Video Article

Measuring Progressive Neurological Disability in a Mouse Model of Multiple Sclerosis

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Abstract

After intracerebral infection with the Theiler's Murine Encephalomyelitis Virus (TMEV), susceptible SJL mice develop a chronic-progressive demyelinating disease, with clinical features similar to the progressive forms of multiple sclerosis (MS). The mice show progressive disability with loss of motor and sensory functions, which can be assessed with multiple apparatuses and protocols. Among them, the Rotarod performance test is a very common behavioral test, its advantage being that it provides objective measurements, but it is often used assuming that it is straightforward and simple. In contrast to visual scoring systems used in some models of MS, which are highly subjective, the Rotarod test generates an objective, measurable, continuous variable (*i.e.,* length of time), allowing almost perfect inter-rater concordances. However, inter-laboratory reliability is only achieved if the various testing parameters are replicated. In this manuscript, recommendations of specific testing parameters, such as size, speed, and acceleration of the rod; amount of training given to the animals; and data processing, are presented for the Rotarod test.

Video Link

The video component of this article can be found at https://www.jove.com/video/54616/

Introduction

Theiler's Murine Encephalomyelitis Virus (TMEV) is a neurotropic single-stranded RNA virus that persistently infects the murine central nervous system (CNS). In susceptible mice, infection with TMEV causes an immune-mediated, chronic-progressive demyelinating disease, known as TMEV-induced demyelinating disease (TMEV-IDD). Experimental infection of mice takes a disease course resembling that seen in progressive forms of multiple sclerosis (MS). TMEV-IDD is characterized by two distinct phases: the acute phase and the chronic phase. The acute phase is a mild, usually subclinical encephalitis[1,2]. The second, chronic phase, beginning about a month after infection, consists of a slowly progressing disability characterized by demyelination, inflammation, and axonal damage[1,2]. The weakness observed in mice is associated with spasticity and, occasionally, severe tonic spasms.

Because there are currently no medications to ameliorate the progressive disability in patients, researchers are particularly attracted by TMEV-IDD, which represents an optimal animal model for monitoring the impact of disease-modifying drugs on disease progression. However, in mice as well as in MS patients, the monitoring of disability progression requires a continuous clinical observation over extended periods of time. In mice, long-term monitoring for disability progression can be accomplished with the Rotarod performance test.

The Rotarod performance test is a standard behavioral test that evaluates motor-associated functions such as coordination, balance, and fatigue in rodents. The mice have to keep their balance on a turning rod, which is rotating under continuous acceleration; the time latency to fall from this rod is recorded. Animals with neurological dysfunction are unable to stay on the rotating rod as long as controls, and they normally drop off when the rotation speed exceeds their motor capacity. The more neurological impairment the animals have, the sooner they fall off the rod, and the shorter the time latency is.

The advantage of the Rotarod test over the traditional visual scoring systems is that it generates an objective, measurable variable-the time latency-which can ultimately be used for statistical analyses to quantify the effects of therapies and experimental procedures[3].

In the Laboratory of Neuroimmunology (LONI) at Dartmouth, mice are subjected to an adaptation protocol, where they are tested prior to TMEV infection in order to familiarize them with the machine and to assess their normal "baseline" balance coordination and motor control[4,5]. Once the baseline is established and the mice are infected with TMEV, they are tested once or twice a week over a period of several months. The actual testing protocol lasts an average of 150 days, thus allowing an assessment of the decline of balance, coordination, and motor control over the entire course of the demyelinating disease.

Several hundred TMEV-IDD and sham-treated mice have been tested so far for neurological dysfunction at Dartmouth. These mice had received various immunomodulatory treatments, but no pharmacological agent has been found to be effective in ameliorating disability progression[6,7].

The present article and the related protocol describe how to characterize the progressive neurological impairment displayed by TMEV-IDD mice. Particularly, the protocol offers recommendations of specific testing parameters believed to be generally suitable for studying neurological disability in TMEV-IDD mice using the Rotarod test. This procedure provides a baseline against which to assess (1) the relevance of this mouse model to progressive MS and (2) its usefulness for testing therapies aimed at treating progressive neurological conditions such as MS. Clearly, the Rotarod performance test and the current optimized testing parameters and protocol are not only useful at detecting progressive neurological disability in the TMEV-IDD mouse model, but are also useful in uncovering impairments in other virus-induced and/or genetic mouse models of neurodegenerative diseases.

Protocol

All animal work utilizes protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Geisel School of Medicine at Dartmouth.

1. The Mouse Model

1. **Induction of TMEV-Induced Demyelinating Disease**
    1. Move the cages containing 4- to 6-week-old female SJL/JHan mice from the rack to a comfortable working space. Mark the mice (*e.g.,* with an ear tag or ear punch) to allow for individual evaluation of clinical and histological disease.
    2. Draw 30 µl of TMEV infecting stock (2 x 10^6 plaque forming units; PFU) in PBS into a 29-gauge insulin syringe and needle.
    3. Prepare the anesthesia gas machine: check the system to ensure the presence of adequate amounts of oxygen and isoflurane for the duration of the procedure.
    4. Turn on the flow meter to 1 L/min. Place the animal into the induction chamber and seal the top. Turn on the vaporizer to 3.5% and monitor the animal until recumbent.
    5. Remove the animal from the chamber and test the mouse by pinching the footpad to ensure adequate anesthesia. Lack of response to a strong pinch indicates adequate anesthesia.
    6. Clean the injection site with 70% isopropyl alcohol.
    7. Inject the 30 µl of TMEV infecting stock into the right cerebral hemisphere by freehand injection (**Figure 1**). The site of injection is approximately halfway between the eye and ear line and just off the midline.
    8. Return the mouse to its holding cage once fully alert and mobile (usually 3 - 5 min).
    9. Euthanize mice by exsanguination or cardiac perfusion 3 to 6 months after TMEV infection, depending on the rapidity of development of the disease.

2. Rotarod Analysis

1. **The Rotarod Apparatus**
    1. Test mice prior to TMEV infection to familiarize them with the machine and to assess their normal baseline balance coordination and motor control.
    2. Start the adaptation protocol on -5 days post infection (dpi; *i.e.,* 5 days before TMEV infection).
    3. Allow the mice to acclimate to the testing room for at least 30 min prior to Rotarod testing, in order to allow them to adjust to the environment.
    4. Make sure that both the Rotarod unit and the computer are plugged in and connected to each other (**Figure 2**).
    5. Pre-set the Rotarod with the -5 dpi training protocol parameters, as described in **Table 1**.
    6. Save the work file with the date and identification information.
    7. Move the cage containing the squad to be tested from the rack to a table adjacent to the Rotarod. Mice are usually tested in squads of 4.
    8. Pick up a mouse by the tail and place it on the rod, facing away from the operator. Repeat for the second through the fourth mouse. If a mouse falls or jumps, place it back in its lane on the Rotarod until all mice are in position. Ignore if any mice turn around to face the operator.
    9. After loading all the mice, press the "Enter" button to start the experiment. Observe the timers start automatically and the rotations per min (rpm) on display for each lane.
        1. As each animal falls from the rod, record the speed of the rod at the time of the fall, as well as the duration of time the animal remained on the rod. The rod will continue to rotate until the last animal has fallen from the rod assembly.
    10. After all the mice have fallen, use a tissue to remove any fecal boli and urine from the rod. The presence of urine and fecal material may affect the ability of mice to grip the rod.
        1. After a 3-min rest, give the mice a second and then a third trial. The maximum time per single trial is 240 sec. Administer a total of 3 trials during each testing day.
    11. Return the mice to their home cage and return them back to the rack. At the end of the experimental session, clean the Rotarod with soap and water to remove all fecal matter off the machine.
    12. Wipe clean the baseplate with ethanol 70%. Spray down the whole machine with chlorine dioxide to disinfect.
    13. On days - 4, - 3, - 2, and - 1 pi, pre-set the Rotarod with the appropriate training protocol parameters, as described in **Table 1**, and repeat steps 2.1.2 to 2.1.12.
    14. After obtaining the baseline measures, infect the mice with TMEV. Allow a 6-day pi recovery period.

Protocol	Testing Day	Frequency	Starting speed (rpm)	Max Speed (rpm)	Acceleration (rpm/sec)	Trials (N x sec)	ITI (min)
Training	- 5 d.p.i	1/day	1	12	01/03	3x240 sec	3
	- 4 d.p.i	1/day	1	13	01/03	3x240 sec	3
	- 3 d.p.i	1/day	1	14	01/03	3x240 sec	3
	- 2; - 1 d.p.i	1/day	5	40	01/03	3x240 sec	3
Experimental	From +7 to +50 d.p.i	2/week	5	40	05/30	3x240 sec	3
	From +51 to +150 d.p.i	1/week	5	40	05/30	3x240 sec	3

Table 1: Rotarod Parameters in Training and Experimental Protocols.

2. **The Rotarod Experimental Protocol**
    1. On +7 dpi, pre-set the Rotarod with the appropriate experimental protocol parameters, as described in **Table 1**. Repeat steps 2.1.2 to 2.1.10.
    2. At the end of trial #3, weigh each mouse and make a note of the body weight on the data sheet. Clean and disinfect the Rotarod as per steps 2.1.11 and 2.1.12.
    3. Test the mice twice a week for the following 6 weeks, as described above. After 6 weeks (in which the mice have likely reached a plateau phase)[8,9], test the mice once a week with the same experimental protocol. The actual testing protocol lasts an average of 150 days, depending on the specific disease course.

3. **Neurological Functional Index**
    1. Export the raw data into a spreadsheet file and analyze the results.
    2. Express data as running time (**Figure 3A**): this is the normal running time plus the passive rotation time minus the rotation delay time (**Table 2**)[10]. Calculate the mean running time of the three trials per day.
    3. Express the data as a neurological functional index (NFI; **Figure 3B**).
        1. Calculate the baseline performance threshold of each individual mouse. The baseline performance threshold is determined as the mean of all running times from day + 15 to + 45 pi[6,7].
        2. Calculate the NFI as the mean of the three most recent average running times divided by the baseline performance threshold of that specific mouse[6,7].
        NOTE: If the tested running times for a mouse on day + 72, + 76, and + 79 pi are 55 sec, 45 sec, and 50 sec, and the baseline time for the same mouse was 135 sec, the NFI for that mouse on +79 dpi will be [(45+50+55)/3]/135 or 0.37.

    4. Express the data as an adjusted NFI (adjNFI; **Figure 3C**): adjust the NFI data by a population value for the single experiment.
        1. Calculate the adjNFI by dividing the NFI value by the average NFI obtained by the sham-treated group on that specific day.

Term	Definition
Normal running time	The total time the mouse spends actively running on the rotating rod, *i.e.*, latency to fall.
Passive rotation time	The amount of time the mouse has remained on the rod in the passive rotation mode.
Rotation delay time	The amount of time the mouse remains on the rod during the passive rotation mode
Passive rotation mode	When the mouse grabs the rod and rotates without having to ambulate.
Total session time	Total amount of time the mouse remains on the rotating rod during the session.
Baseline performance	Pre-damage motor performance assessed to determine the minimum performance threshold.
Neurological function index (NFI)	Clinical index, which compares each mouse motor performance, *i.e.,* running time, at any time to its peak performance.
Adjusted neurological function index (adjNFI)	When a normalization process is applied to adjust NFI data by a population value for the single experiment.
Population value	Average NFI value obtained by the sham-treated group at a specific day.

Table 2: Definitions of Rotarod Parameters Adopted to Quantify Neurological Impairment.

Representative Results

The aim of this representative experiment was to compare the neurological disability induced by the Daniels (DA) strain and BeAn strain of TMEV. For the purposes of the present study, a group of 32 female SJL mice were infected intracranially with TMEV, either the DA strain (n = 16) or the BeAn strain (n = 16), and their clinical signs were monitored over time. An additional group of 20 mice was sham treated (*i.e.,* saline solution was injected intracranially) and served as healthy control group.

The Rotarod performance test was used to evaluate the chronic disease progression in TMEV-infected mice and sham controls. Mice were individually ear marked and subjected to the adaptation protocol daily in the week prior to injection of TMEV and then to the actual testing protocol that lasted 125 days. For each testing day, the running time, defined as the length of time the mouse stayed on the rod, was stored individually for each mouse as the mean of the 3 trials for that day. Rotarod data is also expressed as NFI and as sham-normalized adjNFI.

As shown in **Figure 3A**, infection with TMEV negatively affected the running times of the mice. A repeated-measures two-way ANOVA revealed a significant effect of TMEV infection ($F = 56.76$, $p < 0.0001$) as well as a significant effect of time ($F = 3.26$, $p < 0.0001$) and a time-treatment interaction ($F = 8.065$, $p < 0.0001$). The following Dunnett's multiple comparisons test revealed that both groups of TMEV-infected mice had significantly lower NFI values than those of sham mice ($p \leq 0.01$; **Figure 3B**). The difference was statistically significant starting at day + 61 pi up to the end of the follow-up (**Figure 3B**). There was no difference comparing disability progression in mice infected with the BeAn strain and those infected with the DA strain at all time points ($p > 0.5$; **Figure 3C**).

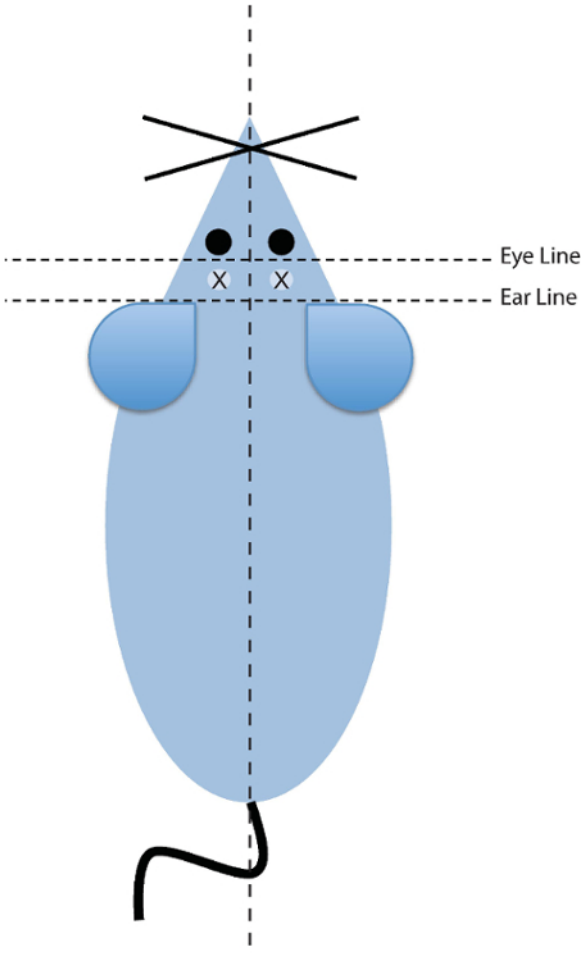

Figure 1: Intracranial Injection. To perform intracranial injections, the mouse is gas-anesthetized and restrained on a solid surface. Up to 30 µl are safely injected into the mouse brain. The site of injection is approximately half way between the eye and ear line and just off the midline. A 30 G needle directly pierces the cranium, and an insulin syringe is conveniently used to prevent the needle from extending too deeply into the brain. Please click here to view a larger version of this figure.

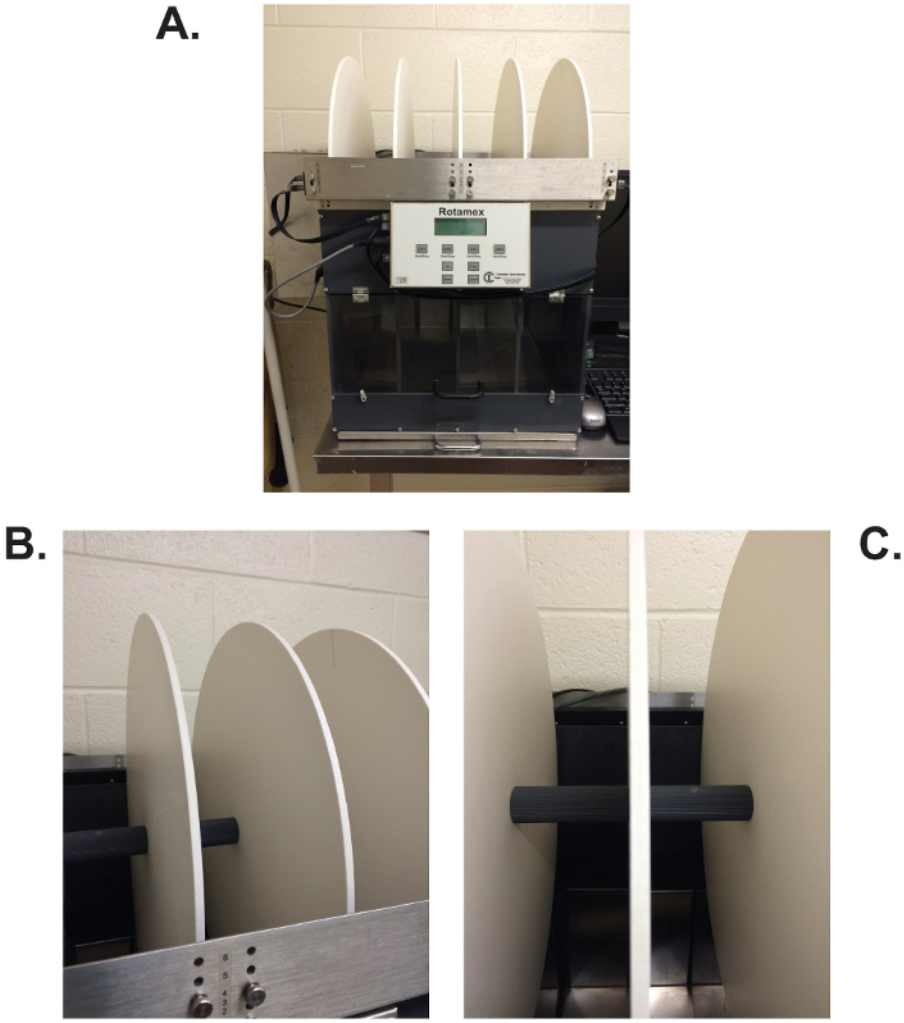

Figure 2: The Rotarod Apparatus. The Rotarod test is a standard neurobehavioral assay in which mice are trained to run on a rotating rod and are timed for how long they can stay on the rod as it slowly speeds up. The rod is suspended above a cage floor, and mice naturally try to stay on the rod to avoid falling to the ground. The drop from the rod to the base of the instrument causes no injury to the mice. **A)** A standard Rotarod apparatus consists of a motor-driven rod with constant or accelerating speeds. **B)** Panels divide the rod into separate lanes, each suited for an individual animal. A total number of 4 mice can be tested during each trial. **C)** The rod is 1.18 in. (about 3 cm) in diameter, and its surface is knurled, allowing the mice to get a better grip. The fall of the mouse is precisely tracked by a photo beam that automatically records the amount of time spent on the rod. The experimental data is recorded in a computer after the mice have all fallen from the rod. Please click here to view a larger version of this figure.

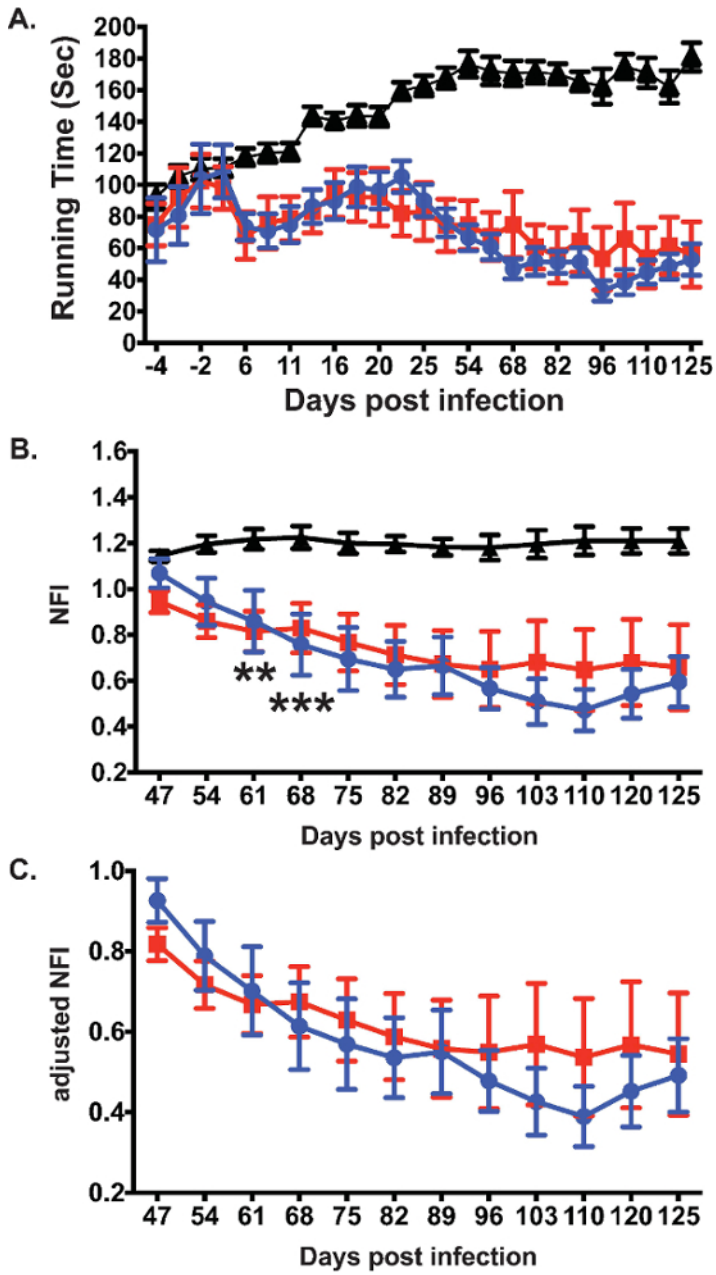

Figure 3: Rotarod Performance Test in TMEV-infected Mice and Sham-controls. The Rotarod test was performed to measure motor function of sham, DA-infected, and BeAn-infected mice. Besides running time (sec) **(A)**, Rotarod data were also expressed as a neurological function index (NFI) **(B)**, and a sham-normalized adjusted NFI (adjNFI) **(C)**. The latency to fall from an accelerating Rotarod was measured in three trials per day. Data are presented as mean ± standard error of the mean (SEM). Blue circle, n = 16 BeAn-infected mice; red squares, n = 16 DA-infected mice; black triangles, n = 20 sham-treated controls. Please click here to view a larger version of this figure.

Discussion

Despite some limitations, the Rotarod performance test represents an important tool for assessing motor function and dysfunction in TMEV-IDD as well as the effect of pharmacological interventions on disability progression in mice.

The Rotarod test was first described in 1957 as a tool for measuring neurological deficits in rodents[11]. Rodents have to walk on a rotating rod, with increasing rotating speed, and try to avoid falling to the ground. The latency to fall is recorded and used as a quantitative end point for motor function: the more neurological impairment the animals have, the sooner they fall off the rod. The clear advantage of this test over the traditional visual scoring systems is that it generates an objective variable that can be used for statistical purposes to quantify the effects of therapies and experimental procedures on disability progression[3]. Visual scoring systems such as the righting reflex test might be preferred as being a simpler and more rapid test to assess locomotor abilities in mice. However, these systems lack the precision of a more quantitative test like the Rotarod performance test, and they should therefore not be used to monitor the natural history or phenotypic changes of mice with mild and progressive

neurological impairment. On the other hand, visual tests like the righting reflex test are particularly adapted to mice that are too young or too affected to be tested on the Rotarod.

In order to avoid distorted assessments of the Rotarod test, the testing parameters (*i.e.,* size, speed, and acceleration of the rotating rod; amount of training given to the animal; and final data processing) must be carefully replicated[3]. Today, the Rotarod performance test is still the most commonly used motor behavioral test, yet there is little consensus on the ideal testing parameters to produce optimal results. We have found studies with TMEV-IDD mice that have used rod diameters from 1.18 to 1.6 in. (3 to 4 cm)[12,13], fixed-speed rates of 5 to 10 rpm[12,13], and acceleration rates of 5 to 10 rpm/min[12-14]. However, it is unknown whether the conclusions about neurological differences would generalize to other test conditions. The present protocol offers recommendations of specific testing parameters that were optimized and are thus suitable for studying progressive neurological disability in the TMEV-IDD mouse model of MS using the Rotarod test. Particularly, before embarking on a Rotarod motor function study in mice, it is recommended that a number of potentially important factors-among others, baseline performance and passive rotation-be considered.

First, regarding baseline performance: exercised and uninfected mice improve their motor performance over time, eventually reaching a stable plateau over several days or weeks[8,9]. This is the result of the normal process of motor learning. However, this improvement is not permanent, and if mice are left untrained for several days, their motor performance on the rotating rod will drop rapidly[9]. Therefore, a baseline performance determination is crucial to differentiate between motor learning and actual recovery after a therapeutic intervention, as well as between a physiological motor performance drop and worsening due to an experimental procedure.

Our optimized Rotarod protocol expects to train TMEV-IDD mice from + 15 to + 45 dpi (*i.e.,* in between the acute encephalitis, which occurs within + 3 to + 12 dpi, and the late chronic demyelinating disease, which develops from + 30 to + 40 dpi)[15]. This allows for the evaluation of motor performance as a result of progressive demyelination and thus excludes any contributing deficits due to the early encephalitis. Ideally, the Rotarod training should be performed prior to disease induction. However, in order to develop a chronic infection of the CNS, SJL mice have to be injected with the virus before their 7th week of age[16]. Since it is generally accepted that the ages of animals that the Rotarod test can be performed on is 8 to 26 weeks[17], an effective training session cannot be completed in mice before TMEV infection.

Training and baseline performance evaluation are also important to exclude the effect of low motivation, low basal performance, and low learning skills. Each animal is different, and these differences need to be considered in a motor performance evaluation. This is best remedied by using a baseline performance normalized index, also known as an NFI. This index also accounts for performance fluctuations that might be observed in mice during different testing sessions. Performance variability is a common issue in behavioral testing, mainly due to factors outside the experimental protocol, such as low motivation and past experiences of the animal. The mean running times for the last three time points of each mouse provides an estimate of the central tendency of the distribution that is expected if performance remains stable. This accounts for day-to-day individual performance variability. Severe neurological disability is then defined by NFI values of less than or equal to 0.3, whereas relatively unaffected mice are scored with NFI values greater than or equal to 0.7. Unfortunately, this strategy does not completely remove the problem that motivational changes might affect motor performance. To further address this problem, mice may be trained with the addition of specific motivating features, such as mild food deprivation prior to training or increased fear of falling caused by adjustable falling heights of 17.5 to 23.5 in. (44.5 to 60 cm).

Second, regarding passive rotations or looping: perhaps the most important threat to the validity of Rotarod data is the occurrence of passive rotations that arise when the mouse can cling on and rotate passively as the rod turns[18]. Although passive rotation is observed on the Rotarod, especially in mice experiencing motor coordination problems, this coping behavior may result in late falling off the rod, which would incorrectly indicate an absence of motor deficits. Therefore, a secondary measure of disturbed motor coordination, which should be considered in the data analysis, is the count of the number of times the mouse turns around the rod without falling off. Also, passive rotation is influenced strongly by task parameters such as the diameter, speed and acceleration degree of the rod[18]. There are no perfect solutions for reducing the risk of passive rotation, but a possible resolution may be the use of a rod with a larger diameter (*i.e.,* 1.18 to 3.15 in. (3 to 8 cm)) or less pronounced ridges so as to slightly reduce the grip of the mouse.

A limitation of the Rotarod performance test is that the motor assessment is limited to a short observation time. Moreover, testing at different time points in the light-dark cycle may alter the outcome of behavioral testing[19]. Thus, in order to overcome these issues, several companies are developing innovative home cage analysis systems that provide 24/7 monitoring of rodent behaviors. These monitoring systems surely represent the newest and most exciting technology available in rodent behavioral research, but are still extremely expensive and not always accessible to research laboratories.

In summary, the Rotarod performance test is a relatively simple, inexpensive, and well-characterized test for long-term evaluation of neurological disability in TMEV-IDD, a murine model of progressive MS. The advantage of this test over the traditional clinical symptoms score systems is that it generates an objective variable to quantify the effects of different drugs, conditions, and procedures on motor function. Given the robustness of the Rotarod performance test, by applying a few simple precautions, as described above, this procedure provides a baseline against which to assess (1) the relevance of the TMEV-IDD mouse model to progressive MS and (2) its usefulness for testing therapies aimed at treating progressive neurological conditions such as MS.

Disclosures

The authors have nothing to disclose.

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