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Original Article

ORIGINAL ARTICLE

Changes in Brain-Derived Neurotrophic Factor Expression Influence Sleep–Wake Activity and Homeostatic Regulation of Rapid Eye Movement Sleep

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Abstract

Study Objectives: Brain-derived neurotrophic factor (BDNF) expression and homeostatic regulation of rapid eye movement (REM) sleep are critical for neurogenesis and behavioral plasticity. Accumulating clinical and experimental evidence suggests that decreased BDNF expression is causally linked with the development of REM sleep-associated neuropsychiatric disorders. Therefore, we hypothesize that BDNF plays a role in sleep–wake (S–W) activity and homeostatic regulation of REM sleep.

Methods: Male and female wild-type (WT; BDNF +/+) and heterozygous BDNF (KD; BDNF +/–) rats were chronically implanted with S–W recording electrodes to quantify baseline S–W activity and REM sleep homeostatic regulatory processes during the light phase.

Results: Molecular analyses revealed that KD BDNF rats had a 50% decrease in BDNF protein levels. During baseline S–W activity, KD rats exhibited fewer REM sleep episodes that were shorter in duration and took longer to initiate. Also, the baseline S–W activity did not reveal any sex difference. During the 3-hour selective REM sleep deprivation, KD rats failed to exhibit a homeostatic drive for REM sleep and did not exhibit rebound REM sleep during the recovery S–W period.

Conclusion: Interestingly, both genotypes did not reveal any sex difference in the quality and/or quantity of REM sleep. Collectively, these results, for the first time, unequivocally demonstrate that an intact BDNF system in both sexes is a critical modulator for baseline and homeostatic regulation of REM sleep. This study further suggests that heterozygous BDNF knockdown rats are a useful animal model for the study of the cellular and molecular mechanisms of sleep regulation and cognitive functions of sleep.

Statement of Significance

Brain-derived neurotrophic factor (BDNF) is ubiquitous in the brain and plays a pivotal role in a myriad of developmental and regulatory functions, including sleep–wake (S–W) activity. Currently, no studies have investigated the influence of biological sex in the causal relationship between alterations of BDNF expression and rapid eye movement (REM) sleep regulation. The results of this study suggest that, regardless of sex, global reductions of BDNF expression in the brain significantly alter S–W architecture and the homeostatic regulation of REM sleep.

Keywords: heterozygous BDNF, REM sleep homeostasis, sleep–wake architecture.

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Introduction

Brain-derived neurotrophic factor (BDNF) activity regulates a variety of functions including neuronal development and plasticity [1–3]. The use of homozygous and heterozygous BDNF rodents has provided substantial evidence to support the neuronal prosurvival functions of BDNF [4, 5]. A number of studies have shown that mice lacking BDNF develop severe sensory deficits and fail to survive past 3 weeks [6, 7]. Comparatively, heterozygous BDNF rodents have a normal lifespan and do not exhibit obvious changes in neuronal density or morphology but do exhibit deficits in neuronal and behavioral plasticity [5, 8–14].

Activity-dependent synaptic plasticity is most associated with hippocampal and amygdala BDNF [15–20]. BDNF activity in the brain is primarily mediated by the tropomysin kinase B (TrkB) receptor, and several studies have shown that hippocampal long-term potentiation (LTP) is induced by BDNF/TrkB signaling [15, 21–25]. This effect was further validated in studies using BDNF knockdown and TrkB knockout mice that revealed learning was impaired due to dysfunctions of BDNF/TrkB signaling [8, 26–28]. Overall, these studies provide evidence that BDNF signaling is a critical component of the cellular mechanisms involved in learning and memory.

Given the importance of BDNF signaling, it is interesting that BDNF expression is significantly altered following sleep deprivation [29–32]. In fact, both animal and human studies using sleep deprivation techniques have shown that disruption of sleep induces cognitive deficits in performance, mood, and memory [33–39]. More specifically, disruptions in rapid eye movement (REM) sleep have been shown to dysregulate the same processes that depend on BDNF, such as learning and memory [35, 40–43]. This functional link led to a suggestion that BDNF plays a critical role in the regulation of REM sleep, and it has been recently shown that BDNF/TrkB signaling in the pedunculopontine tegmentum (PPT) is critical for the development of REM sleep homeostatic drive [31, 44]. Additionally, other studies have suggested that BDNF regulates non-REM sleep [45–48]. Clearly, there is strong evidence to suggest that BDNF plays a critical role in regulating sleep, but the precise nature of this role remains to be unclear. Furthermore, there is mounting evidence that neuropsychiatric disorders are associated with decreased levels of BDNF expression [49–53]. In neuropsychiatric disorders, sleep patterns and sleep quality have been shown to differ among biological sexes [54–60]. However, currently, no studies have investigated the causal relationship between alterations of BDNF expression and biological sex differences in sleep. Therefore, this study was designed to understand the causal role of BDNF in sex-dependent sleep–wake activity and homeostatic regulation of REM sleep.

Methods

Animals and Housing Conditions

The following experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of Tennessee Animal Care Committee (Protocol Number: 2349-UTK). Experiments were conducted on male and female BDNF wild-type (BDNF +/+) and BDNF heterozygous knockdown (BDNF +/–) Sprague Dawley rats, weighing 250–350 g (Sage Labs, Boyertown, PA). Final experiments were performed on a total of 24 rats (six rats/group; four groups: BDNF +/+ male, BDNF +/+ female, BDNF +/– male, and BDNF +/– female). All animals were housed individually in a temperature-controlled room (24°C) with 12-hour light/dark cycle (lights on from 06:00 am to 06:00 pm) and with free access to food, water, and enrichment material. To reduce experimental stress, animals were habituated to daily handling (twice a day for 15 minutes) until the recording session began. Additional care was taken to ensure that the number of animals used was minimized and any potential discomfort was eliminated.

Surgical Procedures for Electrode Implantation

All surgical procedures were performed stereotaxically under aseptic conditions, as described previously [31, 44]. Briefly, while animals were under isoflurane anesthesia (5% induction; 2%–3% maintenance in 100% oxygen), electrodes were chronically implanted to enable recording of cortical electroencephalogram (EEG), hippocampal EEG, and nuchal electromyogram (EMG). All electrodes were secured to the skull with dental acrylic, crimped to miniconnector pins, and brought together in a plastic connector, which was secured to the skull with additional dental acrylic. Upon completion of the surgical procedure, animals were administered saline (5 cc, s.c.) to prevent dehydration. Postoperative pain was controlled with buprenorphine (0.05 mg/kg, I.M.; Cerilliant, Round Rock, TX, USA).

Habituation Recording Session

Following 3–7 days of postsurgical recovery in home cage housing, rats were individually habituated to the recording procedure over the next 8–10 days. Six-hour habituation recording sessions were performed in the sleep–wake (S–W) recording chamber (Pinnacle 8238: 12" diameter × 12" tall; Pinnacle Technology Inc., Lawrence, KS, USA) during normal sleeping hours for rats (09:00 am–03:00 pm) [61]. Each recording chamber supplied free access to food, water, and enrichment material. Additionally, a customized multichannel preamplifier with a flexible cable was secured to the plastic connector mounted on the head of each rat and then mated to a commutator mounted above the cage to allow unrestricted movement. Each habituation recording monitored baseline S–W activity. The last day of habituation recording was determined when the amount of daily variation in the total amount of REM sleep was less than 5% for three consecutive days.

S–W Recording and Selective REM Sleep Restriction Protocol

The preamplifier connected to each rat's head amplified the cortical EEG, hippocampal EEG, and EMG signals by 100×. The signals were received by a commutator, which were then conditioned by an analog filter. EEG signals were sampled at 1 kHz, and band-pass filtered between 0.5 and 100 Hz. EMG signals were sampled at 2 kHz, and band-pass filtered between 10 and 200 Hz. In addition to these physiological signals, the animals' behavior was monitored continuously using a video camera attached above the recording cages. Behavioral and physiological data were synchronously recorded and imaged using Sirenia® Acquisition software (Pinnacle Technology, Inc.). Using Sirenia® Sleep Pro

software (Pinnacle Technology, Inc.), the data were visually scored by an investigator blinded to the treatment conditions. Three behavioral states were distinguished: wakefulness (W), non-REM (NREM) sleep, and REM sleep. The physiological criteria for the identification of these S–W states are described in earlier publications [31, 62]. In the present study, the behavioral states were scored in successive 6-second epochs. For selective REM sleep deprivation, we used a method as previously described and validated in our earlier publications [31, 35, 44]. Briefly, for the purpose of selective REM sleep deprivation, the beginning of each REM sleep episode was identified by observation of ongoing physiological signs (cortical EEG, hippocampal EEG, and EMG) and video of behavioral activity. From the room adjacent to the rat, where the experimenter is observing the physiological signs and videos of animal behavior, the experimenter pressed a mechanical lever within 2–3 seconds of REM sleep onset. This caused the animal's head to be gently lifted (between 1.0 and 1.5 inch), resulting in termination of the animal's REM sleep episode. An important advantage of this selective REM sleep deprivation method is that >75% of REM sleep is successfully eliminated without significantly reducing SWA. Another advantage of this method is that it can induce homeostatic drive for REM sleep within a very short period of time.

Spectral Power Analysis of Slow-Wave Activity

For spectral power analysis, amplified and filtered cortical EEG data were digitized at a sampling frequency of 200 Hz, as described previously [31, 63]. The digitized data were then subjected to a fast Fourier transformation from 0.5 to 100 Hz with an interval of 0.2 Hz. (Sirenia Sleep Pro, Pinnacle Technologies, Inc.). To calculate the total power/hour during total period of time spent in NREM sleep in a 6-hour recording session (bin width of 6 seconds) for the δ wave frequency band (slow-wave activity [SWA]: 0.5–4.5 Hz), the power for the δ frequency band was averaged and expressed as a percentage of the total power within the frequency range of 0.5–100 Hz.

Experimental Design

The first experimental recording session began the day after the last habituation recording session ended. On the first experimental recording day, each rat was brought to the recording cage at 08:55 am and then connected to the S–W recording system. During these recording sessions, each rat was allowed 6 hours (between 09:00 am and 03:00 pm) of undisturbed S–W activity. At the end of these recordings, rats were disconnected from the S–W recording system and transferred back to their home cage. On the second experimental recording day, each rat was again connected to the S–W recording system at 08:55 am and recorded for 6 hours (between 09:00 am and 03:00 pm) of S–W activity. The procedures for the first and second experimental recording days are almost identical except each rat was subjected to a selective REM sleep deprivation protocol during the first three hours of the 6-hour S–W recording session, as described previously [31]. Briefly, within 3–5 seconds of REM sleep onset, each REM sleep episode was terminated using pulley-driven head-lift method. At the conclusion of these experiments, rats were euthanized with an overdose of isoflurane. Immediately following euthanization, brain tissue was collected for the quantification of BDNF using a standard enzyme linked immunosorbent assay (ELISA) technique and tail snips were taken from each rat to determine BDNF sequencing.

Quantification of BDNF Protein Levels and Determination of the BDNF Gene Sequence in Wild-Type and BDNF KD Rats

Brains were quickly removed and snap-frozen as described previously [31]. The frontal cortex (F-CTX) was dissected on an ice-chilled Petri dish and stored in prechilled microcentrifuge tubes at –80°C until further processing. All animals were sacrificed at a fixed time to rule out any variations due to differences in the S–W state at time of death and any diurnal factors contributing to the different levels of BDNF. The amount of BDNF (BDNF/mg total protein) was determined in each rat using a standard ELISA technique as described previously [31, 44].

In order to determine the BDNF gene sequence in the WT and KD rats, DNA was extracted from 4- to 6-mm tail snips using the DNEasy Blood and Tissue Kit (Qiagen, Venlo, The Netherlands), in accordance with the manufacturer's protocol. Extracted DNA was combined with the forward and reverse primers (SAGE labs, Boyertown, PA) and the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Thermo Fisher, Foster City, CA) in order to amplify the BDNF gene using a PCR approach. The PCR conditions were an initial denaturation at 95°C for 5 minutes, followed by 95°C for 30 seconds, 60°C for 30 seconds, 35 cycles of 68°C for 40 seconds, and a final extension at 68°C for 5 minutes. PCR products were prepared for sequencing using Exo-SAP (Affectrix, Thermo Fisher, Foster City, CA). DNA samples were sent to the University of Tennessee Genomics Core for sequencing. The resulting sequence files were aligned and analyzed for percent agreement (99% for all the WT and BDNF KD samples) and deletion location using SeqMan Pro and MegAlign software (DNASTAR, Inc., Madison, WI).

Statistical Analysis

To assess the effects of BDNF genotype and sex on the behavioral and physiological measures of S–W activity, during the first experimental recording day, the percentages of time spent in W, NREM sleep, REM sleep, NREM sleep, and SWA NREM were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. These analyses used time as within subject variable (six levels corresponding to consecutive 1-hour intervals across 6-hour recording sessions) and group as a between-subject variables (BDNF +/+ male, BDNF +/+ female, BDNF +/– male, BDNF +/– female). The number of W, NREM, and REM sleep episodes; mean duration of W, NREM, and REM sleep episodes; and REM sleep latency during the first experimental recording day were all analyzed using one-way ANOVA followed by Bonferroni post hoc tests to determine the individual level of significant differences between groups. These analyses revealed no significant differences between sexes; therefore, we collapsed the groups into genotypes for the second experimental analysis. In order to assess the effects of selective REM sleep restriction on the behavioral and physiological measures of S–W activity, the percentages of time spent in W, NREM sleep, and REM sleep as well as the number of REM sleep episodes were analyzed using two-way ANOVA followed by Bonferroni post

hoc tests, with time as a repeated-measure variable (six levels corresponding to consecutive 1-hour intervals across 6-hour recording sessions) and treatment as a between-subjects variable (two levels corresponding to the two different treatment groups, BDNF +/+ and BDNF –/+). NREM sleep SWA during selective REM sleep deprivation was analyzed using one-way ANOVA followed by Bonferroni post hoc tests. All statistical analyses were performed using Graphpad Prism statistical software (v5.0; Graphpad Software, La Jolla, CA, USA).

Results

KD BDNF Rats Display a 50% Decrease in BDNF Protein Levels and Have a Seven Base-Pair Deletion in the BDNF Gene Sequence

Figure 1A summarizes the baseline levels of BDNF protein in the F-CTX of each group (WT-male, WT-female, KD-male, and KD-female) using ELISA. Overall, the KD rats display ~50% of the BDNF protein levels of the WT rats. The WT-males expressed 10 pg/mg BDNF protein in the F-CTX compared with 5.2 pg/mg BDNF protein levels in KD-males. Similarly, the KD-females expressed 11.1 pg/mg BDNF protein in the F-CTX compared with 4.9 pg/mg BDNF protein levels in KD-females. Also, analysis of the BDNF genotype sequence revealed a seven base-pair deletion in the male and female KD rats. The deletion point was detected by the presence of ambiguous bases. Thus, two base-pairs were detected at one point to indicate that the BDNF gene was heterozygous in the KD rats. This is illustrated by the chromatogram presented in Figure 1B, which shows the appropriate base-pairs for the WT BDNF rats and Figure 1C, a point seven base-pair downstream deletion for the KD rats.

BDNF Genotype But Not Biological Sex Influences S–W Architecture

The hypnograms presented in Figure 2 show representative baseline S–W architecture for the first 6-hour experimental recording session in the male and female, WT, and KD rats. In general, the durations of the S–W cycles in the KD rats are much shorter than the WT rats. The hypnograms show that both the male and female KD rats exhibited fewer and shorter REM sleep episodes than the male and female WT rats. Furthermore, the male and female KD rats displayed greater latency between the beginning of recordings and the first episode of REM sleep than the male and female WT rats. These S–W architectures reveal differences between genotypes but not biological sex (Figure 2).

Influence of BDNF Genotype and Biological Sex on REM Sleep

Figure 3A summarizes the four experimental groups' (WT-male, WT-female, KD-male, and KD-female) baseline values of the total percentage of time spent in REM sleep during the first experimental recording session. Two-way ANOVA indicated significant main effects of group ($F_{(3,20)} = 45$, $p < .001$) and time ($F_{(5,100)} = 2.8$, $p = .021$) but an insignificant group × time interaction ($F_{(3,20)} = 1.5$, $p = .133$) on the total percentages of time spent in REM sleep. Post hoc tests (Bonferonni's multiple comparisons) revealed that, during each hour of the 6-hour recording session, the male KD rats spent significantly less time in REM sleep than the male WT rats (Figure 3A; 1 hour: $t = 5.2$, $p < .001$; 2 hours: $t = 6.7$, $p < .001$; 3 hours: $t = 5.4$, $p < .001$; 4 hours: $t = 4.7$, $p < .001$; 5 hours: $t = 5.6$, $p < .001$; 6 hours: $t = 7.5$, $p < .001$). Similarly, compared with the female WT rats, the female KD rats spent significantly less time in REM sleep in all hours (Figure 3A; 1 hour: $t = 3.5$, $p < .01$; 2 hours: $t = 5.1$, $p < .001$; 3 hours: $t = 3.5$, $p < .01$; 4 hours: $t = 5.3$, $p < .001$; 5 hours: $t = 3.5$, $p < .01$; 6 hours: $t = 5.3$, $p < .001$). Similar post hoc tests on the total percentages of time spent in REM sleep in WT and KD rats did not reveal any significant difference between the males and females. These results indicate that the KD male and female rats spent significantly less time in spontaneously occurring REM sleep than the WT male and female rats.

To further investigate differences in REM sleep, we ran one-way ANOVA between the four groups of animals (WT-male, KD-male, WT-female, KD-female), which revealed significant differences in the total number of REM sleep episodes ($F_{(3,20)} = 19$, $p < .001$), the

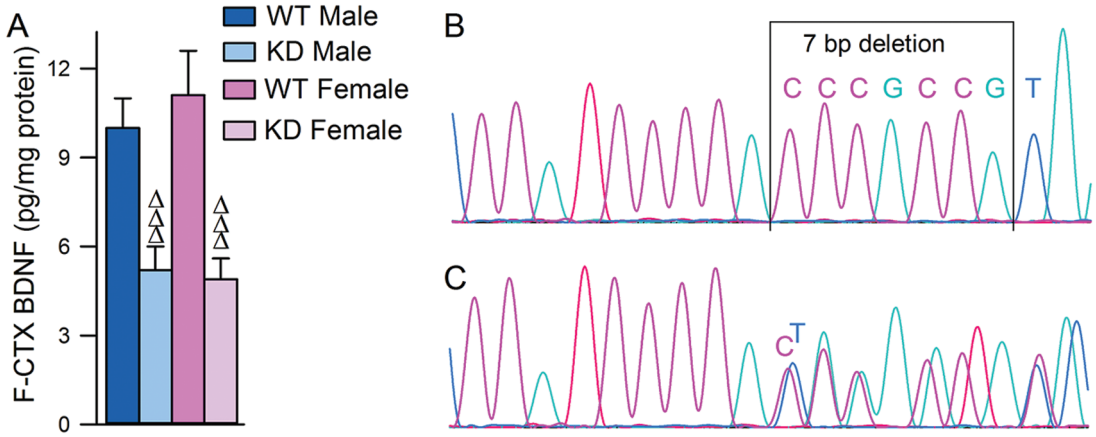

Figure 1. Quantification of baseline BDNF protein levels in the F-CTX and determination of the BDNF gene sequence in BDNF +/+ (WT) and BDNF +/– (KD) rats. (A) Histogram displaying the average amount of total BDNF protein levels (mean ± SD) measured in the F-CTX of WT and KD male and female rats after the first 6-hour experimental recording sessions. Note that compared with the WT male and female rats, KD male and female rats expressed ~50 less BDNF protein in the F-CTX under baseline conditions. (B) Chromatogram representing a 34 base-pair (bp) DNA segment of the BDNF gene in WT male and female rats. (C) Chromatogram representing a 34 bp DNA segment of the BDNF gene with double peaks at the seven bp deletion point in KD male and female rats. Note that the double peaks at the deletion point indicate that the BDNF gene is absent from half of the genetic material sequence.

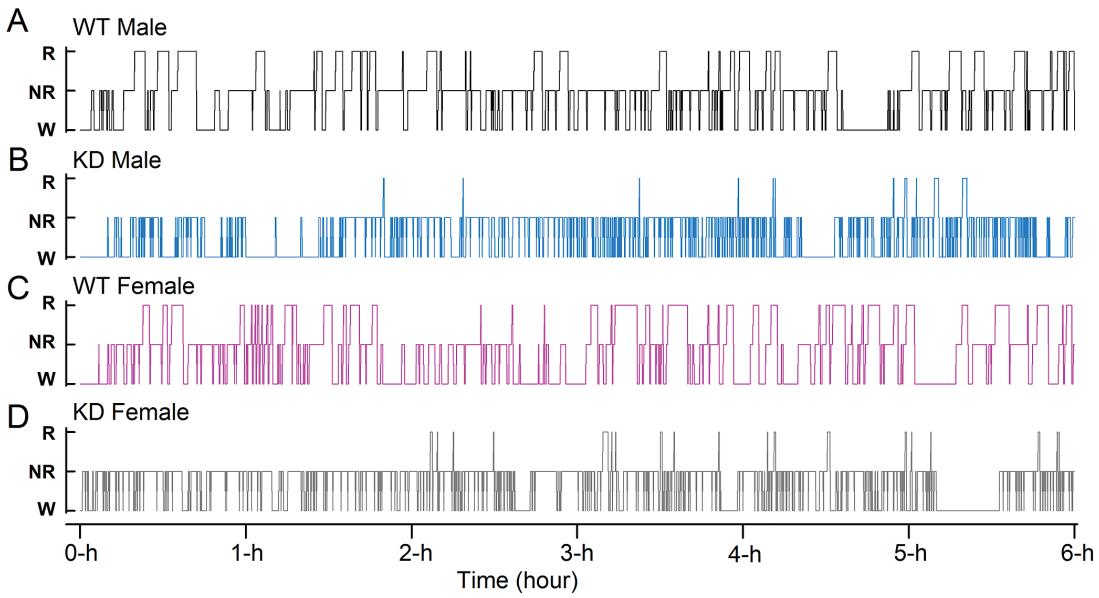

Figure 2. Representative hypnograms reflecting differences in sleep–wake (S–W) architecture between BDNF wild-type (BDNF +/+) and BDNF heterozygous knockdown (BDNF +/–) rats. These continuous 6-hour step histograms plot the occurrence and duration of physiologically and behaviorally defined states of wakefulness (W), NREM sleep (NR), and REM sleep (R) during the first experimental recording session (between 09:00 am and 03:00 pm). (A) represents the S–W architecture in a BDNF +/+ (WT) male, (B) S–W architecture in a BDNF +/– (KD) male, (C) S–W architecture in a BDNF +/+ (WT) female, and (D) S–W architecture of a BDNF +/– (KD) female. Note that both the male and female KD (BDNF +/–) rats exhibited fewer REM sleep episodes and the duration of those REM sleep episodes were shorter compared with the male and female WT (BDNF +/+) rats. Also note that in both WT (BDNF +/+) and KD (BDNF +/–) rats, there are no sex differences in S–W architecture.

mean duration of REM sleep episodes ($F_{(3,20)} = 29$, $p < .001$), and REM sleep latency ($F_{(3,20)} = 7.5$, $p = .002$). Post hoc tests revealed that male KD rats had significantly fewer REM sleep episodes compared with the male WT rats (Figure 3B; $t = 5.4$, $p < .001$). In comparison to WT females, the female KD rats showed similar reductions in number of REM sleep episodes (Figure 3B; $t = 5.0$, $p < .001$). Neither genotype exhibited significant sex differences in number of REM sleep episodes (Figure 3B; WT: $t = 1.0$, $p > .05$; KD: $t = 1.3$, $p > .05$). Post hoc tests on the mean duration of REM sleep episodes revealed that the male KD rats had significantly shorter REM sleep episodes than the male WT rats (Figure 3C; $t = 8.1$, $p < .001$). Similar alterations were found in the females; the average duration of REM sleep episodes was shorter in the KD rats compared with the WT rats (Figure 3C; $t = 4.1$, $p < .01$). Additionally, the mean REM sleep episode duration was slightly shorter in the WT females compared with the WT males (Figure 3C; $t = 3.4$, $p < .05$), but no sex differences were found in the KD rats (Figure 3C; $t = 0.60$, $p > .05$). Post hoc tests also revealed that male KD rats had a significantly longer REM sleep latency than the WT male rats (Figure 3D; $t = 4.1$, $p < .01$). REM sleep latency in the female KD rats was also longer compared with the WT females, but, in contrast to the males, the difference was not significant (Figure 3D; $t = 1.8$, $p > .05$). No sex differences in REM sleep latency were found in either genotype. Overall, the KD rats showed significantly stunted REM sleep resulting from increased latency to REM sleep, decreased mean duration of REM sleep episodes, and reductions in total number of REM sleep episodes.

Wake and Non-REM Sleep Episodes Are Higher in BDNF Heterozygous Rats

Figure 4A summarizes each groups' (WT-male, KD-male, WT-female, KD-female) total percentage of time spent in wakefulness during the first experimental recording session. A two-way ANOVA revealed significant effects of both group and time ($F_{(3,20)} = 8.0$, $p = .001$; $F_{(5,100)} = 4.7$, $p = .001$) but found no significant group × time interaction ($F_{(5,100)} = 1.4$, $p = .181$) on the total percentage of time spent in wakefulness. Post hoc comparisons revealed that, during the first and second hour of the 6-hour recording session, the male KD rats spent significantly more time in wakefulness than the male WT rats (Figure 4A; 1 hour: $t = 2.9$, $p < .05$; 2 hours: $t = 3.0$, $p < .05$). Overall, both male and female KD rats spent more time awake than the WT rats; however, the male KD rats spent significantly more time in wakefulness during the first and second hour of the 6-hour recording session. Surprisingly, the female KD and WT rats did not significantly differ in the percent of time spent in wakefulness (Figure 4A). These results collectively imply that, though some differences were found, the percent of time spent in wakefulness was largely unaffected by sex.

Investigations into other wakefulness parameters using one-way ANOVA between the four groups of animals (WT-male, KD-male, WT-female, KD-female) revealed significant differences in the total number of wake episodes ($F_{(3,20)} = 19$, $p < .001$) but not in the mean duration of wake episodes ($F_{(3,20)} = 2.8$, $p = .065$). Post hoc tests revealed that the male KD rats had a significantly higher total number of wake episodes compared with the male WT rats (Figure 4B; $t = 5.6$, $p < .001$). The female rats showed a similar trend with female KD rats exhibiting significantly more wake episodes than female WT rats (Figure 4B; $t = 5.0$, $p < .001$). Similar post hoc analyses on the mean duration of wake episodes did not reveal any significant genotype or sex difference (Figure 4C). These results indicate that the total amount of wakefulness was not influenced by sex.

A two-way ANOVA on the total percentage of time spent in NREM sleep revealed a significant effect of time ($F_{(5,100)} = 5.8$, $p < .001$), but there was no significant effect of group ($F_{(5,100)} = 1.1$, $p = .372$) or interaction ($F_{(5,100)} = 1.3$, $p = .190$). Post hoc comparisons

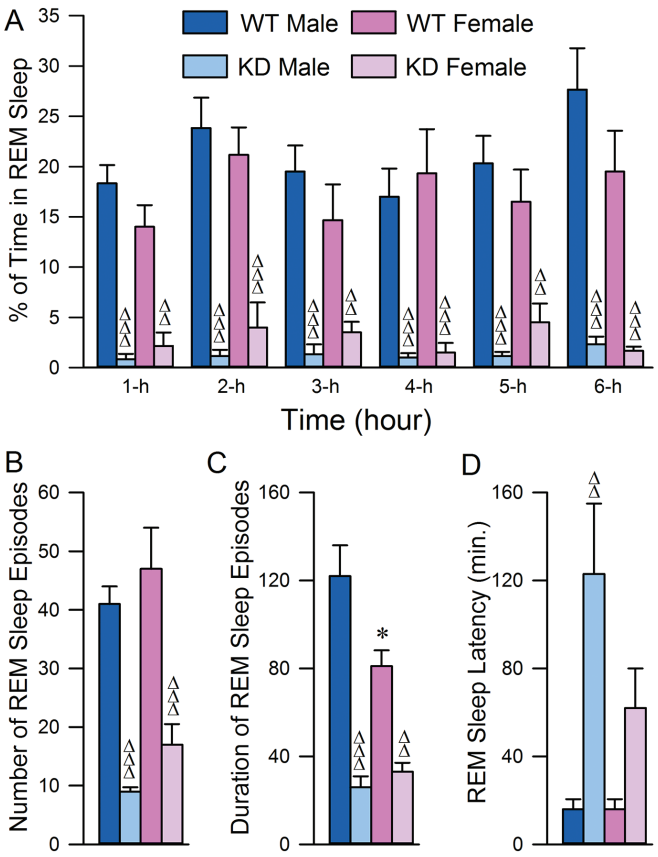

Figure 3. Effects of BDNF genotype (BDNF +/+ and BDNF +/–) and sex (male and female) differences in REM sleep during the first experimental recording session (between 09:00 am and 03:00 pm). (A) Total percentages of time spent in REM sleep (mean ± SE) during consecutive 1-hour intervals. Note that compared with the WT (BDNF +/+), both KD (BDNF +/–) male and female rats spent significantly less time in REM sleep. (B) Total numbers of REM sleep episodes (mean ± SE) during the first experimental recording session. Note that compared with the WT (BDNF +/+), the KD (BDNF +/–) male and female rats displayed fewer REM sleep episodes. (C) Mean duration of REM sleep episodes (in seconds; mean ± SE). Compared with the WT (BDNF +/+), the KD (BDNF +/–) male and female rats had a significantly shorter duration of REM sleep episodes. Interestingly, the duration of REM sleep episodes in the WT (BDNF +/+) male group is significantly longer than the WT (BDNF +/+) female group. (D) REM sleep latency (in minutes; mean ± SE). Note that the KD rats take a significantly longer time to transition into REM sleep compared with WT (BDNF +/+). Asterisk indicates the level of significant (Bonferroni posttests) differences relative to sex (*$p < .05$). Triangle indicates the level of significant (Bonferroni posttests) differences relative to BDNF genotype (ΔΔ, $p < .01$; ΔΔΔ, $p < .001$).

revealed no significant genotype or sex differences on the percentage of time spent in NREM sleep (Figure 4D). However, additional analyses using one-way ANOVA revealed significant differences in both the total number of NREM sleep episodes ($F_{(3,20)} = 20$, $p < .001$) and in the mean duration of NREM sleep episodes ($F_{(3,20)} = 28$, $p < .001$). Post hoc tests revealed that the male KD rats had a significantly higher total number of NREM sleep episodes compared with the male WT rats (Figure 4E; $t = 5.6$, $p < .001$). Likewise, compared with the female WT rats, the female KD rats had significantly more NREM episodes (Figure 4E; $t = 5.2$, $p < .001$). No sex differences in the total number of NREM sleep episodes were found in either genotype (Figure 4E). Post hoc comparisons also revealed that the mean duration of NREM sleep was significantly shorter in the KD males than in WT males (Figure 4F; $t = 7.7$, $p < .001$). KD females showed similar results, with their mean duration of NREM sleep being significantly shorter than the WT females (Figure 4F; $t = 4.8$, $p < .001$). In both genotypes, no sex differences were found in number of NREM sleep episodes. Having documented no large influence of biological sex on these S–W variables, male and female S–W data gathered in the second experimental recording session were combined and grouped based on genotype.

SWA Is Comparable Between WT and KD Rats During Baseline and Selective REM Sleep Deprivation

In order to investigate the effects of sex (male and female) and genotype (WT and KD) on NREM sleep SWA, we compared SWA during baseline and selective REM sleep deprivation recording sessions. The results of the two-way ANOVA on baseline NREM sleep SWA revealed a significant effect on time ($F_{(5,100)} = 12$, $p < .001$) and a significant time × group interaction ($F_{(15,100)} = 2.5$, $p = .003$), but there was no significant effect of group ($F_{(3,20)} = 0.82$, $p = .497$). Bonferroni's post hoc tests did not reveal any significant genotype or sex differences in baseline NREM sleep SWA activity (Figure 5A). In order to analyze the effects of selective REM sleep deprivation on NREM sleep SWA, we used one-way ANOVA between the selective REM sleep deprivation and REM sleep recovery period. The one-way ANOVA did not reveal any significant difference in WT-male ($F_{(5,30)} = 0.89$, $p = .498$), KD-male ($F_{(5,30)} = 0.38$, $p = .859$), WT-female ($F_{(5,30)} = 0.74$, $p = .597$), and KD-female rats ($F_{(5,30)} = 0.13$, $p = .985$). Further analysis using post hoc tests between individual 1-hour intervals did not reveal any significant differences in NREM sleep SWA in WT-male (Figure 5B), KD-male (Figure 5C), WT-female (Figure 5D), and KD-female (Figure 5E) rats.

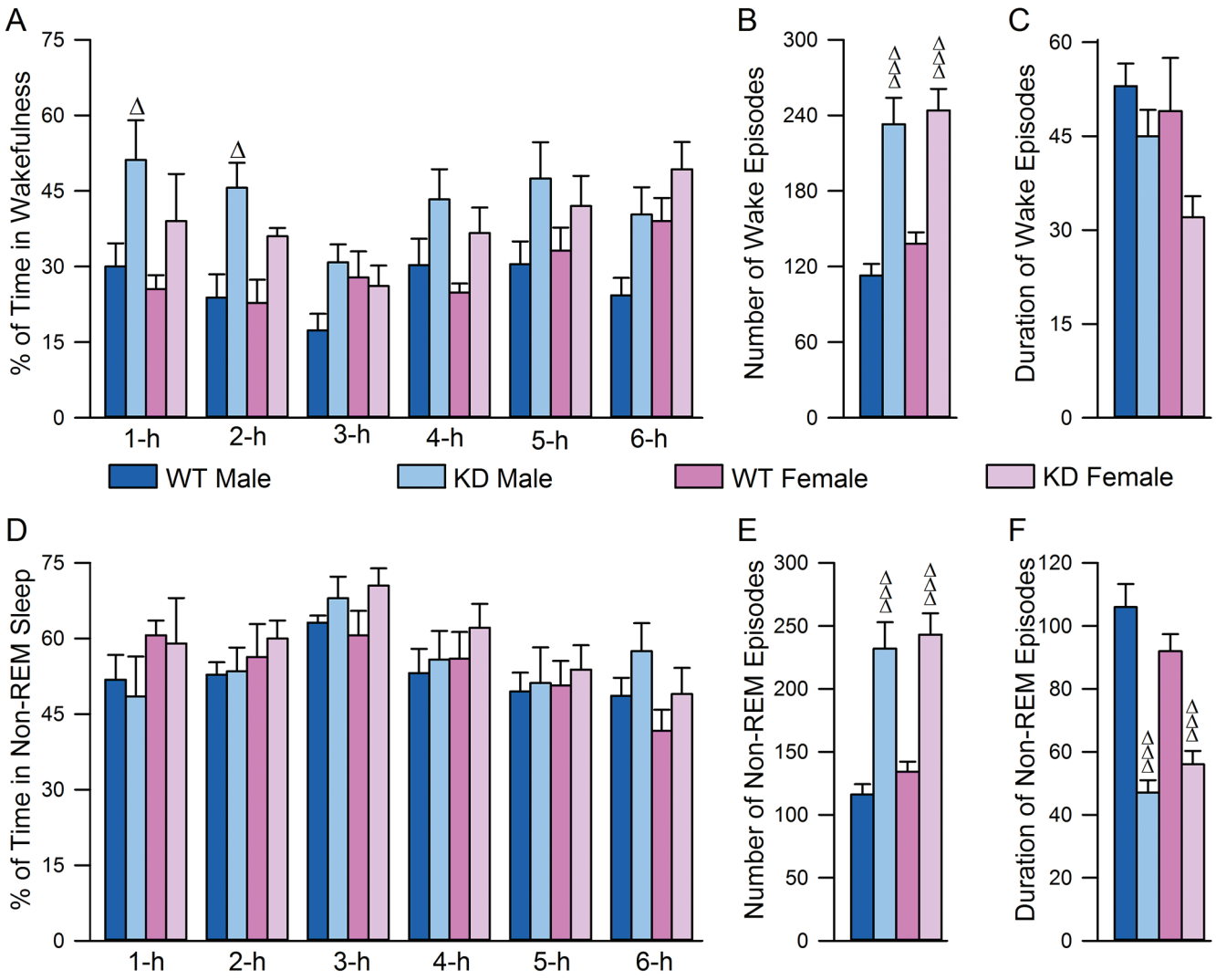

Figure 4. Effects of BDNF genotype (BDNF +/+ and BDNF +/–) and sex (male and female) differences in wakefulness and NREM sleep during the first experimental recording session (between 09:00 am and 03:00 pm). (A) Total percentages of time spent in wakefulness (mean ± SE) during consecutive 1-hour intervals. Note that compared with the WT (BDNF +/+), the KD (BDNF +/–) male spent significantly more time in wakefulness during the first and second hours of the 6-hour recording session. (B) Total numbers of wake episodes (mean ± SE) during the 6-hour recording session. Note that compared with the WT (BDNF +/+), both KD (BDNF +/–) male and female rats exhibited significantly more wake episodes. (C) Mean duration of wake episodes (in seconds; mean ± SE). (D) Total percentages of time spent in NREM sleep (mean ± SE) during consecutive 1-hour intervals. (E) Total numbers of NREM sleep episodes (mean ± SE) during the 6-hour recording session. Note that compared with the WT (BDNF +/+), both KD (BDNF +/–) male and female rats had significantly more NREM sleep episodes. (F) Mean duration of NREM episodes (in seconds; mean ± SE). Note that compared with the WT (BDNF +/+), both KD (BDNF +/–) male and female rats had significantly shorter durations of NREM sleep episodes. Triangle indicates the level of significant (Bonferroni posttests) differences relative to BDNF genotype (Δ, $p < .05$; ΔΔΔ, $p < .001$).

Selective REM Sleep Deprivation Affects S–W Architecture Differently in WT and KD Rats

Figure 6 illustrates the representative effects of selective REM sleep deprivation on S–W architecture in WT and KD rats. The hypnograms show that, as REM sleep was selectively deprived in the WT rats, the number of REM sleep episodes progressively increased (Figure 6A and B). This indicates that the selective REM sleep deprivation protocol induced homeostatic drive for REM sleep. On the other hand, the same REM sleep deprivation protocol did not increase the number of REM sleep episodes in the KD rats (Figure 6C and D). This finding indicates that selective REM sleep deprivation in the KD rats failed to induce homeostatic drive for REM sleep.

Selective REM Sleep Deprivation Attenuates REM Sleep Homeostatic Drive and REM Sleep Recovery in BDNF Heterozygous Rats

In order to investigate the effects of selective REM sleep deprivation on each genotype, we compared the REM sleep data from the baseline S–W recording sessions and the selective REM sleep deprivation recording sessions using two-way ANOVA. For the WT rats, analysis of the total number of REM sleep episodes revealed significant effects of treatment ($F_{(1,22)} = 21$, $p < .001$), time ($F_{(5,110)} = 3.0$, $p = .015$), and a significant treatment × time interaction ($F_{(5,110)} = 4.0$, $p = .002$). Post hoc analyses revealed that the WT rats had a significant increase in the number of REM sleep episodes shown during the selective REM sleep deprivation recording session, compared with baseline

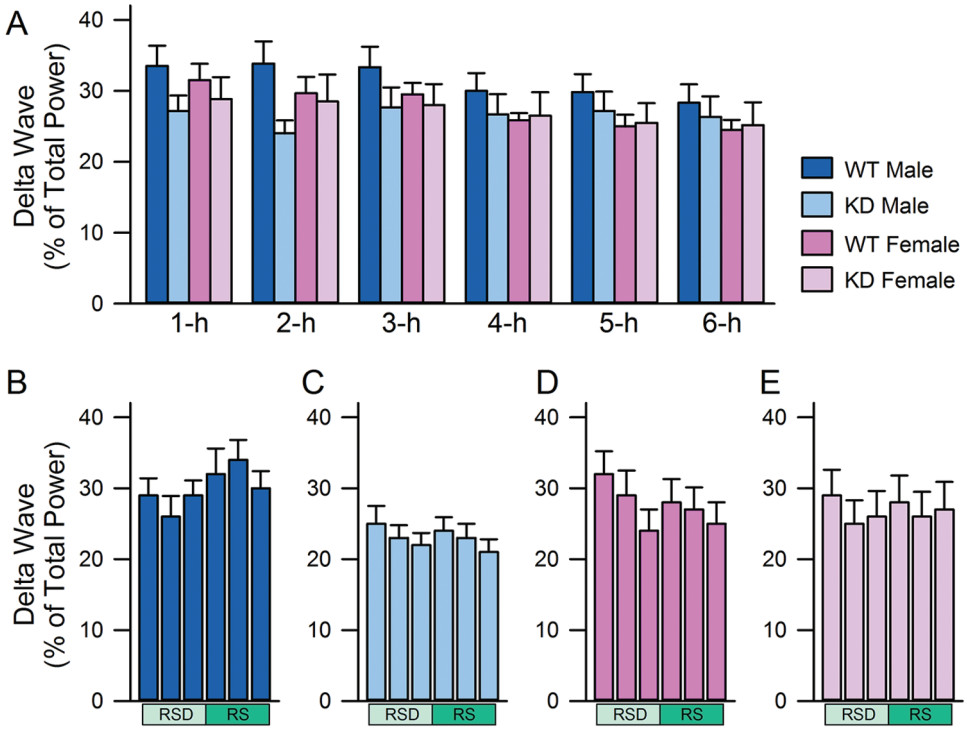

Figure 5. Effects of sex (males and females) and genotype (BDNF +/+ and BDNF +/–) on baseline NREM sleep and selective REM sleep deprivation SWA. Bars represent percentages (mean ± SEM) of total δ power (within the frequency range 0.5–4.5 Hz) during the 6-hour baseline and selective REM sleep deprivation recording sessions. (A) represents baseline NREM sleep SWA during 6-hour baseline recordings between WT-males, KD-males, WT-females, and KD-females. Note that sex nor genotype significantly affected baseline NREM sleep SWA, (B) represents NREM sleep SWA during 3-hour (1–3 hours) selective REM sleep deprivation and 3-hour (3–6 hours) REM sleep recovery periods in WT-male rats, (C) represents NREM sleep SWA during 3-hour (1–3 hours) selective REM sleep deprivation and 3-hour (3–6 hours) REM sleep recovery periods in KD-male rats, (D) represents NREM sleep SWA during 3-hour (1–3 hours) selective REM sleep deprivation and 3-hour (3–6 hours) REM sleep recovery periods in WT-female rats, and (E) represents NREM sleep SWA during 3-hour (1–3 hours) selective REM sleep deprivation and 3-hour (3–6 hours) REM sleep recovery periods in KD-female rats. Note that the selective REM sleep protocol did not significantly affect NREM SWA during the selective REM sleep deprivation or REM sleep recovery period. RSD = selective REM sleep deprivation; RS = REM sleep recovery.

S–W activity (Figure 7A; 2 hours: $t = 4.0$, $p < .001$; 3 hours: $t = 5.8$, $p < .001$). In contrast, the analysis of REM sleep episodes in the KD rats revealed a significant effect of treatment ($F_{(1,22)} = 8.8$, $p = .007$) but not of time ($F_{(5,110)} = 1.4$, $p = .233$) or treatment × time interaction ($F_{(5,110)} = 0.97$, $p = .437$). However, post hoc comparisons revealed all differences to be insignificant (Figure 7B). Overall, these results indicate that selective RSD significantly increased REM sleep episodes in the WT but not the KD rats.

The total percentage of time spent in REM sleep was analyzed using a two-way ANOVA. The results for the WT rats revealed a significant effect of time ($F_{(5,110)} = 25$, $p < .001$) and treatment × time interaction ($F_{(5,110)} = 21$, $p < .001$) but not a significant treatment effect ($F_{(1,22)} = 0.82$, $p = .376$). Post hoc analyses revealed that, when comparing the selective REM sleep deprivation recording sessions to the baseline S–W recording sessions, there was a significant decrease in the percentage of time spent in REM sleep during the first and second hours (Figure 7C; 1 hour: $t = 3.8$, $p < .01$; 2 hours: $t = 5.1$, $p < .001$) followed by a significant increase during the fourth hour ($t = 4.5$, $p < .001$). Here, the data indicate that the selective REM sleep deprivation during the first 3 hours eliminated more than 70% of REM sleep, which demonstrates the effectiveness of the sleep deprivation protocol used for these experiments. Similarly, a two-way ANOVA on the percentage of time spent in REM sleep in the KD rats revealed a significant effect of time ($F_{(5,110)} = 2.8$, $p = .02$) and a treatment × time interaction ($F_{(5,110)} = 4.5$, $p = .001$) but no significant treatment effect ($F_{(1,22)} = 1.1$, $p = .306$). Post hoc analyses revealed a significant increase in percent of time spent in REM sleep during hour 4 of the selective REM sleep deprivation recording sessions ($t = 4.3$, $p < .001$) but not at any other time points (Figure 7D). Taken together, these results demonstrate that the KD rats had significant impairments in the development of REM sleep homeostatic drive.

Selective REM Sleep Deprivation Did Not Increase Wakefulness

To determine the effects of selective REM sleep deprivation on wakefulness, we used a two-way ANOVA to compare the baseline S–W recording sessions and selective REM sleep deprivation recording sessions of each genotype. The results for the WT rats revealed a significant effect of treatment ($F_{(1,22)} = 14$, $p = .001$) and treatment × time interaction ($F_{(5,110)} = 7.4$, $p < .001$) but no significant effect of time ($F_{(5,110)} = 0.79$, $p = .56$) on the percentage of time spent in wakefulness. The results of the post hoc tests revealed that, for both experimental recording sessions, the percent of time spent in wakefulness during the first three hours was comparable (Figure 8A). However, during the 3-hour recovery period, there was a significant decrease in time spent in wakefulness during the selective REM sleep deprivation recording session compared with the baseline S–W recording sessions (Figure 8A: 4 hours: $t = 3.4$, $p < .01$; 5 hours: $t = 4.1$, $p < .001$; 6 hours: $t = 3.9$, $p < .001$). Interestingly, the significant decrease in wakefulness

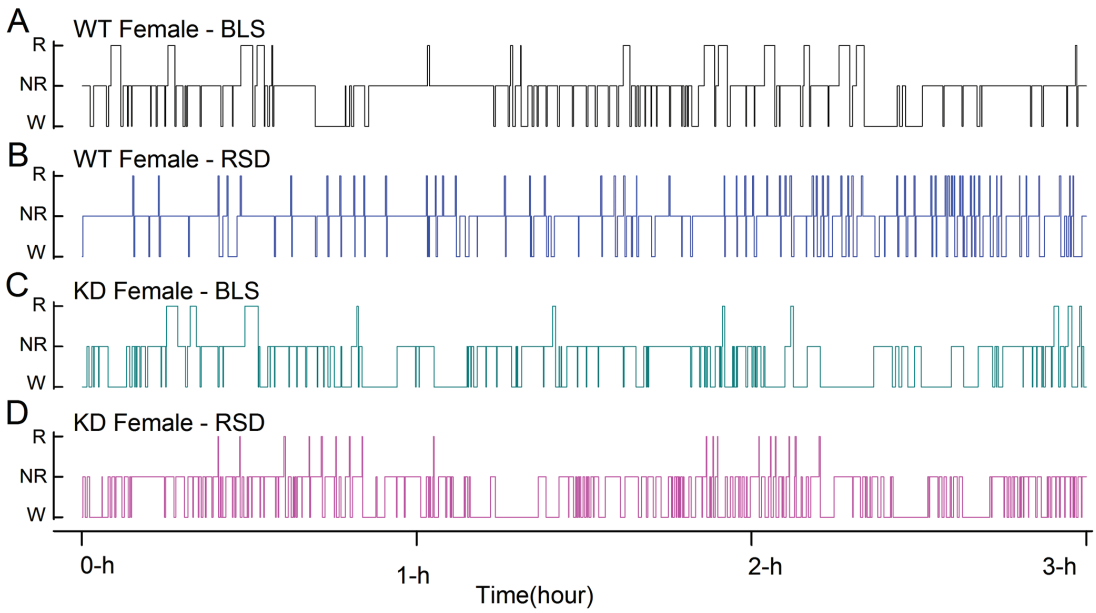

Figure 6. Effects of BDNF genotype (BDNF +/+ and BDNF +/–) and selective REM sleep deprivation on S–W architecture. Representative hypnograms reflecting effects of selective REM sleep deprivation on S–W architecture in BDNF wild type (BDNF +/+) female and BDNF heterozygous knockdown (BDNF +/–) female rats. These continuous 3-hour step histograms plot the occurrence and duration of physiologically and behaviorally defined states of wakefulness (W), NREM sleep (NR), and REM sleep (R). (A) The S–W architecture of a WT (BDNF +/+) female rat during 3-hour undisturbed S–W recordings, (B) the S–W architecture of the same WT (BDNF +/+) female rat during 3-hour selective REM sleep deprivation recordings. Note that as REM sleep was selectively deprived, the number of REM sleep episodes progressively increased. Indicating that this selective REM sleep deprivation protocol induced homeostatic drive for REM sleep. (C) The S–W architecture in a KD (BDNF +/–) female during 3-hour undisturbed S–W recordings, and (D) the S–W architecture of the same KD (BDNF +/–) female during 3-hour selective REM sleep deprivation recordings. Note that the selective REM sleep deprivation protocol did not induce homeostatic drive for REM sleep in the KD (BDNF +/–) female rat. BLS = baseline sleep recording session; RSD = selective REM sleep deprivation recording session.

during the recovery sleep period (between 3 and 6 hours) could be explained by an increase in REM sleep, indicating a strong homeostatic drive was induced by the deprivation protocol. In contrast, the two-way ANOVA on the percentage of time spent in wakefulness in the KD rats did not show any significant effect of time ($F_{(5,110)} = 2.0$, $p = .083$), treatment ($F_{(1,22)} = 0.03$, $p = .859$), or treatment × time interaction ($F_{(5,110)} = 2.3$, $p = .054$). Further post hoc comparisons confirmed that there were no significant differences (Figure 8B). Collectively, these results suggest that the selective REM sleep deprivation protocol did not increase wakefulness.

REM Sleep Deprivation Does Not Affect NREM Sleep in WT and KD Rats

To determine the effects of selective REM sleep deprivation on NREM sleep, we used a two-way ANOVA to compare the baseline S–W recording sessions and selective REM sleep deprivation recording sessions for each genotype. In WT rats, analysis of the percentage of time spent in NREM sleep revealed significant effects of time ($F_{(5,110)} = 5.4$, $p = .002$) and a treatment × time interaction ($F_{(5,110)} = 3.0$, $p = .014$) but no significant effect of treatment ($F_{(1,22)} = 0.02$, $p = .895$). However, post hoc tests revealed these differences to be insignificant (Figure 9A). Likewise, the two-way ANOVA on the percent of time spent in NREM sleep in the KD rats showed significant effects of time ($F_{(5,110)} = 2.8$, $p = .019$) and a treatment × time interaction ($F_{(5,110)} = 2.8$, $p = .02$) but no effect of treatment ($F_{(1,22)} = 0.12$, $p = .736$). Further analysis using post hoc comparisons revealed all differences to be insignificant (Figure 9B). These data indicate that the selective RSD protocol had no significant effect on NREM sleep in either genotype. Additionally, NREM sleep during both experimental recordings in the WT and KD rats was comparable.

Discussion

The principal findings of this study are as follows: (1) during baseline S–W activity, KD rats exhibited an increased number of wakefulness and NREM sleep episodes; (2) KD rats had fewer baseline REM sleep episodes that were shorter in duration; (3) KD rats took a longer time to initiate REM sleep and spent less time in REM sleep during baseline S–W activity; (4) NREM sleep SWA was comparable between WT and KD rats during baseline S–W activity and selective REM sleep deprivation; (5) selective REM sleep deprivation in KD rats did not induce REM sleep homeostatic drive nor did it induce REM sleep rebound during the recovery period; and (6) the total percentages of time spent in wakefulness and NREM sleep during selective REM sleep deprivation remained unaffected. Additionally, biological sex did not influence S–W activity in either the WT or KD rats during baseline or selective REM sleep deprivation recording sessions. Collectively, these results for the first time demonstrated that, regardless of biological sex, global reductions of BDNF expression in the brain significantly alter S–W architecture and the homeostatic regulation of REM sleep.

During the baseline S–W activity recording session, KD rats exhibited a higher total number of wakefulness and NREM sleep episodes. Some studies have shown that an increase in wakefulness causes an up-regulation of BDNF expression in the brain [64–66]. Since BDNF expression is about 50% less in the KD rats, the increase in wakefulness may be a compensatory biological response to increase BDNF. Interestingly, the increased episodes of wakefulness reduced the mean duration of NREM sleep episodes resulting in shorter durations of NREM sleep episodes; however, the total percentages of time spent in both wakefulness and NREM sleep were comparable between WT and KD rats. Here, the results indicate that

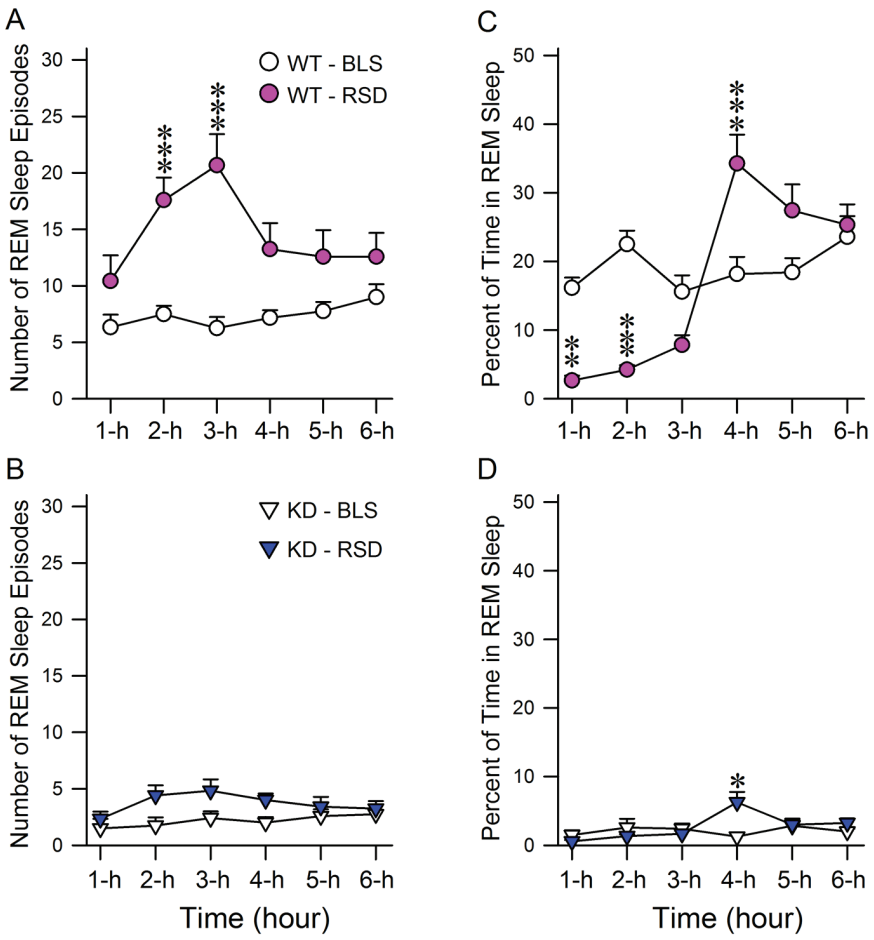

Figure 7. Effects of BDNF genotype (BDNF +/+ and BDNF +/–) and selective REM sleep deprivation on REM sleep homeostatic drive and REM sleep recovery. (A) Total numbers of REM sleep episodes (mean ± SE) in WT (BDNF +/+) rats during 6-hour baseline and selective REM sleep deprivation recording sessions. Note that during the first 3 hours of selective REM sleep deprivation, the number of REM sleep episodes progressively increased, indicating a strong drive for REM sleep. (B) Total numbers of REM sleep episodes (mean ± SE) in KD (BDNF +/–) rats during 6-hour baseline and selective REM sleep deprivation recording sessions. Note, unlike the WT (BDNF +/+) rats, in the KD (BDNF +/–) rats, selective REM sleep deprivation did not induce homeostatic drive for REM sleep. (C) Total percentage of time spent in REM sleep (mean ± SE) in WT (BDNF +/+) rats during 6-hour baseline and selective REM sleep deprivation recording sessions. Note that selective REM sleep deprivation during the first 3 hours eliminated more than 70% of REM sleep, which demonstrates the effectiveness of the selective REM sleep deprivaiton protocol used for these experiments. Also note that, during the first 2 hours after selective REM sleep deprivation protocol, there was a rebound in REM sleep. (D) Total percentages of time spent in REM sleep (mean ± SE) in KD (BDNF +/–) rats during 6-hour baseline and selective REM sleep deprivation recording sessions. Note that, like the WT (BDNF +/+) rat, after the selective REM sleep deprivation protocol, the KD (BDNF +/–) rat had a slight rebound in REM sleep during the first hour of recovery sleep. Asterisk indicates the level of significant (Bonferroni posttests) differences relative to the first experimental recording session (*$p < .05$, **$p < .01$, ***$p < .001$). BLS = baseline sleep recording session; RSD = selective REM sleep deprivation recording session.

this global reduction of BDNF expression did not significantly affect baseline levels of wakefulness and/or NREM sleep activity. Contrary to our results, other studies, using intracerebroventricular (icv) and local application of BDNF, have suggested that BDNF is involved in the regulation of NREM sleep [45, 48].

Previously, it has been shown that homeostatic regulation of REM sleep involves the up-regulation of BDNF expression in the PPT nucleus and dorsal subcoeruleus nucleus (SubCD) [31, 44]. The current study revealed that a global reduction of BDNF expression in KD rats had a profound effect on REM sleep. The results of the baseline S–W activity recording session revealed that KD rats spent significantly less time in REM sleep. Additionally, KD rats exhibited fewer REM sleep episodes that were shorter in duration and took longer to transition into from NREM sleep. These findings suggest that KD rats had difficulty in initiating and maintaining spontaneously occurring REM sleep. Qualitative sleep relies on alternating NREM and REM sleep cycles during total sleep time. In the past, studies have shown that REM sleep expression is dependent on NREM sleep [67, 68]. Thus, one could suggest that the late onset of REM sleep during the baseline S–W activity recording session in the KD rats could be due to shorter durations of NREM sleep episodes. Yet, since the total amount of NREM sleep in the KD rats is comparable to the total amount of NREM sleep in the WT rats, it is unlikely that the reduction in the total amount of REM sleep in the KD rats was affected by the reduction of the mean duration of NREM sleep episodes.

A number of studies have suggested that the intensity of NREM sleep SWA (spectral power in the 0.5–4.5 Hz range, also called δ frequency range) in the cortical EEG is one of the most important physiological substrates for the homeostatic regulation of NREM sleep [69–74]. In support of this suggestion, studies have shown that SWA in NREM sleep typically declines over the course of a daily sleep period and increases in recovery sleep after a period of prolonged waking [71, 73, 75, 76]. Furthermore,

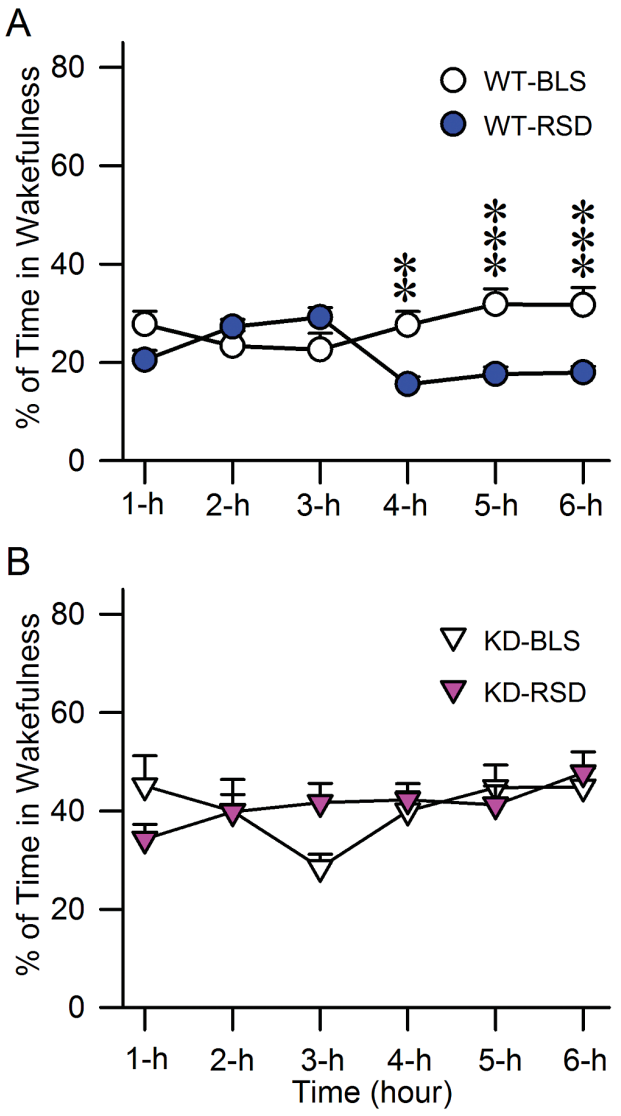

Figure 8. Effects of BDNF genotype (BDNF +/+ and BDNF +/–) and selective REM sleep deprivation on percentage of time spent in wakefulness during baseline and selective REM sleep deprivation recording sessions. (A) Total percentage of time spent in wakefulness (mean ± SE) during 6-hour recordings in WT (BDNF +/+) rats. Note that, during the selective REM sleep deprivation, the amount of wakefulness did not significantly increase, indicating that the selective REM sleep deprivation protocol used successively terminated REM sleep without increasing wakefulness. Also note that, during the unrestricted REM sleep period, the total amount of wakefulness decreased. (B) Total percentage of time spent in wakefulness (mean ± SE) during 6-hour recordings in KD (BDNF +/–) rats. Asterisk indicates the level of significant (Bonferroni posttests) differences relative to the first experimental recording session (**$p < .01$, ***$p < .001$). BLS = first experimental recording session; RSD = second experimental recording session.

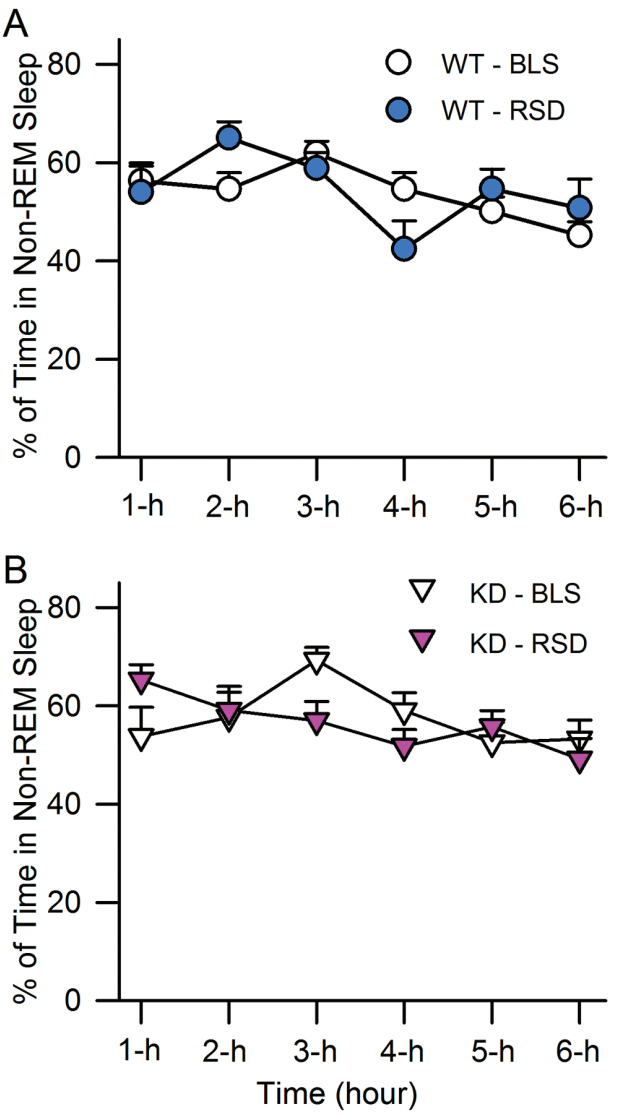

Figure 9. Effects of BDNF genotype (BDNF +/+ and BDNF +/–) and selective REM sleep deprivation on percentage of time spent in NREM sleep during baseline and selective REM sleep deprivation recording sessions. (A) Total percentage of time spent in NREM sleep (mean ± SE) in WT (BDNF +/+) rats during 6-hour recordings. (B) Total percentage of time spent in NREM sleep (mean ± SE) in KD (BDNF +/–) rats during 6-hour recordings. BLS = baseline recording session; RSD = selective REM sleep deprivation recording session.

SWA is reduced in subsequent NREM sleep after a nap and/or excess sleep [77, 78]. Interestingly, BDNF contributes to the regulation of NREM sleep SWA [66]. There is evidence to suggest that the homeostatic regulation of REM sleep is regulated by the homeostatic regulation of NREM sleep [68]. Yet, other studies have suggested that the homeostatic regulatory process of REM sleep is independent of the NREM sleep homeostatic regulatory process [79–81]. In the present study, selective REM sleep deprivation–induced activation of REM sleep homeostatic regulatory processes did not activate NREM sleep SWA. Additionally, BDNF KD rats did not exhibit any deficits in SWA and/or NREM sleep during baseline and/or selective REM sleep deprivation protocol. Therefore, the results of the present study did not support the hypothesis that BDNF is involved in the potentiation of SWA and/or NREM sleep. Since the results of this study do not support the previously mentioned hypothesis, it could be suggested that the homeostatic regulation of REM sleep is not dependent on the homeostatic regulation of NREM sleep, rather these two regulatory processes operate independently.

The results of this study did not reveal any significant sex difference in baseline S–W architecture. However, this observation may be due to a small sample size and should be reconfirmed in a future study using a larger sample size. Despite this limitation, these findings are consistent with a previous study in WT mice that showed no biological sex differences in baseline S–W architecture and/or responses to sleep deprivation [82]. Furthermore, in humans, under controlled

laboratory conditions, baseline sleep patterns are not different between healthy men and women [83, 84]. However, sex differences have been shown to exist under psychological stress [85–87]. Also, we recognize that circadian factors could influence sleep processes; therefore, both experimental recording sessions were performed during the same circadian phase for the WT and KD rats (between 09:00 am and 03:00 pm). Thus, in the present study, the observed differences in S–W architecture between WT and KD rats are not related to sex and/or circadian factors. Additionally, the total percentage of time spent in wakefulness and NREM sleep in the WT and KD rats did not significantly change as a result of the selective REM sleep deprivation. This suggests that our selective REM sleep deprivation protocol induced activation of REM sleep homeostatic regulatory processes without influencing changes in homeostatic regulation of NREM sleep and/or wakefulness.

During the 3-hour selective REM sleep deprivation, the WT rats progressively increased the number of attempts to enter REM sleep indicating a strong drive for REM sleep. Also, during the 3-hour recovery period, the WT rats exhibited a sharp increase in REM sleep episodes to compensate for REM sleep deficits from the preceding selective REM sleep deprivation period. These results are in agreement with the idea that short-term selective REM sleep deprivation is capable of activating the REM sleep homeostatic regulatory processes [31, 44, 88–90]. In contrast to these findings, the 3-hour selective REM sleep deprivation did not induce a homeostatic drive for REM sleep in the KD rats nor did they exhibit REM sleep rebound in the following 3-hour recovery period. These findings demonstrate that REM sleep homeostatic regulatory processes are severely impaired in KD rats. Therefore, it is reasonable to suggest that a normal level of BDNF expression is critical for the homeostatic regulation of REM sleep.

One of the general shortcomings of this study is that baseline sleep and responses to REM sleep deprivation were studied exclusively during the light phase. Therefore, we acknowledge that these genotype differences in the baseline sleep and responses to REM sleep deprivation cannot be generalized for responses that may occur during the dark phase. In the future, a similar study using 24-hour S–W recordings could provide insight into possible circadian differences in sleep responses during the dark phase. Additionally, since the 3-hour selective REM sleep deprivation protocol did not induce homeostatic drive for REM sleep in the KD rats, a future study should consider increasing the duration of the selective REM sleep deprivation to 6 hours.

In summary, the results of this study, for the first time, demonstrate that global reductions of BDNF expression impair spontaneously occurring REM sleep activity during baseline condition and homeostatic regulation of REM sleep. Therefore, any physiological functions that require normal levels of REM sleep and intact REM sleep homeostatic regulatory process will be impaired and/or attenuated by conditions that reduce BDNF levels. This study further suggests that a heterozygous BDNF knockdown rat is a useful animal model for the study of the cellular and molecular mechanisms of sleep regulation and cognitive functions of sleep.

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Disclosure Statement

None declared

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