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Validation of a Walking Wheel Method to Fragment Sleep in Rats

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Background and Objective Many attempts have sought to devise an animal model of sleep fragmentation (SF) to understand the clinical effects of sleep loss, but no study has investigated the usefulness of the walking wheel method to interrupt sleep in rats.

Methods Seven-week-old male Wistar rats were divided into a SF group and an exercise control (EC) group, with five rats in each. SF was achieved with a walking wheel using a 30 s on, 90 s off interval (total walking time 6 h/day). The EC group walked the same distance. Rat multiple sleep latency test was performed to measure sleepiness in rats. Sleep data were collected at baseline, and 4, 12, and 18 days after treatment. Percent (%) time spent awake and in non-rapid eye movement (NREM) and rapid eye movement (REM) sleep, NREM and wake bout number, NREM bout length, and mean sleep latency were analyzed.

Results Numbers of NREM and wake bout were higher in the SF group, whereas the NREM bout length was smaller than in the baseline group. Unlike NREM, REM sleep (%) in the SF group was significantly lower than in the baseline and EC groups. Mean sleep latency was shortened in the SF group compared to the baseline and EC groups. EC did not differ significantly with respect to the amount of sleep time (%), bout number of NREM and wakefulness, NREM bout length, and mean sleep latency, compared to its own baseline.

Conclusions Use of a walking wheel is an effective means of interrupting sleep in rats during a long-term period.

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Key Words Sleep fragmentation, Exercise control, Walking wheel, Rat multiple sleep latency test, Bout.

INTRODUCTION

Sleep is a vital process required for normal body function. Accumulated sleep loss is physiologically and behaviorally harmful to humans and animals.[1] Sleep fragmentation (SF) is caused by the frequent interruption of sleep and is frequently associated with extrinsic or intrinsic events, such as noise, sleep apnea, periodic limb movement, chronic pain syndrome, and intensive care unit syndrome.[2] Experimentally induced-SF causes behavioral and structural alterations including cognitive and spatial memory,[3] loss of N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) in the hippocampal CA1 region,[4] and reduced dentate gyrus neurogenesis.[5,6]

Many researchers have used the sleep deprivation (SD) paradigm to understand the function and effect of sleep loss or disturbance in many species. However, it is not easy to make a long-term total SD (TSD) without the occurrence of uncontrollable episodes of sleep, called microsleep. Even in a relatively short study period of 24 h, microsleeping was present and the percentage amounted to 10-20% by the end of the 24 h,[7] making it difficult to investigate the clinical effects of sleep loss. Many attempts have been made to compare the effects of SF and SD. In terms of hormones,[8] respiratory parameters,[9] psychomotor performance,[10,11] and measure of sleepiness,[12] the two sleep manipulation paradigms exhibited similar effects, implying that SF is as much effective as TSD.

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Based on the findings that SF and SD share a common phenotype in terms of several physiological variables, attention has been focused on the development of an appropriate model regarding SF. Recently, a SF model was validated for animal use by a variety of tools including treadmill, orbital shaker, and sweeping bar.[4-6,13,14] Nevertheless, limitations in the current SF model include the evaluation of sleep patterns of SF rats during a relatively short period (1-7 days),[4,5,14] lack of an appropriate control,[13,14] validation of the model only in mice,[13,14] and significant reductions of non-rapid eye movement (NREM) and REM sleep.[14]

To our knowledge, no study has addressed the use of a walking wheel to fragment sleep in rats. The present study evaluated the effectiveness of an animal model of SF using a walking wheel system during a long-term period.

METHODS

Animals

Seven-week-old male Wistar rats (Orient Bio, Seongnam, Korea) weighing 210-230 g at surgery were used in this study. All animals were maintained in a temperature-controlled recording room with alternating 12-h cycles of light and dark (lights on at 8:00 a.m.). The rats had ad libitum access to food and water. Room temperature (24 ± 2°C) and humidity (50 ± 20%) were monitored continuously. All experimental procedures involving animals were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Committee of the Korea University College of Medicine (KUIACUC-2012-90).

Surgery

The animals were implanted with chronic recording devices for continuous recording of electroencephalography (EEG) and electromyography (EMG). With the animals under isoflurane anesthesia (induction 5%, maintenance 2-2.5%), holes were drilled through the skull bilaterally at -5.0 mm AP and 2.0 mm ML from the bregma. For the EEG, two stainless steel screws were inserted into the holes and a screw fixed in the left frontal bone served as a reference. For the EMG, implanted stainless steel wire electrodes were sutured into the dorsal neck muscle. EEG and EMG electrodes were then connected to a miniature connector and were affixed to the skull with dental cement. After surgery, the animals were placed in clean cages and allowed at least 14 days to recover from surgery.

EEG and EMG Recordings

After the 14-day recovery, the animals were connected to a cable and acclimatized to the processes for 7 days before acquisition of baseline EEG data. EEG and EMG signals were recorded and amplified on a digital polygraph (PowerLab, ADInstruments, Australia) at a sampling rate of 200 Hz. Filter ranges for EEG and EMG were set at 0.3-30 Hz and 10-200 Hz, respectively. After data collection was completed, sleep-wake states were semi-automatically scored into 10-sec epochs as waking, NREM sleep, or REM sleep by examination of EEG and EMG recordings, and were then confirmed by visual scoring using SleepSign software (Kissei Comtec, Nagano, Japan). Each vigilance state was determined after taking into account the visual and power spectrum value as follows: wake, low-amplitude EEG, and high-voltage EMG activities; NREM sleep, high-amplitude slow or spindle EEG, and low-EMG activities; and REM sleep, low-voltage EEG, and EMG activities. We analyzed the EEG and EMG data, scoring it in 10-s epochs as waking, NREM sleep, or REM sleep, and expressed the data as time spent in each state. A "bout" consisted of a minimum of two consecutive 10-s epochs for a given state and ended with any single state-change epoch. To analyze the EEG spectra during NREM sleep, we used a fast Fourier transformation algorithm on all epochs without a visually detectable artifact.

Experimental Design

A repeated measure design using its own baseline level as the control was adopted to assess the effect of SF on sleep parameters. Five rats were simultaneously employed for the sleep recording. Rats were subjected to the SF or EC procedure during the 18 consecutive days following baseline recording and rat multiple sleep latency test (rMSLT). EEG and EMG signals were recorded continuously during 24 h beginning from 8 PM at baseline, and on days 4, 12 and 18. On each recording day, beginning at 8 AM, the cables were connected to each rat for 12 h of acclimatization. On the day of baseline and after completing the 18 day recording, the experimental rats were again subjected to the rMSLT to assess sleepiness. A schematic timeline of our study is shown in Fig. 1.

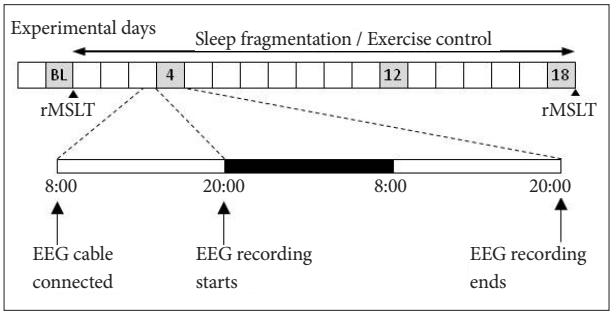

Fig. 1. Schematic experimental time schedule. The rats underwent surgery for sleep monitoring and recovered over a 14-day period. After recovery, the animals were located within a walking wheel for 7-day acclimatization. A 24-h baseline sleep recording was collected beginning from 20:00. rMSLT was performed between 17:00 and 20:00, and then the SF or EC procedure was carried out for 18 consecutive days. During the course of the experiment, 24-h sleep-wake recording was collected at day 4, 12 and 18. rMSLT was conducted again on the next day of SF 18 or EC 18. rMSLT: rat multiple sleep latency test, EEG: electroencephalography, SF: sleep fragmentation, EC: exercise control.

SF

SF was conducted by a rat forced exercise walking wheel system (Model number: 80805A; Lafayette Instruments, Lafayette, IN, USA). This apparatus consisted of six motor-driven individual wheels (internal diameter: 13.38 inches) and a controller box, which allowed the simultaneous rotation of the wheels with a set time interval. Briefly, experimental rats that had recovered from surgery were placed in a wheel cage 7 days before the baseline recording and allowed to acclimate to the novel environment. On recording days, the cables were connected to the socket on the head of each rat and to the data amplification device. Running speed was set at 7 cm/s, taking into account the daily average walking distance of a rat;[6] the selection of 4.5 m/min in this study reflected the fact that the built-in speed setting of the apparatus was only adjustable in 0.5 m increments. The wheel was activated for 30 s and remained stopped for 90 s, providing 30 awakenings per hour, as performed in other SF studies.[4,14] Thus, total walking time of a rat in the SF and exercise control (EC) groups were 6 h/day, which means that the rats in SF and EC groups ran 720 on/off cycles and 36 cycles of on/off cycles per day, respectively.

Exercise Control

The SF method used in this study employed forced movement to interrupt the sleep of the rats. An appropriate control is necessary to avoid confusing the interpretation of experimental results due to the nonspecific effects of movement itself. As previously reported,[4] we offered the same amount of walking distance to the rats, but with a wheel on/off schedule of 10 min on/30 min off, allowing for longer periods of undisturbed sleep. In theory, the condition generates 1.5 awakenings per hour in the movement control group.

rMSLT

The MSLT, which consists of a series of four to five nap opportunities at 2-h intervals during the day, has been recognized as the standard method of quantifying sleepiness in the field of sleep medicine.[15] In our study, we used a rMSLT developed to directly assess sleepiness in the rat, analogous to the human MSLT. rMSLT was conducted between 8 PM and 11 PM at the end of the rat's light/inactive period, as previously reported by McKenna et al.[16] Briefly, rats were stimulated to a gentle sensory noise, such as tapping on the side of the activity wheel, without directly touching the rats. rMSLT consisted of six separate trials, each with 5 min of sleep disturbance followed by 25 min of undisturbed sleep. During the allowed sleep of 25 min, EEG/EMG data were collected. Sleep latency, defined as the moment recording starts until the first 12 continuous 10-sec epochs, was scored as sleep.[17]

Data and Statistical Analyses

Values shown represent the mean ± SEM. To assess the effect of SF procedure on the sleep parameters between and within groups, a two-way repeated measure analysis of variance (ANOVA) with factor 'day' or 'group' was conducted. Whenever significant effects were present ($p < 0.05$) Tukey's post hoc was performed for multiple comparisons of length and number of stage episodes with those of days 4, 12 and 18 of SF or EC within the same experimental groups. An independent t-test was employed for the comparison of values between the SF and EC on the same days. A one-way ANOVA was conducted to evaluate the difference in mean sleep latency at baseline and at day 18 in the SF and EC groups, followed by the Tukey *post hoc* test. All statistical analyses were carried out using SPSS, version 15.0 (SPSS, Chicago, IL, USA). A p value of < 0.05 was considered statistically significant.

RESULTS

Hypnogram

Hypnogram analysis indicated that the SF procedure was effective throughout the study. The SF group consisted of more frequent transitions of the sleep stage than those of baseline and the EC groups, indicating highly fragmented sleep in the rats (Fig. 2). In addition, no significant adaptation to the procedure was observed until day 18 of SF as demonstrated by the shorter mean NREM bout length.

SF Increases Time Spent in Wake Stage

Percent of time spent in wake, NREM, and REM sleep periods was determined by sleep data recorded during a 24-h period at baseline, and on days 4, 12, and 18. During the SF procedures, both the SF and EC groups displayed significant increases in the percentage of time spent in the awake stage (SF: 43.1% to 45.3%, 45.4%, and 47.8% at day 4, 12 and 18, respectively; EC: 42.3% to 46.2%, 46.5%, and 45.8% at day 4, 12 and 18, respectively; Fig. 3A). In all days involving fragmentation, the wake time was significantly higher in both SF and EC groups, compared to the appropriate baseline levels ($p < 0.05$, paired t-test). There was no significance in the day-group interaction ($F = 0.4$, p = NS) as well as group difference ($F = 0.03$, p = NS).

SF Reduces Time Spent in REM Stage in the SF Group

The time spent in the REM stage was significantly reduced over the fragmentation period in the SF group (from 8% to 4.8-5.9%; $F = 26.2$, $p < 0.001$). Repeated measure ANOVA also revealed a significant overall group difference ($F = 17.3$, $p < 0.001$) as well as a significant group-day interaction ($F = 5.7$, $p < 0.05$). The average amount of REM sleep in the SF group was significantly lower than those in the EC group at all days of fragmentation (independent t-test, Fig. 3B). In contrast, no significant decrease in % REM sleep was observed in the EC group during

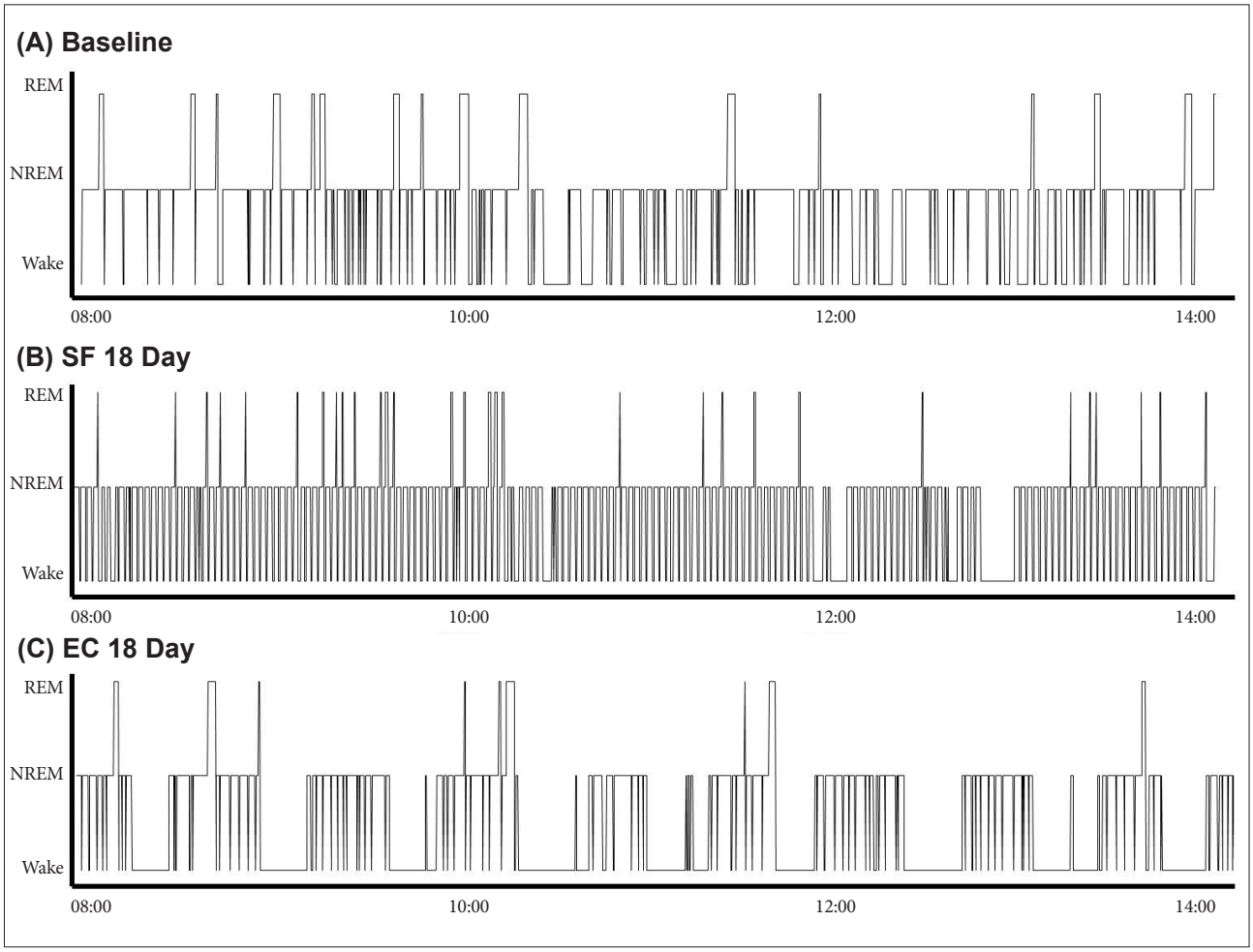

Fig. 2. Representative 6-h hypnogram recorded from the beginning of the 12-h light phase. SF represents highly fragmented sleep patterns compared with the baseline and EC group. A: Baseline group. B: 18 day sleep fragmentation (SF) group. C: 18 day exercise control (EC) group. REM: rapid eye movement, NREM: non-rapid eye movement.

the entire period of the experiment, compared to those in the baseline and SF group (p = NS, Tukey's *post hoc* test and independent t-test).

SF Does Not Affect the Time Spent in NREM Stage

There was no significant day, group, and day-group interaction in the NREM sleep in the repeated measure ANOVA (F = 1.5, F = 0.5, F = 0.4, p = NS, respectively), notwithstanding a trend toward shorter NREM sleep time in both SF and EC groups observed (Fig. 3C).

SF Alters NREM and Wake Bout Number and NREM Bout Length

The bout number and wake and NREM sleep lengths were determined by 24-h recorded sleep data at baseline, and on days 4, 12 and 18. Concerning NREM bout number over the entire experimental period, repeated measure ANOVA revealed significant day and group differences (F = 12.6, p < 0.05 and F = 71.1, p < 0.001, respectively; Fig. 4A) as well as a significant group-day interaction (F = 7.0, p < 0.001). The mean NREM bout number in regards to the 24 h recording from the SF rats was significantly higher than those in the EC group on days 4, 12, and 18 following the procedure (SF: day 4 616.6 ± 7.1, day 12 615.6 ± 10.8, day 18 600.4 ± 8.6; EC: day 4 380.6 ± 29.5, day 12 393.0 ± 23.9, day 18 398.0 ± 12.5, p < 0.001, independent t-test), whereas the baseline values did not differ statistically (429 ± 28.4 vs. 426.4 ± 50.5). The mean wake bout number was also higher on days 4, 12 and 18 compared to those in the EC group (SF: baseline 174.2 4 ± 31.2, day 4 603.2 ± 10.9, day 12 611.8 ± 9.6, day 18 580.4 ± 11.1; EC: baseline 187.6 ± 20.5, day 4 172.2 ± 12.3, day 12 162.2 ± 5.4, day 18 152.8 ± 3.9, p < 0.001, independent t-test) and baseline (p < 0.001, Tukey's *post hoc* test, Fig. 4B). The EC group did not show any significant differences in NREM and wake bout number compared to those in the baseline at all days of fragmentation.

While the NREM and wake bout number were increased by the SF procedure, mean NREM bout length decreased as rats underwent SF on all days of fragmentation, compared to the

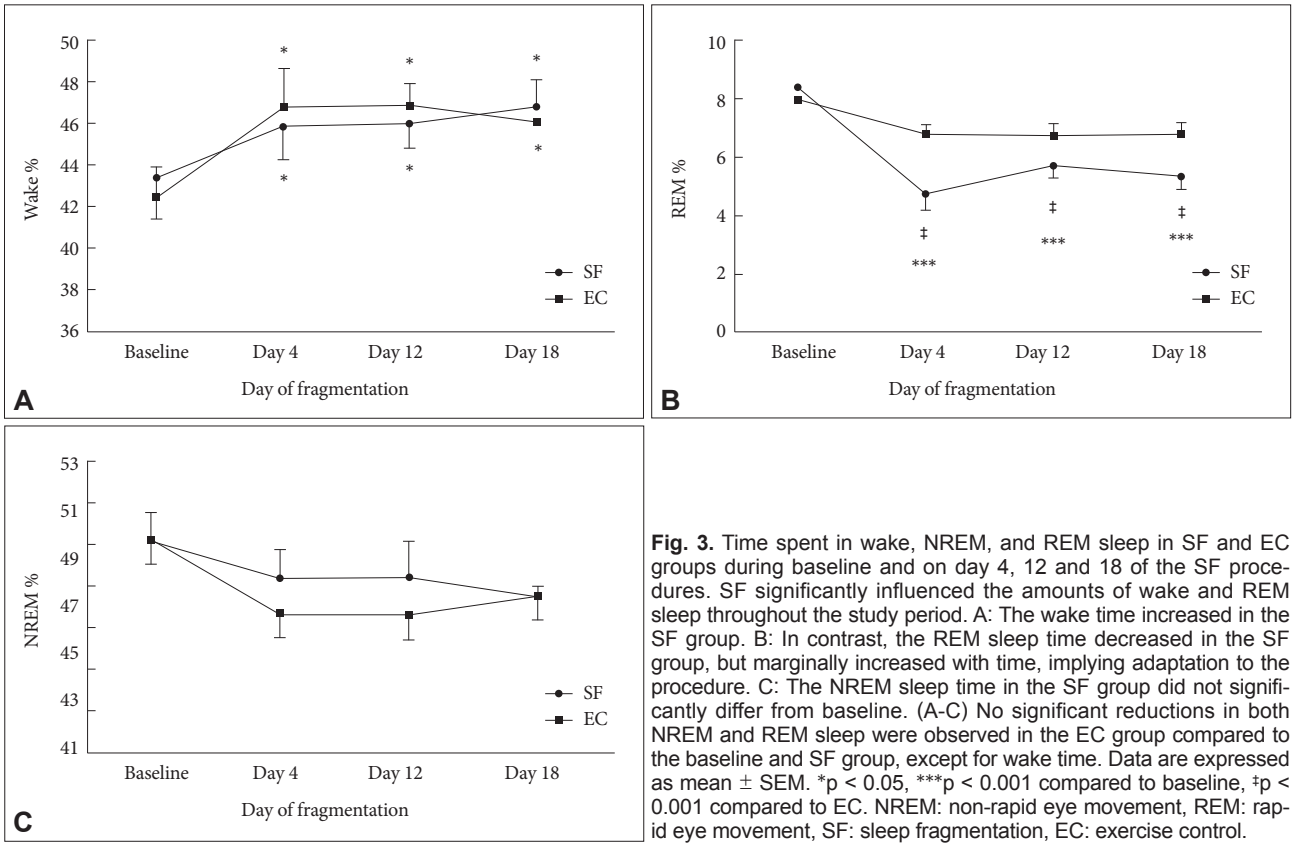

Fig. 3. Time spent in wake, NREM, and REM sleep in SF and EC groups during baseline and on day 4, 12 and 18 of the SF procedures. SF significantly influenced the amounts of wake and REM sleep throughout the study period. A: The wake time increased in the SF group. B: In contrast, the REM sleep time decreased in the SF group, but marginally increased with time, implying adaptation to the procedure. C: The NREM sleep time in the SF group did not significantly differ from baseline. (A-C) No significant reductions in both NREM and REM sleep were observed in the EC group compared to the baseline and SF group, except for wake time. Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.001 compared to baseline, ‡p < 0.001 compared to EC. NREM: non-rapid eye movement, REM: rapid eye movement, SF: sleep fragmentation, EC: exercise control.

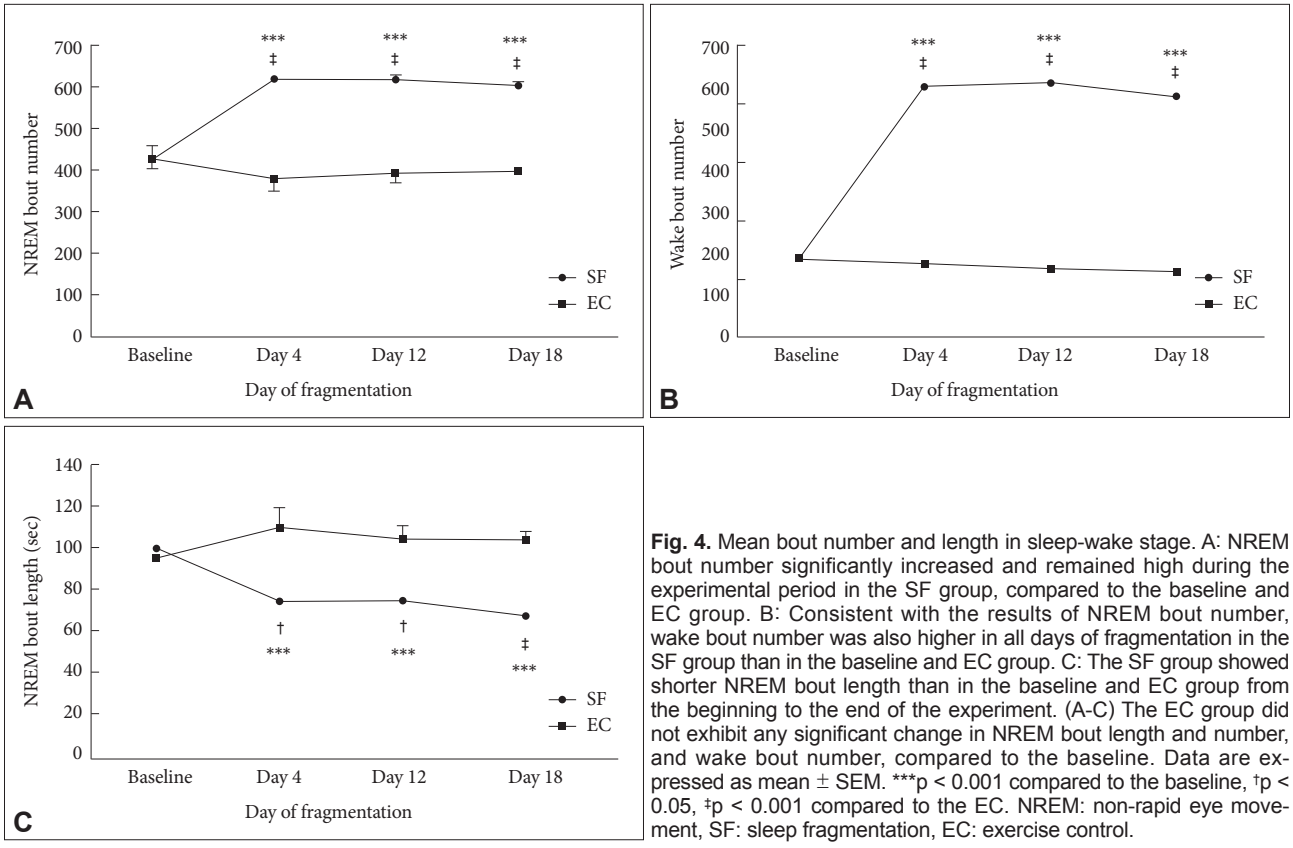

Fig. 4. Mean bout number and length in sleep-wake stage. A: NREM bout number significantly increased and remained high during the experimental period in the SF group, compared to the baseline and EC group. B: Consistent with the results of NREM bout number, wake bout number was also higher in all days of fragmentation in the SF group than in the baseline and EC group. C: The SF group showed shorter NREM bout length than in the baseline and EC group from the beginning to the end of the experiment. (A-C) The EC group did not exhibit any significant change in NREM bout length and number, and wake bout number, compared to the baseline. Data are expressed as mean ± SEM. ***p < 0.001 compared to the baseline, †p < 0.05, ‡p < 0.001 compared to the EC. NREM: non-rapid eye movement, SF: sleep fragmentation, EC: exercise control.

baseline and EC groups (SF: baseline 99.0 ± 3.5 s, day 4 74.2 ± 1.8 s, day 12 74.6 ± 0.6 s, day 18 67.2 ± 1.6 s; EC: baseline 94.4 ± 4.0 s, day 4 108.4 ± 9.8 s, day 12 103.2 ± 6.7 s, day 18 102.4 ± 4.5 s ($p < 0.001$, Tukey's *post hoc* test and independent t-test, Fig. 4C). The mean NREM bout length of the EC did not differ from the value of baseline at all fragmentation days ($p > 0.05$).

Mean Sleep Latency is Reduced by SF

In one-way ANOVA analysis, mean sleep latency (average of the sums from trial 1 to trial 6) was significantly reduced in the SF group compared to those in the baseline and EC groups (baseline 6.7 ± 0.9; SF 2.7 ± 1.0; 5.2 ± 1.3, $F = 15.6$, $p < 0.001$, Tukey *post hoc* test, Fig. 5).

DISCUSSION

The present data demonstrate that the activity wheel method of SF effectively and reliably interrupted sleep in rats during an 18-day assessment period. The numbers of NREM and wake bout were increased in the SF group, compared to the baseline and EC groups, whereas NREM bout length was reduced, reflecting effectively fragmented sleep during SF. Shortened mean sleep latency, assessed by rMSLT, demonstrated that the SF model produced sleepiness in the experimental rats. The percentage of time spent awake was significantly increased in the SF group, but the amount of REM sleep was decreased. NREM sleep in the SF group did not differ from the baseline level, but demonstrated a trend toward reduction.

One of the major clinical symptoms shown in patients complaining of fragmented sleep is excessive daytime sleepiness. Traditionally, the degree of sleepiness in animals has been determined by examining the level of NREM sleep delta power of the experimental group and comparing it with that of the baseline or control groups.[18] It has been established that the more the wake time increases, the greater the elevation of homeostatic sleep pressure, resulting in the accumulation of NREM delta power following sleep manipulations as TSD or selective SD. However, sleepiness can be decoupled from the amount of sleep and intensity during chronic sleep restriction; after chronic sleep restriction, sleepiness increases proportionally, whereas sleep intensity (as demonstrated by NREM delta power) and sleep time do not proportionally reflect homeostatic sleep pressure during the sleep restriction and recovery periods, suggesting that measuring NREM delta power cannot not be a surrogate indicator for sleepiness, especially in a condition of chronic sleep restriction.[19,20] Thus, we employed rMSLT, which is a well-established, objective, and direct method to measure sleepiness in rats,[16] for the comparison of sleepiness between the experimental groups in the present study.

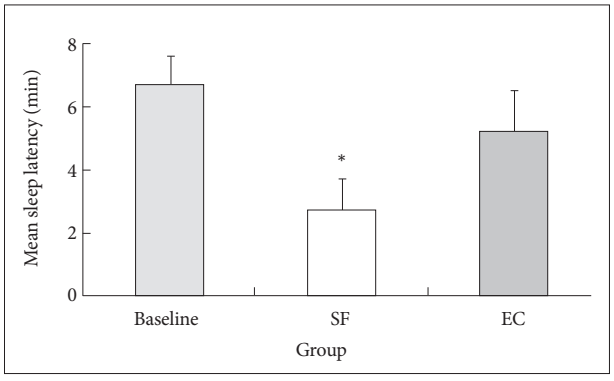

Fig. 5. Mean sleep latency (min) among baseline, 18-day SF and 18-day EC group. rMSLT was conducted twice, before and after the 18 day treatment, in both the SF and EC groups. Values of six trials in the SF and EC groups were merged, averaged, and expressed as a single baseline value to compare it with that of the SF or EC group. The mean sleep latency shortened in the SF group compared to the baseline. Data are expressed as mean ± SEM. *$p < 0.001$ compared to baseline. SF: sleep fragmentation, EC: exercise control, rMSLT: rat multiple sleep latency test.

Consistent with previous studies,[4,14] reduction of REM sleep in the SF group was presently observed, and continued throughout the study. However, notably, it seemed that rats adapted to the fragmentation condition, as evidenced by a mild increase in REM sleep on days 12 and 18, compared to day 4, although a return to the baseline levels was not observed.

It seems that SF itself, not an accompanying decrease in the amount of sleep, led to sleepiness in the SF group, as demonstrated by shortened mean sleep latency. But, the possibility that the loss of REM sleep in the SF group accounted for the reduced sleep latency cannot be excluded. As shown in Fig. 3, REM sleep was reduced to approximately 50% on days 4, 12 and 18 in the SF group as compared to the baseline. Although a direct comparison is difficult, sleep latency was shortened by 52% in rats deprived of REM sleep by the modified multiple platform method as compared to the baseline.[21] Thus, in addition to SF itself, a reduced amount of REM sleep may account for the increased sleepiness in the SF group. Nevertheless, further clarification is necessary.

We assessed the total time spent in sleep-wake stages in the EC group as the control, supplying the similar amount of walking distance to the rats. The EC group did not exhibit significantly different values of % amount of NREM and REM, NREM and wake bout number, NREM bout length, and mean sleep latency, compared to the baseline level, indicating that EC did not change the amount of sleep and did not result in accumulated sleep pressure following the procedure. Notably, although not statistically significant, the EC group revealed a trend toward a decrease of % NREM and REM sleep as compared to the baseline (Fig. 3B and C). Moreover, in accordance with the trend, the % time in the wake period increased in the EC group compared to its baseline without significant increase in sleep latency (Fig. 3A). Thus, it is conceivable that the EC group also underwent a partial sleep loss, leading to accumulated homeostatic sleep pressure. The 30-min resting period, however, was

likely sufficient for the rats to resolve the sleep pressure, as demonstrated by the lack of change of mean sleep latency compared to its baseline value. Therefore, given that the main complaint of individuals with SF is excessive daytime sleepiness, we believe that EC is a useful control for the non-specific movement effects of wheel running-induced SF.

In addition to preserving the amount of NREM sleep and the consistent number of awakenings from the beginning to the end of the experiment, using the walking wheel has a number of advantages. First, forward movement produced by the wheel is more natural to the rats than the orbital shaking method, which adapts horizontal movement. Second, this type of walking wheel system offers flexibility in conducting paradigms such as SF, SD, and forced exercise. Third, up to six rats can be simultaneously tested on separated individual wheels.

Several studies which have been attempting to investigate the influence of SF have employed the treadmill technique to induce SF in animals. To our knowledge, however, only one prior study has used the treadmill technique with a SF protocol of 30 s on/ 90 s off.[4] The study recorded EEG during only 24 h of SF. Because the effectiveness of the SF model of Tartar et al.[4] might be different due to the duration of SF, it is not easy to directly compare the two model systems in terms of sleep parameter. Nevertheless, SF with a 30 s activation/90 s inactivation cycle using a treadmill technique or a walking wheel system produced similar electrographic patterns. First, the % time in the wake stage significantly increased compared to baseline, whereas NREM sleep did not differ from the baseline value, in both treadmill and walking wheel methods. But the amount of REM sleep reduced in both the treadmill and walking wheel system. During the initial period of SF, rats subjected to the treadmill or walking wheel demonstrated reduced REM sleep, but gradually increased over time, although not attaining a baseline value. No significant difference in the amount of REM sleep was observed between the SF and the EC in the previous study, which employed the SF method by a computer-controlled treadmill with a cycle of 3 s activation following the detection of 30 s continuous NREM sleep during 11 days, whereas our walking wheel method did not.

We did not measure the level of stress elicited by the SF procedure. It has been regarded as a confounding variable in some studies attempting to isolate the specific role of SD itself as well as the SF effect,[20,22] in that some stress-mediating factors and sleep disturbance represent common deleterious effects on the body, especially on the brain. For example, elevation in corticosterone induces hippocampal change, including synaptic plasticity modification (i.e., long-term depression and LTP), morphological change, and neurotoxicity,[23] similar to those previously reported for the SF or SD.[4,6,24] Hence, determining the stress level in our SF model will be needed to interpret and compare results from the experiment.

In conclusion, we demonstrate that the SF model using a walking wheel is a reliable and effective method to interrupt sleep in rats during a long-term period. However, although our study demonstrated positive findings, further studies on the stress effects of the model are needed.

Acknowledgments

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Conflicts of Interest

The authors have no financial conflicts of interest.

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