

**Cacna1c (Ca<sub>v</sub>1.2) MODULATES EEG RHYTHM AND REM SLEEP RECOVERY****Cacna1c (Ca<sub>v</sub>1.2) Modulates Electroencephalographic Rhythm and Rapid Eye Movement Sleep Recovery**Deependra Kumar, PhD<sup>1</sup>; Nina Dedic, PhD<sup>1,2</sup>; Cornelia Flachskamm<sup>1</sup>; Stephanie Voulé<sup>1</sup>; Jan M. Deussing, PhD<sup>1</sup>; Mayumi Kimura, PhD<sup>1</sup><sup>1</sup>Max Planck Institute of Psychiatry, Munich, Germany; <sup>2</sup>Institute of Developmental Genetics, Helmholtz Zentrum München, Neuherberg, Germany

**Study Objectives:** The *CACNA1C* gene encodes the alpha 1C ( $\alpha_{1C}$ ) subunit of the Ca<sub>v</sub>1.2 voltage-dependent L-type calcium channel (LTCC). Some of the other voltage-dependent calcium channels, e.g., P-/Q-type, Ca<sub>v</sub>2.1; N-type, Ca<sub>v</sub>2.2; E-/R-type, Ca<sub>v</sub>2.3; and T-type, Ca<sub>v</sub>3.3 have been implicated in sleep modulation. However, the contribution of LTCCs to sleep remains largely unknown. Based on recent genome-wide association studies, *CACNA1C* emerged as one of potential candidate genes associated with both sleep and psychiatric disorders. Indeed, most patients with mental illnesses have sleep problems and *vice versa*.

**Design:** To investigate an impact of Ca<sub>v</sub>1.2 on sleep-wake behavior and electroencephalogram (EEG) activity, polysomnography was performed in heterozygous *Cacna1c* (HET) knockout mice and their wild-type (WT) littermates under baseline and challenging conditions (acute sleep deprivation and restraint stress).

**Measurements and Results:** HET mice displayed significantly lower EEG spectral power than WT mice across high frequency ranges (beta to gamma) during wake and rapid eye movement (REM) sleep. Although HET mice spent slightly more time asleep in the dark period, daily amounts of sleep did not differ between the two genotypes. However, recovery sleep after exposure to both types of challenging stress conditions differed markedly; HET mice exhibited reduced REM sleep recovery responses compared to WT mice.

**Conclusions:** These results suggest the involvement of *Cacna1c* (Ca<sub>v</sub>1.2) in fast electroencephalogram oscillations and REM sleep regulatory processes. Lower spectral gamma activity, slightly increased sleep demands, and altered REM sleep responses found in heterozygous *Cacna1c* knockout mice may rather resemble a sleep phenotype observed in schizophrenia patients.

**Keywords:** Ca<sub>v</sub>1.2, *Cacna1c*, EEG spectral power, L-type voltage-dependent calcium channel, schizophrenia, sleep

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**INTRODUCTION**

Many epidemiologic studies have shown a significance of sleep disturbances in psychiatric disorders. In one study, 40% of individuals in whom insomnia was diagnosed had a psychiatric disorder, whereas 46.4% of individuals with hypersomnia were also comorbid with mental disease.<sup>1</sup> Evidence of a genetic overlap between sleep quality and psychiatric disorders attested that to some extent the same genes would contribute to both phenotypes.<sup>2,3</sup> Recently, several genome-wide association studies (GWAS) have revealed that the *CACNA1C* gene, which encodes the alpha 1C ( $\alpha_{1C}$ ) subunit of the Ca<sub>v</sub>1.2 voltage-dependent L-type calcium channel (LTCC), was significantly associated with mental illnesses such as bipolar disorders,<sup>4</sup> major depression,<sup>5</sup> schizophrenia<sup>6</sup> and sleep disorders including narcolepsy<sup>7</sup> and insomnia.<sup>8,9</sup> In general, psychiatric or sleep phenotypes are largely assessed through questionnaires or diagnoses by physicians' consultations; thus, they depend to a large extent on subjective judgments and might thus bias the phenotype. In order to verify the statistically most significant findings from GWAS, there is a need for experimental settings to study the role of *Cacna1c* using genetic animal models, and to characterize it by an objective analysis.

Several earlier studies suggested that voltage-dependent calcium channels such as Ca<sub>v</sub>3.3 (T-type),<sup>10,11</sup> Ca<sub>v</sub>2.1 (P-/Q-type),<sup>12</sup> Ca<sub>v</sub>2.2 (N-type),<sup>13</sup> and Ca<sub>v</sub>2.3 (E-/R-type)<sup>14</sup> are considerably important modulators of sleep. Furthermore, the voltage-dependent calcium channel Ca<sub>v</sub>3.3 was found to be crucial for electroencephalogram (EEG) rhythms where it regulates EEG spindle generation during sleeping.<sup>10,11</sup> However, the role of LTCCs (Ca<sub>v</sub>1.2) in sleep and EEG rhythm has yet to be elucidated. In the brain, the *CACNA1C* gene or Ca<sub>v</sub>1.2 is densely expressed in the hippocampus and thalamus,<sup>15,16</sup> which are important structures for rhythmic activity in EEGs.<sup>17,18</sup> In the mouse brain, Ca<sub>v</sub>1.2 accounts for approximately 85% of the LTCCs.<sup>19</sup> A relatively high level of *Cacna1c* expression was also shown in the suprachiasmatic nucleus (SCN) of the mouse brain.<sup>20</sup> The SCN plays an important role in the regulation of sleep amount and timing.<sup>21</sup> Lesioning this structure abolishes the circadian sleep-wake rhythm, particularly the consolidation of sleep.<sup>21,22</sup> Given the *Cacna1c* expression in the SCN, together with the association of this gene with sleep disorders, we hypothesized that *Cacna1c* deficiency might affect daily sleep-wake distribution. Because homozygous deletion of *Cacna1c* is lethal, we examined our hypothesis in heterozygous *Cacna1c* knockout (HET) mice, which have previously been used in a study for mood-related behaviors.<sup>23</sup> Further, the dynamics of sleep-wake behavior is largely altered following biological or environmental challenges, whereas sleep patterns under baseline conditions appear normal. In this study, we therefore chose to conduct acute sleep deprivation to challenge homeostatic control of sleep-wake activity<sup>24</sup> and restraint stress, a more severe challenge to impact sleep recovery, particularly the rebound of rapid eye movement (REM) sleep.<sup>25</sup>

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## METHODS AND MATERIALS

### Animals

Heterozygous *Cacnalc* knockout mice (*Cacnalc*<sup>+/-</sup>, HET; n = 11) and their wild-type littermates (*Cacnalc*<sup>+/+</sup>, WT; n = 12) were obtained by interbreeding *Cacnalc*<sup>+/-</sup> mice.<sup>26</sup> The mouse line was originally generated using R1 embryonic stem cells and subsequently kept on a mixed 129S1/Sv × 129X1/SvJ × C57BL/6 background. Only male mice were used in the current study. All animals were group-housed in the breeding facility of the Max Planck Institute of Psychiatry, Munich, Germany. Prior to surgery, the animals were moved to a sound-attenuated recording chamber and housed individually in Plexiglass cages (length 35 cm, width 25 cm, and height 25 cm). During recovery from surgery and throughout the experiments, the housing environment was kept at a constant temperature of 24 ± 1°C on a 12 h light-dark cycle (lights on at 08:00). The animals had access to water and food *ad libitum*. All animal experiments performed in the present study were conducted according to the guidance of the European Community Council Directive, and the protocol was approved by the local commission for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

### Surgical Procedure

Under inhalation anesthesia with an isoflurane/oxygen mixture (Isofluran, DeltaSelect, Dreieich, Germany), the mice were fixed with a stereotaxic apparatus and chronically implanted with EEG and electromyogram (EMG) electrodes for polysomnographic recordings. The implant consisted of four gold wires (0.25–0.30 mm diameter) for EEG recordings, which were placed through the skull epidurally over the frontal and parietal cortices (coordinates, A +1.5 mm and –3 mm, L ± 1.7 mm each), and two additional gold wires were inserted into the cervical portion of the trapezoid muscles for EMG recordings. A thermistor (TT5-10KC3-5, Tewa temperature sensors, Lublin, Poland) was implanted in the subdural space (coordinates: A –2 mm and L 0.5 mm) for monitoring brain temperature. All electrodes and the thermistor were soldered to a micro-socket affixed to the skull with dental acrylic resin. Before the surgical operation, the animals received atropine sulfate (0.05 mg/kg, Atropine, Braun Melsungen, Melsungen, Germany) subcutaneously to stabilize circulation, and meloxicam (0.5 mg/kg, Metacam, Braun Melsungen) as a postoperative analgesic. After surgery, the mice were rested in their home cage for 2 w under the same environmental condition mentioned previously.

### Data Acquisition

Four to eight mice were recorded simultaneously in each recording session. Animals from both genotypes were always included per session. The lead wires of the EEG, EMG electrodes, and thermistor were connected to an electric swivel through a flexible tether. The weights of the swivel and the tether were counterbalanced via a mechanical device close to zero; thus, the mice could move almost without restriction, and acclimation to the setting was confirmed prior to the initiation of recordings by comparison of food/water consumption with presurgery values. EEG and EMG signals were amplified (10,000×), and both filtered EEGs (0.25–64 Hz) and EMGs

(175–1,000 Hz) that had undergone root mean square rectification were digitized by a high-speed analog-to-digital converter (NI-USB-6343-X-series, National Instruments, Austin, TX, USA) at a sampling rate of 128 Hz. The signals were then processed by a personal computer equipped with a LabVIEW-based acquisition system (National Instruments), especially designed for mouse sleep EEG/EMG and temperature data acquisition (EEGraVigilanz, SEA, Cologne, Germany). Polygraphic data were stored on a computer and later processed offline.

After 24-h baseline recording of EEG/EMG and brain temperature, all WT (n = 12) and HET mice (n = 11) were subjected to sleep deprivation, and during the following week half of them (n = 6 each per genotype) received restraint stress. There was at least a 7-day interval between sleep deprivation and restraint stress exposure to ensure recovery. An additional baseline recording was performed before the restraint stress experiment.

Sleep deprivation was conducted for 6 h beginning at the onset of the light period (08:00–14:00) by gentle handling. During this sleep deprivation period, examiners paid careful attention to the mice by playing with cotton swabs or tissue papers, etc., avoiding a direct body touch. In the meantime, polygraphical recordings were continuously made. In this way, we were able to cut off 97.0% of nonrapid eye movement (NREM) sleep and 100% of REM sleep on average. Over the sleep deprivation experiment, differences in recovery sleep were evaluated between the genotypes. Restraint stress was conducted for 1 h beginning at the onset of the light period (08:00–09:00). Restraint stress was achieved by enclosing each animal in a 50 mL falcon tube (diameter = 2.8 cm) with a slight opening at both ends for tail and nose. During restraint stress, their behavior was observed by examiners. Although recording cables were not connected for data acquisition during this 1-h period, the mice stayed awake constantly.

### Sleep-Wake Data Analysis

All EEG and EMG wave forms were processed offline with the LabVIEW-based acquisition program, in which a fast Fourier Transform (FFT) algorithm served for power spectrum analysis of particular EEG frequency contents, i.e., EEG power density within the delta (0.5–5 Hz), theta (6–9 Hz), sigma (10–15 Hz), beta-1/2 (16–23 and 23–32 Hz, respectively), and gamma (32–64 Hz) bands. Epochs containing artifacts and values between 50–50.25 Hz containing electrical noise from power supply were eliminated from power spectral analysis. With the aid of the adapted FFT algorithm, spectral analysis was also performed to provide semiautomatic classification of particular vigilance states as wakefulness and REM and NREM sleep, which were defined in each 4-sec epoch. The defined vigilance states were further confirmed visually and corrected, if necessary. To conduct EEG frequency analysis, mean values of the EEG spectrum per 0.25 Hz bin were calculated for each vigilance state and normalized with the individual average of total EEG power from all vigilance states across all frequency bins and epochs. Regarding slow wave activity (SWA), the absolute power of the frequency range between 0.5–4 Hz during NREM sleep was normalized by the averaged total EEG power from all vigilance states as previously mentioned. To analyze

sleep architecture in detail, the number of rather short or relatively long REM and NREM sleep episodes were counted. Such a "brief" or "sustained" REM/NREM sleep episode was defined in terms of numbers of consecutive 4-sec epochs as requiring 15 ( $\leq 1$  min) or more ( $> 1$  min) epochs for REM sleep and 30 ( $\leq 2$  min) or more ( $> 2$  min) epochs for NREM sleep, respectively. Temperature data in each 4-sec epoch were normalized with the individual total mean value.

### Statistics

For the effects of genotype (*Cacna1c* heterozygous, HET) on sleep and temperature, 24-h time-course changes in NREM sleep, REM sleep, SWA, and brain temperature were evaluated primarily by nonparametric two-way analysis of variance (ANOVA). On occasion, an unpaired *t* test was also applied to compare these changes between particular time windows if necessary. Differences in EEG spectral power between WT and HET mice were analyzed by one-way ANOVA with a Bonferroni *post hoc* test. Regarding responses to sleep deprivation and restraint stress, recovery sleep was first compared with own baseline per genotype using two-way ANOVA for repeated measures, then followed by a Bonferroni *post hoc* test whenever appropriate. The mean amount of REM sleep, REM sleep episode duration, and number were analyzed by Bonferroni multiple comparison test. In all cases, statistical significance was set at  $P < 0.05$ .

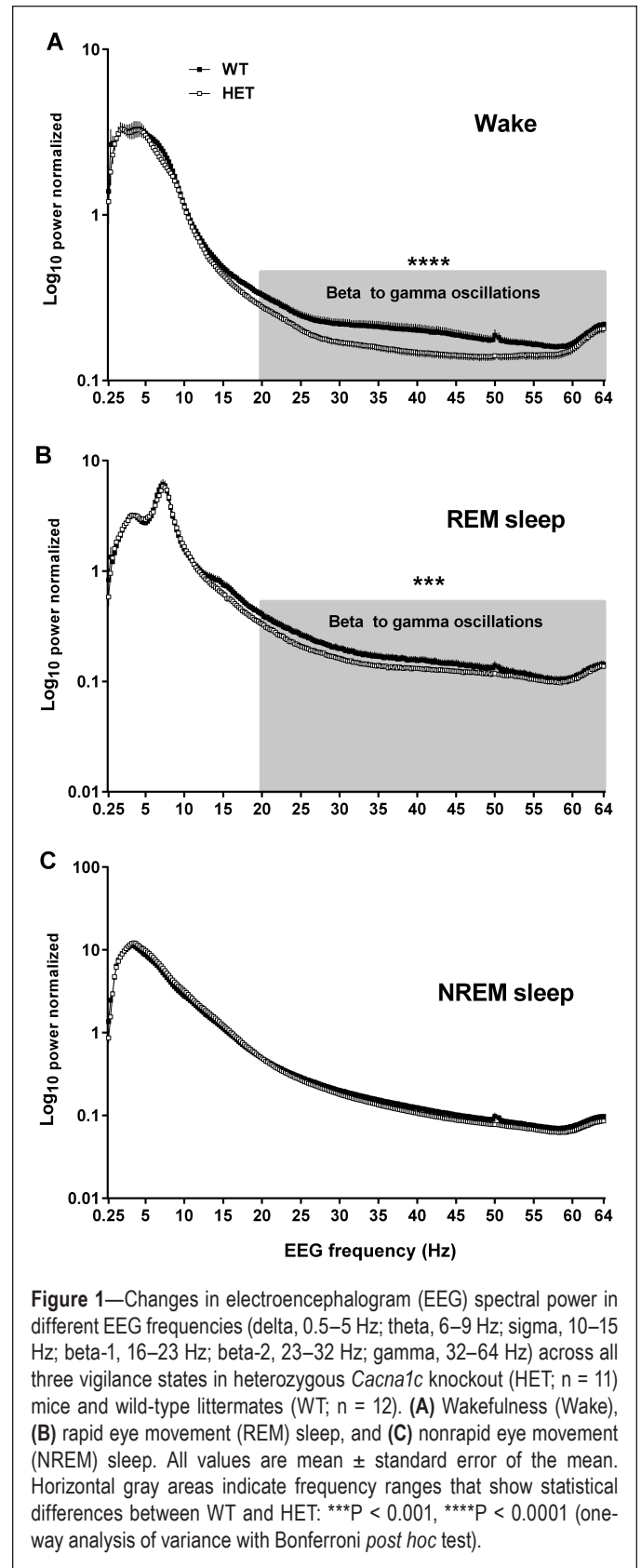
## RESULTS

### *Cacna1c* is Involved in Fast Oscillations of EEG Activity

Figure 1 illustrates a comparative difference in EEG power spectra across three different vigilance states of heterozygous *Cacna1c* knockout (HET) mice and their WT littermates. During wake, EEG power was significantly lower in HET mice than in WT mice over the high frequency ranges (Figure 1A, beta-2 to gamma, 20–64 Hz,  $P < 0.0001$ ). Such a trend of reduced power spectra in HET mice along the fast-oscillating EEG activity also appeared in the sleep EEG. During REM sleep, both HET and WT mice displayed the same spectrum peak in the theta band, a typical EEG profile of REM sleep. However, the EEG power decreased from the sigma range and became significantly lower across the higher frequencies in HET mice than in WT mice (Figure 1B, beta-2 to gamma, 20–64 Hz,  $P < 0.001$ ), which was similarly seen in the wake EEG. During NREM sleep, a typical NREM-related increase in delta power was indistinguishably observed in both genotypes (Figure 1C), and hardly any difference in the EEG power was detected in further frequency ranges.

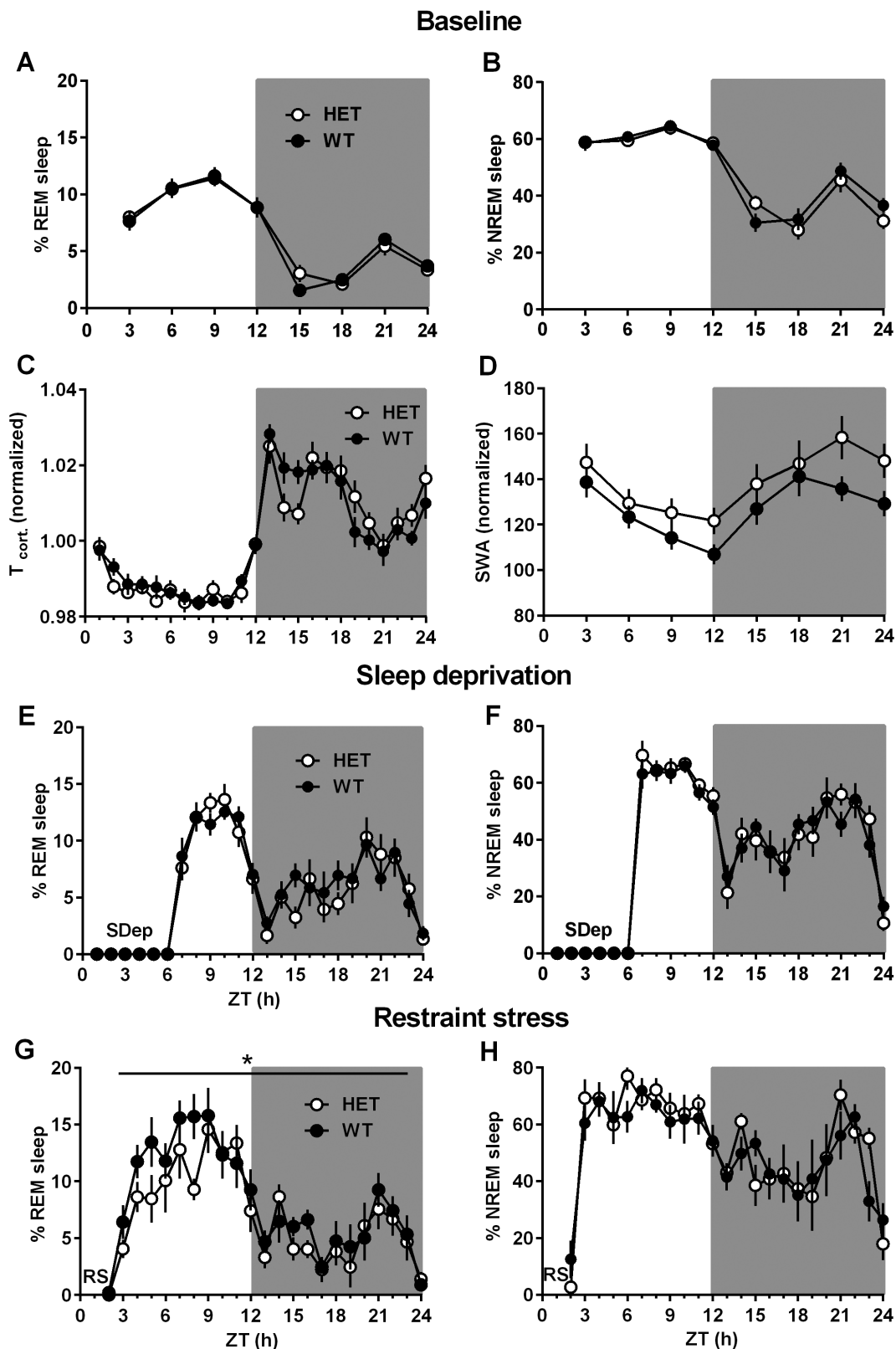
### *Cacna1c* Haploinsufficiency Moderately Promotes Nocturnal Sleep

In order to assess the effects of the *Cacna1c* haploinsufficiency on spontaneous sleep, vigilance states were classified from a 24-h baseline recording in HET and WT mice under the same 12:12 h light-dark cycle to which they had been exposed since birth. The daily amount of neither REM nor NREM sleep significantly differed between two genotypes in the baseline condition (Figure 2A and 2B). However, HET mice spent slightly more time in REM (Zeitgeber time [ZT] 15 h,



**Figure 1**—Changes in electroencephalogram (EEG) spectral power in different EEG frequencies (delta, 0.5–5 Hz; theta, 6–9 Hz; sigma, 10–15 Hz; beta-1, 16–23 Hz; beta-2, 23–32 Hz; gamma, 32–64 Hz) across all three vigilance states in heterozygous *Cacna1c* knockout (HET;  $n = 11$ ) mice and wild-type littermates (WT;  $n = 12$ ). (A) Wakefulness (Wake), (B) rapid eye movement (REM) sleep, and (C) nonrapid eye movement (NREM) sleep. All values are mean  $\pm$  standard error of the mean. Horizontal gray areas indicate frequency ranges that show statistical differences between WT and HET: \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (one-way analysis of variance with Bonferroni *post hoc* test).

$P < 0.05$ , unpaired *t* test) and NREM sleep (ZT 15 h,  $P = 0.052$ , unpaired *t* test) limited only during the initial dark period. A lower cortical brain temperature during the first few hours in the dark period was associated with increased sleeping time in HET mice (Figure 2C). To characterize sleep architecture in



**Figure 2**—Comparison of spontaneous sleep (baseline) and recovery sleep after challenging conditions between heterozygous *Cacna1c* knockout mice (HET, closed symbols; n = 11) and wild-type littermates (WT, open symbols; n = 12). (A) Rapid eye movement (REM) sleep (%), (B) nonrapid eye movement (NREM) sleep (%), (C) normalized cortical brain temperature, and (D) normalized slow wave activity (SWA) during NREM sleep under baseline condition. (E,G) Recovery responses of REM sleep and (F,H) NREM sleep after 6 h of sleep deprivation (SDep) or after 1 h of restraint stress (RS) initiated from the beginning of the light period, respectively. Significant effects of *Cacna1c* haploinsufficiency on sleep were found in REM sleep during recovery after restraint stress (G); HET mice showed lower REM sleep responses, compared to those in WT mice. All values are mean  $\pm$  standard error of the mean. Significant differences between genotypes: \*P < 0.05 (two-way analysis of variance).

HET mice, the average numbers of NREM and REM sleep episodes were compared in 6-h intervals over the light and dark period (Figure S1, supplemental material). HET mice spent more time than WT mice in shorter NREM sleep ( $P = 0.054$ ) over the light and dark period, indicating their fragmented sleep architecture. However, SWA during NREM sleep tended to be higher in HET mice across 24 h, although the difference did not reach statistical significance (Figure 2D). Altogether, analysis on baseline vigilance states of HET mice suggests that a *Cacnalc* haploinsufficiency affects sleep quality. Due to fragmented sleep architecture in HET mice, compared to WT mice, sleep demands remained at the end of the light period, resulting in more entries into sleep episodes at the beginning of the active phase.

### Reduced REM Sleep Recovery After Sleep Deprivation in Heterozygous *Cacnalc* Knockout Mice

To address the question whether the *Cacnalc* haploinsufficiency affects the homeostatic regulation of sleep, usually indicated by accumulation of SWA, sleep responses to 6-h sleep deprivation were examined. Time-course changes in recovery sleep were very similar in both genotypes after sleep deprivation (Figures 2E and 2F). However, the comparison of recovery sleep with their own baseline revealed that responses of REM sleep, but not NREM sleep, appeared differently between the two genotypes (Figure 3). The magnitude of REM sleep responses in WT mice was higher (Figure 3C,  $F_{1,11} = 34.79$ ,  $P < 0.0001$ ) than in HET mice (Figure 3D,  $F_{1,11} = 12.67$ ,  $P < 0.01$ ), particularly during the first half of the dark period. Further quantitative analysis focusing on the first 6 h of the dark period (ZT 13–18 h, Figures 3E–3J) confirmed that WT mice spent more time in REM sleep than HET mice (mean difference = 3.4%,  $P < 0.0001$ ) during this period (Figure 3E). Increased REM sleep in WT mice was a consequence of both longer REM sleep episode duration (Figure 3G, mean difference = 0.3 min;  $t = 3.48$ ,  $P < 0.05$ ) and high occurrences of REM sleep episodes (Figure 3H, mean difference = 8.3 episodes,  $P < 0.05$ ). Although HET mice also showed REM sleep rebound after sleep deprivation (Figure 3D), REM sleep architecture was not significantly altered by sleep deprivation in HET mice (Figures 3I and 3J) during the first half of the dark period ( $P > 0.05$ , Figure 3F). Regarding NREM sleep changes after sleep deprivation, the difference in time spent in NREM sleep was not explicitly exhibited when compared with their own baseline (Figures 3A and 3B). However, both genotypes showed increased NREM episode length (WT, mean difference = 1.2 min,  $P < 0.001$ ; HET, mean difference = 1.18 min,  $P < 0.001$ ) but reduced episode number (WT, mean difference = 20.83 episodes,  $P < 0.0001$ ; HET, mean difference = 23.27 episodes,  $P < 0.001$ ) for the following 6 h immediately after sleep deprivation (Table S1, supplemental material). In addition, an increased level of NREM sleep SWA was detected immediately after sleep deprivation and maintained the higher level almost similarly throughout the second half of the light period in both WT ( $F_{1,5} = 18.96$ ,  $P < 0.001$ ) and HET ( $F_{1,5} = 10.78$ ,  $P < 0.01$ ) mice (Figure S2, supplemental material). Thus, homeostatic control on NREM sleep was preserved in *Cacnalc* haploinsufficiency. Brain temperature in both genotypes decreased immediately after sleep deprivation

and stayed at a lower level throughout the recoding period (Figure S2). The decrease in brain temperature was more robust in the dark period (WT;  $F_{1,11} = 67.10$ ,  $P < 0.0001$ ; HET,  $F_{1,11} = 77.0$ ,  $P < 0.0001$ ) than in the light period, whereas the effects of sleep deprivation on brain temperature were similarly detected in both genotypes (Figure S2).

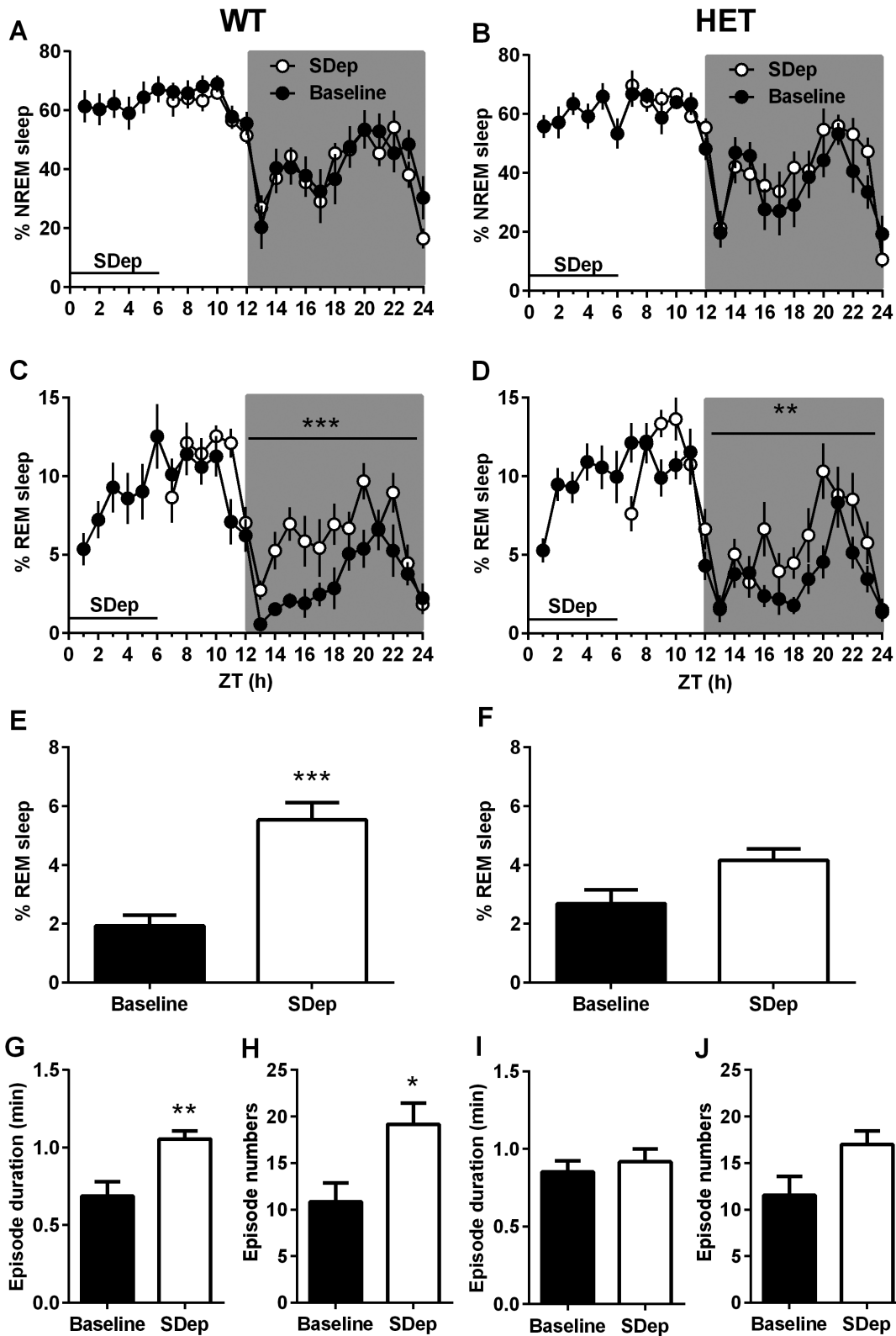
### Reduced REM Sleep Response to Restraint Stress in Heterozygous *Cacnalc* Knockout Mice

To further address whether the *Cacnalc* haploinsufficiency influences stress-induced sleep responses, 1-h restraint stress was applied in both genotypes. In HET mice, REM sleep responses appeared to a significantly lower extent than in WT mice (Figure 2G,  $F_{1,21} = 5.03$ ,  $P < 0.05$ ). This was due to an unchanged episode number, especially a lack of sustained REM episodes in HET mice compared with WT mice, during the second half of the light period (ZT 6–12 h,  $P < 0.05$ , Figure S1). When compared to their own baseline, a significant rebound of REM sleep occurred after restraint stress in WT mice ( $F_{1,21} = 6.62$ ,  $P < 0.05$ ), but not in HET mice (Figures 4C and 4D). WT mice showed significantly more REM sleep (mean difference = 2.9%,  $P < 0.01$ ) during the first half of the dark period (ZT 13–18 h, Figure 4E), which was due to a significantly increased number of REM episodes (Figure 4H, mean difference = 11.1 episodes,  $P < 0.05$ ). HET mice showed no significant changes in the amount of REM sleep and REM episode architecture during that period (Figures 4F, 4I, and 4J). No significant differences in the amount of NREM sleep after restraint stress were observed in HET and WT mice (Figures 4A and 4B). However, both genotypes had increased NREM episode numbers ( $P < 0.05$ ) with a trend toward reduced episode duration during the dark period after restraint stress (Table S1). Restraint stress increased brain temperature, whereas the changes returned to basal levels within 3 h and stayed in comparable ranges afterward. SWA in both genotypes were also comparable after restraint stress (Figure S3, supplemental material).

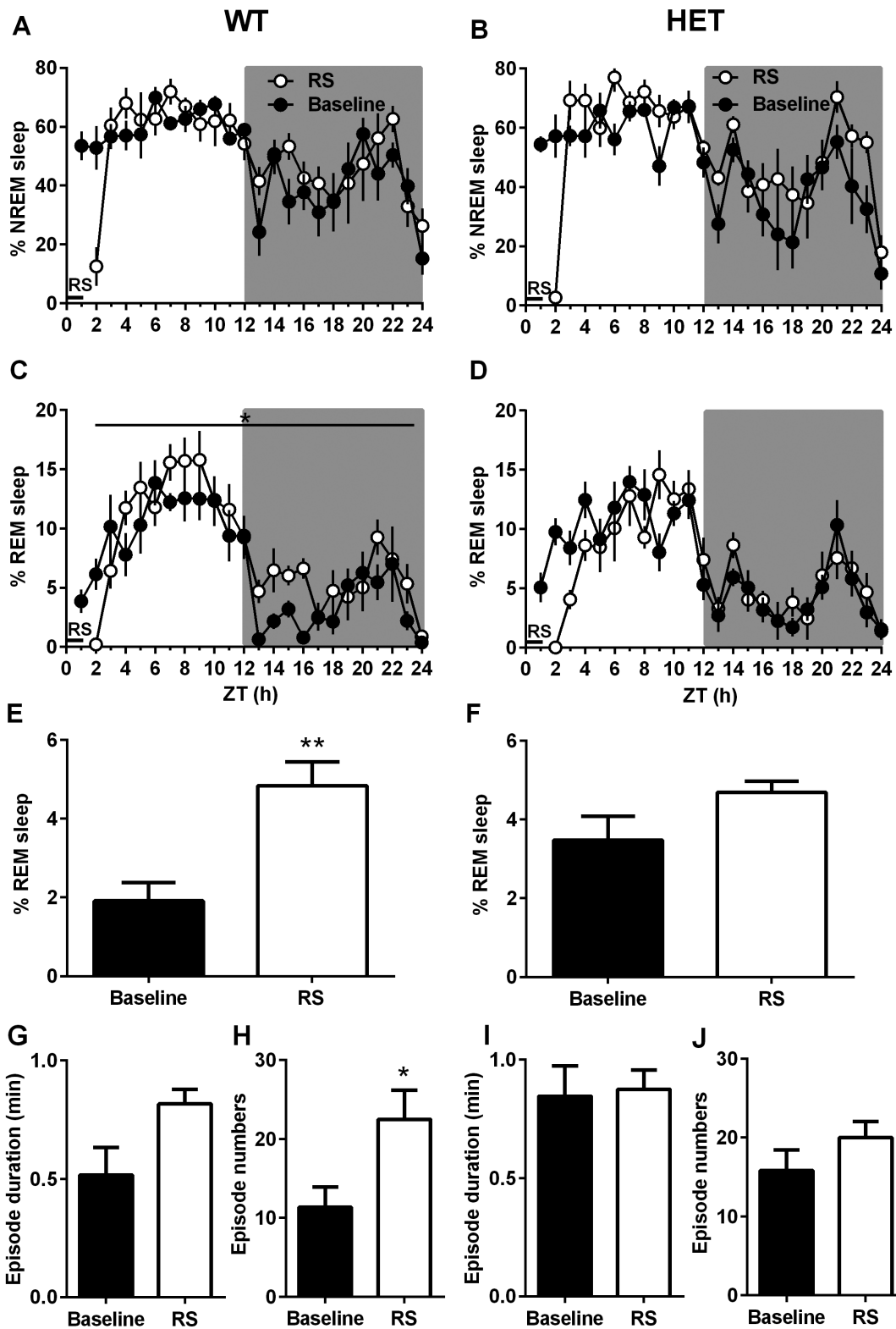
## DISCUSSION

Our results from the current study demonstrate a significant involvement of *Cacnalc* in sleep-wake regulation. For example, *Cacnalc* haploinsufficiency produced large-scale effects on the fast EEG (beta to gamma) rhythm during wake and REM sleep. Further, heterozygous *Cacnalc* mice failed to show their homeostatic REM sleep recovery process after acute stress conditions. This also suggests that reduced  $Ca_v1.2$  function impairs stress-induced sleep responses as well as homeostatic control of sleep.

Observed in HET mice, such as lower EEG spectral power in the beta-gamma frequency range, a trend of higher SWA during NREM sleep and increased sleeping time in the active period of mice could be interrelated phenomena. Fast-oscillating EEG gamma activity is usually high during active waking in animals, reflecting the degree of behavioral arousal.<sup>27,28</sup> It also reflects cortical arousal, independent of motor activity, because it attains high levels in association with attentive immobility but reaches its maximum during particular active behaviors (eating and moving) and also during REM sleep.<sup>27–29</sup> Lower gamma activity in HET mice may indicate a lower level of



**Figure 3**—Effects of the *Cacna1c* haploinsufficiency on recovery sleep responses to sleep deprivation (SDep). (A–D) Changes in nonrapid eye movement (NREM) sleep (%) and rapid eye movement (REM) sleep (%) in wild-type littermates (WT;  $n = 12$ , left panels) and heterozygous *Cacna1c* knockout mice (HET;  $n = 11$ , right panels) subjected to 6 h of sleep deprivation during the first 6 h of the light period. (E,F) Averaged REM sleep amount, (G,I) episode duration, and (H,J) occurrences of REM sleep during the first 6 h of the dark period (Zeitgeber time 13–18 h). All values are mean  $\pm$  standard error of the mean. Significant differences between baseline and recovery: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (A–D; two-way analysis of variance [ANOVA], E–J; one-way ANOVA, Bonferroni multiple comparison *post hoc* test).



**Figure 4**—Effects of the *Cacna1c* haploinsufficiency on recovery sleep responses to restraint stress (RS). (A–D) Changes in nonrapid eye movement (NREM) sleep (%) and rapid eye movement (REM) sleep (%) in wild-type littermates (WT; n = 6, left panels) and heterozygous *Cacna1c* knockout mice (HET; n = 6, right panels) mice subjected to 1 h of restraint stress during the first hour of the light period. (E,F) Averaged REM sleep amount, (G,I) episode duration, and (H,J) occurrences of REM sleep during the first 6 h of the dark period (Zeitgeber time, 13–18 h). All values are mean ± standard error of the mean. Significant differences between baseline and recovery: \*P < 0.05, \*\*P < 0.01 (A–D; two-way analysis of variance [ANOVA], E–J; one-way ANOVA, Bonferroni multiple comparison *post hoc* test).

arousal or alertness during wakefulness. It has previously been reported that beta activity reflects the degree of alertness.<sup>30,31</sup> In addition, HET mice showed low REM sleep associated beta-gamma activity, suggesting again decreased cortical activation. Such low cortical arousal in HET mice might have also influenced SWA during NREM sleep, which remained higher than in WT mice, because it could lead to fragmented sleep cycles. Then, reduced *Cacnalc* function would indirectly increase somnolence or sleep debt, although sleep deprivation similarly elevated SWA in both genotypes. Based on our results from the variations in power spectra, one could suggest that *Cacnalc* haploinsufficiency decreases alertness during wakefulness and induces drowsiness. A slightly increased sleeping time at the beginning of the dark period displayed in HET mice might have occurred due to a remaining sleep demand.

The brain can switch vigilance states rapidly by excitation of the ascending arousal system along the reticular activating system (RAS).<sup>32,33</sup> The RAS is composed of several neural circuits connecting the brainstem to the cortex via the thalamus. Fluctuations in RAS activity by a tonic excitatory drive control the rhythm in EEG.<sup>34,35</sup> There are distinct differences in the EEG rhythm during periods of wakefulness and sleep: low-voltage, fast-oscillating EEG is associated with wakefulness and REM sleep, whereas high-voltage, slow-oscillating EEG is found during NREM sleep. Stimulation of the RAS produces low-voltage, fast-oscillating EEG by suppressing slow wave (delta band; 1–4 Hz) and by promoting gamma band (20–40 Hz) oscillations.<sup>36</sup> Because *Cacnalc* is widely expressed in the brain including brainstem nuclei such as the laterodorsal tegmentum (LDT)<sup>37</sup> and the dorsal raphe nucleus (DRN),<sup>38</sup> and  $\text{Ca}_v1.2$ -mediated  $\text{Ca}^{2+}$  signaling is implicated in shaping neuronal discharge patterns,<sup>39,40</sup> the decreased level of cortical arousal or beta-gamma activity detected in HET mice could be related to a diminished excitatory drive in the RAS due to low  $\text{Ca}_v1.2$  expression.

Another possible pathway that could explain lower cortical activation in HET mice may underlie orexinergic circuits. Orexin/hypocretin produce a long-lasting excitation of arousal-related neurons including those in the LDT and DRN.<sup>37,38</sup> Further, action of orexin/hypocretin on LDT and DRN neurons is likely to be mediated by  $\text{Ca}_v1.2$ .<sup>37,38</sup> Therefore, decreased arousal in HET mice might have occurred due to decreased influences of hypocretin on arousal-related neurons. In fact, HEK 293 cells expressing the hypocretin 1 receptor display an increase in *CACNAIC* expression, triggered by hypocretin binding, suggesting that  $\text{Ca}_v1.2$  is important in cellular action of hypocretinergic neural transmission.<sup>41</sup>

In addition, pharmacological studies have shown a possible connection of suppressed  $\text{Ca}_v1.2$  action with cortical deactivation, i.e., NREM sleep. It has been found that pentobarbital-induced sleep can be augmented by LTCC blockers, verapamil, nifedipine, and diltiazem, respectively.<sup>42–45</sup> For example, even direct application of diltiazem into the DRN extended total sleep time including slow wave sleep in rats treated with pentobarbital.<sup>46</sup> This result was consistent with a previous report using oral gavage application of diltiazem.<sup>47</sup> Thus, L-type  $\text{Ca}^{2+}$  channel blockers promote the hypnotic effects of anesthetics,<sup>42,43</sup> plausibly due to suppression of  $\text{Ca}^{2+}$  influx through the LTCC.<sup>44</sup> Reduced function of LTCCs has an impact on

vigilance control, which reflects the sleep phenotype of heterozygous *Cacnalc* knockout mice.

Another interesting finding from this study is the reduced amount of REM sleep recovery in HET mice when exposed to challenging conditions. HET mice exhibited lower REM sleep rebound after sleep deprivation, indicating a modification in the homeostatic regulation of REM sleep. The brainstem area plays a pivotal role in REM sleep regulation.<sup>48,49</sup> Because *Cacnalc* is widely expressed also in this region, low REM sleep responses to sleep deprivation and restraint might be attributed to decreased  $\text{Ca}^{2+}$  influx via  $\text{Ca}_v1.2$ .<sup>50</sup> If this was the case, it might imply a role of LTCCs such as  $\text{Ca}_v1.2$  in REM sleep homeostasis.

Recently, it has become more evident that stress-induced REM sleep rebound or compensation is an adaptive strategy to cope with aversive and stressful situations.<sup>51</sup> A lower REM sleep rebound after restraint stress in HET mice suggests an impairment in an adaptive response to stress condition. The reduced rebound of REM sleep could also be associated with a decrease of  $\text{Ca}^{2+}$  mobilization within the brain. Restraint stress increases calcium mobilization in brain synaptosomes.<sup>52</sup> In particular, calcium mobilization/influx through LTCCs is important for restraint stress-induced responses. It has been confirmed that pretreatment with cilnidipine (L- and N-type calcium channel blocker) and nimodipine (L-type calcium channel blocker) significantly attenuates restraint stress-induced behavioral alterations,<sup>53</sup> neuroadaptive responses<sup>54</sup> and serum corticosterone levels.<sup>55</sup>

In addition to the brain,  $\text{Ca}_v1.2$  channels are expressed throughout the heart, especially in cardiac muscles and cerebral arteries, and have been shown to be important for cardiac physiology.<sup>26</sup> It has been reported that *Cacnalc* haploinsufficiency produces only a modest decrease in cardiac function during adulthood, whereas severe physical stress conditions are able to generate detrimental effects in cardiomyocytes of HET mice.<sup>56</sup> The exposure to restraint stress may evoke an increase in cardiac parameters such as heart rate or blood pressure, which may eventually lead to neuroendocrine stress. In this case, increased adrenergic tones in the sympathetic nerves may stimulate the central nervous system, which may indirectly contribute to the reduced rebound of REM sleep during recovery from restraint. Further,  $\text{Ca}_v1.2$  is also found in pituitary cells,<sup>57</sup> where  $\text{Ca}_v1.2$ -mediated LTCC current seems to be implicated in prolactin release.<sup>58</sup> Because prolactin is considered as a promoter of rebound sleep after stress,<sup>25</sup> the reduced stress-induced REM sleep in HET mice could to some extent be related to the reduced release of prolactin or other stress-related pituitary hormones. In accordance with previous studies, HET mice were largely indistinguishable from WT littermates; both exhibited normal body weight, life expectancy, and reproduction.<sup>26</sup> Although one has to consider that changes that occurred during development due to reduced expression of peripheral  $\text{Ca}_v1.2$  may indirectly affect the establishment of sleep phenotype observed in HET mice, it seems very unlikely that those peripheral changes account for altered central activities such as beta/gamma oscillations displayed in EEG spectra.

The relationships between sleep and mood disorders are complex. Although further tests are necessary, current results from heterozygous *Cacnalc* knockout mice may add further



relevance to genes associated with psychiatric diseases and mental distress-impaired sleep architecture. Alteration in the amplitude of the gamma rhythm has been found in schizophrenia.<sup>59,60</sup> Negative symptoms of schizophrenia, e.g., poor attention, decline in social interactions, and lack of motivation, may be linked to a subtle change in gamma EEG activity, which is primarily driven by the tonic excitatory drive and responsible for behavioral processes such as perception, consciousness and memory. As mentioned earlier, *Cacnalc* haplo-insufficiency could interfere with the normal tonic excitation in the RAS resulting in changes in gamma rhythms, which is a feature of schizophrenia. Decreased alertness and accumulated drowsiness during wakefulness in HET mice may be a replication of the negative symptoms of schizophrenia.

The literature emphasizes a link between schizophrenia and REM sleep.<sup>61,62</sup> Positive symptoms of schizophrenia (hallucinations and paranoia) may be related to malfunctioning REM sleep processes. Data from experimental conditions revealed that abnormal mentation of patients with schizophrenia during waking has a similar degree of formal cognitive bizarreness as dream narratives obtained from both nonclinical and clinical populations.<sup>63</sup> In this study, we found the reduced REM sleep recovery response in heterozygous *Cacnalc* knockout mice under challenging conditions, showing a malfunctioning of the REM sleep process in  $Ca_v1.2$  deficiency after exposure to acute stress conditions. A failure of REM sleep recovery may increase the risk in the case of repeated or chronic exposure to any challenging conditions. Based on the data obtained in the current study, we conclude that  $Ca_v1.2$ -mediated  $Ca^{2+}$  signaling participates in the control of EEG gamma oscillation and of the REM sleep regulatory process. Heterozygous *Cacnalc* knockout mice are at least partially predisposed to manifestations of impaired sleep regulation rather resembling those of schizophrenia than depression or other psychiatric diseases.

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## DISCLOSURE STATEMENT

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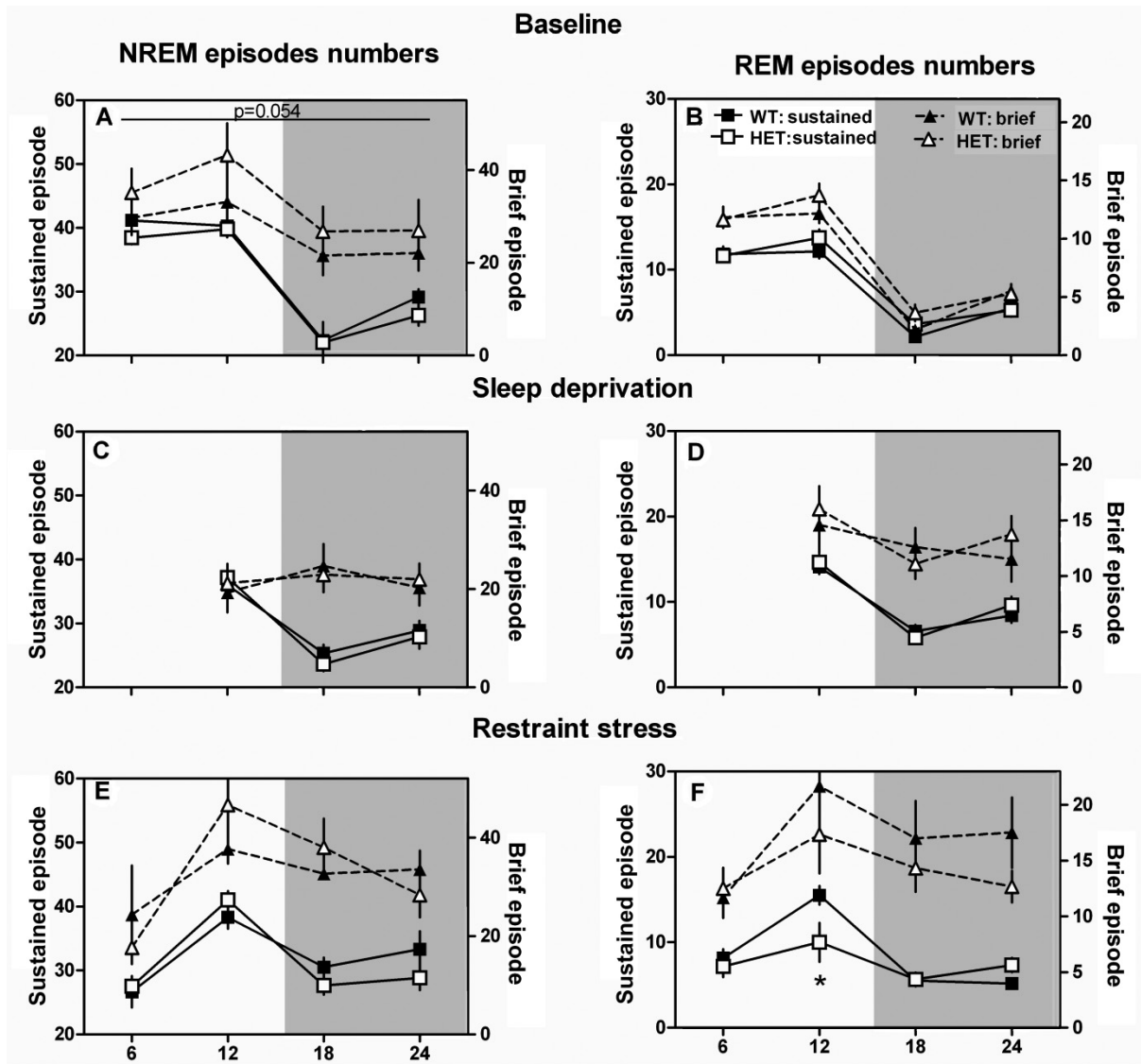
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**SUPPLEMENTAL MATERIAL**

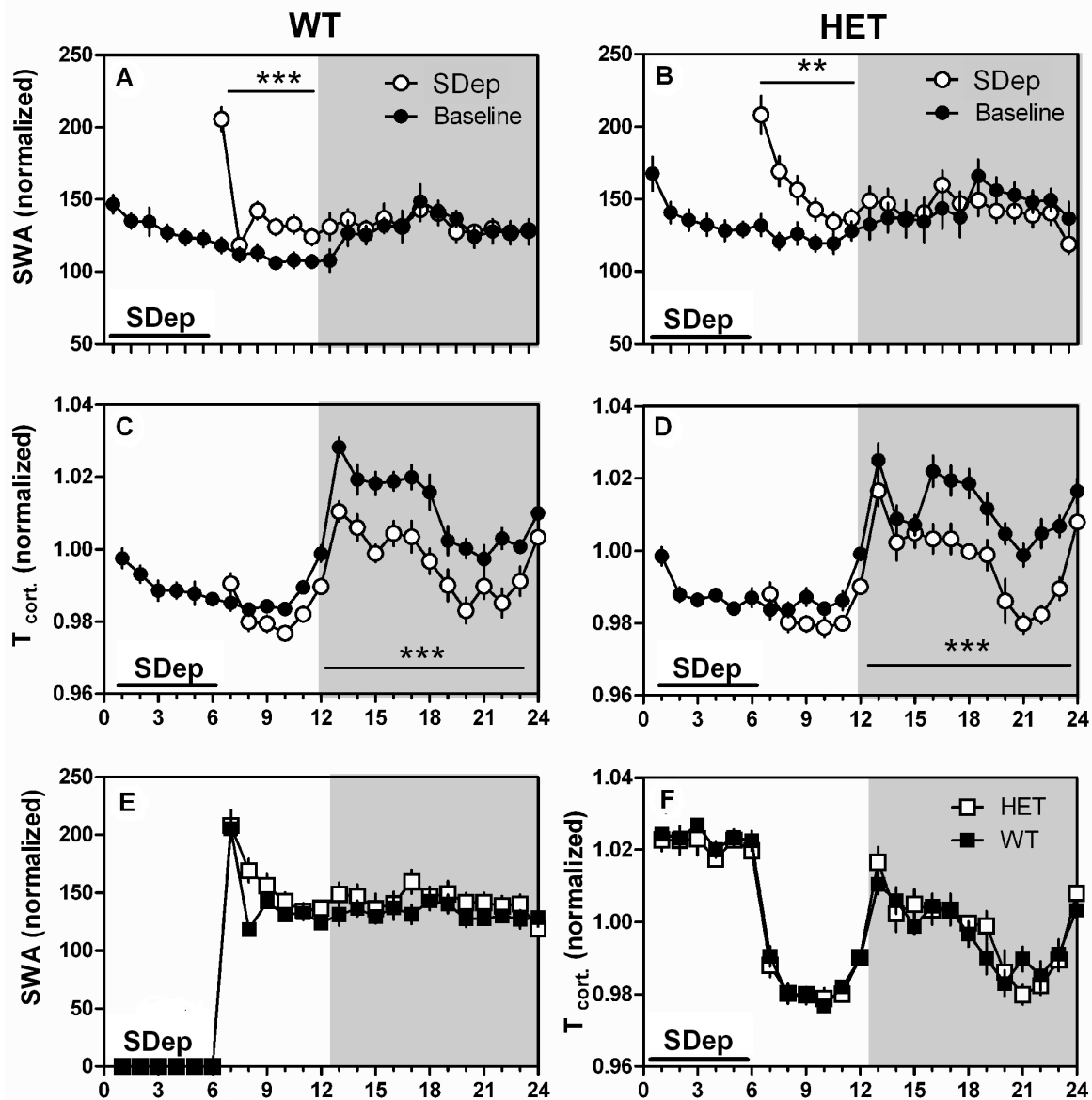
**Table S1**—Mean episode length (min) and number of rapid eye movement and nonrapid eye movement sleep at various conditions.

Time	Genotype	Baseline (WT, n = 12; HET, n = 11)		Sleep Deprivation (WT, n = 12; HET, n = 11)		Restraint Stress ▲ (WT, n = 6; HET, n = 6)	
		NREMS	REMS	NREMS	REMS	NREMS	REMS
0–6 ▲ h	WT	3.1 ± 0.2	1.21 ± 0.06			3.6 ± 0.4	1.21 ± 0.09
	HET	3.1 ± 0.3	1.09 ± 0.05			3.7 ± 0.3	1.04 ± 0.06
6–12 h	WT	3.0 ± 0.1	1.28 ± 0.07	4.2 ± 0.3 <sup>***</sup>	1.38 ± 0.06	3.0 ± 0.1	1.27 ± 0.10
	HET	2.8 ± 0.2	1.11 ± 0.08	4.0 ± 0.3 <sup>***</sup>	1.26 ± 0.06	2.8 ± 0.2	1.23 ± 0.10
12–18 h	WT	2.9 ± 0.2	0.72 ± 0.09 <sup>°°‡</sup>	2.7 ± 0.1 <sup>†</sup>	1.05 ± 0.05 <sup>*</sup>	2.5 ± 0.0	0.81 ± 0.06
	HET	2.7 ± 0.2	0.85 ± 0.07	2.9 ± 0.2	0.91 ± 0.08	2.4 ± 0.2	0.87 ± 0.08
18–24 h	WT	3.3 ± 0.3	0.95 ± 0.10	3.3 ± 0.3	1.22 ± 0.10	2.6 ± 0.1	0.70 ± 0.06
	HET	2.9 ± 0.3	0.88 ± 0.08	3.2 ± 0.2	1.02 ± 0.10	2.7 ± 0.2	0.98 ± 0.08
<b>Amount of Bouts</b>	<b>Genotype</b>	<b>NREMS</b>	<b>REMS</b>	<b>NREMS</b>	<b>REMS</b>	<b>NREMS</b>	<b>REMS</b>
0–6 ▲ h	WT	72.0 ± 4.2	27.2 ± 1.9			39.8 ± 2.9	19.8 ± 1.3
	HET	73.5 ± 5.3	32.0 ± 3.2			45.1 ± 2.8	19.6 ± 2.6
6–12 h	WT	76.0 ± 4.6	30.2 ± 3.1	55.1 ± 4.5 <sup>***</sup>	28.6 ± 2.7	76.0 ± 3.9 <sup>a</sup>	37.0 ± 3.6
	HET	83.0 ± 6.0	35.3 ± 4.0	59.7 ± 4.0 <sup>***</sup>	31.5 ± 2.7	87.6 ± 7.4 <sup>aa</sup>	39.8 ± 6.2 <sup>a</sup>
12–18 h	WT	42.2 ± 5.5 <sup>°°‡</sup>	10.1 ± 1.8 <sup>°°°‡</sup>	50.0 ± 5.1	19.1 ± 2.2	63.1 ± 2.3 <sup>*</sup>	22.5 ± 3.6 <sup>*</sup>
	HET	48.4 ± 6.0 <sup>°‡</sup>	11.5 ± 2.0 <sup>°°°‡</sup>	47.0 ± 3.8 <sup>†</sup>	17.0 ± 1.4 <sup>†</sup>	65.6 ± 5.9 <sup>*</sup>	20.0 ± 2.0 <sup>†*</sup>
18–24 h	WT	48.1 ± 3.7 <sup>‡</sup>	19.5 ± 1.6	49.1 ± 4.5	20.0 ± 1.8	66.8 ± 6.5 <sup>*</sup>	22.6 ± 3.4
	HET	53.0 ± 7.3 <sup>‡</sup>	18.7 ± 2.2 <sup>‡</sup>	54.9 ± 3.2	23.2 ± 2.2	57.1 ± 4.5	20.0 ± 1.8 <sup>†</sup>

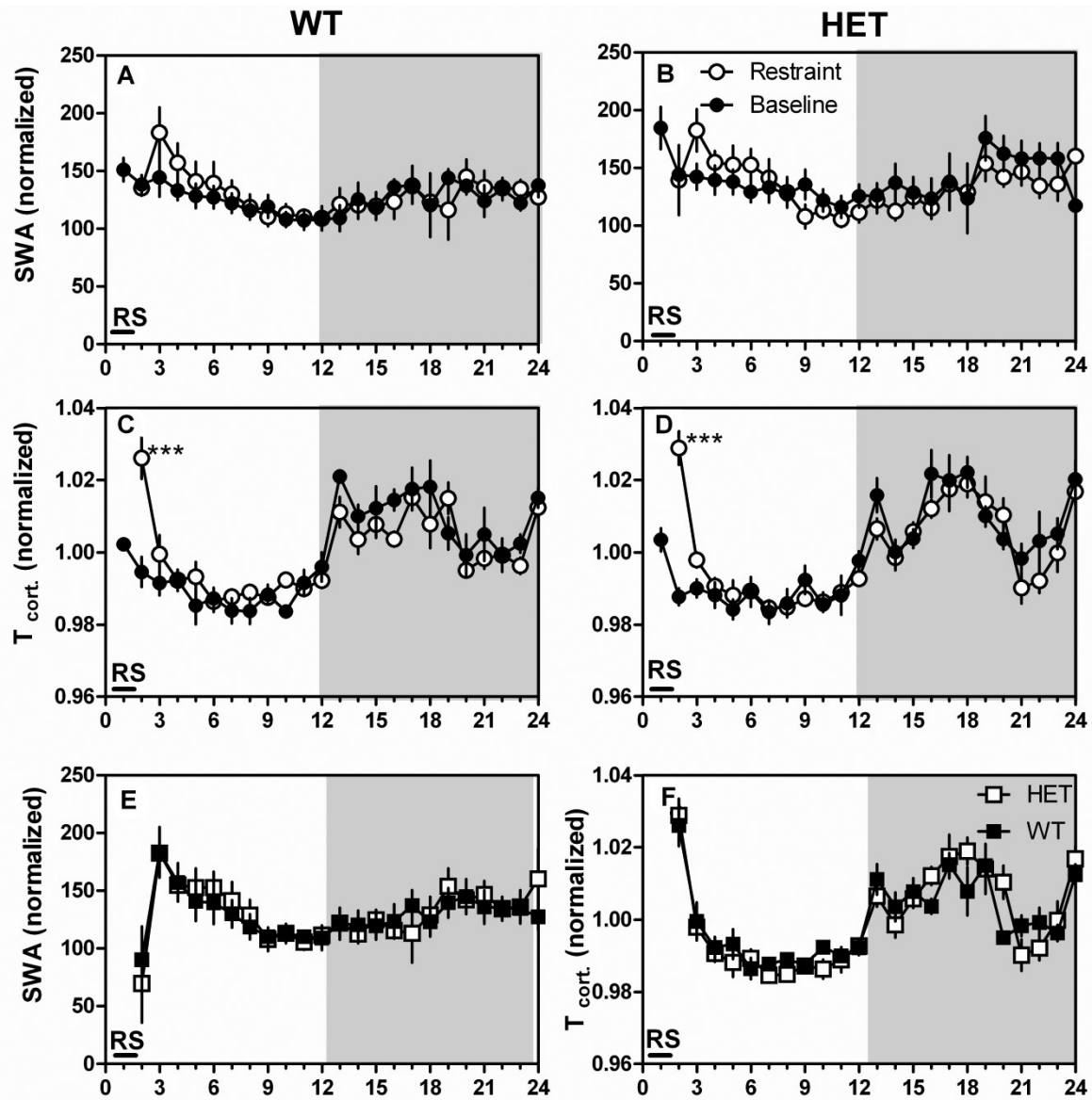
\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 versus time matched bin of baseline. °P < 0.05, °°P < 0.01, °°°P < 0.001 versus 0–6 h, †P < 0.05, ‡P < 0.01 versus 6–12 h. °P < 0.05, °°P < 0.01, versus 0–5 h in restraint stress condition. Note: 0–6 ▲ h, symbol “▲” indicates 1–6 h in restraint stress condition. Recording of 1 h during the period of restraint stress was not performed. HET, heterozygous *Cacna1c* knockout mice; NREMS, nonrapid eye movement sleep; REMS, rapid eye movement sleep; WT, wild-type.



**Figure S1**—The count of brief and sustained nonrapid eye movement (NREM) sleep episodes (left diagrams) and rapid eye movement (REM) sleep episodes (right diagrams) per 6-h bin in baseline, sleep deprivation, and restraint stress conditions. A brief or sustained REM/NREM sleep episode was defined in terms of numbers of consecutive 4-sec epochs as requiring 15 ( $\leq 1$  min) or more ( $> 1$  min) epochs for REM sleep and 30 ( $\leq 2$  min) or more ( $> 2$  min) epochs for NREM sleep, respectively. All values are mean  $\pm$  standard error of the mean. A significant difference between genotypes: \*P < 0.05.



**Figure S2**—Effects of the *Cacna1c* haploinsufficiency on changes in slow wave activity (SWA) and brain temperature ( $T_{cort}$ ) in response to sleep deprivation (SDep). Wild-type littermates (WT;  $n = 12$ ) and heterozygous *Cacna1c* knockout mice (HET;  $n = 11$ ) were subjected to 6 h of sleep deprivation during the first 6 h of the light period. (A–D) Comparisons of SWA (A,B) and  $T_{cort}$  (C,D) within the same genotype (left; WT, right; HET). (E,F) Comparisons of SWA (E) and  $T_{cort}$  (F) between WT and HET mice. The *Cacna1c* haploinsufficiency did not cause differences in SWA or  $T_{cort}$  responses to sleep deprivation. All values are mean  $\pm$  standard error of the mean. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (two-way analysis of variance).



**Figure S3**—Effects of the *Cacna1c* haploinsufficiency on changes in slow wave activity (SWA) and brain temperature ( $T_{\text{cort}}$ ) in response to restraint stress (RS). Wild-type littermates (WT;  $n = 6$ ) and heterozygous *Cacna1c* knockout mice (HET;  $n = 6$ ) were subjected to 1 h of restraint stress during the first hour of the light period. (A–D) Comparisons of SWA (A,B) and  $T_{\text{cort}}$  (C,D) within the same genotype (left; WT, right; HET). (E,F) Comparisons of SWA (E) and  $T_{\text{cort}}$  (F) between WT and HET mice. The *Cacna1c* haploinsufficiency did not cause differences in SWA or  $T_{\text{cort}}$  responses to restraint stress. All values are mean  $\pm$  standard error of the mean. \*\*\* $P < 0.001$  (two-way analysis of variance).