

**BEHAVIOURAL CORRELATES OF EXPERIMENTAL AUTOIMMUNE
ENCEPHALOMYELITIS**

BRIETTA GERRARD
B.Sc., University of Lethbridge

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Department of Neuroscience University of Lethbridge
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*I dedicate this thesis in memory of Grandma Ida, and
Grandma Millie.*

ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system induced in laboratory rodents to mimic human demyelinating diseases such as multiple sclerosis (MS). This thesis applies EAE to studies in behavioural neuroscience by first establishing a literature review of physical and behavioural deficits in various EAE rodent models. Experiment 1 focuses on developing a comprehensive behavioural profile of EAE using a monophasic model of disease in Lewis (LEW) rats immunized with myelin basic protein (MBP). In this experiment, three commonly used EAE assessment scales were compared to determine which scale was the most sensitive to EAE changes. In addition physical and behavioural tasks were used to establish a temporal profile of skilled walking, anxiety, fatigue, and allodynia in EAE rats. In Experiment 2, the influence of chronic stress on the clinical disease course of monophasic MBP-EAE in LEW rats was investigated. Additionally, a longitudinal profile of physical and behavioural symptoms of EAE influenced by chronic stress was established. Furthermore, Experiments 2 and 3 utilized the expression of immune markers, epigenetic changes, and trace element analysis to develop an understanding of the mechanisms behind EAE and the role of chronic stress in this disease. The discussion section of this thesis closes with a summary on the use of LEW rats in these experiments. In addition, a protocol on the methods and troubleshooting of EAE immunizations is presented.

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LIST OF ABBREVIATIONS

| | |
|-----------------|---|
| ANOVA | Analysis of Variance |
| As | Arsenic |
| BBB | Basso, Beattie, Bresnahan |
| B | Boron |
| Ce | Cerium |
| Cl | Chlorine |
| CFA | Complete Freund's Adjuvant |
| CNS | Central Nervous System |
| CNP | Central Neuropathic Pain |
| Co | Cobalt |
| df's | Degrees of Freedom |
| DMD | Disease-Modifying Drugs |
| EAE | Experimental Autoimmune Encephalomyelitis |
| EDSS | Expanded Disability Status Scale |
| EPM | Elevated Plus Maze |
| FL-HL | Forelimb-Hindlimb |
| GP | Guinea Pig |
| GRO/KC | Growth Related Oncogene |
| HPA | Hypothalamus-Pituitary-Adrenal Axis |
| IL | Interleukin |
| K | Potassium |
| LEW | Lewis |
| LHL | Left Hindlimb |
| LSD | Least Significant Difference |
| MBP | Myelin Basic Protein |
| miRNA(s) | MicroRNA(s) |
| Mn | Manganese |
| MOG | Myelin Oligodendrocyte Glycoprotein |
| MRI | Magnetic Resonance Imaging |
| MS | Multiple Sclerosis |
| Ni | Nickel |
| PPMS | Primary Progressive Multiple Sclerosis |
| PRMS | Progressive-Relapsing Multiple Sclerosis |
| Pt | Platinum |
| RHL | Right Hindlimb |
| RM ANOVA | Repeated Measures Analysis of Variance |
| RRMS | Relapsing-Remitting Multiple Sclerosis |
| SEM | Standard Error of the Mean |
| SPMS | Secondary Progressive Multiple Sclerosis |
| Te | Tellurium |
| Tl | Thallium |
| V | Vanadium |

CHAPTER 1

Introduction

1.1 Introduction to Multiple Sclerosis

Multiple Sclerosis (MS) is an unpredictable, chronic inflammatory disease of the central nervous system (CNS), affecting the brain and spinal cord. MS is characterized by the migration of CNS-specific T cells into the brain and spinal cord causing inflammation, neuronal injury, and/or neuronal death which leads to the formation of lesions within the CNS (Losy, 2013; Hurwitz, 2009; Lassmann et al., 2007; Gold et al., 2006). This immune cascade is the cause of marked physical disability in MS. According to a report by Beck et al. (2005) the prevalence rate of MS in Canada is 240/100,000 individuals. Alberta, specifically, has an estimated MS prevalence rate of 357.6/100,000 individuals (Warren et al., 2008). This is one of the highest prevalence rates of MS in the world. To add to this, the prevalence rate of MS in Canada increases each year (Hurwitz, 2009). The average age of onset of MS is approximately 30 years (Sundström et al., 2003; Olofsson et al., 2011) This young age of MS onset represents one of the major causes of reduced capacity to work in Western Societies (Sundström et al., 2003; Olofsson et al., 2011). Therefore, we are in need of early diagnostic techniques and effective therapies to allow individuals living with MS to maintain a high quality of life.

Common clinical subtypes of MS include relapsing-remitting (RRMS), primary progressive (PPMS), secondary progressive (SPMS), and progressive-relapsing (PRMS). RRMS is the most common form of MS, affecting 85% of patients (Miller et al., 2004). This form of MS is characterized by unpredictable occurrences of new symptoms (relapses) followed by recovery to near pre-relapse function (remission). PPMS is defined

as disease progression from onset without any definable periods of relapse or remission (Miller et al., 2004). SPMS is characterized as an initial RRMS disease followed by a switch to progression of disease without any definite periods of relapse or remission (Miller et al., 2004). Approximately 50% of RRMS patients will switch to SPMS within 10 years of their MS diagnosis (Miller et al., 2004). The last form of MS is PRMS. This subtype of MS is characterized by a progressive course of the disease from onset with clear periods of relapse with or without periods of recovery (Miller et al., 2004).

Diagnosis of MS subtype is made by utilizing magnetic resonance imaging (MRI) scans as well as monitoring physical impairments (Alkhawajah, 2011). MRI is useful for determining the location and number of lesions within the CNS. The number of relapses and the number of remission periods are monitored by a neurologist on an annual basis. From a clinical standpoint, it is impossible to predict the disease course of MS (Miller et al., 2004). MS is random in terms of exacerbations and progression. No two cases of MS present the same.

Currently, disease-modifying drugs (DMD) for MS aim to decrease the number of relapses and delay the progression of disease (Markowitz, 2010). DMD such as interferon beta and glatiramer acetate, commonly used to treat RRMS, were measured during clinical trials based on their influence on MRI factors, relapse rate and severity, as well as their effect on the Kurtzke Expanded Disability Status Scale (EDSS) score (Alkhawajah, 2011; See Appendix A). Disability in MS is commonly quantified by the EDSS. A score of 0 on the EDSS indicates normal neurological function, whereas a score of 10 indicates death from MS (Kurtzke, 1983). For perspective, a score of 7 on the EDSS indicates wheelchair bound (Kurtzke, 1983). The EDSS focuses on physical

disability and excludes behavioural symptoms such as cognitive impairment and fatigue (Alkhawajah, 2011).

In addition to physical impairment, MS may cause debilitating co-morbid behavioural symptoms. It is estimated that 40% to 65% of MS patients experience behavioural symptoms (Miller, 2012; Bobholz et al., 2003). Some of the most common behavioural symptoms associated with MS include anxiety (Marie, 2010; Paparrigopoulos et al., 2010), cognitive dysfunction (Jongen et al., 2012), depression (Sariso, 2013; Marie, 2010), fatigue (Lange, 2009; Fisk, 1994), and pain (Michalski, 2011). To date, there is a lack of therapy targeted at treating behavioural impairments of MS. In fact, studies report that interferon beta may worsen or cause depression in MS patients (Plosker, 2011; Frago, 2010). This data emphasizes the urgent need for targeted therapeutics for behavioural symptoms of MS to ultimately increase quality of life for a patient living with MS. Animal models of MS with well-characterized behavioural symptoms therefore are critically important for pre-clinical drug development. In this review, I will discuss the use and validity of experimental autoimmune encephalomyelitis (EAE) as an animal model of MS.

1.2 Experimental Autoimmune Encephalomyelitis as an Animal Model of MS

EAE is an inflammatory disease of the central nervous system induced in laboratory animals to mimic human demyelinating diseases such as multiple sclerosis. EAE, like human MS is characterized by an inflammatory response within the brain and spinal cord, causing demyelination and subsequent axonal injury (Gold et al., 2006). EAE symptoms reflect the anatomical location of the inflammatory lesions within the brain and spinal cord, and may include motor disturbances, loss of body weight, reduced food

and sucrose intake, and/or decreased social interest (Pollak et al., 2000; Pollak et al., 2002).

Active EAE can be induced in susceptible animals by immunizing them with encephalitogenic antigens such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein, or spinal cord tissue emulsified in a modified complete Freund's adjuvant (CFA) (Mannie et al., 2009). In the active model of EAE symptoms should present between 9 and 12 days post-immunization. In addition, passive EAE can be induced by exposing animals to T cells adopted from an active EAE animal (Weissert, 2012). Three to five days after exposure to T cells in the passive EAE model, animals should show signs of EAE. Severity of EAE can range from acute monophasic to chronic relapsing-remitting or chronic progressive (Mannie et al., 2009). The clinical form of EAE depends on the animal species, and the encephalitogenic antigen used. Typically, clinical symptoms of EAE begin with tone loss in the tail ascending to paralysis of the forelimbs (Virley, 2005, Mannie et al., 2009). The pattern of acute EAE onset and spontaneous recovery resembles relapses and remissions of human MS (Mannie et al., 2009). Therefore, the animal model, EAE, is a reasonable scientific tool to investigate pathogenesis and behaviour of human MS.

Although EAE animal models have been used for a few decades, behavioural symptoms of EAE have just begun being characterized and studied. Understanding an EAE animal's behaviour may provide insights into mechanisms behind autoimmune CNS damage and ultimately lead to a better understanding of human MS. From here, we can use EAE animal models to test new prevention methods and therapies. Thus, a comparative study and validation of EAE and MS symptoms is a critical step towards

identifying potential new MS therapeutics. A selected number of the main behavioural manifestations of MS and EAE will be discussed in the following.

1.3 Overview of Motor and Physical Deficits in MS

Seventy percent of MS patients report motor deficits as their most challenging symptoms (Larocca, 2011). Motor impairments associated with MS include ataxia, weakness, and spasticity (Miller et al., 2004). The progression of these three symptoms eventually impedes on the ability to walk. As described previously, the EDSS is the most commonly used scale to assess motor impairment in human MS (Kurtzke, 1983). DMD drugs aim to reduce demyelination caused by the progression of MS, therefore preserving motor capabilities (Markowitz, 2010). Regardless of DMD, 33% of MS patients have difficulty walking within 10 years of disease onset (Scalfari et al., 2010). Therefore, new medications are needed to preserve the motor capabilities in MS patients.

1.3.1 Motor and Physical Deficits in EAE

EAE typically presents with a loss in tail tonicity progressing to forelimb paralysis or death (Virley, 2005; Mannie et al., 2009). A variety of scales are used to classify motor impairments in EAE. The most common scale is the 5 point scale. Based on the 5 point scale: Grade 0, no clinical signs; Grade 1, paralyzed tail; Grade 2, loss in coordinated movements; hind limb paresis; Grade 3, both hind limbs paralyzed; Grade 4: forelimbs paralyzed; Grade 5, moribund (Stromnes et al., 2006). The 5 point scale combines the hindlimbs and forelimbs together making it less sensitive to individual limb deficits. A more sensitive rating scale for EAE impairments was developed at the University of Calgary and is called the 15 Point Scale. The 15 point scale assesses the

state of the tail and each limb individually. For the tail: Grade 0, no clinical signs; Grade 1, partially paralyzed tail; Grade 2, paralyzed tail (Weaver et al., 2005). For each of the limbs: Grade 0, no clinical signs; Grade 1, weak or altered gait; Grade 2, paresis; Grade 3, fully paralyzed limb (Weaver et al., 2005). The sum of the tail grade and 4 individual limb grades are used to determine the score out of 15. Therefore, a fully paralyzed rat would receive a grade of 14. A grade of 15 means mortality. In addition to these EAE rating scales, a variety of behavioural tasks exist to determine locomotor abilities.

Kerschensteiner et al. (2004) explored the locomotor capabilities of a two localized models of EAE in Lewis (LEW) rats immunized with MOG: one EAE model presented with mild symptoms (mean disease score of 0.6 based on the 5 point scale) whereas the other EAE model presented with severe symptoms (mean disease score of 2.2 based on the 5 point scale). Mild EAE animals made a full recovery from EAE by day 14 post immunization whereas severe EAE animals did not make a full recovery by the end of the experiment (Kerschensteiner et al., 2004). Behavioural tasks focusing on motor capabilities utilized in this experiment include the Basso, Beattie, Bresnahan (BBB) locomotor scale, and grid walk (addressing number of errors/hindlimb step) (Kerschensteiner et al., 2004). These behavioural tasks were used at baseline, and then at day 3, 7, 10, 14, 21, and 28 days post EAE induction (Kerschensteiner et al., 2004). Using the BBB locomotor rating scale, which is used to measure mobility, the mild EAE rats developed an average maximal score of 18 (consistent forelimb-hindlimb coordination (FL-HL) during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off) 3 days post EAE development, whereas the severe EAE rats developed an

average maximum score of 8 (sweeping with no weight support or plantar placement of the paw with no weight support) by day 3 post EAE development (Kerschensteiner et al., 2004). By day 21 in this experiment, mild LEW rats were scoring on average 20.5 (consistent plantar stepping and consistent FL-HL coordination) using the BBB scale (Kerschensteiner et al., 2004). Severe EAE animals on the other hand were scoring on average 17 (consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off) by day 21 post EAE induction (Kerschensteiner et al., 2004). Therefore, the motor capabilities of the mild EAE animals are not significantly impaired during any day of disease. In contrast with this, the severe EAE animals show significant motor impairment during symptomatic EAE. Mild EAE animals return to near baseline BBB measures by day 21 post EAE induction, whereas the severe EAE animals are still showing locomotor deficits by day 21. Therefore, the locomotor deficits observed from the severe EAE animals are permanent. The BBB scale is sensitive to coordination, hindlimb movements, stepping and paw placement, tail position, as well as trunk position and stability. A grade of 0 indicates no observable hindlimb movement whereas; a grade of 21 indicates normal locomotion (Basso et al., 1995; See Appendix B). The grid walk tasks requires controlled placement of hindlimbs on grid bars spaced irregularly, therefore addressing skilled walking (Metz et al., 2000; 2002). The mild EAE animals were not significantly different from the controls during any day of disease in regards to the number of grid walk errors (Kerschensteiner et al., 2004). The severe EAE animals on the other hand, made significantly more errors than the controls on day 3, 10, and 14 (Kerschensteiner et al., 2004). Therefore, skilled walking is significantly impaired in

severe EAE animals during symptomatic EAE and not in mild EAE animals.

Buddenberg et al. (2004) further explored the locomotor capabilities of the localized MOG-EAE rat model utilizing the 5 point scale, open field (total distance travelled, number of rearings, and number of rotations), BBB scale, grid walk (addressing number of errors/hindlimb step), narrow beam (utilizing 3 beam sizes and scoring the ability to place the foot), and footprint analysis (angle of foot rotation, the base of support, stride length between left and right foot) at baseline, and then at day 3, 7, 14, 21, and 28 days post EAE induction. All but 3 rats made a full recovery from EAE by day 28 post EAE induction (Buddenberg et al., 2004). The LEW rats in this experiment did not score above grade 3 at the height of their EAE disease based on the 5 point scale (Buddenberg et al., 2004). Therefore, these LEW animals did not progress beyond both hindlimbs paralyzed at the height of their EAE disease. Using the BBB locomotor rating scale, the EAE rats developed a median score of 12 (consistent weight-supporting plantar steps and occasional FL-HL coordination) by day 3 post EAE development (Buddenberg et al., 2004). Therefore, EAE animals show significant impairments in locomotor capabilities in comparison to controls. By day 21 in this experiment, all LEW rats were scoring between 19-21 (consistent plantar stepping and consistent FL-HL coordination) using the BBB scale (Buddenberg et al., 2004). Therefore, these animals returned to near baseline measures by day 21 post EAE induction. Utilizing the open field, which is commonly used to assess spontaneous motor activity in rodents, there were no significant differences between EAE animals and controls in regards to total distance travelled or number of rearings at any point in EAE progression or recovery (Buddenberg et al., 2004). The total number or rotations was significantly different between the EAE animals

and controls at day 3, 7, 14, and 21 (Buddenberg et al., 2004). Therefore, significant differences were seen between the EAE animals and controls prior to the onset of EAE, and during the progression of EAE in regards to number of rotations within the open field. Utilizing the grid walk, and narrow beam, which are used to address skilled walking (Metz et al., 2000; 2002), significant differences were found between the EAE animals and controls within all conditions on day 3, 14, and 21 (Buddenberg et al., 2004). Therefore, skilled walking is significantly impaired in EAE animals prior to the onset of EAE disease and progression of EAE. Lastly, the footprint analysis revealed that EAE animals had significantly rotated hindlimbs at day 7, and significantly reduced base of support by day 14 (Buddenberg et al., 2004). Therefore, foot placement in EAE animals was significantly impaired in comparison to control animals during symptomatic EAE. There were no differences observed between EAE animals and controls in regards to left and right stride distance (Buddenberg et al., 2004). Interestingly, hindlimb rotation and base of support never fully recover by day 30 in EAE animals. Within all other movement tasks, the EAE animals make a full recovery by day 28. Therefore, the footprint analysis may be more sensitive to lingering locomotor deficits that cannot be detected by other motor parameters.

Since Buddenberg et al. study, several other researchers have investigated motor parameters in EAE animals. The rotorod has been used previously in EAE studies to determine locomotor capabilities. The rotorod is used to evaluate motor skill coordination and balance over increasing rotorod speeds (Shiotsuki et al., 2010). Chronic MOG-EAE mice do not show signs of motor impairment utilizing the rotorod prior to EAE onset (Jones et al., 2008; Acharjee et al., 2013; Olechowski et al., 2013). In contrast with this,

significant impairments on the rotorod are seen during symptomatic EAE immunization using the same MOG-EAE model (Jones et al., 2008; Olechowski et al., 2009; Musgrave et al., 2011). All of these MOG-EAE mice developed a maximum disease score of 3 based on the 5 point scale (Jones et al., 2008; Musgrave et al., 2008; Acharjee et al., 2013; Olechowski et al., 2009; 2013). In a mild MOG-EAE model (maximum disease score of 0.5 based on the 5 point scale) no differences were seen between EAE and control animals in regards to rotorod performance during the symptomatic phase of EAE (Peruga et al., 2011). Therefore, severe EAE animals demonstrate significant motor impairments during symptomatic EAE in comparison to mild EAE animals and controls using the rotorod. In addition, the open field was used to address spontaneous activity in the chronic MOG-EAE model. No significant differences were seen between EAE animals and controls in total distance travelled, number of rearings, velocity or movement between quadrants within the activity box prior to the onset of EAE (Jones et al., 2008; Musgrave et al., 2011; Acharjee et al., 2013). Contrasting with this, a significant decrease in total distance travelled, number of rearings, and quadrant changes was observed by EAE animals during symptomatic EAE in comparison to controls within the open field (Jones et al., 2008; Musgrave et al., 2011). All of these MOG-EAE mice developed a maximum disease score of 3 based on the 5 point scale (Jones et al., 2008; Musgrave et al., 2008; Acharjee et al., 2013; Olechowski et al., 2009; 2013). Utilizing the mild MOG-EAE model, as described previously, no significant differences were seen between mild EAE and control animals in regards to total distance travelled within the open field during symptomatic EAE (Peruga et al., 2011). Therefore, EAE animals show significant motor impairments within the open field during symptomatic EAE only when EAE is

severe.

1.4 Overview of Anxiety in MS

Anxiety is characterized by a feeling of unease, nervousness or worry about imminent events. A study by Diaz-Olavarriet et al. (1999) reported that 37% of MS patients suffer from anxiety. The most common anxiety diagnoses associated with MS include obsessive-compulsive disorder, generalized anxiety disorder, and panic attack (Korostil et al., 2007). Anti-depressants are the most common form of treatment for MS-related anxiety. Price et al. (2011) report that antidepressants were not statistically significant in treating anxiety conditions related to MS. In this study antidepressants were effective but their efficacy was questioned (Price et al., 2011). Anxiety ultimately affects the quality of life, especially for a person afflicted with MS. The lack of effective treatment for anxiety in MS suggests that new therapeutics should be investigated. Animal models of MS, such as EAE, may present a clinically relevant avenue for such studies.

1.4.1 Anxiety in EAE

Anxiety-like behaviours in EAE models are not commonly studied and previous results are still controversial. A study completed by Rodrigues et al. (2011) found no signs of anxiety in female C57BL/6 (C6) mice immunized with MOG for chronic-relapsing EAE using the elevated plus maze (EPM) before or during symptomatic EAE. The EPM is widely accepted as a method to test anxiety-like behaviours in rodents (Lister, 1987). This task consists of an elevated plus shaped table with two closed and two open arms. The amount of time spent in a closed arm versus an open arm of the EPM

is measured (Lister, 1987). Rodents typically like to hide in enclosed dark locations when anxious (Lister, 1987). Thus, increased time spent in the closed arm versus the open arm is considered an indicator of anxiety-like behaviour. In opposition with these data, Haji et al. (2012) and Acharjee et al. (2012) report evidence of anxiety-like behaviour in female C6 mice using the same MOG-EAE model as Rodrigues and associates (2011) prior to the onset of symptomatic EAE. In these experiments, C6 mice were tested in the EPM and in an open-field test (Haji et al., 2012; Acharjee et al., 2013). C6 mice with EAE spent more time in the closed arm of the EPM than the controls, which is indicative of anxiety-like behaviour (Haji et al., 2012; Acharjee et al., 2013). In addition, these mice spent more time in the periphery of the open-field box than in the center of the open-field arena (Haji et al., 2012; Acharjee et al., 2013). Rodents with elevated anxiety will typically avoid the bright, open center of an open field arena and spend more time in the periphery of an open-field (Bourin et al., 2007). Like in the EPM, the periphery is enclosed by walls and the animals feel safer in this location. In addition to these results, a study completed by Peruga et al. (2011) using female C6 mice suffering from MOG-EAE found evidence of anxiety-like behaviour during the symptomatic phase of EAE. In this experiment EAE animals displayed a significant increase in their acoustic startle response in a prepulse inhibition task during symptomatic EAE (Peruga et al., 2011). Davis et al. (1993) report that the startle response test is useful for measuring anxiety-like behaviour in mice. Furthermore, the EAE animals showed increased anxiety-like behaviour in a light-dark-box task, which offers animals the choice to explore a brightly lit compartment versus a dark refuge (Peruga et al., 2011).

This limited data set of anxiety-like behaviour in EAE models was obtained using

only one model of EAE. The general lack of data in particular using other EAE models emphasizes the need for further studies investigating anxiety in EAE. This in turn will lead to more avenues to test therapeutics for anxiety that may be useful in the treatment of human MS.

1.5 Overview of Cognitive Dysfunction in MS

Cognitive dysfunction is reported in up to 70% of MS patients regardless of clinical MS subtype (Rao et al., 1991; Wallin et al., 2006). In addition, physical progression of MS is unable to predict degree of cognitive decline (Wallin et al., 2006). Consequently, severe physical disability due to MS does not necessarily associate with increased cognitive impairment. The three most commonly affected cognitive capacities in MS are information processing, information processing speed, and memory (Wallin et al., 2006). These cognitive capacities are very important for everyday life since memory and information processing skills impact an individual's ability to hold a job as well as maintain social relationships. Current therapies for cognitive symptoms of MS include behavioural and/or pharmacological intervention. Targeted behavioural intervention, such as self-generated learning (where MS patients have to come up with an answer without help) has been shown to improve recall and everyday functional capabilities in MS patients (Amato et al., 2012). In addition, computer-based learning has been useful in preserving executive functions in MS patients (Amato et al., 2012). In contrast with this, there are no behavioural treatment options that address processing speed (Amato et al., 2012). In regards to pharmacological intervention, stimulants, potassium channel blockers, and, acetylcholinesterase inhibitors have been used in an attempt to preserve cognition in MS patients. At this point in time, positive results utilizing any of these

medications have yet to be replicated (Amato et al., 2012). Therefore, the literature is inconsistent in regards to the efficacy of pharmacological intervention in MS for cognitive impairment.

1.5.1 Memory Dysfunction in EAE

Cognitive capacities such as memory formation and retention can be studied using EAE animal models. Olechowski et al. (2013) report that C6 mice induced for chronic-relapsing EAE with MOG show evidence of memory impairment using the object recognition task. The object recognition task measures the amount of time a rodent spends exploring a familiar versus a novel object (Antunes et al., 2012). In theory, a rodent should be more interested in exploring a novel object than a familiar object (Antunes et al., 2012). The EAE mice in the Olechowski et al. (2013) study were unable to discriminate between a familiar and a new object. Therefore, the EAE mice spent equal time investigating each object (Olechowski et al., 2013). These data are indicative of an inability to form and retain memories about objects previously visited. In support of this, Acharjee et al. (2013) report that female MOG-EAE C6 mice, as previously described, exhibit memory formation impairment utilizing the fear condition task. The fear condition task exposes an animal until habituation to a tone followed by a foot shock (Acharjee et al., 2013). Later, the animal is exposed to the tone without the foot shock and the amount of time spent in an immobile state is recorded (Acharjee et al., 2013). In this experiment, the EAE mice were not associating the tone with the foot shock and spent significantly less time immobile than the controls after hearing the auditory cue, indicating impaired associative memory.

Other forms of learning are commonly investigated in laboratory rodents using

various tasks for spatial memory. D'Intino et al. (2005) using female Lewis rats immunized with guinea pig spinal cord tissue for chronic-relapsing EAE were significantly slower at recognizing their spatial location in comparison to controls in the Morris water maze task. The water maze task is designed to test spatial learning and memory by measuring the amount of time to navigate the maze using spatial environmental cues in order to find a hidden platform (Sutherland et al., 1983; Morris, 1984). The LEW rats with EAE in this study took longer to find a previously learned location of a platform hidden beneath cloudy water than control animals. In further support of this, male C6 mice immunized for MOG-EAE were tested in the Barnes maze where they also showed significant impairments in spatial learning and memory (Ziehn et al., 2010). The Barnes Maze is an alternative to the water maze in testing spatial learning and memory (Barnes, 1979). Here, animals are placed in the center of a platform with 20 holes located around its circumference and the goal is to find the escape box hidden beneath one of those holes (Barnes, 1979). The escape hole is learned by the animals prior to testing. In this case, the EAE animals took significantly more time to find an escape hole than the controls even though they had previously found the location of the escape route (Ziehn et al., 2010).

Taken together, EAE animals are unable to form and retain memories. In addition, the water and barnes maze data may indicate that processing speed is impaired in EAE animals. Since learning, memory, and processing speed are the most commonly affected cognitive capacities in human MS; EAE animals represent a useful tool to investigate targeted therapeutics to treat these symptoms (Wallin et al., 2006).

1.6 Overview of Depression in MS

Depression is the most commonly reported behavioural comorbidity associated with MS. A study by Diaz-Olavarrieta et al. (1999) reported that 79% of MS patients suffered from depression. This is 2.3 times higher than the rate of depression found in the general population (Patten et al., 2003). There is also a 7.5 times increase in suicide rates in patients diagnosed with MS in comparison to age matched controls from the general population (Sadovnick et al., 1991). Depression, like anxiety, has an effect on overall quality of life. Thus, these statistics may be reflective of the struggle over maintaining a job, receiving an education and/or raising a family while living with MS. Accordingly, the severity of depression at earlier stages of MS serves as a significant prognostic factor for the deterioration of the quality of life among MS patients (Tepavcevic et al., 2013). Within the literature, however, it is difficult to dissociate between reactive depression due to the diagnosis of MS and depression due to the pathogenesis of MS. Hence animal models of MS may serve as a meaningful venue to investigate the causal relationship of depression and MS progression.

1.6.1 Depression in EAE

Pollak et al. (2000) first reported signs of reduced body weight, decreased food and sucrose intake, and loss of social interest as signs of sickness behaviour in EAE rodents. These symptoms of sickness behaviour and anhedonia may be interpreted as a sign of depressive-like behaviour in animals. Like in human depression, depressed animals lack interest in pleasurable activities, followed by a feeling of hopelessness (Castagné et al., 2009). In a behavioural task developed by Musgrave et al. (2011) the amount of time that an animal spent sedentary while gazing at the floor was analyzed

over a 4-minute period in the open field. EAE mice spent significantly more time sedentary while gazing at the floor than the control animals (Musgrave et al., 2011). The EAE mice in this experiment were not interested in interacting with their environment. A study completed by Olechowski et al. (2013) using the same test, confirms these findings. These data may indicate depressive-like behaviour; however, a clear diagnosis of affective and emotional changes in rodent models remains challenging.

Another model of depressive-like behaviour in rodents is learned helplessness. Acharjee et al. (2013) reported that female MOG-EAE C6 mice, as described earlier, become significantly faster immobilized when exposed to a forced swim task and a tail suspension task prior to onset of EAE symptoms. The Porsolt Forced Swim Task and the tail suspension task are commonly used to assess depressive-like behaviour in rodents (Porsolt et al., 1977; Castagné et al., 2009). These tasks expose animals to an inescapable aversive condition and the time it takes for the animal to immobilize serves as a sign of learned helplessness (Castagné et al., 2009). The experiment by Acharjee et al. (2013) indicates that C6 mice exhibit depressive-like symptoms in regards to reduced time spent trying to escape an aversive situation.

A third model that may indicate depressive-like behaviour in EAE is the time spent socializing. Acharjee et al. (2013), as described previously, found evidence that their female EAE mice spent less time socializing than the control mice prior to EAE onset. In this experiment, a mouse was placed in a wire mesh cage within a chamber while an experimental animal was placed in a secondary connecting chamber. The number of entries into the chamber containing the caged mouse as well as the time spent surrounding the wire mesh cage containing the mouse by the experimental animal was

calculated (Acharjee et al., 2013). In this experiment, the EAE mice spent significantly less time entering the chamber containing the caged mouse in addition to less time surrounding the caged mouse than the control mice did. Thus, the authors confirmed their findings of elevated depressive-like behaviours in EAE using an array of tasks to confirm various aspects of depression in their model.

Taken together, EAE animals show signs of depressive-like behaviour as confirmed by a variety of behavioural tasks. Therefore, EAE animals can be used to investigate the mechanism behind human MS depression and potentially create targeted therapeutics to treat these symptoms.

1.7 Overview of Fatigue in MS

70% to 80% of MS patients report feeling fatigued (Markowitz, 2010). The fatigue experienced by MS patients is different from the fatigue experienced by the general population. The mechanism behind fatigue in MS is poorly understood, although it appears to exist without correlation to physical disability (Krupp et al., 1988). Fatigue in MS impedes upon the patient's ability to complete routine physical and cognitive functions (Bakshi, 2003). In addition, fatigue may be brought on by exposure to excess heat (Bakshi, 2003). Fatigue in MS is a complex behavioural symptom because it is associated with a multitude of factors including lack of sleep due to depression and/or pain. Nonetheless, approximately 40% of MS patients report fatigue as their most disabling symptom (Markowitz, 2010). Treatment of fatigue in MS typically includes avoiding heat, physical therapy, taking naps, and utilizing energy conservation techniques (Krupp, 2003). These techniques are not a solution for fatigue in MS but may help to reduce its effect on everyday life. Newer research suggests that medications such as

amantadine or modafinil may decrease MS fatigue (Krupp, 2003). Further research into MS fatigue is needed.

1.7.1 Fatigue in EAE

Although there is a lack of systematic accounts of fatigue in EAE models, a few studies have begun to investigate potentially fatigue-related symptoms using animals. Musgrave et al. (2011), as described previously, report that MOG-EAE animals were unable to maintain their performance on a non-accelerating rotorod as long as control mice during symptomatic EAE. It is possible that through a non-accelerating rod one may be able to detect fatigue-like behaviour. One would expect that a fatigued animal would not be able to continue walking on the rod as long as a control animal. A measurement of fatigue in this experiment would be confounded by the presence of motor disability. Acharjee et al. (2013) also used the rotorod to test motor skills in EAE animals. Data from this study suggests that fatigue is not present before the onset of symptomatic EAE. Acharjee et al. (2013) report no difference between EAE and control mice on the rotorod prior to the onset of EAE.

Additional ways to measure fatigue-like behaviour in EAE may include a manipulation of other currently used behavioural tasks. The open field can be used to determine overall activity (Gould et al., 2009). Reduced overall activity in comparison to controls could be perceived as fatigue-like behaviour. In addition, reduced total number of entries into open and closed arms of the EPM could also be indicative of general changes in the activity level and interpreted as fatigue-like behaviour. Furthermore, reduced endurance in the forced swim task could also be indicative of fatigue. To rule out the confound of learned helplessness, the rodent would need to be habituated to the

forced swim task prior to EAE. Last but not least, Ray et al. (2011) used a running wheel to assess fatigue in a chemotherapy treatment trial for cancer and showed that a fatigued animal spent less time running in the wheel than an animal with vigor. A similar task may be a useful indicator of general activity level for EAE models.

Taken together, limited research exists on fatigue in EAE. A greater understanding of the mechanism behind EAE fatigue may help us understand more about the mechanism behind human MS fatigue. This information in turn, could be used to develop targeted therapeutics for MS fatigue.

1.8 Overview of Pain in MS

67% of MS patients report pain at some point of time during their MS disease course (Miller et al., 2004). This pain is typically located in the extremities and/or trunk and is termed central neuropathic pain (CNP) (Svendsen et al., 2005; Miller et al., 2004). CNP is different from standard pain because it is due to active inflammation and/or damage to the nerve fibers within the CNS (Svendsen et al., 2005). Symptoms of CNP include loss of sensation to stimuli, abnormal sensations (tingling), paroxysmal pain (shooting pain), spontaneous pain (continuous unprovoked pain), evoked pain, hyperalgesia (increased perception of pain), and allodynia (pain in response to an unpainful stimulus) (Baron et al., 2010). Pain in MS is typically treated with pharmacological therapies, such as antidepressants or opioids (Truini et al., 2011). Long term use of either of these medications is not advisable (Truini et al., 2011). Therefore, the development of targeted therapeutics for pain in MS is needed.

1.8.1 Pain Research in EAE

The assessment of pain in EAE involves several paradigms, each using a different noxious stimulus as described in the following.

Mechanical Allodynia. The first area of research in pain and EAE discovered was mechanical allodynia. Pender (1986) was the first researcher to report mechanical allodynia in the tail of female and male LEW rats immunized with MBP or guinea pig spinal cord to induce chronic-relapsing EAE. Pender's rats displayed tail sensitivity to mechanical stimulation at the onset of symptomatic EAE. Mechanical allodynia was determined by vocal response made by the rat in response to a pinch of the tail (Pender, 1986). Olechowski et al. (2009; 2013) and Yuan et al. (2012) report that C6 mice with chronic-relapsing MOG-EAE, show significant mechanical allodynia prior to the onset of symptomatic EAE. These data were determined by the von Frey hair task. The von Frey hair task utilizes calibrated monofilaments to determine sensitivity to mechanical stimulation. Olechowski and associates (2009) applied von Frey hair filaments to the plantar surface of each hindpaw 5 times and recorded the number of nociceptive responses (biting, licking, or shaking). A 60% response rate was considered pain threshold (Olechowski et al., 2009). Mechanical allodynia, however, could not be determined at the peak of EAE or in the chronic phase of disease in this experiment (Olechowski et al., 2009). These observations may be due to the confound of motor impairment making it impossible for the rat to respond to the tactile stimulation. In the same year, Rodrigues et al. (2009) also reported evidence of mechanical allodynia in C6 female mice immunized with MOG for chronic-relapsing EAE prior to onset of symptomatic EAE using von Frey hair filaments. In disagreement with these studies, Lu

et al. (2012), also using the von Frey hair task, did not observe signs of mechanical allodynia.

Cold Allodynia. Olechowski and associates (2009), as described earlier, report that female MOG-EAE C6 mice display signs of cold allodynia prior to, and after onset of EAE symptoms. In this experiment, a drop of acetone was applied to each hindpaw and the reaction time was recorded (Olechowski et al., 2009). Cold allodynia is commonly measured by the acetone evaporation test (Brenner et al., 2012). A drop of acetone applied to the hindpaw, causes a cooling sensation when the acetone evaporates (Brenner et al., 2012). Reactions to the evaporation of acetone included biting, licking, guarding or lifting of the hindpaws. In an additional study, a cold plate was used to test cold allodynia at the level of the tail and hindpaw (Thibault et al., 2009) The time that it took for an animal to lift its hindpaw off the cold plate or the time it took for an animal to remove its tail from the cold stimulus was measured (Thibault et al., 2009). Cold allodynia was reported at the level of the tail prior to EAE onset. In contrast, cold allodynia was reported at the level of the hindpaws prior to, during, and after recovery from symptomatic EAE (Thibault et al., 2009). These data suggest that cold allodynia at the level of the tail is present only in the early stages of the EAE disease course, whereas cold allodynia of the hindlimbs is chronic throughout the EAE course. In the experiment by Thibault and associates (2009) two models of EAE were used in female LEW rats, a chronic-relapsing EAE immunized with MBP and an additional group immunized with MBP as described previously but in addition, this group received a subcutaneous injection of cyclosporine A starting the day of EAE induction, until day 21 post-immunization (Thibault et al., 2009). The findings indicated that cyclosporine A

produced a more severe chronic-relapsing model of EAE and subsequently there was no difference between the two EAE models in this experiment in regards to severity of cold allodynia (Thibault et al., 2009).

Heat Sensitivity. Additional pain studies in EAE report sensitivity to heat. As stated previously, heat may exacerbate signs of fatigue in MS (Bakshi, 2003). Therefore, heat is an important factor to explore in EAE and MS. Accordingly, Aicher and associates (2004) report that female and male SJL/J mice immunized with PLP for chronic-relapsing EAE, both actively and passively, responded significantly faster to exposure to heat on a hot plate. Hyperalgesia of the tail was reported prior to and during EAE in these models of EAE whereas, hindpaw hyperalgesia was only noted at the onset of symptomatic EAE (Aicher et al., 2009). Aicher et al. (2009) also report that female SLJ/J mice had a stronger reaction to the heat stimulus than males. Additional studies by Olechowski et al. (2009) report evidence of hyperalgesia in female C6 mice prior to onset of MOG-induced EAE determined by the hot plate. EAE mice in their experiment were quicker to lift hindlimbs off the hot plate in response to heat compared to controls (Olechowski et al., 2009). More recently, Thibault et al. (2009) report that heat allodynia at the level of the tail was reported only prior to the onset of symptomatic EAE using the tail immersion task. In this test, the tail of a rodent is dipped in temperature-specific water and the latency to withdraw the tail is measured. Lastly, Lu and associates (2012) using female SJL/J and C6 mice immunized with PLP or MOG, respectively, for chronic-relapsing EAE found evidence of heat hyperalgesia only in the chronic phase of disease determined by an infrared beam. The withdrawal latency in response to the heat generated by the infrared beam was recorded. In this experiment, the EAE animals

responded to the heat faster than controls.

Formalin Exposure. Olechowski et al. (2010) exposed C6 mice immunized for chronic EAE with MOG to formalin. In the formalin test, formalin is subcutaneously injected into the hindpaw and the intensity of the pain response is recorded (Coderre et al., 1993). Formalin is a highly noxious substance causing pain for a prolonged period of time (Coderre et al., 1993). Interestingly, Olechowski et al. (2012) noted reduced pain behaviour in EAE mice in comparison to controls in regards to the formalin task. Therefore, EAE mice exhibit altered pain responses in comparison to control animals when exposed to a highly noxious enduring substance. In addition to these results, dysregulation of the glutamatergic system was observed in EAE animals in comparison to controls within this experiment (Olechowski et al., 2010). Therefore, the glutamatergic system plays an important role in mediating long term painful responses in rodents. Further investigation of the glutamatergic system is needed to gain a greater understanding of pain in EAE.

Taken together, EAE animals show evidence of CNP pain as determined by measures of mechanical allodynia, cold allodynia, heat sensitivity, and formalin exposure. Therefore, similar pain responses are noted between human MS patients and EAE animals. Consequently, EAE models represent a useful tool for investigating targeted therapeutics for pain in human MS.

1.9 Summary

Although the understanding of human MS pathogenesis has increased rapidly over the past years, MS patients still face a lack of curative or at least more effective symptomatic treatments. Furthermore, while current DMD for MS aim to decrease the

number of relapses and delay progression of disease (Markowitz, 2010), little is known about their effect on behavioural symptoms of MS. Some of the most common behavioural symptoms experienced by MS patient include anxiety, cognitive impairment, depression, fatigue, and pain. To date, there is a lack of therapy targeted at treating these behavioural comorbidities associated with MS. The creation of new medications will ultimately increase quality of life for a patient living with MS while we continue looking for a cure. Both the search for curative and symptomatic treatments for MS requires the use of pre-clinical animal models that are particularly suited to mimic the behavioural symptoms of MS.

EAE is an inflammatory disease of the central nervous system induced in laboratory animals to mimic human demyelinating diseases such as MS. EAE represents a reasonable scientific tool to investigate the pathogenesis and behaviour of MS-like behaviour. Although EAE has been used for a few decades, the characterization of behavioural symptoms of EAE has just begun. Understanding an EAE animal's behaviour may provide insight into mechanisms behind autoimmune CNS damage and ultimately lead to a better understanding of human MS pathogenesis. From here, we can use EAE animal models to develop and test new prevention methods and therapies for human MS.

1.10 Thesis Objectives and Rationale

The main objective of the thesis is to develop a rat model of EAE as a model of human MS emphasizing behavioural symptoms.

The major objectives of this thesis include:

- 1) Develop and optimize the EAE protocol in female Lewis rats.

2) Assess EAE symptoms in a test battery of motor, sensory, and emotional functions in order to create a comprehensive behavioural profile of progression and recovery.

3) Determine the influence of mild chronic stress on the behavioural and pathological profile of EAE in Lewis rats.

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CHAPTER 2

Experiment 1: Severity of EAE and its Effect on Behavior and Immunity

2.1 Abstract

Multiple Sclerosis (MS) is an unpredictable neurological condition that causes debilitating behavioural symptoms such as emotional instability, fatigue, and sensory loss. Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model to resemble the hallmark neuropathological features of human multiple sclerosis. Although physical deficits in EAE have been well characterized, little is known about behavioural symptoms of this disease. Here, we used female Lewis rats immunized with myelin basic protein (MBP) to produce monophasic EAE for comprehensive behavioural assessment. We compared three commonly used locomotor rating scales to monitor the severity of EAE and established a temporal profile of skilled walking ability, anxiety-like behaviours, fatigue, and allodynia. The behavioural profile highlighted the progression, severity and recovery of EAE. Interestingly, inflammatory markers existed beyond the recovery of motor symptoms. In turn, these inflammatory markers programmed the expression of microRNAs that were shown to be differentially regulated in human MS. These findings provide a framework for the development of a standardized behavioural assessment approach in rodent EAE models. Furthermore, the findings confirm the validity of the EAE model to mimic major neurobehavioural features of human MS.

2.2 Introduction

Multiple Sclerosis (MS) is an unpredictable neurological condition characterized by central nervous system inflammation, neuronal injury and/or neuronal death (Losy, 2013; Hurwitz, 2009; Lassmann et al., 2007; Gold et al., 2006). MS is characterized by the infiltration of immune cells into the central nervous system (CNS) causing an inflammatory response (Mancall, 2010). Specifically, high levels of anti-myelin basic protein (MBP), interleukins such as IL-6, IL-10, and IL-12 are implicated in MS pathogenesis (Olsson et al., 1990; Mancall, 2001). In addition, recent research has indicated that cytokine activity may regulate the expression of microRNA (miRNA) in chronic disease. In particular, the expression of miR-23, miR-146a, and miR-155 are induced by circulating cytokines (Junker et al., 2009). Furthermore, MS was shown to be associated with differential expression of miR-34a, miR-146a, miR-155, miR-326 (Junker et al., 2009; Tufekci et al., 2011; Koch et al., 2013). Therefore, miRNA may represent a useful biomarker for the diagnosis of MS. In addition, miRNA changes in MS represent a new avenue of targeted therapeutics for this disease.

MS is characterized by debilitating motor impairments (Miller et al., 2004) and additional behavioural comorbidities such as anxiety (Marie et al., 2010), fatigue (Lange et al., 2009), and sensory loss (Rae-Grant et al., 1999). It is estimated that 40% to 65% of MS patients experience behavioural impairments (Bobholz et al., 2003). Currently, little is known about the neuropathological origins of these symptoms (Amato et al., 2006). In addition, therapeutics used to treat these behavioural comorbidities are often ineffective (Krupp, 2003; Price et al., 2011; Truini et al., 2011; Amato et al., 2012).

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used

animal model of MS (Mannie et al., 2009). EAE mimics inflammatory processes found within the central nervous system causing subsequent demyelination and axonal injury implicated in human MS (Gold et al., 2006). While we know CNS damage in EAE causes various physical deficits, little is known about the behavioural symptoms associated with this process (Acharjee et al., 2013). Therefore, EAE represents a useful tool to further investigate the mechanism behind MS as well as behavioural parameters associated with this disease.

In this study, we investigated the outcomes of MBP-induced EAE in rats using a comprehensive behavioural test battery to assess a detailed temporal profile of functional loss and recovery. The outcomes of three commonly used locomotor rating scales were compared along with skilled walking and behavioural comorbidities, such as anxiety, fatigue, and pain. In addition, cytokine levels, hair mineral accumulation as a measure of metabolic change, and associated patterns of microRNA (miRNA) were analyzed in order to further profile the mechanism behind MS pathogenesis.

2.3 Materials and Methods

2.3.1 Subjects and Housing

Twenty 8-10 week old female Lewis (LEW) rats purchased from Charles River were used. The animals were housed in pairs under standard environmental conditions (12:12 hour light/dark cycle with lights on at 7:30 AM). Animals had access to food and water *ad libitum*. Rats were monitored daily for weight loss and neurological signs. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the local animal welfare committee at the University of Lethbridge.

2.3.2 Induction of EAE

Depending on the myelin peptide sequence used and dosage of that specific peptide sequence, the progression and severity of EAE can be variable. For that reason, we induced EAE in LEW rats with two different peptide portions of guinea pig (GP) MBP: GP MBP 69-87 (YGSLPQKSQRSQDENPVVH) and GP MBP 69-88 (YGSLPQKSQRSQDENPVVHF). GP MBP₆₉₋₈₇ was obtained from the Peptide Synthesis Facility, University of Calgary (Calgary, AB) whereas; GP MBP₆₉₋₈₈ was a generous gift from Dr. MD. Mannie (East Carolina University). Both of these protein segments produce monophasic EAE. In addition, to the different peptide portions of GP MBP, two different dosages of each peptide sequence was used: 50 µg or 100 µg. These manipulations were necessary in order to determine whether or not the progression and severity of EAE was influenced by peptide sequence and dosage, and if so, if this has any effect on behavioural symptoms of EAE. Experimental groups were: (1) 50 µg MBP₆₉₋₈₇ -inoculated (GP69-87 50 µg n = 4); (2) 100 µg MBP₆₉₋₈₇ -inoculated (GP69-87 100 µg n = 4); (3) 50 µg MBP₆₉₋₈₈ -inoculated (GP69-88 50 µg n = 4); (4) 100 µg MBP₆₉₋₈₈ -inoculated (GP69-88 100 µg n = 4); and (5) control (Control n = 4). EAE was induced by subcutaneous immunization at the base of the tail with the above described GP MBP emulsified in Freund's adjuvant (Difco Laboratories, BD Bioscience). Freund's adjuvant was supplemented with 4-mg/ml heat killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, BD Biosciences) to make complete Freund's adjuvant (CFA). The final concentration of CFA in the emulsion was 1mg/ml. Control rats were treated with CFA as described above.

2.3.3 EAE Assessment

2.3.3.1 Classic 5-Point Rating Scale

The Classic 5-Point Scale was used to assess EAE clinical disease. Signs of EAE were graded on the following 5 point scale: Grade 0, no clinical signs; Grade 1, paralyzed tail; Grade 2, loss in coordinated movements; hind limb paresis; Grade 3, both hind limbs paralyzed; Grade 4: forelimbs paralyzed; Grade 5, moribund (Stromnes et al., 2006).

2.3.3.2 Extended 15-Point Scale

The Extended 15-Point Scale was developed by the Yong laboratory as a detailed rating scale for EAE motor symptoms (Weaver et al., 2005). Clinical signs of EAE were graded on a scale ranging from 0 to 15 for the tail function and each limb separately. For the tail: Grade 0, no clinical signs; Grade 1, partially paralyzed tail; Grade 2, paralyzed tail (Weaver et al., 2005). For each of the limbs: Grade 0, no clinical signs; Grade 1, weak or altered gait; Grade 2, paresis; Grade 3, fully paralyzed limb (Weaver et al., 2005). The sum of the tail grade and four individual limb grades were used to determine the score out of 15. Therefore, a fully paralyzed rat would receive a grade of 14. A grade of 15 indicates mortality.

2.3.3.3 Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale

The 21-point BBB locomotor rating scale was originally developed for spinal cord injury models and is based on mobility (Basso et al., 1995). This ranking scale is sensitive to coordination, hindlimb movements, stepping and paw placement, tail position, as well as trunk position and stability. A grade of 0 indicates no observable hindlimb movement whereas a grade of 21 indicates normal locomotion (Basso et al.,

1995; See Appendix B). In this test, rats were placed in an open field measuring 1.5 meters in diameter and allowed to freely explore for 4 minutes. During this time, the experimenter observed rat locomotion and recorded their score.

2.3.4 Behavioural Testing

2.3.4.1 Ladder Rung Walking Task

The ladder rung task was used to demonstrate the effect of EAE on skilled motor movements (Metz et al., 2002; 2009). This task was used before EAE immunizations (baseline), day 5 after immunization, onset of EAE, symptom peak of EAE, first day of full recovery from EAE, and 10 days post full recovery. Prior to baseline, the rats were trained to cross a 1 m horizontal ladder with a variety of irregularly spaced rungs. The rungs were placed at random 0.5 cm to 5 cm apart. The ladder design was maintained for all testing days. During testing, the rats were filmed crossing the ladder rung in 3 sessions. Steps and errors of the left and right hindlimbs on the ladder rung were determined based on a 7-category rating scale (Metz et al., 2002; 2009; See Appendix C). Using the foot fault scoring system, an error is considered a score of 0, 1, or 2 (Metz et al., 2002; 2009).

2.3.4.2 Elevated Plus Maze

The Elevated Plus Maze (EPM) was used to test the emotional state of animals (Lister, 1987). This task was used before EAE immunizations (baseline), day 5 after immunization, onset of EAE, symptom peak of EAE, first day of full recovery from EAE, and 10 days post full recovery. Rats were allowed to freely explore a Plexiglas plus-shaped maze that consists of open and closed arms for 5 minutes. The apparatus consists

of two opposed open arms measuring 50×10 cm, crossed at right angle with two opposed arms of the same size. The latter are enclosed by walls 40 cm high, except for the entrance. The four arms delimited a central area of 10 cm^2 . The whole apparatus is elevated 50-cm above the floor. The total time (in seconds) spent in the closed arm was calculated. In addition, the total number of entries into all arms or specifically the open arms was calculated.

2.3.4.3 Open Field Test

Rats were placed in the middle of a clear, plastic box (36 x 36 cm) with surrounding infrared sensors before EAE immunizations (baseline), day 5 after immunization, onset of EAE, symptom peak of EAE, first day of full recovery from EAE, and 10 days post full recovery. Rats were left in the open field for a total of 10 minutes in conditions of low noise and dim lighting. Total horizontal distance (in cm) was tracked by the infrared sensor system. Total horizontal distance travelled was used as an indicator of overall activity level or fatigue.

2.3.4.4 Mechanical Allodynia

A set of calibrated von Frey hair monofilaments were used daily to assess sensitivity to punctate mechanical stimuli (Olechowski et al., 2009; 2013). Rats were placed in a clear Plexiglas chamber on an elevated wire mesh screen. Calibrated von Frey hair filaments were applied to the plantar surface of each hind paw in the ascending order of bending force (range: 2.0 g – 100.0 g). Each hair was applied 5 times per paw, and the number of nocifensive responses (vigorous shaking, prolonged lifting, licking or biting

of the stimulated paw) was recorded. The monofilament which produced nocifensive responses greater than 60% of the time was taken as the “threshold.”

2.3.5 Tissue Collection

Rats were anesthetized and blood samples were collected by cardiac puncture on day thirty-two or thirty-three post EAE immunization. The rats were then sacrificed by intracardiac infusion of 0.2 ml of sodium pentobarbital (Euthansol, CDMV Inc., Québec, Canada). After cardiac arrest, the animals were decapitated and the spinal cord was collected and flash-frozen for further analysis. In addition hair samples were collected for further analysis.

2.3.5.1 Blood Analysis

Following blood collection, samples were kept on ice for 30 min and then centrifuged at 1000 g for 10 min at 4°C to obtain plasma. Plasma samples were analyzed by Eve Technologies (Calgary, AB) to determine the level of 23 cytokines. In addition, the concentration of anti-MBP was confirmed by Mitogen Advanced Diagnostic (Calgary, AB).

2.3.5.2 miRNA Expression Analysis

MicroRNAs expression analysis was done using Illumina GAIIx genomic analyzer (PlantBiosis, Lethbridge). Briefly, base calling and demultiplexing was completed using CASAVA 1.8.1 software pipeline with default settings. Short read quality was examined using FastQC software. Adapters were trimmed using cutadapt software (<http://code.google.com/p/cutadapt/>). FastQC quality check was performed after trimming. MiRNA detection and counting was performed using standalone MicroRazerS

version 1.0 (Emde et al 2010). Statistical comparisons were done using DESeq bioconductor package (Anders & Huber 2010).

2.3.5.3 Hair Sample Analysis

Approximately 0.5 g of hair was collected from the abdomen and back from each rat. The hair samples were stored in 2-ml Eppendorf tubes at room temperature. Hair trace elementary analysis was performed by CanAlt Health Laboratories (Ontario, Canada). Hair samples were cut into small pieces using clean stainless steel scissors. About 300 ± 5 mg was transferred into tarred, labelled centrifuge tubes, and the exact weight was recorded. To each sample digestion tube, 3.0 ml of reagent-grade nitric acid (HNO₃) was added. Samples were incubated for 25 minutes. Samples were then subjected to acid microwave digest, in order to stabilize the elements of interest. The digestate solution was analyzed for amounts of mineral element and trace metals by inductively coupled plasma mass spectrometry. Sample results were quantified by comparison with calibration solutions of known concentrations. To control for metal trace contamination, fabric was cut with the same pair of scissors and used as control for hair sample analysis.

2.3.6 Statistical Analysis

Statistical analysis was carried out using SPSS version 21.0 software (IBM, USA). Statistical differences were compared between mild EAE, severe EAE, and control animals by one way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LSD) post-hoc tests, repeated measures analysis of variance (RM ANOVA)

with LSD post-hoc tests, and paired t-test as necessary. Statistical significance was set at 0.05. All bar and line graphs were plotted as mean \pm standard error of mean (SEM).

2.4 Results

2.4.1 Severity Of EAE Was Affected By Protein Segment And Not Dosage

Using the classic 5-point scale (Fig. 1A) the disease course of rats immunized for EAE with 50 μ g GP69-87 showed clinical deficits that developed between day 12 and 14 post-immunization. Symptoms began as a partially paralyzed tail (Grade 0.5) and progressed to a fully paralyzed tail (Grade 1.0) by day 13. All rats that received GP69-87 made a full recovery from EAE by day 17. Rats immunized for EAE with 100 μ g GP69-87 developed signs of EAE between day 11 and 12 post-immunization. Initially this group of rats presented with a partially paralyzed tail (Grade 0.5) and progressed to a fully paralyzed tail or loss in coordinated movement; hind limb paresis (Grade 1.0 or 2.0, respectively) by day 13. This group of rats also made a full recovery by day 17. Rats immunized for EAE with 50 μ g GP69-88 developed EAE between day 8 and 12 post-immunization. Early clinical signs of EAE in this group began with a partially paralyzed tail (Grade 0.5) and progressed to a loss in coordinated movement; hind limb paresis (Grade 2.0) by day 13. This group of rats made a full recovery from EAE by day 16. Rats immunized for EAE with 100 μ g GP69-88 developed EAE on day 12 post-immunization. These rats initially presented with a fully paralyzed tail (Grade 1.0) and progressed to a loss in coordinated movements; hind limb paresis or both hind limbs paralyzed (Grade 2.0 or 3.0, respectively) by day 14. This group of rats made a full recovery from EAE by day 18. Overall, there were no group differences between rats who received either dosage of GP69-87 and control rats ($p > 0.05$, RM ANOVA, LSD post-hoc test). On the other

hand, rats who received either dosage of GP69-88 had a significant difference in clinical disease progression in comparison to controls ($p < 0.01$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore degrees of freedom (df's) were corrected with Greenhouse-Geisser. This test revealed a significant effect of day ($F(2.381, 35.713) = 26.289$, $p = 0.000$), and day X group interaction ($F(9.523, 35.713) = 3.155$, $p = 0.006$). Rats immunized with 50 μg GP69-88 developed EAE the earliest (day 8) followed by rats who received 100 μg GP69-87 (day 11) while the other 2 experimental groups developed EAE day 12 or later. Rats immunized for EAE with 100 μg GP69-88 developed the most severe maximum disease score and recovered the latest.

Using the Extended 15-Point Scale (Fig. 1B) rats immunized for EAE with 50 μg GP69-87 developed symptoms beginning with a partially paralyzed tail (Grade 1.0) and progressed to a fully paralyzed tail or a weak/altered gait (Grade 2.0 or 3.0, respectively). Rats immunized for EAE with 100 μg GP69-87 initially presented with a partially paralyzed tail (Grade 1.0) and progressed to a weak or altered gait (Grade 3.0). Rats immunized for EAE with 50 μg GP69-88 developed a partially paralyzed tail (Grade 1.0) and progressed to a fully paralyzed tail and paresis of one limb (Grade 5.0). Rats immunized for EAE with 100 μg GP69-88 presented with a fully paralyzed tail (Grade 2.0) and progressed to paresis of one limb and weak/altered gait of a second limb (Grade 5.0 or 6.0, respectively). Day of onset, maximum disease score and recovery are the same per group as previously listed in the 5 point scale analysis. Overall, there were no group differences between rats who received either dosage of GP69-87 and control rats ($p > 0.05$, RM ANOVA, LSD post-hoc test). On the other hand, rats who received either

dosage of GP69-88 had a significant difference in clinical disease progression in comparison to controls ($p < 0.01$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df's were corrected with Greenhouse-Geisser. This test revealed a significant effect of day ($F(2.202, 33.035) = 19.682$, $p = 0.000$), and day X group interaction ($F(8.809, 33.035) = 2.533$, $p = 0.025$).

Using the BBB locomotor rating scale (Fig. 1C) rats immunized for EAE with 50 μg GP69-87 developed symptoms beginning with consistent frontlimb-hindlimb (FL-HL) coordination during gait; and toe clearance occurred consistently during forward limb advancement; predominant paw position was parallel at initial contact and lift off (Grade 19.0) and progressed to consistent FL-HL coordination; and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position was parallel to the body at initial contact (Grade 15.0). Rats immunized for EAE with 100 μg GP69-87 initially presented with a consistent coordinated gait; consistent toe clearance; predominant paw position was parallel at initial contact and lift off; but trunk instability and tail was consistently up (Grade 20.0) and progressed to consistent FL-HL coordination; and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position was parallel to the body at initial contact (Grade 15.0). Rats immunized for EAE with 50 μg GP69-88 developed a consistent coordinated gait; consistent toe clearance; predominant paw position was parallel at initial contact and Lift off ; but trunk instability and tail consistently up (Grade 20.0) and progressed to occasional weight supported plantar steps, no forelimb-hindlimb (FL-HL) coordination (Grade 10.0). Rats immunized for EAE with 100 μg GP69-88 presented with a consistent FL-HL coordination during gait; and toe clearance occurred frequently during forward

limb advancement; predominant paw position was parallel at initial contact and lift off or consistent FL-HL coordination during gait; and toe clearance occurred consistently during forward limb advancement; predominant paw position was parallel at initial contact and rotated at lift (Grade 17.0 or 18.0, respectively) and progressed to extensive movement of two joints and slight movement of the third or extensive movement of all three joints of the HL (Grade 6.0 or 7.0, respectively). Day of onset, maximum disease score and recovery are the same per group as previously listed in the 5 point scale analysis. Overall, there were no group differences between rats who received either dosage of GP69-87 and control rats in regards to BBB analysis ($p>0.05$, RM ANOVA, LSD post-hoc test). On the other hand, rats who received either dosage of GP69-88 had a significant difference in clinical disease progression in comparison to controls ($p<0.05$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a significant effect of day ($F(2.086,31.290)=12.339$, $p=0.000$), and day X group interaction ($F(8.344,31.290)=1.922$, $p=0.089$).

There were no significant differences between the 50 μ g or 100 μ g dose of GP69-87 using any of the above listed scales ($p>0.05$, RM ANOVA, LSD post-hoc test). Due to this, these two groups were combined and will be referred to as "mild" EAE in the following sections. In addition, there were no significant differences between the 50 μ g or 100 μ g dose of GP69-88 using any of the above described scales ($p>0.05$, RM ANOVA, LSD post-hoc test). Due to this, these two groups were combined and will be referred to as "severe" EAE in the following sections.

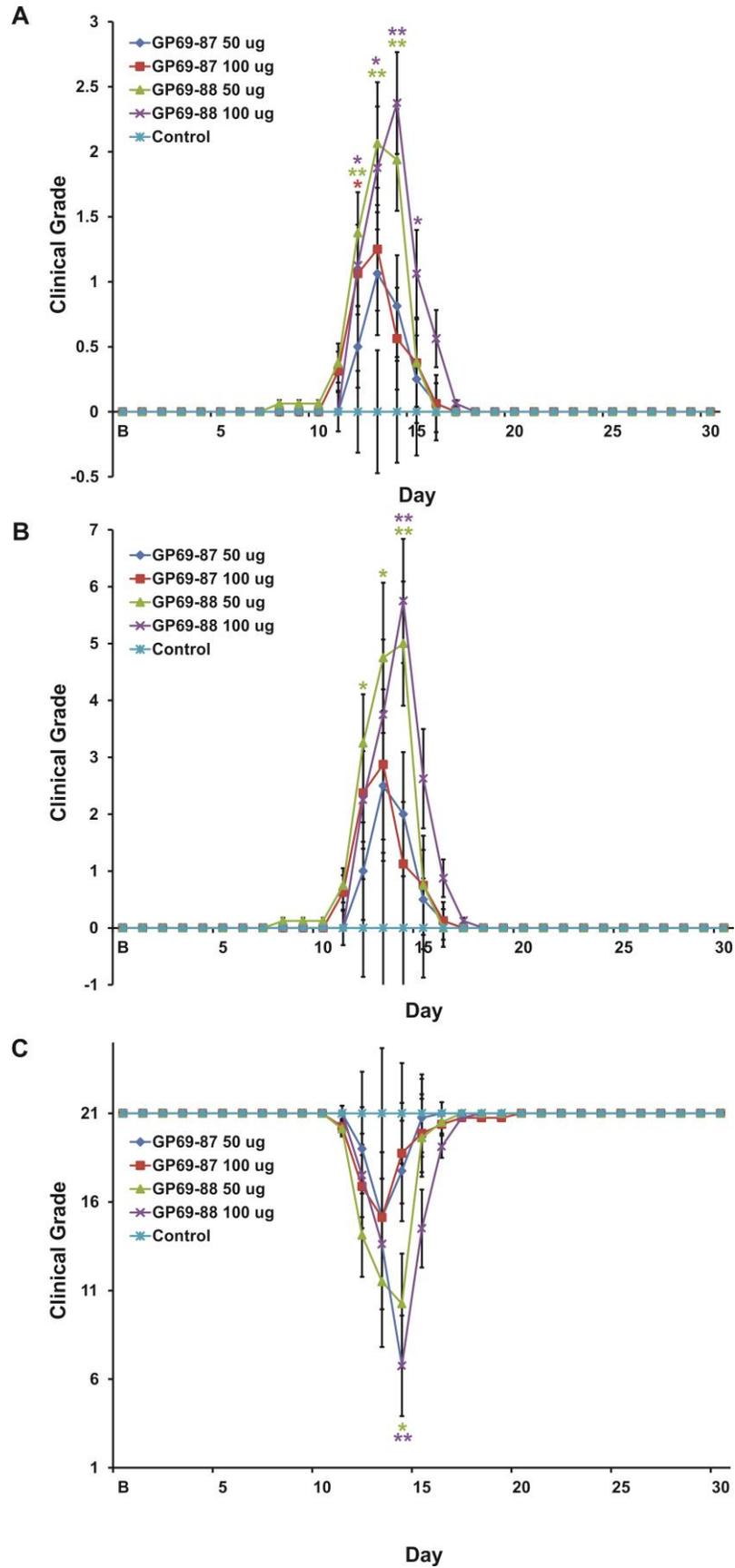


Fig. 1: Progression of clinical deficits in GP69-87 50 µg, GP69-87 100 µg, GP69-88 50 µg, GP69-88 100 µg, and control rats. **(A)** 5-point scale. Overall there were no group differences between rats who received either dosage of GP69-87 and control rats ($p > 0.05$, RM ANOVA, LSD post-hoc test). Rats who received either dosage of GP69-88 were significantly different in comparison to controls ($p < 0.01$, RM ANOVA, LSD post-hoc test). **(B)** Extended 15-point scale. Overall there were no group differences between rats who received either dosage of GP69-87 and control rats ($p > 0.05$, RM ANOVA, LSD post-hoc test). A significant difference was observed between rats who received either dosage of GP69-88 in comparison to controls ($p < 0.01$, RM ANOVA, LSD post-hoc test). **(C)** BBB Scale. Overall there were no group differences between rats who received either dosage of GP69-87 and control rats ($p > 0.05$, RM ANOVA, LSD post-hoc test). Rats who received either dosage of GP69-88 were significantly different in comparison to controls ($p < 0.05$, RM ANOVA, LSD post-hoc test). Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. * indicates significance between GP69-87 50 µg and control rats; * indicates significance between GP69-87 100 µg and control rats; * indicates significance between GP69-88 50 µg control rats; and * indicates significance GP69-88 100 µg and control rats. Error bars represent \pm SEM.

2.4.2 Mild and Severe EAE Impaired Skilled Walking Ability

In the ladder rung walking paradigm, there were no overall significant changes in the foot fault score of the left hindlimb (LHL) (Fig. 2A) of mild EAE animals ($p = 0.622$, RM ANOVA, LSD post-hoc test), or severe EAE animals ($p = 0.405$, RM ANOVA, LSD post-hoc test), in comparison to control animals. Mild EAE animals were not significantly different than severe EAE animals ($p = 0.693$, RM ANOVA, LSD post-hoc test). Therefore, severity of EAE did not affect the LHL foot fault score. Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed no significant effect of phase ($F(2.037, 32.588) = 2.001$, $p = 0.151$), or phase X group interaction ($F(4.073, 32.588) = 1.957$, $p = 0.124$). Foot fault score significantly increased in mild EAE animals ($F(2, 17) = 5.025$, $p = 0.009$), and severe EAE animals ($F(2, 17) = 5.025$, $p = 0.011$), in comparison to controls during the recovery phase.

Right hindlimb (RHL) foot fault scores (Fig. 2B) did not differ overall between mild EAE ($p=0.655$, RM ANOVA, LSD post-hoc test), or severe EAE animals ($p=0.495$, RM ANOVA, LSD post-hoc test), in comparison to control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.187$, RM ANOVA, LSD post-hoc test). Therefore, severity of EAE did not affect the RHL foot fault score. Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed no significant effect of phase ($F(1.977,31.633)=2.469$, $p=0.101$), or phase X group interaction ($F(3.954,31.633)=1.575$, $p=0.206$). Furthermore, a paired-samples t -test was conducted to compare phases of disease within each animal group. There was a significant decrease found between mild EAE baseline and mild post-phase of disease ($p=0.003$, paired t -test). Interestingly, the RHL foot fault score significantly increased in mild EAE animals in comparison to severe EAE animals during the pre-phase of EAE ($F(2,17)=3.555$, $p=0.024$).

Hindlimb foot placement (Fig. 2C) accuracy was analyzed by the number of errors per step. There were no overall significant changes between the LHL of mild EAE ($p=0.685$, RM ANOVA, LSD post-hoc test), or severe EAE animals ($p=0.705$, RM ANOVA, LSD post-hoc test), in comparison to control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.965$, RM ANOVA, LSD post-hoc test). Therefore, severity of EAE did not affect the error rate of the LHL. Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed no significant effect of phase ($F(2.137,34.190)=2.722$, $p=0.077$), or phase X group interaction ($F(4.274,34.190)=2.324$, $p=0.073$). Interestingly, significantly less errors were made by the LHL of mild EAE animals in comparison to

controls during the recovery phase of disease ($F(2,17)=5.061$, $p=0.001$). In contrast with mild EAE animals, severe EAE animals made significantly more errors in comparison to control animals during the recovery phase of disease ($F(2,17)=5.061$, $p=0.009$). Lastly, a significant decrease in LHL errors was seen between the peak phase, and recovery phase of disease in severe EAE animals ($p=0.044$, paired t-test).

There were no overall significant changes in error rate between the RHL (Fig. 2D) of mild EAE ($p=0.585$, RM ANOVA, LSD post-hoc test), or severe EAE animals ($p=0.945$, RM ANOVA, LSD post-hoc test) in comparison to control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.460$, RM ANOVA, LSD post-hoc test). Therefore, severity of EAE did not affect the error rate of the RHL. Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a significant phase interaction ($F(1,988,31.806)=3.716$, $p=0.036$) but not a significant phase X group interaction ($F(3.976,31.806)=1.680$, $p=0.179$). Interestingly, there was a significant increase in errors made by mild EAE animals in comparison to severe EAE animals during the pre-phase of disease ($F(2,17)=3.146$, $p=0.023$).

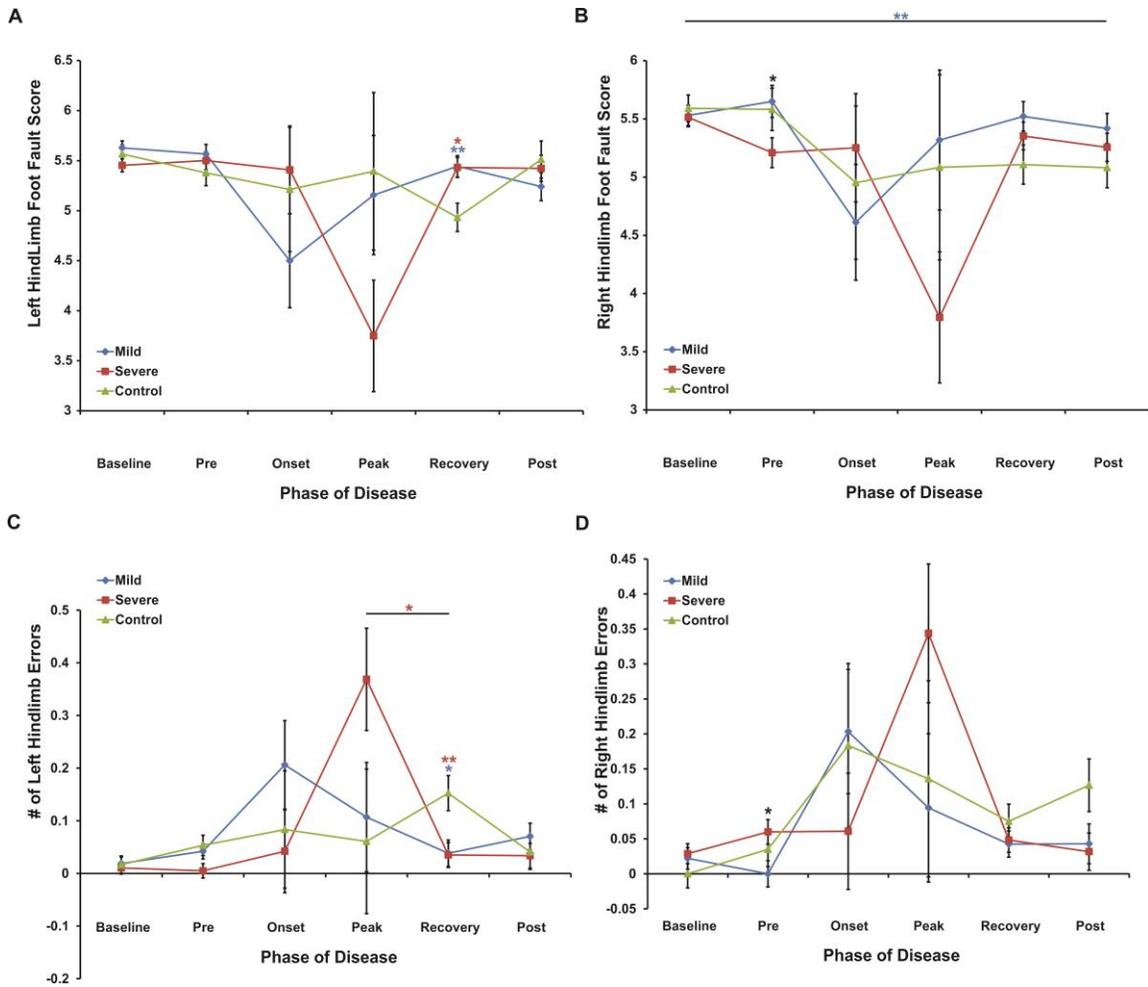


Fig. 2: Ladder rung walking task analysis in mild EAE, severe EAE, and control rats (**A**) Left hind limb foot fault score. Overall no significant changes were observed between mild EAE animals ($p=0.622$, RM ANOVA, LSD post-hoc test), and severe EAE animals ($p=0.405$, RM ANOVA, LSD post-hoc test), in comparison to control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.693$, RM ANOVA, LSD post-hoc test). Control animals did not develop EAE. (**B**) Right hindlimb foot fault score. Overall mild EAE animals ($p=0.655$, RM ANOVA, LSD post-hoc test), and severe EAE animals ($p=0.495$, RM ANOVA, LSD post-hoc test), were not significantly different in comparison to control animals. In addition, mild EAE animals were not significantly different from severe EAE animals ($p=0.187$, RM ANOVA, LSD post-hoc test). Control animals did not develop EAE. (**C**) Left hindlimb Error rate. There were no overall significant changes between mild EAE ($p=0.685$, RM ANOVA, LSD post-hoc test), or severe EAE animals ($p=0.705$, RM ANOVA, LSD post-hoc test), in comparison to control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.965$, RM ANOVA, LSD post-hoc test). Control animals did not develop EAE. (**D**) Right hindlimb error rate. There were no overall significant changes between mild EAE ($p=0.585$, RM ANOVA, LSD post-hoc test), and severe EAE animals ($p=0.945$, RM ANOVA, LSD post-hoc test) in comparison to control animals. Mild EAE

animals were not significantly different from severe EAE animals ($p=0.460$, RM ANOVA, LSD post-hoc test). Control animals did not develop EAE. Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between mild EAE and control rats; * indicates significance between severe EAE and control rats; and * indicates significance between mild and severe EAE rats. Error bars represent \pm SEM.

2.4.3 EAE Increased Anxiety-like Behaviour

The EPM was used to measure anxiety-like behaviour in rodents (Lister, 1987). In this task, an overall group difference was observed between mild EAE animals ($p=0.007$, RM ANOVA, LSD post-hoc test), and severe EAE animals ($p=0.006$, RM ANOVA, LSD post-hoc test), in comparison to control animals for time spent in the closed arm (Fig. 3A). There was no overall group difference between mild, and severe EAE animals ($p=0.972$, RM ANOVA, LSD post-hoc test). This suggests that increased severity of EAE is not associated with an increase in anxiety-like behaviour. Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a significant phase ($F(3.271,52.333)=12.889$, $p=0.000$), and phase X group interaction ($F(6.542,52.333)=3.9310$, $p=0.002$). Severe EAE animals spend significantly less time in the closed arm during baseline in comparison to controls ($F(2,17)=3.054$, $p=0.026$). In contrast with this, mild EAE animals spend significantly more time in the closed arm during onset ($F(2,17)=6.207$, $p=0.005$), peak ($F(2,17)=5.606$, $p=0.013$), and recovery ($F(2,17)=21.364$, $p=0.000$) in comparison to control animals. Likewise, severe EAE animals spend significantly more time in the closed arm during onset ($F(2,17)=6.207$, $p=0.005$), peak ($F(2,17)=5.606$, $p=0.005$), and recovery ($F(2,17)=21.364$, $p=0.000$) in comparison to control animals. A paired-samples t-test was conducted to compare phases of disease within each animal group. Mild EAE animals ($p=0.023$, paired t-test), and severe EAE animals ($p=0.000$, paired t-test) spend

significantly more time in the closed arm during the recovery phase in contrast with the post-phase of disease. In addition, severe EAE animals spend significantly more time in the closed arm during the baseline phase of disease in comparison to the post-phase ($p=0.002$, paired t-test). These results indicate that mild and severe EAE animals display anxiety-like behaviour. Lastly, a paired comparison revealed a significant difference in time spent in the closed arm during baseline and pre ($p=0.019$, paired t-test); and post and baseline ($p=0.000$, paired t-test) for control animals. This is to be expected. Control animals should demonstrate reduced anxiety over time when being exposed to the same environment continuously.

Furthermore, the number of entries into the open arm (Fig. 3B) was assessed for anxiety-like behaviour. There was an overall group difference between mild EAE ($p=0.006$, RM ANOVA, LSD post-hoc test), and severe EAE animals ($p=0.005$, RM ANOVA, LSD post-hoc test), in comparison to control animals for number of entries into the open arm. Mild EAE animals were not significantly different from severe EAE animals ($p=0.968$, RM ANOVA, LSD post-hoc test). These results further suggest that increased EAE severity is not associated with increased anxiety. A significant phase ($F(5,80)=11.743$, $p=0.000$), and phase X group interaction ($F(10,80)=5.623$, $p=0.002$) was observed. Mild EAE animals make significantly fewer open arm entries in comparison to control animals during baseline ($F(2,17)=3.784$, $p=0.027$), onset ($F(2,17)=14.748$, $p=0.000$), peak ($F(2,17)=8.987$, $p=0.004$), and recovery ($F(2,17)=25.050$, $p=0.000$). Likewise, severe EAE animals make fewer entries into the open arm during baseline ($F(2,17)=3.784$, $p=0.019$), onset ($F(2,17)=14.748$, $p=0.000$), peak ($F(2,17)=8.987$, $p=0.001$), and recovery ($F(2,17)=25.050$, $p=0.000$) in comparison

to control animals. A paired-samples t-test was conducted to compare phases of disease within each animal group. Severe EAE animals made fewer entries into the open arm during the pre phase of disease ($p=0.028$, paired t-test) in comparison to baseline. In contrast, mild EAE animals ($p=0.033$, paired t-test), and severe EAE animals ($p=0.001$, paired t-test) made significantly more entries into the open arm during the post-phase of disease in comparison to the recovery phase. In addition to this, severe EAE animals made significantly more entries into the open arm during the post-phase of disease in comparison to the baseline phase ($p=0.013$, paired t-test). This suggests that a recovery from anxiety is made after EAE symptoms disappear. Lastly, a paired comparison revealed a significant difference in open arm entries during post and baseline for control animals ($p=0.002$, paired t-test). This is to be expected. Control animals should demonstrate reduced anxiety over time when being exposed to the same environment continuously.

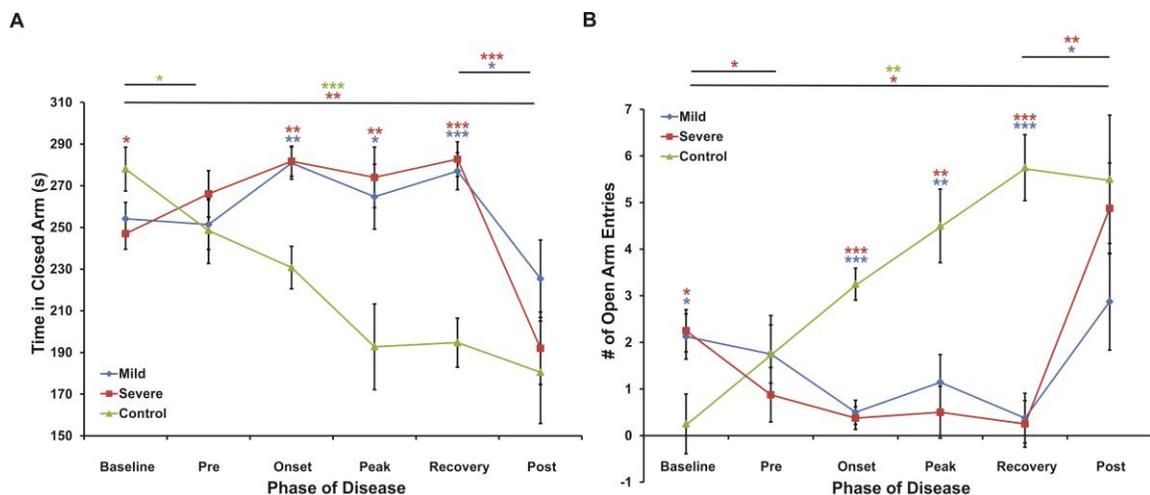


Fig. 3: Anxiety-like behaviour in mild EAE, severe EAE, and control rats. (A) Time spent in the closed arm of the EPM. Overall mild EAE animals ($p=0.007$, RM ANOVA, LSD post hoc test), and severe EAE animals ($p=0.006$, RM ANOVA, LSD post hoc test)

were significantly different from control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.972$, RM ANOVA, LSD post hoc test). **(B)** Number of open arm entries in the EPM. Overall mild EAE animals ($p=0.006$, RM ANOVA, LSD post hoc test), and severe EAE animals ($p=0.005$, RM ANOVA, LSD post hoc test) were significantly different from control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.968$, RM ANOVA, LSD post hoc test). Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between mild EAE and control rats; * indicates significance between severe EAE and control rats; and * indicates significance between mild and severe EAE rats. Error bars represent \pm SEM.

2.4.4 EAE Reduced General Activity Level Indicating Fatigue

The EPM was used to determine overall activity level which could be used as an indicator of fatigue. There was an overall group difference between mild EAE animals ($p=0.003$, RM ANOVA, LSD post-hoc test), and severe EAE animals ($p=0.000$, RM ANOVA, LSD post-hoc test) in comparison to controls for number of arm entries (Fig. 4A). Conversely, mild EAE animals were not significantly different from severe EAE animals ($p=0.480$, RM ANOVA, LSD post-hoc test). A significant phase ($F(5,80)=16.874$, $p=0.000$), and phase X group interaction ($F(10,80)=5.937$, $p=0.000$) was observed. Mild EAE animals made fewer arm entries than control animals during onset ($F(2,17)=8.987$, $p=0.001$), peak ($F(2,17)=15.625$, $p=0.000$), and recovery ($F(2,17)=37.596$, $p=0.000$). Similarly, significantly less arm entries were made by severe EAE animals in comparison to controls during onset ($F(2,17)=8.987$, $p=0.001$), peak ($F(2,17)=15.625$, $p=0.000$), and recovery ($F(2,17)=37.596$, $p=0.000$). A paired-samples t-test was conducted to compare phases of disease within each animal group. Mild EAE animals ($p=0.012$, paired t-test), and severe EAE animals ($p=0.000$, paired t-test) made significantly more arm entries during the post-phase of disease in comparison to the recovery phase. In addition to this, severe EAE animals made significantly more arm

entries during the post-phase of disease than at baseline ($p=0.015$, paired t-test). These results indicate that mild and severe EAE animal cohorts were much less active than the control cohort. This can be interpreted as fatigue-like behaviour. Lastly, a paired comparison revealed a significant difference in arm entries during baseline and pre ($p=0.024$, paired t-test); and post and baseline ($p=0.009$, paired t-test) for control animals. This is to be expected. Control animals should become accustomed to the EPM overtime and become increasingly interested in exploring other arms.

In addition, the open field was used to measure fatigue-like behaviour. This was also inferred from overall activity level. There was an overall group difference between mild EAE animals ($p=0.013$, RM ANOVA, LSD post-hoc test), and severe EAE animals ($p=0.009$, RM ANOVA, LSD post-hoc test) in comparison to controls for distance travelled (Fig. 4B). Mild EAE animals were not significantly different from severe EAE animals ($p=0.831$, RM ANOVA, LSD post-hoc test). This further suggests that severity of EAE does not impede upon fatigue-like behaviour. Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a significant phase interaction ($F(2.541,38.115)=33.627$, $p=0.000$) but not a significant phase X group interaction ($F(5.082, 38.115)=0.777$, $p=0.574$). Mild EAE animals were significantly less active than control animals during pre ($F(2,17)=3.146$, $p=0.036$), onset ($F(2,17)=6.296$, $p=0.005$), recovery ($F(2,17)=13.087$, $p=0.001$), and post-phase of disease ($F(2,17)=2.893$, $p=0.042$). Likewise, severe EAE animals were significantly less active than controls during the pre ($F(2,17)=3.146$, $p=0.035$), onset ($F(2,17)=6.296$, $p=0.004$), peak ($F(2,17)=2.921$, $p=0.027$), recovery ($F(2,17)=13.087$, $p=0.000$), and post-phase of disease ($F(2,17)=2.893$, $p=0.043$). A paired-samples t-test

was conducted to compare phases of disease within each animal group. There was a significant reduction in activity level during the pre phase of disease in comparison to the baseline phase for mild EAE animals ($p=0.008$, paired t-test), and severe EAE animals ($p=0.000$, paired t-test) in comparison to controls. Likewise mild EAE animals ($p=0.005$, paired t-test), and severe EAE animals ($p=0.012$, paired t-test) were significantly less active during onset than in the pre phase of disease. Lastly, mild EAE animals ($p=0.012$, paired t-test), and severe EAE animals ($p=0.000$, paired t-test) were significantly less active in the post-phase of disease in comparison to the baseline phase. Contrasting with the previous statements, severe EAE animals were significantly more active in the post-phase of disease in comparison to the recovery phase of disease ($p=0.004$, paired t-test). These results indicate that mild and severe EAE cohorts were much less active than the control cohort and therefore, more fatigued. Lastly, a paired comparison revealed a significant difference during baseline and pre ($p=0.000$, paired t-test); and post and baseline ($p=0.005$, paired t-test) for control animals. This suggests that control animals habituate to the open field overtime, thereby becoming less active.

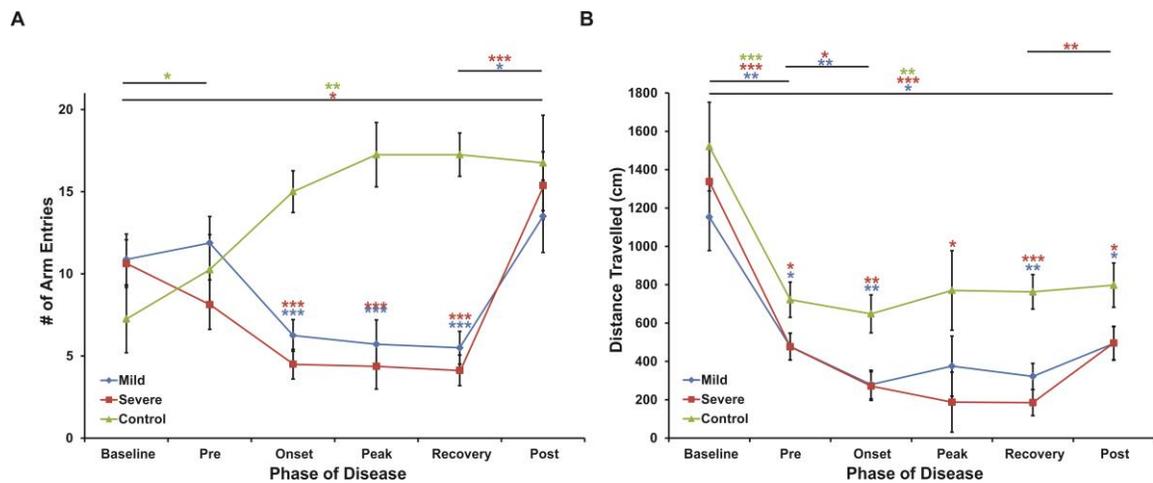


Fig. 4: Fatigue-like behaviour in mild EAE, severe EAE, and control rats. **(A)** Number of arm entries in the EPM. Overall mild EAE animals ($p=0.003$, RM ANOVA, LSD post hoc test), and severe EAE animals ($p=0.000$, RM ANOVA, LSD post hoc test) were significantly different from control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.254$, RM ANOVA, LSD post hoc test). **(B)** Distance travelled in the open field. Overall the mild EAE animals ($p=0.013$, RM ANOVA, LSD post hoc test), and severe EAE animals ($p=0.009$, RM ANOVA, LSD post hoc test) were significantly different from control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.831$, RM ANOVA, LSD post hoc test). Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between mild EAE and control rats; * indicates significance between severe EAE and control rats: and * indicates significance between mild and severe EAE rats. Error bars represent \pm SEM.

2.4.5 EAE Modulated Mechanical Allodynia

Pain threshold was inferred from sensitivity to mechanical stimulation of the hindpaw (Fig.5). Mild EAE rats ($p=0.093$, RM ANOVA, LSD post-hoc test), and severe EAE rats ($p=0.060$, RM ANOVA, LSD post-hoc test) showed no overall significant difference in comparison to control rats in regards to pain threshold. In addition, mild EAE animals were not significantly different from severe EAE animals ($p=0.064$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a significant phase interaction ($F(2,17)=5.232$, $p=0.010$) but not a significant phase X group interaction ($F(4,105,34.895)=0.930$, $p=0.459$). Interestingly, severe EAE animals showed a significant increase in pain threshold in comparison to controls during the peak phase of disease ($F(2,17)=2.817$, $p=0.039$). A paired comparison revealed that mild EAE animals ($p=0.002$, paired t-test), and severe EAE animals ($p=0.000$, paired t-test) had a significantly reduced pain threshold during the pre phase of disease in comparison to baseline measurements. Furthermore, a significantly reduced pain threshold was seen during the post-phase of disease in comparison to the baseline phase of disease in mild

EAE animals ($p=0.004$, paired t-test), and severe EAE animals ($p=0.001$, paired t-test). Contrasting with the two previous statements, mild EAE animals ($p=0.041$, paired t-test), and severe EAE animals ($p=0.030$, paired t-test) showed a significant increase in pain threshold during the peak phase in comparison with disease onset. Lastly, mild EAE animals had a significantly reduced pain threshold during the recovery phase of disease in comparison to severe EAE animals ($F(2,17)=3.666$, $p=0.026$).

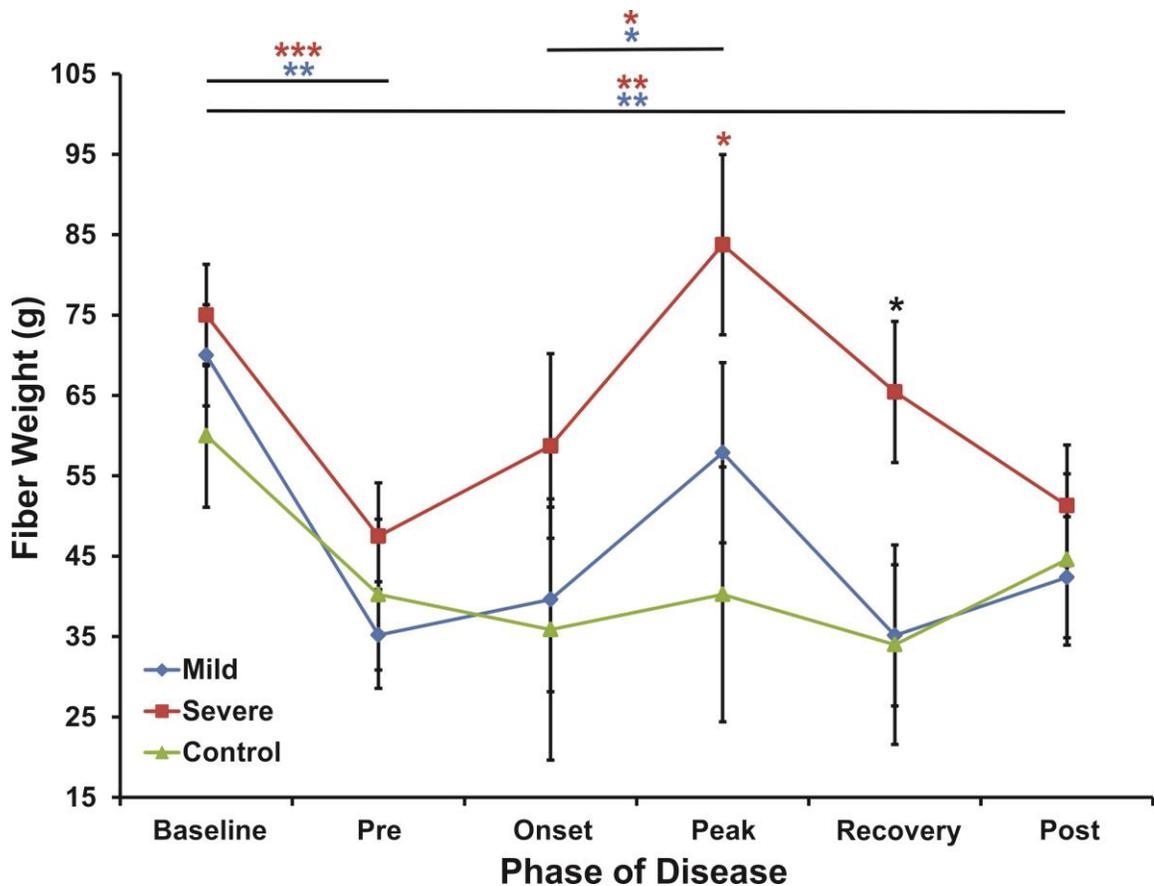


Fig. 5: Sensitivity to mechanical stimulation determined by fiber weight (g) in mild EAE, severe EAE, and control rats. Overall mild EAE animals ($p=0.093$, RM ANOVA, LSD post hoc test), and severe EAE animals ($p=0.060$, RM ANOVA, LSD post hoc test) were not significantly different from control animals. Mild EAE animals were not significantly different from severe EAE animals ($p=0.064$, RM ANOVA, LSD post hoc test). Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between mild EAE and control rats; * indicates significance between severe EAE and

control rats: and * indicates significance between mild and severe EAE rats. Error bars represent \pm SEM.

2.4.6 EAE Altered Plasma Levels of IL-1 β , IL-10, IL-12, and Anti-MBP

The concentration of 23 cytokines in the blood of mild EAE, severe EAE, and control animals were assessed. Of the 23 cytokines tested, only 8 were reliably quantified, and of these, only interleukin 1-beta (IL-1 β) (Fig. 6A), interleukin 10 (IL-10) (Fig. 6B), and interleukin 12 (IL-12) (Fig. 6C) showed significance. The concentration of IL-1 β was significantly increased in mild EAE animals in comparison to severe (F(2,17)=7.740, p=0.004) and control animals (F(2,17)=7.740, p=0.005). In addition, the concentration of IL-10 was significantly increased in mild EAE animals in comparison to severe EAE animals (F(2,17)=3.767, p=0.005). Furthermore, IL-12 was significantly increased in mild EAE animals in comparison to severe (F(2,17)=4.962, p=0.030) and control animals (F(2,17)=4.962, p=0.011).

The fluorescence intensity of anti-myelin basic protein in blood plasma from mild EAE, severe EAE, and control animals was quantified (Fig. 6D). The fluorescence intensity was significantly increased in mild EAE (F(2,16)=4.611, p=0.020), and severe EAE animals (F(2,16)=4.611, p=0.011) in comparison to control animals.

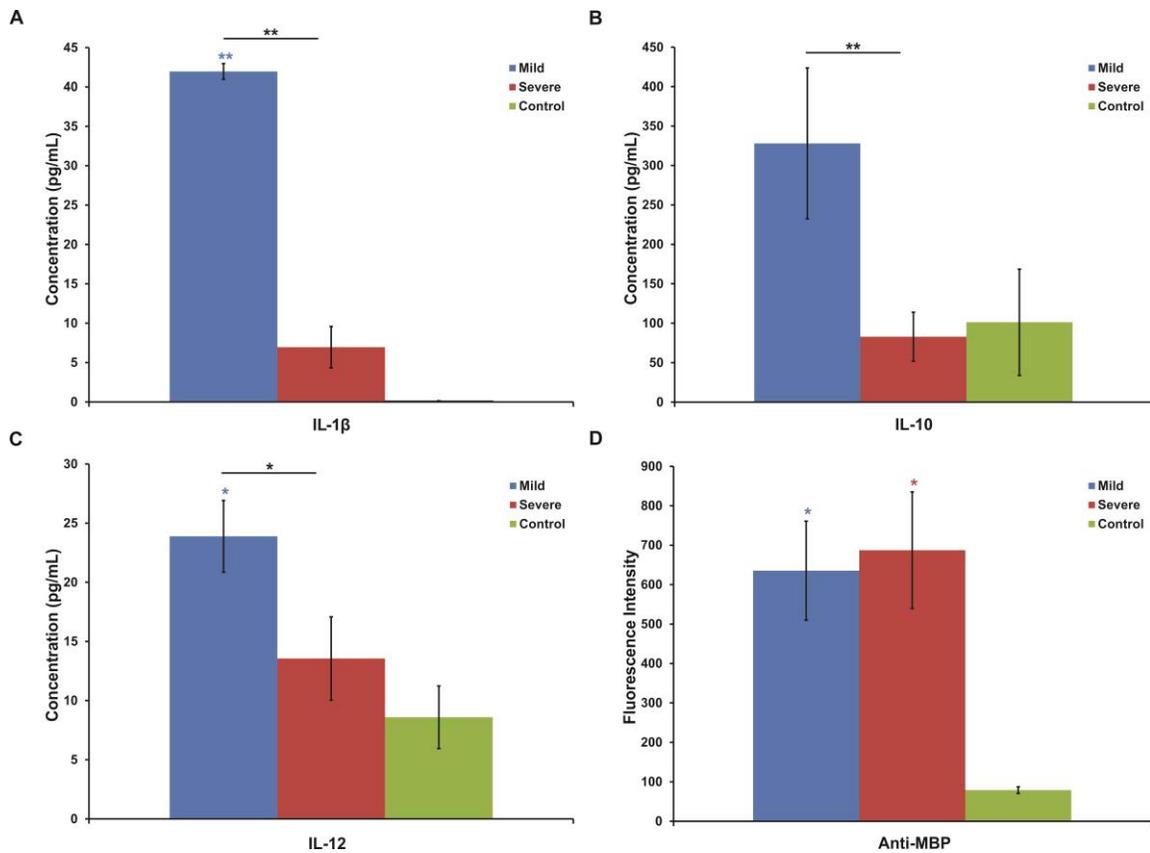


Fig. 6: Blood analysis in mild EAE, severe EAE, and control rats. (A) IL-1 β . (B) IL-10. (C) IL-12. (D). Anti-MBP. The levels of IL-1 β , and IL-12 were significantly elevated in mild EAE animals in comparison to controls. In addition, the levels of IL-1 β , IL-10, and IL-12 were significantly elevated in mild EAE animals in comparison to severe EAE animals. Asterisks indicate significances: * p <0.05, ** p <0.01, *** p <0.001. * indicates significance between mild EAE and control rats; * indicates significance between severe EAE and control rats: and * indicates significance between mild and severe EAE rats. Error bars represent \pm SEM.

2.4.7 EAE Differentially Regulated miR-146a, miR-150, and miR-155

miRNA analysis (Fig. 7) revealed that miR-146a was up-regulated in mild (F(2,6)=29.231, p =.001) and severe EAE animals (F(2,6)=29.231, p =0.000) in comparison to control animals. In addition, miR-155 was also up-regulated in mild (F(2,6)=29.052, p =0.001) and severe EAE animals (F(2,6)=29.052, p =0.000) in comparison to controls. Contrasting with miR-146a, and miR-155, miR-150 was

significantly down-regulated in mild ($F(2,6)=295.378$, $p=0.000$) and severe EAE animals ($F(2,6)=295.378$, $p=0.000$) in comparison to controls. Interestingly, miR-150 is down-regulated in mild EAE animals in comparison to severe EAE animals ($F(2,6)=295.378$, $p=0.010$).

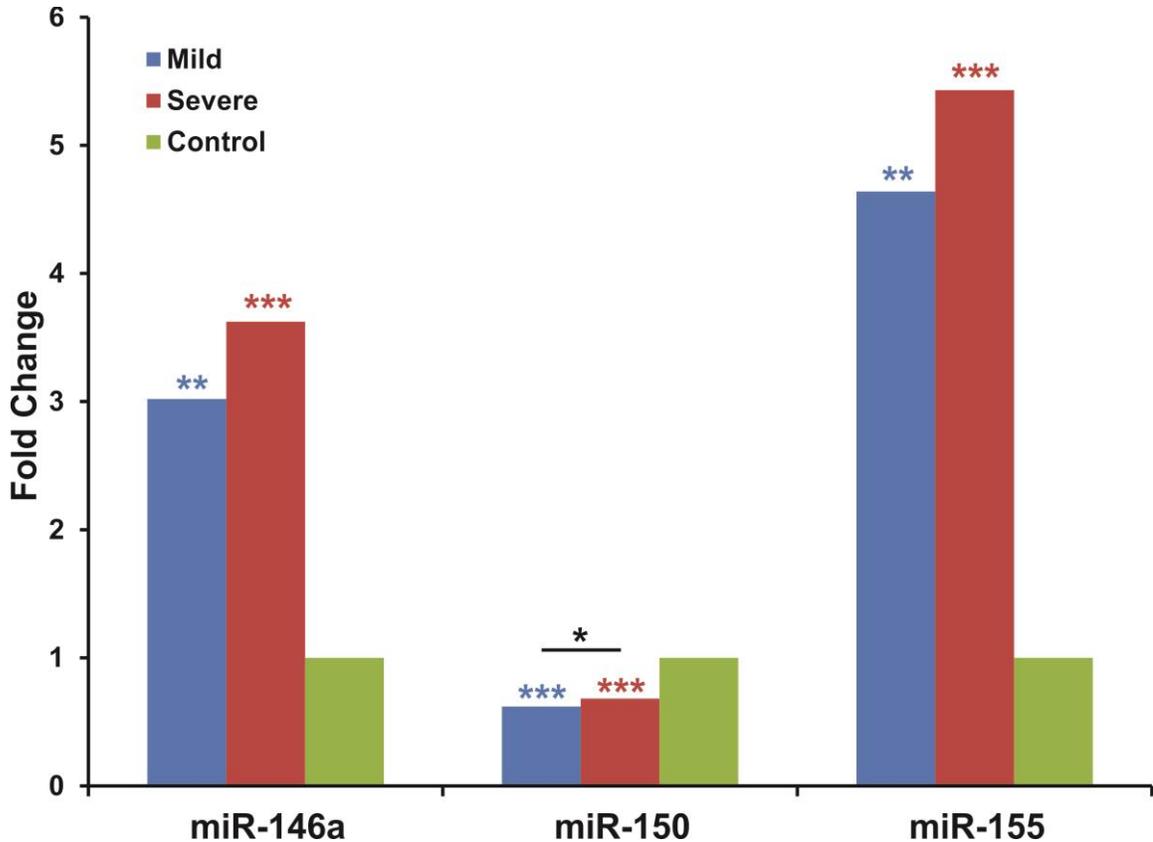


Fig. 7: miRNA analysis from spinal cord tissue in mild EAE, severe EAE, and control rats. miR-146a, and miR-155 expression were elevated in both EAE cohorts in comparison to control animals. Conversely, the expression of miR-150 was down-regulated in both EAE cohorts in comparison to control animals. Interestingly, miR-150 was significantly down-regulated in mild EAE animals in comparison to severe EAE animals. Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between mild EAE and control rats; * indicates significance between severe EAE and control rats: and * indicates significance between mild and severe EAE rats. Error bars represent \pm SEM.

2.4.8 EAE Modified Content Levels of B, Co, Mn, Hg, Pt, Te, and Tl

Hair trace elementary analysis (Fig 8) in mild EAE animals revealed reduced content of manganese (Mn) ($F(2,17)=4.402$, $p=0.018$), platinum (Pt) ($F(2,17)=5.100$, $p=0.010$), tellurium (Te) ($F(2,17)=6.317$, $p=0.003$), and thallium (Tl) ($F(2,17)=7.247$, $p=0.001$) when compared to control animals. In contrast with Mn, Pt, Te, and Tl, content levels of cobalt (Co) increased in mild EAE animals in comparison to controls ($F(2,17)=4.014$, $p=0.046$). In addition, hair element analysis revealed reduced content levels of boron (B) ($F(2,17)=7.487$, $p=0.001$), mercury (Hg) ($F(2,17)=4.881$, $p=0.006$), and Tl ($F(2,17)=7.247$, $p=0.001$) in severe EAE animals in comparison to control animals. Reduced levels of Mn ($F(2,17)=4.402$, $p=0.032$), Pt ($F(2,17)=5.100$, $p=0.029$), and tellurium (Te) ($F(2,17)=6.317$, $p=0.003$) were found in mild EAE animals in comparison to severe EAE animals. Co, on the other hand, increased in mild EAE animals in comparison to severe animals ($F(2,17)=4.014$, $p=0.020$).

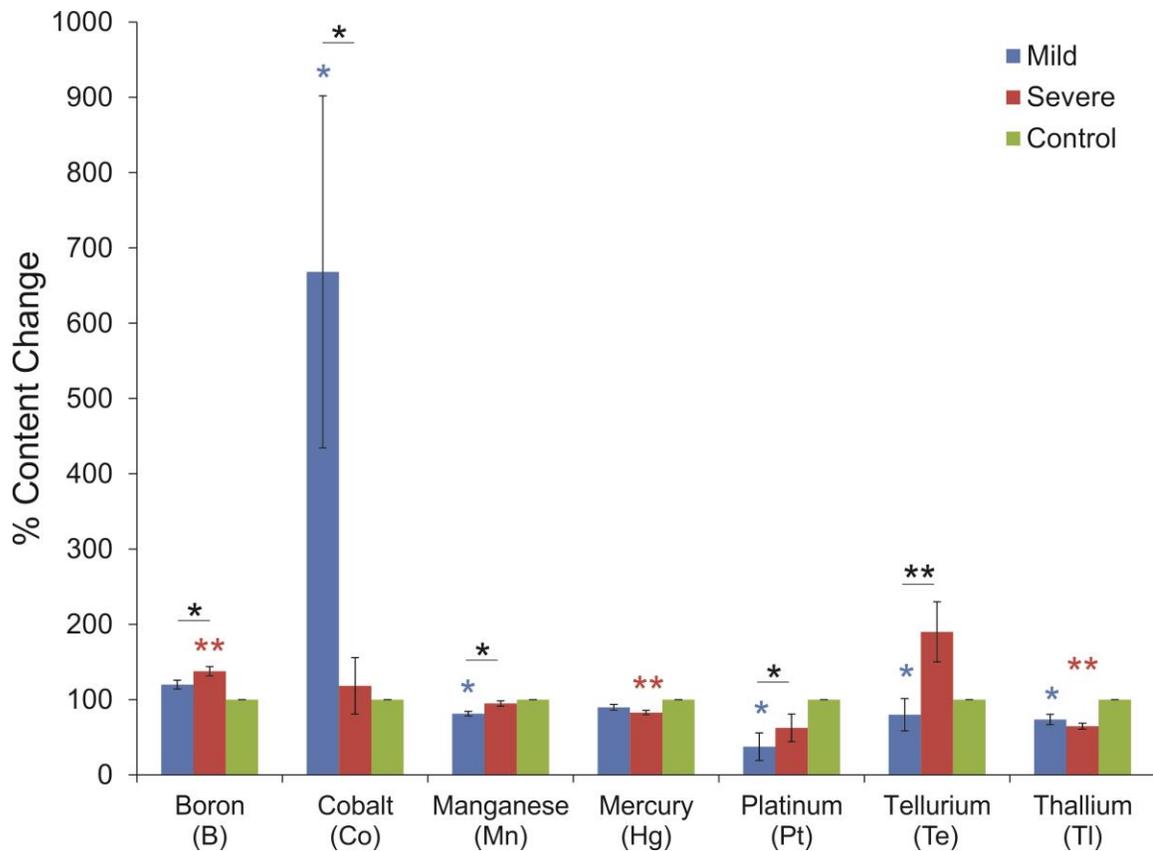


Fig. 8: Hair trace element analysis in mild EAE, severe EAE, and control rats. Increased content levels of B, Co, and Tl were observed in mild EAE animals in comparison to control rats. Conversely, decreased content levels of Mn, Pt, and Tl were found in mild EAE animals in comparison to controls. B content level was significantly increased in severe EAE animals in comparison to controls. Hg, and Tl content levels were significantly decreased in severe EAE animals in comparison to controls. Co content level significantly increased in mild EAE animals in comparison to severe EAE animals. Lastly B, Mn, Pt, and Tl content levels were significantly decreased increased in mild EAE animals in comparison to severe EAE animals. Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. * indicates significance between mild EAE and control rats; * indicates significance between severe EAE and control rats: and * indicates significance between mild and severe EAE rats. Error bars represent \pm SEM.

2.5 Discussion

In this study, behavioural, immunological, and epigenetic outcomes in mild EAE, and severe EAE were compared to control rats. Specifically, movement, anxiety, fatigue and pain were investigated as well as changes in cytokine levels, miRNA expression, and

the content level of trace elements within the hair of experimental rats in comparison to controls. The progression and recovery from EAE was assessed by the 5 point scale, 15 point scale and BBB scale. These assessment scales indicated that all EAE animals developed EAE between 8 and 14 days post immunization. All EAE animals recovered from clinical disease by day 17 post immunization. EAE animals immunized with GP69-88 developed an exacerbated disease course in comparison to animals immunized with GP69-87. Dosage of MBP did not significantly affect EAE severity. Therefore, animals immunized with GP69-87 were referred to as “mild” EAE animals, whereas animals immunized with GP69-88 were referred to as “severe” EAE animals. Fine motor performance was not significantly impaired in mild or severe EAE animals in comparison to controls. Although, a trend in motor disruption is observed in the ladder rung walk task during EAE onset in mild EAE animals. In comparison to mild EAE animals, the motor performance of severe EAE animals appears to be disrupted during the peak phase of EAE. Severity of EAE did not significantly impact motor performance. Mild and severe EAE animals display signs of anxiety and fatigue in comparison to control animals. Interestingly, there were no significant differences overall between the mild and severe EAE animals in measures of anxiety, or fatigue. Mild and severe EAE animals did not demonstrate significant mechanical allodynia in comparison to controls. Furthermore, EAE elevated the level of IL-1B, IL-12, and anti-MBP while decreasing the level of IL-10. miR-146a, miR-150, and miR-155 were differentially expressed in mild and severe EAE animals in comparison to controls. Our analysis of trace elements revealed increased content levels of boron, and cobalt in mild and severe EAE animals. In comparison to boron and cobalt, reduced content of manganese, mercury, platinum, and

thallium were observed in mild and severe EAE animals.

In terms of this experiment there were no significant differences between the mild EAE animals and controls utilizing the 5 point, 15 point or BBB scale. In contrast with the mild EAE animals, the severe EAE animals were significantly different than the control animals utilizing the 5 point, 15 point, and BBB scale. Using ANOVA, the p-value was less than 0.01 using the 5 and 15 point scale to compare severe EAE animals to controls. The BBB scale on the other hand, developed a p-value, using ANOVA, less than 0.05 comparing the severe EAE animals in comparison to the controls. Therefore, the 5 and 15 point scale showed more significance when comparing the severe EAE animals to the control animals. Surprisingly, using ANOVA, the 5-point scale showed the most significance when comparing the 5 point scale to the 15 point scale. Therefore, the 5 point scale appears to demonstrate the most significance using ANOVA in this experiment to assess EAE development and recovery. Perhaps this monophasic model of EAE does not require a complex assessment scale to measure disease activity. Most rats begin to recover from EAE 3 days after disease onset; therefore disease onset and recovery happen quickly. Due to the short period of disease seen in this EAE model, symptoms change rapidly; therefore making a less complex disease assessment scale appropriate.

Interestingly, dosage of MBP did not affect EAE severity utilizing the 5 point, 15 point, or BBB scale. Mannie and associates (1990) reported similar findings in their investigation of GP MBP in rat EAE. They noticed a ceiling effect around 25 μ g of GP MBP. In contrast with MBP dosage, the protein segment of MBP seemed to have a significant effect on EAE disease severity. In MBP-EAE experiments using LEW rats,

the 75-84 peptide sequence of GP MBP needs to be conserved in order to observe an encephalitogenic response (Mannie et al., 1990). Due to the fact that both MBP segments utilized in this experiment maintained this requirement, the supply source had to of affected the EAE outcome. Miller and associates (2007) and Olechowski and associates (2009) report that clinical progression of EAE varies between laboratories due to the supply source of myelin protein. Therefore, it is important to find a reliable supplier of MBP for EAE experiment.

The present data suggests that mild and severe EAE animals display signs of anxiety and fatigue. This is in line with human MS studies where up to 80% of patients report symptoms of anxiety and/or fatigue (Diaz-Olavarriet et al., 1990; Markowitz, 2010). Interestingly, the clinical severity of EAE is not correlated with the severity of anxiety or fatigue symptoms in this experiment. This information is in line with previous studies in human MS where the severity of behavioural symptoms did not correlate with the severity of physical disease (Janardhan et al., 2002; Lobentanz et al., 2004; Acharjee et al., 2013). In human MS, 67% of patients' report sensory impairment at some point of time during their disease course (Rae-Grant et al., 1999; Miller et al., 2004). This finding is in contrast with the data in this experiment because mild and severe EAE animals did not display significant signs of mechanical allodynia in comparison to controls. Interestingly, both mild and severe EAE animals used in this experiment show a trend towards a decrease in pain threshold during the pre phase of disease, followed by an increase in pain threshold during onset and peak phase of disease. These pain threshold findings are in line with previous EAE studies demonstrating an increased sensitivity to pain during the pre phase of disease followed by a decreased sensitivity to pain during

clinical EAE in mice using both tactile allodynia and formalin exposure (Olechowski et al., 2009; 2010; 2013). It is important to note that mild EAE animals were able to mobile properly during the peak phase of disease as confirmed by the ladder rung walking task. Therefore, the mild EAE animals increase in pain threshold is not confounded by motor impairment. In contrast with this, the mobility capabilities of severe EAE animals were impaired during the peak phase of disease in this experiment as suggested by the ladder rung walking task. Therefore, the increased pain threshold in severe EAE animals may be a reflection of the inability to mobile properly therefore making withdrawal reactions hard to carry out. Severity of EAE was not associated with severity of mechanical allodynia. Once again, this is comparable to human MS where the severity of disease does not often correlate with the severity of behavioural symptoms (Janardhan et al., 2002; Lobentanz et al., 2004; Acharjee et al., 2013).

The underlying cellular mechanisms that mediate the development of physical and behavioural symptoms in EAE were investigated through the analysis of immune cells. Specifically, blood samples collected during the post phase of EAE were analyzed for 23 cytokines, and anti-MBP. Cytokine analysis revealed that the level of IL-1 β , and IL-12 were elevated in mild and severe EAE animals in comparison to control animals. IL-1 β , and IL-12 play a role in the development of cell mediated immunity (Mancall, 2001). IL-1 β is a proinflammatory cytokine that is produced in response to pathogens (in regards to this experiment, the pathogen would be the EAE immunization) (Wesa et al., 2001). The production of IL-1 β is believed to be responsible for motor disturbances, loss of body weight, reduced food and sucrose intake, and/or decreased social interest in EAE animals (Cartmell et al., 2001; Acharjee et al., 2013). In turn, an increase in the concentration of

IL-1 β is associated with the up-regulation of IL-12 (Wesa et al., 2001). IL-12 drives cell-mediated immunity by controlling the T helper 1 response (Mancall, 2001; Wesa et al., 2001). Therefore, IL-1 β , and IL-12 play a role in the demyelination and axonal damage seen within this EAE model. These findings are comparable to human MS where IL-1 β and IL-12 have been found in acute brain MS plaques (Brosnan et al., 1995; Winghagen et al., 1995; Herx et al., 2001). Interestingly, high levels of IL-1 β and IL-12 exist in both EAE groups after a full recovery from EAE has been made. Therefore, inflammatory markers of EAE exist beyond recovery from symptomatic disease. IL-10, in comparison to IL-1 β , and IL-12 was reduced in mild EAE animals in comparison to controls. IL-10 is an anti-inflammatory cytokine that inhibits the production of T cells and killer cells (D'Andrea et al., 1993; Ozenci et al., 1999). In addition, IL-10 suppresses the secretion of IL-12 (D'Andrea et al., 1993; Ozenci et al., 1999). Therefore, IL-10 protects against IL-12 thereby reducing demyelination and axonal damage seen in EAE and MS. Lastly, anti-MBP was the final immune factor analyzed from plasma samples collected during this experiment. The fluorescence intensity was elevated in mild and severe EAE animals in comparison to controls. In human MS, 59% of patients are seropositive for anti-MBP (Olsson et al., 1990; Egg et al., 2001). Therefore anti-MBP is associated with human MS but there are other factors contributing to disease pathogenesis as well. Taken together, IL-1 β , IL-10, IL-12, and anti-MBP mediate the immune response responsible for CNS damage in EAE and human MS.

Furthermore, the present study analyzed miRNA from spinal cord tissue in mild and severe EAE animals in comparison to controls. miRNA analysis revealed that miR-150 was significantly down-regulated in mild and severe EAE animals in comparison to

controls. miR-150 is implicated in the innate immune and plays a role in B cell differentiation (Tufekci et al., 2011). In contrast with mRNA-150, miR-146a and miR-155 are up-regulated in mild and severe EAE animals in comparison to control animals. Up regulation of miR-146a, and miR-155 are found in active MS lesions (Junker et al., 2009; Koch et al., 2013). Specifically, miR-146a, and miR-155 reduce CD47 (“don’t eat me” signals) in brain cells. The reduction of CD47 is associated with demyelination and axonal damage (Junker et al., 2009). Specifically, miR-146a is believed to regulate T cell activation (Jurkin et al., 2011; Koch et al., 2013) whereas miR-155 is believed to control the release of macrophages and microglia (Junker et al., 2009). Interestingly, the expression of miR-155 is up-regulated when there is an increase in the secretion of IL-1 β (Kutty et al., 201). Therefore, an inflammatory immune cascade could cause epigenetic changes which in turn could cause an increase in susceptibility to autoimmune diseases, such as MS.

Last but not least, trace element analysis from body hair revealed increased levels of boron and cobalt in mild and severe EAE animals in comparison to controls. Elevated organ levels of aluminum, arsenic, boron, mercury, and titanium are linked with neuropathological conditions (Mezzetti et al. 1998). In contrast with boron, a high level of cobalt within the soil in an Ukrainian study was associated with fewer diagnoses of MS (Zapadniuk, 1992). Therefore, exposure to boron and cobalt are differentially associated with the development of MS. Manganese, mercury, platinum, and thallium, in contrast with boron and cobalt, decreased in content in mild and severe EAE animals in comparison to controls. Elevated soil levels of iron, manganese, mercury, and thallium are implicated in neurodegeneration (Al Hammouri et al., 2011; Farina et al., 2012).

Unfortunately, these elements were not elevated in this study. Perhaps the level of manganese, mercury, and thallium found within this study are reflective of neurodegeneration that happened during EAE development. Hair follicles grow for approximately 7-20 days in rats (Ambeskovic et al., 2013). Therefore, the hair samples collected at euthanasia may not have been present while the rats developed and recovered from EAE. Most interestingly, tellurium which was significantly elevated in severe EAE animals is an indicator of demyelination and remyelination of the peripheral nerves (Toews et al., 1999). Unfortunately, this element was not elevated in mild EAE animals. Therefore, the results in regards to the role of tellurium in EAE are ambiguous. Once again, the level of tellurium within the sampled body hair may be indicative of demyelination and remyelination that happened during EAE development and recovery. The levels of tellurium may have been affected by the period in which hair was sampled. In regards to human MS, differential levels of copper, iodine, manganese, sulfur, selenium, vanadium, and zinc have been implicated in this disease (Ryan et al., 1978; Smith et al., 1989). Further research is needed in order to understand the role of trace elements in EAE and MS.

2.6 Conclusions

Rats immunized with GP69-88 for EAE developed an exacerbated disease course in comparison to animals immunized with GP69-87. Unexpectedly, the 5 point scale represented the most sensitive measure of EAE development and recovery. Interestingly, results determined from EAE by the 5 point scale were not predictive of significant motor impairment in mild or severe EAE animals. A trend did suggest that mild EAE animals developed signs of motor impairment during EAE onset, whereas severe EAE animals

developed signs of motor impairment during the peak phase of disease. Conversely, behavioural symptoms such as anxiety, and fatigue were significantly elevated in mild and severe EAE animals in comparison to controls. The severity of EAE was not associated with the severity of these behavioural symptoms. Symptoms of mechanical allodynia were not significant in mild and severe EAE animals in comparison to controls. A trend suggested that mild and severe EAE animals display a decrease in pain threshold during the pre phase of EAE followed by an increase in pain threshold during onset and peak phase of disease. In addition, EAE elevated the level of IL-1 β , IL-12, and anti-MBP while decreasing the level of IL-10. In turn the secretion of these cytokines may affect the expression of miRNA. Specifically, miR-146a, miR-150, and miR-155 were differentially expressed in mild and severe EAE animals in comparison to controls. Our analysis of trace elements revealed increased content levels of boron, and cobalt in mild and severe EAE animals. In comparison to boron and cobalt, reduced content of manganese, mercury, platinum, and thallium were observed in mild and severe EAE animals. These elements may represent useful indicators of MS development and neuropathology.

Taken together, this study validated the hypothesis that EAE animals develop comparable physical and behavioural symptoms as seen in human MS. Therefore, this EAE model represents a useful tool to investigate targeted therapeutics for physical and behavioural aspects of human MS. In addition, this model of EAE may be useful to develop targeted therapeutics for epigenetic factors related to MS. This data in turn, may be useful for other neurological conditions associated with behavioural deficits and epigenetic markers, such as Parkinson's disease and Alzheimer's disease.

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CHAPTER 3

Experiment 2: Chronic Stress Modulates the Course of EAE

3.1 Abstract

Multiple sclerosis (MS) is characterized by central nervous system inflammation, neuronal injury and neuronal death. The causal factors contributing to onset and severity of MS are not well understood, however, stress was suggested to modulate the inflammatory processes and symptoms of this disease. Here we investigated in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, the influence of chronic stress on behavioural progression and severity of EAE. Lewis rats were immunized for monophasic EAE with MBP₆₉₋₈₈ and were exposed to chronic stress starting 7 days prior to immunization and continuing daily until day 30. The findings revealed that chronic stress modulated the course of EAE. Stress in EAE animals exaggerated EAE-induced deficits in overground locomotion and skilled walking. Interestingly, symptoms of anxiety, fatigue, and pain were not escalated in EAE + stress animals in comparison to EAE animals. In addition, stress elevated the level of interleukin 1-beta while decreasing the levels of growth related oncogene and leptin. Using trace elementary analysis of body hair to investigate metabolic homeodynamics, EAE was associated with reduced arsenic content levels in body hair, which were not further affected by stress. Stress in EAE, however, was associated by elevated chlorine, and vanadium levels. Arsenic, chlorine, and vanadium are implicated in immune function. Furthermore, spinal cord microRNA (miRNA) expression in EAE revealed elevated miR-16, miR-21, miR-142-3p, miR-142-5p, miR-146a, and miR-155 expression. Stress further elevated miR-146 and miR-155 levels, two miRNA that have been

recognized as biomarkers in human MS. These findings suggest that stress exerts synergistic effects by worsening EAE symptoms and exaggerating EAE hallmarks of inflammation, metabolism and gene regulation. Thus, stress may represent a significant risk factor affecting inflammatory processes leading to symptomatic deterioration in MS.

3.2 Introduction

Multiple sclerosis (MS) is characterized by central nervous system inflammation, neuronal injury and neuronal death. The causal factors contributing to onset and severity of MS are not well understood, however, stress was suggested to modulate the inflammatory processes and symptoms of this disease. Patients afflicted with MS frequently report that stress triggers relapses and worsens their symptoms (Ackerman et al., 2002; Buljevac et al., 2003). Interestingly, the severity or type of stressor did not correlate with the severity of MS relapse (Ackerman et al., 2002). What's more, a study completed by Mohr et al. (2000) reported the development of new lesions within the brain 8 weeks after exposure to a psychological stressor in MS patients. Therefore, stress represents one of the most potent modulators of immune function and inflammatory processes in MS.

Depending on its duration and severity, stress can disrupt immune functions and autoimmunity processes (Godbout et al., 2006; Harpaz et al., 2013). In various animal models, chronic stress in particular has been associated with an increased vulnerability to infectious disease and autoimmunity (Godbout et al., 2006; Harpaz et al., 2013). As a response to the perception of chronic stress, the hypothalamic-pituitary-adrenal (HPA) axis increases the secretion of glucocorticoids such as cortisol which inhibits the activation, proliferation and recruitment of immune cells (Barnes, 2006). In the short

term, excess glucocorticoids suppress the immune system and reduce inflammation within the peripheral nervous system (Pérez-Nievas et al., 2010). O'Connor et al. (2003) challenge this thought, suggesting that glucocorticoids do not always cause a reduction in inflammation and may in response to a specific stressor, actually cause inflammation. For example, work in an animal model demonstrated that the level of proinflammatory cytokine interleukin-1 beta (IL-1 β) does not change within various brain regions in response to a predator (Plata-Salamán et al., 2000). In addition Dinkel et al. (2003) report that excess glucocorticoids increased the inflammatory response within the hippocampus in response to a neuronal injury. Therefore, exposure to a stressful stimulus does not always induce an anti-inflammatory cascade within the brain.

Epigenetic programming may represent one avenue behind immune system modification in human MS. For example, Leung et al. (2010) report that microRNAs (miRNA) change in response to chronic stress. Specifically, miR-16 is associated with exposure to stress in humans (Katsuura et al., 2012).

Experimental autoimmune encephalomyelitis (EAE), the most commonly used animal model of MS, mimics the hallmark neuropathological features of human MS (Gold et al., 2006). Therefore, this model represents a useful tool to investigate the effect of chronic stress in MS. In addition, we can investigate the effect of chronic stress on behavioural comorbidities of MS; emphasizing anxiety, fatigue, and pain. In this study, we hypothesize that chronic stress will exacerbate clinical signs of EAE in rats. In addition, behavioural comorbidities associated with human MS will be worsened due to chronic stress exposure. Lastly, miRNA analysis may demonstrate mechanisms behind immune system modification in EAE or MS.

In this study, we investigated the outcomes of MBP-induced EAE rats exposed to mild chronic stress using a comprehensive behavioural test battery to assess a detailed temporal profile of functional loss and recovery. The outcomes of the 5 point scale, skilled walking, and behavioural comorbidities, such as anxiety, fatigue, and pain were compared. In addition, cytokine levels, hair mineral accumulation as a measure of metabolic change, and associated patterns of miRNA were analyzed in order to further profile the mechanism behind MS pathogenesis and the effects of mild chronic stress.

3.3 Materials and Methods

3.3.1 Subjects and Housing

Thirty one 8 -10 week old female Lewis (LEW) rats purchased from Charles River were used. The animals were housed in groups of two or three under standard environmental conditions (12:12 hour light/dark cycle with lights on at 7:30 AM). Animals had access to food and water *ad libitum*. Surgical and experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care at the University of Lethbridge.

3.3.2 Stress Treatment

Stress was induced in 15 LEW rats by restraint for 20 min a day for 37 consecutive days. The stress procedure began one week prior to EAE immunization and continued daily until day 30 post immunization. Animals were placed in a transparent Plexiglas cylinder (5 cm inner diameter) that maintained them in a standing position without compression of the body (Metz et al., 2005). The restraint was considered mild

compared to other experiments (McEwen, 1999, 2000). Ventilation was possible through perforated ends of the container.

3.3.3 Induction of EAE

17 LEW rats were immunized with guinea pig (GP) myelin basic protein (MBP) (segment 69-88: YGSLPQKSQRSQDENPVVHF) obtained from GenScript (Piscataway, NJ) for monophasic EAE. Experimental groups were: (1) MBP-inoculated (EAE n = 9); (2) inoculated with MBP and submitted to stress for 37 days (EAE + Stress n = 8); (3) stress for 37 days (Stress n = 7); and (4) control (Control n = 7). EAE was induced by subcutaneous immunization at the base of the tail with GP MBP₆₉₋₈₈ emulsified in Freund's adjuvant (Difco Laboratories, BD Bioscience). Freund's adjuvant was supplemented with 4-mg/ml heat killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, BD Biosciences) to make complete Freund's adjuvant (CFA). The final concentration of CFA in the emulsion was 1mg/ml. Stress and control rats were treated with CFA as described above.

3.3.4 EAE Assessment

Rats were monitored daily for weight loss and neurological signs. The Classic 5 Point Scale was used to assess EAE clinical disease. Signs of EAE were graded on the following 5 point scale: Grade 0, no clinical signs; Grade 1, paralyzed tail; Grade 2, loss in coordinated movements; hind limb paresis; Grade 3, both hind limbs paralyzed; Grade 4: forelimbs paralyzed; Grade 5, moribund (Stromnes et al., 2006).

3.3.5 Behavioural Testing

3.3.5.1 Ladder Rung Walking Task

The ladder rung task was used to demonstrate the effect of EAE on skilled motor movements (Metz et al., 2002; 2009). This task was used before EAE immunizations (baseline), day 5 after immunization, onset of EAE, symptom peak of EAE, first day of full recovery from EAE, and 10 days post full recovery. Prior to baseline, the rats were trained to cross a 1 m horizontal ladder with a variety of irregularly spaced rungs. The rungs were placed at random 0.5 cm to 5 cm apart. The ladder design was maintained for all testing days. During testing, the rats were filmed crossing the ladder rung in 3 sessions. Steps and errors of the left and right hindlimbs on the ladder rung were determined based on a 7-category rating scale (Metz et al., 2002; 2009; See Appendix C). Using the foot fault scoring system, an error is considered a score of 0, 1, or 2 (Metz et al., 2002; 2009).

3.3.5.2 Elevated Plus Maze

The Elevated Plus Maze (EPM) was used to test the emotional state of animals (Lister, 1987). This task was used before EAE immunizations (baseline), day 5 after immunization, onset of EAE, symptom peak of EAE, first day of full recovery from EAE, and 10 days post full recovery. Rats were allowed to freely explore a Plexiglas plus-shaped maze that consists of open and closed arms for 5 minutes. The apparatus consists of two opposed open arms measuring 50 × 10 cm, crossed at right angle with two opposed arms of the same size. The latter are enclosed by walls 40 cm high, except for the entrance. The four arms delimited a central area of 10 cm². The whole apparatus is elevated 50-cm above the floor. The total time (in seconds) spent in the closed arm was

calculated. In addition, the total number of entries into all arms or specifically the open arms was calculated.

3.3.5.3 Open Field Test

Rats were placed in the middle of a clear, plastic box (36 x 36 cm) with surrounding infrared sensors before EAE immunizations (baseline), day 5 after immunization, onset of EAE, symptom peak of EAE, first day of full recovery from EAE, and 10 days post full recovery. Rats were left in the open field for a total of 10 minutes in conditions of low noise and dim lighting. Total horizontal distance (in cm) was tracked by the infrared sensor system. Total horizontal distance travelled was used as an indicator of overall activity level or fatigue.

3.3.5.4 Mechanical Allodynia

A set of calibrated von Frey hair monofilaments were used daily to assess sensitivity to punctate mechanical stimuli (Olechowski et al., 2009; 2013). Rats were placed in a clear Plexiglas chamber on an elevated wire mesh screen. Calibrated von Frey hair filaments were applied to the plantar surface of each hind paw in the ascending order of bending force (range: 2.0 g – 100.0 g). Each hair was applied 5 times per paw, and the number of nocifensive responses (vigorous shaking, prolonged lifting, licking or biting of the stimulated paw) was recorded. The monofilament which produced nocifensive responses greater than 60% of the time was taken as the “threshold.”

3.3.6 Tissue Collection

Rats were anesthetized and blood samples were collected by cardiac puncture on day thirty-one or day thirty-three post EAE immunization. Following this, the rats were

sacrificed by intracardiac infusion with 0.2 ml of sodium pentobarbital (Euthansol, CDMV Inc., Québec, Canada). After cardiac arrest, the animals were decapitated using a guillotine and the spinal cord was collected and flash-frozen for further analysis. In addition hair samples were also collected and stored for further analysis.

3.3.6.1 Blood Analysis

Following blood collection, samples were kept on ice for 30 min and then centrifuged at 1000 g for 10 min at 4°C to obtain plasma. Plasma samples were analyzed by Eve Technologies, Calgary, AB to determine the level of 23 cytokines. In addition, the concentration of anti-MBP was confirmed by Mitogen advanced Diagnostic, Calgary, AB.

3.3.6.2 miRNA Expression Analysis

MicroRNAs expression analysis was done using Illumina GAIIX genomic analyzer (PlantBiosis, Lethbridge). Briefly, base calling and demultiplexing was completed using CASAVA 1.8.1 software pipeline with default settings. Short read quality was examined using FastQC software. Adapters were trimmed using cutadapt software (<http://code.google.com/p/cutadapt/>). FastQC quality check was performed after trimming. MiRNA detection and counting was performed using standalone MicroRazerS version 1.0 (Emde et al 2010). Statistical comparisons were done using DESeq bioconductor package (Anders & Huber 2010).

3.3.6.3 Hair Trace Elementary Analysis

Approximately 0.5 g of hair was collected from the abdomen and back post-mortem from each rat. The hair samples were stored in 2-ml Eppendorf tubes at room

temperature. Hair trace elementary analysis was performed by CanAlt Health Laboratories (Ontario, Canada). Hair samples were cut into small pieces using clean stainless steel scissors. About 300 ± 5 mg was transferred into tarred, labelled centrifuge tubes, and the exact weight was recorded. To each sample digestion tube, 3.0 ml of reagent-grade nitric acid (HNO₃) was added. Samples were incubated for 25 minutes. Samples were then subjected to acid microwave digest, in order to stabilize the elements of interest. The digestate solution was analyzed for amounts of mineral element and trace metals by inductively coupled plasma mass spectrometry. Sample results were quantified by comparison with calibration solutions of known concentrations. To control for metal trace contamination, fabric was cut with the same pair of scissors and used as control for hair sample analysis.

3.3.7 Statistical Analysis

All bar and line graphs were plotted as mean \pm standard error of mean (SEM) and statistical analysis was carried out using SPSS version 21.0 software (IBM, USA). Statistical differences were compared between EAE, EAE + stress, stress, and control animals by one way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) post-hoc test, repeated measures analysis of variance (RM ANOVA) with LSD post-hoc test, and paired t-test as necessary. Statistical significance was set at 0.05.

3.4 Results

3.4.1 Chronic Stress Exacerbates Clinical Severity of EAE

LEW rats immunized with MBP₆₉₋₈₈ were scored daily using the 5 point scale for signs of neurological impairment (Fig. 1). The first assessment made, addressed observable differences between the two EAE cohorts. EAE animals developed clinical signs of disease between day 10 and day 16 post immunization. EAE + stress animals developed EAE one day earlier, day 9 and showed symptoms until day 16 post immunization. On average EAE + stress animals presented signs of disease slightly earlier (day 11 vs. day 11.4, respectively) and severity of EAE was greater between day 11 and 14 post immunization in comparison to EAE group. EAE animals initially presented with partial paralysis of the tail (Grade 0.5) and later progressed to a paralyzed tail and/or loss in coordinated movements; hind limb paresis (Grade 1, or 2, respectively). EAE + stress animals initially presented with partial paralysis of the tail (Grade 0.5) and progressed to loss in coordinated movements; hind limb paresis and/or both hind limbs paralyzed (Grade 2, or 3, respectively). Therefore, EAE + stress animals presented with a more severe form of EAE than EAE animals. There were significant overall group differences between EAE ($p < 0.01$, RM ANOVA, LSD post-hoc test) and EAE + stress animals ($p < 0.01$, RM ANOVA, LSD post-hoc test) in comparison to stress or control animals. Conversely, there was no overall group difference between EAE and EAE + stress animals ($p = 0.053$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore degrees of freedom (df's) were corrected with Greenhouse-Geisser. This test revealed a significant effect of day

($F(2.451,66.168)=21.424$, $p=0.000$), and day X group interaction ($F(7.352,66.168)=7.827$, $p=0.000$). Stress and control animals did not present with signs of EAE.

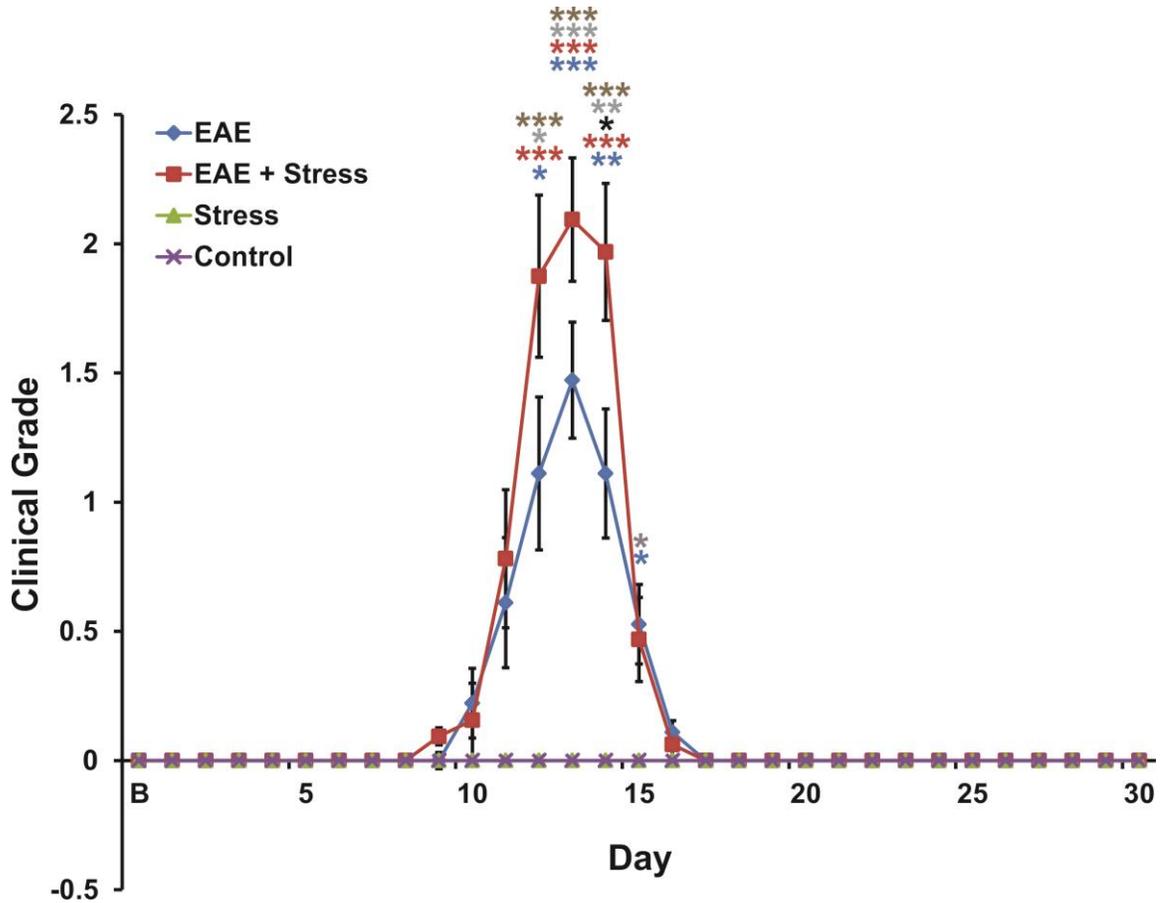


Fig. 1: Progression of clinical deficits in EAE, EAE + stress, stress, and control cohorts of rats. There were significant overall group differences between EAE ($p<0.01$, RM ANOVA, LSD post-hoc test) and EAE + stress animals ($p<0.01$, RM ANOVA, LSD post-hoc test) in comparison to stress or control animals. There was no overall group difference between EAE, and EAE + stress animals ($p=0.053$, RM ANOVA, LSD post-hoc test). Control and stress animals did not develop EAE. Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.4.2 Chronis Stress Impacts Fine Motor Skills

The ladder rung task was used to demonstrate the effect of EAE on skilled motor movements (Metz et al., 2002; 2009). Figure 2A addresses LHL Foot fault scores. Overall there were no significant group differences between EAE animals in comparison to stress, or control animals ($p > 0.05$, RM ANOVA, LSD post-hoc test). EAE + stress animals, on the other hand, were significantly different from EAE ($p = 0.29$, RM ANOVA, LSD post-hoc test) and control animals ($p = 0.026$, RM ANOVA, LSD post-hoc test). EAE + stress animals were not however, significantly different in comparison to the stress animals ($p = 0.100$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(1.681, 42.033) = 3.587$, $p = 0.008$), and phase X group interaction ($F(1.681, 42.033) = 5.330$, $p = 0.001$). Interestingly, Stress animals had a significantly reduced LHL foot fault score in comparison to EAE + stress animals ($F(3, 27) = 3.587$, $p = 0.022$), and controls ($F(3, 27) = 3.587$, $p = 0.004$) at baseline. Acute stress appears to affect fine motor movement of the LHL. EAE + stress animals had significantly reduced LHL foot fault scores in comparison to EAE ($F(3, 27) = 6.203$, $p = 0.002$), stress ($F(3, 27) = 6.203$, $p = 0.002$), and controls ($F(3, 27) = 6.203$, $p = 0.001$) during the peak phase of disease. Therefore, skilled motor movements of the LHL were more affected in EAE + stress animals in comparison to EAE animals during the peak phase of disease. A paired-samples t-test was conducted to compare phases of disease within each animal group. There was a significant difference in the LHL foot fault scores for onset and peak ($p = 0.049$, paired t-test); peak and recovery ($p = 0.046$, paired t-test); recovery and post

($p=0.018$, paired t-test); and post and baseline ($p=0.000$, paired t-test) for EAE + stress animals.

Figure 2B addresses RHL foot fault scores. There were no overall group differences between EAE animals in comparison to stress or control animals in regards to RHL foot fault scores ($p>0.05$, RM ANOVA, LSD post-hoc test). EAE + stress animals, on the other hand were significantly different in comparison to EAE ($p=0.030$, RM ANOVA, LSD post-hoc test), stress ($p=0.003$, RM ANOVA, LSD post-hoc test), and control animals ($p=0.035$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(1.867,46.683)=6.239$, $p=0.005$), and phase X group interaction ($F(5.602,46.683)=5.483$, $p=0.000$). EAE + stress animals had significantly decreased RHL foot fault scores in comparison to EAE ($F(3,27)=7.760$, $p=0.001$), stress ($F(3,27)=7.760$, $p=0.001$), and control animals ($F(3,27)=7.760$, $p=0.000$) during the peak phase of disease. Therefore, skilled motor movements of the RHL were more affected in EAE + stress animals in comparison to EAE animals during the peak phase of disease. In contrast with this, significantly increased RHL scores were observed during the pre ($F(3,27)=2.813$, $p=0.008$), and recovery ($F(3,27)=2.940$, $p=0.032$) phase of disease in EAE + stress animals in comparison to controls. A disease phase comparison demonstrated a significant increase in RHL foot fault scores during the recovery phase of disease in comparison to the peak phase for EAE + stress animals ($p=0.028$, paired t-test). Therefore, the RHL motor abilities of EAE + stress animals recovered rapidly after disease amelioration. EAE animals had significantly increased RHL foot fault scores in comparison to control animals during the recovery phase ($F(3,27)=2.940$, $p=0.009$). In

addition a significant increase in RHL foot fault scores was seen during the recovery phase of disease in comparison to the peak phase of disease in EAE animals ($p=0.041$, paired t-test). Therefore, the RHL motor capabilities of EAE animals recovered rapidly after symptomatic EAE.

Figure 2C demonstrates LHL error rate. There were no overall significant group differences between EAE and stress or control animals ($p>0.05$, RM ANOVA, LSD post-hoc test). Overall, EAE + stress animals were significantly different than the EAE ($p=0.006$, RM ANOVA, LSD post-hoc test), stress ($p=0.006$, RM ANOVA, LSD post-hoc test), and control animals ($p=0.001$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(1.631,40.773)=8.662$, $p=0.001$), and phase X group interaction ($F(4.893,40.773)=9.3343$, $p=0.000$). Specifically, EAE + stress animals made significantly more LHL errors than EAE ($F(3,27)=4.50$, $p=0.000$), stress ($F(3,27)=4.50$, $p=0.000$), and controls ($F(3,27)=4.50$, $p=0.000$) in the peak phase of disease. In contrast with this, EAE + stress animals made significantly less LHL errors than stress animals during the recovery phase of disease ($F(3,27)=2.607$, $p=0.029$). This was followed by an increase in LHL errors made by EAE + stress animals during the post phase of disease in comparison to control animals ($F(3,27)=1.886$, $p=0.040$). Therefore, EAE + stress animals had significantly more trouble making correct LHL foot placements in comparison to the other groups. A paired-samples t-test was conducted to compare phases of disease within each animal group. There was a significant difference in the LHL error rate for onset and peak ($p=0.016$, paired t-test); peak and recovery ($p=0.014$, paired t-test); and recovery and post ($p=0.040$, paired t-test) for EAE + stress animals.

Figure 2D demonstrates RHL error rate. There were no overall significant differences in number of RHL errors between EAE and stress or control animals ($p > 0.05$, RM ANOVA, LSD post-hoc test). EAE + stress animals on the other hand, made significantly more RHL errors overall than EAE ($p = 0.011$, RM ANOVA, LSD post-hoc test), stress ($p = 0.001$, RM ANOVA, LSD post-hoc test), and control animals ($p = 0.001$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(1.637, 40.929) = 7.207$, $p = 0.004$), and phase X group interaction ($F(4.911, 40.929) = 6.093$, $p = 0.000$). EAE + stress animals made significantly more RHL errors than EAE ($F(3, 27) = 8.548$, $p = 0.001$), stress ($F(3, 27) = 8.548$, $p = 0.000$), and controls ($F(3, 27) = 8.548$, $p = 0.000$) during the peak phase of disease. EAE animals on the other hand, made significantly more RHL errors during baseline in comparison to the post phase of disease ($p = 0.045$, paired t-test). Lastly, significantly less RHL errors were made during the recovery phase of disease in comparison to the peak phase by EAE + stress animals ($p = 0.023$, paired t-test). Therefore EAE + stress animal's RHL skilled motor movements were significantly more affected than EAE animals.

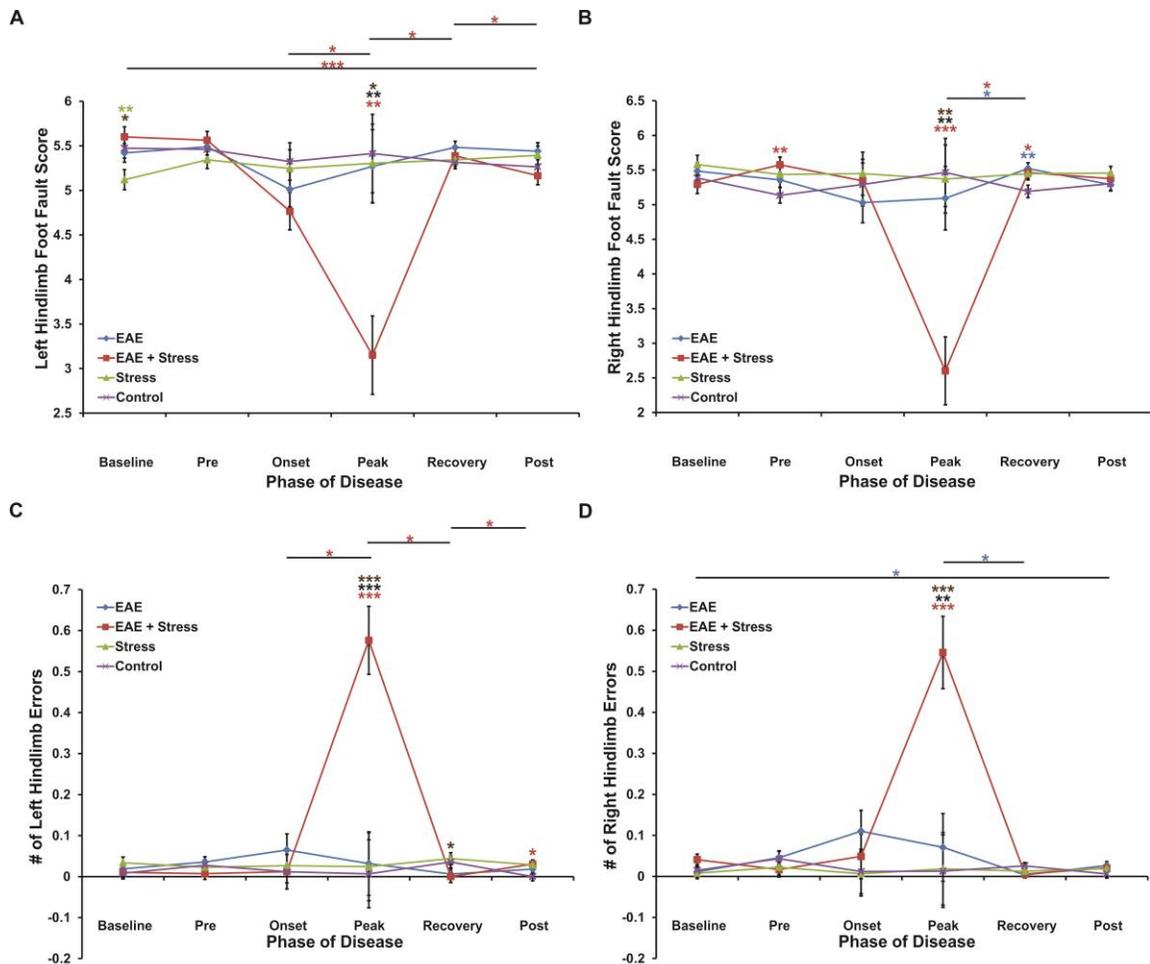


Fig. 2: Ladder rung walking task analysis in EAE, EAE + stress, stress, and control cohorts of rats. **(A)** Left hindlimb foot fault score. Overall there were no significant group differences between EAE animals in comparison to stress, or control animals ($p > 0.05$, RM ANOVA, LSD post-hoc test). EAE + stress animals were significantly different than EAE ($p = 0.29$, RM ANOVA, LSD post-hoc test) and control animals ($p = 0.026$, RM ANOVA, LSD post-hoc test). EAE + stress animals were not significantly different in comparison to stress animals ($p = 0.100$, RM ANOVA, LSD post-hoc test). **(B)** Right hindlimb foot fault score. There were no overall group differences between EAE animals in comparison to the stress or control animals ($p > 0.05$, RM ANOVA, LSD post-hoc test). EAE + stress animals were significantly different from the EAE ($p = 0.030$, RM ANOVA, LSD post-hoc test), stress ($p = 0.003$, RM ANOVA, LSD post-hoc test), and control animals ($p = 0.035$, RM ANOVA, LSD post-hoc test). **(C)** Left hindlimb Error rate. There were no overall significant differences between EAE ($p > 0.05$, RM ANOVA, LSD post-hoc test) and stress or control animals. EAE + stress animals were significantly different than the EAE ($p = 0.006$, RM ANOVA, LSD post-hoc test), stress ($p = 0.006$, RM ANOVA, LSD post-hoc test), and control animals ($p = 0.001$, RM ANOVA, LSD post-hoc test). **(D)** Right hindlimb error rate. EAE animals were not significantly different from stress or control animals ($p > 0.05$, RM ANOVA, LSD post-hoc test). EAE + stress

animals were significantly different than the EAE ($p=0.011$, RM ANOVA, LSD post-hoc test), stress ($p=0.001$, RM ANOVA, LSD post-hoc test), and control animals ($p=0.001$, RM ANOVA, LSD post-hoc test). Asterisks indicate significances: $*p<0.05$, $**p<0.01$, $***p<0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.4.3 Chronic Stress Does Not Increase Anxiety in EAE Animals

The EPM is used to measure anxiety-like behaviour in rodents (Lister, 1987).

Figure 3A demonstrates time spent in the closed arm in seconds. Overall EAE animals ($p=0.035$, RM ANOVA, LSD post-hoc test), and EAE + stress animals ($p=0.041$, RM ANOVA, LSD post-hoc test) were significantly different from control animals in regards to time spent in the closed arm. EAE ($p=0.489$, RM ANOVA, LSD post-hoc test) and EAE + stress animals ($p=0.505$, RM ANOVA, LSD post-hoc test) were not significantly different from the stress animals. In addition EAE animals were not significantly different in comparison to EAE + stress animals ($p=0.996$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(3.467,86.678)=7.729$, $p=0.000$), and phase X group interaction ($F(10.401,86.678)=6.093$, $p=0.000$). EAE ($F(3,27)=3.640$, $p=0.030$), and EAE + stress animals ($F(3,27)=3.640$, $p=0.004$) spent significantly more time in the closed arm during the peak phase of disease in comparison to controls. In addition, EAE animals spent significantly more time in the closed arm in comparison to stress ($F(3,27)=11.314$, $p=0.001$), and control animals ($F(3,27)=11.314$, $p=0.000$) during the recovery period. Likewise, EAE + stress animals spent significantly more time in the closed arm in comparison to stress ($F(3,27)=11.314$, $p=0.000$), and control animals

($F(3,27)=11.314$, $p=0.000$) during the recovery period. Using a paired comparison, EAE animals spent significantly less time in the closed arm during the post phase of disease in comparison to the recovery phase ($p=0.015$, paired t-test). Furthermore, EAE ($F(3,27)=4.094$, $p=0.010$), and EAE + stress animals ($F(3,27)=4.094$, $p=0.003$) spent significantly more time in the closed arm during the post phase of disease in comparison to controls. A paired comparison revealed that EAE + stress animals spent significantly less time in the closed arm during the post phase of disease in comparison to the recovery phase ($p=0.010$, paired t-test). Interestingly, control animals spent significantly less time in the closed arm during the post phase of disease in comparison to stress animals ($p=0.001$, t-test). In addition, stress animals spent significantly less time in the closed arm during recovery than in the peak phase of disease ($p=0.006$, paired t-test). Therefore, EAE + stress animals exhibit a temporary recovery from anxiety during the recovery phase of disease and then make a relapse. Chronic stress is associated with an increase in anxiety behaviour in rats (Chiba et al., 2012). Last but not least, control animals spent significantly more time in the closed arm during baseline than the post phase of disease ($p=0.020$, paired t-test). This is to be expected. Control animals should demonstrate reduced anxiety over time when being exposed to the same environment continuously.

Furthermore, the number of entries into the open arm (Fig. 3B) was calculated as an indicator of anxiety-like behaviour. Overall, EAE animals were significantly different than control animals in regards to open arm entries ($p=0.045$, RM ANOVA, LSD post-hoc test). Conversely, EAE animals were not significantly different in comparison to EAE + stress ($p=0.838$, RM ANOVA, LSD post-hoc test) or stress animals ($p=0.380$, RM ANOVA, LSD post-hoc test). Similarly, EAE + stress animals were not significantly

different from stress or control animals ($p > 0.05$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(3.372, 84.293) = 7.424$, $p = 0.000$), and phase X group interaction ($F(10.115, 84.293) = 3.238$, $p = 0.001$). EAE animals made significantly fewer entries into the open arm during peak ($F(3, 27) = 3.651$, $p = 0.014$), recovery ($F(3, 27) = 8.016$, $p = 0.003$), and post phase ($F(3, 27) = 3.119$, $p = 0.031$) than control animals. During the peak ($F(3, 27) = 3.651$, $p = 0.006$), and recovery phase of disease ($F(3, 27) = 8.016$, $p = 0.003$), EAE + stress animals made fewer open arm entries than control animals. Likewise, less entries were made into the open arm by EAE ($F(3, 27) = 8.016$, $p = 0.001$), and EAE + stress animals ($F(3, 27) = 8.016$, $p = 0.001$), in comparison to the stress animals during the peak phase of disease. A paired-samples t -test was conducted to compare phases of disease within each animal group. There was a significant difference in the number of open arm entries during pre and onset ($p = 0.032$, paired t -test); and recovery and post ($p = 0.009$, paired t -test) for EAE animals. EAE + stress animals made significantly more open arm entries during the post phase of disease in comparison to the recovery phase of disease ($p = 0.014$, paired t -test). Interestingly, the stress animals made significantly less entries into the open arm during the post phase of disease in comparison to controls ($F(3, 27) = 3.119$, $p = 0.007$). Lastly, the stress animals made significantly more open arm entries during the recovery phase of disease in comparison to the peak phase ($p = 0.042$, paired t -test). Therefore, EAE + stress animals exhibit a temporary recovery from anxiety during the recovery phase of disease and then make a relapse. Like previously stated, chronic stress is associated with an increase in anxiety behaviour in rats (Chiba et al., 2012).

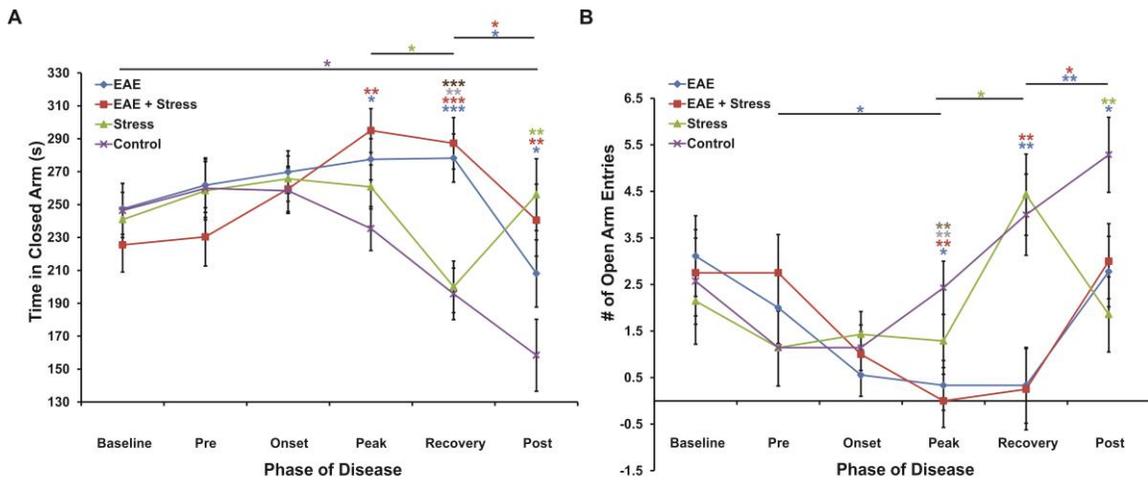


Fig. 3: Anxiety-like behaviour in EAE, EAE + stress, stress, and control cohorts of rats. **(A)** Time spent in the closed arm of the EPM. Overall EAE animals ($p=0.035$, RM ANOVA, LSD post-hoc test), and EAE + stress animals ($p=0.041$, RM ANOVA, LSD post-hoc test) were significantly different from control animals. EAE ($p=0.489$, RM ANOVA, LSD post-hoc test) and EAE + stress animals ($p=0.505$, RM ANOVA, LSD post-hoc test) were not significantly different from stress animals. In addition EAE animals were not significantly different in comparison to EAE + stress animals ($p=0.996$, RM ANOVA, LSD post-hoc test). **(B)** Number of open arm entries in the EPM. Overall, EAE animals were significantly different than control animals ($p=0.045$, RM ANOVA, LSD post-hoc test). Conversely, EAE animals were not significantly different in comparison to EAE + stress ($p=0.838$, RM ANOVA, LSD post-hoc test) or stress animals ($p=0.380$, RM ANOVA, LSD post-hoc test). Similarly, EAE + stress animals were not significantly different from stress or control animals ($p>0.05$, RM ANOVA, LSD post-hoc test). Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.4.4 Chronic Stress Does Not Increase Fatigue in EAE Animals

Total arm entries in the EPM (Fig. 4A) were used to determine overall activity level which could be used as an indicator of fatigue. Overall EAE animals ($p=0.026$, RM ANOVA, LSD post-hoc test) and EAE + stress animals ($p=0.018$, RM ANOVA, LSD

post-hoc test) were significantly different from control animals in regards to number of arm entries. EAE ($p=0.105$, RM ANOVA, LSD post-hoc test), and EAE + stress animals ($p=0.074$, RM ANOVA, LSD post-hoc test) were not significantly different in comparison to stress animals. In addition, EAE animals were not significantly different in comparison to EAE + stress animals ($p=0.805$, RM ANOVA, LSD post-hoc test).

Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(3.565,89.135)=9.982$, $p=0.000$), and phase X group interaction ($F(10.696,89.135)=4.099$, $p=0.000$). EAE animals made significantly less arm entries during onset than control animals ($F(3,27)=2.445$, $p=0.021$). In addition, EAE animals made significantly fewer arm entries in the peak ($F(3,27)=5.554$, $p<0.05$), and recovery phase of disease ($F(3,27)=13.392$, $p<0.01$) in comparison to stress and control animals. Likewise, EAE + stress animals made significantly fewer arm entries in the peak ($F(3,27)=5.554$, $p<0.01$), and recovery phase of disease ($F(3,27)=13.392$, $p<0.001$) in comparison to stress and control animals. A paired-samples t-test was conducted to compare phases of disease within each animal group. There was a significant difference in the number of arm entries during pre and onset ($p=0.036$, paired t-test); onset and peak ($p=0.041$, paired t-test); and recovery and post ($p=0.001$, paired t-test) for EAE animals. In addition, a paired comparison revealed significant differences during pre and onset ($p=0.037$, paired t-test); onset and peak ($p=0.007$, paired t-test); and recovery and post ($p=0.006$, paired t-test) for EAE + stress animals. Interestingly, stress animals made significantly less arm entries during the post phase of disease in comparison to controls ($p=0.045$, t-test). Additionally, stress animals made significantly more arm entries during the recovery phase of disease in comparison

to the peak phase ($p=0.023$, paired t-test). In our experiment, chronic stress seems to cause fatigue.

In addition, the open field was used to measure fatigue-like behaviour. Fatigue behaviour is inferred from distance travelled (Fig. 4B) in the open field. EAE animals were not significantly different from EAE + stress, stress, or control animals in regards to distance travelled ($p>0.05$, RM ANOVA, LSD post-hoc test). EAE + stress animals, on the other hand, were overall significantly different from control animals ($p=0.032$, RM ANOVA, LSD post-hoc test). Conversely, EAE + stress animals were not significantly different from the stress animals ($p=0.516$, RM ANOVA, LSD post-hoc test).

Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(2.685,87.587)=21.468$, $p=0.000$), and phase X group interaction ($F(8.054,87.587)=2.378$, $p=0.025$). At disease onset, control animals were significantly more active than stress animals ($F(3,27)=1.941$, $p=0.034$). Therefore, acute stress causes fatigue in our animals. Additionally, EAE animals travel significantly less distance than control animals during the peak ($F(3,27)=8.087$, $p=0.001$), and recovery phase of disease ($F(3,27)=8.087$, $p=0.011$). EAE animals were also less active than stress animals in the peak phase ($F(3,27)=8.087$, $p=0.043$). Similarly, EAE + stress animals travel significantly less distance than control animals during the peak ($F(3,27)=8.087$, $p=0.000$), and recovery phase of disease ($F(3,27)=8.087$, $p=0.002$). Significantly less distance is travelled by EAE + stress animals in comparison to the stress animals during the peak phase ($F(3,27)=8.087$, $p=0.008$). Interestingly, EAE + stress animals were significantly less active than EAE ($F(3,27)=4.315$, $p=0.026$), and control animals ($F(3,27)=4.315$, $p=0.004$) during the post phase of disease. In addition,

stress animals were significantly less active than control animals during the post phase ($F(3,27)=4.315, p=0.015$). This data suggests that chronic stress causes chronic fatigue. A paired-samples t-test was conducted to compare phases of disease within each animal group. There was a significant difference in distance travelled during pre and onset ($p=0.003$, paired t-test); recovery and post ($p=0.007$, paired t-test); and post and baseline ($p=0.035$, paired t-test) for EAE animals. Additionally, a significant difference was seen during baseline and pre ($p=0.015$, paired t-test); peak and recovery ($p=0.037$, paired t-test); recovery and post ($p=0.009$, paired t-test); and post and baseline ($p=0.014$, paired t-test) for EAE + stress animals. As expected, significant changes were seen during baseline and pre ($p=0.001$, paired t-test); pre and onset ($p=0.015$, paired t-test); and post and baseline ($p=0.001$, paired t-test) for the stress animals. Furthermore, a significant increase in distance travelled occurred during the baseline phase of disease in comparison to the post phase of disease for control animals ($p=0.027$, paired t-test). This data suggests that stress and control animals habituate to the open field overtime, therefore becoming less active.

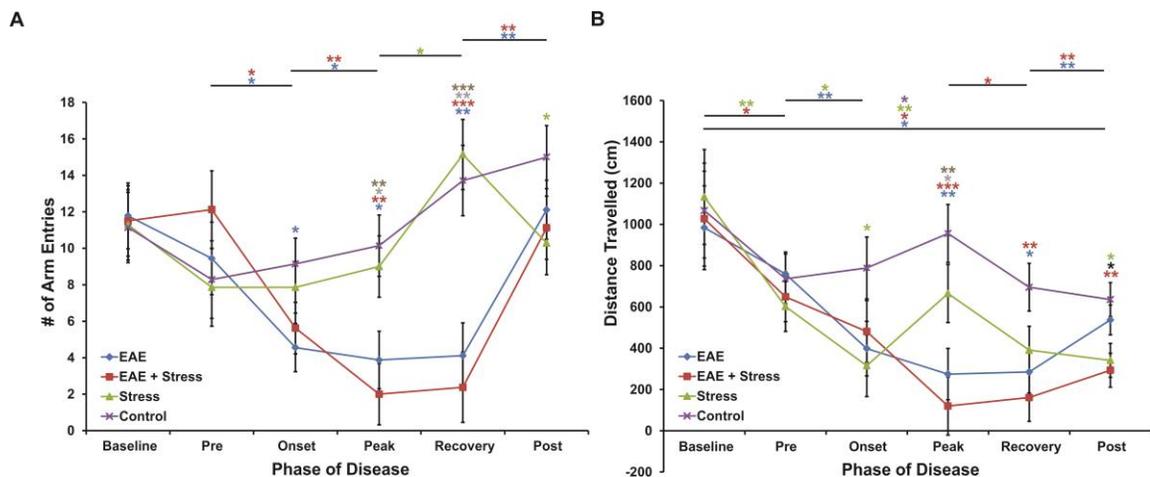


Fig. 4: Fatigue-like behaviour in EAE, EAE + stress, stress, and control cohorts of rats. **(A)** Number of arm entries in the EPM. Overall EAE animals ($p=0.026$, RM ANOVA, LSD post-hoc test) and EAE + stress animals ($p=0.018$, RM ANOVA, LSD post-hoc test) were significantly different from control animals. EAE ($p=0.105$, RM ANOVA, LSD post-hoc test), and EAE + stress animals ($p=0.074$, RM ANOVA, LSD post-hoc test) were not significantly different in comparison to stress animals. In addition, EAE animals were not significantly different in comparison to EAE + stress animals ($p=0.805$, RM ANOVA, LSD post-hoc test). **(B)** Distance travelled in the open field. EAE animals were not significantly different from EAE + stress, stress, or control animals in regards to distance travelled ($p>0.05$, RM ANOVA, LSD post-hoc test). EAE + stress animals, on the other hand, were overall significantly different from the control animals ($p=0.032$, RM ANOVA, LSD post-hoc test). Conversely, EAE + stress animals were not significantly different from stress animals ($p=0.516$, RM ANOVA, LSD post-hoc test). Asterisks indicate significances: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.4.5 Chronic Stress Does Not Increase Mechanical Allodynia in EAE Animals

Pain threshold was inferred from sensitivity to mechanical stimulation of the hindpaw (Fig. 5). There were no overall group differences between any of the animal cohorts ($p>0.05$, RM ANOVA, LSD post-hoc test). Mauchley's W indicated a violation of sphericity, therefore df 's were corrected with Greenhouse-Geisser. This test revealed a phase ($F(3.352,87.146)=9.550$, $p=0.000$), and phase X group interaction ($F(10.055,87.146)=4.879$, $p=0.000$). Interestingly, stress animals showed an increased sensitivity to pain at baseline in comparison to controls ($F(3,27)=1.805$, $p=0.035$). Perhaps exposure to an acute stressor caused an increased sensitivity to pain. EAE animals showed a significant decrease in pain threshold during the pre phase of disease in comparison to stress animals ($F(3,27)=2.183$, $p=0.027$). Conversely, an increased sensitivity to pain is seen during the peak phase of disease by EAE animals ($F(3,27)=5.323$, $p<0.05$), and EAE + stress animals ($F(3,27)=5.323$, $p<0.05$) in

comparison to stress and control animals. This work is in line with previous studies demonstrating an increased sensitivity to pain prior to the onset of clinical EAE followed by a decreased sensitivity to pain during symptomatic disease in mice using both tactile allodynia and formalin exposure (Olechowski et al., 2009; 2010; 2013). Interestingly, during the post phase of disease, EAE animals demonstrate a reduced pain threshold in comparison to stress animals ($F(3,27)=1.517, p=0.046$). Perhaps overtime, exposure to chronic stress increased pain threshold. Lastly, a paired-samples t-test was conducted to compare phases of disease within each animal group. Significant changes in pain sensitivity were seen during baseline and pre ($p=0.040$, paired t-test); onset and peak ($p=0.040$, paired t-test); and peak and recovery phase of disease ($p=0.003$, paired t-test) by EAE animals. Likewise, EAE + stress animals showed a significant difference in pain threshold during pre and onset ($p=0.032$, paired t-test); onset and peak ($p=0.040$, paired t-test); and peak and recovery phases of disease ($p=0.004$, paired t-test). In further support of chronic stress causing an increase in pain sensitivity overtime, stress animals showed a significant difference in pain threshold during baseline and pre ($p=0.013$, paired t-test), and post and baseline phases of disease ($p=0.001$, paired t-test).

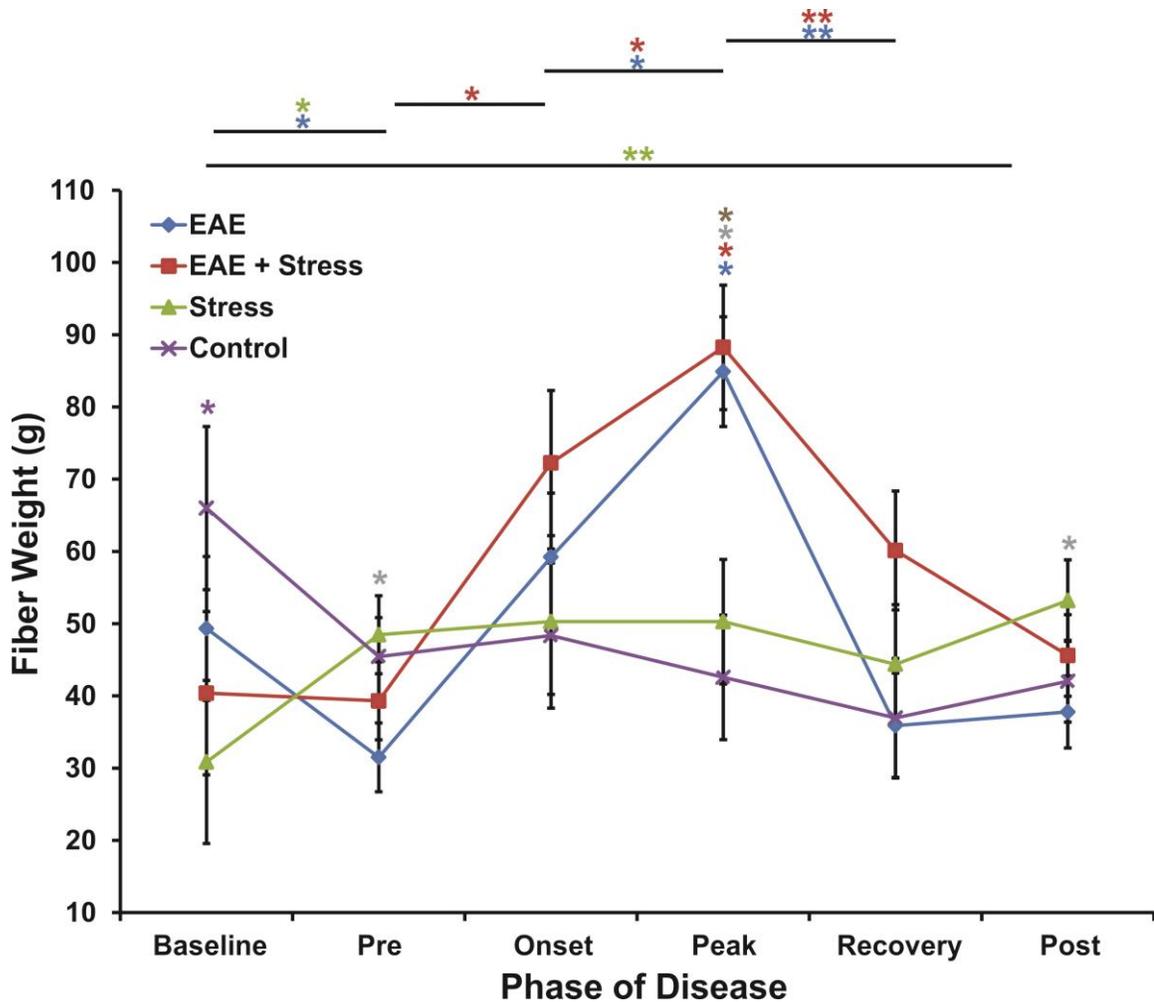


Fig. 5: Sensitivity to mechanical stimulation determined by fiber weight (g) in EAE, EAE + stress, stress, and control cohorts of rats. There were no overall group differences between any of the animal cohorts ($p > 0.05$, RM ANOVA, LSD post-hoc test). Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.4.6 Stress Elevated IL-1 β Levels While Reducing GRO/KC and Leptin Levels

Eve Technologies (Calgary, AB) quantified the concentration of 23 cytokines in the blood of EAE, EAE + stress, stress, and control animals. Of the 23 cytokines tested, only 7 were reliably quantified, and of these, only interleukin 1-beta (IL-1 β), growth

related oncogene (GRO/KC), and leptin showed significance. The concentration of IL-1 β (Fig. 6B) was significantly decreased in EAE ($F(3,14)=6.586$, $p<0.01$), and control animals ($F(3,14)=6.586$, $p<0.05$) in comparison to EAE + stress, and stress animals. Conversely, EAE ($F(3,14)=37.809$, $p<0.01$), and control animals ($F(3,14)=37.809$, $p<0.01$) had a significant increase in GRO/KC (Fig. 6A) concentrations in comparison to EAE + stress, and stress animals. On the other hand, leptin (Fig. 6C) concentrations significantly decreased in stress animals in comparison to EAE ($F(3,14)=2.556$, $p=0.048$), and control animals ($F(3,14)=2.556$, $p=0.037$).

Mitogen Advanced Diagnostics (Calgary, AB) quantified the fluorescence intensity of anti-MBP (Fig. 6D) in blood plasma from EAE, EAE + stress, stress, and control animals. The fluorescence intensity is significantly elevated in EAE cohort in comparison to the stress ($F(3,14)=4.295$, $p=0.014$), and control animals ($F(3,14)=4.295$, $p=0.008$).

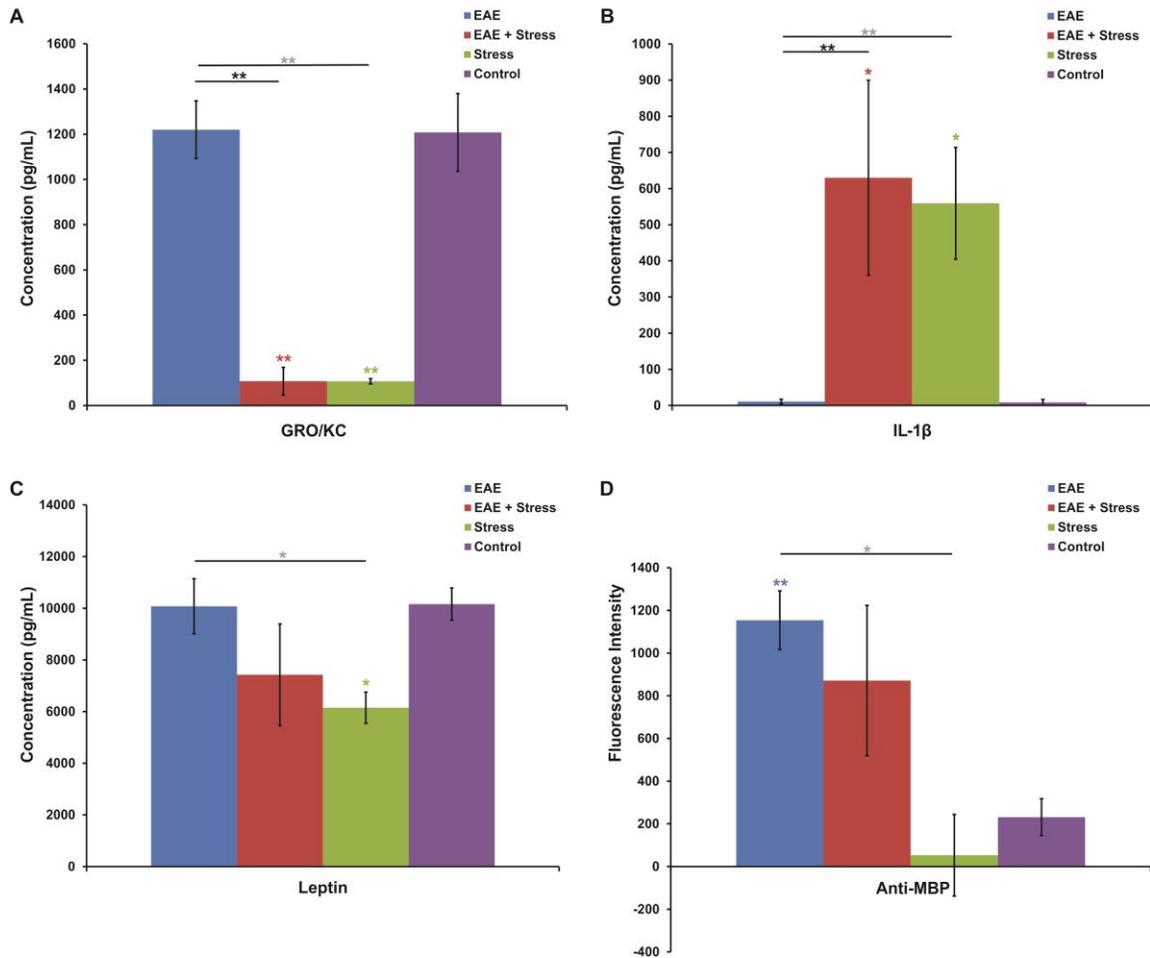


Fig. 6: Blood analysis in EAE, EAE + stress, stress, and control cohorts of rats. (A) GRO/KC. (B) IL-1 β . (C) Leptin. (D). Anti-MBP. The concentration of IL-1 β was elevated in stress animals whereas the concentration of GRO/KC and leptin were decreased in stress animals. Anti-MBP was elevated in EAE and EAE + stress animals in comparison to controls. Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.4.7 EAE Elevated the Expression of miR-16, miR-21, miR-142-3p, miR-142-5p, miR-146a, and miR-155

miRNA analysis (Fig. 7) revealed that miR-21 ($F(3,8)=6.051$, $p < 0.05$), miR-142-3p ($F(3,8)=4.255$, $p < 0.05$), miR-142-5p ($F(3,8)=4.299$, $p < 0.05$), miR-146a

($F(3,8)=26.039$, $p<0.01$), and miR-155 ($F(3,8)=14.830$, $p<0.01$) were up-regulated in EAE, and EAE + stress animals in comparison to controls. Similarly, miR-16 was up-regulated in EAE + stress animals in comparison to controls ($F(3,8)=15.970$, $p=0.002$). Expression of miR-16 ($F(3,8)=15.970$, $p=0.045$), miR-146a ($F(3,8)=26.039$, $p=0.034$), and miR-153 ($F(3,8)=11.887$, $p=0.022$) were down-regulated in EAE animals in comparison to EAE + stress animals. EAE and EAE + stress animals had up-regulated levels of miR-16 ($F(3,8)=15.970$, $p<0.01$), miR-21 ($F(3,8)=6.051$, $p<0.05$), miR-142-3p ($f(3,8)=4.255$, $p<0.05$), miR-146a ($F(3,8)=26.039$, $p<0.01$), and miR-155 ($F(3,8)=14.830$, $p<0.01$) in comparison to stress animals. In addition EAE animals had an increase in the expression of miR-142-5p in comparison to stress animals ($F(3,8)=4.299$, $p=0.041$). In contrast with the previous statement, miR-153 ($F(3,8)=11.887$, $p<0.05$), and miR-219a ($F(3,8)=6.179$, $p<0.05$) was significantly down-regulated in EAE and EAE + stress animals in comparison to controls. Lastly, miR-153 ($F(3,8)=11.887$, $p=0.016$), and miR-219a ($F(3,8)=6.179$, $p=0.039$) was significantly up-regulated in stress animals in comparison to controls.

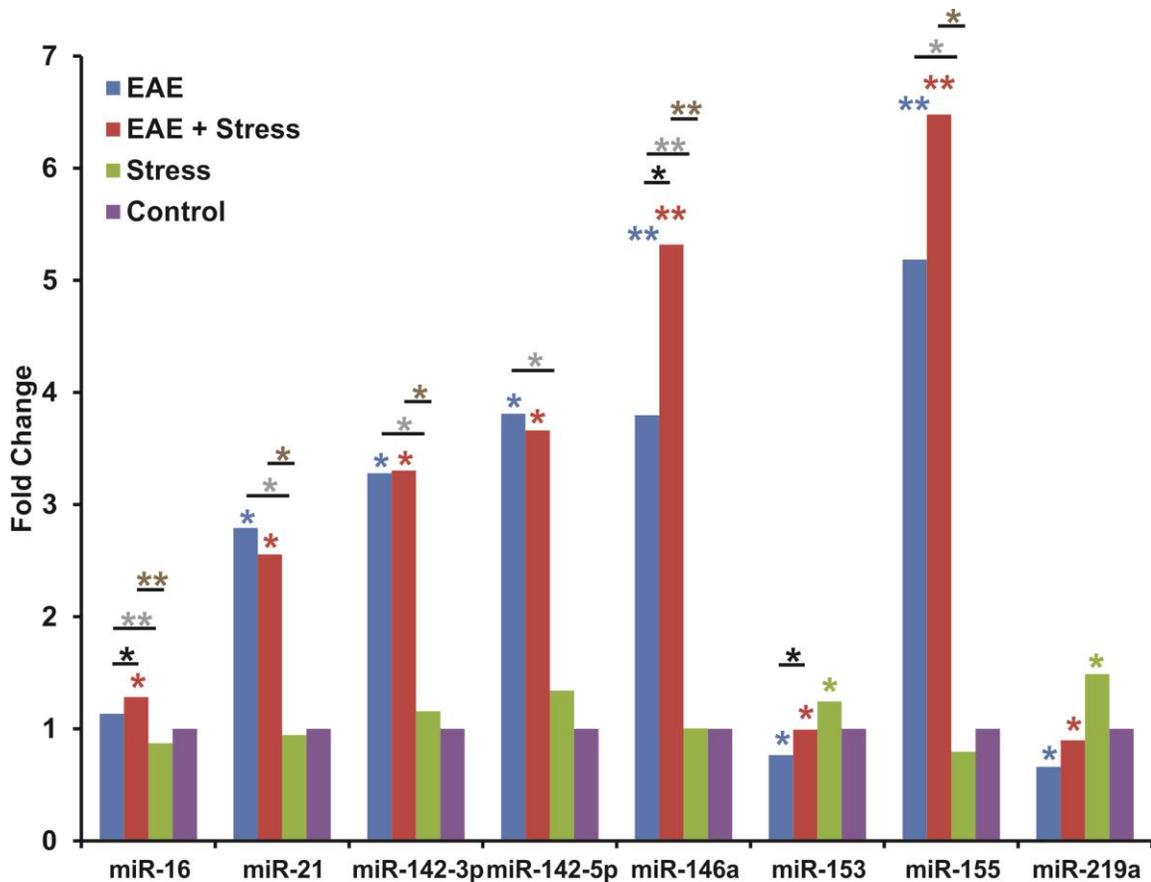


Fig. 7: miRNA analysis from spinal cord tissue in EAE, EAE + stress, stress, and control cohorts of rats. miR-16, miR-21, miR-142-3p, miR-142-5p, miR-146a, miR-146 and miR-155 expression were elevated in both EAE cohorts in comparison to stress and control animals. Conversely, the expression of miR-153, and miR-219 were down-regulated in both EAE cohorts in comparison to stress and control animals. Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.4.8 Chronic Stress Alters Levels of Chlorine and Vanadium

Hair trace elementary analysis (Fig. 8) in EAE animal revealed increased content of Potassium (K) ($F(3,14)=5.102$, $p=0.009$), and decreased content of Arsenic (As) ($F(3,14)=6.044$, $p=0.028$), and Cerium (Ce) ($F(3,14)=12.756$, $p=0.000$) when compared to control animals. Decreased levels of As ($F(3,14)=6.044$, $p=0.024$), and Chlorine (Cl)

($F(3,14)=5.517$, $p=0.022$) were also observed in EAE animals in comparison to stress animals. EAE + stress animals had increased levels of Cl ($F(3,14)=5.517$, $p=0.014$), and decreased levels of Ce ($F(3,14)=12.756$, $p=0.000$) in comparison to controls. EAE + stress animals also had decreased levels of Nickel (Ni) ($F(3,14)=3.253$, $p=0.013$), and K ($F(3,14)=5.102$, $p=0.037$) in comparison to stress animals. Cl ($F(3,14)=5.517$, $p=0.005$), and As ($F(3,14)=6.044$, $p=0.001$) levels were increased in EAE + stress animals in comparison to EAE animals. Conversely, Ni levels were increased in EAE animals in comparison to EAE + stress animals ($F(3,14)=3.253$, $p=0.034$). Vanadium (V) levels were decreased in EAE ($F(3,14)=4.811$, $p=0.005$), and control animals ($F(3,14)=4.811$, $p=0.005$) in comparison to stress animals. On the other hand, levels of Ce decreased in stress animals in comparison to controls ($F(3,14)=12.756$, $p=0.000$). Cl ($F(3,14)=5.517$, $p=0.037$), and K ($F(3,14)=5.102$, $p=0.003$) levels were increased in stress animals in comparison to control animals.

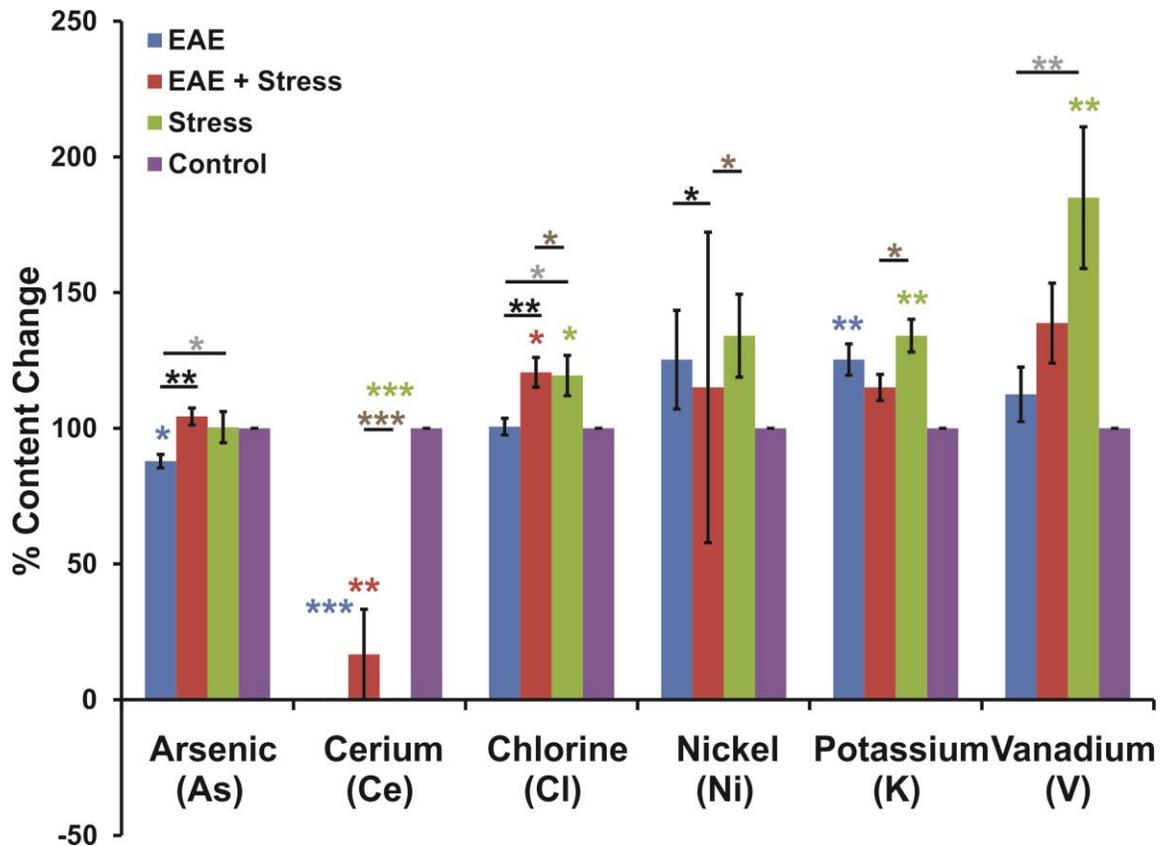


Fig. 8: Hair trace element analysis in EAE, EAE + stress, stress, and control cohorts of rats. Decreased content of As was observed in EAE animals in comparison to all other animal groups. Conversely, increased content of Cl and V were found in both stress cohorts in comparison to EAE and control animals. Asterisks indicate significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. * indicates significance between EAE and control rats; * indicates significance between EAE + stress and control rats; * indicates significance between stress and control rats; * indicates significance between EAE and EAE + stress rats; * indicates significance between EAE and stress rats; and * indicates significance between EAE + stress and stress rats. Error bars represent \pm SEM.

3.5 Discussion

In this study, behavioural, immunological, and epigenetic outcomes in EAE, EAE + stress, and stress rats were compared to non-stress control rats. Specifically, movement, anxiety, fatigue and pain were investigated as well as changes in cytokine levels, miRNA expression, and the content level of trace elements within the hair of experimental rats in comparison to controls. The time course assessed by the 5 point scale indicated that EAE

animals exposed to chronic stress develop symptoms of clinical disease on average one day earlier and present with an exacerbated disease course. In addition, stress worsened fine motor performance among EAE + stress animals compared to the effects of EAE alone during the peak phase of disease. Interestingly, there were no significant differences overall between EAE + stress animals in comparison to EAE animals in measures of anxiety, fatigue, and pain. In fact, EAE animals and not EAE + stress animals were significantly different from controls in one measure of anxiety (number of open arm entries). In addition, EAE + stress animals were significantly different from controls in one measure of fatigue (total distance travelled) whereas EAE animals were not. Furthermore, stress elevated the level of IL-1 β , while decreasing the level of GRO/KC, and leptin. miR-16, miR-21, miR-142-3p, miR142-5p, mir-153, miR-146a, miR-155, and mir-219 were differentially expressed in EAE and EAE + stress animals in comparison to controls. Our analysis of trace elements revealed decreased levels of arsenic in EAE animals. Furthermore, elevated levels of chlorine and vanadium were found in stress animals.

The present data are in line with previous observations showing that stress has long-lasting effects on motor and sensory behaviour (Metz et al., 2001, 2005) and outcomes in animal models of human neurodegenerative disease (Smith et al., 2008). The behavioural findings suggest that clinical severity of EAE is not correlated with the severity of anxiety or fatigue in EAE. This information is in line with previous studies in human MS where the severity of behavioural symptoms did not correlate with the severity of physical disease (Janardhan et al., 2002; Lobentanz et al., 2004; Acharjee et al., 2013). The significance seen between EAE + stress animals and controls in regards to

total distance travelled could be explained by EAE + stress animals' inability to mobile properly as determined by the ladder rung task. All of the fatigue behavioural parameters involved motor movement. Furthermore, an increase in pain threshold during the peak phase of disease is observed in both EAE cohorts in comparison to controls. EAE animals were able to mobile properly during the peak phase of disease as confirmed by the ladder rung walking task. Therefore, EAE animals increase in pain threshold is not confounded by motor impairment. The increased pain threshold in EAE + stress animals on the other hand, may be a reflection of the inability to mobile properly therefore making withdrawal reactions hard to carry out. The present pain threshold findings are in line with previous studies demonstrating a decreased sensitivity to pain during clinical EAE in mice using both tactile allodynia and formalin exposure (Olechowski et al., 2009; 2010; 2013). Once again, a significant difference between EAE cohorts in regards to pain threshold was not observed. Taken together, these findings illustrate that anxiety, fatigue, and pain disturbances can be detected in MBP₆₉₋₈₈ immunized animals. Furthermore, chronic stress does not exacerbate symptoms of anxiety, fatigue, or pain in EAE animals. Therefore, behavioural impairments of EAE are not necessarily correlated with clinical severity of disease.

The underlying cellular mechanisms that mediate the development of behavioural comorbidities in stress and EAE were investigated through the analysis of immune cells. Specifically, blood samples collected during the post phase of EAE were analyzed for 23 cytokines, and anti-MBP. Cytokine analysis revealed that the level of IL-1 β was elevated in EAE + stress and stress animals in comparison to EAE and control animals. A previous experiment in rats exposed to high-light open-field developed an increase in IL-

1 β levels within the hippocampus (Badowska-Szalewska et al., 2013). Therefore, increased IL-1 β levels may be associated with exposure to chronic stress. Interestingly, previous studies on periodontal disease in rats report increased serum IL-1 β levels were associated with increased disease activity (Peruzzo et al., 2008). This is in line with the present study. EAE + stress animals had elevated levels of IL-1 β and exacerbated clinical disease activity. In contrast with IL-1 β , GRO/KC and leptin levels were decreased in EAE + stress and stress animals in comparison to EAE animals and controls. Acute stress is believed to cause an increase in leptin within the periphery nervous system which is responsible for reducing circulating glucocorticoid levels (Bornstein et al., 1997). Our study suggests that chronic stress may modulate the role of circulating blood levels of leptin. A study completed by de Oliveira et al. (2013) report just that: chronic stress desynchronizes the serum level of leptin in rats (de Oliveira et al., 2013). Therefore, exposure to chronic stress modulates the level of leptin and may actually increase inflammation within the body. Little is known about the GRO/KC in human MS. However, elevated levels of GRO/KC have been reported in response to acute psychological stress in an animal model of diabetes and gastritis (Yeo et al., 2012; Li et al., 2013). Our data is in opposition with this. GRO/KC levels were reduced in EAE + stress and stress animals. Perhaps chronic stress modulates the expression of GRO/KC as well. Furthermore, anti-MBP level was the last factor analyzed from plasma samples collected from experimental animals and controls. The fluorescence intensity was elevated in EAE and EAE + stress animals in comparison to controls. Interestingly, the fluorescence intensity was only significantly elevated in EAE animals and not EAE + stress animals in comparison to controls. In human MS, 59% of patients are seropositive

for anti-MBP (Olsson et al., 1990; Egg et al., 2001). Therefore anti-MBP is associated with human MS but there are other factors contributing to disease pathogenesis as well.

Taken together, the above listed cytokines likely represent a response to chronic stress and not EAE disease activity. Perhaps these findings are due to the time point in which blood samples were collected. All EAE animals made a full recovery from clinical disease by the post phase. Anti-MBP on the other hand, may be a useful biomarker to determine a diagnosis of EAE or human MS.

The present miRNA analysis revealed that miR-16, miR-21, miR-142-3p, miR-142-5p, miR-146a, and miR-155 were up-regulated in EAE and EAE + stress animals in comparison to controls. miR-153, and miR-219 on the other hand are down-regulated in EAE and EAE + stress animals in comparison to controls. The role of miR-16, and miR-153 in MS is unknown. However, miR-16, and miR-153 have been associated with various kinds of cancer (Lu et al., 2005; Ying et al., 2013; Zhang et al., 2013). Interestingly, miR-16 has been associated with stress in humans (Katsuura et al., 2012). Like in MS, stressful life events have been associated with an increased risk of cancer (Lillberg et al., 2003). Expression of miR-21, miR-142-3p, miR-142-5p, miR-146a, and miR-155 are increased in active human MS lesions (Junker et al., 2009; Keller et al., 2010, Koch et al., 2013). The expression of miR-219 on the other hand, is increased in inactive MS lesions (Junker et al., 2009). miR-21, miR-142, miR-146a, and miR-155 are believed to target CD47 cells (Junker et al., 2009). CD47 cells release a “do not eat me” signal (Gardai et al., 2006). miR-155, specifically, is known to down-regulate the expression of CD47 cells (Junker et al., 2009). Within the central nervous system, up-regulation of miR-155 would cause a reduction in CD47 cells therefore causing a

lesion. miR-219 is associated with oligodendrocyte regulation and myelin maintenance (Li et al., 2012). Therefore miR-219 may promote healing within inactive MS lesions. Interestingly, miR-146a and miR-155 are regulated by the expression of cytokines (Junker et al., 2009). Therefore, an inflammatory immune cascade could cause epigenetic changes which in turn could cause an increase in susceptibility to autoimmune diseases, such as MS.

Within this study, increased levels of arsenic, chlorine, potassium, and vanadium were found in EAE and EAE + stress animals in comparison to controls. Hair levels of the elements copper, iodine, manganese, sulfur, selenium, vanadium, and zinc have been implicated in human MS patients (Ryan et al., 1978; Smith et al., 1989). High levels of arsenic are associated with elevated levels of inflammatory molecules as well as an increased risk of developing a neurological disorder (Mezzetti et al., 1998; Rodriguez et al. 2003; Wu et al., 2003; Wright et al. 2006; Ambeskovic et al., 2013). The level of cerium on the other hand was decreased in EAE and EAE + stress animals. Interestingly, cerium is thought to regulate the expression of inflammatory cytokines (Sang et al., 2013). Specifically, cerium is believed to up-regulated IL-1 β (Sang et al., 2013). Perhaps the level of cerium found in our rats is reflective of the inflammatory cascade that happened during symptomatic EAE. Hair follicles grow for approximately 7-20 days in rats (Ambeskovic et al., 2013). Therefore, the hair samples collected at euthanasia would have been present while the rats developed and recovered from EAE. The content level of cerium may have decreased overall due to the rats recovering from EAE. Furthermore, elevated levels of chlorine and vanadium were found in stress animals. Chlorine and vanadium are proposed to modify the immune system, specifically increasing the level of

circulating B and T cells as well as altering the function of macrophages (Exon et al., 1987; Mravcová et al., 1993). Additionally, exposure to chlorine is associated with various neurobehavioral deficits in humans including: depression, dizziness, fatigue, irritability, memory loss, inability to concentrate, poor balance, and loss of strength (Kilburn, 1995; 1996). Therefore, increased levels of chlorine and vanadium may be indicative of a modified immune response and subsequent neurobehavioural deficits. Currently, there is little literature on trace element analysis and MS. Further research in this area is needed to understand the role of trace elements in human MS.

3.6 Conclusions

Mild chronic stress exacerbates the clinical severity of EAE, which in turn, impacts locomotor capabilities. Interestingly, mild chronic stress does not influence the severity of behavioural deficits including: anxiety, fatigue, and pain in EAE. In addition, mild chronic stress modulated the level of circulating cytokines such as IL-1 β , GRO/KC, and leptin within the blood. These differentially secreted cytokines in turn, may regulate the expression of miRNA. Specifically, the expression of miR-146a, and miR-155 are believed to be controlled by cytokine level. miR-146a, and miR-155 are implicated in human MS. Lastly, trace elementary analysis of body hair revealed reduced arsenic content levels in body hair of EAE animals, which was not further affected by stress. Stress in EAE, however, was associated with elevated chlorine, and vanadium levels. Arsenic, chlorine, and vanadium are implicated in immune regulation.

Taken together, this monophasic model of EAE in LEW rats represents a useful tool to investigate targeted therapeutics for physical and behavioural aspects of human MS. In addition, this model of EAE may be useful to develop targeted therapeutics for

epigenetic factors related to MS. This data in turn, may be useful for other neurological conditions associated with behavioural deficits and epigenetic markers, such as Parkinson's disease and Alzheimer's disease.

3.7 References

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CHAPTER 4

General Discussion

4.1 Summary

The main objective of this research was to develop a rat model of EAE as a model of human MS. Although EAE has been well described in terms of physical impairments, little is known about possible behavioural symptoms. The first chapter of this thesis focuses on outlining what is known about physical and behavioural deficits of EAE animals. Within Chapter 2, female Lewis (LEW) rats were used to profile the development and recovery of motor, emotional, and sensory symptoms of EAE. Chapter 3 exposes EAE animals to chronic stress in order to determine how this might affect their disease course and subsequently the effect of stress on behaviour. In Chapters 2 and 3, immune cells, the expression of miRNA, and trace element analysis of hair were used to investigate the mechanism behind rat EAE.

Most behavioural investigations in EAE models have been performed in mice. Although the use of standardized behavioural tests in mice has received more attention over the last decade, advanced and comprehensive behavioural test batteries for laboratory rats are more readily available. The size of a rat makes it easier to perform various behavioural manipulations and utilize invasive techniques (Teegarden, 2012; Weissert, 2012). Therefore rats represent a suitable animal species to investigate behavioural profiles of diseases, such as EAE. LEW and Dark Agouti, are the most common rat strains used to study EAE (Mannie et al., 2009). LEW rats were used in this experiment because Dark Agouti rats often develop arthritis in response to EAE immunization (Mannie et al., 2009). The development of arthritis would impede on the

ability to determine behavioural symptoms of EAE.

Our data suggests that increasing EAE severity is associated with a decline in fine motor skill capabilities. This is to be expected. A severe form of EAE would encompass more motor paralysis and this should in turn impact the ability to create fine motor movements. Interestingly, measure of anxiety, fatigue, and pain are not affected by the severity of EAE. This information is in line with previous studies in human MS where the severity of behavioural symptoms did not correlate with the severity of physical disease (Janardhan et al., 2002; Lobentanz et al., 2004; Acharjee et al., 2013). This data suggests that a mechanism beyond demyelination is associated with these symptoms.

A study completed by Acharjee et al. (2013) suggested that mice develop signs of anxiety, cognitive loss, and depression before the onset of EAE. This data indicates that a mechanism beyond demyelination mediates some behavioural aspects of EAE. During the pre phase of EAE demyelination has not yet begun. Acharjee et al. (2013) report elevated cytokine and glucocorticoid levels within their EAE mice in comparison to controls during the pre phase of disease. Interestingly, the production of interleukin 1-beta (IL-1 β) is believed to be responsible for motor disturbances, loss of body weight, reduced food and sucrose intake, and/or decreased social interest in EAE animals (Cartmell et al., 2001; Acharjee et al., 2013). Within the previously described experiment, EAE mice displayed an elevated concentration of IL-1 β prior to EAE onset. Therefore cytokines such as IL-1 β may mediate behavioural symptoms of EAE.

Furthermore, current research suggests that miR-146a and miR-155 are regulated by the expression of cytokines (Junker et al., 2009). Interestingly, these two miRNA are highly associated with MS (Junker et al., 2009). MiR-146a, and miR-155, are predicted to

target CD47 cells (Junker et al., 2009). miR-155 specifically, is implicated in the demyelination process within the CNS because it decreases the expression of CD47 cells (Junker et al., 2009). Therefore, an inflammatory immune cascade could cause epigenetic changes which in turn could cause an increase in susceptibility to autoimmune diseases, such as MS.

Trace element analysis within this thesis revealed differential content levels of boron, cobalt, manganese, mercury, platinum, and thallium in the body hair of EAE animals in comparison to controls. Additionally, trace element analysis in the chronic stress and EAE chapter revealed differential levels of arsenic, cerium, chlorine, potassium, and vanadium in EAE and stressed EAE animals in comparison to controls. Exposure to boron and cobalt may play a role in susceptibility to MS (Zapadniuk, 1992; Mezzetti et al. 1998). Arsenic, chlorine and vanadium, on the other hand, are associated with regulation of the inflammatory response (Exon et al., 1987; Mravcová et al., 1993; Mezzetti et al., 1998; Rodriguez et al. 2003; Wu et al., 2003; Wright et al. 2006; Ambeskovic et al., 2013; Sang et al., 2013). Furthermore, toxic levels of manganese, mercury, platinum, and thallium are associated with neurodegeneration (Al Hammouri et al., 2011; Farina et al., 2012). Interestingly levels of manganese, mercury, platinum, and thallium were decreased in EAE animals in comparison to controls in this study. Perhaps these contrasting results and the difference in trace element expression between chapters two and three are due to the time point that body hair samples were collected. Hair follicles grow for approximately 7-20 days in rats (Ambeskovic et al., 2013). Our experiment lasted for 30 or more days. The hair samples collected during euthanasia may not have expressed the elements present during symptomatic EAE. In the future, hair

samples should be collected prior to the onset of EAE and during symptomatic EAE in order to gain a greater understanding of the role of trace elements in EAE and human MS. In addition, utilizing these time points to collect hair samples in a future chronic stress and EAE study may further describe the relationship between stress and the development of EAE.

4.2 Working with EAE

While EAE represents an invaluable tool to investigate MS, it does not model all aspects of the human disease. Therefore, the EAE model should be chosen with specific research questions in mind. The animal species as well as the encephalitogenic antigen used can greatly influence the EAE disease course (Weissert, 2012). For example, some EAE models present with a relapsing-remitting disease course, whereas others are more chronic (Mannie et al., 2009). In addition, some EAE models are inflammatory in nature, whereas others result in demyelination of the spinal cord and/or brain (Mannie et al., 2009). Therefore, specific EAE models are suited for immunology studies, while others are suited for neuroscience (Weissert, 2012).

4.2.1 The Encephalitogenic Antigen

Upon determining which EAE model to use, the composition of myelin protein is of foremost importance for the success of the experiment. Whether the animal model requires myelin basic protein, myelin oligodendrocyte glycoprotein, proteolipid protein, or spinal cord tissue, the purity level should be 98% or higher (Weissert, 2012). Furthermore the protein segment of the encephalitogenic antigen is important. There are encephalitogenic boundaries that need to be maintained in order to successfully induce

EAE (Mannie et al., 1990). These boundaries depend on the animal species and protein source used. Therefore, these parameter need to be investigated before EAE experiments begin. In addition, it is recommended that a large batch of myelin protein is ordered at one time from the same supplier. Variation in the EAE disease course exists between laboratories using the same protocol and protein due to the provider of the encephalitogenic antigen (Olechowski et al., 2009). Tiny production variations when creating the encephalitogenic antigen can greatly influence the disease course and severity of EAE. Once a reliable protein supplier is found, it is recommended to continually use their encephalitogenic antigen.

4.2.2 Creating the Adjuvant

There is no difference between utilizing incomplete Freund's or complete Freund's adjuvant. Either way, the adjuvant will have to be supplemented with bacterium to create modified complete Freund's adjuvant. The recommended bacterium is: *Mycobacterium tuberculosis* H37Ra (Acharjee et al., 2013, Olechowski et al., 2013). Furthermore, the final concentration of bacterium in the adjuvant commonly ranges from 4mg/mL to 6mg/mL (Mannie et al., 2009; Acharjee et al., 2013, Olechowski et al., 2013). Increasing the bacterium dosage typically increases the severity of EAE. It may take time to determine the proper bacterium dosage for the EAE model being utilized in your lab.

4.2.3 Creating the Emulsion

First off, it may take 1-2 hours to create the emulsion. Plan accordingly. Before you begin the procedure, all containers and tools that may be used while creating the emulsion should be autoclaved. Unwanted bacterial species can modify the EAE disease

course by acting as adjuvants (Shaw et al., 1964, Mannie et al., 2009). Afterwards, 1 mg of the purified encephalitogenic antigen needs to be dissolved in 1 mL distilled water. It is not necessary to utilize more than 1 mg of the encephalitogenic antigen because there is a ceiling effect (Mannie et al., 1990). From here, the modified complete Freund's adjuvant should be mixed with the encephalitogenic antigen solution in a 1:1 ratio. This process can either be performed by hand utilizing luer-lok syringes and a stopcock, or with a vortex, or sonicator machine (Mannie et al., 2009). If using a vortex or sonicator, the emulsion should be placed on ice in-between 10 second bouts (Mannie et al., 2009). The complete emulsion should be viscous and not separate when exposed to an aqueous surface (Mannie et al., 2009). The emulsion can be created the night before and stored in the fridge (Stromnes et al., 2006).

4.2.4 EAE Immunization

Typically, a total volume of 100 μ l of the emulsion is spread subcutaneously between two injection sites: either side of the base of the tail (Mannie et al., 2009; Acharjee et al., 2013, Olechowski et al., 2013). Previously, the emulsion was injected in each hind limb foot pad. It is no longer acceptable to do this (Weissert et al., 2009). Injecting through the footpads causes unnecessary irritation and can impede on the results of behavioural assessment. It is important to make sure the injection is subcutaneous. If not, animals can develop internal abscesses at the injection site. In addition, it is advisable to utilize a new needle per animal injection. This is not a requirement of most animal welfare committees but it reduces injection site irritation. Using a dull needle can cause itching or an external abscess after EAE immunization.

4.2.5 Repeat EAE Immunizations

There is a limited time window in which reimmunization for EAE will be successful if the first attempt does not work. Rodents become resistant to reinduction of EAE by 5 weeks post first immunization (Willenborg, 1979; Hinrichs et al., 1981). Reinduction of EAE prior to 5 weeks post first immunization, leads to an earlier disease onset with reduced disease duration and symptoms (Willenborg, 1979). It is important to note that with the passing of each additional week post initial EAE immunization, the EAE success rate will decline (Willenborg, 1979). Rodents do not redevelop EAE after the 5 week mark due to the development of specific antigen-reactive cells against the myelin protein used (Willenborg, 1979; Hinrichs et al., 1981). Therefore, great care should be provided when creating the initial EAE emulsion. It is much harder to reinduce EAE if the first attempt was unsuccessful.

4.3 Conclusion

From my knowledge, this is the first experiment that profiled longitudinal behavioural parameters of EAE in LEW rats. In addition, this is the first experiment that investigated longitudinal behavioural parameters of LEW rats immunized for EAE exposed to chronic stress. Furthermore, developing the EAE model at the University of Lethbridge came with various obstacles. In this discussion, a protocol for EAE development was outlined.

Taken together, this thesis validated the hypothesis that EAE animals develop comparable behavioural symptoms as seen in human MS. Specifically, EAE animal's shows signs of motor impairment, anxiety, fatigue, and pain. Fine motor skills are increasing impaired with increasing EAE severity. Anxiety, fatigue, and pain on the other

hand, are not affected by the severity of EAE. Interestingly, chronic stress exacerbates the severity of EAE, and impedes on fine motor skills but does not significantly affect, anxiety, fatigue, or pain symptoms. Furthermore, cytokines appear to regulate the expression of miRNA that are associated with EAE and MS. Trace element analysis of rat body hair indicated that arsenic, boron, cerium, chlorine, cobalt, manganese, mercury, platinum, potassium, thallium, and vanadium are associated with EAE susceptibility as well as immune regulation and neurodegeneration. Everything included, MBP-EAE in LEW rats represents a useful tool for investigating the mechanism behind the pathogenesis of human MS. These findings may offer new therapeutic avenues to treat MS in humans. Specifically, MBP-EAE in LEW rats could be used to develop targeted behavioural therapeutics for human MS. Furthermore, MBP-EAE in LEW rats could be used to investigate targeted therapeutics for epigenetic changes associated with EAE and MS.

4.4 References

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APPENDIX A: Kurtzke Expanded Disability Status Scale

- 0.0:** Normal Neurological Exam
- 1.0:** No disability, minimal signs on 1 FS
- 1.5:** No disability, minimal signs on 2 of 7 FS
- 2.0:** Minimal disability in 1 of 7 FS
- 2.5:** Minimal disability in 2 FS
- 3.0:** Moderate disability in 1 FS; or mild disability in 3 - 4 FS, though fully ambulatory
- 3.5:** Fully ambulatory but with moderate disability in 1 FS and mild disability in 1 or 2 FS; or moderate disability in 2 FS; or mild disability in 5 FS
- 4.0:** Fully ambulatory without aid, up and about 12hrs a day despite relatively severe disability. Able to walk without aid 500 meters
- 4.5:** Fully ambulatory without aid, up and about much of day, able to work a full day, may otherwise have some limitations of full activity or require minimal assistance. Relatively severe disability. Able to walk without aid 300 meters
- 5.0:** Ambulatory without aid for about 200 meters. Disability impairs full daily activities
- 5.5:** Ambulatory for 100 meters, disability precludes full daily activities
- 6.0:** Intermittent or unilateral constant assistance (cane, crutch or brace) required to walk 100 meters with or without resting
- 6.5:** Constant bilateral support (cane, crutch or braces) required to walk 20 meters without resting
- 7.0:** Unable to walk beyond 5 meters even with aid, essentially restricted to wheelchair, wheels self, transfers alone; active in wheelchair about 12 hours a day
- 7.5:** Unable to take more than a few steps, restricted to wheelchair, may need aid to transfer; wheels self, but may require motorized chair for full day's activities
- 8.0:** Essentially restricted to bed, chair, or wheelchair, but may be out of bed much of day; retains self care functions, generally effective use of arms
- 8.5:** Essentially restricted to bed much of day, some effective use of arms, retains some self care functions
- 9.0:** Helpless bed patient, can communicate and eat
- 9.5:** Unable to communicate effectively or eat/swallow
- 10.0:** Death due to MS

APPENDIX B: Basso, Beattie, Bresnahan Locomotor Rating

Description of the Categories

In general the 21 point scale follows the recovery progression. The first categories; categories 0 - 7, evaluates HL joint movements of the early stage of recovery.

0. No observable hind limb (HL) movement
1. Slight movement of one or two joints, usually the hip &/or knee
2. Extensive movement of one joint or Extensive movement of one joint and slight movement of one other joint
3. Extensive movement of two joints
4. Slight movement of all three joints of the HL (hip, knee & ankle)
5. Slight movement of two joints and extensive movement of the third
6. Extensive movement of two joints and slight movement of the third
7. Extensive movement of all three joints of the HL

The second section, categories 8-13, evaluates stepping and coordination in the intermediate phase of recovery.

8. Sweeping with no weight support or Plantar placement of the paw with no weight support
9. Plantar placement of the paw with weight support in stance only (i.e. when stationary) or Occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping
10. Occasional weight supported plantar steps, no FL-HL (forelimb-hindlimb) coordination
11. Frequent to consistent weight supported plantar steps and no FL-HL coordination
12. Frequent to consistent weight supported plantar steps and occasional FL-HL coordination
13. Consistent weight supported plantar steps and frequent FL-HL coordination

The third section, categories 14-21, evaluates the fine details of paw usage during locomotion

14. Consistent weight supported steps, consistent FL-HL coordination; and, Predominant paw position during locomotion is rotated or Frequent plantar stepping, consistent FL-HL coordination and occasional dorsal stepping
15. Consistent FL-HL coordination; and No toe clearance or occasional toe clearance during forward limb advancement; Predominant paw position is parallel to the body at initial contact
16. Consistent FL-HL coordination during gait; and Toe clearance occurs frequently during forward limb advancement; Predominant paw position is parallel at initial contact and rotated at lift off
17. Consistent FL-HL coordination during gait; and Toe clearance occurs frequently during forward limb advancement; Predominant paw position is parallel at initial contact and lift off
18. Consistent FL-HL coordination during gait; and Toe clearance occurs consistently during forward limb advancement; Predominant paw position is parallel at initial contact and rotated at lift of
19. Consistent FL-HL coordination during gait; and Toe clearance occurs consistently during forward limb advancement; Predominant paw position is parallel at initial contact and lift off
20. Consistent coordinated gait; consistent toe clearance; Predominant paw position is parallel at initial contact and Lift off ; but Trunk instability & Tail consistently up
21. Coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability & tail consistently up

APPENDIX C: Ladder Rung Walking Task Foot Fault Score

Rating scale for foot placement in the skilled ladder rung walking test

| Category | Type of foot mis-placement | Characteristics |
|----------|----------------------------|---|
| 0 | Total Miss | Deep fall after limb missed the rung |
| 1 | Deep Slip | Deep fall after limb slipped off the rung |
| 2 | Slight Slip | Slight fall after limb slipped off the rung |
| 3 | Replacement | Limb replaced from one rung to another |
| 4 | Correction | Limb aimed for one rung but was placed on another Or: Limb position on same rung was corrected |
| 5 | Partial Placement | Limb placed on rung with either digits/toes or wrist/heel |
| 6 | Correct Placement | Midportion of limb placed on rung |

Foot Fault Score

