Published in final edited form as:

J Neuroimmunol. 2010 March 30; 220(1-2): 79–89. doi:10.1016/j.jneuroim.2010.01.015.

Chronic restraint stress during early Theiler's virus infection exacerbates the subsequent demyelinating disease in SJL mice: II. CNS disease severity

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Abstract

Theiler's murine encephalomyelitis virus (TMEV) infection is a well-characterized model of multiple sclerosis (MS). Previous research has shown that chronic restraint stress (RS) during early TMEV infection exacerbates behavioral signs of disease. The present data suggest RS-induced increases in CNS inflammation, demyelination, and axonal degeneration may underlie this exacerbation. In addition, we report that males exhibit greater CNS inflammation and higher numbers of demyelinating lesions while females show greater susceptibility to RS-induced exacerbation. These findings indicate RS during early TMEV infection increases CNS lesion formation during the late phase and suggest the effects of RS are sex-dependent.

1.0 Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease characterized by inflammatory cell infiltration of the brain and spinal cord. Central nervous system (CNS) inflammation is accompanied by demyelination and axonal degeneration resulting in sensory and motor impairments (Mohr & Dick, 1998; Stinissen et al., 1997). Individual differences in disease vulnerability and disease course have been linked with complex genetic traits (Dyment et al., 2004) as well as sex differences (Cottrell et al., 1999). In addition, exposure to environmental factors, such as stressful life events (Ackerman et al., 2002, 2003; Brown et al., 2006a/b; Buljevac et al., 2003; Mohr et al., 2000; Mohr & Cox, 2001) or life-threatening situations (Grant et al., 1989), as well as infection with a number of viruses (Gilden, 2005; Fazakerley & Walker, 2003; Sibley et al., 1985) including herpes (Challoner et al., 1995), measles (Jacobson et al., 1985) and Epstein-Barr (Gottlieb, 2003; Martyn et al., 1993) have

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also been linked with the onset and exacerbation of MS. Although human clinical studies have played an important role in identifying these relationships, animal models of MS are needed to experimentally investigate the role of genetic and environmental factors in determining disease onset and progression.

Theiler's virus-induced demyelination (TVID) provides a well-characterized model in which mice develop MS-like disease following intracerebral inoculation with Theiler's murine encephalomyelitis virus (TMEV) (Lipton, 1975; Oleszak et al., 2004). Viral infection during adolescence has been linked with an increased risk for developing MS in humans (Acheson, 1977; Gilden, 2005; Gottlieb, 2003; Kurtzke & Hyllestad, 1987; Sospedra & Martin, 2005), suggesting that the TVID model is a relevant model to study MS (Friedman & Lorch, 1985). TMEV infection is characterized by a biphasic CNS inflammatory disease. During the early phase of infection, the virus infects primarily neurons and glia of the gray matter resulting in central nervous system (CNS) inflammation (Njenga et al., 1997) and behavioral signs of encephalitis and polio-like symptoms (Campbell et al., 2001; Johnson et al., 2004, 2006; Sieve et al., 2004, 2006). In contrast, the late phase of disease is characterized by inflammatory demyelination of the white matter with macrophages/monocyte and glial infection. Although resistant strains of mice effectively clear the virus from the CNS during the early phase, susceptible strains fail to produce an effective immune response resulting in the viral persistence that is essential for triggering the process of demyelination in the spinal cord (Blakemore et al., 1988, Lipton & Melvold, 1984; Rodriguez et al., 1983; Oleszak et al., 2004). Several processes mediate demyelination during the late phase of disease including direct virus-induced lysis of oligodendrocytes (Roos & Wollman, 1988), delayed-type hypersensitivity responses (Rodriguez & Sriram, 1988), cytotoxic T cell activity (Clatch et al., 1987; Welsh et al., 1987) and autoimmunity (Borrow et al., 1998; Miller et al., 1997). While genetic factors regulate susceptibility to demyelination (Brahic & Bureau, 1998; Brahic et al., 2005), other factors that influence the early immune response play a critical role in determining the degree of viral persistence and thereby the severity of demyelination (Aubagnac et al., 1999; Brahic et al., 2005; Borrow et al., 1992; Rodriguez et al., 1996; Sieve et al., 2004).

We have previously shown that restraint stress applied during the first four weeks of TMEV infection disrupts the development of the immune response to the virus, resulting in increased behavioral symptom severity, increased mortality and dysregulated inflammatory response within the CNS during the early phase (Campbell et al., 2001; Steelman et al., 2009; Welsh et al., 2004; Young et al., 2008). Moreover, viral clearance is impaired due to reduced CNS inflammatory cell infiltrate and decreased cytokine/chemokine expression during early infection leading to greater viral persistence (Mi et al., 2004, 2006a/b). Disruption of the early immune response by restraint stress speeds the onset and increases clinical signs of disease, exacerbates motor impairment and leads to increased CNS inflammation during the subsequent demyelinating phase of disease (Sieve et al., 2004). Sieve also examined whether disease progression varied according to the sex of the animal and whether sex differentially interacted with stress. Although males exhibited greater behavioral impairments during chronic TMEV infection compared to females regardless of stress exposure, unexpectedly the males did not show corresponding increases in inflammation. However, the negative finding regarding inflammation may have been attributable to methodological limitation. One limitation of that study was the method used to evaluate lesions focused entirely on the presence or absence of inflammatory cells in the spinal cord using hematoxylin and eosin (H&E) staining (for methods see Sieve et al., 2004). This method may lack sensitivity and it does not allow for direct assessment of demyelination. To address these limitations, the present study extends the previous findings by providing a comprehensive analysis using multiple markers of CNS disease, including inflammation, demyelination, axonal loss, astrogliosis, and viral replication in male and

female mice exposed to chronic stress during early TMEV infection. In addition, we sought to further our understanding of the specific mechanisms by which sex and stress impact TMEV-induced behavioral impairments. The data herein indicate that both sex and stress exert powerful influences on disease processes contributing to TVID.

2.0 Materials and Methods

2.1. Subjects

A 2 (male vs. female) $\times$ 2 (stressed vs. unstressed) design was employed with 6 subjects in each condition (N = 24). Male (n = 12) and female (n = 12) SJL/JCrHsd mice were obtained from Harlan (Houston, TX) at 3 weeks of age. All mice were housed 3 per cage with food and water available *ad libitum*; males and females were housed in separate rooms with separate ventilation systems. All subjects were evaluated during both the early (first 4 weeks post-infection) and the late (from 4 weeks post-infection until sacrifice at 135 days pi) phase of disease. One mouse died during the course of restraint stress (male/stressed), so this subject was excluded from the microscopic analysis presented here. Two age-matched non-infected mice were sacrificed at the same age and served as comparison subjects for microscopic analyses, but these mice were not used for statistical analyses. All animals were housed in accordance with Texas A&M University and National Institutes of Health animal care guidelines.

2.2. Restraint stress

Infected mice were restrained in ventilated 60 mL plastic syringes in their home cages (Sheridan et al., 1991; Sieve et al., 2004, 2006). Half of all mice were restrained for 8 hours (h) in their cages one night prior to infection (day -1 post-infection [D-1 pi]) and for 8 h per night, five nights per week, for the following 4 weeks according to the method described previously (Sieve et al., 2004; Campbell et al., 2001). The other half of the mice remained unrestrained in their home cages throughout the experiment. The two age-matched non-infected mice also remained unrestrained in their home cage throughout the experiment.

2.3. Infection

Following the first night of restraint, all mice were inoculated with 5×10^4 pfu of the BeAn strain of TMEV intracranially into the right cerebral cortex (Welsh et al., 1987; Campbell et al., 2001). The BeAn strain of Theiler's virus (obtained from Dr. H.L. Lipton, Department of Neurology, Northwestern University, Chicago, IL) was propagated and amplified in BHK-21 cells. The culture supernatant containing infectious virus was divided into aliquots and stored at -80°C until inoculation.

2.4. Behavioral Measures

All mice were evaluated for behavioral signs of disease during the early (< 4 weeks pi) and late phases of TMEV infection (>4 weeks pi) up through day 135 pi. A detailed presentation of the behavioral data collected from these subjects has been presented elsewhere (see Sieve et al., 2004) and this data is summarized in Table 1. During the early phase of infection, subjects were monitored twice weekly for signs of illness, specifically anhedonia was monitored using sucrose preference changes as determined by the percent sucrose solution consumed and clinical signs of illness/encephalitis (acute phase clinical scores) are used for the present analyses. Previous findings have been mixed with some showing behavioral changes during early infection and others showing no significant behavioral signs of early infection (Lipton, 1975; Sieve et al., 2004, 2006), however we have previously shown significant behavioral changes during early infection. Behavioral impairment during the late phase of disease was measured weekly by rotarod performance (motor task) and late phase

clinical scoring to evaluate the motor impairment (see Sieve et al., 2004, 2006). Spontaneous activity (horizontal and vertical) was measured on D57, D77, and D105 pi, using the open field paradigm described by Sieve et al. (2004, 2006). Only those behavioral measures significantly altered by the experimental manipulation(s) were used for correlation with the present microscopic findings.

2.5. Preparation of spinal cord tissue

Mice were euthanized at D135 pi with pentobarbital, perfused via the left ventricle with PBS followed by 10% formalin in phosphate buffer pH 7.2, and processed as described by Campbell et al. (2001). The present study focused on evaluating lesions in the spinal cord as previous research has shown that the spinal cord is regularly affected during the late phase of the disease (Blakemore et al., 1988; Njenga et al., 1997; Sieve et al., 2004, 2006). Vertebral columns containing intact spinal cord were removed and sectioned transversely into 12 pieces. All segments were embedded in paraffin blocks and stored at 4°C until sectioning. Spinal cords were serial sectioned (5 μ m) on a microtome at room temperature, mounted on individual slides (1 slide for each animal containing 12 spinal sections). Sections were cleared of paraffin with xylene and rehydrated in graded alcohol baths. Two raters, blind to subjects' conditions, scored each serial section using StereoInvestigator software.

2.6. Hematoxylin & Eosin staining and evaluation method for inflammation

Sections were stained with H&E (Sheehan & Hrapchak, 1987). Other sections from these same subjects had been previously evaluated based on a categorical rating system for lesion expression with scores ranging from 0 (*negative for lesion*) to 4 (*very prominent*) (see Campbell et al., 2001; Sieve et al., 2004). However, recent research from our laboratory has established a more quantitative method for measuring lesion extent and severity (see Meagher et al., 2007). The TMEV model is characterized by inflammatory lesions of the spinal cord (Blakemore et al., 1988); lesions evaluated included perivascular cuffing (perivascular accumulation of lymphocytes and macrophages) within the parenchyma, accumulation of lymphocytes and microglia/macrophages within the parenchyma (referred to as microgliosis), and meningitis (accumulation of lymphocytes and macrophages in the meninges). Each section was scored by two raters blind to condition using StereoInvestigator software to measure meningitis and parenchymal inflammation. Meningitis was determined by measuring the total perimeter length (μ m) and then determining the percent perimeter affected by lesion. Parenchymal inflammation was determined by first measuring the total area (μ m²) of each section and then the percent of the total area showing parenchymal inflammation. The two independent rater scores were averaged, and this average result was used for all analyses. Statistical analyses of the inflammatory lesions of the spinal cord involved separate ANOVA conducted for the percent of the perimeter affected by meningitis, the total area of parenchymal inflammation (including both areas occupied by microgliosis and perivascular cuffs) and the total number of perivascular cuffs within each section.

2.7. Weil's staining and evaluation method for demyelination

Demyelination was evaluated following staining of sections with the Weil's method for myelin (Weil, 1928). The Weil's stain technique requires hematoxylin followed by differentiation with iron alum. Sections were allowed to air-dry and then cover slipped. Two independent raters used StereoInvestigator software to separately identify and outline areas of demyelination (all measurements were completed at 10 $\times$ magnification). Each rater then used these tracings to assess 1) the percent area of the total white matter affected by demyelination and 2) the total number of individual lesions present. The average of the two raters' values were used for all analyses. These tracings were then overlaid onto serial

sections from the same subject stained for axons, astroglia, or viral antigen so that axonal loss, glial activation and the presence of TMEV antigen expressing cells could be evaluated both within areas of identified demyelination and in normal white matter.

2.8. Holmes silver impregnation and evaluation method axonal degeneration/loss

Axonal degeneration was evaluated in normal control and infected mice following impregnation of sections according to the Holmes method (Holmes et al., 1947). Sections were impregnated in a 1/20,000 solution of silver nitrate for 16 hours at 37°C and developed in a solution of 1% hydroquinone in 10% sodium sulphite. Sections were allowed to air dry and then cover slipped. For each section, selected areas known to be demyelinated, as identified in adjacent serial sections stained with the Weil's myelin stain, were evaluated. Sections were scored on a 0–5 scale to record the degree of axonal degeneration and/or loss as defined by the proportion of axons that were swollen or missing within areas of demyelination. This measure included both axonal swelling, an indication of axonal degeneration, as well as reduction in the number of impregnated axons. It should also be noted that status spongiosus (a spongiform lesion of the white matter) is a characteristic lesion of this demyelinating process and can often result from axonal loss as well as from splitting of the myelin sheath and separation of the nervous tissue parenchyma. The total evaluation of axonal injury included the evaluation of individual areas of demyelination compared to areas of normal white matter within individual Holmes impregnated sections. The following criteria were used to evaluate each area of demyelination or normal white matter: 0 = 0% axonal degeneration/100% of axons intact; 1 = 20% axonal degeneration/80% of axons intact; 2 = 40% axonal degeneration/60% of axons intact; 3 = 60% axonal degeneration/40% of axons intact; 4 = 80% axonal degeneration/20% of axons intact; 5 = 100% axonal degeneration/0 % of axons intact. Two raters used StereoInvestigator software to assess the total degree of axonal degeneration within all identified areas. The two scores were averaged, and this average value was used for all analyses.

2.9. Glial Fibrillary Acidic Protein (GFAP) staining and evaluation method

GFAP staining for evaluation of astrocytic response was performed using a MACH2 Universal HRP Polymer Detection Kit (MACH2, BioCare Medical, Concord, CA). Slides were incubated with anti-GFAP (1:500) for 1 h at room temperature. After incubation, slides were incubated in secondary antibody (1:100) in PBS for 1 h at room temperature. DAB (3,3'-diaminobenzidine) (Dako Cytomation, Carpinteria, CA) was used in the color reaction at the site of the target antigen. GFAP expression was determined using StereoInvestigator software to measure brightness within areas of demyelination and within areas of normal white matter [scale from 0 (no staining)-256 (very dark staining)]. The average brightness within demyelinated areas was calculated for each section and then compared to the density of non-lesioned/normal areas of white matter in the same sections.

2.10. TMEV immunostaining

Antigen retrieval for TMEV detection in tissue sections was performed by heating slides in a coplin jar containing an EDTA buffer (Trilogy, Cell Marque Corporation, Rocklin, CA) in a 97° C water bath for 20 minutes. Slides were incubated in a 1:10 primary antibody solution for 60 minutes at room temperature. The rabbit anti-TMEV IgG primary antibody was isolated from serum using a cyanogen bromide-activated sepharose column and purified through dialysis of the antibody solution. After primary incubation, sections were rinsed in PBS, 1% normal donkey serum (in PBS) and 1:100 biotinylated secondary antibody for 2 h. Sections were then rinsed in PBS, incubated in ABC complex (Elite Vector) for 1 h, rinsed with PBS and then incubated in DAB (3,3'-diaminobenzidine) (Dako Cytomation, Carpinteria, CA) with nickel.

2.11. Statistical analyses

Data are presented as mean $\pm$ SEM. Analysis of variance (ANOVA) was used to evaluate differences across conditions (e.g. sex and restraint effects). Trend analysis was employed to further evaluate relationships within the data where appropriate (i.e. when the range of scores for a particular measure was restricted). In all cases where trend analysis was employed, groups were numbered 1–4 with 1 = female/unstressed, 2 = female/stressed, 3 = male/unstressed, and 4 = male/stressed, based on our hypotheses. Relationships between histological measures were all completed using standard *Pearson* correlation coefficient scores (r) as were relationships between histological variables and behavioral measures with the exception of clinical scores. Clinical score is a nonparametric measure so a more appropriate nonparametric correlation measure, *Kendall's tau*, was used to determine the relationship between histological measures and clinical score.

3.0. Results

3.1. Inflammation was significantly increased in spinal cords of stressed subjects

Figure 1 (*panels A-E*) depicts representative sections of the spinal cord from each group of infected mice (female/unstressed, female/stressed, male/unstressed, male/stressed) as well as an uninfected control. Lesions included meningitis, microgliosis and perivascular cuffing that involved the cervical, thoracic and lumbar levels of the spinal cord (see higher magnification in Figure 2). Meningitis (indicated by arrows) and microgliosis/perivascular cuffing (indicated by round-end pointers) are prominent in female/stressed, male/unstressed, and male/stressed conditions but less so in the female/unstressed mice. Figure 2 (*panels A-D*) is a higher magnification view of a specific area of the sections from Figure 1 (indicated by arrows in Figure 1). The extent of meningitis and parenchymal inflammation seen in Figure 2 is reflective of that seen throughout the experiment, with female/unstressed (Figure 2, section A) subjects showing significantly less inflammatory cell infiltration of the meninges and parenchyma. As illustrated in Figure 3, female/unstressed subjects exhibited less meningitis and parenchymal inflammation than subjects in all other groups. A 2 (sex) $\times$ 2 (stress) ANOVA revealed a significant main effect of sex on the percent meningitis, $F(1, 19) = 7.70$, $p < 0.05$ (see *panel A*). The main effect of stress was marginally significant, $F(1, 19) = 3.49$, $p = 0.077$. Bonferroni mean comparisons indicated that stress significantly increased the percent meningitis for females ($p < 0.05$) but not males ($p \geq 0.05$). To further evaluate the relationships within the data, we used the more powerful technique of trend analysis. A significant linear trend was found indicating that male/stressed subjects had the highest percent meningitis while female/unstressed subjects had the lowest percent meningitis ($p < 0.001$; 95% confidence interval: lower bound = 4.796, upper bound = 22.061). In addition, female/unstressed subjects had less area occupied by inflammation (area of parenchymal inflammation/total area of section) compared to all other conditions (see *panel B*). A 2 (sex) $\times$ 2 (stress) ANOVA confirmed a significant main effect of sex, $F(1, 19) = 4.41$, $p < 0.05$. Bonferroni mean comparisons did not show a significant effect of stress for females or males (all $p > 0.05$). However, a significant linear trend indicated that the male/stressed subjects had the greatest area of total inflammation while the female/unstressed subjects had the least ($p < 0.05$; 95% confidence interval: lower bound = 0.101, upper bound = 1.077).

3.2. Demyelination was significantly increased in spinal cords of stressed subjects

Separate analyses were conducted for the area of demyelination and the number of lesions present. Demyelination was found in cervical, thoracic and lumbar sections, and lesions occurred with equal frequency in ventral and lateral funiculi of affected sections. Figure 4 illustrates the areas of demyelination as well as normal white matter in the four groups of mice examined. Female/unstressed subjects (*panel A*) exhibited less demyelination

compared to all other groups (female/stressed-*panel B*, male/unstressed-*panel C*, male/stressed-*panel D*). While stress increased the severity of demyelination in both males and females, the sex difference was abolished for stressed males and females (see *panels B* and *D*). As illustrated in Figure 5, stress significantly increased the degree of demyelination. A 2 (sex) $\times$ 2 (stress) ANOVA confirmed a significant main effect of stress on the percent of white matter showing demyelination, $F(1, 19) = 7.71, p < 0.05$ (see *panel A*). No other significant main effects or interactions were present. Bonferroni mean comparisons confirmed a significant effect of stress for both males ($p < 0.05$) and females ($p < 0.01$). Trend analysis was also conducted. A significant linear trend existed, indicating that subjects in the male/stressed condition showed the highest percent demyelination while female/unstressed subjects exhibited the lowest percent demyelination ($p < 0.05$; 95% confidence interval: lower bound = 0.590, upper bound = 6.497). The male/stressed group also showed the highest number of total lesions. A 2 (sex) $\times$ 2 (stress) ANOVA confirmed a significant main effect of sex on lesion number, $F(1, 19) = 4.524, p < 0.05$ (see *panel B*). No other significant main effects or interactions existed. Bonferroni mean comparisons confirmed that stress significantly increased lesion number for males ($p < 0.05$) but had no significant effect on lesion numbers in females ($p > 0.05$). Trend analysis was also conducted, and a significant linear trend indicated that male/stressed subjects exhibited higher numbers of lesions than any other group ($p < 0.05$; 95% confidence interval: lower bound = 1.005, upper bound = 8.997).

3.3. Axonal degeneration was increased in demyelinated areas in stressed mice

Figure 6 depicts representative sections from a male/stressed subject. Axonal degeneration was increased by stress exposure but not significantly affected by sex. As illustrated in Figure 7, stress exposure resulted in greater axonal degeneration within areas of demyelination regardless of sex. A 2 (sex) $\times$ 2 (stress) ANOVA revealed a main effect of stress that approached significance, $F(1, 18) = 4.045, p = 0.06$. No other significant main effects or interactions were detected, all $F(1, 18) \leq 0.45, p \geq 0.513$. Bonferroni mean comparisons revealed that stress significantly increased axonal degeneration for males but not females. A significant linear trend indicated that male/stressed subjects had more axonal degeneration than all other conditions ($p < 0.05$, 95% confidence interval: lower bound = -0.000646, upper bound = 0.829).

3.4. Glial activation coincided with areas of demyelination

GFAP expression was greater within areas of demyelination compared to areas of normal white matter (data not shown). A 2 (sex) $\times$ 2 (stress) $\times$ 2 (lesion vs. control area) ANOVA confirmed a difference in GFAP expression between areas of normal white matter (control) and areas of demyelination, $F(1, 18) = 36.407, p < 0.001$. GFAP staining was not affected by sex or stress; no other significant main effects or interactions were present, all $F(1, 18) \leq 0.973, p \geq 0.337$. Bonferroni mean comparisons indicated that stress did not significantly increase astrogliosis for males or females. Trend analysis did not detect a significant trend for differences in astrogliosis. It is worth noting that areas of demyelination tend to have a high number of vacuoles resulting in greater luminance from these areas (i.e. more light passes through the “holes” and increases the brightness of these areas). While there were no effects of stress or sex on the extent of astrogliosis as determined by this method, the areas of demyelination were significantly darker indicating more intense GFAP staining in spite of the increased presence of vacuoles within the areas of interest.

3.5. Cells expressing TMEV antigen were detected within and surrounding areas of demyelination

In susceptible strains of mice, TMEV persists in macrophages, monocytes and oligodendroglia within CNS white matter and contributes to the development of

autoimmune-mediated demyelination (Brahic et al., 1981; Lipton & Dal Canto, 1977). As illustrated in Figure 8, cells staining positive for TMEV antigen, many of which were identified as putative macrophages, were found in and around areas of demyelination. Other much smaller mononuclear cells that did not stain positively were interpreted to be lymphocytes. Virus-positive cells were largely concentrated in and around areas of demyelination. The distribution of TMEV antigen-expressing cells differed based on the stage of demyelination within a particular section. As can be seen in Figure 9A, areas that were suspected to be in a stage preceding demyelination contained virus-positive cells throughout the area affected. In contrast, the TMEV-positive cells primarily surrounded clearly defined areas of demyelination (Figure 8B).

3.6. Relationships between histological and behavioral measures

3.6.1. Relationships among histological measures—The percent meningitis and percent area of demyelination were highly positively correlated, $r = 0.592$, $p < 0.01$. This relationship was not surprising given that the demyelination is dependent on an immune-mediated process within the CNS. In addition, percent area of demyelination was positively correlated with average axonal loss, $r = 0.433$, $p < 0.05$.

3.6.2. Inflammation-behavioral outcome correlations—A variety of behavioral measures of early and late phase disease were correlated with inflammation (see Table 2). Inflammation was correlated with several measures of disease severity in both the early and late phases. It was anticipated that behavioral measures would correlate with measures of inflammation within the brain and spinal cord as we have previously shown a relationship between symptom severity and extent of inflammation (Sieve et al., 2004; Johnson et al., 2006; Meagher et al., 2007). Both total area of inflammation, *Kendall's tau* = 0.369, $p < 0.05$, and the percent perimeter with meningitis, *Kendall's tau* = 0.453, $p < 0.01$, were positively correlated with early phase clinical scores taken during the fourth week of infection (D 22 pi). In addition, a significant negative correlation existed between sucrose preference scores at D 24 pi and the total area of inflammation in late phase, $r = -0.389$, $p < 0.05$. This finding suggests that early phase clinical scores serve as a behavioral indicator of the early phase viral load and thereby serve as a predictor of inflammatory lesion formation in the late phase disease. Both horizontal activity and vertical activity at D50 pi were negatively correlated with the severity of meningitis during late phase TMEV infection, $r = -0.377$, $p < 0.05$ and $r = -0.366$, $p < 0.05$, respectively. However, later activity levels on days 77 and 105 pi were not significantly correlated with severity of meningitis or with total inflammation, but this is likely due to an overall reduction in activity for all subjects as the late phase progressed. Finally, a significant positive correlation existed between late phase clinical scores (D 130 pi) and severity of meningitis, *Kendall's tau* = 0.287, $p < 0.05$.

3.6.3. Demyelination-behavioral outcome correlations—Demyelination described in the late stage of the disease in this study was strongly correlated with clinical scores during both early and late disease phase; additionally, demyelination was highly negatively correlated with two measures of hind limb motor coordination and balance, rotarod performance and vertical activity. As can be seen in Table 2, clinical scores taken during early infection (D 22 pi) were positively correlated with the eventual severity of demyelination (percent area of total white matter with demyelination), *Kendall's tau* = 0.668, $p < 0.001$. While D 22 pi is too early for demyelination to have developed, it is possible that this correlation is due to another variable related to both behavioral signs of early disease and demyelination (e.g. high viral load in early disease and resultant increased viral lysis of oligodendrocytes in late phase disease). Vertical activity, but not horizontal activity, occurring soon after the initiation of late phase (D50 pi) was negatively correlated with severity of demyelination, $r = -0.473$, $p < 0.05$. Also, as seen in the inflammation-

activity correlation, horizontal and vertical activity measures taken longer after the initiation of the late phase (Days 77 and 105 pi) showed no significant correlation with severity of demyelination. As with inflammation, it appears that all subjects exhibited significantly reduced activity levels at these later time points. Clinical scores during the late phase (D 130 pi) were positively correlated with the severity of demyelination, *Kendall's tau* = 0.411, $p < 0.01$. A significant negative correlation existed between rotarod speed (124 pi) and severity of demyelination, $r = -0.656$, $p < 0.001$. In addition, subjects' latency to fall from the rotarod was negatively correlated with the severity of demyelination, $r = -0.549$, $p < 0.01$.

4.0. Discussion

Previous data suggest that both stress exposure and sex play a significant role in determining the severity of the behavioral changes associated with TMEV infection (Johnson et al., 2004; Sieve et al., 2004, 2006), but the impact of these factors on demyelination has been inconclusive. The present study evaluated the impact of sex and stress on inflammatory demyelinating lesion formation during TVID. In a previous study, Sieve et al. (2004) showed that male mice and mice exposed to chronic restraint stress during early TMEV infection exhibited more severe clinical signs of disease and more impaired motor coordination during the late phase of disease than females and unstressed controls. Contrary to these behavioral findings, stress exposure during this time increased CNS inflammatory cell infiltration during the late phase of disease but there was no discernible difference in inflammation between males and females. The current study used additional histological measures of TVID and a more rigorous method of quantifying inflammatory cell infiltration to evaluate whether sex and stress impact behavioral symptoms through alterations in disease processes. Our results confirm prior research indicating that stress exacerbates CNS disease severity (inflammation and demyelination). Though males show increased disease severity regardless of stress exposure, we demonstrated that restraint stress differentially affects female SJL mice, resulting in more severe CNS inflammation (non-suppurative meningitis and parenchymal inflammation) and more extensive demyelination (% demyelination) compared to unstressed females.

Putative macrophages and activated astrocytes were found to be closely associated with areas of demyelination, a finding in agreement with previous research (Peña Rossi et al., 1997; Pozner et al., 2004; Rubio et al., 2006; Zheng et al., 2001). We also found that virus-positive cells were distributed centrally within newly forming lesions and in the periphery of more mature/developed lesions. Astrocytes were activated within areas of demyelination, though neither sex nor stress affected the extent of astrogliosis. With the exception of the astrocytic response, the above results suggest that sex and stress alter the progression and severity of behavioral impairments and CNS damage occurring in the late phase of TMEV infection. We hypothesize that sex and stress impair early viral clearance thereby increasing the vulnerability to and/or severity of TVID. Impaired viral clearance could increase lesion formation through a number of pathways, both direct (e.g. increased viral lysis of oligodendrocytes) and indirect (e.g. increased baseline inflammation within the CNS due to a persistent viral infection that increases recruitment of macrophages and reactivation of virus in astrocytes and oligodendrocytes to cause demyelination). Based on the present results, future studies should investigate the mechanisms mediating the more severe behavioral and histological manifestations of late disease observed in males (compared to females) and following stress exposure in females. The present results may help shed light on the relationship between stressful life events and relapse (Mohr et al., 2000), as well as on the underlying mechanisms responsible for the more rapid disease course in men (Noseworthy et al., 2000).

4.1 Stress-induced alterations in TMEV-induced demyelinating disease

The development of TVID involves the early infection of neurons followed by viral persistence in glia and macrophages and the recruitment of TMEV-specific T cells and antibody formation directed against CNS tissue resulting in autoimmunity. Stress exposure before and during the initial viral infection can result in significant immunosuppression during a time when an effective immune response is required to limit viral replication (Campbell et al., 2001; Mi et al., 2004; Sieve et al., 2004, 2006; Welsh et al., 2004; Young et al., 2008). Supporting this, we have previously shown that restraint stress increases circulating corticosterone (CORT) levels, downregulates the expression of chemokines and cytokines, and impair NK cell function, resulting in diminished immune cell infiltration into the CNS and impaired viral clearance (Campbell et al., 2001; Mi et al., 2004, 2006; Steelman et al., 2009; Welsh et al., 2004; Young et al., 2008). Restraint also results in significant lymphopenia and thymic atrophy, which may further impair viral clearance and, subsequently, eventually permit the formation of autoreactive T and B cells. Because stress is immunosuppressive during this period, the virus persists in the CNS and leads to eventual development of the demyelinating phase of the disease.

4.2. Sex-related differences in immune response and disease vulnerability

Previous studies investigating sex differences in the development of TVID report contradictory findings. One study by Hill et al. (1998) reported that female mice showed greater disease susceptibility during the transitional period between the early encephalitic phase (day 14 pi) and the onset of the demyelination (day 27 pi) compared to males. In contrast, Alley et al. (2003) found that males exhibited higher viral titers during early disease and more severe late phase motor impairments, but only minimal increases in spinal cord demyelination that did not explain differences in motor impairment when focusing on late phase motor impairment and demyelination (day 45 pi and at 5–6 months pi). Sieve et al. (2004) and the current study examined early and late disease, including viral replication, immune responses, motor function, as well as various measures of CNS disease in male and female SJL mice. Our work shows a repeated pattern of increased disease severity in males, which is consistent with the MS literature (Noseworthy et al., 2000).

Sex differences in disease course may be determined by multiple factors, including differential regulation of the immune response by sex hormones (Kappel et al., 1990). Androgens have been linked with increased susceptibility to viral infection by upregulating viral receptors (Lyden et al., 1987), and altering the inflammatory response to infection (Huygen & Palfliet, 1984; Araneo et al., 1991; Quinn et al., 1993). Across a variety of models, females show more robust humoral and cellular immune responses (Gaillard & Spinedi, 1998), which could contribute to more effective viral clearance during early infection and altered development of autoimmunity during late disease (Whitacre et al., 1999). In the present study, females exhibited less severe disease during the late phase, which would be expected if viral clearance were more effective. This is in agreement with other work showing that estrogen can be neuroprotective by reducing inflammation, demyelination and axonal loss during the late disease (Fuller et al., 2005, 2007).

Given the increased effectiveness of the female immune response to viral infection, it may seem counterintuitive that females are much more likely to be diagnosed with MS. However, females may also differ from males in their sensitivity to and/or risk for stress exposure. Along this line, females tend to show higher levels of circulating corticosterone following stress exposure compared to males (Homo-Delarche et al., 1991; Gaillard & Spinedi, 1998; Sieve et al., 2004; Turner, 1990). An increased corticosterone response could translate to greater immunosuppression and failure to clear neurotropic viral infections as well as, potentially, increased risk for chronic demyelinating disease due to higher viral load.

4.3. Conclusions

Human MS research suggests that a variety of factors including sex, stress, and pathogen exposure play an important role in determining vulnerability, predicting disease course and in the risk for relapse. Although MS diagnosis rates are higher for women, men are at increased risk for the more severe primary-progressive presentation of the disease (Noseworthy et al., 2000). While the mechanisms mediating the severe functional impairments observed in men with MS remain to be elucidated, recent data from our laboratory (Meagher et al., 2010) and others (Alley et al., 2003; Fuller et al., 2005, 2007) suggests that females exhibit more effective viral clearance resulting in lower viral load, as well as reduced inflammation and demyelination, during late disease than in males. Given that several retrospective studies indicate life stress often precedes disease onset (Ackerman et al., 2002; Grant et al., 1989; Warren et al., 1982), the present data may help shed light on the mechanisms by which stress impacts disease course in men and women with MS.

Acknowledgments

This research was supported by grants to C.J.R.W. and M.W.M from the National Multiple Sclerosis Society RG 3128 and NIH/NINDS R01 39569 as well as NIH/NINDS R01-NS060822 awarded to M.W.M and C.J.R.W. Dr. A.N. Sieve is currently affiliated with the University of North Texas Health Science Center, Department of Molecular Biology and Immunology. The authors would like to thank Torry Dennis and Laura Gravens for their assistance with data collection.

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Figure 1.

Representative spinal cord sections from each condition illustrating degree of inflammation (sections A-D: all animals viral inoculated, section E: uninfected control, H & E stain-4× magnification). Subjects in the female/unstressed condition (*section A*) exhibited significantly less non-suppurative meningitis (arrows) and parenchymal inflammation/lymphocyte/microglia accumulation and perivascular cuffing (indicated by round-end pointers) compared to the female/stressed (*section B*), male/unstressed (*section C*), and male/stressed (*section D*) conditions. All infected conditions (*sections A-D*) had evidence of meningitis and inflammation compared to no lesions present in a section taken from an uninfected/unstressed age-matched comparison (*section E*).

Figure 2.

Higher magnification of designated areas in Figure 1 (indicated by arrows in Figure 1 sections A-D) corresponding to each condition illustrating non-suppurative meningitis and parenchymal inflammation (all animals viral inoculated, H & E stain-10× magnification). Meningitis can be seen in all sections (*sections A-D*) as collections of darkly stained cells in the meninges (outer edges of the section); in addition, infected mice in all conditions showed accumulation of lymphocytes/macrophages/microglia (darkly stained cells located throughout the section-*sections B and D*) and accumulation of lymphocytes/macrophages surrounding blood vessels within the parenchyma (perivascular cuffing-*section B*). Female/unstressed mice (*section A*) exhibited significantly less inflammatory cell infiltration of the meninges and parenchyma compared to all other conditions (*sections B-D*).

A. SEVERITY OF MENINGITIS

B. SEVERITY OF PARENCHYMAL INFLAMMATION

Figure 3.

Statistical evaluation of H & E stained spinal cord sections from animals-all viral inoculated. All data is presented as mean $\pm$ SEM. Stress significantly increased both meningitis and total inflammation (noted by a single asterisk)(based on ANOVA). When analyzed separately using planned comparisons stress was shown to increase inflammation in females, but not males; the significant difference between the female/unstressed and female/stressed conditions is noted by two asterisks. *Panel A* depicts the percent meningitis in the meninges covering the circumference of the section The female/unstressed condition had less percent perimeter with meningitis than the female/stressed, male/unstressed, and the male/stressed conditions. *Panel B* depicts the percent of parenchymal inflammation [sum of areas of

accumulated lymphocytes/total area of white matter]. Subjects in the female/unstressed condition had the smallest area of total inflammation compared to the female/stressed, male/unstressed, and male/stressed conditions.

Figure 4.

Representative spinal cord sections from each condition illustrating demyelination involving the lateral and/or ventral funiculi (all animals viral inoculated, Weil's stain-4 $\times$ magnification). The demyelinated (light and unstained; arrows) areas adjacent to normal myelinated tissue (black/gray) in the lateral and ventral funiculi are particularly visible in sections B (female/stressed) and D (male/stressed). Subjects in the female/unstressed condition (*section A*) showed less demyelination than female/stressed (*section B*), male/unstressed (*section C*), and male/stressed (*section D*). Stress significantly increased demyelination as can be seen when the minimal demyelination in sections A and C (female/unstressed and male/unstressed, respectively) is compared to the more extensive demyelination in sections B and D (female/stressed and male/stressed, respectively). Male/unstressed subjects (*section C*) showed more demyelination than female/unstressed subjects (*section A*) as indicated by the arrows pointing to larger total areas of demyelination.

A. PERCENT AREA OF DEMYELINATION

B. NUMBER OF DEMYELINATED AREAS

Figure 5.

Measures of white matter affected by demyelinating lesions. All data are presented as mean $\pm$ SEM, asterisk denotes a significant difference. Stress significantly increased the area of demyelination (sum of the areas of demyelination/total area of white matter) and the number of lesions present within the white matter; significant differences between stressed and unstressed groups are denoted by an asterisk. *Panel A.* When analyzed separately using planned comparisons, stress was shown to increase the percent demyelination for both males and females. *Panel B.* When analyzed separately using planned comparisons, stress was shown to increase the number of individual lesions present in males but not females;

significant difference between male/unstressed and male/stressed conditions is noted by two asterisks.

A. WEIL'S STAIN (DEMYELINATION)

B. HOLMES STAIN (AXONS)

Figure 6.

Section A is a representative Weil's stained section ($4\times$ magnification; animal viral inoculated) from a male/stressed subject illustrating two areas of very early demyelination (solid lines) and an area of normal white matter for comparison (dashed line); *section B* depicts the Holmes silver nitrate impregnated serial section from the same subject ($4\times$ magnification) with the inset frame illustrating axonal degeneration within one of the illustrated areas of very early demyelination (Holmes stained, $40\times$ magnification [oil]). The arrow heads indicate examples of swollen and degenerating axons within the areas of demyelination; arrows indicate normal/intact axons within the same area.

EXTENT OF AXONAL DEGENERATION WITHIN AREAS OF DEMYELINATION

Figure 7.

Axonal degeneration measured within areas of demyelination (Holmes silver nitrate impregnation). All scoring was performed at 20× magnification; each area of demyelination (or normal white matter) was assigned a score on a scale from 0–5 when compared to the surrounding normal white matter with 0 representing no axonal degeneration or loss. All data are presented as mean ± SEM, asterisk denotes a significant difference as a result of stress exposure based on the ANOVA. Planned comparisons revealed that stress increased axonal degeneration for male, but not female subjects; double asterisks denote the significant difference between the male/stressed and male/unstressed conditions.

Figure 8.

Representative spinal cord section from a female/stressed subject illustrating the presence of TMEV antigen in putative macrophages associated with an area interpreted to be a stage preceding demyelination and area of well-defined demyelination (immunohistochemical staining for TMEV antigen, 4× with 20× magnification insets, animal viral inoculated). Arrows indicate cells identified as being virus-positive (dark staining for TMEV-antigen). The distribution of TMEV antigen-expressing cells interpreted to be macrophages differed based on the stage of demyelination determined by the extent of lesion formation at a particular location. *Inset A.* Lesion suspected to be a stage preceding demyelination containing many dark brown immunostained cells interpreted to be macrophages. This pattern of TMEV antigen-expressing cells was more frequently seen in male subjects. *Inset B.* Lesion identified by well-defined demyelination surrounded by TMEV antigen-expressing cells, interpreted to be macrophages; this pattern occurred more frequently in female subjects. Note that the macrophages are more peripheral than central at this stage of lesion development.

Table 1

Average Scores for Behavioral Measures of Disease Severity

			Unstressed		Stressed	
<i>Acute Phase</i>			Male	Female	Male	Female
Sucrose Preference	Mean		78.2%	74.9%	47.6%	82.6%
	SEM		0.173	0.022	0.215	0.008
Clinical Score (Illness)	Mean		0.08	0.0	0.60	0.41
	SEM		0.480	0.00	0.167	0.076
<i>Chronic Phase</i>						
Clinical Score	Mean		3.04	2.92	4.6	3.71
	SEM		0.199	0.096	0.456	0.109
Spontaneous Activity	Horizontal	Mean	13.383	18.83	14.88	20.88
	SEM		0.973	1.887	2.262	1.781
Vertical	Mean		0.811	1.23	0.993	0.93
	SEM		0.138	0.191	0.317	0.229
Speed	Mean		1.83	2.0	1.6	1.58
	SEM		0.152	0.236	0.219	0.248
Rotarod	Latency	Mean	18.83	16.1	24.33	22.58
	SEM		3.404	5.294	3.554	5.070

The early phase of TMEV infection is defined as up to 4 weeks post-infection, corresponding to the period of restraint in the present experiment. The late phase is defined as from 4 weeks to 135 days post-infection for the present experiment. Restraint stress during the early phase significantly increased behavioral impairments during both early and late disease phases. Males showed more severe behavioral impairments than females during both the early and late phase.

Table 2

Correlations between Behavioral Measures taken during acute and chronic TMEV disease and Inflammation/Demyelination following restraint stress

	Inflammation	Demyelination
<i>Acute phase</i>		
Sucrose Preference	-0.389 ^{a*}	---
Clinical Score	0.369 ^{a*} , 0.453 ^{b*}	0.668 ^{***}
<i>Chronic phase</i>		
Vertical Activity	-0.366 ^{b*}	-0.473 [*]
Horizontal Activity	-0.377 ^{b*}	---
Clinical Score	0.287 ^{b*}	0.411 [*]
Rotarod (speed & latency)	---	-0.656 (s) ^{***} , -0.549 (l) ^{**}

* indicates significant correlations with $p < 0.05$

** indicates significant correlations with $p < 0.01$

*** indicates significant correlations with $p < 0.001$

^a indicates inflammation measure is total area of inflammation

^b indicates inflammation measure is percent perimeter with meningitis

CNS inflammation was significantly correlated with all behavioral measures of disease except rotarod performance. demyelination was highly correlated with clinical scores in both the early and late disease phase as well as two measures of motor coordination and balance, vertical activity and rotarod performance.