Social stress alters the severity and onset of the chronic phase of Theiler’s virus infection

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Abstract

Social stress alters the acute phase of Theiler’s virus infection (TMEV), a model of multiple sclerosis. Stress applied prior to infection had deleterious disease outcomes, while stress applied concurrent with infection was protective. The current study examined multiple behavioral (motor impairment, open field activity) and immunological measures (IL-6, antibodies to virus and myelin proteins) in both the acute and chronic phases of TMEV. It was found that stress applied prior to infection exacerbated disease outcomes, while concurrent application was protective in both disease phases.

1. Introduction

Multiple sclerosis (MS) is a common demyelinating condition of the central nervous system (CNS), effecting 350,000 people in the United States alone, with a prevalence rate of 60–200/100,000 (Sospedra and Martin, 2005). The etiology of MS remains unknown, but studies have implicated both genetic and environmental factors (Kurtzke and Hyllested, 1987; Noseworthy et al., 2000; Sospedra and Martin, 2005). For example, adolescent exposure to certain viruses is associated with later development of MS (Kurtzke and Hyllested, 1987). Additionally, MS patients often report elevated levels of stress prior to initial diagnosis and/or disease exacerbation (Ackerman et al., 2000; Grant et al., 1989; Mohr et al., 2000; Warren et al., 1982). However, not all studies show negative outcomes associated with stress–MS interactions. For example, severe stressors have been found to lower the rate of relapses of MS or fail to effect lesion development (Mohr et al., 2000; Nisipeanu and Korczyn, 1993). Taken as a whole, these findings indicate that stressor characteristics may be a factor in determining the complex effects of stress on MS symptom development.

Theiler’s virus infection (TMEV) in mice is a biphasic disease that causes an acute CNS inflammatory phase followed by a chronic neuroinflammatory/autoimmune demyelination phase similar to MS. The chronic phase of the disease has many similarities, both behaviorally and physiologically with progressive MS (Lipton, 1975; Oleszak et al., 2004). Our laboratory has previously shown that the nature (e.g. restraint vs. social stress) and timing of the stress determines its impact on disease development. Specifically, if social stress is applied prior to infection, the disease is exacerbated. In contrast when social stress is applied concurrently with infection, disease severity is reduced compared to infected, non-stressed animals (Johnson et al., 2004). However, if restraint stress is applied concurrent with infection, the disease is again exacerbated (Campbell et al., 2001; Sieve et al., 2004). These data demonstrate the complex relationship of stress during the acute, inflammatory phase of TMEV. However, little is known regarding the impact of stress during the chronic demyelinating phase of TMEV infection. Thus, the current study sought to examine the effects of social stress applied during the acute phase of
TMEV on the chronic, MS-like phase of the disease. This was accomplished by examining the timing of social stress in relation to infection in the acute phase (as in previous studies, Johnson et al., 2004) and then following these animals through to the chronic phase of the disease as well. By following animals through both phases of the disease, we were able to determine how social stress applied immediately before and after initial infection alters both phases of the disease, as well as examine how acute phase measures relate to chronic phase development.

Past studies have demonstrated that the social stressor, social disruption (SDR), alters the disease process of acute TMEV infection in several ways. First, SDR applied prior to infection (PRE-SDR) induced glucocorticoid resistance (GCR) and elevated CNS inflammation (Johnson et al., 2004). In contrast to the PRE-SDR animals, when SDR was applied concurrently with infection (CON-SDR), GCR failed to develop and the inflammation was not significantly different than that observed in non-stressed animals (NON-SDR). In addition, SDR leads to GCR in splenic macrophages and elevated levels of IL-6 (in the absence of infectious stimuli) (Avitsur et al., 2002; Avitsur et al., 2001; Stark et al., 2002). Interestingly, GCR has also been found to develop in the parents of pediatric cancer patients, leading to alterations in IL-6 regulation (Miller et al., 2002). Thus, IL-6 elevations (possibly due to GCR-related dysregulation of macrophages) may be a factor in the development of the effects of SDR in TMEV infection. The IL-6 elevations found in previous studies due to SDR may not be solely due to GCR-induced dysregulation of macrophages, as there are many sources of IL-6, including neuronal and adipose tissue (Murphy et al., 1999; Papanicolaou and Vgontzas, 2000). In addition, IL-6 may also impact the development of GCR (although it is not necessary based on previous work, Stark et al., 2002). From previous studies, we know that GCR develops differentially in the PRE-SDR and CON-SDR groups. The PRE-SDR animals develop GCR, while the CON-SDR animals do not. Since PRE-SDR animals also develop greater inflammation and GCR results in greater pro-inflammatory cytokine production, GCR induced IL-6 from macrophages appears to be a plausible explanation for the differences in disease development across the two groups.

The current study extends our prior work by examining how the timing of SDR at the time of infection alters the development of the chronic phase of TMEV infection. Here, we exposed TMEV infected male Balb/cJ mice to PRE-SDR, CON-SDR or NON-SDR. Mice were then examined regularly during early infection for behavioral signs of the acute disease. We also examined the immunomodulator, IL-6 at day 9 post-infection (pi). Following the acute phase, mice were monitored monthly until behavioral signs of the chronic disease were noted and then weekly measures were taken to examine the development of chronic phase disease. In addition, antibody levels to TMEV and myelin components were measured monthly.

2. Methods

2.1. Animals

Male Balb/cJ mice (n=18) were bred in-house and weaned at post-natal day 21 (pnd 21). Mice were placed with littermates, three per cage and maintained on a 12-h light/dark cycle (0500/1700h) with ad libitum access to food and water. Each cage was assigned to one of three SDR conditions (PRE-SDR, CON-SDR, NON-SDR) based on several factors. First, conditions were counter-balanced based on weight. Second, cages from the same dam were not placed in the same experimental condition to reduce the possible littermate confounds when explaining group differences. Littermates were used in this study primarily because they are less likely to become aggressive within the cage over the long time period required for a chronic phase study of Theiler’s virus infection in Balb/cJ mice. Additional cages of littermates were left uninfected and unstressed to provide control data for several assays (e.g. footprint stride length, IL-6 ELISA, RIAs). Intruders for SDR were active male breeders 6–8 months of age, selected from the breeding colony based on latency to attack peers within 30 s and adolescents within 2 min on three separate occasions. All animal protocols were in accordance with NIH Guidelines for Care and Use of Laboratory Animals and were approved by the Texas A&M Laboratory Animal Care and Use Committee.

2.2. Social disruption (SDR) stress

For the stressed mice, intruders were introduced into the cage of resident mice at dark cycle onset (1700h) for a period of 2h for a total of 6 SDR sessions. SDR occurred for three consecutive sessions, then one night off, followed by three additional consecutive sessions (Avitsur et al., 2001; Stark et al., 2001), in a separate procedure room. Fig. 1 provides a time line of the experimental procedures. NON-SDR mice remained in the colony room, undisturbed for the duration of SDR. SDR sessions were monitored to ensure that the intruder attacked the residents and that the residents demonstrated submissive behaviors. Intruders that did not attack within 10 min of session initiation were replaced and the session continued for the remaining 2h. This procedure occurred for the week prior to infection for the PRE-SDR group, while the procedure occurred beginning the same day as infection and continued for 1 week post-infection for the CON-SDR group.

2.3. Virus/infection

The BeAn strain of Theiler’s virus (obtained from Dr. H.L. Lipton, Department of Microbiology and Immunology, University of Chicago, Chicago, IL) was propagated and amplified in lung tumor cells. The culture supernatant
containing infectious virus was aliquoted, titrated and stored at −70°C before use (Welsh et al., 1987). Mice were anesthetized on day 0 pi/pnd 37 with isoflurane (Vedco Inc., St. Joseph, MO) and injected intracranially (ic) into the right mid-parietal cortex (depth approximately 1.5 mm) with 5 × 10^4 pfu of the BeAn strain of Theiler’s virus in a 20-μl volume as previously described (Campbell et al., 2001; McGavern et al., 1999, 2000; Rose et al., 1998; Theil et al., 2000). Inoculation for all subjects occurred at 2100 h, either following the last SDR session (PRE-SDR) or the first SDR session (CON-SDR).

2.4. Behavioral assessment of motoric impairment and illness

Multiple measures of psychomotor behavior were examined. These included: hind limb impairment ratings, locomotor activity (in the vertical and horizontal planes) and footprint stride length. Behavioral assessments were collected for the acute phase through day 28 pi. Many studies find that viral clearance is maximal between days 21 and 28 pi; thus, the distinction between acute and chronic phase disease in the past has been based on the underlying immunological process (Oleszak et al., 2004). Day 28 pi was used as the latest acute phase collection time point based on the previous literature. In the current study, all groups then began to exhibit behavioral signs of recovery compared to non-infected age matched control levels for several weeks until, by day 77 pi, all mice were at the age-matched control levels on all behavioral measures. All groups remained at this level until approximately day 126 pi, when the PRE-SDR animals began to deviate significantly from the other groups (including the age-matched controls). At this point and beyond, measures were collected for chronic phase assessment.

2.4.1. Behavioral ratings of illness

During the first 28 days pi, mice were examined every other day for signs of encephalitis and hind limb impairment (HLI). From day 28 pi through 177 pi, measures were taken weekly (see Fig. 1). Illness raters were blind to the subject’s experimental conditions and were trained to an inter-rater reliability criterion of \( r = 0.90 \) on this measure. All subjects underwent the exact same scoring procedure independent of symptoms or experimental condition. A separate numeric score was given for each hind limb individually, based on the symptoms of impairment the mice display (0=healthy, 1=slight weakness in grip, 2=clear weakness in grip, 3=slight paralysis, 4=moderate paralysis, 5=complete paralysis with muscle tone, 6=complete paralysis with no muscle tone; Johnson et al., 2004). These separate scores were then added together and the combined score analyzed and presented here in.

2.4.2. Open field activity monitoring

Weekly 20-min sessions were used to measure horizontal and vertical locomotion activity. Assessment began on day 7 pi and continued through day 177 pi. Six Versamax open field chambers (Omnitech), equipped with two banks of eight photocells on each wall, were used to measure horizontal and vertical locomotion. These open field boxes are interfaced with a digital multiplexor (Coulbourn E61-58) located in an adjacent room. Testing was conducted in the dark between 1500 and 1700 h. White noise (64 dB) was continually present to mask extraneous disturbances.

2.4.3. Stride length

Footprint stride length and spread were assessed similar to the method of McGavern et al. (1999, 2000). Briefly, hind limbs were painted with blue finger paint while forelimbs were painted with red finger paint. Mice were then allowed to walk down a 2.5" by 36" runway lined with paper to record limb placement. This has been shown to provide a reliable and valid measure of virally mediated nerve damage and demyelination (McGavern et al., 1999, 2000). To minimize space, only hind limb data is presented, as the forelimb data was similar.

2.4.4. Body weight

Body weight was measured every 7 days beginning at weaning as an assessment of illness. Failure to gain weight normally may be taken as indication of illness and/or failure to thrive.

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**Fig. 1.** Timeline showing the timing of procedures and data collection.
2.5. Assays on serum

2.5.1. Blood collection

Mice were individually transported (in a small plastic container with air holes) 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. While it is possible that the mild stress and tissue injury inherent in the bleeding procedure may influence several of our dependent measures, this variable is equated across groups. Hence, any differences observed between groups cannot be attributable to the bleeding procedure alone. Furthermore, age-matched uninfected and unstressed controls were included, which allowed the data for IL-6, antibodies and the behavioral measures of impairment to be compared to age-matched controls (see Statistical analysis).

2.5.2. IL-6 ELISA

IL-6 levels, in sera collected at day 9 pi, were determined using an ELISA assay (R&D Systems Madison, WI), following manufacturers instructions.

2.5.3. Antibody responses to Theiler’s virus and myelin components

RIAs were used to measure sera antibodies against Theiler’s virus (whole virus, identical to that used for infection), myelin basic protein (recombinant bovine, MBP, Sigma, St. Louis, MO), myelin oligodendrocyte glycoprotein peptide (synthetic purified, MOG33-55, Sigma, St. Louis, MO) and proteolipid protein peptide (synthetic purified, PLP139-151, AnaSpec Inc., CA) using previously described procedures (Dolimbek et al., 2002; Sieve et al., 2004; Young et al., 1983). It is important to note that the RIA was developed using radiolabeled protein A, which binds to the Fc portion of immunoglobulin. Therefore, the level of radioactivity (counts per minute, cpm) measured is equated with antibody level (Dolimbek et al., 2002; Young et al., 1983). Antibody levels were assessed at days 42, 140 and 170 pi.

2.6. Statistical analysis

Data are presented as mean±S.E.M. Analysis of variance (ANOVA) was used to evaluate differences across SDR and infection conditions, and repeated measure ANOVA were used for analysis over time as appropriate. These analyses were followed by post hoc mean comparisons using Duncan’s new multiple range test. The design of this study examined only TMEV infected animals; therefore, basal levels of several measures were not available for ANOVA analysis (e.g. stride length, antibodies). To address these issues, we ran a small number (n=3) of age-matched control mice, subtracted these levels prior to analysis and noted in Results as appropriate. Pearson correlation coefficients (r) were used to determine inter-rater reliability.

In addition, a correlation analysis was performed to examine the relationship between acute phase behavioral and immunological measures and chronic phase behavioral and physiological/immunological measures. Because we compared the relationship between early time points to later time points, prediction of chronic phase development can be inferred. These data are also presented as Pearson correlation coefficients (r).

3. Results

3.1. Acute phase measures (days 0–28 pi)

We collected a battery of behavioral and immunological measures throughout both the acute and chronic phases of the disease. Overall, the acute phase measures replicated our previous findings: PRE-SDR has a detrimental effect on multiple behavioral assessments of the disease, while CON-SDR is protective (compared to NON-SDR and PRE-SDR).

3.1.1. Effect of timing of SDR on acute phase hind limb impairment

Motoric impairment is the hallmark of both the early, polio-like disease in the Balb/cJ strain as well as the chronic MS-like phase of TMEV infection. Several motoric impairment measures were taken, the first of which was HLI, a scoring system that has previously been shown to be sensitive to the changes in impairment in early TMEV infection in Balb/cJ mice (Johnson et al., 2004). The effect of timing of SDR and TMEV infection on HLI was assessed through day 27 pi for the acute phase. PRE-SDR exacerbated infection related hind limb impairment, while CON-SDR reduced this impairment (Fig. 2A). ANOVA confirmed a main effect of the timing of SDR, F(2,1)=24.158, p<0.0001. In addition, an interaction between time and SDR indicates that HLI levels changed differently over time across the groups, F(28,210)=2.013, p<0.01. Post hoc analysis determined that the PRE-SDR animals have the greatest impairment at all time points (significant through day 14 pi except at day 5 pi, compared to NON-SDR). In addition, CON-SDR animals had significantly less impairment from day 5 pi through day 21 pi.

3.1.2. Effect of timing of SDR on acute phase stride length

A second measure of motoric impairment, stride length, at day 20 pi has previously been shown to be reduced in the PRE-SDR animals, while the CON-SDR and NON-SDR animals had no deficit (Johnson et al., 2004). Here, stride length at day 20 pi was again assessed, with controls subtracted from all groups to provide a change from baseline measure. Analysis was otherwise conducted similar to McGavern et al. (2000), averaging the first six readable step lengths, and then subtracting this number from age-matched...
controls. These data are presented in Fig. 2B. An ANOVA confirmed a main effect of SDR, \( F(2,15)=12.073, p<0.001 \). Post hoc means comparison showed that the PRE-SDR animals had significantly greater deficit compared to both NON-SDR and CON-SDR (as seen previously). In addition, post hoc analysis also showed that the CON-SDR animals had significantly less deficits compared to the NON-SDR animals.

3.1.3. Effect of timing of SDR on acute phase body weights

Consistent with our previous work, reduction in overall body weight gain over time was also found (Johnson et al., 2004). The PRE-SDR animals failed to gain weight at the same rate as CON-SDR and NON-SDR animals (data not shown). ANOVA analysis confirmed a main affect for SDR, \( F(2,15)=3.742, p<0.05 \); however, no interaction with time occurred, \( p>0.05 \). Thus, the body weight deficits incurred by day 7 pi were maintained across time, including through the chronic phase (data not shown).

3.1.4. Effect of SDR on open field activity

Analyses of open field activity found deficits in both horizontal and vertical activity due to SDR treatment (Fig. 3). An ANOVA confirmed main effects of SDR on overall horizontal activity, total distance, movement numbers, movement time, vertical time and overall vertical activity, \( F’s(2,15)>3.8, p’s<0.05 \) (Fig. 3A–F, respectively). Post hoc means-comparisons showed that PRE-SDR animals were significantly less active compared to CON-SDR animals, on all measures noted, but significant reductions compared to NON-SDR animals only occurred on vertical measures.

3.1.5. Effect of SDR on day 9 pi IL-6 levels

IL-6 levels were examined in sera collected at day 9 pi (Fig. 4). Once again, age-matched control average levels were subtracted from each animal prior to analysis, in order to control for baseline levels. The age-matched control average was 9.6 pg/ml (note that the average of control mice run by manufacturer is 7.8 pg/ml, with a range of 0–20 pg/ml). An ANOVA found a main effect of SDR on acute phase IL-6 levels, \( F(2,15)=47.989, p<0.0001 \). Post hoc analysis showed that the PRE-SDR animals had significantly greater IL-6 levels compared to both NON-SDR and CON-SDR, while CON-SDR animals had significantly less IL-6 compared to the NON-SDR animals.

3.2. Chronic phase measures of motor function

As in the acute phase, we examined several measures of motor function in the later phase of the disease, including HLI, stride length and open field measures. Chronic phase was determined to have begun when animals began to diverge from the age-matched controls on any measure. This occurred first in the PRE-SDR animals, around day 126 pi. The NON-SDR group began to diverge after day 140 pi. Thus, we report chronic phase measures taken just prior to the NON-SDR group’s divergence to demonstrate the development of the disease, beginning around day 135 pi. As in the acute phase, these measures converge to show that the PRE-SDR animals developed a more severe chronic disease, while the CON-SDR animals continued to be protected.

3.2.1. Effect of SDR on chronic phase hind limb impairment

HLI was assessed regularly, from day 28 pi; however, the animals were recovering or recovered until day 138 pi; therefore, only results from day 138 pi onwards are shown here. This time point was chosen because the PRE-SDR group began to show levels of impairment that differed significantly from age-matched controls. Fig. 5A shows that PRE-SDR animals developed severe HLI suddenly, around day 138 pi, and maintained the same level throughout the chronic phase. The CON-SDR animals did not develop significant impairment for another month (day 165 pi). An ANOVA revealed a main effect of SDR, \( F(2,15)=10.48, p<0.001 \).
p < 0.001, as well as an interaction with time [F(10,75) = 2.651, p < 0.05]. Post hoc analysis indicated that the CON-SDR animals had significantly less impairment. By day 172 pi, all groups had become nearly equally impaired.

3.2.2. Effect of SDR on chronic phase stride length

Stride length was assessed at day 140 pi, due to the development of HLI around day 138 pi in the PRE-SDR group. Once again, these data were subtracted from controls. Fig. 5B shows that the PRE-SDR animals again had the greatest deficits, while the CON-SDR animals had the least. ANOVA analysis confirmed a main effect of SDR, F(2,14) = 76.893, p < 0.01. Post hoc analysis showed that the PRE-SDR animals had significantly decreased stride length compared to both the NON-SDR and CON-SDR animals, while the CON-SDR animals again had significantly less deficits compared to the NON-SDR animals.

3.2.3. Effect of SDR on chronic phase horizontal and vertical behavior

Fig. 6 depicts the effect of SDR conditions on horizontal and vertical locomotor activity, collapsed across days. Deficits are apparent in both horizontal and vertical activities in the PRE-SDR animals compared to the other two groups (Fig. 6). An ANOVA confirmed that most horizontal measures (activity, distance, movement numbers, Fig. 6A–C, respectively) had significant main effects for SDR, F’s(2,14) < 4.5, p’s < 0.05. Post hoc differences are noted in Fig. 6 by an asterisk. In addition, the vertical measures also had significant main effects for SDR, F’s(2,14) < 6.5, p’s < 0.01, shown in Fig. 6D, E and F for activity, movement numbers and time, respectively.

In summary, we assessed multiple measure of motor function, and found significant deficits on these measures for the PRE-SDR animals, while the CON-SDR animals were the least affected on these measures.
3.3. Chronic phase measures of immune functions

Several measures of immune and autoimmune indicators in the chronic phase of the disease were also assessed. These included the level of antibody to TMEV, as well as antibody levels to myelin components (as a measure of autoimmune responses): MBP, MOG33-55 and PLP139-151, and spleen weights. Overall, the PRE-SDR animals had the largest spleens, greatest antibody levels to virus and greatest autoimmune antibody levels.

3.3.1. Effect of SDR on spleen weights

Fig. 7A shows that the PRE-SDR animals had significantly increased spleen weights upon sacrifice. The main effect of SDR was confirmed by an ANOVA, $F(2,14)=4.064, p<0.05$. Post hoc analysis showed that the PRE-SDR animals had significantly greater spleen weights compared to the NON-SDR animals.

3.3.2. Effect of SDR on antibody to Theiler’s virus

The levels of antibody to Theiler’s virus levels were greatest in the PRE-SDR animals (Fig. 7B). Once again, averaged age-matched control data was subtracted prior to analysis ($\bar{x}$=217.8, 219.6 and 197.4 cpm at days 42, 140 and 170 pi, respectively). ANOVA analysis on these data showed a main effect of SDR, $F(2,15)=146.084, p<0.0001$. Post hoc analysis found that the PRE-SDR animals had significantly greater levels of antibody to virus compared to both NON-SDR and CON-SDR animals, and the CON-SDR animals had the lowest level of all three antibodies.

3.3.3. Effect of SDR on the autoimmune response to myelin epitopes

Fig. 8A–C present the results of the analysis of antibody levels to myelin (MOG33-55, MBP and PLP139-151, respectively), as indicators of autoimmune responses in each group. As with the TMEV antibodies, in order to control for basal levels, the averages of age-matched controls were subtracted prior to analysis ($\bar{x}$ for MOG33-55, MBP and PLP139-151 were, respectively, 218.4 cpm, 214.2 cpm and 210.6 cpm). The PRE-SDR animals had the greatest antibody titers to myelin at all time points examined (days 42, 140 and 170 pi); thus, only the main effect of SDR, collapsed over time, is reported. An ANOVA revealed a main effect for SDR, $F(2,15)=111.884, 370.648, 56.026$ (MOG33-55, MBP and PLP139-151, respectively), $p’s<0.001$. Post hoc analysis found that the PRE-SDR animals had the greatest levels of all three antibodies, while the CON-SDR animals had the lowest level of all three antibodies.
3.4. Acute phase measures that predict chronic phase development and severity

A correlation analysis was conducted and several acute phase measures were found to predict the severity of chronic phase measures. Table 1 summarizes the behavioral results for acute measures that predict chronic onset measures, all correlations reported are at $p<0.05$ and items in bold are at $p<0.01$ significance levels. Measures taken at day 7 pi, including HLI, body weight and open field activity measures (total distance, vertical activity and vertical time), predict chronic onset levels, taken at day 136 pi of HLI, body weight, foot print stride length and open field activity measures (horizontal activity, movement numbers, vertical activity, vertical movement numbers and vertical time). For the sake of brevity, only the onset correlations are presented; however, this pattern of results is similar at later chronic disease time points as well. In addition, IL-6 (taken at day 9 pi) and antibody levels to virus (TMEV) and myelin proteins (MOG33-55 and MBP taken at day 42 pi) were also correlated with the behavioral measures mentioned above. These correlations set a pattern of results that demonstrate that many behavioral and immunological measures taken early in

![Fig. 6. The effect of PRE-SDR and CON-SDR on open field activity in chronic Theiler's virus infection. PRE-SDR had significantly reduced horizontal activity (A), horizontal movement numbers (B), horizontal distance traveled (C), vertical activity (D), vertical movement numbers (E) and vertical time (F) compared to CON-SDR. In addition, PRE-SDR also had significantly less activity compared to NON-SDR on horizontal activity (A), horizontal distance (C) and vertical movement numbers (E). Asterisks indicate significant differences.](image)
the disease significantly predict many aspects of the onset of behavioral symptoms of the chronic disease.

Table 2 summarizes the results of acute phase measures that predict physiological and immunological measures from the chronic phase. Once again, many acute behavioral as well as immunological measures predicted chronic phase physiological changes in the spleen, thymus (note that thymus weights were not significantly different across groups, data not shown, as a whole they correlated with several acute measures). In addition, both the behavioral and immunological acute measures significantly and consistently predicted the chronic phase immunological measures (TMEV, MOG33-55 and MBP).

4. Discussion

In the past, our laboratory found that PRE-SDR exacerbated, while CON-SDR alleviated the severity of the acute phase of TMEV infection. The current study replicated and extended these findings to show that PRE-

SDR stress caused a more severe disease course in both the acute phase and chronic phase of TMEV infection (compared to both NON-SDR and CON-SDR). In addition, CON-SDR again appeared to alleviate the severity of the acute phase and this also led to a less severe chronic phase of TMEV infection for these animals. Finally, we found that several acute behavioral and immunological measures were significant predictors of the chronic phase onset and development. These results replicate and extend our previous work that demonstrated that the timing of SDR was an important factor in determining the direction of the
effect on TMEV infection disease development (Johnson et al., 2004). In addition, these results add to our understanding of the effects of social stress on the development of both the acute and chronic phases of TMEV infection, and how the disease course in the acute phase correlates with the chronic phase.

Several indicators of immune function were investigated in the early phase of TMEV. During the acute phase, at day 9 pi, we examined circulating levels of the pro-inflammatory cytokine IL-6. PRE-SDR was shown to elevate IL-6 levels above the other two groups. Moreover, examination of the correlations to chronic phase measures found that acute phase IL-6 levels are one of the best predictors of chronic phase onset and development. Previous work using SDR also found elevated sera IL-6 levels in sera at both days 1 and 6 of SDR (Stark et al., 2002). In addition, several studies have found that IL-6 is elevated acutely due to TMEV infection (Mi et al., submitted for publication; Palma et al., 2003). In addition, IL-6 can be a potent inflammatory stimulus, which may explain the elevated inflammation in the PRE-SDR animals in our original study (Johnson et al., 2004). Recently, we have also found that IL-6 may be necessary for the negative effects of PRE-SDR to occur (Johnson et al., 2005). The role of this cytokine in SDR-related changes in TMEV infection is clearly of interest for further investigation.

Following infection with TMEV, viral specific antibodies develop. If the virus persists, which is necessary for the development of the chronic phase of the disease, then antibodies to TMEV would also persist (Oleszak et al., 2004). Thus, the finding that social stress systematically altered the level of antibody to virus in the current study has several possible interpretations. One possibility is that the higher levels found in the PRE-SDR group could be an indicator of a more effective anti-viral antibody response and the lower antibody level in the CON-SDR animals could indicate a failure to develop an appropriate immune response. However, this interpretation is inconsistent with the overall pattern of results. An alternative interpretation is that the higher levels of antibody observed in the PRE-SDR animals indicate increased viral levels, stimulating greater antibody production, while the lower levels in the CON-SDR animals indicate lower viral levels, stimulating less antibody production. Based on both the current data (behavioral and immunological) and the viral clearance data from our previous acute phase study, the later interpretation has more support (Johnson et al., 2004).

Table 1
Correlations of acute phase measures and chronic phase behavioral development measures

<table>
<thead>
<tr>
<th>Chronic onset (day 136 pi) behavioral measures</th>
<th>Hind limb impairment</th>
<th>Body weight</th>
<th>Stride length</th>
<th>Activity—horizontal</th>
<th>Movement number—horizontal</th>
<th>Activity—vertical</th>
<th>Movement number—vertical</th>
<th>Time—vertical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7 pi</td>
<td>0.453</td>
<td>0.367</td>
<td>0.578</td>
<td>-0.406</td>
<td>-0.559</td>
<td>-0.335</td>
<td>-0.494</td>
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<tr>
<td>Body weight</td>
<td>-0.341</td>
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<td></td>
<td>0.353</td>
<td></td>
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<tr>
<td>Total distance</td>
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<td>0.392</td>
<td>0.468</td>
<td>0.45</td>
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<td></td>
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<tr>
<td>Activity—vertical</td>
<td>-0.333</td>
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<tr>
<td>Time—vertical</td>
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<tr>
<td>Day 9 pi</td>
<td>IL-6</td>
<td>0.456</td>
<td>0.361</td>
<td>-0.52</td>
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<td></td>
</tr>
<tr>
<td>Antibody to PLP139-151</td>
<td>-0.389</td>
<td>-0.355</td>
<td>0.357</td>
<td>0.375</td>
<td>0.509</td>
<td>0.558</td>
<td>0.569</td>
<td></td>
</tr>
<tr>
<td>Antibody to TMEV</td>
<td>-0.367</td>
<td>0.468</td>
<td>-0.435</td>
<td>0.377</td>
<td>0.68</td>
<td>0.688</td>
<td>0.616</td>
<td>0.728</td>
</tr>
</tbody>
</table>

All shown are p < 0.05, bold p < 0.01.

Table 2
Correlations of acute phase measures and chronic phase immunological measures

<table>
<thead>
<tr>
<th>Chronic phase physiological/immunological measures</th>
<th>Spleen</th>
<th>Seminal vesicles</th>
<th>Thymus</th>
<th>Antibody to TMEV at day 140 pi</th>
<th>Antibody to TMEV at day 170 pi</th>
<th>Antibody to MOG33-55 at day 140 pi</th>
<th>Antibody to MOG33-55 at day 170 pi</th>
<th>Antibody to MBP at day 140 pi</th>
<th>Antibody to MBP at day 170 pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7 pi</td>
<td>0.382</td>
<td>0.530</td>
<td>0.502</td>
<td>-0.490</td>
<td>-0.521</td>
<td>-0.575</td>
<td>-0.638</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody to TMEV</td>
<td>0.557</td>
<td>0.588</td>
<td>0.518</td>
<td>0.484</td>
<td>0.419</td>
<td>0.587</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity—vertical</td>
<td>0.587</td>
<td>0.598</td>
<td>0.537</td>
<td>0.508</td>
<td>0.428</td>
<td>0.606</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time—vertical</td>
<td>0.587</td>
<td>0.598</td>
<td>0.537</td>
<td>0.508</td>
<td>0.428</td>
<td>0.606</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 9 pi</td>
<td>IL-6</td>
<td>0.387</td>
<td>0.799</td>
<td>0.765</td>
<td>-0.694</td>
<td>-0.755</td>
<td>-0.825</td>
<td>-0.837</td>
<td></td>
</tr>
<tr>
<td>Antibody to PLP139-151</td>
<td>-0.552</td>
<td>-0.420</td>
<td>-0.799</td>
<td>0.765</td>
<td>-0.694</td>
<td>-0.755</td>
<td>-0.825</td>
<td>-0.837</td>
<td></td>
</tr>
<tr>
<td>Antibody to TMEV</td>
<td>-0.463</td>
<td>-0.341</td>
<td>0.880</td>
<td>0.864</td>
<td>0.993</td>
<td>0.975</td>
<td>0.770</td>
<td>0.897</td>
<td></td>
</tr>
<tr>
<td>Antibody to MOG33-55</td>
<td>-0.370</td>
<td>0.371</td>
<td>0.880</td>
<td>0.864</td>
<td>0.993</td>
<td>0.975</td>
<td>0.770</td>
<td>0.897</td>
<td></td>
</tr>
<tr>
<td>Antibody to MBP</td>
<td>0.439</td>
<td>0.664</td>
<td>0.625</td>
<td>0.647</td>
<td>0.740</td>
<td>0.864</td>
<td>0.808</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All shown are p < 0.05, bold p < 0.01.
interpretation is also consistent with prior psychoneuroimmunology research on antibody measures of latent viruses, such as Epstein-Barr virus (EBV). This work has shown that EBV antibody levels provide a valid measure of cell-mediated immunity and that antibody levels increase after exposure to a range of immunosuppressive psychosocial stressors (Esterling et al., 1992; Glaser et al., 1985, 1993, 1991; Kiecolt-Glaser et al., 1987, 1994). After exposure to stress, EBV reactivates and releases viral antigens into circulation, which in turn causes a humoral antibody response. Likewise, the immunosuppressive effects of PRE-SDR may allow TMEV to replicate at higher levels and release more viral antigen into circulation, to which a humoral antibody response emerges. By analogy, if levels of antibodies against EBV antigens provide an indirect measure viral replication and cell-mediated immune function, then levels of antibody against TMEV may also provide a similar read-out of viral replication and cell-mediated immune function. Fig. 9 depicts a theoretical model of the development of viral titers and antibody to virus production in the SDR/TMEV infected mice. The titer levels are based on our previous studies that demonstrated that CON-SDR animals cleared a significant portion of virus from day 7 to day 21 pi (going from approximately 10^5 to less than 10^3 pfu/mg of tissue, while the PRE-SDR animals had lower levels at day 7 pi (approximately 10^5 pfu/mg of tissue), but failed to clear any virus by day 21 pi (Johnson et al., 2004).

Balb/cJ mice are considered susceptible to the chronic phase of Theiler’s virus based on one prior study (Nicholson et al., 1994). Our work differs from this single previous study using TMEV infection in Balb/cJ mice in several ways. Primarily, our laboratory typically uses 1–5 × 10^5 pfu, while Nicholson et al. (1994) used 2.9 × 10^6, approximately 100-fold more concentrated virus than is used in our own laboratory. This great difference in the titer of virus used for infection may explain why we have significant elevations in auto-antibody responses, but failed to find significant histo-

logical indications of either inflammation or demyelination (data not shown, details on procedures used may be found in Johnson et al., 2004; Bancroft and Stevens, 1982, respectively). The infection protocol differences may also account for these difference across our study and Nicholson et al. (1994) in the behavioral onset of chronic phase indications (days 130–165 pi vs. days 90–120 pi, respectively).

Nicholson and colleagues found that 8 out of 10 mice developed a slight “wobbliness” (on a scale of 0–2, with 2 being extreme movement difficulties) around days 90–120 pi. Of the eight mice that developed this behavioral sign of chronic disease, four developed some histological signs of inflammatory. In contrast, although we did not observe significant levels of inflammation (even in the two mice that developed complete paralysis), 100% of our PRE-SDR mice and 67% of the NON-SDR animals had clear behavioral signs of neurological impairment (hind limb impairment, reduced vertical movements and stride length), and 100% of these two groups had significantly elevated antibodies to myelin proteins compared to age-matched controls by day 140 pi.

Another possible explanation for our findings may be that Balb/cJ mice develop a more pronounced polio-like disease during acute TMEV infection relative to other susceptible strains of mice. Many strains (SJL, B.10, CBA) do not show pronounced hind limb impairment during the acute phase of the disease. In contrast, Balb/cJ mice develop impairment in the hind limbs that may include partial or complete paralysis within the first weeks of disease that is alleviated after 30–45 days pi followed by a return of hind limb impairment during late disease. Polio and post-polio syndrome follow a similar pattern. Polio causes paralysis in the legs that can dissipate over time if the person survives. Although some recovery of function occurs early in life, many persons with polio develop unexplained weakness and motor impairment in one or more limbs as they age, a condition known as post-polio syndrome (Chasens and Umlauf, 2000; Dalakas, 1995).

We propose that the Balb/cJ mice may have developed a post-polio like syndrome rather than MS-like demyelination and paralysis during late TMEV infection. Some evidence supporting this hypothesis exist: 88% of the time the hind limb that developed the most severe impairment in the acute phase also developed the most severe impairment in the chronic disease. Thus, initially infecting mice with a relatively low viral titer may have induced a post-polio-like chronic phase, whereas a higher titer may lead to MS-like chronic phase. We do not know if motor neuron loss outside of the CNS occurred at this time, although this would add a great deal of evidence for this theory. In addition, while the mice in the present study have slightly, but significantly elevated IL-6 levels, post-polio syndrome researchers have not examined IL-6 specifically, although other inflammatory cytokines are elevated, so it is possible that IL-6 may be important as well (Gonzalez et al., 2002).

Given the paucity of studies using Balb/cJ mice, further
investigation will be required before any final conclusions may be drawn.

The current study also differs form other TMEV studies using other strains of mice. The auto-antibodies to myelin epitopes that developed in the Balb/cJ mice were not consistent with studies using the highly susceptible SJL strain. In the SJL strain, PLP139-151 is the most important epitope in TMEV infection (Olson et al., 2002, 2004). The current study examined the antibodies to PLP139-151, MBP, and MOG33-55, and found that generally, the PRE-SDR mice had the greatest levels of auto-antibodies to myelin components. In contrast to SJL mice, the Balb/cJ mice used here had an attenuated PLP139-151 antibody response. Furthermore, PLP139-151 was the only physiological measure that was not correlated with other behavioral and physiological measures in the chronic phase. Typically, the chronic phase onset in SJL mice is around day 45 pi, compared to days 90–160 pi in the Balb/cJ mice. Thus, these strains (SJL and Balb/cJ) have obviously varied disease development and PLP139-151 may not be the primary epitope of interest when assessing chronic phase autoimmune responses in Balb/cJ mice.

Overall, the effect size differences, particularly between CON-SDR and NON-SDR, in this study were greater between the groups than in our previous work (Johnson et al., 2004). In our previous studies, CON-SDR animals had similar hind limb impairment and stride length to NON-SDR animals. Here, the CON-SDR animals had significantly less deficits in hind limb impairment scores and stride length through day 21 pi. Several possible factors may be contributing to these data. The earlier studies used mice that were shipped from the breeder to arrive at pnd 21. Although dams were sent with animals this young, arrival at pnd 21 requires weaning at pnd 18, causing early weaning stress. In addition, these animals were exposed to shipping, adding another possible level of background stress. Thus, in our previous work, all groups had possible background levels of stress due to early weaning and shipping that may have masked the effect of SDR, yielding a smaller overall effect size in our previous study. The current mice were bred in-house, thus avoiding both the extra shipping stress period and possible pre-weaning stress, allowing larger effect sizes to be found.

The current study has clinical implications for the viral etiology theory of MS development. One theory of MS etiology is that adolescent viral exposure may be responsible for the later autoimmune development (Kurtzke, 1993; Noseworthy et al., 2000; Sospedra and Martin, 2005). For instance, several viruses have been thought to be the underlying cause of MS, including Epstein Barr and Herpes-6 (Gutierrez et al., 2002; Levin et al., 2003). But further analysis indicated that many, if not all of the population, have antibodies to these pathogens (Herman et al., 2001). Alternatively, a recent review has found retrospective reporting indicates that the timing of stress around infection may be an important factor (Mohr and Pelletier, 2006). The present animal study provides the first experimental evidence that the timing of the stressor in relationship to infection determines the impact on later disease course. Therefore, a multi-factorial model may be more accurate, one that takes into account both the exposure to the viruses associated with MS, as well as the stress level prior to and at the time of viral exposure.

In conclusion, these results further clarify the adverse effects of PRE-SDR on TMEV infection, as we have now shown that elevated symptoms in the acute phase are associated with a more severe chronic phase. In addition, we have shown that those animals that had lower symptoms (CON-SDR animals) and reduced acute phase severity had delayed onset and reduced severity of chronic phase symptoms. Thus, if we are able to intervene in the acute phase to reduce acute phase measures of disease, we can confidently hypothesize that this will have a protective effect on chronic phase outcomes.

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