Published in final edited form as:

Exp Neurol. 1999 July ; 158(1): 171–181. doi:10.1006/exnr.1999.7082.

Quantitative Assessment of Neurologic Deficits in a Chronic Progressive Murine Model of CNS Demyelination

Dorian B. McGavern^{*}, Laurie Zoecklein[†], Kristen M. Drescher^{‡,†}, and Moses Rodriguez^{‡,†,*}

[‡]Department of Neurology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

[†]Department of Immunology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

^{*}Molecular Neuroscience Program, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Abstract

The precise factors involved in the development of a progressive motor dysfunction, a hallmark of immune-mediated demyelinating diseases such as multiple sclerosis, are not well defined. The ability to identify neurologic deficits that result in impaired motor performance early in disease may allow for the identification of therapeutic interventions that slow or eliminate the progression toward a permanent dysfunction. Here we describe the use of three objective, quantitative functional assays (spontaneous activity box, rotarod, and footprint analysis) to detect early neurologic deficits following the initiation of a demyelinating disease with Theiler's murine encephalomyelitis virus (TMEV). The results show that the assays are capable of revealing neurologic deficits at the early stages of the demyelinating disease process. These findings are the first to objectively characterize neurologic function in an animal model of progressive CNS demyelination.

Keywords

Theiler's virus; rotarod; footprints; spontaneous activity; multiple sclerosis; neural

INTRODUCTION

The ability to objectively detect neurologic deficits in a murine model of progressive central nervous system (CNS) demyelination provides a useful system for experimental intervention. Human demyelinating diseases of the CNS that are immune-mediated, such as multiple sclerosis (MS), often result in a progressive motor dysfunction (1); however, the contributions of various factors to this functional impairment (i.e., demyelination, axonal damage, components of the immune response) are not completely understood. Therefore, the objective detection of motor deficits in an animal model of MS provides a unique opportunity to dissect out which factors give rise to the early deficits that eventually progress towards a permanent dysfunction.

Intracerebral injection of Theiler's murine encephalomyelitis virus (TMEV) in susceptible mice results in a biphasic disease characterized by an acute neuronal infection followed by chronic immune-mediated demyelination of the CNS (2). In this model, susceptibility and viral persistence maps to the class I MHC D region (3–5). Mice of the susceptible

H-2^{f,p,q,r,s,v} haplotypes are unable to clear virus from the CNS, which results in pathological changes and neurologic deficits similar to those seen in MS (6, 7). Animals of a resistant haplotype (H-2^{b,d,k}) clear the virus during the acute phase (within 21 days) and do not show any signs of demyelination or neurologic deficits. Resistant mice do, however, suffer from the same neuronal damage as susceptible mice during the acute phase of disease.

It is very difficult to detect early neurologic deficits in the TMEV model using traditional subjective assays that assess the dysfunction in mice based on appearance. Subjective assessment of susceptible SJL/J mice by Murray *et al.* at 90 days postinfection determined that a minority of the animals was symptomatic (8). To enhance our ability to objectively detect deficits at earlier time points, we utilized three independent quantitative assays of clinical function: (a) computerized spontaneous activity monitoring box, (b) rotarod, and (c) footprint analysis. We have previously used the activity monitoring box to assess neurologic deficits in susceptible SJL/J (H-2^s) mice (9). In addition, the rotarod assay has been shown to be a sensitive measure of motor balance, coordination, and control (10–13). Several studies have demonstrated that mice with deficiencies in components of CNS myelin perform poorly on the rotarod when compared to wild type controls (10, 13). Footprint analysis has also been shown to be a sensitive indicator of neurologic deficits in rodents, and the methodology is well-established (14). Studies have shown alterations in stride following spinal cord injury (15), neuropathies (16), and sciatic nerve injury (17). Therefore, the use of all three assays in concert should provide an accurate clinical picture of disease progression in infected mice.

Despite the fact that neurologic testing has been utilized to detect deficits in various rodent model systems (10–17), a comprehensive study of neurologic deficits in a progressive demyelinating disease of the CNS has never been reported. Such a study may have direct relevance to demyelinating diseases in the CNS of humans. In this paper, we describe the progression of TMEV-induced chronic demyelinating disease in two highly susceptible strains of mice, SJL/J (H-2^s) and B10.Q (H-2^q). SJL/J mice were selected for study, as they have been described as a prototypic susceptible strain (18). In addition, B10.Q were selected as a second susceptible strain based on previous findings which determined that susceptibility to viral persistence resulted from their H-2^q haplotype on a C57BL background (3). The comparison between resistant C57BL/6 mice and susceptible B10.Q mice was especially relevant because the genetic background of these mice is identical except for the MHC haplotype. Disease progression was monitored in individual mice using the three independent assays of neurologic function. As controls, infected susceptible strains of mice were directly compared to age-matched, sham-infected mice of an identical genotype and to infected, resistant C57BL/6J (H-2^b) mice, which do not show demyelination.

RESULTS

Decreased Spontaneous Activity in Susceptible Strains of Mice

Assessment of nocturnal spontaneous activity serves as an excellent measure of natural behavior in mice. In a previous study, we demonstrated that the measurement of nocturnal vertical spontaneous activity was a sensitive indicator of neurologic deficits in TMEV-

infected SJL/J mice (9). The sensitivity of this assay is most likely a reflection of its ability to assess hindlimb rearing in mice. As the hindlimbs of susceptible mice often become stiff or paralyzed during the chronic stages of TMEV infection, the rearing response is consequently reduced. In contrast, horizontal activity is a measurement of the level of spontaneous walking about the cage and may not be as severely affected during the progression of disease in susceptible mice. This is due to the fact that it is easier for mice with hindlimb deficits to walk than to engage in spontaneous rearing.

In the present study, we assessed both the nocturnal horizontal and the vertical spontaneous activity in C57BL/6J, B10.Q, and SJL/J mice at two stages of the disease (Fig. 1). As was expected, mice of the resistant C57BL/6J background showed no significant decreases in horizontal (Fig. 1A) or vertical (Fig. 1B) activity during the two chronic time ranges when measurements were taken. In contrast, susceptible B10.Q (Figs. 1C and 1D) and SJL/J (Figs. 1E and 1F) mice showed reductions in horizontal and vertical activity at both the 55–71 days postinfection (d.p.i.) and the 98–128 d.p.i. time ranges. Consistent with our previous findings (9), measurement of vertical activity proved to be the most sensitive indicator of neurologic deficits in both susceptible strains of mice. Infected B10.Q mice had a 46% reduction in vertical activity between 55 and 71 d.p.i., which progressed to a 78% reduction between 98 and 128 d.p.i. (Fig. 1D). In comparison, infected SJL/J mice showed a marked 78% reduction in vertical activity between 55 and 71 d.p.i. and an 87% reduction between 98 and 128 d.p.i. (Fig. 1F).

To allow direct comparisons to be made between each of the experimental strains, ratios of infected/uninfected (sham) nocturnal activities were calculated for all groups of mice (Figs. 1G and 1H). These ratios were calculated to control for the variability in the spontaneous activity of individual strains of mice. A ratio approximately equal to one or greater signifies no significant difference in nocturnal activities between infected and sham-infected controls. Consistent with the results described above, the horizontal (Fig. 1G) and vertical (Fig. 1H) activity ratios for resistant C57BL/6J mice were approximately one at both of time ranges assessed. In contrast, significant reductions in horizontal activity were observed in susceptible B10.Q and SJL/J mice when compared to the C57BL/6J mice (Fig. 1G). A 32% decrease in horizontal activity was observed in B10.Q mice between 55 and 71 d.p.i. that was not statistically different from the 40% reduction seen in SJL/J mice. Between 98 and 128 d.p.i. B10.Q mice did not experience significant changes in horizontal activity from 55 to 71 d.p.i., whereas SJL/J mice progressed to a 65% reduction in horizontal activity when compared to C57BL/6J mice. Marked reductions in vertical activity were detected when both strains of susceptible mice were compared to resistant C57BL/6J mice (Fig. 1H). Vertical activity was reduced by 58% in B10.Q mice 55–71 d.p.i., while SJL/J mice had a 75% reduction. Measurements between 98 and 128 d.p.i. revealed an 86% reduction of vertical activity in both B10.Q and SJL/J mice, confirming the sensitivity of this assay in detecting subtle neurologic deficits.

Disruption of Motor Coordination in Susceptible Strains of Mice

As a more sensitive indicator of neurologic deficits, we utilized the rotarod assay as an objective measure of motor balance, coordination, and control (10–13). We assessed the

performance of sham-infected and infected C57BL/6J, B10.Q, and SJL/J mice at varying time points using the accelerated rotarod assay (Fig. 2). Testing at 24, 48, and 93 d.p.i. for C57BL/6J mice revealed no statistically significant decreases in rotarod performance of infected mice when compared to sham-infected mice at any of the time points assessed (Fig. 2A). In contrast, compared to the sham-infected controls, infected B10.Q mice first showed a decrease (26%) in rotarod performance at 48 d.p.i. A decrease of 50% was subsequently observed at 93 d.p.i. that was statistically lower than the 48 d.p.i. measurement (Fig. 2B). Decreases in performance were also observed in SJL/J mice; however, the rate of progression of the disease was different (Fig. 2C). Deficits were observed as early as 24 d.p.i. for infected SJL/J mice (42% decrease), suggesting that a component of motor function was lost during the acute phase of the disease. Decreases in performance were also observed at 48 d.p.i.; however, these deficits were not statistically different from those observed at 24 d.p.i. By 93 d.p.i. a 57% decrease was seen for infected SJL/J mice that was significantly lower than the 24 and 48 d.p.i. time points.

It is apparent that both age and intracerebral injection can influence rotarod performance, as sham-infected mice from all strains showed a reduction from the baseline measurements (Figs. 2A–2C). To control for these factors and allow for interstrain comparisons, ratios of infected/uninfected (sham) rotarod performance were calculated at all time points for each strain (Fig. 2D). These results indicate that B10.Q mice did not have a decreased rotarod performance at 24 d.p.i. when compared to C57BL/6J mice, whereas SJL/J mice had a 50% decrease. By 48 d.p.i. B10.Q mice showed a reduction comparable to SJL/J mice; however, the deficits in SJL/J mice were not statistically different from those observed at 24 d.p.i.. At the 93 d.p.i. time point, both B10.Q and SJL/J mice exhibited a 62% decrease from C57BL/6J mice, which was significantly reduced from their respective 48 d.p.i. performances. In concert, these data indicate that despite the fact that susceptible B10.Q and SJL/J mice have similar deficits at 48 and 93 d.p.i., the onset of these functional impairments originated much earlier in the progression of disease for SJL/J mice.

Although all susceptible mice were infected with equivalent viral titers, the disease progression in individual animals occurred at different rates. This heterogeneity may have resulted from several factors that include anatomical variability of early viral infection and/or the efficiency of the immune response in limiting virus spread. To demonstrate the heterogeneity of disease progression and emphasize the advantage of using the accelerated rotarod assay in its detection, individual rotarod performances of B10.Q (Fig. 2E) and SJL/J (Fig. 2F) were plotted at each of the time points assessed. The time course for infected B10.Q mice revealed that only 3 of 10 mice at 24 d.p.i. had a rotarod performance below the lowest sham-infected control. By 48 d.p.i. this number had increased to 6 of 9 mice, and by 93 d.p.i. to 6 of 7 mice. This reduction in the total number of mice resulted from 1 death between 24 and 48 d.p.i. and 2 deaths between 48 and 93 d.p.i.. In contrast, the time course for susceptible SJL/J mice revealed that 7 of 10 mice at 24 d.p.i. had a reduced rotarod performance, which increased to 9 of 10 at both 48 and 93 d.p.i.. Collectively, these data emphasize that the ability to detect deficits from averaged rotarod data at various time points postinfection may be dependent on the heterogeneity of disease progression for a given strain. For example, decreased rotarod performances were easily detected at 93 d.p.i. in both B10.Q and SJL/J mice, as the factors that contribute to the development of neurologic

deficits were sufficient to generate a motor dysfunction in nearly all mice. In contrast, the factors that contribute to neurologic deficits were not as pronounced in 24 d.p.i. B10.Q mice, but were present in SJL/J mice.

Alterations in Stride Detected in Susceptible Mice

As footprint analysis has been shown to be a sensitive indicator of functional impairments in rodent model systems (15–17), we set out to determine if alterations in stride were a sensitive indicator of progressive neurologic deficits. We developed footprint analysis methodology to measure forelimb/hindlimb length and width of stride (Fig. 3) in sham-infected and infected C57BL/6J, B10.Q, and SJL/J mice. As the width of stride parameter did not reveal any significant deficits between any of the strains in the time course, data are only shown for the forelimb and hindlimb length of stride (Fig. 4). A time course of C57BL/6J mice showed no significant alterations in the length of stride between sham-infected and infected mice at any time point postinfection (Fig. 4A). In contrast, examination of footprints for infected B10.Q and SJL/J mice revealed a statistically significant decrease in forelimb and hindlimb stride length at 94 d.p.i. when compared to the uninfected baseline (0 d.p.i.) measurements (Figs. 4B and 4C). This decrease was not observed in B10.Q or SJL/J sham-infected controls.

It is evident from the data for sham-infected mice in Figs. 4A–4C that age, and possibly the intracerebral injection, affected stride length. The trend for all sham-infected controls suggested that stride length increased with age. However, this trend was not observed for infected B10.Q and SJL/J mice (Figs. 4B and 4C). In fact, either a significant decrease or no change from the baseline measurement was observed for both strains. To eliminate the factors of age and intracerebral injection and allow for interstrain comparisons, ratios of infected/uninfected (sham) hindlimb stride lengths were calculated at all time points (Fig. 4D). A ratio equal to one or greater signifies that there was no significant difference between the infected and the sham-infected group at a given time point. Forelimb stride length ratios were not shown, as the results were identical to the hindlimb stride length ratios.

Hindlimb stride length ratios for C57BL/6J mice revealed no significant differences in stride length, evidenced by ratios of approximately one at all time points. The hindlimb stride length ratios for the B10.Q mice are consistent with the results above in that they reveal a statistically significant decrease in stride at 94 d.p.i. compared to baseline (0 d.p.i.) measurements. In contrast, ratios for SJL/J mice resulted in detection of decreased hindlimb stride length as early as 23 d.p.i.. Decreased stride length was also detected at 50 d.p.i.; however, this was not statistically different from the 23 d.p.i. measurements. A decrease in stride length was observed at 94 d.p.i., and this was statistically reduced from the 23 and 50 d.p.i. measurements. The deficits observed in SJL/J mice at 94 d.p.i. were comparable to those observed in B10.Q mice at the same time point.

Progressive Demyelination in Susceptible Mice

A number of factors can theoretically contribute to the progression of neurologic deficits in susceptible B10.Q and SJL/J mice during the chronic stages of disease. These include persistent virus replication, chronic inflammation, and morphologic changes in the CNS

white matter. One obvious factor is the progressive demyelination that occurs in the CNS of these mice. To document the increasing lesion size in susceptible mice, we examined the spinal cord white matter in a separate group of C57BL/6J, B10.Q, and SJL/J mice at several time points postinfection (Fig. 5). As was expected, no demyelination was detected in the spinal cord white matter of infected C57BL/6J mice at any time point (Figs. 5A–5C). In contrast, demyelination was detected at 17 d.p.i. (Fig. 5D), 45 d.p.i. (Fig. 5E), and 114 d.p.i. (Fig. 5F) in B10.Q mice. The demyelinating lesions increased in size from 17 to 114 d.p.i. SJL/J mice also showed small focal lesions as early as 15 d.p.i. (Fig. 5G). These lesions became significantly larger at 45 d.p.i. (Fig. 5H) and 100 d.p.i. (Fig. 5I).

DISCUSSION

In this study we demonstrated several methods to quantitatively assess disease progression in two strains of mice susceptible to TMEV-induced demyelination. Each of the assays provided information pertaining to the neurologic function of susceptible mice at various time points postinfection. In addition, the sensitive methodology determined that nearly 90% the susceptible mice were symptomatic at a time point (i.e., 90 d.p.i.) when subjective clinical inspection determined that approximately 12% of the mice were affected (8). We believe that the methods are reproducible and can be used to routinely determine a profile of function in rodents with a wide variety of neurologic disorders. As larger scale studies would become labor-intensive if all three assays were used to assess neurologic function, we recommend that one assay, such as rotarod or footprint analysis, be selected to limit the time investment. Because mice infected with TMEV progress from having minor disruptions in gait to profound neurologic dysfunction (i.e., spasticity and paralysis), each of these assays allow an objective characterization of disease progression.

While functional assays, such as the rotarod, have classically been utilized in pharmacological studies to demonstrate the effects of sedation on motor coordination, an emerging body of literature indicates that these assays are also useful in detecting deficits in models of neurologic damage where some component of motor function is disrupted (9–13, 15–17). For example, the spinal cord injury field has gone to great efforts to objectively characterize degrees of functional recovery following CNS damage and to correlate this functional recovery with pathological observations (19, 20). In contrast, very little is known of how the pathological changes observed in a multifocal demyelinating disease of the CNS contribute to neurologic deficits. Therefore, the results described in this study define objective criteria for future research designed to determine specific factors that lead to neurologic deficits in demyelinating disease. It is possible that a chronic immune response directed against a persistent viral infection in the CNS could alter functional performance through systemic effects. This could affect performance on the rotarod or measures of spontaneous activity and gait. However, the hindlimb gait abnormalities (including spasticity and paralysis) commonly observed in chronically infected susceptible strains of mice suggest that the neurologic deficits detected by the functional assays result primarily from the underlying CNS pathology. Additionally, it is possible to dissect out the systemic effects by correlating individual functional scores (using rotarod or footprint analysis) with sensitive indicators of CNS pathology. For example, a strong correlation between rotarod

performance and a sensitive measure of CNS demyelination would demonstrate that the amount of CNS demyelination determines the severity of motor dysfunction.

Three independent assays of neurologic function were used in this study to characterize disease progression in TMEV-infected C57BL/6J, B10.Q, and SJL/J mice. The results documented no detectable signs of neurologic deficit in resistant C57BL/6J mice, while susceptible B10.Q and SJL/J mice showed a progressive decrease in neurologic function over time. The study also demonstrated that the onset of the progressive disease was unique in B10.Q and SJL/J mice. Deficits were revealed as early as 24 d.p.i. in SJL/J, whereas B10.Q mice did not show deficits until 48 d.p.i. These results indicate that the onset of neurologic impairments may be induced in the acute phase of the disease for SJL/J mice, while in the chronic phase of the disease for B10.Q mice.

Variations in disease progression were also observed in B10.Q and SJL/J mice. Rotarod performances revealed that a smaller percentage of B10.Q mice (3 of 10) had deficits early in the course of disease (24 d.p.i.), whereas most SJL/J mice (7 of 10) were affected by this time point. By 94 days postinfection, nearly all B10.Q and SJL/J showed clinical signs using the three independent assays, and the severity of these deficits were comparable in both strains. The largest variation in disease progression was observed between 23 and 94 d.p.i. B10.Q mice did not show decreases in stride until 94 d.p.i., while SJL/J revealed deficits at 23 d.p.i. The trend for decreased stride length in SJL/J mice was identical to the trend for decreased rotarod performance. This was not the case for B10.Q mice. Alterations in stride were only observed at 94 d.p.i., while decreases in rotarod performance were observed at 48 d.p.i. Additionally, three deaths occurred during the course of B10.Q infection, while no deaths were observed for SJL/J mice.

Collectively, these results indicate that the functional assays utilized were very sensitive in detecting differences in disease progression between the two susceptible strains of mice. These results also indicate that the underlying factors contributing to a variable presentation of neurologic deficits can be determined by further analysis of these two strains. We have confirmed that demyelination is progressive in both SJL/J and B10.Q with both strains showing small, focal demyelinating lesions as early as 15 d.p.i. It is possible that these focal demyelinating lesions contribute to the early deficits in SJL/J mice, yet this possibility may not be the only explanation considering the exceptionally small lesion size and the fact that an increased lesion size at 45 d.p.i. did not result in increased deficits. Additionally, B10.Q mice also had similar lesions at 15 d.p.i., but no detectable neurologic deficits. An alternative hypothesis is that the early deficits observed in SJL/J mice resulted from the acute neuronal infection in the brain and spinal cord gray matter.

In addition to demyelination, it is also possible that secondary injury to axons contributes to the neurologic deficits observed at the later time points assessed in the time course. We have previously shown significant axonal degeneration at 270 d.p.i. in SJL/J mice (9). Therefore, it is possible that signs of axonal degeneration exist at early stages in the disease. A recent study showed that axonal damage was present in proteolipid protein-deficient mice at a time point when deficits were detected using the rotarod assay (13). It is also becoming widely accepted that axonal damage contributes to the permanent disability observed in MS patients

(21, 22). Each of these findings supports the hypothesis that axonal degeneration following demyelination may result in a permanent motor dysfunction in susceptible strains of mice infected with TMEV.

Using the sensitive quantitative assays described in this study, we now have the unique ability to determine which factors (i.e., axonal loss, severity and location of the demyelinating plaques) are most important in causing neurologic deficits in susceptible strains of mice. Detailed studies are underway to correlate functional performance with underlying immune-mediated pathologic abnormalities and to determine if the heterogeneity in disease progression coincides with any of the aforementioned parameters. More specifically, the relative contributions of axonal damage and demyelination to motor deficits will be determined by calculating both axonal area distributions and percentages of spinal cord demyelination in animals with varying degrees of neurologic dysfunction. As susceptible mice at any given time point postinfection do not necessarily suffer from the same degree of neurologic impairment, this variability can be utilized to determine which pathologic variable (i.e., axonal loss or demyelination) impairs one mouse more than another. We also have the ability to utilize these assays to assess the benefit of potential treatment strategies that limit demyelination and axonal damage or enhance the natural reparative process of remyelination.

MATERIALS AND METHODS

Mice

C57BL/6J ($n = 20$) (prototypic resistant strain), B10.Q ($n = 19$), and SJL/J ($n = 20$) (prototypic susceptible strain) were purchased from The Jackson Laboratories (Bar Harbor, ME) and tested in the functional assays. Mice at 8 weeks of age were injected intracerebrally with 2×10^6 PFU ($n = 10$ mice per strain) of the Daniel's strain of TMEV in a 10 μ l volume. Sham, age-matched controls ($n = 9$ – 10 mice per strain) were intracerebrally injected with 10 μ l of PBS. Following injection, mice were individually monitored at multiple time points using the three independent functional assays.

Spontaneous activity monitoring

We have previously used the Digiscan activity monitoring system (Omnitech Electronics; Columbus, OH) for the detection of clinical deficits in mice (9). The apparatus consists of an acrylic cage ($40 \times 40 \times 30.5$ cm) supported by a metal frame that holds two sets of photocells. The device measures the number of discrete horizontal and vertical movements by tabulating the number of projected infrared beam interruptions and was used following rotarod/footprint analysis. At the first time range assessed, six infected mice were randomly selected and compared to six age-matched, sham-infected controls for each strain. Because only two activity boxes were available, individual assays consisted of placing three sham-infected and three infected mice in separate activity boxes for 80 consecutive hours exposed to identical environmental conditions: (a) freely accessible food and water, (b) a normal 12-h light/dark cycle, (c) 70°F ambient temperature. This was done a second time with a different pairing to allow a total of six sham-infected and six infected mice to be analyzed per strain. These pairings were kept constant for subsequent time range assessments. The broad time

ranges shown in Fig. 1 represent the number of days needed to analyze all strains (C57BL/6, B10.Q, and SJL/J) of mice using only two activity monitoring boxes. This experimental design controlled for all confounding variables, which included age, intracerebral injection, and environmental factors. Means and standard errors for horizontal and vertical nocturnal activities were calculated by averaging the hourly infrared beam interruptions from 6 PM to 5 AM over the 3-day analysis period. Ratios of horizontal nocturnal activities (infected/sham-infected) were calculated by dividing the mean nocturnal horizontal activities for the infected animals by the mean nocturnal horizontal activity for the age-matched, sham-infected controls. The same calculation was done to determine vertical activity ratios. Statistical differences between mean activities for infected and sham-infected controls were calculated using the Student *t* test. Interstrain comparisons between horizontal and vertical nocturnal activity ratio data were made using an ANOVA. Pairwise comparisons were made using the Student–Neuman–Keuls method ($P<0.05$).

Rotarod analysis

The Rotamex rotarod (Columbus Instruments; Columbus, OH) is a device used to assess balance, coordination, and motor control. The apparatus consists of a suspended rod powered by a variable speed motor capable of running at a constant velocity (fixed-speed rotarod) or a constant accelerated rate (accelerating rotarod). The rod is suspended 28.5 cm over a shock grid, which is set at 0.5 mA during the training period. The rotarod is capable of monitoring the performance of four mice at the same time through the use of lane dividers. Automation of the system is achieved through the use of a computer that records the velocity of the rod and the time at which a mouse falls and interrupts an infrared beam. All mice were exposed to 3 days of training prior to the start of the time course. This was done to thoroughly familiarize all animals with the apparatus. The training protocol consisted of three trials (each separated by 3–4 min) per day at the following speeds: Day 1—12 rpm for 3 min; Day 2—13 rpm for 3 min; Day 3—14 rpm for 3 min. This was followed by the collection of preinjection baseline data for all animals on days 4 and 5. Day 4 measurements consisted of three trials at 14 rpm for 3 min. All animals were able to complete this assay without falling. Day 5 measurements were used as the experimental baseline and involved testing performance on an accelerated (10 rpm/min) rotarod assay (start speed, 10 rpm; end speed, 70 rpm; time, 6 min; No. of trials, 3). The rpm at the time of fall was collected for all mice during each of the three trials and then averaged. This was used as the baseline for individual mice. Following the collection of baseline data, mice were injected with either PBS ($n = 9$ to 10 mice per strain) or TMEV ($n = 10$ mice per strain). Collection of experimental data at various time points postinfection consisted of measurement by a constant speed assay on Day 1 (14 rpm for 3 min; No. of trials, 3) followed by an accelerated assay on Day 2 (start speed, 10 rpm; end speed, 70 rpm; time, 6 min; No. of trials, 3). At all time points the constant speed assay was used as the familiarization assay and the accelerated speed assay was used as the experimental assay. Mean experimental data for all groups at various time points were calculated by first subtracting the start speed (10 rpm) from all individual accelerated measurements. The percentage preserved from the baseline-accelerated performances was then calculated for all mice individually to control for baseline differences in the ability of mice. These percentages were then averaged for each experimental group and used to generate standard errors. Ratios

of infected/sham-infected accelerated measurements were calculated by dividing the infected-accelerated performances by the mean sham-infected performance at a given time point. Statistical differences between sham-infected and infected data at all time points were calculated by using a two-way repeated measures ANOVA. Statistical differences between ratio data were calculated using an ANOVA. Pairwise comparisons for both were made using the Student–Newman–Keuls method ($P<0.05$).

Footprint analysis

Footprints were analyzed at pre- and postinjection time points without prior training. The forelimb and hindlimb paws of mice were painted with red and blue nontoxic, washable activity paint (RoseArt Industries; Livingston, NJ), respectively. Mice were then placed at the start of a plexiglass-defined walkway (90.5 cm long, 6.2 cm wide, and 22.6 cm high) and expected to walk along a strip of standard white paper. Prints were then digitized using a Hewlett Packard color scanner (ScanJet 4c) and analyzed using a program written for the KS400 image analysis software (Kontron Elektronik GmbH, Munich) on a pentium platform. Forelimb and hindlimb length and width of stride were obtained from a minimum of six steps per mouse by centering the opposite corners of a box on two consecutive forelimb or hindlimb prints (see Fig. 3). The computer program automatically calculated the distances using the length and width of the boxes shown in Fig. 3. Preinjection measurements were collected for all mice and used as the baseline. Mean data were calculated by first subtracting the baseline of each mouse from its measurements at the various time points postinjection. These changes from baseline () were then averaged and used to generate standard errors. Ratios of infected/sham-infected measurements were calculated by dividing the infected distances by the mean sham-infected distance at each time point. To calculate statistical differences, a one-way repeated measures ANOVA was used to compare the groups at various time points to their baseline measurements. Statistical comparisons between ratio data were performed using an ANOVA. Pairwise comparisons for both were done using the Student–Newman–Keuls method ($P<0.05$).

Analysis of spinal cord demyelination

A separate group of mice (not used in the functional assays) was anesthetized with 10 mg of pentobarbital (ip) and perfused via intracardiac puncture with Trump's fixative (phosphate-buffered 4% formaldehyde with 1% glutaraldehyde, pH 7.2). The spinal cord was then removed, sectioned coronally into 1-mm blocks, post-fixed with osmium tetroxide, and embedded in Araldite (Polysciences; Warrington, PA). One-micrometer-thick cross-sections were cut from 10 blocks and stained with 4% paraphenylenediamine. Photography was performed using an Olympus AX70 scope (4× objective) fitted with a SPOT cooled color digital camera.

Acknowledgments

This work was supported by the National Institutes of Health Grants RO1 NS24180 and RO1 NS32129 and the generous contributions of Mr. and Mrs. Eugene Applebaum. D.B.M. is supported by a predoctoral NRSA from the National Institute of Mental Health (Grant 1F31ME12120). K.M.D. is a fellow of the National Multiple Sclerosis Society.

References

1. Adams, RD., Victor, M., Ropper, AH. Multiple sclerosis and allied demyelinating diseases. In: Wonsiewicz, MJ., Navrozov, M., editors. Principles of Neurology. McGraw-Hill; New York: 1998. p. 902-927.
2. Lipton HL. Theiler's virus infection in mice: An unusual biphasic disease leading to demyelination. Infect Immun. 1975; 11:1147-1155. [PubMed: 164412]
3. Rodriguez M, Leibowitz J, David C. Susceptibility to Theiler's virus-induced demyelination: Mapping of the gene within the H-2D region. J Exp Med. 1986; 163:620-631. [PubMed: 3005466]
4. Rodriguez M, David C. Demyelination induced by Theiler's virus: Influence of the H-2 haplotype. J Immunol. 1985; 135:2145-2148. [PubMed: 2991380]
5. Clatch RJ, Melvold RW, Miller DH, Lipton HL. Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease in mice is influenced by the H-2D region: Correlation with TMEV-specific delayed-type hypersensitivity. J Immunol. 1985; 135:1408-1414. [PubMed: 3925009]
6. Dal Canto MC, Lipton HL. Multiple sclerosis—Animal model: Theiler's virus infection in mice. Am J Pathol. 1977; 88:497-500. [PubMed: 195474]
7. Dal Canto MC, Lipton HL. Recurrent demyelination in chronic central nervous system infection produced by Theiler's murine encephalomyelitis virus. J Neurol Sci. 1979; 42:391-405. [PubMed: 512673]
8. Murray PD, McGavern DB, Lin X, Njenga MK, Leibowitz J, Pease LR, Rodriguez M. Perforin-dependent neurologic injury in a viral model of multiple sclerosis. J Neurosci. 1998; 18:7306-7314. [PubMed: 9736651]
9. Rivera-Quinones C, McGavern D, Schmelzer JD, Hunter SF, Low PA, Rodriguez M. Absence of neurological deficits following extensive demyelination in a class I-deficient murine model of multiple sclerosis. Nature Med. 1998; 4:187-193. [PubMed: 9461192]
10. Kuhn PL, Petroulakis E, Zazanis GA, McKinnon RD. Motor function analysis of myelin mutant mice using a rotarod. Int J Dev Neurosci. 1995; 13:715-722. [PubMed: 8787862]
11. Rozas G, Labandeira Garcia JL. Drug-free evaluation of rat models of parkinsonism and nigral grafts using a new automated rotarod test. Brain Res. 1997; 749:188-199. [PubMed: 9138718]
12. Klugmann M, Schwab MH, Puhlhofer A, Schneider A, Zimmermann F, Griffiths IR, Nave K. Assembly of CNS myelin in the absence of proteolipid protein. Neuron. 1997; 18:59-70. [PubMed: 9010205]
13. Griffiths IR, Klugmann M, Anderson T, Yool D, Thomson C, Schwab MH, Schneider A, Zimmerman HM, McCulloch M, Nadon N, Nave K. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. Science. 1998; 280:1610-1613. [PubMed: 9616125]
14. Klapdor K, Dulfer BG, Hammann A, Josef van der Staay F. A low-cost method to analyse footprint patterns. J Neurosci Methods. 1997; 75:49-54. [PubMed: 9262143]
15. Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME. Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature. 1995; 378:498-501. [PubMed: 7477407]
16. Wietholter H, Eckert S, Stevens A. Measurement of atactic and paretic gait in neuropathies of rats based on analysis of walking tracks. J Neurosci Methods. 1990; 32:199-205. [PubMed: 2385137]
17. Medinaceli L, Freed WJ, Wyatt RJ. An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp Neurol. 1982; 77:634-643. [PubMed: 7117467]
18. Lipton HL, dal Canto MC. Chronic neurologic disease in Theiler's virus infection of SJL/J mice. J Neurol Sci. 1976; 30:201-207. [PubMed: 978224]
19. Kunkel-Bagden E, Dai HN, Bregman BS. Methods to assess the development and recovery of locomotor function after spinal cord injury in rats. Exp Neurol. 1993; 119:153-164. [PubMed: 8432357]
20. Fehlings MG, Tator CH. The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. Exp Neurol. 1995; 132:220-228. [PubMed: 7789460]

21. Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. *Brain*. 1997; 120:393–399. [PubMed: 9126051]
22. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med*. 1998; 338:278–285. [PubMed: 9445407]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIG. 1.

Measurement of horizontal (A, C, E, G) and vertical (B, D, F, H) nocturnal spontaneous activity following TMEV infection. (A, B) No detectable decreases in horizontal (A) or vertical (B) activity were observed for 55- to 71-day infected or 98- to 128-day infected C57BL/6J mice when compared to age-matched, sham-infected controls. (C, D) Analysis of susceptible B10.Q mice revealed a statistically significant decrease in horizontal activity (C) for 98- to 128-day infected mice. Additionally, a marked decrease in vertical activity (D) was observed for 55- to 71-day infected and 98- to 128-day infected B10.Q mice. (E, F) By comparison, analysis of the susceptible SJL/J mice revealed significant decreases in horizontal (E) and vertical (F) activity for 55- to 71-day infected and 98- to 128-day infected mice. (G, H) Ratios of infected/uninfected (sham) horizontal (G) and vertical (H) nocturnal spontaneous activities were calculated to allow for interstrain comparisons. A ratio approximately equal to one signifies no difference between the infected group and the

respective sham-infected controls. Horizontal activity ratios (G) at 55–71 and 98–128 days were reduced in both B10.Q and SJL/J mice when compared C57BL/6J mice. Vertical activity ratios (H) were also markedly reduced in B10.Q and SJL/J mice at 55–71 and 98–128 days when compared to C57BL/6J mice. (Asterisks are used to denote statistical significance. Infected mice were compared to their respective sham-infected controls for A–F. Susceptible strains of mice were compared to C57BL/6 mice for G and H.) (Bars, mean $\pm$ standard error after analysis of six mice per group.)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIG. 2.

Assessment of motor coordination following TMEV infection using the accelerated rotarod assay. The baseline performance in all animals is designated as 100%. (A) Analysis of rotarod performance between infected and age-matched, sham-infected C57BL/6J at 24, 48, and 93 d.p.i. revealed no significant decreases for the infected group. (B) In contrast, deficits were detected for infected B10.Q mice at 48 and 93 d.p.i., but not at 24 d.p.i. (C) Decreased rotarod performances were also observed at 24, 48, and 93 d.p.i. for infected SJL/J mice. (D) Ratios of infected/uninfected (sham) rotarod performances were calculated for all strains to allow interstrain comparisons. Ratios equivalent to one or greater signify that there is no difference between the infected group and the respective sham-infected controls. Results indicate that both B10.Q and SJL/J mice had significant reductions in rotarod performance at 48 and 93 d.p.i. when compared to C57BL/6J mice, but only SJL/J mice showed differences at 24 d.p.i. (Bars, the mean $\pm$ standard error after analysis of 9–10 mice per group.) (E, F) Individual rotarod performances are shown for B10.Q (E) and SJL/J (F) mice to demonstrate the heterogeneity of disease progression during the time course. (Asterisks are used to denote statistical significance. Infected mice were compared to their respective sham-

infected controls for A–C. Susceptible strains of mice were compared to C57BL/6 mice for D.)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIG. 3. Methodology used for footprint analysis. The parameters assessed in all strains of mice included forelimb stride length (a) and width (b) and hindlimb stride length (c) and width (d). Gray (originally red) is used to designate the forelimb prints, while black (originally blue) is used to designate the hindlimb prints.

FIG. 4.

Measurement of forelimb and hindlimb stride length following TMEV infection.

(A) Analysis of forelimb and hindlimb stride length in C57BL/6J mice revealed no significant stride decreases for infected mice when compared to sham-infected controls. Data are presented as the change () from baseline measurements. (B) In contrast, a significant decrease in forelimb and hindlimb stride length was detected in 94-day infected B10.Q mice when compared to baseline measurements. This trend was not detected in sham-infected controls. (C) Decreases in forelimb and hindlimb length were also detected in 94-day infected SJL/J mice when compared to baseline measurements. (D) Ratios of infected/uninfected (sham) hindlimb stride lengths were calculated to remove the factors of age and injection from the infected group. Ratios equivalent to or greater than one signify no difference between the infected group and the respective sham-infected controls. Infected C57BL/6J mice demonstrated no significant alterations in hindlimb stride length at any of the time points assessed. In contrast, infected B10.Q mice demonstrated alterations in stride length at 94 d.p.i., while infected SJL/J mice demonstrated alterations in stride length at 23, 50, and 94 d.p.i. (Bars, the mean $\pm$ standard error after analysis of 9–10 mice per group.

Asterisks are used to denote statistical significance. All mice were compared to their baseline measurements for A–D.)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIG. 5.

Progressive demyelination in susceptible strains of mice. Demyelination was not detected in infected C57BL/6J mice at 24 d.p.i. (A), 45 d.p.i. (B), or 100 d.p.i. (C). However, demyelination was detected in infected B10.Q mice at 17 d.p.i. (D, black arrows), 45 d.p.i. (E), and 114 d.p.i. (F). Additionally, the lesions at 17 d.p.i. (D) were small and focal and progressively increased in size at 45 d.p.i. (E) and 114 d.p.i. (F). Small focal demyelinating lesions (black arrows) were also detected at 15 d.p.i. (G) in infected SJL/J mice. These lesions became progressively larger at 45 d.p.i. (H) and 100 d.p.i. (I).