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 MS. 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. The aim of the current study was to examine the effects both voluntary wheel running and forced treadmill exercise on clinical disability and hippocampal brain-derived neurotrophic factor (BDNF) in SJL mice with relapsing-remitting EAE. The results demonstrated no significant effects of exercise, delivered during remission after initial disease onset, on clinical disability scores or levels of hippocampal BDNF in mice with relapsing-remitting EAE. Further, our results demonstrated no significant increase in citrate synthase (CS) activity in the gastrocnemius and soleus muscles of mice in the running wheel and treadmill conditions compared to 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. Klaren et al. *J Neurosci Res* 94(10): 907-914, 2016.

INTRODUCTION

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 results in impairments and symptoms such as muscle spasticity and weakness, walking and balance dysfunction, and reduced quality of life (QOL; Lublin, 2005).

There is an abundance of research from clinical trials demonstrating the safety and beneficial effects of physical activity and exercise training in persons with MS (Pilutti et al., 2014; Motl & Pilutti, 2012). Further, 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

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mechanisms of action for treatment effects on MS pathophysiology (Robinson et al., 2014). EAE is a T-helper (Th) 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, resulting in primary demyelination of axonal tracks, subsequent impaired axonal conduction in the CNS, and progressive hind limb paralysis (Robinson et al., 2014; Kuerten & Angelov, 2008; Wekerle, 2008).

Previous research that has 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 to attenuate clinical disability and synaptic and dendritic defects of EAE in mice with chronic EAE (Rossi et al., 2009). A more recent study identified forced swimming exercise in mice with chronic EAE to increase brain-derived neurotrophic factor (BDNF) production and decrease demyelination in both the brain and spinal cord, as well as attenuate the clinical presentation of EAE from onset to peak disease (Bernardes et al., 2013). To our knowledge, no study has examined the effects of exercise using a model similar to the relapsing-remitting course of MS (RRMS). We believe this is important as 85% of persons with MS are initially diagnosed with RRMS (Canadian Agency for Drugs and Technologies in Health, 2013). Results from studies using a chronic EAE model may differ from a relapsing-remitting EAE model and therefore may have different implications for persons with RRMS. Further, 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 as the beneficial treatment effects of exercise in MS can only be documented after diagnosis. We believe 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.

The purpose of this study was to investigate 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 relapsing-remitting model of EAE. We hypothesized voluntary wheel running and treadmill exercise to attenuate clinical disability scores and increase levels of hippocampal BDNF compared to sedentary mice with relapsing-remitting EAE.

MATERIALS AND METHODS

Animals

The experimental procedures described herein were approved by the Institutional Animal Care and Use Committee and were performed in accordance with 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 as previous research has demonstrated a greater severity of EAE in female SJL mice compared to male (Papenfuss et al., 2004). Further, the majority of EAE research, specifically exercise in EAE research, have used female mice (Klaren et al, 2014). All mice were housed in individual cages with food and water ad libitum, on a 12h light/dark cycle with lights on at 10:00h and controlled temperature (20-21°C).

Active induction of RR-EAE

Complete Freund's adjuvant (CFA) was prepared by combining 25ml incomplete Freund's adjuvant (Difco:DF0639606) and 100mg Mycobacterium tuberculosis (inactivated and desiccated; Difco:DF3114338), resulting in a final concentration of 4mg/ml. 10mg PLP139-151 (Genemed Synthesis:SP522985) was diluted in PBS (pH=7.4) and then added dropwise to an equivalent volume of CFA while vortexing in between each drop. The CFA and PLP139-151 emulsion was then vortexed for 45min until appropriate consistency. Prior to injection, mice were anesthetized with 4% isoflourane and then were subcutaneously injected with 100μL CFA

emulsion containing 200μg Mycobacterium tuberculosis and 75μg PLP139-151 distributed over 2 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 & Goverman, 2006; Miller et al., 2010) with slight modification. Specifically, severity was assessed on a 0 to 5 scale as follows: grade 0, normal mouse with no overt signs of disease; grade 0.5, tail weakness or hind limb weakness but not both; grade 1, tail paralysis; grade 1.5, tail paralysis and slight wobbly gait; grade 2, tail paralysis, loss of righting reflex and fore/hind limb weakness (wobbly gait); grade 2.5, partial fore/hind limb paresis; grade 3, one complete limb paralysis; grade 3.5, one complete limb paralysis and weakness in the other limb; grade 4, two limb paralysis; grade 4.5, two limb paralysis and weakness in fore limb; grade 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 will experience this acute episode for variable times as the disease is relapsing and remitting (McCarthy et al., 2012). The point where disease reaches its highest score is referred to as the peak of acute disease. After the initial episode or a subsequent relapse, mice will 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 was defined as a sustained increase (>2 days) of at least one full grade in clinical score after the animal had previously improved 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 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 using 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 initial episode of clinical disability. Each mouse was randomized the first day the clinical score decreased by 0.5 from 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 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 Inc.) that provided 24-hour access for the mice to voluntarily run. Mice randomized into the forced treadmill running condition were subjected to 5 days/week running on a motorized treadmill (DC5, Jog-a-Dog) at 5% grade, 14 m/min, for 30 minutes. Mice were encouraged to run with gentle prodding, and mice that refused to comply for >10 seconds were removed. Foam pads were placed at the back of each lane to lessen the risk of injury. Running volume (m) in the treadmill condition was calculated by multiplying the number of minutes each mouse ran per day by 14 m/min. The average running volume was then calculated over 36 days of treadmill running. Running volume (m) in the running wheel condition was calculated by multiplying the total wheel revolutions per day by the circumference of the running wheel (m). The average running volume was then calculated over 50 total days of wheel running.

Euthanasia, tissue extraction, protein isolation and quantification.

Mice were brought to a surgical plane of anesthesia on days 69 and 71 by intraperitoneal (i.p.) injection of Ketamine/Xylazine (Ketamine: 1.7mg per 20g body weight; Xylazine: 0.26mg per 20g body weight) in a total volume of 0.1ml. Mice were transcardially perfused through the left ventricle with sterile phosphate buffered saline (PBS; pH=7.4), decapitated and the brains removed. Next, using a sterile razor blade, the brain was bisected sagittally and the hippocampus was immediately dissected using sterile forceps,

then flash frozen in liquid nitrogen and stored in a -80° freezer. The spinal column was removed and fixed overnight at 4°C in 4% paraformaldehyde (PFA), then cryoprotected with 30% sucrose in PBS. The following day, the spinal cords were frozen in optimal cutting temperature (OCT) compound and stored in a -80° freezer. Gastrocnemius and soleus muscle from both hind limbs were dissected with scissors, flash frozen in liquid nitrogen, and stored in a -80° freezer.

BDNF ELISA

Hippocampal samples were removed from the -80° freezer and placed on ice. A protein lysis buffer was prepared containing Tris-HCl (50mM, pH=8.0), NaCl,(150mM), 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), sodium orthovanadate (1mM), NaF (1mM), 0.1% protease inhibitor cocktail (PIC; DMSO solution), and phenylmethanesulfonyl fluoride (PMSF; 1mM). 200µl of lysis buffer was added to each sample, placed on ice for 10min, and all samples were sonicated until a homogenous mixture developed. Samples were centrifuged at 12,000×g for 10min, and protein-containing supernatants were removed and placed in 1.5ml tubes. Protein levels of each sample were quantitated by Bradford assay according to manufacturer's instructions (Micro BCA Protein Assay Kit, Thermo Scientific). Levels of BDNF in the hippocampus were determined (range: 31.2–2000pg/ml) using a mouse-specific ELISA kit according to the manufacturer's instructions (Abnova).

Citrate synthase assay

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

Immunohistochemistry

The spinal cords were segmented in transverse planes with the aid of a cryostat (Leica CM 1950) and the transverse sections of 10-20µm thickness were placed on slides (Fisher Super Frost Plus) and stored in a -80° freezer. To determine levels of demyelination, myelin was stained with oil red O staining (Sigma-Aldrich) as described previously (Steelman et al., 2012). Briefly, sections were incubated for 45-60min at 37°C, rehydrated with distilled water for 5min, treated with 100% propylene glycol for 2min, then soaked in oil red O for 24–48h. 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) for 7min. Afterwards excess stain was removed by washing slides with distilled water and slides were mounted using Fluoromount-G (Southern Biotech) and premium cover glass (Fisher). Slides were imaged using a NanoZoomer slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan) and analyzed with NDP.view software. The number of lesions was counted in each section and the percent of total demyelination was estimated. When available, scores of multiple sections (2–5) 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 version 21 (RRID:SCR_002865). Mixed-factor analysis of variance (ANOVA) was used to identify differences in clinical disability scores and body

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

Variable	Treatment condition				
	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.

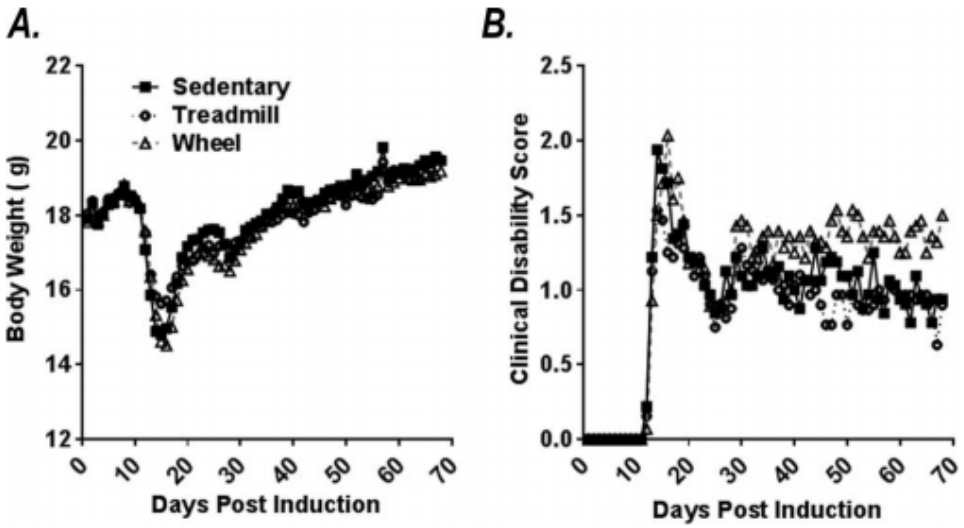

Fig. 1. Exercise did not affect EAE severity or disease trajectory. **A**: Average body weight post-EAE induction in sedentary (n = 15), running wheel (n = 15), and treadmill (n = 16) conditions. **B**: Average clinical disability score post-EAE induction in sedentary (n = 15), running wheel (n = 15),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

mass among treatment conditions (i.e., running wheel, treadmill, and sedentary) over 68 days post-induction of EAE. Between-subjects ANOVAs were 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 (pg/mg), and CS activity (μmol/min/mg protein). A between-subjects ANOVA was also used to identify differences in running volume (average distance ran (m)/day) between running wheel and treadmill conditions. The average number of lesions per section was analyzed with a one-way ANOVA and average number of lesions per section for each region was analyzed with a two-way ANOVA. Demyelination was analyzed with a non-parametric Kruskal-Wallis test. Pearson product-moment correlations (r) were used to examine associations between running volume in treadmill and running wheel conditions and CS activity and levels of hippocampal BDNF. Statistical significance was determined as $p<0.05$.

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 illustrated in Table 1. Regardless of condition, disease onset occurred between days 12-21 post-induction ($F(1,44)=1.6$, $p=0.21$). 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 were randomized into condition by day 25 post-

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.

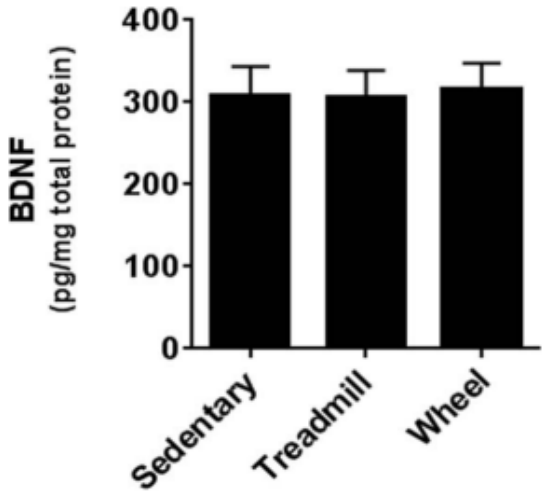

Fig. 2. Exercise did not alter hippocampal BDNF. Levels of hippocampal BDNF in sedentary (n = 10), running wheel (n = 10), and treadmill (n = 10) groups at day 69 and 71 post-EAE induction as determined by ELISA. Results are mean ± SE.

induction (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 score and are shown in Figures 1A and 1B, respectively. The mixed-factor ANOVA (Group × 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 2. 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 were also not different among conditions (F(1,44)=0.02; p=0.98). Further, 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).

Levels of hippocampal BDNF

The concentrations of hippocampal BDNF (pg/mg) did not differ among conditions (F(1,27)=0.05; p=0.95) (Table 3 and Figure 2).

Running volume, muscle weights, and CS activity

Table 3 demonstrates running volume (m/day), combined gastrocnemius and soleus muscle mass (g), and CS activity (μmol/min/mg protein) among treatment conditions. Running volume between groups differed such that mice in the running wheel condition ran approximately 1500 m per day compared to approximately 200 m per day in the treadmill condition (F(1,29)=10.9, p<0.05). There was no differences in gastrocnemius and soleus muscle mass (g) 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 to the sedentary and running wheel conditions (Fig. 3).

Running volume, CS activity, and levels of hippocampal BDNF

Pearson product-moment correlations (r) demonstrated an overall correlation between running volume (m/day) and levels of hippocampal BDNF (pg/mg) (r=0.49, p<0.05). However, when analyzed by condition, this correlation remained significant only for the running wheel condition (r=0.81, p<0.05), but not the treadmill condition (r=-0.06, p=0.88). Pearson

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

Variable	Treatment condition				
	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.

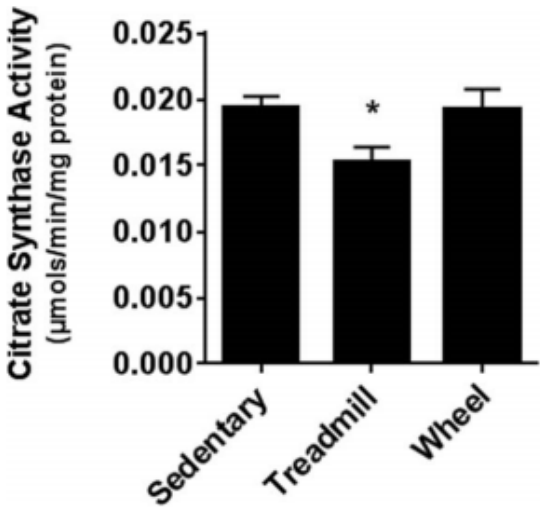

Fig. 3. Muscle citrate synthase activity differed among the conditions. Citrate synthase activity in sedentary (n = 10), running wheel (n = 10), and treadmill (n = 10) conditions at day 69 and 71 post-EAE induction. Results are mean ± SE. *$P < 0.05$.

product-moment correlations demonstrated a significant overall correlation between running volume (m/day) and CS activity (μmol/min/mg protein) ($r=0.54$, $p<0.05$). When analyzed by condition, this correlation was stronger, albeit non-significant, in the running wheel condition ($r=0.48$, $p=0.16$) compared to 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 the extent of demyelination ($H=1.16$, $p=0.56$) (Fig. 4D).

DISCUSSION

The current study demonstrated no significant effects of forced treadmill exercise or voluntary wheel running on clinical disability 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., myelin oligodendrocyte glycoprotein (MOG)35-55) improved overall clinical disability compared with a sedentary condition (Rossi et al., 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 & White, 2013). However, a more recent study demonstrated that C57Bl/6 mice with chronic EAE in a forced swimming condition delivered for four weeks pre EAE induction and over 10 or 14 days immediately post EAE induction (MOG35-55) had attenuated disease severity and an increase in BDNF in the brain and spinal cord compared to 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 as these behaviors are not initiated during the onset of disease. Therefore, the goal of the current study was to use a novel, more applicable, model of relapsing-remitting EAE with physical activity treatment delivered during the remission period after the initial onset of EAE, to determine if the physical activity affected disease trajectory.

Contrary to our hypotheses that voluntary wheel running and forced treadmill exercise

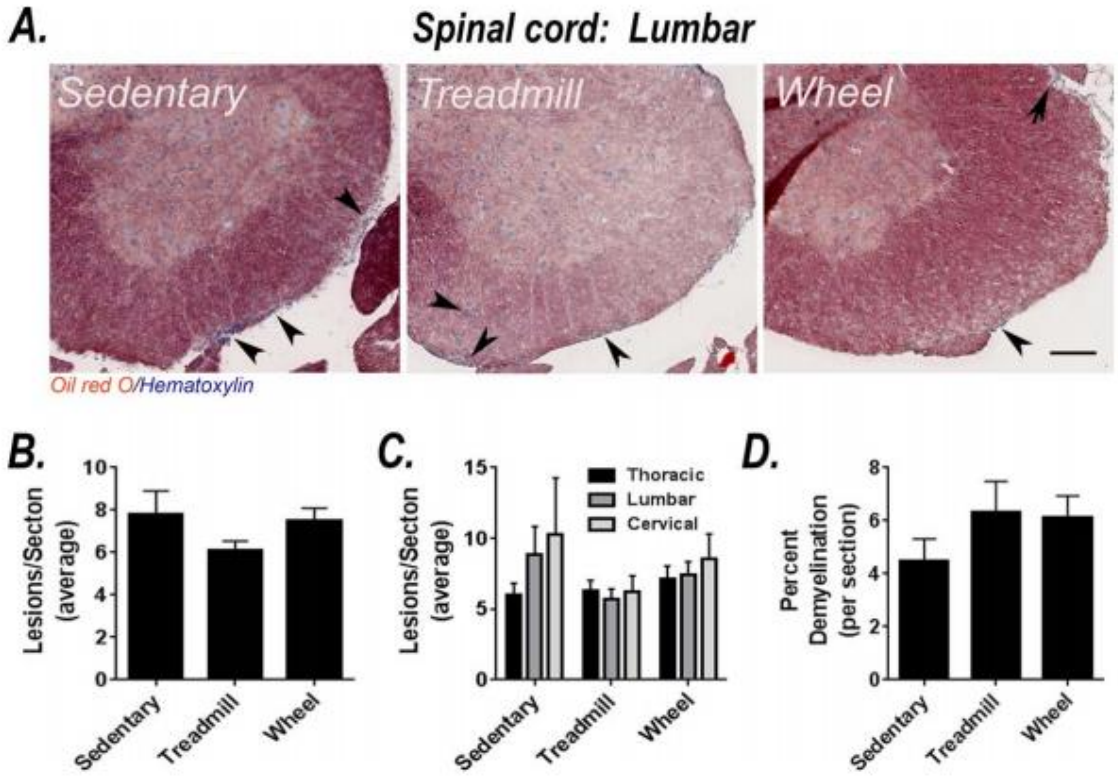

Fig. 4. Exercise did not affect spinal cord pathology. **A**: Representative transverse sections of the ventral horn from the lumbar region of spinal cords in the sedentary, treadmill, and wheel conditions were stained for myelin with oil red O (red) and for nuclei with hematoxylin (blue). Arrowheads 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.]

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, or hippocampal BDNF. It is possible 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 citrate synthase (CS) activity in the gastrocnemius and soleus muscles of mice in the sedentary condition compared to the running wheel condition and lower levels of CS activity in the treadmill condition compared to both the sedentary and running wheel conditions. An increase in CS activity is commonly reported as a result of exercise training due to an increase in mitochondrial content (Holloszy, 1967). Thus, our results are in contradiction as 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). Further, previous studies have proposed the idea that specific strains of mice may not respond to exercise with increases in CS activity (Fernandez-Verdejo et al., 2014; Liu et al., 2009); 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 to the sedentary

condition. Our results do suggest higher amounts of wheel running and treadmill exercise are positively associated with levels of hippocampal BDNF, and this is consistent with previous research (Berchtold et al., 2005). The low levels of activity could further be associated with fatigue, as muscle weakness is a common symptom in EAE (Baxter, 2007), or depression, as previous evidence has demonstrated a depressive-like behavioral syndrome in mice with EAE (Pollak et al., 2002). Further, 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 model. Altogether, our results indicate 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, and 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 if 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. Further, a research design employing the relapsing-remitting model of EAE is warranted to compare the effects of exercise across animal models as to date, research on exercise in EAE is not yet conclusive. Lastly, more information on the pathophysiological effects of exercise, such as BDNF, 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. Firstly, while previous research demonstrates 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 of the treadmill condition as not all mice were compliant with the protocol (i.e., 5 days/week at 5% grade, 14 m/min, for 30 minutes) 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, as the volume of exercise may have been too low to detect any differences in the exercise conditions compared to the sedentary condition. Further, 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). Lastly, it may be important to assess other neurodegenerative parameters (i.e., astroctyes or microglia activation) and inflammatory markers (i.e., leukocyte infiltration and recruitment, levels of chemokines and cytokines, and rupture of blood-brain barrier), that have previously been identified as alterations of the disease course (Frohman et al., 2008).

4.1 Conclusion

In summary, the current study demonstrates the amount and/or intensity of forced treadmill exercise and voluntary wheel running by mice with relapsing-remitting EAE, during remission after the initial disease onset, may need 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 no known or potential conflict 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 manuscript for important intellectual content: REK, BDP, JH, JAW, RWM.

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