et al., 2010; G A W Rook & Brunet, 2005; Villeneuve et al., 2018). The potential causes of depletion of gut microbiome diversity in industrialized countries includes the utilization of antibiotics, clean water, improved medical sanitization, and processed foods (Shen & Wong, 2016). In order to validate this hypothesis, it is necessary to understand the molecular mechanisms by which these commensal microorganisms have been able to promote immune health of their human hosts historically.

Though improved hygiene has led to fewer incidences of infectious diseases, it may also be a potential cause of inflammatory disorders (Ege, 2017; Shen & Wong, 2016) due to decreased immunoregulation (G. A. W. Rook & Brunet, 2005). As the hygiene hypothesis has been further examined, the term "hygiene" is an inadequate term for the cause of poor immunity. The root of the issue is reduction of gut microbiota diversity, due to improved hygienic practices, a lack of exposure to infection during early childhood development, and sanitary medical practices (Björkstén, 2009; A. H. Liu, 2015). Without proper immunoregulation by microorganisms, it is possible to develop an immune disorder mediated by an imbalance between T helper 1 (Th1) and T helper 2 (Th2) responses (G. A. W. Rook & Brunet, 2005). The Th1 pathway protects against viruses and intracellular pathogens by releasing inflammatory molecules such as IFN-γ, IL-2, and TNF-β. The Th2 pathway is designed to protect against extracellular pathogens by stimulating an antibody response, facilitating amplification of eosinophils (granule-containing, inflammatory leukocytes), and inhibiting normal phagocytic cell

functioning (Kidd, 2003; Parham, 2015; Romagnani, 2000). An imbalance between Th1 and Th2 mediated immunity can cause immune dysregulation. A system polarized towards Th1-mediated responses provokes the pathogenesis of specific inflammatory diseases such as autoimmune disorders, while a system dominated by Th2-mediated responses provokes the development of allergic reactions through the humoral upregulation of IgE antibodies (Romagnani, 2000). Effector $CD4^+$ T regulatory (T_{reg}) cells modulate $CD4^+$ T helper cells, including those associated with both Th1 and Th2 responses. Commensal microbiota have an integral role in activating T_{reg} cells (Kou & Parker, 2018). In the absence of these microbiota, there is an inappropriate induction of both Th1 and Th17 responses, which promotes inflammation through amplification of inflammatory cytokines and Th17-mediated activation of eosinophils (Dias & Banerjee, 2013; Kou & Parker, 2018; Graham A. W. Rook, 2012; Graham A. W. Rook, Lowry, & Raison, 2013). The ability of commensal microorganisms, such as helminth parasites, to modulate the immune system of their host provides evolutionary advantages to both the microorganism as well as the host.

Because mammals co-evolved with helminths, the parasites established immunological benefits for the hosts as a way of protecting themselves from expulsion. Immunoglobulin E antibodies (IgE) bind and cross-link cell surface receptors (FcεRI) on mast cells, basophils, and activated eosinophils, all of which lead to the release of histamine and other inflammatory mediators that cause vasodilation and smooth muscle contraction to attempt to eject the parasite (Parham, 2015). Though these attempts at expulsion are usually unsuccessful, they limit the diseases that can be induced by the parasites. The host's immune response to all helminth worms includes the induction of Th2-mediated responses in order to generate IgE antibodies (Zhou et al., 2016). This Th2 response is generated through T_{reg} cells, alternatively activated macrophages, and dendritic cells (Maizels, 2004; Matisz et al., 2018). The presence of helminths induces the production of the Th2 cytokine, IL-4, which stimulates alternatively activated macrophages. This subset of macrophages plays a role in suppressing Th1 responses through the differentiation of T_{reg} cells (Kreider, Anthony, Urban, & Gause, 2007). Dendritic cells that have been exposed to helminths activate anti-inflammatory Th2 cytokines (Matisz et al., 2018). Dendritic cells activate Th2 cytokines when helminth-generated antigens are phagocytosed by dendritic cells and presented on the dendritic cell surface by major histocompatibility complex class II molecules(Connor, Tang, Camberis, Le Gros, & Ronchese, 2014; Matisz et al., 2018).

These molecules interact with additional immune cells and suppress the immune response. T_{reg} cells, alternatively activated macrophages, and dendritic cells all contribute to the shift towards an anti-inflammatory, Th2-mediated phenotype in the host.

In the absence of a diverse microbiome, the Th2 segment of the immune system can develop in an unregulated fashion, promoting overactive and autoreactive T cells, which can ultimately lead to allergies and autoimmune disorders, respectively. Introduction of benign helminths to the gut microbiome could potentially educate the immune system to restore the Th1/Th2 balance and promote an anti-inflammatory phenotype (Johnstton et al., 2010; Kou & Parker, 2018; Williamson et al., 2016). In this study, we used the benign helminth, *Hymenolepis diminuta*. This species of helminth co-evolved with rodents and does not harm the host (Makki et al., 2011; Sulima et al., 2018), which makes it a potential therapeutic treatment for immune dysregulation. *H. diminuta* have been observed to ameliorate colitis, inflammation of the lining of the colon, (Johnston et al., 2010; Lopes, Shute, Wang, & McKay, 2018; Melon, Wang, Phan, & McKay, 2010) and improve cognitive functioning (Williamson et al., 2016) in rodents. Helminthic therapy could be useful in combating the poor immunoregulation deriving from the depletion of the gut microbiome.

Methods and Results

Experiment 1: Two 4-0 Suture CCI

Methods

Subjects

Pathogen-free Sprague Dawley rats aged 10-12 weeks upon arrival were used for every experiment (Envigo). Rats were housed in pairs in Plexiglas cages with food and water available *ad libitum*. The Vivarium was temperature controlled at 23 ± 3˚C and light-controlled with a 12 hour light-dark cycle. All methods were approved by the Institutional Animal Care and Use Committee of the University of Colorado Boulder.

Production of Hymenolepis diminuta cytercercoids (HDCs)

Grain beetles (*Tenebrio molitor*) were used as intermediate hosts for the lifecycle of *H. diminuta*. Newly hatched beetles do not naturally contain HDCs, so to begin the production of HDCs, beetles must be fed *H. diminuta* eggs. Beetles were kept in plastic containers without a supply of food or water for two days. This assured that beetles were sufficiently hungry when they were introduced to *H. diminuta* eggs within rat fecal pellets. Then, fresh fecal pellets containing *H. diminuta* eggs were fed to the beetles for one day. At this point, the grain beetles were returned to housing in plastic containers with oats and celery. Five to seven weeks later, *Hymenolepis diminuta* cystercoids (HDCs) were ready to be harvested.

Harvesting of HDCs and HDC Colonization

HDCs were harvested from the abdomen of grain beetles. To access HDCs, the head, thorax, wings, and legs were removed from the beetle. The abdomen was placed in 0.06% saline, and contents of the abdomen were removed using a scalpel. HDCs were viewed under a 40x magnification, and doses of 5 HDCs were aliquoted with plastic transfer pipettes. Doses were fed to the rats from transfer pipettes. HDCs in the 0.06% saline solution are pictured below (Figure 1). Control rats were fed doses of the vehicle (0.06% saline) from the plastic transfer pipettes.

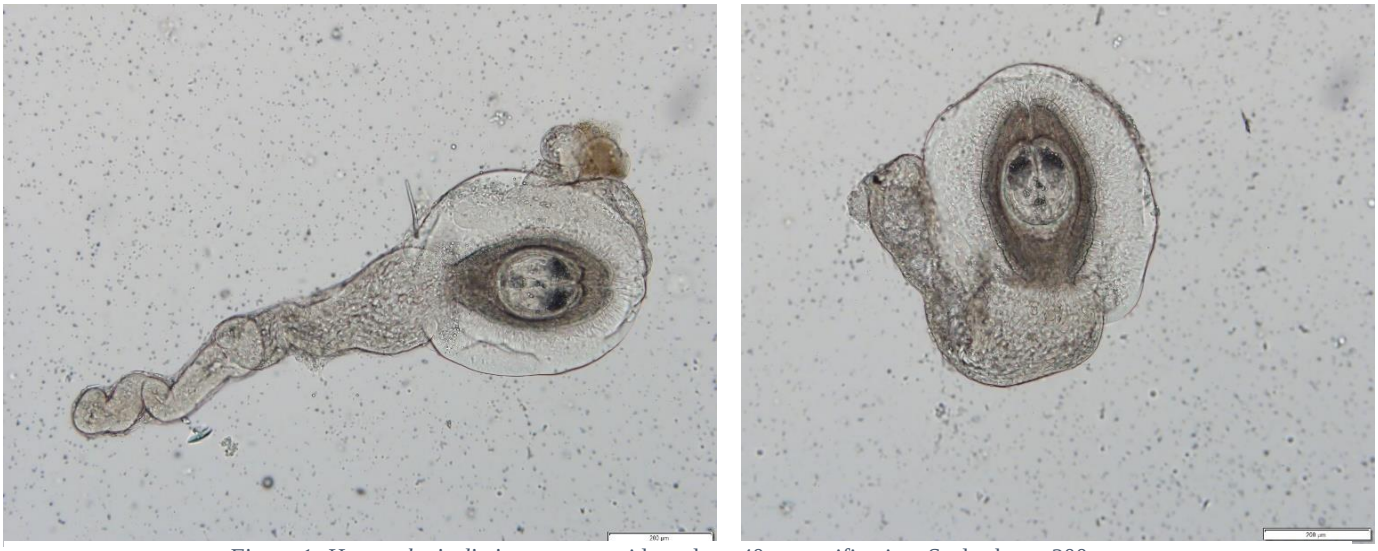

Figure 1: Hymenolepis diminuta cystercoids under a 40x magnification. Scale shows 200µm.

Fecal Float Test

Fecal float tests were performed on each rat to assure that rats had been colonized by the HDCs. Three weeks after the rats were fed HDCs, the *H. dimunta* cystercoids matured into full helminth worms in the small intestines of the rats. At this point, the worms began producing *H. diminuta* eggs that appeared in the fecal pellets of the rats. To determine whether eggs were present, rats were single housed for 4 hours. One fresh pellet from each rat was placed in a saturated saline solution in a test tube. A stir rod was used to mix the pellet with the solution to create a thin slurry. Saturated saline solution was added to the test tube until a meniscus formed above the test tube rim, and the solution sat for 15 minutes to allow eggs to rise to the top of the solution. Glass slides were gently pressed against the meniscus to wet mount a sample onto the slide. The sample was viewed at a 100x magnification to view the *H. diminuta* eggs. Figure 2 shows a sample of *H. diminuta* eggs.

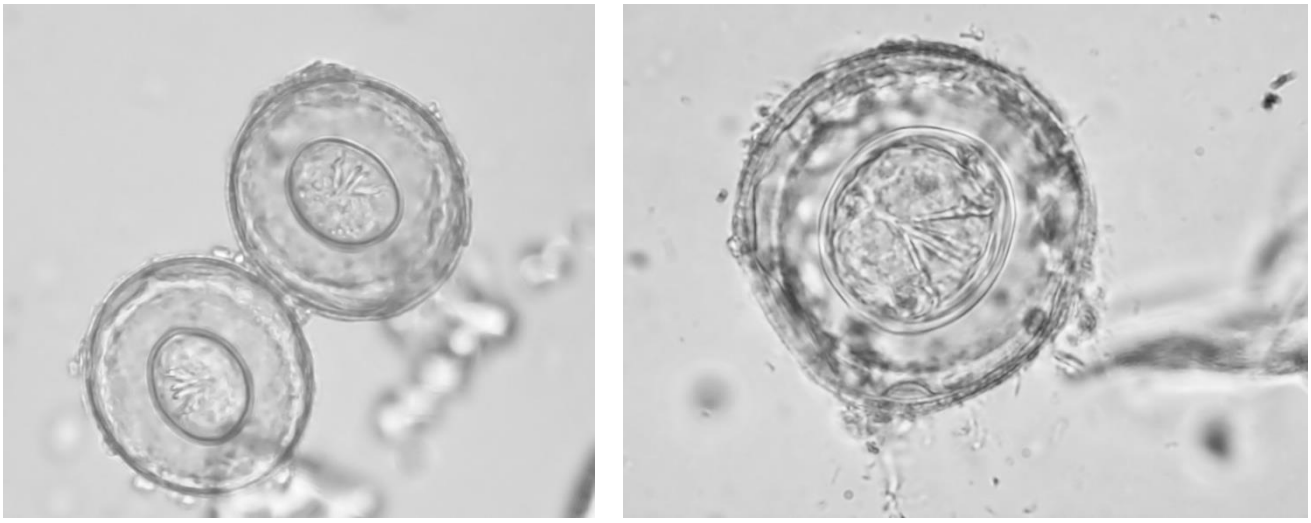

Figure 2. H. diminuta eggs. The picture on the left was taken at a 40x magnification, and the picture on the right was taken at a 100x magnification.

Chronic Constriction Injury Surgery

A CCI surgery of the left sciatic nerve was used to generate neuropathic pain. Animals were exposed to isoflurane to induce anesthesia. The skin nearest to the sciatic nerve was shaved and treated with 70% ethanol, and the surgery was performed aseptically. The sciatic nerve was isolated and partially exposed, and two sterile chromic gut sutures (cuticular 4-0 WebGut, Patterson Veterinary, Devens, MA) were tied around the sciatic nerve. The knots were gently tied so that the sutures would not slide along the nerve. When the sutures were tied, the muscle was sewn together using silk suture (3-0 ETHICON PERMA-HAND, Patterson Veterinary, Devens, MA). Finally, staples were used to close the incision of the skin. All animals were monitored following the surgery, and they were returned to their home cages when they could move autonomously. Recovery was monitored daily for one week following CCI surgeries.

Mechanical Allodynia

Rats were given three habituation sessions to the testing environment for 60 minutes each prior to CCI surgeries. Testing was conducted prior to surgeries as a baseline, then every week after surgery, as well as the tenth day after surgery. On the days of testing, animals were habituated to the experimental setting for 20-30 minutes prior to testing. A set of 10 calibrated

Semmes-Weinstein monofiliments (von Frey hairs; Stoelting, Wood Dale, IL) were pressed perpendicularly to the right and left hind paws randomly for 6 second intervals (Chacur et al., 2001; P. M. Grace et al., 2016). This determined the threshold intensity of the filament that elicited a paw withdrawal. The logarithmic stiffness of the filaments was between 3.61 (0.40 g) to 5.18 (15.14 g). Paw withdrawal responses were used to generate absolute threshold values (50% probability of withdrawal) through a Gaussian integral psychometric function (Harvey, 1986; Treutwein & Strasburger, 1999) which could be analyzed statistically on the PsychoFit program (Milligan et al., 2001).

Fear Conditioning

Conditioning consisted of exposing rats to the conditioning context, which was a clear plastic chamber (26 L x 21 W x 24H, cm) with a mesh screen top and an open bottom. This chamber was placed inside an Igloo ice chest (54 L x 30 W x 27 H, cm) with a white interior that was turned on its side. The bottom of the chamber was placed over a removable set of stainless steel rods (1.5 mm in diameter, spaced 1.2 cm apart) connected to a power supply (Coulbourn Instruments, Allentown, PA). Rats were conditioned in pairs using two identical ice chests, conditioning chambers, and rods. Rats were allowed to freely explore the conditioning chamber for 2 minutes. Following this two-minute period, a 15-second tone was played through a speaker, then a 2-second, 1.5 mA shock was delivered to the animals from stainless steel rods beneath their feet. Each of the conditioning chambers was cleaned with water before animals were exposed to the chambers. Animals were transported between their home cages and conditioning chambers in a black bucket with a black lid (Barrientos et al., 2015).

Contextual memory of the rats was tested 72 hours later. First, rats were brought in pairs to the conditioning chamber in the same black bucket. Rats remained in this context, and freezing behavior was observed in order to measure contextual memory. Freezing behavior was used as a sign of associating the conditioning context with a fear-based memory of the shock. Rats were placed in the conditioning context for 6 minutes, and their freezing behavior (either freezing or active) was scored every 10 seconds. Freezing was characterized by complete absence of movement except shallow breathing. Following this 6-minute interval, rats were returned to their home cages in the black bucket.

Novel-context, tone freezing was tested approximately 2 hours after contextual memory was tested (3 days after conditioning). Rats were transferred from their home cages to a novel context in a silver, metal bucket with a metal lid. The novel context consisted of a green, round, plastic container (35.5 diameter x 26.5 H, cm) with a layer of bedding on the floor. Rats were placed in this novel context, and freezing was scored every 10 seconds for 3 minutes. Then, the tone came on for 3 minutes, and freezing behavior was scored every 10 seconds. The tone was the exact same as the tone from conditioning. Conditioning and scoring during all portions of fear conditioning tests were carried out by blind observers.

Juvenile Social Exploration (JSE)

Rats were placed in individual, standard plastic cages that were identical to their home cages with a wire lid and bedding. These cages were located in an experimental testing room. After 1 hour in the new cages, a juvenile rat was placed in the cage with the adult experimental rat. Rats were tested individually. A blind observer recorded the amount of time that the adult rat initiated interaction with the juvenile over a two-minute interval. This interaction was characterized by sniffing, pinning, and grooming of the juvenile rat by the adult (John P. Christianson et al., 2010; J.P. Christianson et al., 2008). This test was performed prior to CCI surgeries as a baseline and 6 weeks post-CCI surgeries.

Statistics

GraphPad Prism Version 8 (San Diego, CA) was used for all statistical analysis and graph creation. For mechanical allodynia, absolute threshold values were calculated using a one-way ANOVA followed by Tukey's post hoc test, which verified that there were no baseline differences between groups. Group differences after CCI surgeries were determined using a two-way ANOVA and Sidak's post hoc test. Data is represented as mean ± SEM. Fear conditioning and JSE data were analyzed using repeated measures ANOVAs, and statistical significance was set at $p < 0.05$.

Results

Experiment 1 provided promising data regarding improvement in mechanical allodynia as well as contextual fear conditioning for helminth-treated animals; no significant improvement was seen in juvenile social exploration. The Maier Watkins Laboratory has previously shown that CCI surgeries induce neuropathic pain and mechanical allodynia (P. M. Grace et al., 2018). Animals were colonized with *H. diminuta* or administered saline (vehicle) 6 weeks before CCI surgeries. Relative levels of allodynia were assessed before CCI surgeries as a baseline and weekly following CCI. Testing was conducted until 13 weeks after CCI surgeries, and there were 7 animals per group. Colonization with *H. diminuta* attenuated both severity and longevity of mechanical allodynia (Figure 3). Chronic pain resolved in helminth-treated animals in approximately 13 weeks.

It has been shown that CCI surgeries decrease contextual memory compared to rats that receive a sham surgery, in which the sciatic nerve is exposed but no sutures are tied (Qian et al., 2018). Significant differences were observed between helminth-treated and control animals in contextual fear conditioning but not in novel-context, tone freezing (Figure 4). Naïve rats showed a similar average level of freezing to that of the helminth-treated rats for contextual memory, which suggested that the memory impairment induced by CCI was counteracted by helminth immunoregulation. All Pavlovian fear conditioning tests were conducted 6 weeks after CCI surgeries. Contextual memory and tone freezing were assessed 72 hours after conditioning. Helminth colonization significantly improved context-based memory; however, all rats showed approximately equal levels of freezing in response to the tone.

JSE was conducted 6 weeks after CCI, which was the same week that fear conditioning occurred. No differences in interaction time with the juvenile rat were observed between helminth-treated and control animals (Figure 5). Naïve, control, and helminth-colonized animals interacted with the juvenile rat for equivalent amounts of time. This demonstrated that neither helminth colonization nor CCI surgeries had an impact on social interaction.

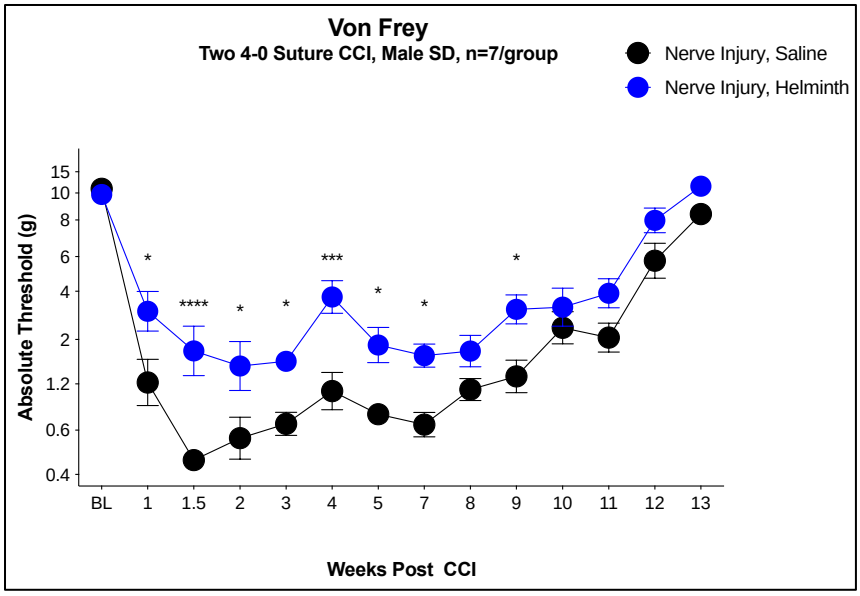

Figure 3: Rats were colonized with *H. diminuta* six weeks prior to CCI surgeries. *Von* Frey thresholds were measured after helminth colonization, but prior to CCI. Thresholds were evaluated at weekly intervals following CCI surgeries. Helminth-treated animals attenuated mechanical allodynia by comparison to control animals following CCI (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). Data are shown as mean ± SEM.

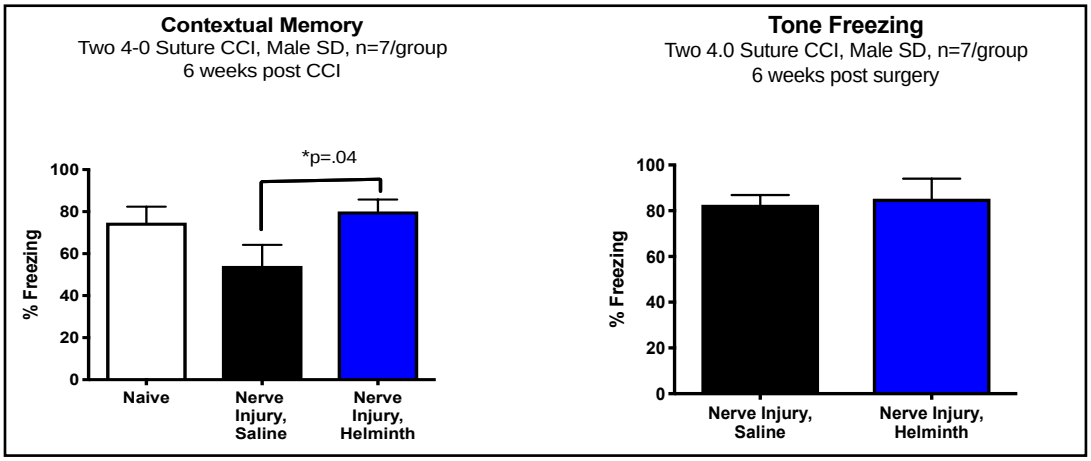

Figure 4: Rats underwent Pavlovian Fear Conditioning 12 weeks after colonization with *H. diminuta* and 6 weeks after CCI. Contextual memory and tone freezing were tested 4 days after conditioning. Helminth-colonized rats showed significant improvement in contexutal memory by comparison to control rats (*p = 0.04). Control rats showed impaired performance compared to naïve rats that did not receive CCI surgeries, verifying that the CCI treatment decreases hippocampal-dependent memory. Naïve rats did not undergo helminth colonization or CCI surgery. No significant difference between helminth-treated rats and control rats was displayed during the novel context, tone freezing test. Data are shown as mean ± SEM.

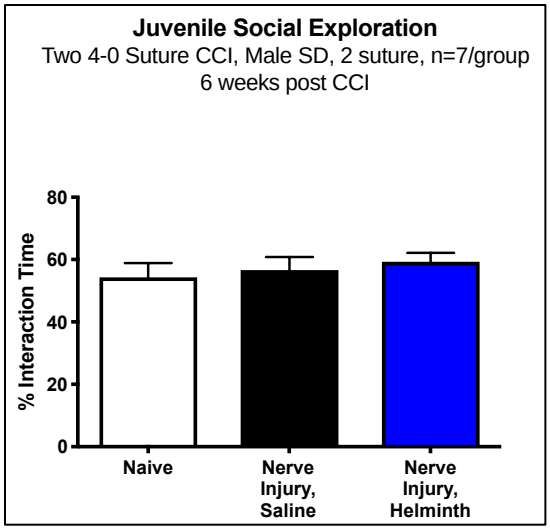

Figure 5: JSE was conducted 12 weeks after colonization with *H. diminuta* and 6 weeks after CCI. Naïve rats were not colonized, and they did not receive a CCI surgery. No significant group differences between naïve, control, or helminth-colonized rats were observed. Neither the CCI surgery nor helminth colonization produced effects in social interaction behavior. Data are shown as mean ± SEM.

Experiment 2: Two 4-0 Suture CCI Followed by Morphine Injections

Methods

HDC colonization, fecal float tests, CCI surgeries, mechanical allodynia measurements, fear conditioning, and JSE were performed as stated in Experiment 1. Additionally, morphine injections were administered 10 days after CCI surgeries.

Morphine Injections

Beginning 10 days after CCI surgeries, animals were given twice-daily sub cutaneous (s.c.) injections of morphine (5mg/kg) for 5 days. Morphine was gifted from the National Institute on Drug Abuse Drug Supply Program (NDSP) Division of Therapeutics and Medical Consequences, Research Triangle Institute, NC. It has been previously shown that introduction of morphine following a CCI surgery prolongs neuropathic pain for months after morphine exposure has stopped (P. Grace et al., 2019; P. M. Grace et al., 2018).

Results

The experimental design for this study was identical to Experiment 1 regarding colonization, CCI surgeries, and behavioral testing. In addition to these procedures, subcutaneous (s.c.) morphine injections were administered 10 days after CCI surgeries in order to determine whether helminth colonization could combat the combined effects of CCI and morphine injections on mechanical allodynia (Figure 6). There were 5 animals in each treatment group. No significant differences between any of the groups were observed. Unexpectedly, morphine injections did not worsen neuropathic pain, even within the groups of control rats. Additionally, the helminth-treated rats did not attenuate neuropathic pain, regardless of exposure to morphine.

There were also no group differences for contextual fear conditioning or tone freezing (Figure 7). Morphine injections did not produce significant group differences in contextual fear conditioning nor novel-context tone freezing tests. Fear conditioning was performed 6 weeks after CCI surgeries and 4 weeks after morphine injections ended. No significant differences were observed in JSE testing in any group (Figure 8). JSE was performed during the same week as fear conditioning. Neither helminth colonization nor morphine injections yielded significant behavioral results in any testing.

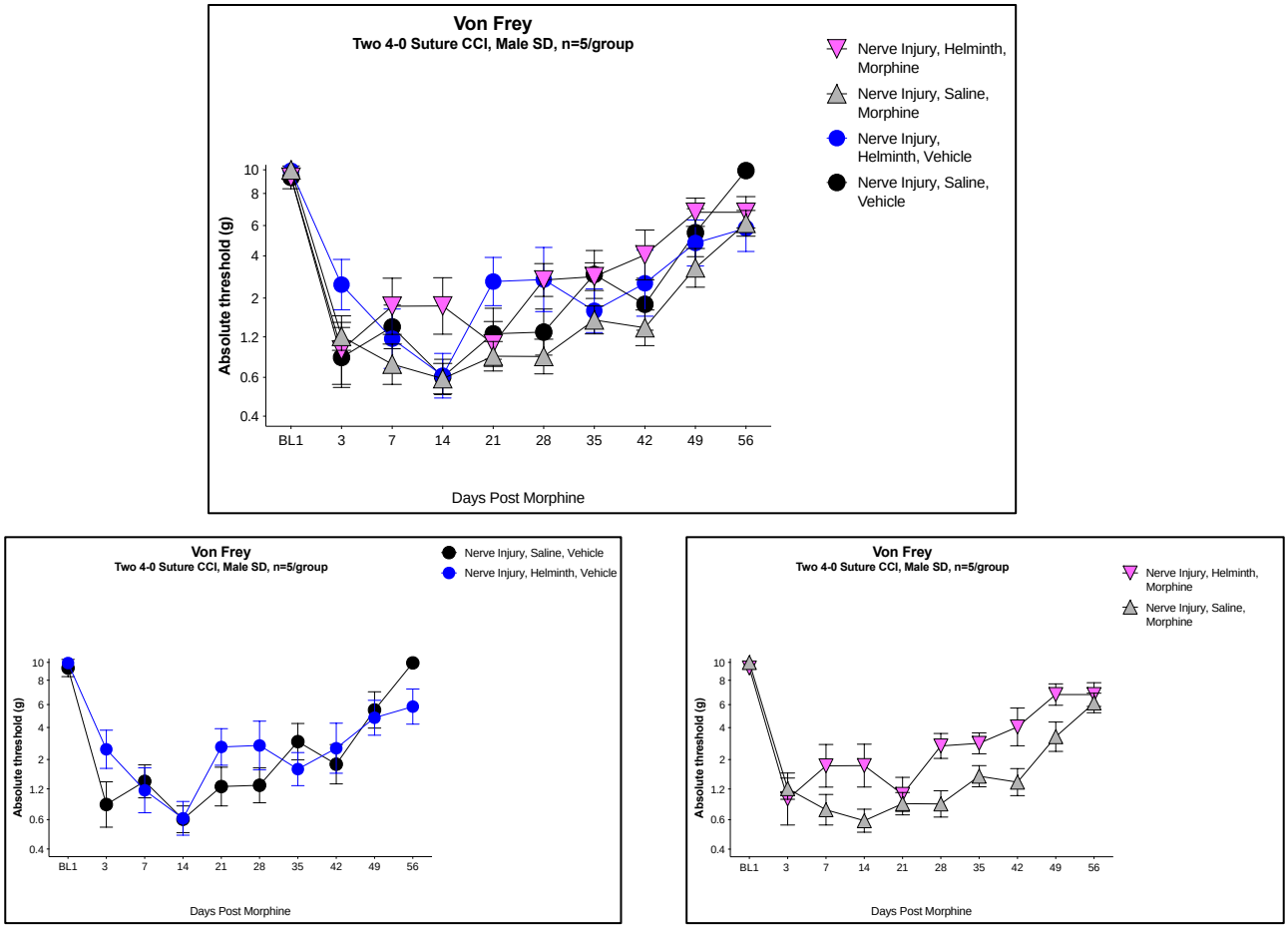

Figure 6: Von Frey testing for mechanical allodynia following CCI and s.c. morphine injections. The baseline test occurred prior to CCI surgeries. A second baseline was taken 9 days after CCI surgeries, which was 1 day prior to morphine injections. Tests were conducted weekly after the 5 day timecourse of injections had finished. No significant differences were observed between any of the groups. Data are shown as mean ± SEM.

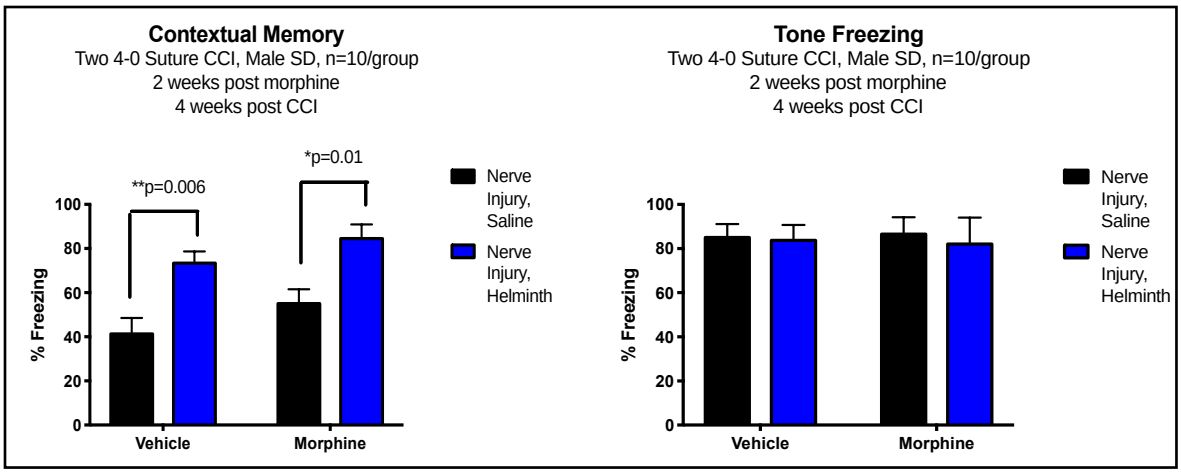

Figure 7: Rats underwent Pavlovian Fear Conditioning 10 weeks after colonization with *H. diminuta*, 4 weeks after CCI, and 2 weeks after morphine injections ended. Contextual memory and tone freezing were tested 4 days after conditioning. Within the group of rats that received saline injections, helminth-colonized rats showed significant improvement in contexutal memory by comparison to control rats (**p = 0.006). This was also true with the rats that received morphine injections (*p = 0.01). No significant difference between helminth-treated rats and control rats was displayed during the novel context, tone freezing test regarless of morphine administration. Data are shown as mean ± SEM.

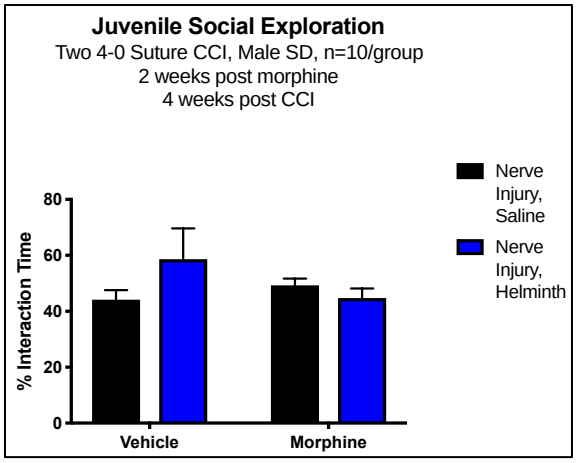

Figure 8: JSE was conducted 10 weeks after colonization with *H. diminuta*, 4 weeks after CCI, and 2 weeks after the morphine s.c. injection timecourse ended. The helminth-treated animals showed a significant increase in interaction time by comparisson to the control animals in the saline group (*P = 0.02). No significant difference was observed in the animals that were admistered morphine. Data are shown as mean ± SEM.

Experiment 3: One 6-0 Suture CCI

Methods

HDC colonization, fecal float tests, mechanical allodynia measurements, and JSE were performed in an identical manner to Experiment 1. CCI surgeries were also performed identically to Experiment 1, but instead of receiving a two suture CCI with 4-0 WebGut suture, these rats received a one suture CCI with 6-0 WebGut suture. A single 6-0 suture induces a lower level of neuropathic pain than two 4-0 sutures. Conditioning and contextual memory tests were completed as they were in Experiment 1; however, no novel context tone-freezing tests were conducted.

Results

To investigate whether helminth immunoregulation was able to attenuate neuropathic pain from a less intense chronic pain model than the two 4-0 suture CCI, pain was induced through a one 6-0 suture CCI. There were 10 rats in each treatment group. No significant differences in relative levels of mechanical allodynia between helminth-colonized and control animals were observed after CCI (Figure 9). Baseline tests were completed before helminth colonization and immediately before CCI surgeries to assure that there was no group difference prior to the induction of neuropathic pain.

No differences in contextual fear conditioning (Figure 10) or JSE (Figure 11) were observed during this experiment. The tone freezing test was not performed for this experiment because no group differences were seen in previous experiments. Fear conditioning was performed 3 weeks after CCI surgeries, and JSE was performed 4 weeks after CCI surgeries. Even with less neuropathic pain, helminth immunoregulation did not attenuate pain or improve contextual memory or social interaction compared to control rats.

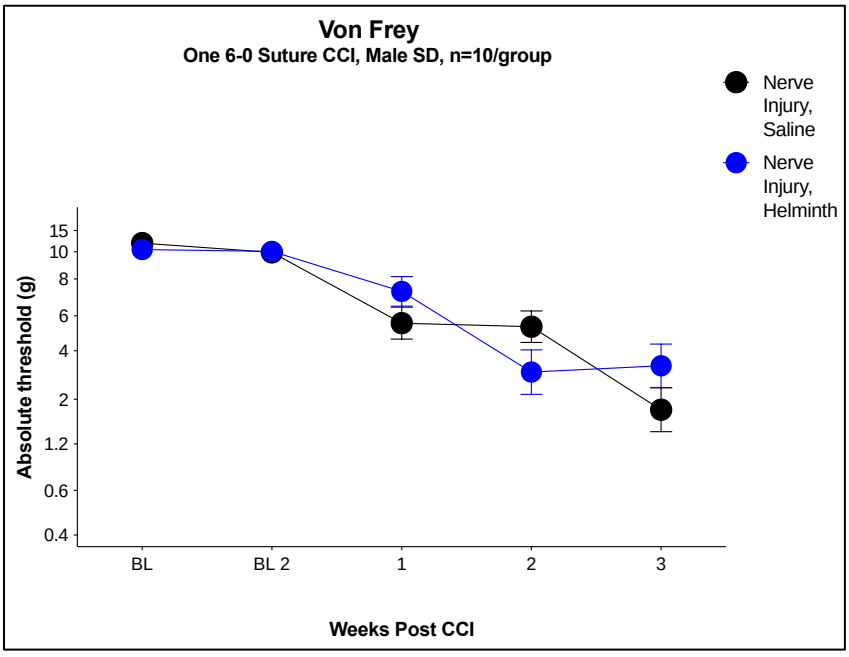

Figure 9: Von Frey testing for mechanical allodynia following a one 6-0 suture CCI. One baseline tests occurred prior to helminth colonization and the second baseline test occurred prior to CCI surgeries. Tests were conducted weekly following CCI. No significant differences were observed between helminth treated and control animals. Data are shown as mean ± SEM.

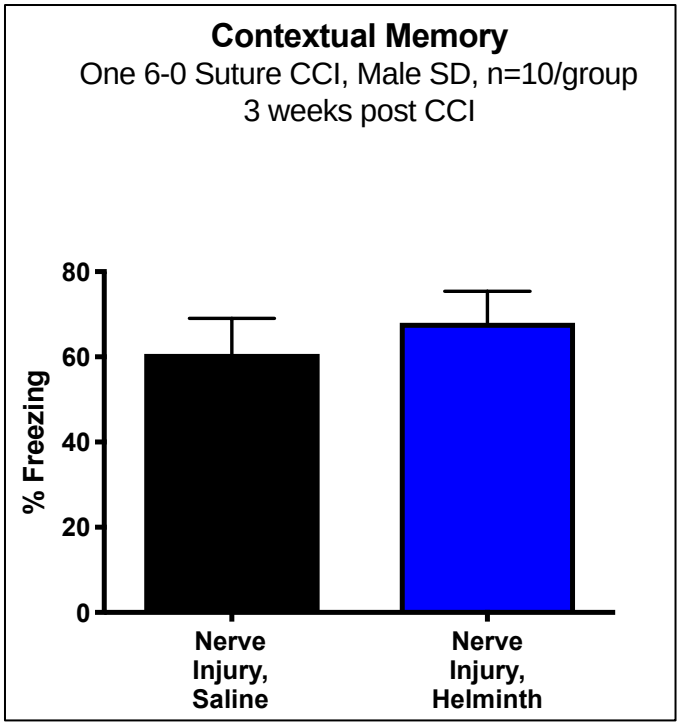

Figure 10: Rats underwent Pavlovian Fear Conditioning 9 weeks after colonization with *H. diminuta* and 3 weeks after CCI. Contextual memory was tested 4 days after conditioning. No significant difference in contextual memory between helminth-treated rats and control rats was displayed. Data are shown as mean ± SEM.

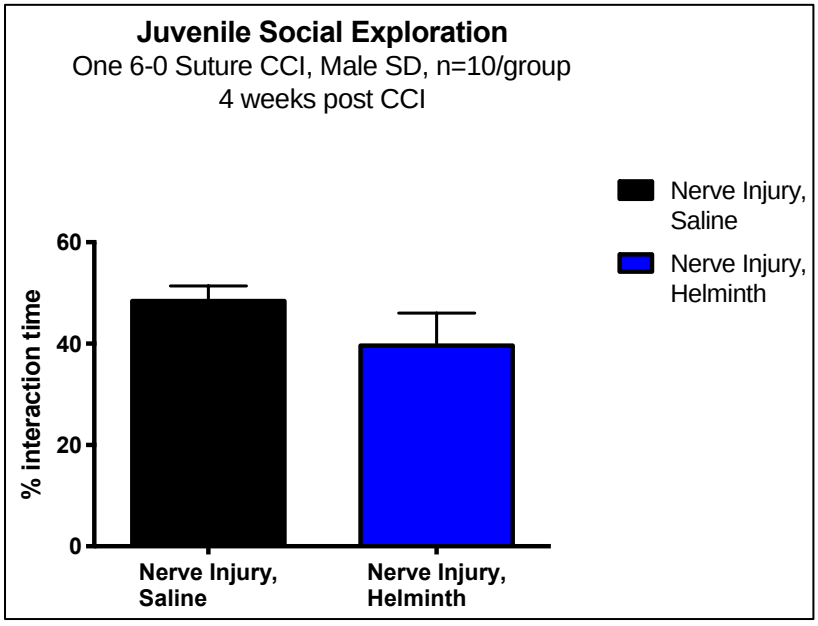

Figure 11: JSE was conducted 10 weeks after colonization with *H. diminuta* and 4 weeks after CCI. No significant differences between helminth-colonized or control animals were observed. Data are shown as mean ± SEM.

Experiment 4: Two 4-0 Suture CCI Following Voluntary Wheel Running

Methods

HDC colonization, fecal float tests, mechanical allodynia measurements, CCI surgeries, and JSE were performed in an identical manner to Experiment 1. Conditioning and contextual memory tests were completed as they were in Experiment 1; however, no novel context tone-freezing tests were conducted. Additionally, half of the rats in each group (helminth-colonized and control) were allowed to run on wheels in their cages for the 6 weeks following colonization.

VWR

Rats were housed individually with wheels in their cages, and control animals were kept single-housed in cages without wheels. Rats with wheels had unlimited ability to run voluntarily for 6 weeks. This time period coincided with the 6 weeks that the *H. diminuta* eggs matured into worms. Wheel revolutions were collected through Vital View software (Mini Mitter, Bend, OR), and this information was used to determine the distance traveled. It has been previously shown that voluntary exercise prior to a CCI surgery attenuates a pro-inflammatory response and neuropathic pain (P. M. Grace et al., 2016; Speaker et al., 2014).

Results

Voluntary wheel running and helminth colonization were both used as treatments to determine whether they were able to decrease neuropathic pain development and increase contextual memory and social interaction. There were 10 animals in each treatment group, for a total of 40 rats in this study. There were no significant effects between any of the groups in Von Frey testing (Figure12), contextual fear conditioning (Figure 13), or JSE (Figure 14). Even in the control groups, VWR did not attenuate pain, which was unexpected, since this treatment had been verified in previous studies. Additionally, helminth treatment did not improve neuropathic pain, contextual memory, or JSE behavior, regardless of access to running wheels.

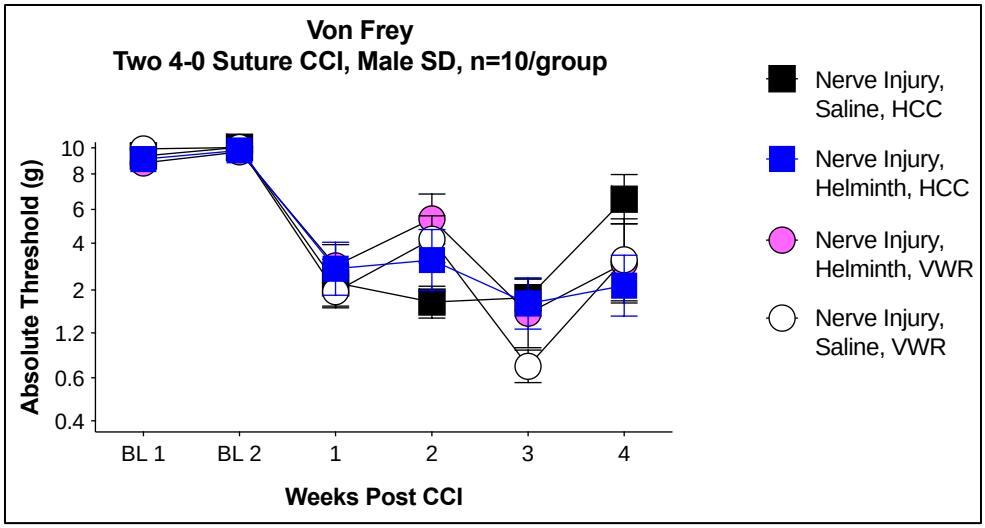

Figure 12: Von Frey testing for mechanical allodynia following a two 4-0 suture CCI. The first baseline test occurred prior to voluntary wheel-running, and the second baseline occurred the day before CCI surgeries. Tests were conducted weekly following CCI. No significant differences were observed between any of the groups. Neither helminth colonization nor VWR attenuated mechanical allodynia. Data are shown as mean ± SEM.

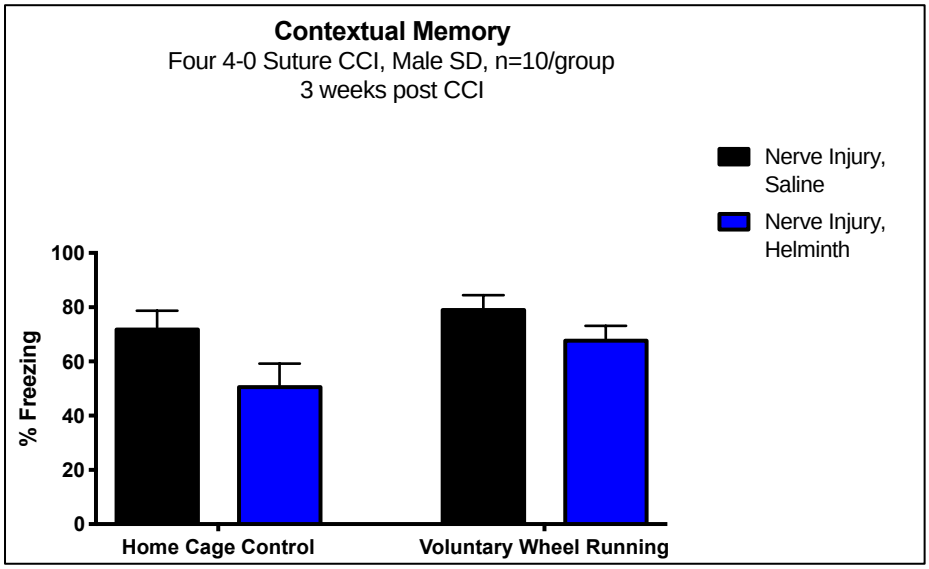

Figure13: Rats underwent Pavlovian Fear Conditioning 9 weeks after colonization with *H. diminuta*, 3 weeks after the 6-week VWR timecourse ended, and 3 weeks after CCI. Contextual memory and was tested 4 days after conditioning. No significant differences in contextual memory between any of the groups were observed. Data are shown as mean ± SEM.

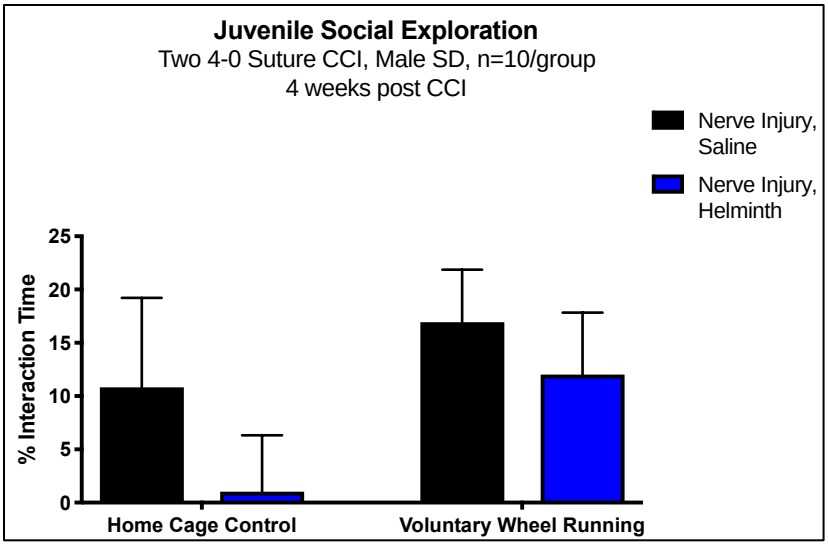

Figure 14: JSE was conducted 10 weeks after colonization with *H. diminuta* and 4 weeks after CCI. No significant group differences any groups were observed. Data are shown as mean ± SEM.

Experiment 5: Four 4-0 Suture CCI

Methods

HDC colonization, fecal float tests, and mechanical allodynia measurements were performed in an identical manner to Experiment 1. CCI surgeries were also performed identically to Experiment 1, but instead of receiving a two suture CCI with 4-0 WebGut suture, these rats received a four suture CCI with 4-0 WebGut suture. Conditioning and contextual memory tests were completed as they were in Experiment 1; however, no novel context tone-freezing tests were conducted. JSE was not conducted during this experiment.

Results

The aim of this experiment was to investigate whether a helminth treatment attenuates pain and improves contextual memory and social interaction behavior following the induction of a pain model with four 4-0 sutures, which was more intense than the two 4-0 suture CCI that was used in Experiment 1. There were 10 animals in each treatment group. No significant effects were observed for Von Frey Testing (Figure 15) or contextual memory (Figure 16). The helminth-colonized rats showed slightly higher attenuation of allodynia and slightly improved

contextual memory; however, none of these results were statistically significant. JSE was not performed for this experiment, because no significant effects were observed in previous experiments.

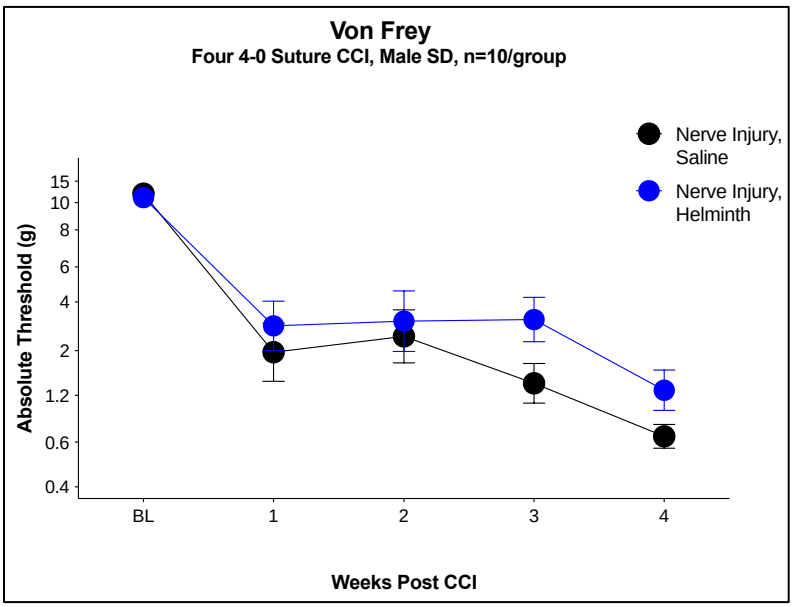

Figure 15: Von Frey testing for mechanical allodynia following a two 4-0 suture CCI. The baseline test occurred prior to CCI surgeries. Tests were conducted weekly following CCI. No significant differences were observed between helminth-treated and control animals. Data are shown as mean ± SEM.

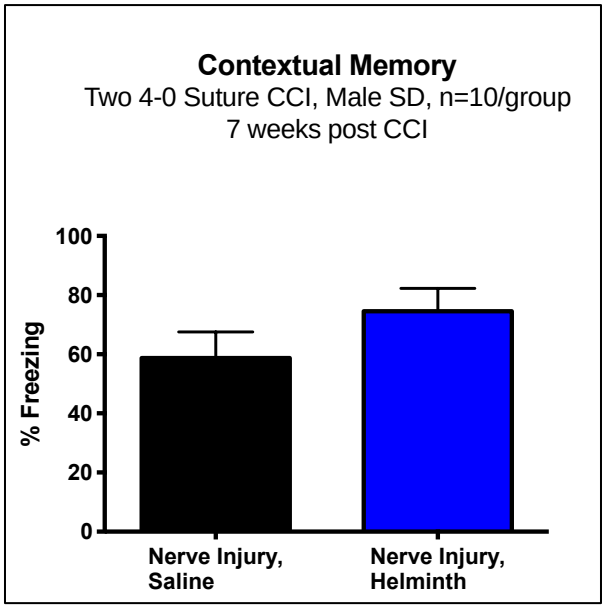

Figure 16: Rats underwent Pavlovian Fear Conditioning 13 weeks after colonization with *H. diminuta* and 7 weeks after CCI. Contextual memory and was tested 4 days after conditioning. No significant difference in contextual memory between helminth-treated and control animals was observed. Data are shown as mean ± SEM.

Experiment 6: Two 4-0 Suture CCI, Females

Methods

HDC colonization, fecal float tests, CCI surgeries, and mechanical allodynia measurements were performed in an identical manner to Experiment 1. Conditioning and contextual memory tests were completed as they were in Experiment 1; however, no novel context, tone freezing tests were conducted. JSE was not conducted during this experiment. Additionally, female Sprague Dawley rats were the subjects of this experiment, instead of male rats. Females were the subjects because it has been shown that females have a differential balance of T cells; they have a greater number of infiltrating T_{reg} cells than males (Fernandes et al., 2018; Tipton, Baban, & Sullivan, 2012). With a greater number of T_{reg} cells infiltrating the spinal cord, the upregulation of T_{reg} cells from helminth colonization may have a greater influence on neuropathic pain in females than male.

Results

Female Sprague Dawley rats were the subjects of this experiment, which was designed to investigate the effects of sex differences on behavior following helminth immunoregulation and CCI surgeries. There was no significant difference between groups during mechanical allodynia testing (Figure 17). However, the control animals showed significant improvement in contextual memory comparted to the helminth-treated group (Figure 18). This was the reverse effect of Experiment 1, and this could potentially be due to the abnormal behavior of the female rats during this task. They froze an abnormally large percentage of the time, so freezing behavior may not be an accurate representation of memory in this case. JSE was not performed, since no significant effects were seen in previous experiments.

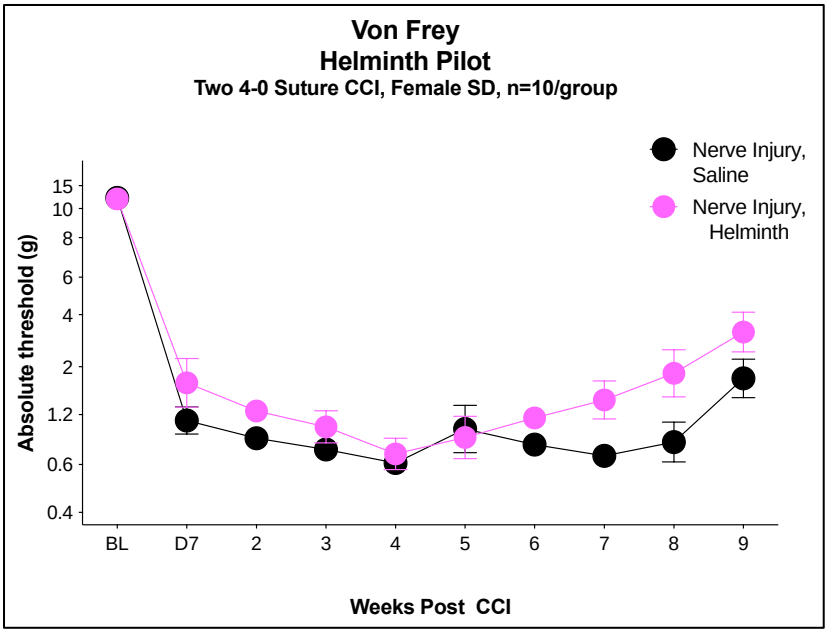

Figure 17: Von Frey testing for mechanical allodynia following a two 4-0 suture CCI in female animals. The baseline test occurred prior to CCI surgeries. Tests were conducted weekly following CCI. No significant differences were observed between helminth-treated and control animals. Data are shown as mean ± SEM.

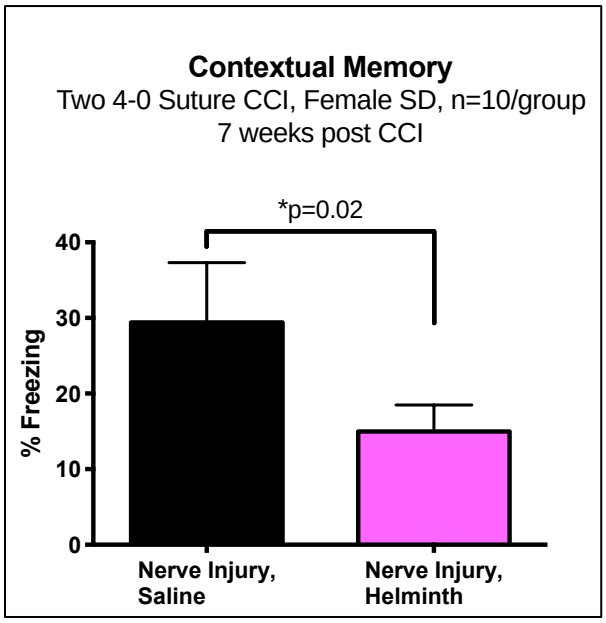

Figure 18: Rats underwent Pavlovian Fear Conditioning 9 weeks after colonization with *H. diminuta* and 7 weeks after CCI. Contextual memory and was tested 4 days after conditioning. No significant differences in contextual memory between helminth-treated and control animals was observed. Data are shown as mean ± SEM.

Experiment 7: Analysis of Hippocampus mRNA

Methods

HDC colonization and fecal float tests were conducted as stated in Experiment 1.

Hippocampus Extraction

Six weeks after colonization with *H. diminuta* eggs, rats were euthanized using i.p. 65 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA). Transcardial perfusion was performed using cold 0.9% saline. Following decapitation, the brain was removed, and the whole hippocampus was isolated. The hippocampus was flash frozen in liquid nitrogen and stored at -80˚C.

Total RNA Extraction

The phenol:chloroform extraction method was used to isolate samples of total RNA from hippocampus tissue (Chomczynski & Sacchi, 1987). Tissue was homogenized in 800µL of Trizol reagent (Invitrogen, Carlsbad, CA) for 30 seconds. Following homogenization, samples were incubated at room temperature for 5 minutes. Then, 160µL of chloroform was added, and the samples were vortexed for 2 minutes. Centrifugation (11,900 x g) was performed for 15 minutes at 4˚C. The supernatant was removed from the tube without disrupting the nucleic acid phase in the bottom of the tube, and 8µL of Glycogen (Invitrogen) was added to the tube, and the sample was vortexed to allow the glycogen to bind RNA. 400µL of isopropyl alcohol was added to allow the RNA to precipitate, and the samples were vortexed and incubated for 10 minutes at room temperature. Samples underwent centrifugation (11,900 x g) for 10 minutes at 4˚C, then the RNA pellet was washed twice using 1mL of 75% ethanol and centrifugation (7,500 x g) for 5 minutes at 4˚C. The sample was allowed to air dry until the ethanol had evaporated, then the pellet was resuspended in 11µL of nuclease-free water. The total RNA sample was stored at -80˚C.

cDNA synthesis

SuperScript II First Strand Synthesis System for RT-PCR (Invitrogen) was used to reverse transcribe total RNA into cDNA. RNA samples were transferred to 1mL microcentrifuge tubes, and 1µL of random hexamer primers (5ng/µL) and 1µL dNTPs (mM) were added. Samples were placed in the iCycler, where they were incubated at 65˚C for 5 minutes. Then, samples were placed on ice, and 4µL of RT 5X first strand buffer and 2µL DTT (0.1M) were added to the samples. Samples were incubated at 25˚C for 2 minutes. Lastly, 1µL of SuperScript II RT (200U/µL) was added to the sample, and the reverse transcription reaction occurred through a series of incubations (25˚C for 10 minutes, 42˚C for 50 minutes, then 70˚C for 15 minutes). The incubation at 70˚C inactivated the reaction. The samples were removed from the iCycler and stored at -20˚C.

Quantitative Real-Time Polymerase Chain Reaction (aRT-tPCR)

The Quantitect SYBR Green PCR Kit (Quiagen, Valencia, CA) was used for PCR amplification of cDNA. Each well of the plate contained 25µL of mastermix (13µL SYBR, 1µL forward primers, 1µL reverse primers, 10µL H_2O, and 1µL of cDNA sample). Samples of cDNA were vortexed before they were added to the wells. Reactions were run in duplicate using 96-well plates, and PCR products were measured with the MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA). The PCR conditions were 95˚ for 15minutes, 94˚ for 15 seconds, 57˚ for 30 seconds, 72˚ for 30 seconds, and 65˚ for 30 seconds. There were 40 cycles of the 94˚, 57˚, and 72˚ steps before progressing to the 65˚ step. The relative gene expression was quantified using the $2^{-\Delta\Delta}$CT method (Livak & Schmittgen, 2001). The average CT of the internal control, β-actin, was subtracted from the average CT of the experimental CT values for the genes of interest (ΔCT). Then, the highest ΔCT of each gene was used as a calibrator, and this value was subtracted from the mean ΔCT of experimental values (ΔΔCT). The $2^{-\Delta\Delta}$CT method gives the fold change of gene expression normalized to the housekeeping gene (β-actin) expression. Protocols for total RNA isolation, cDNA synthesis, and qRT-PCR can be found in previous publications (Frank, Fonken, Dolzani, et al., 2018).

Tissue Preparation and Bradford Assay

An extraction buffer solution (Invitrogen) and protease inhibitors (Sigma) were added to hippocampal tissue, and the samples were sonicated. Samples were centrifuged for 10 minutes at 14,000 x g and 4˚C, and the supernatants were pipetted out and placed in new tubes (Frank, Fonken, Annis, Watkins, & Maier, 2018). A Bradford assay was performed in order to analyze total protein concentration in each sample. Coomassie Brilliant Blue G-250 was added to protein samples and after 2 minutes, the absorbance of the samples at 595nm was measured (Bradford, 1976) with a spectrophotometer. Absorbance data was used to determine the relative protein concentration of each sample.

Enzyme-Linked Immunosorbent Assay (ELISA)

A rat IL-10 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) was used to determine the protein levels of IL-10 in whole hippocampus tissue (García-Miguel et al., 2018). Samples of 50µL of each protein sonicate were added to wells in the multiplex plate, and the plate was incubated for 2 hours at room temperature. 100µL of conjugate were added to the wells, and the plate was allowed to incubate for 2 more hours at room temperature. Then, 100µL of the substrate solution were added to each well and the plate was allowed to incubate for 30 minutes at room temperature. During this incubation, all light was blocked from the plate. Finally, the reaction was stopped by adding 100µL of the stop solution to each well, and absorbance of each sample was measured using a spectrophotometer set to 450nm. Absorbance data was used to determine levels of IL-10 protein in each sample. Data are not shown because the levels of IL-10 protein were too low to be detected.

Table 1: PCR Primer Specifications

All primer sequences were designed by Matthew Frank at the University of Colorado, Boulder.

Gene	Primer Sequence (5'-3')
B-actin	F: AGAGGCATCCTGACCCTGAA R: GCTCATTGTAGAAAGTGTGGT
IL-10	F: TTACCTGCAGTCCTGGTCTT R: TGCAGGTCTGAGTCTTCAG
IL-10R	F: TTACCTGCAGTCCTGGTCTT R: TGCAGGTCTGAGTCTTTCAG
IL-4	F: GAACTCACTGAGAAGCTGCA R: GAAGTGCAGGACTGCAAGTA
IL-4R	F: TGGTACAACCACTTCCAGCT R: TGCTGAAGTAACAGGTCAGG
IL-13	F: AGACCAGAAGACTTCCCTGT R: TCAATATCCTCTGGGTCCTG
TGF-β	F: TACTGCTTCAGCTCCACAGA R: TGTCCAGGCTCCAAATGTAG

Results

The purpose of this experiment was to determine whether colonization with *H. diminuta* altered the cytokine expression in the brain. Relative levels of mRNA expression were measured using qRT-PCR. There were 8 animals in each group. Figure 19 shows mRNA expression of IL-10, IL-10 receptor, IL-13, TGF-β, IL-4 and IL-4 receptor. Levels of IL-10 mRNA were significantly increased ($P < 0.0001$), while levels of the IL-10 receptor were significantly decreased ($P < 0.02$). Upregulation of IL-10 expression contributes to an anti-inflammatory cytokine milieu, and the downregulation of IL-10R expression may be reflective of an increased release of IL-10. There was also a significant decrease in levels of IL-13 mRNA ($P < 0.0001$). Though IL-13 is typically an anti-inflammatory cytokine, it has also been linked to airway inflammation, which is characteristic in asthma, and *H. diminuta* have been known to decrease the risk of asthma. Levels of TGF-β, IL-4 and IL-4R mRNA showed no significant differences between helminth-colonized and control animals. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.

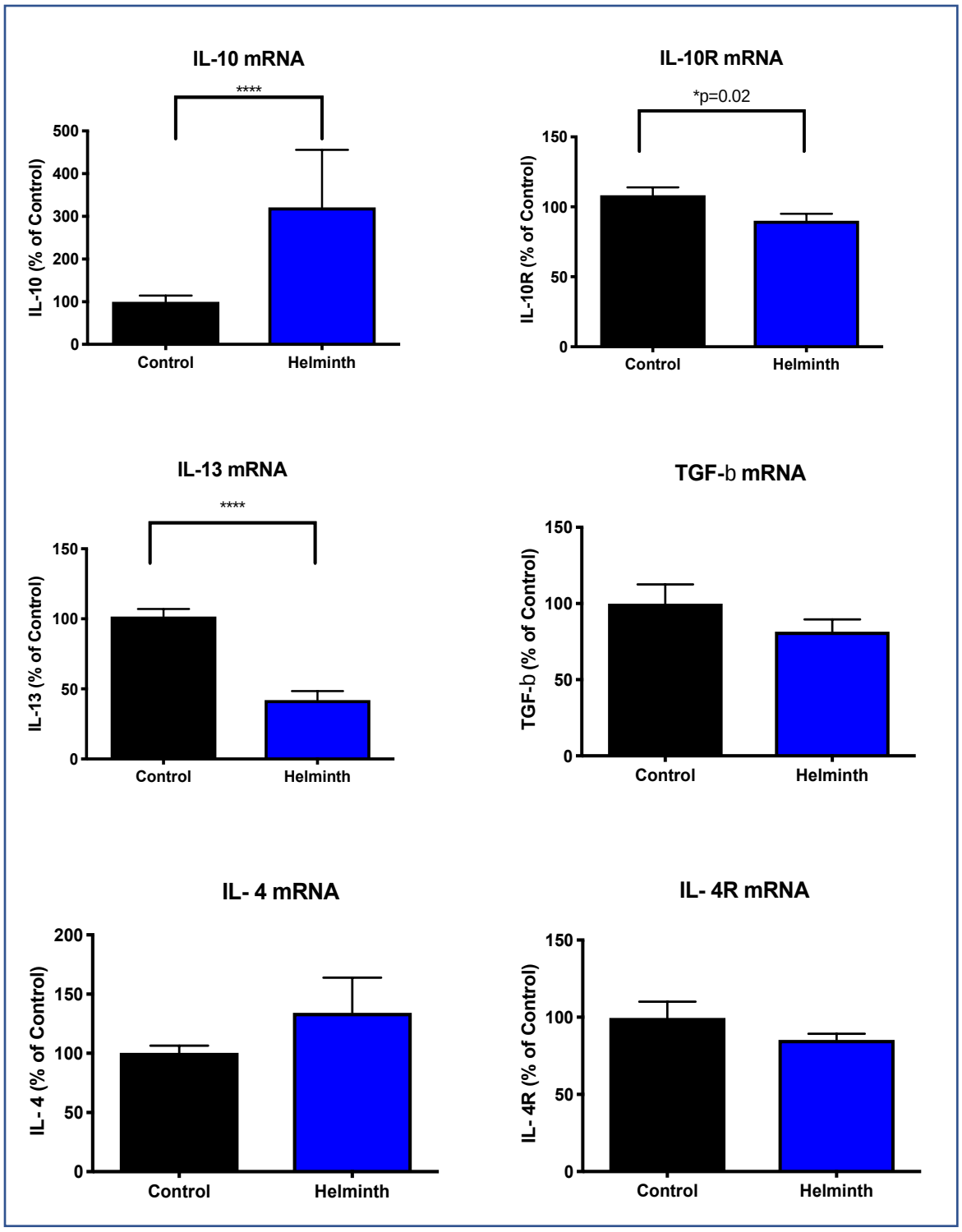

Figure 19: All qRT-PCR was performed on hippocampus tissue. IL-10 mRNA expression increased significantly in helminth-treated animals (****P < 0.0001). IL-10R (P = 0.02) and IL-13 (P < 0.0001) mRNA decreased significantly in helminth-treated animals compared to control animals. There were no significant differences in IL-4, IL-4R, and TGF-β mRNA levels between groups (* P < 0.05, ****P < 0.0001). Real time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Discussion

These current studies suggest that *H. diminuta* may not be an effective therapy for chronic pain; however, they are a promising treatment for neuroinflammation in the brain. The results from Von Frey, Pavlovian fear conditioning, and JSE behavioral tests following CCI surgeries are inconclusive. Each of the animals experienced mechanical allodynia as expected; however, only the first experiment showed significant differences between helminth-colonized and control groups. In all other experiments, helminth-colonized groups and control groups mirrored each other, which indicates that helminth immunoregulation is not effective for alleviating chronic pain from a peripheral nerve injury. Regardless of the type and number of sutures that were used for CCI surgeries, no consistent group differences were observed. The two 4-0 suture CCI (Figure 3, Figure 6, Figure 12, Figure 17), four 4-0 suture CCI (Figure 15), and one 6-0 suture CCI (Figure 9) demonstrated approximately equal intensity and longevity of mechanical allodynia between helminth-colonized and control animals throughout all studies after Experiment 1. Interestingly, animals that received morphine did not experience prolonged mechanical allodynia following CCI (Figure 6), even though this had been observed in previous studies (P. Grace et al., 2019). This trend was true for both the helminth-colonized and control groups. Neuropathic pain for animals that received saline injections resolved slightly faster compared to the animals that received morphine injections, but this was not a significant effect. Additionally, mechanical allodynia did not seem to be attenuated by voluntary wheel running (Figure 12). There was no consistent group separation during this study, so Von Frey testing was terminated four weeks after CCI surgeries. Because the known paradigms regarding the effects of morphine and VWR on chronic pain were not observed, replication studies could be useful to determine whether these treatments are producing the expected results according to previous studies. Female rats are known to have elevated levels of infiltrating T_{reg} cells (Fernandes et al., 2018; Tipton et al., 2012), so it was expected that increasing the number of activated T_{reg} cells would increase immunoregulation and decrease T cell-induced inflammation. However, there was no significant difference in chronic pain levels or longevity between helminth-colonized and control animals (Figure17).

Similar to Von Frey results, fear conditioning behavioral results were inconsistent and often showed no significant difference between treatment groups. The first two experiments

indicated that helminth-colonization improved contextual memory compared to control animals (Figure 4, Figure 7). For Experiment 2, helminth colonization improved contextual memory for animals that were administered saline injections, as well as the animals that received morphine injections, so helminth immunoregulation was able to reverse the behavioral memory impairment of neuroinflammation from CCI surgeries and morphine. All experiments following Experiment 2 did not show any significance between treatment groups for contextual memory tests. No experiments showed significant group differences for novel context tone-freezing tests because all animals froze to the tone, demonstrating that they had developed a long-term association between the tone stimulus and the foot shock (Figure 4, Figure 7). Juvenile social exploration tests never produced differential behavioral results between groups (Figure 5, Figure 8, Figure 11, Figure 14). Data represented in Figure 5 revealed that CCI surgeries had no affect on social interaction, and this could explain why increased immunoregulation from the helminth treatment would not impact JSE.

In order to explore the molecular basis of helminth immunoregulation, qRT-PCR was performed to quantify the relative cytokine mRNA expression in the hippocampus (Figure 19). *H. diminuta* treatment upregulated mRNA expression of IL-10 and downregulated IL-10R, and both of these contribute to an anti-inflammatory cytokine milieu. Levels of IL-10 protein were undetectable in all samples of whole hippocampus tissue. IL-13 mRNA was downregulated, and this was expected, because IL-13 is responsible for airway inflammation and asthma (Corren, 2013). Though IL-13 can also play a role in anti-inflammatory processes, helminths are known to promote immunoregulation, which prevents the development of asthma and allergies (Parfrey et al., 2017; Graham A. W. Rook, 2012). TGF-β, IL-4, and IL-4R mRNA levels were not significantly impacted by *H. diminuta* treatment. IL-4 has been implicated in improved hippocampal-dependent memory (Chen et al., 2004, p. 4; Derecki et al., 2010). There was no consistent improvement in learning and memory behavior, and there was no significant upregulation of IL-4 or downregulation of its receptor in the hippocampus, so both behavioral results and molecular analysis support that *H. diminuta* colonization does not improve memory.

Potential future directions for studying the effects of *H. diminuta* colonization include further analysis of cytokine expression in the brain and investigation of the behavioral effects of helminths in relation to stress and anxiety. Protein levels of cytokines in the hippocampus should

be analyzed to assure that changes in mRNA expression truly drive changes in protein expression. Differential mRNA expression within the dorsal and ventral hippocampus should be analyzed to determine whether each portion of the hippocampus is impacted by helminth immunoregulation. The ventral hippocampus is responsible for stress and emotion while the dorsal hippocampus is connected to cognition (Fanselow & Dong, 2010), so knowledge of cytokine expression in each portion of the hippocampus could guide further behavioral studies toward treatment of stress and anxiety disorders and/or cognitive disorders. Analysis of mRNA and protein levels in the amygdala would be beneficial, because the amygdala plays a role in the development of neuropsychiatric disorders (Schumann, Bauman, & Amaral, 2011), and *H. diminuta* treatments are known to improve symptoms of neuropsychiatric disorders (Kou & Parker, 2018). If differences in cytokine expression are seen in the amygdala and ventral hippocampus, behavioral tests should be administered to determine the effects of *H. diminuta* colonization on anxiety and depression. An inescapable tailshock method could be used to induce learned helplessness and depressive symptoms in rats (Maier & Seligman, 2016), and behavioral tests could be utilized to determine behavioral effects of helminth treatment. Fear conditioning could be performed to determine the amount of time that it takes for extinction of the conditioned fear because this behavioral test targets the amygdala (Barad, Gean, & Lutz, 2006), while contextual fear conditioning targets the hippocampus. JSE could also be performed following inescapable tailshock to determine whether helminth immunoregulation is able to improve social interaction after induced stress, which is known to impair social interaction (Helmreich et al., 2012).

In examining the effectiveness of *H. diminuta* as a treatment for neuroinflammation, the clinical concerns of helminthic therapy must be considered. The immunosuppressive state that is elicited by helminth treatment has been linked to risks of carcinogenesis (Grange et al., 2008; Pastille et al., 2017; Zambirinis, Pushalkar, Saxena, & Miller, 2014). The process of immunosurveillance involves the circulation of immune cells that search for and destroy harmful pathogens, and it is responsible for the destruction of tumor cells (Nicolazzo, Gradilone, Carpino, Gazzaniga, & Raimondi, 2019). Helminth-induced activation of T_{reg} cells causes decreased immunosurveillance due to the upregulation of anti-inflammatory cytokines (Hewitson et al., 2009). This immunosuppression can allow tumor cells to evade the immune system so that they can survive and proliferate (Pastille et al., 2017), and this mechanism mimics one of the

primary ways that tumor cells naturally evade the immune system. Tumor cells induce the activation of anti-inflammatory cytokines, such as IL-10, and this signal inhibits cytotoxic T cells, natural killer cells, and dendritic cells from destroying tumor cells (Feng et al., 2018; Mannino et al., 2015). However, chronic inflammation is often characteristic of tumor microenvironments, so helminth immunosuppression may be useful in cancer prevention and treatment (Diakos, Charles, McMillan, & Clarke, 2014; Nakamura & Smyth, 2017; Oikonomopoulou et al., 2014). Additionally, helminth colonization induces the polarization of alternatively activated (M2) tumor-associated macrophages in order to fight parasitic infection (Hallowell et al., 2017; Lee et al., 2015; C.Y. Liu et al., 2013). These M2 macrophages promote the epithelial-mesenchymal transition, which is major contributing factor in the development tumor growth and metastasis (C.Y. Liu et al., 2013; Yu, Chien, & Chang, 2016). Before any clinical research using *H. diminuta* colonization is possible, further investigation is necessary to determine whether exposure to these helminths can cause anti- or pro-carcinogenic effects. This information would be useful in creating immunotherapies that harness the beneficial aspects and avoid the negative consequences of *H. diminuta*.

The results from this project partially support the tenets of the hygiene hypothesis. Though behavioral results following CCI surgeries were inconclusive, molecular investigation of cytokine levels in the hippocampus showed promotion of an anti-inflammatory cytokine milieu due to the upregulation of IL-10 and downregulation of its receptor. These promising results guide future research toward investigation of cytokine levels in other brain regions, such as the amygdala. Additionally, the absence of consistent behavioral effects and presence of molecular changes in the brain suggest that the immunoregulation induced by *H. diminuta* may primarily influence the brain. This supports further research that investigates the effects of helminth colonization on neuropsychiatric disorders. Finally, the potential unintended effects of *H. diminuta* colonization, such as increased carcinogenesis, must be examined in order to avoid the side effect of tumor growth and metastasis when establishing helminth immunotherapy.

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References

Akkoc, T., Eifan, A. O., Ozdemir, C., Yazi, D., Yesil, O., Bahceciler, N. N., & Barlan, I. B. (2008). Mycobacterium vaccae Immunization to OVA Sensitized Pregnant BALB/c Mice Suppressed Placental and Postnatal IL-5 and Inducing IFN-γ Secretion. *Immunopharmacology and Immunotoxicology*, *30*(1), 1–11. https://doi.org/10.1080/08923970701812159

Austin, P. J., Kim, C. F., Perera, C. J., & Moalem-Taylor, G. (2012). Regulatory T cells attenuate neuropathic pain following peripheral nerve injury and experimental autoimmune neuritis. *Pain*, *153*(9), 1916–1931. https://doi.org/10.1016/j.pain.2012.06.005

Austin, P. J., & Moalem-Taylor, G. (2010). The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. *Journal of Neuroimmunology*, *229*(1–2), 26–50. https://doi.org/10.1016/j.jneuroim.2010.08.013

Barad, M., Gean, P.-W., & Lutz, B. (2006). The Role of the Amygdala in the Extinction of Conditioned Fear. *Biological Psychiatry*, *60*(4), 322–328. https://doi.org/10.1016/j.biopsych.2006.05.029

Barrientos, R. M., Thompson, V. M., Arnold, T. H., Frank, M. G., Watkins, L. R., & Maier, S. F. (2015). The role of hepatic & splenic macrophages in E. coli-induced memory impairments in aged rats. *Brain, Behavior, and Immunity*, *0*, 60–67. https://doi.org/10.1016/j.bbi.2014.07.004

Bień, J., Sałamatin, R., Sulima, A., Savijoki, K., Bruce Conn, D., Näreaho, A., & Młocicki, D. (2016). Mass spectrometry analysis of the excretory-secretory (E-S) products of the model cestode Hymenolepis diminut a reveals their immunogenic properties and the presence of new E-S proteins in cestodes. *Acta Parasitologica*, *61*(2), 429–442. https://doi.org/10.1515/ap-2016-0058

Bilbo, S. D., Wray, G. A., Perkins, S. E., & Parker, W. (2011). Reconstitution of the human biome as the most reasonable solution for epidemics of allergic and autoimmune diseases. *Medical Hypotheses*, *77*(4), 494–504. https://doi.org/10.1016/j.mehy.2011.06.019

Björkstén, B. (2009). The hygiene hypothesis: do we still believe in it? *Nestle Nutrition Workshop Series. Paediatric Programme*, *64*, 11–18; discussion 18-22, 251–257. https://doi.org/10.1159/000235780

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Chacur, M., Milligan, E. D., Gazda, L. S., Armstrong, C., Wang, H., Tracey, K. J., … Watkins, L. R. (2001). A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats. *Pain*, *94*(3), 231–244.

Chen, L., Grabowski, K. A., Xin, J., Coleman, J., Huang, Z., Espiritu, B., … Huang, H. (2004). IL-4 Induces Differentiation and Expansion of Th2 Cytokine-Producing Eosinophils. *The Journal of Immunology*, *172*(4), 2059–2066. https://doi.org/10.4049/jimmunol.172.4.2059

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, *162*(1), 156–159. https://doi.org/10.1006/abio.1987.9999

Christianson, John P., Ragole, T., Amat, J., Greenwood, B. N., Strong, P. V., Paul, E. D., … Maier, S. F. (2010). 5-hydroxytryptamine 2C receptors in the basolateral amygdala are involved in the expression of anxiety after uncontrollable traumatic stress. *Biological Psychiatry*, *67*(4), 339–345. https://doi.org/10.1016/j.biopsych.2009.09.011

Christianson, J.P., Benison, A. M., Jennings, J., Sandsmark, E. K., Amat, J., Kaufman, R. D., … Maier, S. F. (2008). The sensory insular cortex mediates the stress-buffering effects of safety signals but not behavioral control. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(50), 13703–13711. https://doi.org/10.1523/JNEUROSCI.4270-08.2008

Connor, L. M., Tang, S.-C., Camberis, M., Le Gros, G., & Ronchese, F. (2014). Helminth-conditioned dendritic cells prime CD4+ T cells to IL-4 production in vivo. *Journal of Immunology (Baltimore, Md.: 1950)*, *193*(6), 2709–2717. https://doi.org/10.4049/jimmunol.1400374

Corren, J. (2013). Role of interleukin-13 in asthma. *Current Allergy and Asthma Reports*, *13*(5), 415–420. https://doi.org/10.1007/s11882-013-0373-9

Cragg, J. J. (2018). Prevalence of chronic pain among individuals with neurological conditions. *Health Reports*, *29*(82), 8.

Dahlhamer, J. (2018). Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults — United States, 2016. *MMWR. Morbidity and Mortality Weekly Report*, *67*. https://doi.org/10.15585/mmwr.mm6736a2

Derecki, N. C., Cardani, A. N., Yang, C. H., Quinnies, K. M., Crihfield, A., Lynch, K. R., & Kipnis, J. (2010). Regulation of learning and memory by meningeal immunity: a key role for IL-4. *The Journal of Experimental Medicine*, *207*(5), 1067–1080. https://doi.org/10.1084/jem.20091419

Descalzi, G., Mitsi, V., Purushothaman, I., Gaspari, S., Avrampou, K., Loh, Y.-H. E., … Zachariou, V. (2017). Neuropathic pain promotes adaptive changes in gene expression in brain networks involved in stress and depression. *Science Signaling*, *10*(471). https://doi.org/10.1126/scisignal.aaj1549

Diakos, C. I., Charles, K. A., McMillan, D. C., & Clarke, S. J. (2014). Cancer-related inflammation and treatment effectiveness. *The Lancet. Oncology*, *15*(11), e493-503. https://doi.org/10.1016/S1470-2045(14)70263-3

Dias, P. M., & Banerjee, G. (2013). The role of Th17/IL-17 on eosinophilic inflammation. *Journal of Autoimmunity*, *40*, 9–20. https://doi.org/10.1016/j.jaut.2012.07.004

Dlugovitzky, D., Stanford, C., & Stanford, J. (2011). Immunological basis for the introduction of immunotherapy with Mycobacterium vaccae into the routine treatment of TB. *Immunotherapy*, *3*(4), 557–568. https://doi.org/10.2217/imt.11.6

Ege, M. J. (2017). The Hygiene Hypothesis in the Age of the Microbiome. *Annals of the American Thoracic Society*, *14*(Supplement_5), S348–S353. https://doi.org/10.1513/AnnalsATS.201702-139AW

Fairweather, D., & Cihakova, D. (2009). Alternatively activated macrophages in infection and autoimmunity. *Journal of Autoimmunity*, *33*(3–4), 222–230. https://doi.org/10.1016/j.jaut.2009.09.012

Fanselow, M. S., & Dong, H.-W. (2010). Are The Dorsal and Ventral Hippocampus functionally distinct structures? *Neuron*, *65*(1), 7. https://doi.org/10.1016/j.neuron.2009.11.031

Feng, L., Qi, Q., Wang, P., Chen, H., Chen, Z., Meng, Z., & Liu, L. (2018). Serum levels of IL-6, IL-8, and IL-10 are indicators of prognosis in pancreatic cancer. *The Journal of International Medical Research*, *46*(12), 5228–5236. https://doi.org/10.1177/0300060518800588

Fernandes, R., Garver, H., Harkema, J. R., Galligan, J. J., Fink, G. D., & Xu, H. (2018). Sex Differences in Renal Inflammation and Injury in High-Fat Diet-Fed Dahl Salt-Sensitive Rats. *Hypertension (Dallas, Tex.: 1979)*, *72*(5), e43–e52. https://doi.org/10.1161/HYPERTENSIONAHA.118.11485

Fernando, M. R., Reyes, J. L., Iannuzzi, J., Leung, G., & McKay, D. M. (2014). The Pro-Inflammatory Cytokine, Interleukin-6, Enhances the Polarization of Alternatively Activated Macrophages. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0094188

Frank, M. G., Fonken, L. K., Annis, J. L., Watkins, L. R., & Maier, S. F. (2018). Stress disinhibits microglia via down-regulation of CD200R: a mechanism of neuroinflammatory priming. *Brain, Behavior, and Immunity*, *69*, 62–73. https://doi.org/10.1016/j.bbi.2017.11.001

Frank, M. G., Fonken, L. K., Dolzani, S. D., Annis, J. L., Siebler, P. H., Schmidt, D., … Lowry, C. A. (2018). Immunization with Mycobacterium vaccae induces an anti-inflammatory milieu in the CNS: Attenuation of stress-induced microglial priming, alarmins and anxiety-like behavior. *Brain, Behavior, and Immunity*, *73*, 352–363. https://doi.org/10.1016/j.bbi.2018.05.020

Frank, M. G., Fonken, L. K., Watkins, L. R., Maier, S. F., & Lowry, C. A. (2018). Could Probiotics Be Used to Mitigate Neuroinflammation? *ACS Chemical Neuroscience*. https://doi.org/10.1021/acschemneuro.8b00386

García-Miguel, M., Riquelme, J. A., Norambuena-Soto, I., Morales, P. E., Sanhueza-Olivares, F., Nuñez-Soto, C., … Chiong, M. (2018). Autophagy mediates tumor necrosis factor-α-induced phenotype switching in vascular smooth muscle A7r5 cell line. *PLoS ONE*, *13*(5). https://doi.org/10.1371/journal.pone.0197210

Grace, P., Galer, E., Strand, K., Corrigan, K., Berkelhammer, D., Maier, S., & Watkins, L. (2019). Repeated Morphine Prolongs Postoperative Pain in Male Rats. *Anesthesia & Analgesia*, *128*(1), 161–167. https://doi.org/10.1213/ANE.0000000000003345

Grace, P. M., Fabisiak, T. J., Green-Fulgham, S. M., Anderson, N. D., Strand, K. A., Kwilasz, A. J., … Watkins, L. R. (2016). Prior voluntary wheel running attenuates neuropathic pain. *Pain*, *157*(9), 2012–2023. https://doi.org/10.1097/j.pain.0000000000000607

Grace, P. M., Hutchinson, M. R., Maier, S. F., & Watkins, L. R. (2014). Pathological pain and the neuroimmune interface. *Nature Reviews. Immunology*, *14*(4), 217–231. https://doi.org/10.1038/nri3621

Grace, P. M., Strand, K. A., Galer, E. L., Rice, K. C., Maier, S. F., & Watkins, L. R. (2018). Protraction of neuropathic pain by morphine is mediated by spinal damage associated molecular patterns (DAMPs) in male rats. *Brain, Behavior, and Immunity*, *72*, 45–50. https://doi.org/10.1016/j.bbi.2017.08.018

Grange, J. M., Bottasso, O., Stanford, C. A., & Stanford, J. L. (2008). The use of mycobacterial adjuvant-based agents for immunotherapy of cancer. *Vaccine*, *26*(39), 4984–4990. https://doi.org/10.1016/j.vaccine.2008.06.092

Gundra, U. M., Girgis, N. M., Ruckerl, D., Jenkins, S., Ward, L. N., Kurtz, Z. D., … Loke, P. (2014). Alternatively activated macrophages derived from monocytes and tissue macrophages are phenotypically and functionally distinct. *Blood*, *123*(20), e110–e122. https://doi.org/10.1182/blood-2013-08-520619

Hallowell, R. W., Collins, S. L., Craig, J. M., Zhang, Y., Oh, M., Illei, P. B., … Horton, M. R. (2017). mTORC2 signalling regulates M2 macrophage differentiation in response to helminth infection and adaptive thermogenesis. *Nature Communications*, *8*, 14208. https://doi.org/10.1038/ncomms14208

Harvey, L. O. (1986). Efficient estimation of sensory thresholds. *Behavior Research Methods, Instruments, & Computers*, *18*(6), 623–632. https://doi.org/10.3758/BF03201438

Helminth parasites – masters of regulation - Maizels - 2004 - Immunological Reviews - Wiley Online Library. (n.d.). Retrieved March 10, 2019, from https://onlinelibrary.wiley.com/doi/abs/10.1111/j.0105-2896.2004.00191.x

Helmreich, D. L., Tylee, D., Christianson, J. P., Kubala, K. H., Govindarajan, S. T., O'Neill, W. E., … Maier, S. F. (2012). Active behavioral coping alters the behavioral but not the endocrine response to stress. *Psychoneuroendocrinology*, *37*(12), 1941–1948. https://doi.org/10.1016/j.psyneuen.2012.04.005

Hernández-Pando, R., Aguilar, D., Orozco, H., Cortez, Y., Brunet, L. R., & Rook, G. A. (2008). Orally administered Mycobacterium vaccae modulates expression of immunoregulatory molecules in BALB/c mice with pulmonary tuberculosis. *Clinical and Vaccine Immunology: CVI*, *15*(11), 1730–1736. https://doi.org/10.1128/CVI.00286-08

Hewitson, J. P., Grainger, J. R., & Maizels, R. M. (2009). Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. *Molecular and Biochemical Parasitology*, *167*(1–9), 1–11. https://doi.org/10.1016/j.molbiopara.2009.04.008

Johnston, M. J. G., Wang, A., Catarino, M. E. D., Ball, L., Phan, V. C., MacDonald, J. A., & McKay, D. M. (2010). Extracts of the rat tapeworm, Hymenolepis diminuta, suppress macrophage activation in vitro and alleviate chemically induced colitis in mice. *Infection and Immunity*, *78*(3), 1364–1375. https://doi.org/10.1128/IAI.01349-08

Kidd, P. (2003). Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Alternative Medicine Review: A Journal of Clinical Therapeutic*, *8*(3), 223–246.

Kou, H. H., & Parker, W. (2018). Intestinal worms eating neuropsychiatric disorders? Apparently so. *Brain Research*, *1693*(Pt B), 218–221. https://doi.org/10.1016/j.brainres.2018.01.023

Kreider, T., Anthony, R. M., Urban, J. F., & Gause, W. C. (2007). Alternatively activated macrophages in helminth infections. *Current Opinion in Immunology*, *19*(4), 448–453. https://doi.org/10.1016/j.coi.2007.07.002

Lee, W. J., Tateya, S., Cheng, A. M., Rizzo-DeLeon, N., Wang, N. F., Handa, P., … Kim, F. (2015). M2 Macrophage Polarization Mediates Anti-inflammatory Effects of Endothelial Nitric Oxide Signaling. *Diabetes*, *64*(8), 2836–2846. https://doi.org/10.2337/db14-1668

Lees, J. G., Duffy, S. S., Perera, C. J., & Moalem-Taylor, G. (2015). Depletion of Foxp3+ regulatory T cells increases severity of mechanical allodynia and significantly alters systemic cytokine levels following peripheral nerve injury. *Cytokine*, *71*(2), 207–214. https://doi.org/10.1016/j.cyto.2014.10.028

Lightowlers, M. W., & Rickard, M. D. (1988). Excretory-secretory products of helminth parasites: effects on host immune responses. *Parasitology*, *96 Suppl*, S123-166.

Liu, A. H. (2015). Revisiting the hygiene hypothesis for allergy and asthma. *Journal of Allergy and Clinical Immunology*, *136*(4), 860–865. https://doi.org/10.1016/j.jaci.2015.08.012

Liu, C.-Y., Xu, J.-Y., Shi, X.-Y., Huang, W., Ruan, T.-Y., Xie, P., & Ding, J.-L. (2013). M2-polarized tumor-associated macrophages promoted epithelial–mesenchymal transition in pancreatic cancer cells,

partially through TLR4/IL-10 signaling pathway. *Laboratory Investigation*, *93*(7), 844–854. https://doi.org/10.1038/labinvest.2013.69

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Lopes, F., Shute, A. J., Wang, A., & McKay, D. M. (2018). A77 TAPEWORM PARASITE HYMENOLEPIS DIMINUTA PROTECTS YOUNG MICE FROM EXPERIMENTAL COLITIS BY A RECALL MEMORY RESPONSE WITH WORM ANTIGEN. *Journal of the Canadian Association of Gastroenterology*, *1*(suppl_2), 121–121. https://doi.org/10.1093/jcag/gwy009.077

Maier, S. F., & Seligman, M. E. P. (2016). Learned Helplessness at Fifty: Insights from Neuroscience. *Psychological Review*, *123*(4), 349–367. https://doi.org/10.1037/rev0000033

Makki, M., Shahbazi, F., Teimoori, S., Rokni, M., Abaei, M., Mobedi, I., … Mowlavi, G. (2011). Establishment of Hymenolepis diminuta Life Cycle to Provide Parasite Mass Production. *Iranian Journal of Parasitology*, *6*(2), 60–63.

Mannino, M. H., Zhu, Z., Xiao, H., Bai, Q., Wakefield, M. R., & Fang, Y. (2015). The paradoxical role of IL-10 in immunity and cancer. *Cancer Letters*, *367*(2), 103–107. https://doi.org/10.1016/j.canlet.2015.07.009

Matisz, C. E., Geuking, M. B., Lopes, F., Petri, B., Wang, A., Sharkey, K. A., & McKay, D. M. (2018). Helminth Antigen-Conditioned Dendritic Cells Generate Anti-Inflammatory Cd4 T Cells Independent of Antigen Presentation via Major Histocompatibility Complex Class II. *The American Journal of Pathology*, *188*(11), 2589–2604. https://doi.org/10.1016/j.ajpath.2018.07.008

McKenney, E. A., Williamson, L., Yoder, A. D., Rawls, J. F., Bilbo, S. D., & Parker, W. (2015). Alteration of the rat cecal microbiome during colonization with the helminth Hymenolepis diminuta. *Gut Microbes*, *6*(3), 182–193. https://doi.org/10.1080/19490976.2015.1047128

Melon, A., Wang, A., Phan, V. C., & McKay, D. M. (2010). Infection with Hymenolepis diminuta Is More Effective than Daily Corticosteroids in Blocking Chemically Induced Colitis in Mice. *Journal of Biomedicine & Biotechnology*. https://doi.org/10.1155/2010/384523

Milligan, E. D., O'Connor, K. A., Nguyen, K. T., Armstrong, C. B., Twining, C., Gaykema, R. P., … Watkins, L. R. (2001). Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *21*(8), 2808–2819.

Nakamura, K., & Smyth, M. J. (2017). Targeting cancer-related inflammation in the era of immunotherapy. *Immunology and Cell Biology*, *95*(4), 325–332. https://doi.org/10.1038/icb.2016.126

Nicolazzo, C., Gradilone, A., Carpino, G., Gazzaniga, P., & Raimondi, C. (2019). Molecular Characterization of Circulating Tumor Cells to Study Cancer Immunoevasion. *Methods in Molecular Biology (Clifton, N.J.)*, *1884*, 247–258. https://doi.org/10.1007/978-1-4939-8885-3_17

Oikonomopoulou, K., Brinc, D., Hadjisavvas, A., Christofi, G., Kyriacou, K., & Diamandis, E. P. (2014). The bifacial role of helminths in cancer: involvement of immune and non-immune mechanisms. *Critical Reviews in Clinical Laboratory Sciences*, *51*(3), 138–148. https://doi.org/10.3109/10408363.2014.886180

Okada, H., Kuhn, C., Feillet, H., & Bach, J.-F. (2010). The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clinical and Experimental Immunology*, *160*(1), 1–9. https://doi.org/10.1111/j.1365-2249.2010.04139.x

Parfrey, L. W., Jirků, M., Šíma, R., Jalovecká, M., Sak, B., Grigore, K., & Pomajbíková, K. J. (2017). A benign helminth alters the host immune system and the gut microbiota in a rat model system. https://doi.org/10.1371/journal.pone.0182205

Parham, P. (2015). *The Immune System* (4th ed.). 711 Third Avenue, New York, NY 10017, USA: Garland Science.

Pastille, E., Frede, A., McSorley, H. J., Gräb, J., Adamczyk, A., Kollenda, S., … Westendorf, A. M. (2017). Intestinal helminth infection drives carcinogenesis in colitis-associated colon cancer. *PLoS Pathogens*, *13*(9), e1006649. https://doi.org/10.1371/journal.ppat.1006649

Qian, Y., Xia, T., Cui, Y., Chu, S., Ma, Z., & Gu, X. (2018). The role of CaMKII in Neuropathic Pain and Fear Memory in Chronic Constriction Injury in Rats. *The International Journal of Neuroscience*, 1–27. https://doi.org/10.1080/00207454.2018.1512986

Ren, D., Beinart, D., Pi, C., Poulton, S., Holzknecht, Z. E., Swanson, C., & Parker, W. (2017). Returning to the Wild: Restoring Natural Levels of Immune Function to Laboratory Animals. *Journal of Applied Life Sciences International*, *14*(3), 1–13.

Romagnani, S. (2000). T-cell subsets (Th1 versus Th2). *Annals of Allergy, Asthma & Immunology: Official Publication of the American College of Allergy, Asthma, & Immunology*, *85*(1), 9–18; quiz 18, 21. https://doi.org/10.1016/S1081-1206(10)62426-X

Rook, G A W, & Brunet, L. R. (2005). Microbes, immunoregulation, and the gut. *Gut*, *54*(3), 317–320. https://doi.org/10.1136/gut.2004.053785

Rook, Graham A. W. (2012). Hygiene Hypothesis and Autoimmune Diseases. *Clinical Reviews in Allergy & Immunology*, *42*(1), 5–15. https://doi.org/10.1007/s12016-011-8285-8

Rook, Graham A. W., Lowry, C. A., & Raison, C. L. (2013). Microbial 'Old Friends', immunoregulation and stress resilience. *Evolution, Medicine, and Public Health*, *2013*(1), 46–64. https://doi.org/10.1093/emph/eot004

Schumann, C. M., Bauman, M. D., & Amaral, D. G. (2011). Abnormal structure or function of the amygdala is a common component of neurodevelopmental disorders. *Neuropsychologia*, *49*(4), 745–759. https://doi.org/10.1016/j.neuropsychologia.2010.09.028

Shen, S., & Wong, C. H. (2016). Bugging inflammation: role of the gut microbiota. *Clinical & Translational Immunology*, *5*(4), e72. https://doi.org/10.1038/cti.2016.12

Smyth, K., Morton, C., Mathew, A., Karuturi, S., Haley, C., Zhang, M., … Parker, W. (2017). Production and Use of Hymenolepis diminuta Cysticercoids as Anti-Inflammatory Therapeutics. *Journal of Clinical Medicine*, *6*(10), 98. https://doi.org/10.3390/jcm6100098

Speaker, K. J., Cox, S. S., Paton, M. M., Serebrakian, A., Maslanik, T., Greenwood, B. N., & Fleshner, M. (2014). Six weeks of voluntary wheel running modulates inflammatory protein (MCP-1, IL-6, and IL-10) and DAMP (Hsp72) responses to acute stress in white adipose tissue of lean rats. *Brain, Behavior, and Immunity*, *39*, 87–98. https://doi.org/10.1016/j.bbi.2013.10.028

Stanford, J., Stanford, C., & Grange, J. (2004). Immunotherapy with Mycobacterium vaccae in the treatment of tuberculosis. *Frontiers in Bioscience: A Journal and Virtual Library*, *9*, 1701–1719.

Strachan, D. P. (1989). Hay fever, hygiene, and household size. *BMJ : British Medical Journal*, *299*(6710), 1259–1260.

Sulima, A., Savijoki, K., Bień, J., Näreaho, A., Sałamatin, R., Conn, D. B., & Młocicki, D. (2018). Comparative Proteomic Analysis of Hymenolepis diminuta Cysticercoid and Adult Stages. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.02672

Tipton, A. J., Baban, B., & Sullivan, J. C. (2012). Female spontaneously hypertensive rats have greater renal anti-inflammatory T lymphocyte infiltration than males. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *303*(4), R359-367. https://doi.org/10.1152/ajpregu.00246.2012

Treutwein, B., & Strasburger, H. (1999). Fitting the psychometric function. *Perception & Psychophysics*, *61*(1), 87–106.

Villeneuve, C., Kou, H. H., Eckermann, H., Palkar, A., Anderson, L. G., McKenney, E. A., … Parker, W. (2018). Evolution of the hygiene hypothesis into biota alteration theory: what are the paradigms and where are the clinical applications? *Microbes and Infection*, *20*(3), 147–155. https://doi.org/10.1016/j.micinf.2017.11.001

Williamson, L. L., McKenney, E. A., Holzknecht, Z. E., Belliveau, C., Rawls, J. F., Poulton, S., … Bilbo, S. D. (2016). Got worms? Perinatal exposure to helminths prevents persistent immune sensitization and cognitive dysfunction induced by early-life infection. *Brain, Behavior, and Immunity*, *51*, 14–28. https://doi.org/10.1016/j.bbi.2015.07.006

Yu, C.-C., Chien, C.-T., & Chang, T.-C. (2016). M2 macrophage polarization modulates epithelial-mesenchymal transition in cisplatin-induced tubulointerstitial fibrosis. *BioMedicine*, *6*(1), 5. https://doi.org/10.7603/s40681-016-0005-5

Zambirinis, C. P., Pushalkar, S., Saxena, D., & Miller, G. (2014). Pancreatic cancer, inflammation, and microbiome. *Cancer Journal (Sudbury, Mass.)*, *20*(3), 195–202. https://doi.org/10.1097/PPO.0000000000000045

Zhang, L., Jiang, Y., Cui, Z., Yang, W., Yue, L., Ma, Y., … Qian, A. (2016). Mycobacterium vaccae induces a strong Th1 response that subsequently declines in C57BL/6 mice. *Journal of Veterinary Science*, *17*(4), 505–513. https://doi.org/10.4142/jvs.2016.17.4.505

Zhang, M., Mathew, A. J., & Parker, W. (2018). Production of Hymenolepis diminuta in the Laboratory: An Old Research Tool with New Clinical Applications. *Methods in Molecular Biology (Clifton, N.J.)*, *1799*, 27–38. https://doi.org/10.1007/978-1-4939-7896-0_3

Zhou, D., Zhang, H., Bai, Z., Zhang, A., Bai, F., Luo, X., … Lu, Z. (2016). Exposure to soil, house dust and decaying plants increases gut microbial diversity and decreases serum immunoglobulin E levels

in BALB/c mice. *Environmental Microbiology*, *18*(5), 1326–1337. https://doi.org/10.1111/1462-2920.12895