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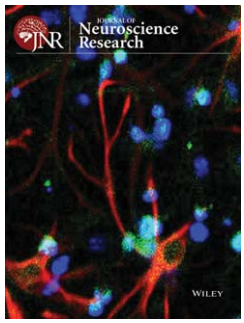
Effects of Exercise in a Relapsing-Remitting Model of Experimental Autoimmune Encephalomyelitis



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Previous research has examined the effects of exercise in experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis. However, all previous studies have utilized a chronic model of EAE, with exercise delivered prior to or immediately after induction of EAE. To our knowledge, no study has examined the effects of exercise delivered during a remission period after initial disease onset in a relapsing-remitting model of EAE (RR-EAE). The current study examines the effects of both voluntary wheel running and forced treadmill exercise on clinical disability and hippocampal brain-derived neurotrophic factor (BDNF) in SJL mice with RR-EAE. The results demonstrate no significant effects of exercise delivered during remission after initial disease onset on clinical disability scores or levels of hippocampal BDNF in mice with RR-EAE. Furthermore, our results demonstrate no significant increase in citrate synthase activity in the gastrocnemius and soleus muscles of mice in the running wheel or treadmill conditions compared with the sedentary condition. These results suggest that the exercise stimuli might have been insufficient to elicit differences in clinical disability or hippocampal BDNF among treatment conditions.

Key words: EAE; exercise; BDNF; RRID:MGI_2663948; RRID:SCR_002865

Multiple sclerosis (MS) is an immune-mediated neurological disease of the central nervous system (CNS). This neurological disease involves periods of inflammation, axonal demyelination and transection, and neurodegeneration within the CNS (Frohman et al., 2006). The extent and location of damage within the CNS result in impairments and symptoms such as muscle spasticity and weakness, walking and balance dysfunction, and reduced quality of life (Lublin, 2005).

An abundance of research from clinical trials demonstrates the safety and beneficial effects of physical activity and exercise training in persons with MS (Motl and Pilutti, 2012; Pilutti et al., 2014). Furthermore, previous research has examined the pathophysiological effects of physical activity and exercise in the animal model of MS, experimental autoimmune encephalomyelitis (EAE; Klaren et al., 2014). EAE is the most commonly used model to study mechanisms of action for treatment effects on MS pathophysiology (Robinson et al., 2014). EAE is a T-helper-cell-mediated autoimmune disease characterized by T-cell and monocyte infiltration in the CNS associated with local inflammation (Robinson et al., 2014). The autoimmune molecular targets identified have been proteins expressed by myelin-producing oligodendrocytes in the CNS, such as proteolipid protein (PLP), myelin basic protein, and myelin oligodendrocyte glycoprotein (MOG), resulting in primary demyelination of axonal tracks, subsequent impaired axonal conduction in the CNS, and progressive hindlimb paralysis (Kuerten and Angelov, 2008; Wekerle, 2008; Robinson et al., 2014).

Previous researchers who have studied the effects of exercise in EAE have used a model similar to the chronic, progressive course of MS. For example, one study identified voluntary wheel running as attenuating clinical disability and synaptic and dendritic defects of EAE in mice with chronic EAE (Rossi et al., 2009). A more recent

SIGNIFICANCE:

The current study examines the effects of both voluntary and forced exercise in SJL mice with relapsing-remitting experimental autoimmune encephalomyelitis. This study is novel in that the exercise treatment conditions were delivered during a remission period after the initial onset of disease; this would be most similar to the delivery of exercise training in persons with multiple sclerosis (MS) because the beneficial effects of exercise in MS can be documented only after diagnosis. Our results demonstrate no significant effects of exercise delivered during remission after the initial disease onset on clinical disability or levels of hippocampal brain-derived neurotrophic factor.

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study identified forced-swimming exercise in mice with chronic EAE to increase brain-derived neurotrophic factor (BDNF) production and to decrease demyelination in both the brain and the spinal cord as well as to attenuate the clinical presentation of EAE from onset to peak disease (Bernardes et al., 2013). To the best of our knowledge, no study has examined the effects of exercise using a model similar to the relapsing-remitting (RR) course of MS (RRMS). We believe that this is important because 85% of persons with MS are initially diagnosed with RRMS (Canadian Agency for Drugs and Technologies in Health, 2013). Results from studies that used a chronic EAE model may differ from an RR-EAE model and, therefore, may have different implications for persons with RRMS. Furthermore, no study has examined the effects of exercise delivered during a remission period after the initial onset of disease. This would be most similar to the delivery of exercise training in persons with MS because the beneficial treatment effects of exercise in MS can be documented only after diagnosis. We believe that it is important to investigate how exercise might influence disease course and identify specific pathophysiological mechanisms responsible for the benefits of exercise demonstrated in persons with RRMS.

This study investigates the effects of both voluntary wheel running and forced treadmill exercise delivered during the initial remission on clinical disability scores and levels of hippocampal BDNF in a model of RR-EAE. We hypothesize that voluntary wheel running and treadmill exercise will attenuate clinical disability scores and increase levels of hippocampal BDNF compared with those of sedentary mice with RR-EAE.

MATERIALS AND METHODS

Animals

Experimental procedures described herein were approved by the institutional animal care and use committee and were performed in accordance with the guidelines of the National Institutes of Health. Six- to eight-week-old female SJL mice (RRID:MGI_2663948, n = 47) were obtained from Harlan Laboratories (Indianapolis, IN). Only female mice were used because previous research has demonstrated a greater severity of EAE in female SJL mice compared with male mice (Papenfuss et al., 2004). Furthermore, most EAE research, specifically exercise in EAE research, has used female mice (Klaren et al., 2014). All mice were housed in individual cages with food and water ad libitum and controlled temperature (20–21°C) under a 12-hr light/dark cycle with lights on at 10:00 < AM.

Active Induction of RR-EAE

Complete Freund's adjuvant (CFA) was prepared by combining 25 ml incomplete Freund's adjuvant (DF0639606; Difco, Detroit, MI) and 100 mg mycobacterium tuberculosis (inactivated and desiccated; DF3114338; Difco), resulting in a final concentration of 4 mg/ml. $PLP_{139–151}$ (10 mg; SP522985; Genemed Synthesis, San Antonio, TX) was diluted in phosphate-buffered saline (PBS; pH 7.4) and then added dropwise to an equivalent volume of CFA while vortexing between each drop. The CFA and $PLP_{139–151}$ emulsion was then vortexed for 45 min, until an appropriate consistency developed. Prior to injection, mice were anesthetized with 4% isoflurane and then were subcutaneously injected with 100 μl CFA emulsion containing 200 μg mycobacterium tuberculosis and 75 μg $PLP_{139–151}$ distributed over two spots across the flank (McCarthy et al., 2012). Mice were weighed and monitored daily for disability. Clinical disability was evaluated by a blinded assessor. Active EAE was scored as described previously (Stromnes and Goverman, 2006; Miller et al., 2010), with slight modification. Specifically, severity was assessed on a scale from 0 to 5: 0, normal mouse with no overt signs of disease; 0.5, tail weakness or hindlimb weakness but not both; 1, tail paralysis; 1.5, tail paralysis and slightly wobbly gait; 2, tail paralysis, loss of righting reflex, and fore-/hindlimb weakness (wobbly gait); 2.5, partial fore-/hindlimb paresis; 3, one complete limb paralysis; 3.5, one complete limb paralysis and weakness in the other limb; 4, two limb paralysis; 4.5, two limb paralysis and weakness in forelimb; 5, moribund state/death by EAE. The first clinical episode is referred to as *acute-phase disease*, which is preceded by pronounced weight loss. Mice experience this acute episode for variable times as the disease is relapsing and remitting (McCarthy et al., 2012). The point at which disease reaches its highest score is referred to as the peak of acute disease. After the initial episode or a subsequent relapse, mice experience a recovery (remission). If the recovery lasts for at least 2 days and drops by at least one grade level, the recovery is deemed an authentic remission. A relapse is defined as a sustained increase (>2 days) of at least one full grade in clinical score after the animal had previously improved by at least a full clinical score and had stabilized for at least 2 days. The on-treatment severity index was used as a measure of clinical disability over the duration of the experiment (Theien et al., 2003). This was calculated by adding the clinical scores for each mouse beginning on the initial day of treatment (i.e., access to treadmill or running wheel) and dividing this number by the number of scores examined. Body mass was also measured daily with a scale sensitive to 0.1 g. Mice were sacrificed at days 69 and 71 post-EAE induction.

Randomization of Conditions

Mice were randomized into sedentary (n = 16), voluntary wheel running (n = 15), or forced treadmill exercise (n = 16) treatment conditions following the initial episode of clinical disability. Each mouse was randomized on the first day the clinical score decreased by 0.5 from the initial peak score. The criteria for beginning treatment occurred when mice demonstrated a clinical score of 1.5 (for mice with an initial peak clinical score ≥2) or when mice demonstrated a clinical score that was 0.5 less than the peak score (for mice with an initial peak score ≤1.5). Mice randomized into the sedentary condition remained in the same standard cage. Mice randomized into the activity wheel condition were housed in cages with a running wheel (SOF-860; Med Associates, St. Albans, VT) that provided 24-hr access for the mice to run voluntarily. Mice randomized into the forced treadmill running condition were subjected to 5 days/week of running on a motorized treadmill (DC5; Jog-a-Dog, Ottawa Lake, MI) at a 5% grade, 14 m/min, for 30 min. Mice were encouraged to run with gentle prodding, and mice

that refused to comply for >10 sec were removed. Foam pads were placed at the back of each lane to lessen the risk of injury. Running volume (meters) in the treadmill condition was calculated by multiplying the number of minutes for which each mouse ran per day by 14 m/min. The average running volume was then calculated over 36 days of treadmill running. Running volume (meters) in the running wheel condition was calculated by multiplying the total wheel revolutions per day by the circumference of the running wheel (meters). The average running volume was then calculated over 50 total days of wheel running.

Euthanasia, Tissue Extraction, and Protein Isolation and Quantification

Mice were brought to a surgical plane of anesthesia on days 69 and 71 by intraperitoneal injection of ketamine/xylazine (ketamine 1.7 mg/20 g body weight, xylazine 0.26 mg/20 g body weight) in a total volume of 0.1 ml. Mice were transcardially perfused through the left ventricle with sterile PBS (pH 7.4) and decapitated, and the brains were removed. Next, the brain was bisected sagitally with a sterile razor blade, and the hippocampus was immediately dissected with sterile forceps, flash frozen in liquid nitrogen, and stored in a –80°C freezer. The spinal column was removed and fixed overnight at 4°C in 4% paraformaldehyde and then cryoprotected with 30% sucrose in PBS. On the following day, the spinal cords were frozen in optimal cutting temperature compound and stored in a –80°C freezer. Gastrocnemius and soleus muscles from both hindlimbs were dissected with scissors, flash frozen in liquid nitrogen, and stored in a –80°C freezer.

BDNF ELISA

Hippocampal samples were removed from the –80°C freezer and placed on ice. A protein lysis buffer was prepared containing Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), sodium orthovanadate (1 mM), NaF (1 mM), 0.1% protease inhibitor cocktail (PIC; DMSO solution), and phenylmethanesulfonyl fluoride (1 mM). Lysis buffer (200 μl) was added to each sample, samples were placed on ice for 10 min, and all samples were sonicated until a homogenous mixture developed. Samples were centrifuged at 12,000*g* for 10 min, and protein-containing supernatants were removed and placed in 1.5-ml tubes. Protein levels of each sample were quantitated by Bradford assay according to the manufacturer's instructions (Micro BCA protein assay kit; Thermo Fisher Scientific, Waltham, MA). Levels of BDNF in the hippocampus were determined (range 31.2–2,000 pg/ml) with a mouse-specific ELISA kit according to the manufacturer's instructions (Abnova, Taipei City, Taiwan).

Citrate Synthase Assay

As a measure of muscular activity and mitochondrial content resulting from exercise training (Holloszy, 1967), we chose to analyze citrate synthase (CS) levels in muscle samples. Gastrocnemius and soleus muscle samples were removed from the –80°C freezer and placed on ice. A protein lysis buffer was prepared containing Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1% PIC. Lysis buffer (500 μl) was added to each sample, samples were placed on ice for 10 min, and all samples were homogenized until a homogenous mixture developed. Samples were transferred to 1.5-ml tubes and centrifuged at 12,000*g* for 10 min, and protein-containing supernatants were removed and placed in 1.5-ml tubes. Protein levels of each sample were quantitated by Bradford assay according to the manufacturer's instructions (Micro BCA protein assay kit, Thermo Scientific). Levels of CS activity (micromoles/minute/milligram protein) in the gastrocnemius and soleus muscles were determined with a mouse-specific kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Immunohistochemistry

The spinal cords were segmented in transverse planes with the aid of a CM 1950 cryostat (Leica, Wetzlar, Germany), and the transverse sections of 10–20-μm thickness were placed on slides (Superfrost Plus; Thermo Fisher Scientific) and stored in a –80°C freezer. To determine levels of demyelination, myelin was stained with oil red O staining (Sigma-Aldrich, St. Louis, MO) as described previously (Steelman et al., 2012). Briefly, sections were incubated for 45–60 min at 37°C, rehydrated with distilled water for 5 min, treated with 100% propylene glycol for 2 min, and then soaked in oil red O for 24–48 hr. Excess stain was removed by washing the slides with 85% propylene glycol, followed by a wash with distilled water. Next, sections were stained with hematoxylin (Sigma-Aldrich) for 7 min. Afterward, excess stain was removed by washing slides with distilled water, and slides were mounted with Fluoromount-G (Southern Biotechnology, Birmingham, AL) and premium cover glass (Thermo Fisher Scientific). Slides were imaged with a NanoZoomer slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan) and analyzed in NDP.view software. The number of lesions was counted in each section, and the percentage of total demyelination was estimated. When available, scores of multiple sections (two to five) within the thoracic, lumbar, and cervical sections were averaged. The final score from each region represents the average scores from each treatment condition.

Statistical Analysis

Data were analyzed in SPSS v.21 (RRID:SCR_002865; IBM, Armonk, NY). Mixed-factor ANOVA was used to identify differences in clinical disability scores and body mass among treatment conditions (i.e., running wheel, treadmill, and sedentary) over 68 days postinduction of EAE. Between-subjects ANOVA was used to identify differences among treatment conditions in disease onset variables, day of randomization, clinical disability scores of each relapse, number of relapses, cumulative clinical disability scores, on-treatment severity index, gastrocnemius and soleus muscle mass, levels of BDNF (picograms/milligram), and CS activity (micromoles/minute/milligram protein). Between-subjects ANOVA was also used to identify differences in running volume (average distance run [meters]/day) between running wheel and treadmill conditions. The average number of lesions per section was analyzed by

TABLE I. Day of Disease Onset, Clinical Disability Scores, and Day of Randomization Among Treatment Conditions*

	Treatment condition				
Variable	Sedentary (n = 16)	Running wheel (n = 15)	Treadmill (n = 16)	F value	*P* value
Day of disease onset	13.6 (1.9)	13.7 (1.4)	14.8 (2.8)	1.6	0.21
Day of initial disease episode peak clinical disability score	16.3 (2.3)	15.9 (1.8)	16.8 (2.9)	0.54	0.59
Peak clinical disability score of initial disease episode (mdn, IQR)	2.5 (1.0)	2.5 (1.0)	2.5 (1.0)	0.34	0.71
Day randomized	18.3 (2.4)	18.3 (1.8)	18.6 (2.6)	0.09	0.91

*Values are mean (SD) unless otherwise noted.

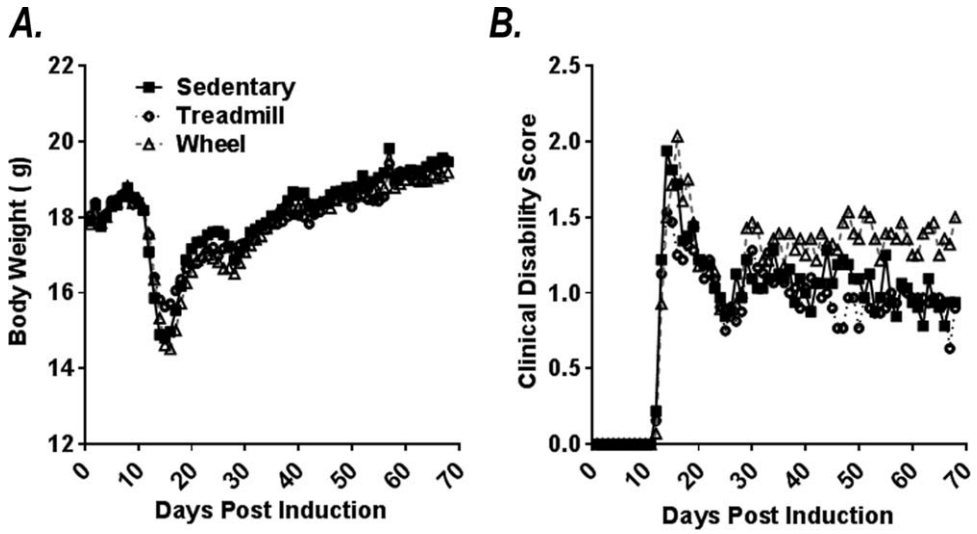

Fig. 1. Exercise did not affect EAE severity or disease trajectory. **A**: Average body weight post-EAE induction in sedentary (n = 15), running wheel (n = 15), and treadmill (n = 16) conditions. **B**: Average clinical disability score post-EAE induction in sedentary (n = 15), running wheel (n = 15), and treadmill (n = 16) conditions. Indicators of variation in A and B were removed for clarity.

one-way ANOVA, and average number of lesions per section for each region was analyzed by two-way ANOVA. Demyelination was analyzed by nonparametric Kruskal-Wallis test. Pearson product-moment correlations (*r*) were used to examine associations between running volume in treadmill and running wheel conditions and between CS activity and levels of hippocampal BDNF. $P < 0.05$ was considered statistically significant.

RESULTS

Disease Equivalence and Randomization

The average day of disease onset, day of initial disease episode peak score, peak clinical disability score of initial disease episode, and day of randomization into conditions are shown in Table I. Without respect to condition, disease onset occurred between days 12 and 21 postinduction ($F_{1,44} = 1.6$, $P = 0.21$). The day of peak clinical disability score at disease episode ($F_{1,44} = 0.54$, $P = 0.59$) and peak clinical disability score of initial disease episode ($F_{1,44} = 0.34$, $P = 0.71$) were similar among conditions. All mice had been randomized into condition by day 25 postinduction ($F_{1,44} = 0.09$, $P = 0.91$).

Effects of Voluntary Wheel Running and Forced Treadmill Exercise on Clinical Disability Severity

Body weights and clinical disability scores are shown in Figure 1A,B. The mixed-factor ANOVA (group-$\times$time) indicated no differences in body weight ($F_{1,43} = 0.83$, $P = 0.44$) or clinical disability score ($F_{1,43} = 1.68$, $P = 0.20$) among conditions over 68 days post-EAE induction. The average peak clinical disability scores at each relapse, total number of relapses, cumulative sum of clinical disability scores, and average on-treatment severity index for each condition are shown in Table II. The mean peak clinical disability scores of relapse 1, relapse 2, and relapse 3 were not different among conditions ($F_{1,41} = 0.32$, $P = 0.73$; $F_{1,41} = 0.77$, $P = 0.47$; $F_{1,41} = 0.50$, $P = 0.63$, respectively). The total number of relapses also was not different among conditions ($F_{1,44} = 0.02$, $P = 0.98$). Furthermore, the cumulative sum of clinical disability scores and the average on-treatment severity index were not different among conditions ($F_{1,44} = 1.8$, $P = 0.18$ and $F_{1,44} = 0.84$, $P = 0.44$, respectively).

TABLE II. Effect of Voluntary Wheel Running and Forced Treadmill Running on RR-EAE*

Variable	Treatment condition				
	Sedentary (n = 16)	Running wheel (n = 15)	Treadmill (n = 16)	F value	*P* value
Peak clinical disability score of relapse 1 (median, IQR)	2.0 (1.5)	2.5 (1.5)	2.5 (1.5)	0.32	0.73
Peak clinical disability score of relapse 2 (median, IQR)	2.0 (0.5)	2.5 (0.5)	2.0 (1.5)	0.77	0.47
Peak clinical disability score of relapse 3 (median, IQR)	2.0 (0.0)	2.5 (0.5)	1.5 (0.5)	0.50	0.63
Number of relapses	1.8 (0.8)	1.9 (1.0)	1.9 (1.0)	0.02	0.98
Cumulative sum of clinical disability scores	52.8 (21.6)	65.3 (26.9)	47.9 (30.3)	1.8	0.18
On-treatment severity index	1.0 (0.4)	1.3 (0.5)	1.1 (0.8)	0.84	0.44

*Values are mean (SD) unless otherwise noted. The on-treatment severity index was calculated by adding the clinical scores for each mouse beginning on the initial day of treatment (i.e., access to treadmill or running wheel) and dividing this number by the number of scores examined.

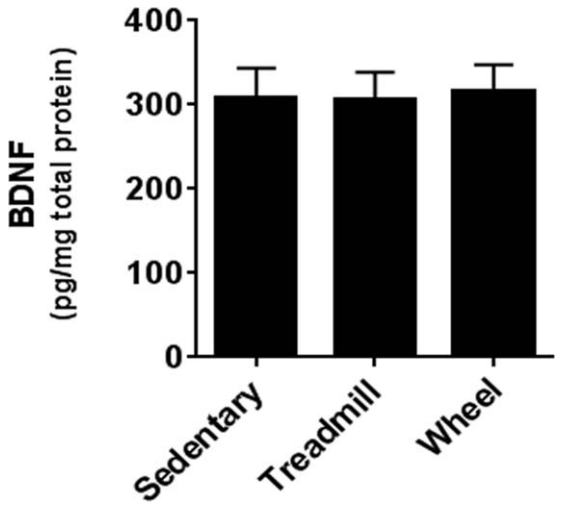

Fig. 2. Exercise did not alter hippocampal BDNF. Levels of hippocampal BDNF in sedentary (n = 10), running wheel (n = 10), and treadmill (n = 10) groups at day 69 and 71 post-EAE induction as determined by ELISA. Results are mean ± SE.

Levels of Hippocampal BDNF

The concentrations of hippocampal BDNF (pg/mg) did not differ among conditions ($F_{1,27} = 0.05$, $P = 0.95$; Fig. 2, Table III).

Running Volume, Muscle Weights, and CS Activity

Table III shows running volume (meters/day), combined gastrocnemius and soleus muscle mass (grams), and CS activity (micromoles/minute/milligram protein) among treatment conditions. Running volume among groups differed such that mice in the running wheel condition ran approximately 1,500 m per day compared with approximately 200 m per day in the treadmill condition ($F_{1,29} = 10.9$, $P < 0.05$). There were no differences in gastrocnemius and soleus muscle mass (grams) among conditions ($F_{1,27} = 0.53$, $P = 0.59$). However, CS activity differed among conditions ($F_{1,27} = 3.9$, $P < 0.05$) such that CS activity in the treadmill condition was lower compared with the sedentary and running wheel conditions (Fig. 3).

Running Volume, CS Activity, and Levels of Hippocampal BDNF

Pearson product-moment correlations demonstrated an overall correlation between running volume (meters/day) and levels of hippocampal BDNF (picograms/milligram; $r = 0.49$, $P < 0.05$). However, when analyzed by condition, this correlation remained significant for the running wheel condition ($r = 0.81$, $P < 0.05$) but not for the treadmill ($r = –0.06$, $P = 0.88$) condition. Pearson product-moment correlations demonstrated a significant overall correlation between running volume (meters/day) and CS activity (micromoles/minute/milligram protein; $r = 0.54$, $P < 0.05$). When analyzed by condition, this correlation was stronger, albeit nonsignificant, in the running wheel condition ($r = 0.48$, $P = 0.16$) compared with the treadmill condition ($r = 0.07$, $P = 0.85$).

Immunohistochemistry

Histopathological analysis of the spinal cord demonstrated that exercise treatment had no effect on the degree of pathology in lesions per region ($F_{4,69} = 0.79$, $P = 0.54$; Fig. 4A), total number of lesions per section ($F_{2,75} = 1.5$, $P = 0.22$; Fig. 4B,C), or extent of demyelination ($H = 1.16$, $P = 0.56$; Fig. 4D).

DISCUSSION

The current study demonstrates no significant effects of forced treadmill exercise or voluntary wheel running on clinical disability scores or levels of hippocampal BDNF in mice with RR-EAE. To the best of our knowledge, this is the first study to examine effects of both forced and voluntary exercise delivered during remission after the initial onset of disease in an RR-EAE model.

Limited research has sought to examine the effects and pathophysiological mechanisms of both forced and voluntary exercise in EAE. Indeed, the entirety of this research has been applied in a chronic model of EAE most similar to the progressive course of MS, with exercise typically delivered immediately post-EAE induction for variable durations. For example, data from a previous study indicated that voluntary exercise (e.g., wheel running) delivered over 50 days immediately post-EAE induction (i.e., $MOG_{35–55}$) improved overall clinical disability compared with a sedentary condition (Rossi et al.,

TABLE III. Running Volume, Muscle Weights, CS Activity, and Levels of BDNF Among Treatment Conditions*

	Treatment condition				
Variable	Sedentary (n = 16)	Running wheel (n = 15)	Treadmill (n = 16)	F value	*P* value
Running volume/day (m)	NA	1,497.6 (1,601.2)	177.6 (102.3)	10.9	0.003
Gastrocnemius and soleus muscle weights (g)	0.25 (0.03)	0.26 (0.04)	0.26 (0.04)	0.53	0.59
CS activity (μmol/min/mg protein)	0.019	0.019	0.015	3.9	0.03
BDNF (pg/mg)	332.4	342.3	332.2	0.05	0.95

*Values are mean (SD) unless otherwise noted. NA, not applicable.

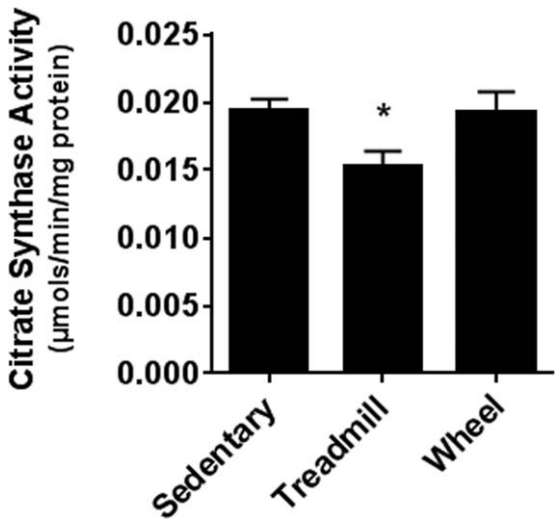

Fig. 3. Muscle citrate synthase activity differed among the conditions. Citrate synthase activity in sedentary (n = 10), running wheel (n = 10), and treadmill (n = 10) conditions at day 69 and 71 post-EAE induction. Results are mean ± SE. *$P < 0.05$.

2009). In the Lewis rat model of chronic EAE, no significant differences in either total brain BDNF protein or clinical disability scores were observed between forced treadmill running and sedentary groups (Patel and White, 2013). However, a more recent study demonstrated that C57Bl/6 mice with chronic EAE in a forced-swimming condition delivered for 4 weeks pre-EAE induction and over 10 or 14 days immediately post-EAE induction ($MOG_{35–55}$) had attenuated disease severity and an increase in BDNF in the brain and spinal cord compared with a sedentary, control condition (Bernardes et al., 2013). Collectively, the heterogeneity of previous research (e.g., exercise modality and duration) has made conclusions on outcomes (i.e., clinical disability and protein expression) and translation for the application of exercise and physical activity in humans difficult to interpret because these behaviors are not initiated during the onset of disease. Therefore, the current study used a novel, more applicable, model of RR-EAE with physical activity treatment delivered during the remission period after the initial onset of EAE to determine whether the physical activity affected disease trajectory.

Contrary to our hypotheses that voluntary wheel running and forced treadmill exercise would attenuate disease progression and modulate levels of hippocampal BDNF, we found no significant differences in either clinical disability, including number and severity of relapses and hippocampal BDNF. It is possible that the amount of activity in the treadmill and wheel running conditions was insufficient to elicit any differences in hippocampal BDNF or clinical disability in comparison with the sedentary condition. Our results indicated similar levels of CS activity in the gastrocnemius and soleus muscles of mice in the sedentary condition compared with the running wheel condition and lower levels of CS activity in the treadmill condition compared with both the sedentary and running wheel conditions. An increase in CS activity is commonly reported as a result of exercise training because of an increase in mitochondrial content (Holloszy, 1967). Thus, our results are in contradiction because one would expect higher levels of CS activity in the running wheel and treadmill conditions. However, a previous study demonstrated no increase in CS activity in Balb/c mice that ran 10,000–35,000 m/week on a running wheel (Fernandez-Verdejo et al., 2014). The mice in the current study ran ~10,500 m/week in the running wheel condition and ~1,000 m/week in the treadmill condition; therefore, the volume and/or intensity of activity in both conditions may be insufficient to influence CS activity or levels of hippocampal BDNF that may be related to clinical disability (Bernardes et al., 2013). Furthermore, previous articles have proposed the idea that specific strains of mice may not respond to exercise with increases in CS activity (Liu et al., 2009; Fernandez-Verdejo et al., 2014); perhaps the SJL strain of mice with EAE is unresponsive to exercise effects as measured by CS activity. The low rates of activity in the running wheel and treadmill conditions could further explain why there were no differences in hippocampal BDNF compared with the sedentary condition. Our results do suggest that higher amounts of wheel running and treadmill exercise are positively associated with levels of hippocampal BDNF, and this is in line with previous research (Berchtold et al., 2005). The low levels of activity could further be associated with fatigue, given that muscle weakness is a common symptom in EAE (Baxter, 2007), or depression, given that previous research has demonstrated a depressive-like behavioral syndrome in mice with EAE (Pollak et al., 2002). Furthermore, fatigue is a characteristic symptom in RRMS and has been associated with decreased physical activity (Motl et al., 2012); this association may be communal in the RR-EAE

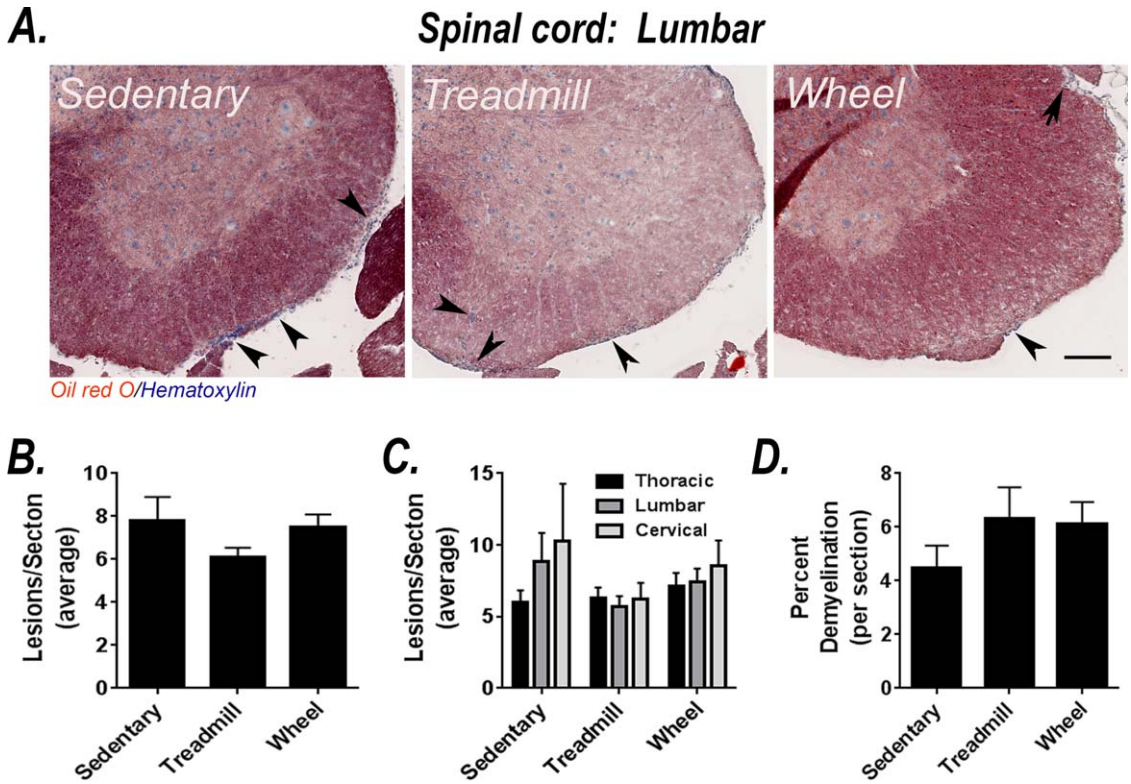

Fig. 4. Exercise did not affect spinal cord pathology. **A**: Representative transverse sections of the ventral horn from the lumbar region of spinal cords in the sedentary, treadmill, and wheel conditions were stained for myelin with oil red O (red) and for nuclei with hematoxylin (blue). Arrowheads indicate areas where lesions are present. **B**: Average number of lesions per section for each condition. **C**: Average number of lesions per section in each spinal cord region for each condition. **D**: Estimated percentage of demyelination per section for each condition. All data are mean ± SE and are derived from multiple sections per animal (n = 3–6). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

model. Altogether, our results indicate that the volume of forced treadmill exercise and voluntary wheel running undertaken after onset of MS provide insufficient physiological stimuli to influence CS activity, hippocampal BDNF, or clinical disability.

The continued investigation of exercise effects in EAE should focus on identifying the benefits or possible disease-modifying effects of exercise when the treatment (i.e., wheel running or forced exercise) is delivered post-EAE induction. Future research could also examine varied doses and time points of exercise. For example, it may of interest to examine whether frequency (i.e., days of access to running wheel and treadmill), intensity (speed and incline of treadmill), duration, and time point of exercise initiation are significant components of disease-modifying effects of exercise. Furthermore, a research design employing the RR-EAE model is warranted to compare the effects of exercise across animal models because research on exercise in EAE is not yet conclusive. Finally, more information on the pathophysiological effects of exercise, such as BDNF level, is required to help identify specific mechanisms involved in exercise.

The strengths of this study include the large number of animals and duration (i.e., ~60 days), presence of a blinded assessor for clinical disability scores, and inclusion of both voluntary wheel running and forced treadmill exercise. However, this study is not without limitations. First, although previous research has demonstrated various responses of the SJL mouse to exercise, such as endothelial responses (Kim et al., 2015), we are not aware of research that has examined responses specific to this study (i.e., BDNF and CS). Therefore, future research may be warranted to identify responses to exercise in the SJL mouse without EAE. Another limitation is the amount of exercise in the treadmill condition because not all mice were compliant with the protocol (i.e., 5 days/week at 5% grade, 14 m/min, for 30 min), and this may confound the true effects of exercise on clinical disability. Another limitation is overall volume of exercise in the running wheel and treadmill conditions; the volume of exercise may have been too low to detect any differences in the exercise conditions compared with the sedentary condition. Furthermore, we did not evaluate BDNF levels in other neural substrates or in the serum, and these levels may

have differed in response to exercise (Bernardes et al., 2013) or during the relapse phases (Sarchielli et al., 2002). Finally, it may be important to assess other neurodegenerative parameters (i.e., astrocytes or microglia activation) and inflammatory markers (i.e., leukocyte infiltration and recruitment, levels of chemokines and cytokines, and rupture of the blood–brain barrier) that have previously been identified as alterations of the disease course (Frohman et al., 2008). In summary, the current study demonstrates that the amount and/or intensity of forced treadmill exercise and voluntary wheel running by mice with RR-EAE during remission after the initial disease onset may be required to be above a certain threshold for any observable benefits for clinical disability scores or levels of hippocampal BDNF.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no known or potential conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: REK, AJS, BDP, JAW, RWM; Acquisition of data: REK, AJS, BDP, US, JH; Statistical analysis and interpretation of data: REK, BDP, JH, JAW, RWM; Drafting of the manuscript: REK, AJS, JAW, RWM; Critical revision of the article for important intellectual content: REK, BDP, JH, JAW, RWM.

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