

Chronic restraint stress during early Theiler's virus infection exacerbates the subsequent demyelinating disease in SJL mice

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Received 15 March 2004; received in revised form 1 June 2004; accepted 15 June 2004

Abstract

Chronic restraint stress, administered during early infection with Theiler's virus, was found to exacerbate the acute central nervous system (CNS) viral infection and the subsequent demyelinating phase of disease (an animal model of Multiple Sclerosis (MS)) in SJL male and female mice. During early infection, stressed mice displayed decreased body weights and spontaneous activity; while increased behavioral signs of illness and plasma corticosterone (CORT) levels. During the subsequent chronic demyelinating phase of disease, previously stressed mice had greater behavioral signs of the chronic phase, worsened rotarod performance, and increased inflammatory lesions of the spinal cord. In addition, mice developed autoantibodies to myelin basic protein (MBP), proteolipid protein peptide (PLP139-151), and myelin oligodendrocyte glycoprotein peptide (MOG33-55).

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Keywords: Theiler's murine encephalomyelitis virus (TMEV); Restraint stress; Demyelination; Multiple Sclerosis; Myelin-reactive autoantibodies

1. Introduction

Multiple Sclerosis (MS) is one of the most common demyelinating conditions of the central nervous system (CNS), effecting 350,000 people in the United States alone (Anderson et al., 1992). MS is characterized by infiltration of inflammatory cells, focal demyelination, and loss of axons in the CNS, which commonly leads to a loss of sensation and function in the limbs, incontinence, and eventually paralysis (Mohr and Dick, 1998). Psychological stress has been linked to the development of MS in human patients (for review see, Mohr and Cox, 2001), and has likewise been shown to have a profound impact on animal

models of MS, such as Experimental Allergic Encephalomyelitis (EAE) and Theiler's virus induced demyelination (TVID) (Levine et al., 1962; Levine and Saltzman, 1987; Griffin and Whitacre, 1990; Bukilica et al., 1991; Griffin et al., 1993; Dowdell et al., 1999; Campbell et al., 2001; Welsh et al., 2004). However, the exact nature of the interaction between stress and these demyelinating conditions is poorly understood.

In MS patients, stress frequently precedes the initial development of the disease, as well as exacerbations in symptomatology and lesion development (Warren et al., 1982; Grant et al., 1989; Mohr et al., 2000; Mohr and Cox, 2001; Ackerman et al., 2003). However, whereas moderate chronic stressors (such as daily hassles) lead to exacerbation, relatively brief severe stressors appear to alleviate symptoms (Mohr et al., 2000; Mohr and Cox, 2001; Ackerman et al., 2003; Nisipeanu and Korczyn, 1993).

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Similarly, the relationship between stress and disease activity is complex in animal models of MS. For example, administration of stressors following the induction of EAE has been found to suppress many indices of disease (Levine et al., 1962, 1987; Griffin and Whitacre, 1990; Bukilica et al., 1991; Griffin et al., 1993; Dowdell et al., 1999). In contrast, chronic stress administered prior to and during acute Theiler's virus infection tends to exacerbate the development of disease (Campbell et al., 2001; Johnson et al., 2004; Satterlee et al., 2001; Welsh et al., 2004; Mi et al., 2004).

Theiler's murine encephalomyelitis virus (TMEV) typically causes an asymptomatic acute viral infection of the CNS (Theiler, 1934). However, under certain conditions (such as stress-induced immunosuppression) behavioral signs of early infection develop (Campbell et al., 2001; Welsh et al., 2004; Johnson et al., 2004). If the virus persists in the CNS, this is followed by an inflammatory demyelinating condition of the CNS (Lipton, 1975). Restraint (RST) stress administered during the first 4 weeks of Theiler's virus infection exacerbated this early infection: increasing behavioral signs of infection, weight loss, mortality rates, and viral load in the CNS, while decreasing inflammation in the brain at day 7 pi (Campbell et al., 2001). This effect appears to be mediated by two major stress systems: the hypothalamic pituitary adrenal axis (HPA) and the sympathetic medullary axis (SAM) (Satterlee et al., 2001; McCullough et al., 2002; Faulkner et al., 2003). Although the effects of stress on this early viral infection have been investigated, it is unclear whether stressed-induced exacerbation of acute infection alters the severity of the subsequent chronic demyelinating phase of the disease.

To further investigate this issue, the present study examined whether administration of chronic RST stress during acute infection with Theiler's virus alters the course of the chronic demyelinating disease. Previously, we have found that chronic RST stress exacerbates acute TMEV infection in male CBA mice (Campbell et al., 2001). However, susceptibility to Theiler's virus is under genetic control, and also varies according to the sex of mice (Lipton, 1975; Kappel et al., 1990). Whereas CBA mice display an intermediate susceptibility to the development of TVID, SJL mice are highly susceptible to persistent CNS infection with Theiler's virus and the development of TVID. In some studies, female SJL mice are known to have greater susceptibility to disease as compared to males, a pattern that is similar to that found in human MS patients (Kappel et al., 1990; Hill et al., 1998), while under different housing conditions, male mice develop more severe symptomatology of disease (Alley et al., 2003). Thus, disease progression across sex and strain varies, and may differentially interact with RST stress. To investigate the effects of chronic RST stress during acute infection on the subsequent TVID, we used male and female SJL mice. SJL mice are the most commonly used strain to study the chronic demyelinating phase of TVID, and the time-course and progression of the

demyelinating disease is well characterized (for reviews see: Miller and Gerety, 1990; Tsunoda et al., 1997; Kim and Palna, 1999; Oleszak et al., 2004). Thus, we evaluated the effects of restraint and sex on behavioral, histological, and immunological manifestations of acute and chronic disease.

2. Materials and methods

2.1. Subjects

Male ($n=12$) and female ($n=12$) SJL mice were obtained from Harlan (Houston, TX) at 3 weeks of age. All mice were housed three per cage with food and water available ad libitum. Male and female mice were housed in separate rooms with separate ventilation systems. They were allowed to acclimate to their environment for 1 1/2 weeks prior to infection, during which time they were handled by all experimenters at least twice and baseline measures were obtained. All animals were housed in accordance with Texas A&M University and National Institutes of Health animal care guidelines.

2.2. Infection

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 aliquoted and stored at -70°C before use (Welsh et al., 1987). As in previous studies, mice were inoculated with 5×10^4 p.f.u. of the BeAn strain of Theiler's virus intracranially into the right cerebral cortex (Welsh et al., 1987; Campbell et al., 2001) at 4.5 weeks of age.

2.3. Restraint stress

Mice were restrained in their home cages, in 60 ml plastic syringes, drilled with holes for ample ventilation (Sheridan et al., 1991; Campbell et al., 2001). RST occurred for a duration of 8 h, during the dark cycle, for five successive nights per week, with 2 days off in between weeks. The duration of RST was determined by a pilot study to be the maximum amount of RST stress that uninfected SJL mice of this age could tolerate.

2.4. Behavioral measures

2.4.1. Behavioral scoring

During the acute phase of Theiler's virus infection, mice were observed and given a numerical score for behavioral indications of encephalitic-like symptoms: 0=no behavioral signs of illness, 1=ruffled fur, 2=ruffled fur and slightly hunched posture, 3=ruffled fur, very hunched posture, and lethargic, 4=moribund (Campbell et al., 2001). During the chronic phase of disease, mice were observed and given a

numerical score for behavioral signs of the chronic phase: 0=no behavioral impairment, 1=weakness in hind limbs, 2=slightly wobbly gait, 3=definitely wobbly gait, 4=very wobbly gait, hunched posture, and loss of righting reflex, 5=all of the previously mentioned symptoms and incontinence, 6=moribund (Borrow et al., 1998).

2.4.2. Sucrose preference

As an additional index of illness, sucrose preference was measured during the acute phase of the disease. Preference for a sweet solution such as sucrose has been shown to decrease following immune challenge with lipopolysaccharide and GP120 administration (Barak et al., 2002; Yirmiya et al., 1994; Yirmiya, 1996). However, other studies indicate that chronic stress increases preference for sweet food and food intake (Badiani et al., 1996; Ely et al., 1997). Mice were given the option of 2% sucrose solution or tap water for the week prior to infection, and for the following 4 weeks. The position of the sucrose and water bottles was alternated daily, to prevent any place preference. Sucrose preference was calculated by dividing the intake of the sucrose solution, by the total fluid intake.

2.4.3. Rotarod

Mice were placed on a rod (4 cm in diameter, and 20 cm in length) located 20 cm from a padded platform rotating at 6 rpm. Every 30 s, the speed of rotation was increased by 3 rpm (9, 12, 15, 18, 21, 24, 27, 30 rpm). The latency and speed at which the mouse fell from the rod was recorded. Each mouse was run through two trials each session. McGavern et al. (1999) have found this test to be sensitive to the motor impairments produced by TVID, which are observed with demyelinating lesions in the spinal cord.

2.4.4. Spontaneous activity

Spontaneous activity has been found to be reduced by immune challenge (lipopolysaccharide, GP120) and Theiler's virus induced demyelinating lesions in the spinal cord (Yirmiya et al., 1994; McGavern et al., 1999; Barak et al., 2002). McGavern et al. (1999) found that spontaneous activity decreased in Theiler's infected animals as compared to uninfected controls during the chronic phase of Theiler's virus infection. A modified version of Yirmiya et al. (1994) and Ossenkopp et al. (1994) was used to monitor spontaneous activity. To measure activity, mice were placed individually in a 9 in. (width)×15 in. (length)×24 in. (height) open field coated with 1/4 in. of the same bedding used in their home cages. They were videotaped from 24 in. above the floor, for a 10-min session. The tapes were scored for overall locomotion and frequency of specific behaviors, by experimenters blind to the subjects' conditions. In order to determine locomotion, a grid was placed over the television screen, covering the image of the floor of the open field (five squares across, and three squares down). Passage of the head and shoulders into a new square was considered a square entry. The total number of square

entries per minute was analyzed. During a separate scoring session, the frequency per minute of jumping, leaning, and rearing was recorded to measure vertical activity. Overall horizontal activity was operationalized as the total number of interior and exterior square entries. Overall vertical activity was operationalized as the sum of jumping, rearing and leaning. Separate statistical analyses of the individual subscales of horizontal (interior and exterior grid entries) and vertical (jumping, rearing, and leaning) yielded the same pattern of results as analyzing our horizontal and vertical summary measures.

2.5. Assays on plasma

2.5.1. Blood collection

Mice were individually transported to an adjacent room and bled via the saphenous vein, within 2 min of cage disturbance to minimize stress artifacts. The legs were shaved 12 h earlier. The order of blood collection was counterbalanced across conditions. After the bleeding procedure, mice were placed in a recovery cage separate from their home cage, until all of the mice had been bled.

2.5.2. Corticosterone

Plasma corticosterone (CORT) was measured by radioimmunoassay (RIA) as described in Keith et al. (1978). Following centrifugation and separation, plasma samples were stored at -80°C until analyzed. The CORT level in 10 μl of plasma was determined using a ^{125}I -RIA kit (ICN Biomedicals, Costa Mesa, CA).

2.5.3. Antibody responses to Theiler's virus and myelin proteins

RIAs were used to test mouse plasma for antibodies against Theiler's virus, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein peptide (MOG33-55) and proteolipid protein peptide (PLP139-151) using previously described procedures (Young et al., 1983; Dolimbek et al., 2002). PLP139-151 is the major encephalitic peptide recognized by SJL mice (McRae et al., 1992). This technique was developed because conventional ELISA tests were not sensitive enough to detect these antibodies in the plasma from our mice. The RIA was developed using radiolabeled protein-A which binds to the Fc portion of immunoglobulin. Consequently, the level of radioactivity measured equated with the antibody level. As Fig. 6 shows, the antibody levels are fairly low, and by a dilution of 1/160 are not detectable.

Briefly, the plates were washed with Tween 20 (0.05% v/v) in RO H₂O and rinsed with RO H₂O. Washed flexible u-shaped, 96-well polyvinyl chloride plates (Costar, Cambridge, MA) were coated with 100 μl of carbonate buffer (pH 9.6) containing Theiler's virus (1.0×10^7 p.f.u./100 μl). Likewise, to bind MBP or myelin peptides to the plates, 100 μl assay buffer (made up from two parts: 495 ml of part A: 0.08 M Trizma HCl, 0.03 M Trizma base and 0.15 M NaCl

at a final pH of 7.2, and 5 ml of part B: 1.0% nonfat dry milk (NFDM) and 0.5% Tween-20 in reverse osmosis (RO) H₂O containing either 1.0 µg of either MBP (from bovine; Sigma, Saint Louis, MO 63103 USA), MOG33-55 (Sigma), or PLP139-151 (AnaSpec, California) was added to the wells. The plates were incubated at 4 °C for 24 h and then washed and rinsed again as previously described. The plates were blocked with 3.0% NFDM in phosphate PBS (pH 9.0), 200 µl/well, for 1 h at 37 °C. Following washing, mouse test serum, negative control mouse serum or positive control serum from mouse (mouse anti-Theiler's virus antisera), or goat polyclonal IgG anti-MBP and goat polyclonal IgG anti-MOG antiserum (Santa Cruz Biotechnology, California), respectively were diluted 1/40 in assay buffer, and added to the wells. Positive control Theiler's virus antisera were acquired from pooled serum of three SJL mice (Jackson Laboratories) that had received a total of three intraperitoneal (IP) injections of UV-inactivated BeAn (concentration of 1×10^5 p.f.u./100 µl PBS).

Following the serial dilutions, the plates were then incubated for 1 h at 37 °C and then washed and 100 µl of rabbit anti-mouse IgG (H+L) (diluted 1/500 from stock) (Accurate Chemical and Scientific, New York) was added to each of the wells in the plates. The plates were incubated for 1 h at 37 °C, washed with Tween 20 (0.05% v/v) in RO H₂O and rinsed with RO H₂O. Subsequently, 100 µl of ¹²⁵I-Protein-A (1×10^5 cpm/100 µl assay buffer) was added to each well, and the plates were incubated at room temperature for 1 h. They were then washed and rinsed with Tween 20 and RO water (as described above). Once the plates were dry, every well was cut out and counts were determined by using a micromedic 4/200 plus automatic gamma counter.

2.6. Histological analysis

Mice were euthanized at 135 days pi with pentobarbital, perfused via the left ventricle with PBS followed by 10% formalin in phosphate buffer pH 7.2, and processed as described in Campbell et al. (2001). Coronal spinal cord sections were stained with Hematoxylin and Eosin. An experimenter blind to the subjects' conditions scored sections for the severity (number of cell layers in the meninges or perivascular cuffs) and area (percentage on meninges with inflammation and the number of perivascular cuffs) of inflammation. The TMEV model is characterized by inflammatory demyelinating lesions in the spinal cord (Blakemore et al., 1988). From our analysis of serial sections of spinal cord stained with Wiles myelin stain and hematoxylin and eosin (to visualize inflammation), we have found that demyelination is observed in the areas of inflammation (Sieve et al., in preparation).

2.7. Procedure

A 2 (sex) × 2 (stress) design was employed. Six subjects were placed in each group, counterbalanced by weight upon

arrival, for a total of 24 subjects. All mice were infected. Half of all mice were RST stressed one night prior to infection, and for the following 4 weeks. Previous studies from our laboratory (Campbell et al., 2001; Welsh et al., 2004) have found that restraint-induced changes in behavioral signs of illness, weight loss, NK cell activity, CNS viral titers, and histological CNS inflammation were selective to infected animals. Therefore, in the current study only infected animals were used to reduce animal numbers. During acute infection mice were regularly weighed, behaviorally scored for behavioral encephalitic-like symptoms, and had their food, water, and sucrose intake monitored. An additional measure of illness behavior, activity monitoring was taken on D3/4, D10/11, and D17/18 post-infection (pi). Animals were bled via the saphenous vein of the leg on days -5, 1, 7, 16, 24, and 45 pi for CORT analysis. Blood was collected within 2 min of cage disturbance, to minimize any stress artifacts. When RST stressed, animals were bled immediately following the nightly RST session. During the chronic phase of disease, once behavioral signs of the chronic phase began to appear, animals were behaviorally scored for signs of the chronic phase weekly, were tested on the rotarod weekly, and underwent activity monitoring on days 57, 77 and 105 pi. Animals were bled via the saphenous vein of the leg on days 69, 100, and 127 pi for antibody (Ab) to virus and Ab to myelin protein analyses. Mice were euthanized at day 135 pi with pentobarbital and perfused with PBS followed by 10% formalin.

2.8. Statistical analysis

Analyses of variance (ANOVAs) were conducted on the data. Where possible, baseline measures were used as a covariate, and analyses of covariance (ANCOVAs) were conducted instead. Bonferroni *t*-tests, Duncan's multiple range tests, and means comparisons were used for post hoc analyses. Correlation matrices (Pearson's bivariate) were computed on select dependent measures to calculate the inter-relationships between the dependent variables. A *p* value of 0.05 or less was considered significant in all cases.

3. Results

3.1. Acute phase

3.1.1. Body weights

As depicted in Fig. 1A, there were significant effects of sex and RST stress on body weights. Independent of condition and day post-infection, male mice weighed more than female mice, all *F*s > 49.60, all *p*s < 0.05. Over time, RST stressed mice displayed decreased body weights as compared to infected nonrestrained mice, *F*(6,119) = 2.768, *p* < 0.05. This weight difference only existed during the 4-

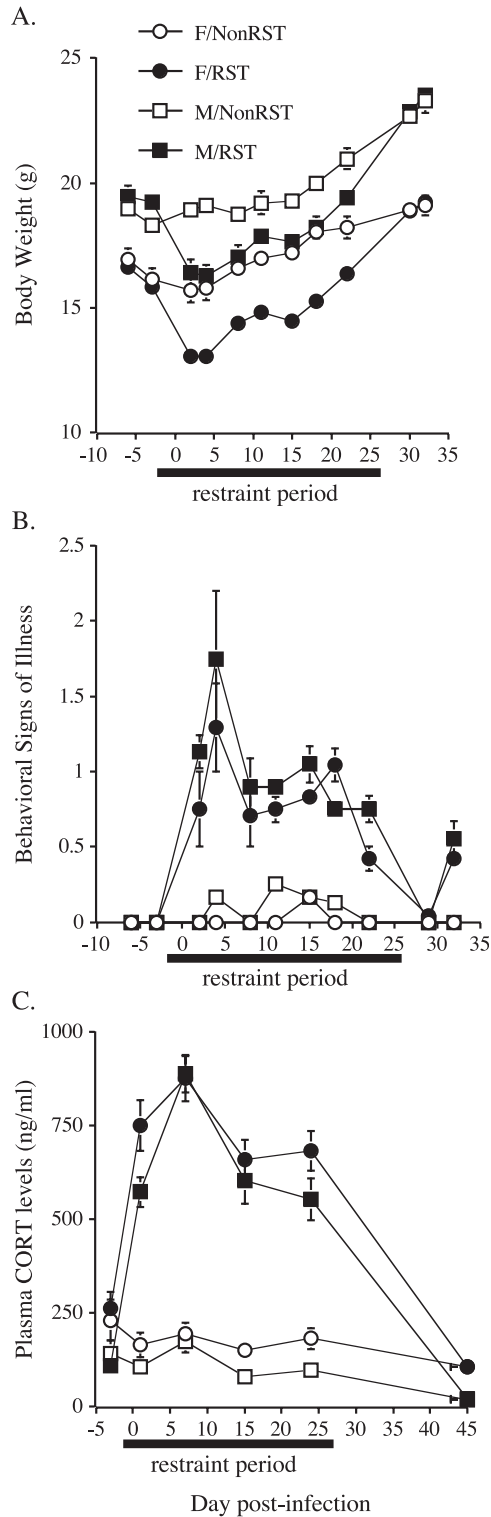


Fig. 1. As shown in panel A, RST stress decreased body weights in male and female SJL mice during the restraint period (days 1–24 pi). Following the cessation of restraint, there were no differences in body weights between the RST and nonrestrained (NonRST) groups. Panel B depicts the behavioral signs of illness over days pi. RST increased behavioral signs of illness in the acute phase in male and female SJL mice, during the restraint period (days 1–24 pi). As shown in panel C, RST stress increased plasma CORT levels in male and female SJL mice during the restraint period (days 1–24 pi). Following the cessation of restraint, there were no differences between the RST and NonRST groups. All data are expressed as the mean±S.E.M.

week period of RST stress; upon the cessation of RST stress, there was no significant effect of RST stress on body weights, $F(1,19)=0.150$, $p>0.05$.

3.1.2. Food intake

No significant effect of RST or sex was detected for food intake (data not shown).

3.1.3. Sucrose preference

There were no baseline differences in sucrose preference. However, during the RST stress period, RST stressed mice had a significantly greater preference for the sucrose solution than nonrestrained infected mice, $F(1,4)=8.208$, $p<0.05$. There were no significant effects of sex or any significant interactions with stress condition. Though a decrease in sucrose preference is associated with increased illness, stress has been found to increase the preference for sweet foods (Badiani et al., 1996; Ely et al., 1997). Here, the effect of stress seems to be overshadowing any potential effect of increased illness on sucrose preference (Table 1).

3.1.4. Behavioral signs of illness

As depicted in Fig. 1B, there was a main effect of RST stress and day pi on behavioral scores, as well as a day pi by stress interaction, all $F_s>3.19$, all $p_s<0.05$. No other differences were found, all $p_s>0.05$. RST stressed mice displayed increased behavioral scores as compared to infected nonrestrained mice. This behavioral score difference also only persisted during the 4-week period of RST stress. Once again, cessation of RST resulted in no significant effect of RST stress on behavioral scores, during the acute phase. One mouse died throughout the course of restraint stress (male, restrained). A score of 4 (the highest rating on this scale, which is given for moribund or mortality) was included on the day of the death, and then the deceased animal was removed from all measures for all of the subsequent time points.

3.1.5. Plasma CORT levels

There was a baseline CORT level difference, such that female mice had higher CORT levels than male mice 3 days prior to infection, $F(1, 20)=7.557$, $p<0.05$. No other baseline differences were found. As depicted in Fig. 1C, during the RST stress period, there were main effects of sex, stress, and day pi and a significant stress by day pi

Table 1

Sucrose preference is represented as the average daily sucrose solution intake for a cage divided by the total fluid intake for a cage

		Restraint	Nonrestraint
Male	Mean	74.1%	70.9%
	S.E.M.	2.72	0.25
Female	Mean	80.8%	70.2%
	S.E.M.	1.54	0.25

Sucrose preference was greater for restraint stressed infected animals as compared to nonrestrained infected animals.

interaction, such that over time RST stressed mice had higher CORT levels than infected nonrestrained mice, all $F_s > 5.14$, all $p_s < 0.05$. Females continued to have higher CORT levels than males throughout the RST stress period, $F(1,19) = 5.55$, $p < 0.05$. Following the cessation of RST stress, there was no effect of stress condition. Similar to baseline CORT, a significant effect of sex was observed for post-infection CORT with females having higher levels compared to males, $F(1,19) = 33.726$, $p < 0.05$.

3.1.6. Spontaneous activity

There were significant main effects of stress and day pi on vertical activity (the sum of leaning, rearing and jumping frequencies), both $F_s > 6.90$, $p_s < 0.05$. Stressed animals had decreased activity as compared to nonrestrained mice. Over time, independent of stress condition, vertical activity also decreased. No other differences were found. On the horizontal activity measure, a similar pattern was observed. There were significant main effects of stress, day pi, and a stress by day pi interaction, all $F_s > 5.15$, all $p_s < 0.05$. RST stressed animals had decreased horizontal activity as compared to nonrestrained mice. The nonrestrained mice decreased horizontal activity over time, while horizontal activity levels remained low in the stressed animals. See Fig. 2A and B for horizontal and vertical activity, respectively.

3.1.7. Summary

The present study replicates the pattern of results previously observed in male CBA mice (Campbell et al.,

2001; Satterlee et al., 2001; Faulkner et al., 2003; Welsh et al., 2004) but in a mouse strain with a greater susceptibility to Theiler's virus (SJL mice) as well as in females. However, unlike our prior study (Campbell et al., 2001), we did not see as severe mortality or behavioral signs of illness. In the current study, only 1 of the 24 SJL mice died (a male, RST, infected). A possible explanation for this difference across studies could be the change in the duration of the nightly RST stress period. To study the SJL mice in the chronic phase, and to reduce mortality due to stress alone (which was observed in SJL mice), the RST stress session was shortened from the 12 h previously used with CBA mice to 8 h in the present study. Even with this change, RST stress still had a significant effect on both male and female SJL mice during the acute phase of Theiler's virus infection, decreasing body weights and activity levels (an indication of increased illness; Barak, 2002), while increasing behavioral signs of illness, plasma CORT levels, and sucrose preference (consistent with previous findings that stress increased the preference for sweet food; Badiani et al., 1996; Ely et al., 1997).

3.2. Chronic phase

3.2.1. Behavioral data

RST stress exacerbated rotarod performance and behavioral signs of the chronic phase during the chronic phase of the disease. However, overall vertical and horizontal activity levels did not appear to be sensitive to the stress-induced

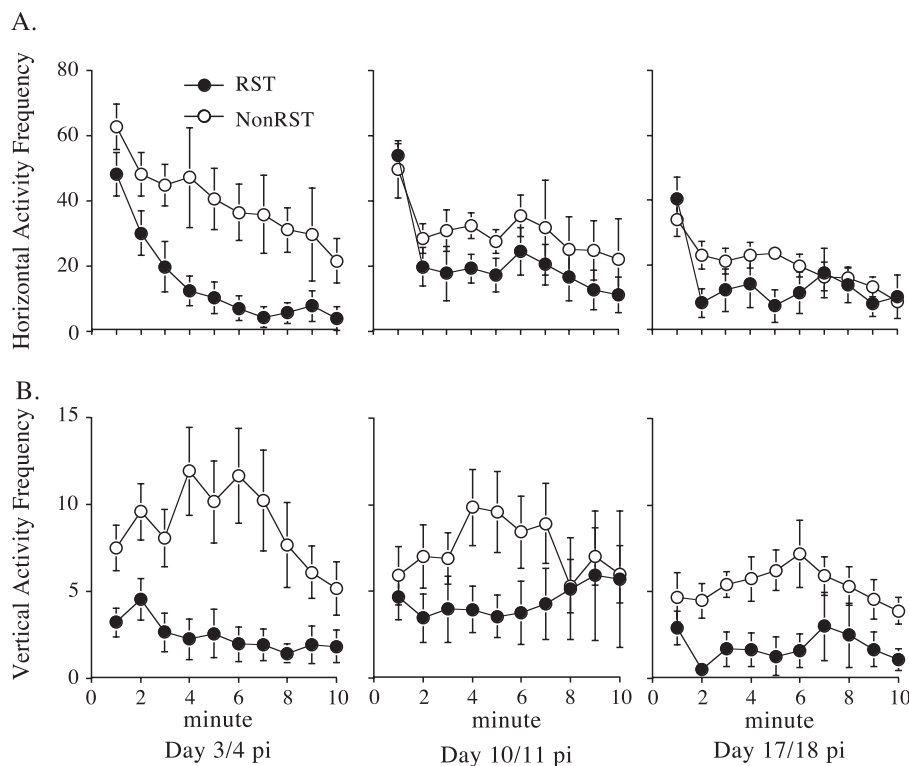


Fig. 2. RST stress decreased horizontal activity (panel A) and vertical activity (panel B) in male and female SJL mice on days 3/4, 10/11, and 17/18 pi. All data are expressed as the mean \pm S.E.M.

exacerbation of disease progression. The shorter duration of our session (10 min versus the 72 h used by McGavern et al., 1999) may have decreased our ability to detect a significant effect of disease on this measure.

As depicted in Fig. 3A, there were significant effects of stress, day pi, and a stress by day pi interaction on behavioral signs of the chronic phase, such that previously RST stressed mice had increased behavioral scores as compared to infected nonrestrained mice, all $F_s > 2.21$, all $p_s < 0.05$. Bonferroni t -tests confirmed that RST stressed mice had higher behavioral scores at day 45 pi (showing an earlier onset of behavioral symptoms in stressed mice) and 59–134 pi, all $p_s < 0.05$, but there were no differences between these groups at days 38 and 52 pi, both $p_s > 0.05$. Although there was a significant main effect of sex, with males consistently having higher behavioral scores than

females, $F(1,19)=4.65$, $p < 0.05$, there was no sex by stress interaction.

Similarly, there were significant main effects of sex, stress, and day pi on rotarod performance, such that RST stressed mice spent less time on the rotarod than infected nonrestrained mice, all $F_s > 5.28$, all $p_s < 0.05$ (Fig. 3B). Females had higher latencies to fall from the rotarod, and as time progressed rotarod latencies decreased. There were also significant stress by day pi, and stress by day pi by sex interactions, both $F_s > 2.12$, both $p_s < 0.05$. Bonferroni t -tests confirmed that independent of sex, previously RST stressed mice had significantly worsened rotarod performance at days 46 and 68–95 pi, all $p_s < 0.05$, but these groups were not different on this measure at days 60, 124, and 131 pi, all $p_s > 0.05$. A separate set of Bonferroni t -test was used to investigate the stress by day pi by sex interaction. For males, RST stress impaired rotarod performance on days 46, 74, 81, and 88 pi only, all $p_s < 0.05$. For females, RST impaired rotarod performance on days 68, 81, 88, and 95 pi, but enhanced performance on days 124 and 131 pi as compared to nonrestrained mice, all $p_s < 0.05$. No other differences were significant.

However, some behavioral measures (spontaneous activity and body weights) were not sensitive to the stress-induced exacerbation of the chronic disease. There were main effects of sex and minute, and a day pi by minute interaction on horizontal activity, all $F_s > 2.03$, all $p_s < 0.05$, but no effects of stress. In general, females were more active on this measure, and activity levels were greatest during the first minutes, and subsequently declined. See Fig. 4A and B for horizontal and vertical activity levels across time. Consistent with horizontal activity, there were no effects of stress on vertical activity. There was, however, a main effect day pi, $F(1,19)=51.06$, $p < 0.05$. Across all groups, vertical activity decreased as the disease progressed. Several factors may contribute to the behavioral deficits observed in infected SJL/J mice during the chronic stage of disease, including persistent virus replication, inflammation, and progressive demyelination that occurs in the CNS of these mice. However, the decrease in vertical activity that we observed in all infected mice is consistent with the difference in activity found in other studies between uninfected control mice and infected mice displaying histological signs of demyelination (McGavern et al., 1999). Nevertheless, without an uninfected control group, we cannot definitively conclude that the decrease in vertical activity observed in the present study is attributable to infection.

Body weights also did not show an effect of stress, but merely significant main effects of sex and day pi, such that females consistently weighed less than males, and all mice had normal weight gain with age, both $F_s > 6.81$, both $p_s < 0.05$ (data not shown).

Taken together, the behavioral data collected during the chronic phase for SJL mice (behavioral signs of the chronic phase and rotarod performance) suggests that RST stress during the acute phase not only exacerbated the acute phase

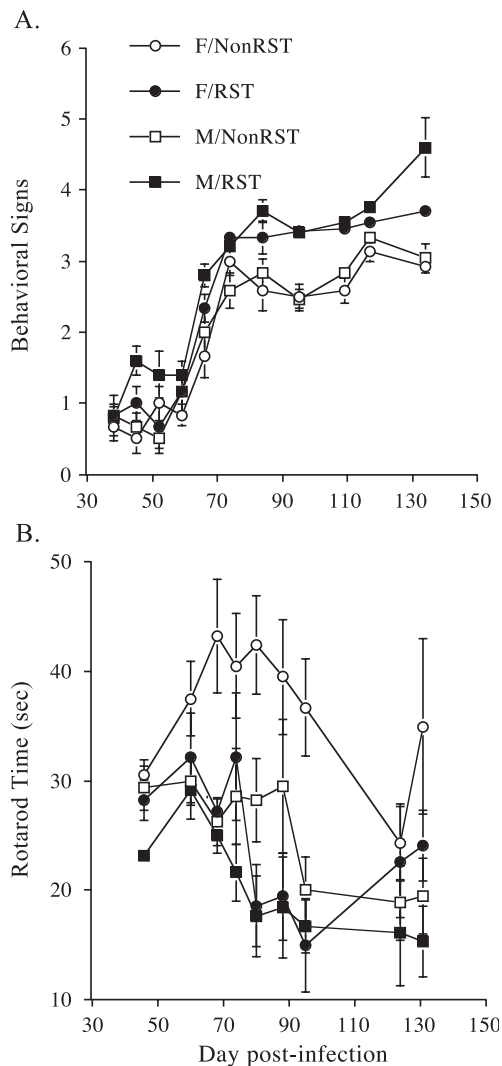


Fig. 3. Throughout the chronic phase, previously RST stressed male and female mice had increased behavioral signs of demyelination (panel A). Previously RST stressed male and female mice also had decreased performance on the rotarod task (panel B). All data are expressed as the mean \pm S.E.M.

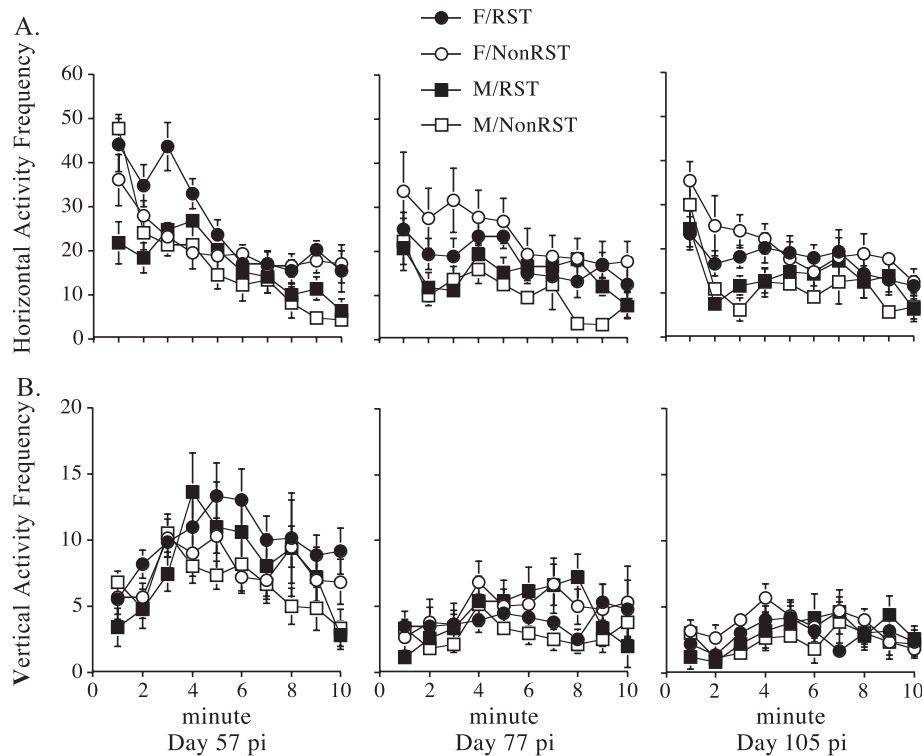


Fig. 4. Previous RST stress did not alter horizontal activity (panel A) or vertical activity (panel B) in male or female SJL mice on days 57, 77, or 105 pi. However, females were consistently more active than males on both measures. Consistent with previous reports on TVID, vertical activity decreased in all groups as the disease progressed. All data are expressed as the mean \pm S.E.M.

of the disease, but also the later chronic demyelinating condition. It is possible that the lack of sensitivity of activity levels to the stress-induced exacerbation is due to the limited amount of time sampled (10 min). Other researchers have used a time frame as great as 72 h (McGavern et al., 1999), which was not possible for practical reasons in this experiment. Nonetheless, consistent with McGavern et al. (1999), activity levels significantly decreased on both horizontal and vertical measures as the chronic demyelinating phase progressed.

3.2.2. Histological analysis of lesions

Analyses of the inflammatory lesions within the spinal cord confirmed what was suggested by the behavioral data: in the chronic phase of the disease, SJL mice, independent of sex, were exacerbated by previous RST stress (Fig. 5A–D). A main effect of stress was found on both perivascular cuffing measures (number of cuffs per spinal cord section and average number of cell layers in those cuffs; Fig. 5B and D, respectively), as well as on both measures for meningitis (percentage of meninges inflamed per spinal cord section and the average number of cell layers within the meninges; Fig. 5A and C, respectively), all $F_s > 9.28$, all $p_s < 0.05$. Previously, RST stressed mice had increased inflammatory lesions within the spinal cord as compared to the infected nonrestrained mice. No sex differences were detected. Additionally, both

perivascular cuffing and meningitis scores correlated highly with behavioral scores in SJL mice, both $F_s > 22.09$, both $p_s < 0.05$ (Fig. 5E and F).

3.2.3. Plasma antibody analyses

Plasma Ab levels to myelin components (MOG33-55, PLP139-151, and MBP) and to Theiler's virus were measured at days 69, 100, and 127 pi. As depicted in Fig. 6A, a significant main effect of day pi and a significant day pi by sex interaction were found for Ab to MOG33-55, both $F_s > 3.04$, both $p_s < 0.05$. Bonferroni t -tests confirmed that at day 69 pi females had significantly more Ab to MOG33-55 than males, $p < 0.05$. Previous RST stress did not impact this variable, and no other differences were found, all $p_s > 0.05$. A significant main effect of sex, and a stress by sex interaction were found for Ab to MBP levels, both $F_s > 5.28$, both $p_s < 0.05$ (Fig. 6B). No other effects were significant. Females had higher levels of Ab to MBP than males, but this main effect was qualified by the sex by stress interaction. Mean comparisons determined that only when nonrestrained, females had higher levels than males, $p < 0.05$. Additionally, in females only, previous RST stress reduced Ab to MBP levels as compared to nonrestrained mice, $p < 0.05$. This effect of stress condition did not exist in males. Similarly, as seen in Fig. 6C, a significant main effect of day pi was found on Ab to PLP139-151, $F(2, 28) = 6.91$, $p < 0.05$, such that Ab

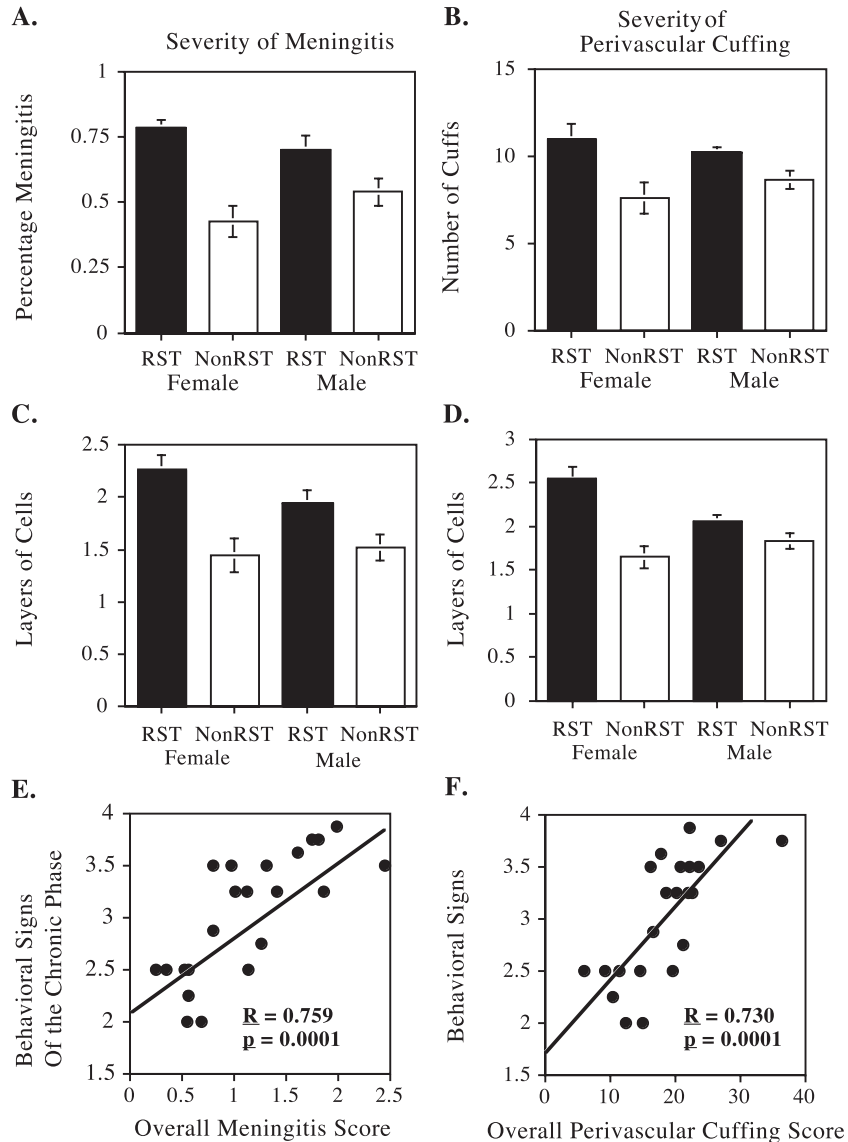


Fig. 5. Previous RST increased all measures of inflammatory lesions in the spinal cord in male and female SJL mice: percentage of spinal cord with meningitis (panel A), layers of cells in the meninges (panel C), number of perivascular cuffs in a spinal cord section (panel B), and number of layers of cells in a cuff (panel D). Data are expressed as the mean \pm S.E.M. Behavioral signs of chronic disease were highly correlated with the severity of meningitis and perivascular cuffing. Overall, perivascular cuffing was computed by multiplying the number of cuffs by the number of cell layers in the cuffs. Overall meningitis was computed by multiplying the percentage of meninges affected by the number of cell layers in the meninges.

levels to PLP139-151 increased over this time period for all animals independent of sex or stress condition. No other significant effects for PLP139-151 were found. As seen with PLP139-151, a significant main effect of day pi was found on Ab to Theiler's virus, $F(2, 28)=6.03$, $p<0.05$, (Fig. 6D). No other significant differences were found for Ab to Theiler's virus levels, all $ps>0.05$. Thus, antibody levels do not reflect the stress-induced exacerbation of disease, suggesting that changes in antibody production do not mediate the adverse effects of restraint. Nevertheless, these findings are important because they characterize the autoantibody response and observed autoantibodies to PLP139-151, MOG33-55 and MBP in the late demyelinating phase of the disease.

3.3. Inter-relationships between acute and chronic phase dependent variables

A correlation matrix was computed to investigate the relationship between dependent measures taken during the acute phase of infection with measures taken during the chronic demyelinating phase of disease (Table 2). The acute phase measures of illness and stress were highly correlated with each other. For example, highest CORT level, initial weight loss within the first few days of infection/restraint, highest acute phase behavioral score, as well as horizontal and vertical activity on day 3 pi were all highly correlated, all $ps<0.05$. The same pattern of results was obtained when correlations were conducted for all of

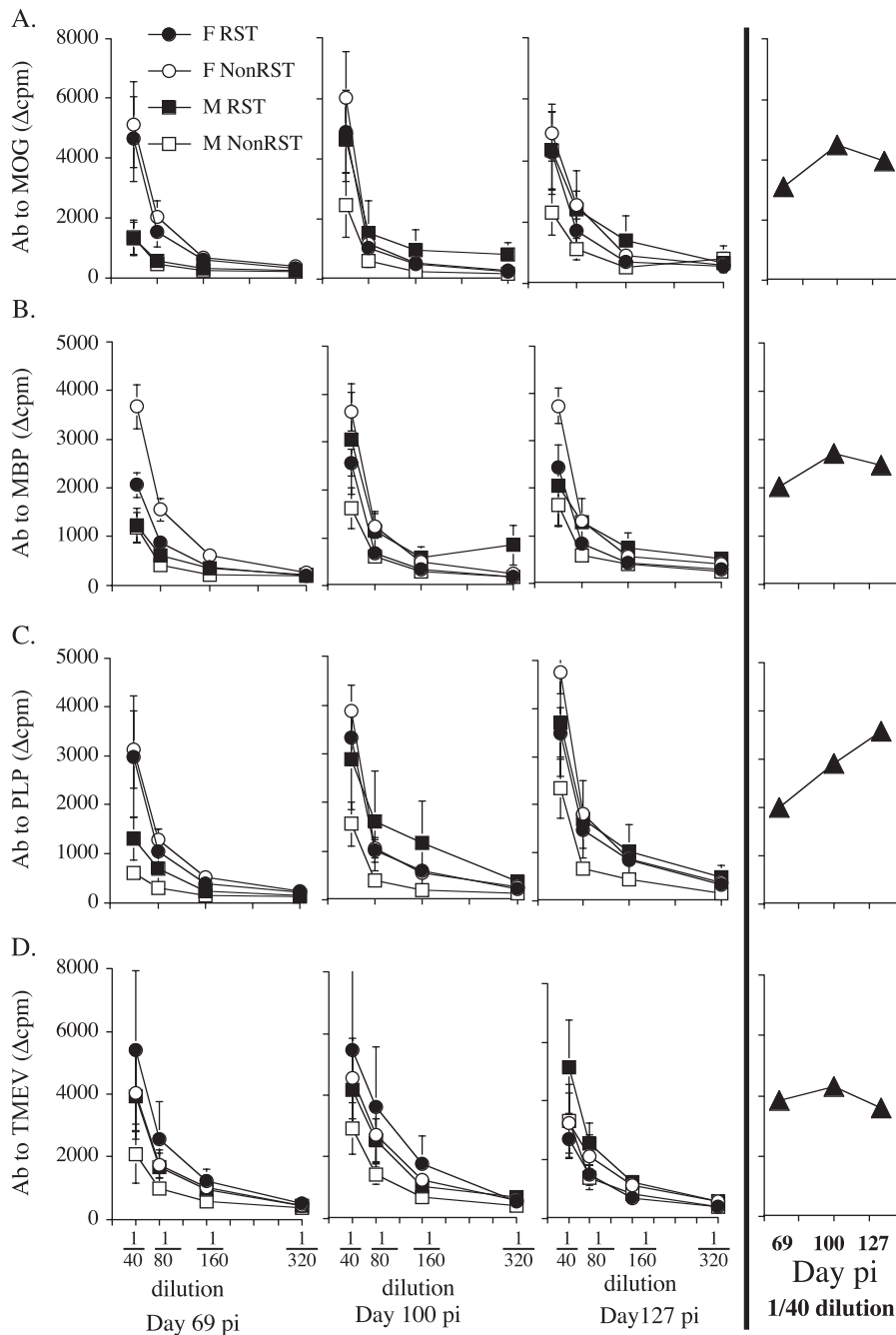


Fig. 6. At day 69 pi, females had greater Ab to MOG33-55 levels than males (panel A). No other sex or stress differences were detected for MOG33-55. Nonrestrained females had higher levels of Ab to MBP than males, and in females only, previous RST stress reduced Ab to MBP levels as compared to nonrestrained mice (panel B). No other sex or stress differences were detected for MBP. No sex or stress differences were detected for PLP139-151 (panel C) or Theiler's virus (panel D). On the right side of the figure, Ab levels from the 1/40 dilution are collapsed across groups and presented over day pi. Ab levels to MOG, PLP, and increased from day 69 pi to day 127 pi. All data are expressed as the mean \pm S.E.M.

the individual time points throughout the acute phase (data not shown). Baseline weight and baseline CORT level did not correlate well with the measures taken during the restraint stress period, but they did correlate highly with each other, such that as mouse weight increased, plasma CORT levels decreased.

Measures of illness and stress during the acute phase were also shown to be predictive of chronic phase disease

progression, on rotarod performance, behavioral signs of the chronic phase, and inflammatory lesions in the spinal cord. Highest acute behavioral score, initial weight loss, highest CORT level, and activity measures at day 3 pi were consistently correlated with behavioral signs of the chronic phase and histological lesions, and were frequently correlated with rotarod performance, all $ps < 0.05$. However, these acute phase measures were not shown to be

Table 2
Correlations between acute and chronic phase dependent variables

Dependent measure	Day pi	Baseline weight	Baseline CORT	High behavioral score	Initial weight loss	High CORT	Horizontal activity D3pi	Vertical activity D3pi
ACUTE PHASE								
Baseline weight	-3	1.0						
Baseline CORT	-3	-.747*	1.0					
High behavioral score	NA	.060	.064	1.0				
Initial weight loss	NA	.029	-.110	-.807*	1.0			
High CORT	NA	.022	.096	.687*	-.863*	1.0		
Horizontal activity	3	-.074	-.021	-.839*	.654*	-.676*	1.0	
Vertical activity	3	-.140	.128	-.686*	.663*	-.640*	.856*	1.0
CHRONIC PHASE								
Behavioral score	38	-.062	-.074	.106	.002	.105	-.127	-.188
Behavioral score	45	.319	-.311	.434*	-.408*	.492*	-.329	-.266
Behavioral score	52	-.005	.023	.079	-.229	.181	.106	.005
Behavioral score	59	.267	-.250	.070	-.080	.286	.063	.108
Behavioral score	66	.297	-.179	.410*	-.269	.466*	-.360^	-.296
Behavioral score	74	-.087	.111	.397^	-.513*	.479*	-.248	-.214
Behavioral score	84	.359^	-.140	.533*	-.578*	.559*	-.397^	-.414*
Behavioral score	95	.123	-.125	.745*	-.794*	.800*	-.643*	-.729*
Behavioral score	109	.320	-.299	.683*	-.661*	.780*	-.665*	-.489*
Behavioral score	117	.209	-.111	.348^	-.426*	.546*	-.327	-.229
Behavioral score	134	.446*	-.213	.559*	-.573*	.646*	-.440*	-.451*
Rotarod time	46	-.237	.072	-.366^	.322	-.499*	.118	.104
Rotarod time	60	-.417*	.319	-.094	.215	-.089	-.085	.200
Rotarod time	68	-.316	.276	-.379^	.282	-.318	.413^	.461*
Rotarod time	74	-.493*	.461*	-.167	.241	-.179	.091	.262
Rotarod time	81	-.306	.214	-.623*	.549*	-.549*	.488*	.587*
Rotarod time	88	-.238	.200	-.495*	.519*	-.426*	.285	.393^
Rotarod time	95	-.240	.182	-.385^	.381^	-.410*	.368^	.423*
Rotarod time	124	-.263	.353^	-.125	.128	-.018	-.027	.206
Ab to MOG33-55	69	-.385^	-.101	-.189	-.093	-.024	.120	-.049
Ab to MOG33-55	100	-.022	-.350	-.038	-.245	.068	.098	-.111
Ab to MOG33-55	127	.029	-.334	-.051	-.234	.095	.027	-.197
Ab to MBP	69	-.585*	.363	-.324	.154	-.256	.478*	.142
Ab to MBP	100	-.189	.172	.162	-.140	.042	.105	-.029
Ab to MBP	127	-.398^	.404^	-.029	.028	-.148	.247	.152
Ab to PLP139-151	69	-.360	.044	.117	-.205	.061	-.009	-.153
Ab to PLP139-151	100	-.196	.113	.064	-.226	.083	.042	-.020
Ab to PLP139-151	127	-.028	.058	.045	-.134	.041	.283	.180
Ab to Theiler's virus	69	.081	-.203	.222	-.138	.145	-.123	-.204
Ab to Theiler's virus	100	.102	-.207	.201	-.103	.086	-.069	-.176
Ab to Theiler's virus	127	.528*	-.354	.143	-.018	.052	-.053	-.091
Number of cuffs	135	.417*	-.255	.348^	-.217	.272	-.139	-.137
Layers in cuffs	135	-.010	.057	.440*	.461*	.493*	-.289	-.261
Percent meningitis	135	.064	-.101	.466*	-.515*	.652*	-.332	-.346^
Layers in meninges	135	-.072	.292	.639*	-.635*	.744*	-.461*	-.380^

Baseline and acute phase variables are presented by column in the top row (baseline body weight, baseline CORT, highest behavioral score, initial weight loss, highest CORT, horizontal activity, and vertical activity), while acute and chronic phase variables are presented in rows by day post-infection. During the acute phase, measures of illness and stress were highly correlated with each other. In addition, acute phase measures of illness and stress were predictive of chronic phase measures of disease progression, including rotarod, behavioral indications of chronic disease, and histological inflammatory lesions in the spinal cord. Gray boxes and * indicate significant correlations at $p < 0.05$, whereas the symbol ^ denotes marginally significant correlations at $p < 0.10$.

predictive of autoantibody or viral antibody levels in the chronic phase. Baseline weight and CORT levels were also, but to a much lesser degree, predictive of some elements of chronic phase disease. Thus, there appear to be pre-existing individual, or "trait", differences that predicted some aspects of chronic disease, as well as stress-induced, or "state", differences that predicted other manifestations of chronic disease.

4. Discussion

The present study provides evidence that stress experienced prior to the onset of a demyelinating condition, such as MS, worsens the development of the disease. RST stress applied during the first 4 weeks of Theiler's virus infection hastened the onset and exacerbated the subsequent demyelinating phase of disease. Both male and female SJL mice

that were previously stressed displayed increased behavioral and histological indications of disease: exacerbated behavioral signs of chronic disease, decreased rotarod performance, and increased inflammatory lesions within the spinal cord. Moreover, acute phase measures of illness and stress predicted chronic phase measures of disease progression, including rotarod, behavioral indications of chronic disease, and inflammatory lesions in the spinal cord.

These findings provide experimental evidence that stress administered during early infection can exacerbate the subsequent inflammatory demyelinating phase of disease. Though this does not coincide with studies using EAE, which show no effect of stress prior to disease induction, and a suppression during disease induction (Levine et al., 1962; Griffin and Whitacre, 1990; Griffin et al., 1993; Dowdell et al., 1999; Levine and Saltzman, 1987; Bukilica et al., 1991), it is similar to that reported by MS patients (Mohr and Cox, 2001). Thus, this study provides some of the first experimental evidence in an animal model that corresponds to the correlational evidence in human MS populations. As early as 1877, Charcot reported that stressful experiences precipitated the onset of MS (Charcot, 1877). Mohr and Cox (2001) reviewed the effects of stress on multiple sclerosis. Many studies since then have found that MS patients, as compared to healthy controls, or patients with other neurological disorders, report more stressful experiences prior to initial symptomatology. Additionally, longitudinal studies find stress to increase the chances of exacerbation of MS (Mohr and Cox, 2001; Ackerman et al., 2003). The type of stress, however, may be important. While relatively moderate and chronic stressors seem to follow the pattern of stress-induced exacerbation of disease, severe stressors (e.g., war, see Nisipeanu and Korczyn, 1993) have been found to lower the rate of relapses of MS. Mohr et al. (2000) found increased conflict, disruption of routine, and daily hassles predicted the risk of developing new brain lesions 8 weeks later, while there was no effect of severe stress on brain lesion development. Yet, Ackerman et al. (2003) found exacerbations in MS symptoms to be more likely following stressful life events, independent of the type of stressor. Because the relationship between stress and MS in humans is complex, this research domain is likely to benefit from the investigation of stress using animal models.

The current study also replicates, in male and female SJL mice, the previous findings in male CBA mice: RST stress exacerbates early Theiler's virus infection (Campbell et al., 2001). Both male and female SJL mice displayed decreased body weights, and increased behavioral signs of illness during the first 4 weeks of infection. However, mortality rates were lower in the current study when compared to Campbell et al. (2001). This may be related to the reduced amount of RST stress administered in the current study (8 h instead of 12 h per night). The duration of restraint was reduced from 12 to 8 h to reduce mortality due to stress alone, which was observed in SJL mice, but not in CBA

mice. In addition, the present study extends this line of work by showing that RST stress exacerbated another index of illness behavior during acute disease: activity monitoring. RST stressed animals displayed decreased activity levels as compared to infected nonrestrained mice, providing converging behavioral evidence that early viral infection is exacerbated by stress. Furthermore, this increase in illness was associated with an increase in CORT levels throughout the 4 weeks of RST stress.

Another aim of this study was to investigate whether stress-induced changes in disease course varied by sex. We expected that CORT levels in all RST stressed mice would be elevated, possibly to a greater degree in females. Female rodents are known to have a greater, and more long-lasting HPA activation in response to stress (Turner, 1990; Homodelarhe et al., 1991; Griffin et al., 1993; Gaillard and Spinedi, 1998), and this could have modulated the effects of stress on Theiler's virus infection. Indeed, females not only displayed a higher basal CORT level, but also greater stress-induced levels of CORT. However, this sex difference in CORT levels did not translate into sex by stress interaction on Theiler's virus infection. While stress exacerbated infection, it did not do so to a greater degree in females even with their significantly higher CORT levels.

Yet, sex did impact the disease process, independent of stress condition. Though sex differences were found, the pattern of results is complex. In the early viral infection, there were no sex differences in behavioral signs of illness, activity monitoring, or sucrose preference. However, in later disease, males had greater behavioral signs of chronic disease, poorer rotarod performance, and reduced activity levels as compared to females, but there were no sex differences in spinal cord lesions. While males had greater behavioral signs of disease, females had higher levels of autoantibody levels to MOG33-55 and MBP at certain time points in the late disease. The pattern of results across other studies is similarly complicated. While Alley et al. (2003) found SJL males to develop a greater severity of disease than females, Hill et al. (1998) found female SJL mice to have a greater incidence and severity of disease. In SJL mice infected with TMEV, female mice displayed greater inflammation and demyelination of the brain and spinal cord as compared to males (Hill et al., 1998).

Multiple factors may underlie the divergent findings on sex differences in the development of TVID, including strain of Theiler's virus, and environmental variations. Alley et al. (2003) and Hill et al. (1998) used the DA strain of Theiler's virus while the current study used the BeAn strain. Another possible explanation may lie in variations in housing conditions. All of the mice in the current study were housed in groups of three; whereas in Alley et al. (2003) males were individually housed while females were group housed. Because social isolation is a significant stressor (Banerjee, 1972), the sex differences observed in the Alley study may be attributable to this variable. Thus,

the effect of sex in Theiler's virus infection requires further investigation.

The findings that stress exacerbates the early viral infection (Campbell et al., 2001; current study) and the later demyelinating disease (current study) in Theiler's virus infection contrast with the effects of stress on the other commonly used rodent model of multiple sclerosis, EAE. The differences in how stress affects EAE and Theiler's virus infection may lie in their immunological mechanisms of demyelination and neuronal destruction. The mechanism of EAE pathogenesis involves the induction of autoreactive T cells. Stimulated and activated CD4+ T cells increase adhesion molecules, enter the CNS, and secrete proinflammatory Th1 cytokines, leading to the recruitment of mononuclear cells. B cell secretion of anti-myelin antibody (Ab), in concert with macrophage/glia secreted cytotoxic factors, leads to demyelination (Tsunoda and Fujinami, 1996). In this exclusively autoimmune-mediated model, stress induced suppression of the immune system, and thus alleviation of the disease process, would be an expected result. Indeed, when stress does alter the course of EAE it tends to reduce various aspects of the disease. For example, prior studies indicate that 19 daily sessions of inescapable tail-shock (80, 5 s, 1 mA) or noise stress (60, 5 s, 90 dB fire alarm) have no effect when administered prior to EAE induction, while stressor exposure following EAE induction had a protective effect (Bukilica et al., 1991). Specifically, tail-shock reduced the incidence and duration of EAE, delayed disease onset, and decreased the severity of clinical and histological symptoms. Noise stress on the other hand, only delayed the disease onset. Other studies found restraint stress in rats to likewise delay EAE onset and decrease the incidence and severity of disease (Levine et al., 1962; Griffin and Whitacre, 1990; Griffin et al., 1993; Dowdell et al., 1999). These and other studies have also shown that the alleviation of EAE by restraint appears to be mediated by the HPA axis, rather than the sympathetic nervous system (Dowdell et al., 1999; Levine and Saltzman, 1987).

Theiler's virus induced demyelination (TVID), though similar to EAE in the resulting demyelination, behavioral signs of chronic disease, and histological lesions, involves a different immunopathological pathway (for reviews see: Dal Canto et al., 1995; Tsunoda and Fujinami, 1996; Oleszak et al., 2004). In the first few weeks following inoculation with a live TO strain of Theiler's virus, CNS neurons are infected with the virus. During this acute phase of the infection, Theiler's virus specific cellular and humoral immunity removes the virus from the gray matter, inducing apoptosis of the virally infected neurons. Viral infection persists in glia and macrophages located in the white matter throughout the chronic phase of the disease (Brahic et al., 1981). During this phase, additional macrophages, Theiler's virus specific T cells and antibody are recruited to the CNS, inducing oligodendrocyte apoptosis, inflammation, and demyelination (Tsunoda et al., 1997). Multiple mechanisms of demyelination have been found in TVID, including: direct

viral lysis of oligodendrocytes (Roos and Wollmann, 1984), bystander demyelination mediated by virus specific DTH T cells (Clatch et al., 1987), cytotoxic T cell reactivity (Rodriguez and Sriram, 1988), and autoimmune mediated demyelination (Welsh et al., 1987; Miller et al., 1997; Borrow et al., 1998). Following the immune response directed against Theiler's virus, epitope spreading leads to CD4+ T cells reactive to myelin components (Miller et al., 1997; Borrow et al., 1998). Virus-specific T cell responses cause bystander destruction of myelin. Recruited and CNS-resident antigen presenting cells process and present these endogenous myelin epitopes to autoreactive T cells (Miller et al., 1997). Antibody to myelin is also present at this later stage of the disease (Welsh, et al., 1987). In the current study, antibody levels to virus and myelin proteins (MOG33-55, PLP139-151, MBP) were measured at days 69, 100, and 127 pi. Autoantibodies to whole myelin membranes (Welsh et al., 1987) and MBP (Rauch et al., 1987) have been previously detected in mice infected with Theiler's virus. In the current study, we further dissected the autoantibody response and observed autoantibodies to PLP139-151, MOG33-55, and MBP in the late demyelinating phase of the disease. To the best of our knowledge, this represents the first report of specific autoantibodies directed against these determinants in TVID and as such validates Theiler's virus infection as an excellent model of MS. It is interesting to note that T cell responses to these proteins also develop during late TVID (Miller et al., 1997).

When comparing the effects of stress on EAE and TVID, it is important to note that the stressors are being applied during different phases in the immunological response of the disease process. In EAE, a purely autoimmune mediated model, the stressors are applied either before or during the autoimmune components of the disease. With research conducted on Theiler's virus infection thus far, the stressor has been applied before and during the acute viral infection, when the immune system is attempting to remove the virus from the CNS (Campbell et al., 2001; Welsh et al., 2004; Mi et al., 2004). It is possible that if the stress-induced suppression of the immune system occurred during the chronic demyelinating disease, when autoimmune processes are in place, rather than the preceding acute viral infection, that a similar pattern to EAE would be discovered (Lipton and Dal Canto, 1976, 1977). Stress at that point in the disease process may alleviate rather than exacerbate the TVID. Research is currently underway to address this issue.

There are at least two potential pathways by which stress during early infection could exacerbate Theiler's virus induced demyelination. One possibility is that stress alters the functioning of the immune system. Biron (1998, 1999) proposes that altering the early immunological events in a viral infection may have a cascading effect on later infection. The initial responses to the virus influence the innate immune response and the production of innate components, which in turn influence the development of the adaptive immunity directed towards the viral infection.

Early cytokine responses can activate protective mechanisms within cells as well as natural killer cells (NK cells) and macrophages. This innate cytokine and cellular response can shape the endogenous T cell responses. Thus, altering the initial cytokine and innate immune responses to Theiler's virus with RST stress may subsequently alter the adaptive immune responses to the virus and myelin in the chronic phase of disease.

Consistent with this view, we have found that male CBA mice subjected to RST during acute infection exhibit thymic atrophy, decreased NK cell activity in the spleen, and decreased numbers of lymphocytes in the blood, all of which may contribute to the increased viral load found in the CNS of RST stressed animals (Campbell et al., 2001; Welsh et al., 2004). Further research has found chemokine changes in the brain and spleen in response to RST stress (Mi et al., 2004). On day 7 post Theiler's virus infection, Mi et al. (2004) found Ltn, IP-10 and RANTES to be elevated in the spleen and brain, and that RST stress significantly decreased these levels. These chemokines have been shown to be involved in the chemoattraction of inflammatory cells to the CNS (Salmaggi et al., 2002; Palma and Kim, 2001; Hoffman et al., 1999; Murray et al., 2000; Theil et al., 2000). Indeed, stress during early Theiler's virus infection has been found to decrease inflammation in the CNS at D7p.i. (Mi et al., 2004; Campbell et al., 2001), followed by an increase in inflammation at D24p.i. (Campbell et al., 2001). Would there be similar cellular changes in the chronic demyelinating phase of Theiler's virus infection when RST stressed in the early viral infection? Additionally, chronic restraint stress may not only result in persistent immunological changes that last throughout the chronic phase of disease, but these immunological changes may be due to a chronic stress-induced persistent alteration in neuroendocrine function, including the activity of the hypothalamic pituitary adrenal axis (McEwen, 1998). Both of these possibilities require further investigation. However, data from this study suggest that humoral immunity may be unchanged in the chronic phase when mice are RST stressed during early viral infection. We did detect specific autoantibodies directed against MOG33-55, PLP139-151, and MBP in TVID, further validating Theiler's virus infection as an excellent model of MS. However, overall antibody levels did not reflect the stress-induced exacerbation of disease. This may reflect insufficient sensitivity on this measure, or be an indication that stress-induced changes in the disease process do not include antibody production.

A second possibility lies within the effects of stress on the viral load within the CNS. RST stress has been previously found to decrease Theiler's virus clearance from the brain and spinal cord during the first 4 weeks of infection (Campbell et al., 2001). This suppression of viral clearance is thought to be at least in part due to a stress-induced corticosterone mediated immunosuppression. Previous studies (Satterlee et al., 2001) have found that

administration of corticosterone as a substitute for restraint stress lead to a similar worsening of the acute viral infection. Following acute viral infection, Theiler's virus levels normally drop to undetectable levels until the onset of the chronic demyelinating phase of disease, where a resurgence of viral replication can be seen (Welsh et al., 1987, 1989). Did the stress-induced increase in viral load in the acute phase translate into a higher viral load at the onset of the chronic phase? Did this increased level of viral replication lead to increased myelin destruction and enhanced autoimmune demyelination? Borrow et al. (1992) found that removing CD8 T cells prior to acute viral infection resulted in a higher viral load in the CNS and more severe demyelinating disease. Stress-induced immunosuppression may likewise be exacerbating the demyelinating disease through this mechanism.

Future studies intend to investigate the potential mechanisms underlying the stress-induced exacerbation of the chronic demyelinating phase of Theiler's virus infection. Though many avenues of research still exist in this area, the present study provides strong experimental evidence that parallels correlational evidence in human Multiple Sclerosis patients that stress worsens the development of a demyelinating condition.

Acknowledgements

The authors acknowledge Lin Bustamante for excellent technical assistance with the preparation of H&E sections and Robin Johnson and Thomas Prentice for critical review of this manuscript. The authors would also like to thank Jessica Harrison, Justin Amaro, Patrick Bridegam, and Jennifer Cowart for all of their hard work on this project. This research was funded by grants to C.J.R.W. and M.W.M. from the National Multiple Sclerosis Society RG 3128 and NIH/NINDS R01 39569.

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