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Neuropathic pain impairs sleep architecture, non-rapid eye movement sleep, and reticular thalamic neuronal activity

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Abstract

Background: Neuropathic pain (NP) is a chronic and debilitating condition frequently comorbid with insomnia. However, the alterations in sleep architecture under NP conditions and the mechanisms underlying both pain and sleep disturbances remain poorly understood. The reticular thalamic nucleus (RTN) plays a crucial role in non-rapid eye movement sleep (NREMS) and pain processing, but its involvement in NP-related sleep disruptions has not been fully elucidated.

Methods: To investigate sleep-related electrophysiological changes in NP, we performed continuous 24-hour electroencephalogram/electromyogram (EEG/EMG) recordings in rats exhibiting allodynia following L5-L6 spinal nerve lesions. Additionally, we assessed the in vivo neuronal activity of the RTN in both NP and sham-operated control rats. Spectral analyses were conducted to examine alterations in sleep oscillatory dynamics. Reticular thalamic nucleus neuronal responses to nociceptive pinch stimuli were classified as increased, decreased, or unresponsive.

Results: Neuropathic pain rats exhibited a significant reduction in NREMS (–20%, $P < .001$) and an increase in wakefulness (+ 19.13%, $P < .05$) compared to controls, whereas rapid eye movement sleep (REMS) remained unchanged. Sleep fragmentation was pronounced in NP animals ($P < .0001$), with frequent brief awakenings, particularly during the inactive/light phase. Spectral analysis revealed increased delta and theta power during both NREMS and REMS. Reticular thalamic nucleus neurons in NP rats displayed a higher basal tonic firing rate, along with increased phasic activity (number of bursts), although the percentage of spikes in bursts remained unchanged.

Conclusions: Neuropathic pain is characterized by disrupted sleep architecture, reduced NREMS, and heightened RTN neuronal firing activity with partial compensation of burst activity. Given that RTN burst activity is essential for optimal NREMS, its disruption may contribute to NP-induced sleep impairments. These findings suggest that altered EEG/EMG signals, alongside dysregulated RTN neuronal activity, may serve as potential brain markers for NP-related insomnia.

Keywords: sleep; pain; L5-L6 model; EEG; insomnia; reticular thalamus.

Significance Statement

This study provides novel insights into the relationship between neuropathic pain (NP), sleep disruption, and reticular thalamic nucleus (RTN) activity. Using an established NP model in rats, we demonstrate that NP is associated with reduced non-rapid eye movement sleep (NREMS), increased sleep fragmentation, and elevated delta and theta power during sleep. Notably, in vivo electrophysiological recordings revealed a global increase in RTN tonic firing, with only partial adaptation of burst parameters, suggesting a maladaptive compensatory response. These findings support the hypothesis that RTN dysfunction contributes to NP-induced sleep disturbances and highlight its potential as a key hub linking chronic pain and insomnia. Understanding these mechanisms may pave the way for targeted therapeutic strategies to restore sleep architecture and mitigate NP-related symptoms.

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INTRODUCTION

Neuropathic pain (NP) is a chronic pain condition caused by a lesion or a disease of the somatosensory system, which includes peripheral fibers and central nervous system and affects 7%-10% of the population.[1] Neuropathic pain is characterized by sensory abnormalities such as unpleasant abnormal sensation (dysesthesia), pain response to a stimulus that would not normally evoke pain (allodynia), and an increased response to painful stimuli (hyperalgesia).[2] Chronic pain condition has a negative impact on the quality of life, including sleep disturbance and sleep deprivation (up to 48% of the patients).[3-6] Indeed, poor sleep occurred in patients with a wide variety of pain disorders including musculoskeletal,[7,8] post-herpetic trigeminal neuropathy,[9] post-surgery NP, HIV, multiple sclerosis, trigeminal neuralgia, cancer, trauma/accident, and diabetes.[10] Further, both chronic pain and insomnia can lead to other mental and physical comorbidities such as depression and type 2 diabetes.[3]

The mechanisms underlying NP and comorbid insomnia are still under investigation. Studies suggest that NP disrupts the sleep-wake cycle pattern, but, in turn, sleep disturbances have been correlated with exacerbated pain sensation and increased risk of developing chronic widespread pain.[11-16] One major limitation in studying the pathophysiology of NP and insomnia is the lack of reliable markers for assessing the quality and severity of both conditions. Although some studies have proposed that the electroencephalogram (EEG) and electromyogram (EMG) recordings can serve as objective biomarkers for chronic NP,[17] research applying these techniques to simultaneously study NP and insomnia remains limited.[18,19] Recent findings demonstrated that sleep fragmentation and non-rapid eye movement (NREM) sleep disruption occur in NP mice and can be prevented by genetic ablation of nociceptors in peripheral sensory neurons.[20] However, the brain circuits involved in NP-related sleep disturbances and specifically the brain regions contributing to insomnia and nociception have yet to be fully identified.

Interestingly, emerging evidence suggests that the reticular thalamic nucleus (RTN), a key regulator of NREMS,[21,22] may also play a critical role in regulating nociception.[23-25] The RTN is the major source of thalamic inhibition, regulates thalamocortical interactions that are critical for sensory processing, attention, and cognition.[23,26] Reticular thalamic nucleus dysfunction has been linked to sensory abnormality, attention deficit, and sleep disturbance across multiple neurodevelopmental disorders.[27,28]

The RTN is a cluster of GABAergic neurons that receive inputs directly from the cortex and thalamus and send inhibitory outputs to the thalamic relay nuclei, which project back to the cortex[29]; RTN is relevant for translational studies because it is evolutionarily conserved across species, including rodents and humans.[23] Given its dual involvement in sleep regulation and pain sensory processing, the RTN represents a potential neural substrate linking NP and insomnia, making it a candidate for further investigation as a therapeutic target for addressing comorbid pain and sleep disturbances in human patients. For this reason, the present study aimed to investigate 24-hour EEG/EMG activity in freely moving rats with NP (L5-L6 lesion) and corresponding sham control animals. Additionally, we conducted in vivo electrophysiological recordings of RTN neurons to characterize potential changes in their activity following NP. We hypothesized that RTN neuronal dysfunction could be associated with both NP-related insomnia and pain processing, representing a shared neural mechanism contributing to these comorbid conditions.

MATERIALS AND METHODS

Animals

Wistar rats (male) weighting 140-160 g at the beginning of the experiment were housed under standard conditions, at a constant temperature of 22 °C, with food and water provided ad libitum and under a 12-h light/dark cycle (lights on at 7:00 AM; lights off at 7:00 PM). All experimental procedures and surgeries were approved by the Animal Ethics Committee of local institutional committee for animal use and care (protocol# 7181, Comparative Medicine and Animal Resources Centre, McGill University), following the Canadian Council of Animal Care and Canadian Institute of Health Research for animal care and scientific use.

L5-L6 Spinal Nerve Ligation and Measurement of Mechanical Allodynia

Neuropathic pain was induced according to Kim and Chung's method[30] and based on established protocols previously validated in our laboratory.[31] Briefly, animals were anesthetized with isoflurane (5% induction; 2% maintenance). To access the left lumbar spinal nerve, an incision is made at the level of the posterior iliac crest. The left L5 and L6 spinal nerves were exposed, identified, and tightly ligated distal to the dorsal root ganglion. In control sham-operated rats, the same incision was made but the nerves were exposed without further manipulation. The incision was closed using 3.0 vicryl suture, and animals were allowed to recover for 14 days. Rats showing motor deficits such as paw dragging, foot dropping, lack of coordination, deformities of the hind paw, or growth of the toenail (less than 5%) were eliminated from the study[31] (Figure 1A).

Assessment of Mechanical Allodynia

Fourteen days after the surgery, mechanical allodynia was assessed using the up-and-down method,[32] (Figure 1A). All animals were placed on a chamber test (clear plastic with mesh-bottomed cage) and allowed to adapt for 45 minutes in their cage. Calibrated von Frey filaments for rats (Stoelting, ranging from 3.61 [0.407 g] to 5.46 [26 g] bending force) were applied to the mid-plantar surface of the hind paw. If there was no response, the next filament with higher force was tested. If there was a positive response (lifting and/or shaking the hind paw), the next lower filament was then tested. This continued until 6 consecutive responses had been obtained. The mean 50% paw withdrawal threshold (g) was calculated for each rat using Dixon's formula.[33] Only animals with a paw withdrawal threshold less than 4 g were considered allodynic and were further implanted with EEG/EMG electrodes for 24-hour electrical recording; non-allodynic rats were excluded[31] (Figure 1A).

EEG/EMG Implantation

Sham and neuropathic rats were deeply anesthetized with isoflurane (5% for induction, 2%-3% for maintenance) and placed in a stereotaxic frame. For EEG monitoring, 3 stainless-steel epidural electrodes were positioned through 1.5 mm burr holes at −2 mm anteroposterior (AP) and −3 mm lateral (L), −4.5 mm AP and +3 mm L, −7 mm AP and −3 mm AP according to bregma (coordinates have been chosen according to the Rat Brain atlas of Paxinos and Watson). For the EMG signal, 3 flexible stainless-steel wires were implanted into the neck muscle. Then, wires and connectors were fixed to the skull using dental acrylic (Coltene/Whaledent Inc.). According to our well-established laboratory procedures, rats were given a 5-day recovery period, which was sufficient for restoring a normal sleep/wake cycle.[34,35]

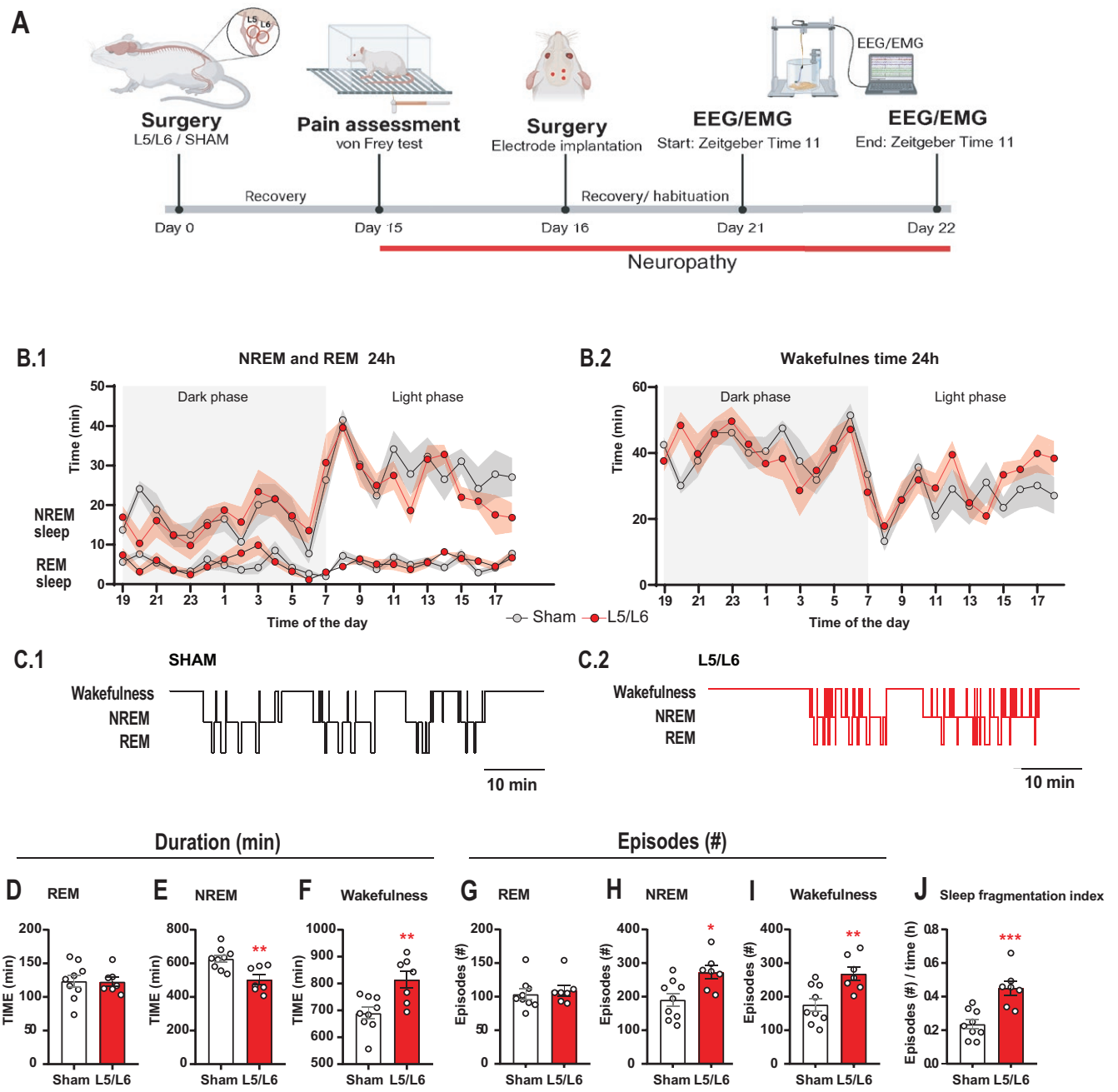

Figure 1. Analysis of the time REMS, NREMS, and wakefulness episodes in sham vs L5-L6 lesioned rats. (**A**) Schematic timeline illustrating the timing of L5-L6 induction and behavioral experiments. The duration of REMS and NREMS (**B.1**), and wakefulness (**B.2**) is represented hour by hour during 24 hours in sham control (gray) and L5/L6 (red)-operated rats. Data are expressed as mean ± SEM, 2-way ANOVA for RM. Representative hypnograms of sham (**C.1**) and L5/L6 (**C.2**) were used during the 60-min recording during the light phase. Twenty-four-hour analysis of duration of REMS (**D**), NREMS (E), and wakefulness (**F**) and the number of episodes in REMS (**G**), NREMS (**H**), and wakefulness (**I**) by sham control and L5-L6-operated animals. Sleep fragmentation index is shown in **J**. Data are expressed as mean ± SEM. Sham $n = 9$, L5-L6 $n = 7$. $^{*}P < .05$ $^{**}P < .01$ $^{***}P < .001$ vs Sham. Unpaired t-test. NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep.

EEG/EMG Data Analysis

Twenty-four hours after surgery, rats were placed for habituation in the recording room from 12:00 PM to 9:00 PM daily. During this time, the rats were placed in the recording chambers and connected to a flexible 6-flat cable (3M Scotchflex®), in a freely moving manner. No recordings were performed, but tolerance to the cable and sleep behavior were observed. On day 5, (4 days after habituation) EEG/EMG recordings were performed for 24 h, starting at Zeitgeber time 11 (6:00 PM). Electroencephalogram/electromyogram signals were amplified with a total gain of 10 000 and then digitalized by a CED 1401 interface system, processed on-line, and analyzed offline by Spike 2 software, in parallel with analog-to-digital samplings of amplified (Grass, P55) polygraphic signals (EEG; sampling rate, 100 or 200 Hz). Consecutive 10-second epochs were subjected to a Fast Fourier Transform, and EEG power spectra density was computed in the frequency range of 0-64 Hz.[34]

Analysis of EEG and EMG Data

Analysis was made offline using the spike 2 software (CED). Three classical sleep stages were identified using EEG and EMG recordings. Wakefulness was determined by a sustained activity of EMG and low amplitude and high frequency of EEG. Non-rapid eye movement sleep was characterized by high amplitude δ wave (1-4 Hz) and low muscle activity. Rapid eye movement sleep was marked with muscle atonia as observed in EMG and low amplitude θ wave in EEG (4-8 Hz). The frequency range for the sigma band was 11–15 Hz. Only periods longer than 10 seconds were marked for further analysis of awake, REMS and NREMS, in order to eliminate the transitional period such as drowsiness (Figures 1B-I and 2). Power spectra were expressed as a percentage change relative to sham control animals (Figure 3). Sleep fragmentation index (SFI) was calculated as follows: total number of awakenings/shifts from sleep (NREMS and REMS) divided by the total sleep time in hours (Figures 1J and 2I and R).

In Vivo Extracellular Recording of Reticular Thalamic Neurons

In vivo single-unit extracellular recordings of pain-sensitive and pain-insensitive neurons in RTN were performed in both sham-operated and L5-L6 spinal nerve-ligated animals following our previous work.[34] Rats were anesthetized with urethane (1.2 g/kg, i.p.) and positioned in a stereotaxic frame (David Kopf Instruments). Following a midline scalp incision, a burr hole was drilled at the following stereotaxic coordinates corresponding to the RTN: –2.56 mm AP, –3.5 mm ML, and 5.0–6.5 mm ventral relative to bregma (Paxinos and Watson, 2006). Body temperature was maintained using a heated pad under the rat body. Extracellular single-unit recordings were performed using a single-barreled glass micropipette (R&D Scientific Glass; tip diameter, 1 to 3 µm) filled with 2% pontamine sky blue dye in sodium acetate (2 M, pH 7.5; electrode impedance, 5–10 MΩ).

Single-unit activity was filtered (AC, 0.2–2 kHz), amplified (Bak Electronics Model RP-I), and fed to an oscilloscope (BK Precision; 20 MHz, 1522) and an audio monitor. Spike shapes were digitalized by a CED 1401 interface system, processed on-line, and analyzed offline by Spike 2 software, in parallel with analog-to-digital samplings of amplified (Grass, P55) polygraphic signals (EEG; sampling rate, 100 or 200 Hz). Using an amplitude spike discriminator, single-unit activity was isolated. Reticular thalamic nucleus neurons were recognized by their long burst (50 ms) and accelerando-decelerando bursting firing pattern,[36] followed by a tonic tail.[37,38] The first 30 seconds immediately after detecting the neuron, the signal was not recorded to eliminate mechanical artifacts due to electrode displacement. The spontaneous single-spike activity of the neurons was then recorded for at least 5 minutes. Firing and burst neuronal activity were analyzed offline with Spike 2 software to measure the basal firing rate and number of bursts. The burst of RTN neurons consisted of a discharge of at least 4 spikes,[39] with an onset defined by a maximum inter-spike interval of 20 ms and a pre-burst and post-burst interval of 100 ms.[38] The longest interval allowed within a burst was 70 ms to include the tonic tail, which is merely the end of a spike barrage.[38] Neurons displaying no burst activity were automatically excluded from the analysis.

Statistical Analysis

Data analysis was done using GraphPad Prism statistical software version 5.04 (SYSTAT Software, Inc.). A 2-tailed unpaired *t*-test was performed to compare neuropathic (L5-L6) and sham-operated (control) animals regarding the total duration and number of episodes of REMS, NREMS, and wakefulness, as well as sleep fragmentation, sleep latency, and neuronal activity parameters, including bursting characterization and firing rate. To evaluate hourly variations in REMS and NREMS durations across the light and dark phases and to evaluate firing rate changes between baseline and during pinch-evoked activity in RTN neurons, a 2-way repeated-measures (RM) ANOVA was conducted. Post hoc analyses were performed using the Bonferroni-corrected *t*-test comparisons. Data are expressed as mean ± SEM, and statistical significance was set at $P < .05$.

RESULTS

NP Decreased the Duration of NREMS Sleep Across 24 Hours

To evaluate the impact of NP induced by L5-L6 nerve ligation on sleep architecture, we first analyzed sleep-wake patterns across the 24-hour light/dark cycle in freely moving rats following the experiment timeline in Figure 1A. Figure 1B-J illustrates the effect of NP on REMS, NREMS, and wakefulness durations in NP rats ($n = 7$) compared to sham-operated controls ($n = 9$). Hour-by-hour analyses of REMS and NREMS (Figure 1B.1) and wakefulness (Figure 1B.2) duration across the 24-hour light/dark cycle were performed along with representative hypnograms from a sham-operated rat (Figure 1C.1) and an NP rat (Figure 1C.2), highlighting qualitative sleep disruption caused by NP. For REMS duration (Figure 1B.1), 2-way ANOVA for repeated measures showed no interaction between time and condition ($F_{(23,332)} = 1.445$; $P = .0876$), a significant effect of time ($F_{(23,332)} = 2.214$; $P = .0013$), and no effect of condition ($F_{(1,14)} = 0.0731$; $P = .7908$). Similarly, no interaction between time and condition ($F_{(23,332)} = 1.146$; $P = .2934$), a significant effect of time ($F_{(7.553,105.7)} = 8.163$; $P < .0001$), and no effect of condition ($F_{(1,14)} = 0.3109$; $P = .5860$) was found for the hour-by-hour analysis of NREMS duration (Figure 1B.1). Finally, for the hour-by-hour duration of wakefulness (Figure 1B.2), there was no interaction between time and condition ($F_{(23,332)} = 1.272$; $P = .1833$), but a significant effect of time ($F_{(7.389,103.5)} = 6.381$; $P < .0001$), and no effect of condition ($F_{(1,14)} = 0.2866$; $P = .6008$) was found. We then performed the analysis of the sleep stages over the entire 24-hour period and found that REMS duration did not differ significantly between sham and NP animals (Figure 1D; $t_{(14)} = 0.07$, $P = .94$).In contrast, NP animals displayed a significant reduction in NREMS duration compared to sham controls (Figure 1E; $t_{(14)} = 3.53$, $P = .003$). Accordingly, NP animals also displayed a significant increase in wakefulness throughout the 24-hour period compared to the sham group (Figure 1F; $t_{(14)} = 3.35$, $P = .004$).

NP Increased the Number of NREMS and Wakefulness Episodes and SFI During 24 Hours

We next analyzed the number of REMS, NREMS, and wakefulness episodes over the 24-hour period (Figure 1G-I). No significant differences were observed in the number of REMS episodes between NP and sham-operated rats (Figure 1G; $t_{(14)} = 0.51$, $P = .61$). In contrast, NP animals exhibited a significant increase in the number of NREMS episodes compared to sham controls (Figure 1H; $t_{(14)} = = 2.92$, $P = .01$). Similarly, it was found that NP animals displayed a significantly higher number of episodes of wakefulness over the 24-hour period (Figure 1I; $t_{(14)} = 3.32$, $P = .005$). Consistently, the SFI was markedly elevated in L5-L6 NP rats (Figure 1J; $t_{(14)} = 4.360$, $P = .0007$), further confirming disrupted sleep architecture in NP conditions.

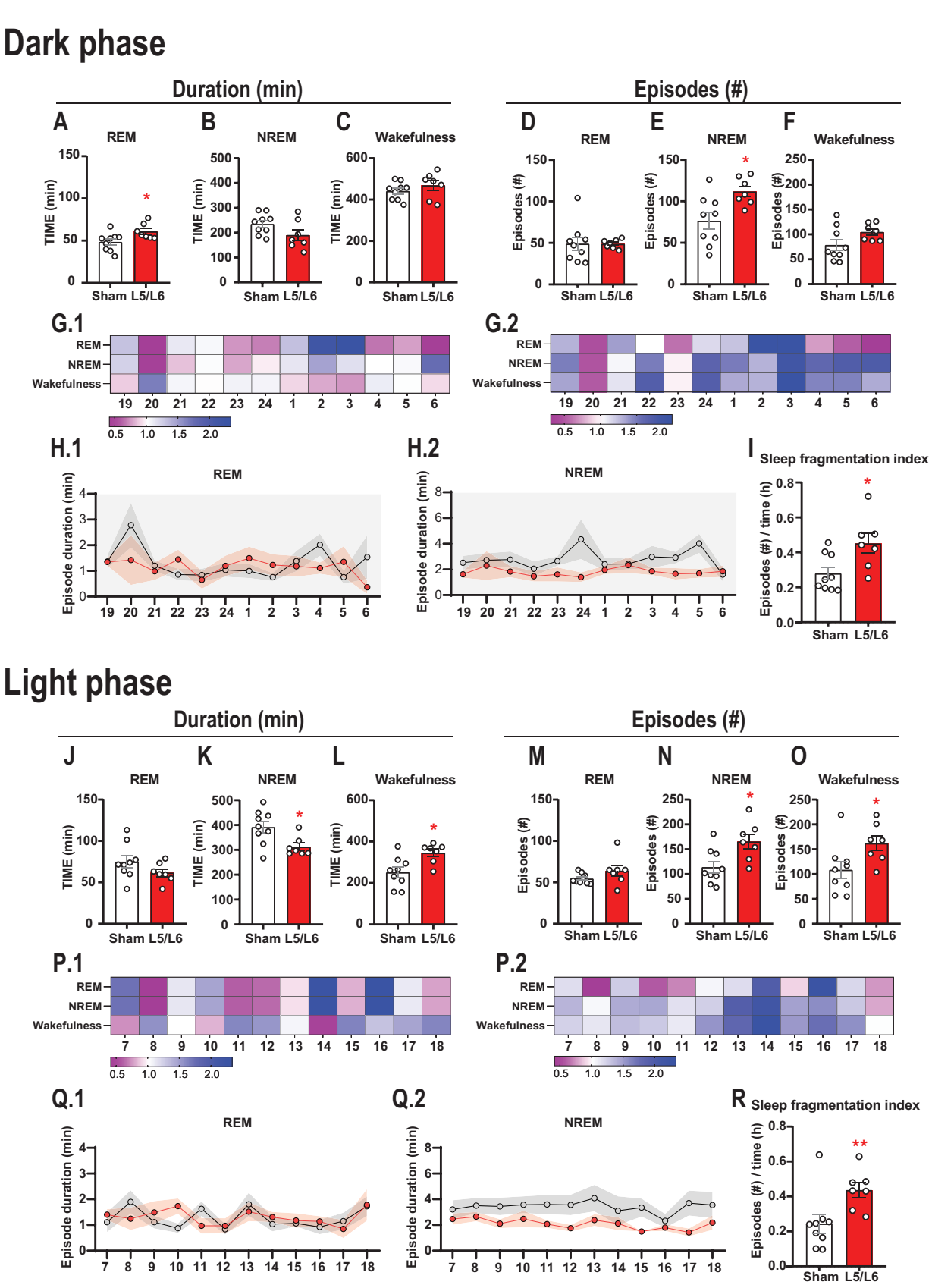

Figure 2. Twelve-hour analysis of the REMS, NREMS, and wakefulness episodes in sham vs L5-L6. **TOP**: The results during the dark/active phase (from 7 PM to 7 AM) are shown from **A** to **I. BOTTOM**: The results during the light/inactive phase (from 7 AM to 7 PM) are shown in **J to R**. Twelve-hour analysis of the duration of REMS (**A,J**), NREMS (**B,K**), and wakefulness (**C,L**) and the number of episodes in REMS (**D,M**), NREMS (**E,N**), and wakefulness (**F,O**) in sham control (white column) and L5-L6-operated animals (red column) during the dark (**TOP**) and light phase (**BOTTOM**), respectively. Heat map representing the mean of the distribution of REMS, NREMS, and wake episodes by hour during the dark (**G1,2**) and light (**P1,2**) periods as a function of duration (left) and number of episodes (right) normalized by the mean of sham control determined by a scoring of 10-second epoch. The length of episodes of REMS, **H.1** (dark phase) and **Q.1** (light phase), and NREMS **H.2** (dark phase) and **Q.2** (light phase) are presented hour by hour. Sleep fragmentation index in both the dark (**I**) and Light (**R**) phases are shown on the right. Data are expressed as mean ± SEM. Sham $n = 9$, L5-L6 $n = 7$. *$P < .05$ **$P < .01$ vs Sham. Unpaired *t*-test. NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep.

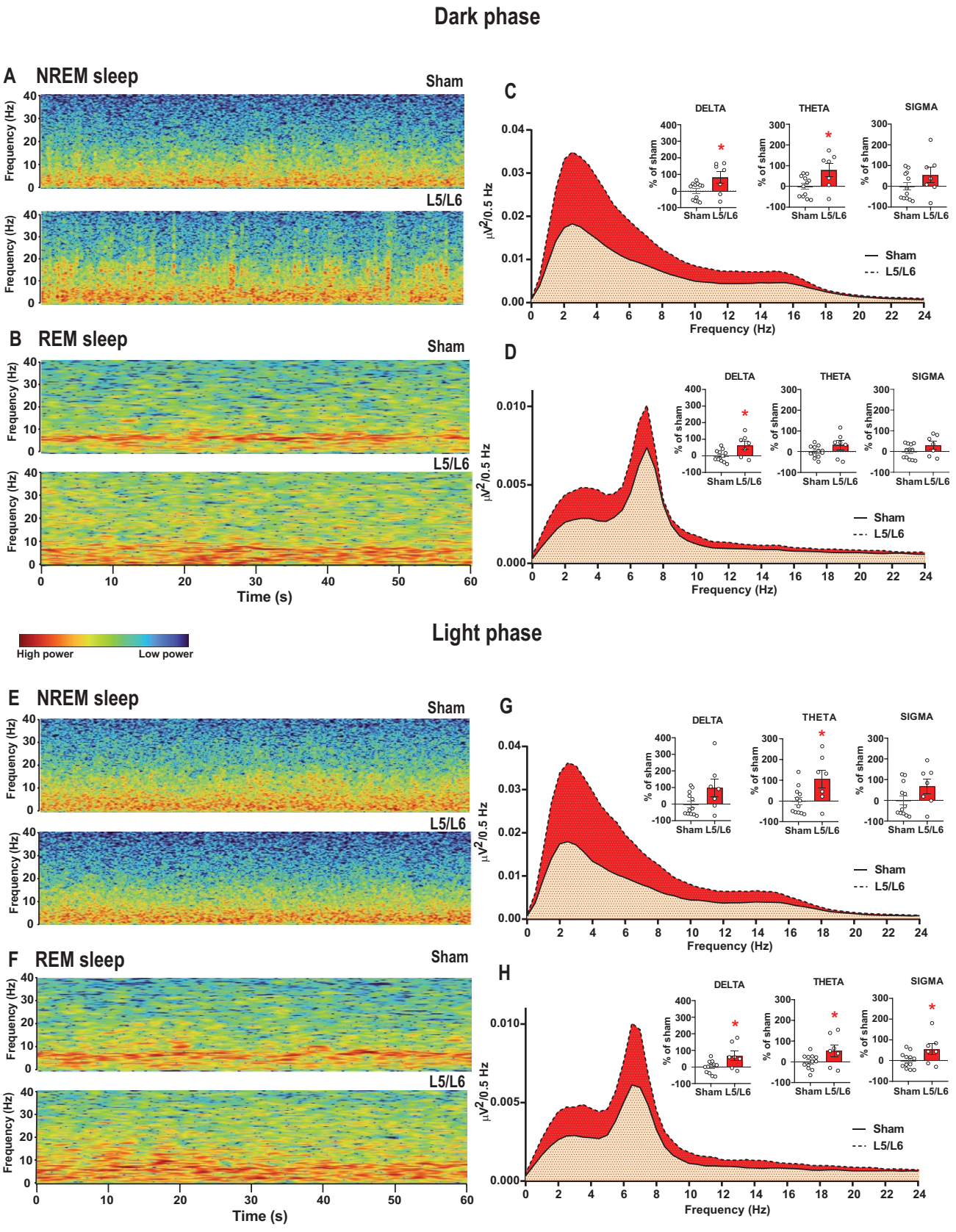

Figure 3. Effect of neuropathic pain during the dark (TOP) and light (BOTTOM) phases with analysis of power spectra. (Left) Spectrogram comparing power spectral density during NREMS (**A, E**) and REMS (**B, F**) between sham and L5/L6 animals. (**Right top**) Delta, theta, and sigma waves during NREMS (**C**) and REMS (**D**) during the dark phase. (**Right bottom**) Delta, theta, and sigma waves during NREMS (**G**) and REMS (**H**) in the dark phase in sham (beige) and L5/L6 animals (red). Data are expressed as a percentage of change. Data are expressed as mean ± SEM. Sham $n = 9$, L5-L6 $n = 6$. *$P < .05$ vs sham control group. NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep.

NP Increased the Duration of REMS in the Dark/Active Phase

To further characterize the altered sleep structure across the 24-hour light/dark cycle, we separated our analysis into the dark (active) and light (inactive) phases. We investigated the impact of NP on the duration of the three vigilance states during the dark/active phase (Figures 2A-I). Independent *t*-test analysis revealed that NP significantly increased the duration of REMS compared to the sham group (Figure 2A; $t_{(14)} = 2.46$, $P = .02$). In contrast, no significant differences were observed in the duration of NREMS (Figure 2B; $t_{(14)} = 1.76$, $P = .09$) or wakefulness (Figure 2C; $t_{(14)} = 1.02$, $P = .32$) between the groups.

NP Decreased the Duration of NREMS Sleep in the Light/Inactive Phase

During the light phase (Figure 2J-R), NP did not alter REMS duration compared to sham animals (Figure 2J; $t_{(14)} = 1.47$, $P = .16$). However, NREMS duration was significantly reduced in NP animals compared to the sham group (Figure 2K; $t_{(14)} = 2.71$, $P = .01$). Additionally, NP rats exhibited an increased duration of wakefulness compared to sham controls (Figure 2L; $t_{(14)} = 2.95$, $P = .01$).

NP Increased the Number of NREMS Episodes During the Dark/Active Phase

The number of REMS episodes during the dark/active phase did not differ between NP and sham rats (Figure 2D; $t_{(14)} = 0.01$, $P = .98$). In contrast, NP rats exhibited a significant increase in the number of NREMS episodes compared to the sham group (Figure 2E; $t_{(14)} = 2.8$, $P = .013$). The number of wakefulness episodes did not differ between the two groups (Figure 2F; $t_{(14)} = 1.86$, $P = .08$).

NP Increased the Number of NREMS and Wakefulness Episodes During the Light/Inactive Phase

During the light/inactive phase, the number of REMS episodes did not differ between NP and sham animals (Figure 2M; $t_{(14)} = 1.57$, $P = .15$). In contrast, NP rats displayed a significant increase in the number of NREMS (Figure 2N; $t_{(14)} = 2.76$, $P = .015$) and wakefulness (Figure 2O; $t_{(14)} = 2.31$, $P = .03$) episodes compared to sham animals.

NP Did not Alter the Length of REMS or NREMS Episodes During Both the Dark and Light Phases

We next examined the average duration of REMS and NREMS episodes on an hourly basis during the dark phase (Figure 2H.1 and H.2, respectively). Two-way RM ANOVA for REMS episode duration revealed no significant interaction between time and NP condition ($F_{(11,154)} = 1.15$; $P = .32$), and no effects of NP condition ($F_{(1,14)} = 0.51$; $P = .48$) and time ($F_{(3.96,55.46)} = 1.19$; $P = .32$) (Figure 2H.1). Similarly, NREMS episode duration analysis during the dark phase indicated no significant interaction ($F_{(11,154)} = 1.67$; $P = .08$), and no effects of condition ($F_{(1,14)} = 2.25$; $P = .15$) and time ($F_{(4.04,\ 56.65)} = 1.06$; $P = .38$) (Figure 2H.2). During the light phase, two way RM ANOVA for REMS episode duration revealed no significant interaction between factors ($F_{(11,154)} = 1.02$; $P = .42$), and no effect of condition ($F_{(1,14)} = 0.02$; $P = .88$) and time ($F_{(4.88,68.35)} = 1.48$; $P = .20$) (Figure 2Q.1). Similarly, NREMS episode duration analysis yielded no interaction condition × time ($F_{(11,154)} = 0.69$; $P = .74$), and no effects of time ($F_{(4.69,\ 65.67)} = 1.01$; $P = .41$) and condition ($F_{(1,14)} = 2.78$; $P = .11$) (Figure 2Q.2).

NP Increased the SFI During the Light and Dark Phases

Given the observed alterations in sleep parameters, including sleep duration and the number of sleep episodes, we calculated the SFI during the light and the dark phases. Independent *t*-test analysis during the dark phase (Figure 2I) revealed a significantly higher SFI in NP rats compared to the sham group ($t_{(14)} = 2.67$, $P = .018$). Similarly, SFI was higher in NP compared to sham during the light phase (Figure 2R; $t_{(14)} = 2.68$, $P = .017$).

NP Induced an Increase in Theta Waves During NREMS in Both Light and Dark Phases

We also analyzed the potential changes induced by NP on the power spectra (delta, theta, and sigma waves) of NREMS during both light and dark phases. The quality of the changes in power spectra is shown in Figure 3A and E for NREMS and Figure 3B and F for REMS. During the dark phase, *t*-test analysis of NREMS showed that delta power ($t_{(17)} = 2.43$, $P = .026$) and theta power ($t_{(17)} = 2.56$, $P = .02$) were both significantly higher in the NP group compared to the sham group, while there were no significant differences between the two groups in the percentage of sigma power ($t_{(17)} = 1.45$, $P = .16$; Figure 3C inset). In the light phase, theta power ($t_{(17)} = 2.66$, $P = .016$) was significantly higher in the NP group compared to the sham group, while only a tendency for higher delta power in NP was present ($t_{(17)} = 2.04$, $P = .056$), with no significant change in sigma power ($t_{(17)} = 1.70$, $P = .10$; Figure 3G inset).

NP Induced an Increase in Delta, Theta, and Sigma Waves During REMS in the Light Phase

We further analyzed the changes induced by NP on the power spectra (delta, theta, and sigma waves) of REMS during both light and dark phases (Figure 3B and F). During the dark phase (Figure 3D), REM sleep analysis showed that the delta power ($t_{(17)} = 2.80$, $P = .012$) was significantly higher in NP animals compared to the sham group, while there were no significant differences between the two groups in theta ($t_{(17)} = 1.63$, $P = .12$) or sigma ($t_{(17)} = 1.57$, $P = .13$) power, as shown in Figure 3D inset. During the light phase (Figure 3H), REMS analysis showed that delta ($t_{(17)} = 2.60$, $P = .018$), theta ($t_{(17)} = 2.17$, $P = .04$) and sigma ($t_{(17)} = 2.23$, $P = .039$) powers were significantly higher in NP animals compared to the sham group.

NP Increased the Basal Tonic and Phasic Burst-Firing Activity in RTN Neurons

To investigate whether the neuronal activity of RTN neurons was altered in NP, we recorded spontaneous neuronal activity in the RTN using in vivo single-unit extracellular electrophysiological recordings in anesthetized rats (Figure 4A). The electrophysiological recordings primarily targeted the anterior dorsal RT area, which somatotopically corresponds to the hind paw.[40] Analysis of tonic firing activity revealed a significant increase in basal neuronal activity in NP animals compared to sham controls (Figure 4B; $t_{(55)} = 4.47$, $P < .0001$; Sham $n = 30$ neurons from 15 rats, NP $n = 27$ neurons from 17 rats). Similarly, the firing and burst-firing properties of RTN neurons were affected by NP (Table 1A). Neuropathic pain rats exhibited a greater number of bursts over a period of 180 seconds compared to sham-operated animals ($t_{(55)} = 2.95$, $P = .004$). The number of spikes per burst was also higher in NP rats than in sham-operated animals ($t_{(55)} = 2.27$, $P = .02$). Likewise, burst rate (Hz), burst frequency, and the coefficient of variation of burst activity were significantly elevated in NP animals ($t_{(55)} = 3.08$, $P = .003$; $t_{(55)} = 2.29$, $P = .02$; $t_{(55)} = 1.95$, $P = .05$, respectively) compared to sham rats. In contrast, the percentage of spikes in bursts did not differ between the 2 groups ($t_{(55)} = 1.46$, $P = .15$).

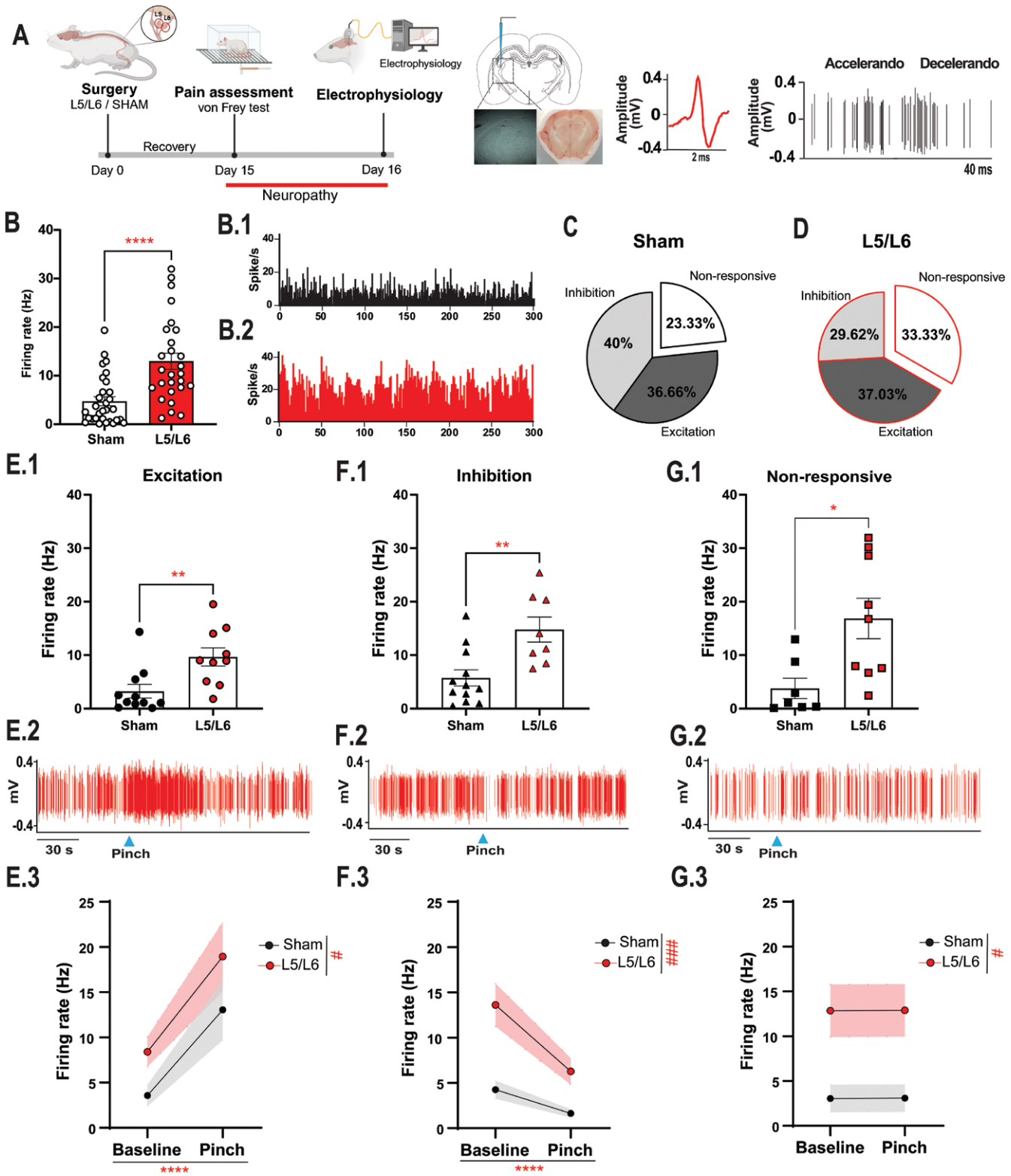

Figure 4. Spontaneous electrical activity of neurons in the reticular thalamus nucleus (RTN). (**A**) Experimental schema (top). Representative coronal section of rat brain with the photomicrograph of the recording site in the RTN with the typical spike waveform of GABAergic RT neurons. The white arrow indicates the site of the electrode recording labeled with sky blue dye (middle), with the neuronal wave and the accelerando-decelerando pattern of RTN GABAergic neurons (right). Mean firing rate (**B**), histogram (300 seconds) of the spontaneous firing rate activity in sham control (**B.1**) and L5/L6 rats (**B.2**). Percentages of neurons that excite, inhibit, or do not have a reaction to mechanical painful stimuli on sham (C) and L5/L6 rats (**D**) were calculated. Spontaneous firing rate activity in sham and L5-L6-operated rats, according to the 3 subpopulations: excitability (**E.1**), inhibition (**F.1**), and not responsive after pinch (G.1) is shown. Student's unpaired 2-tailed *t*-test $^{*}P < .05$ $^{**}P < .01$ vs Sham. The firing rate histogram of a single RTN neuron showing the pinch-evoke excitation (**E.2**), pinch-evoke inhibition (**F.2**), and pinch non-response (**G.2**) is shown. Quantification of the firing rate between spontaneous firing activity before and during the pinch was compared for the pinch-evoke excitation (**E.3**) pinch-evoke inhibition (**F.3**) and pinch non-response (**G.3**). Two-way ANOVA for RM analysis. $^{*}P < .05$, $^{***}P < .001$, $^{****}P < .0001$ vs sham control, $^{\#}P < .05$, $^{\#\#\#}P < .001$ vs baseline. Data are expressed as mean ± SEM. Sham $n = 38$, L5/L6 $n = 33$.

Table 1. Spontaneous burst-firing activity of reticular thalamic neurons after L5-L6 spinal nerve ligation.

	Number of burst (3 minute)	Spikes in burst	Spikes per burst (%)(%)	Burst rate (Hz)	Burst duration (ms)	Burst events	Var. Coef. (%)
A. Groups Sham (n = 30) L5/L6 (n = 27)	38.83 ± 8.08 82.63 ± 12.81 [a]	695.1 ± 208.6 1593 ± 346.4[b]	33.51 ± 4.74 45.25 ± 6.65	0.21 ± 0.04 0.44 ± 0.06[a]	320.9 ± 66.67 480.2 ± 94.87	1474 ± 291.3 2458 ± 330.4[b]	150.2 ± 14.89 114.2 ± 10.25
Statistics	**t = 2.95, P = .004**	**t = 2.27, P = .02**	t = 1.46, P = .15	**t = 3.08, P = .003**	t = 1.40, P = .16	**t = 2.24, P = .02**	**t = 1.95, P = .056**
B. Excitation Sham (n = 11) L5/L6 (n = 10)	21.73 ± 8.65 73.0 ± 21.18[b]	253.7 ± 116.4 591.4 ± 203.0	29.38 ± 7.36 33.04 ± 8.23	0.11 ± 0.04 0.38 ± 0.10[b]	298.4 ± 63.31 243.7 ± 41.34	695.6 ± 191.5 1593 ± 296.6[b]	152.9 ± 21.36 128.4 ± 20.13
Statistics	**t = 2.32, P = .03**	t = 1.47, P = .15	t = 0.33, P = .74	**t = 2.5, P = .02**	t = 0.70, P = .48	**t = 2.58, P = .01**	t = 0.83, P = .41
C. Inhibition Sham (n = 12) L5/L6 (n = 8)	33.92 ± 7. 72 74.63 ± 17.39[b]	593.9 ± 241.6 1568.0 ± 553.2	26.50 ± 5.76 44.37 ± 11.92	0.23 ± 0.06 0.39 ± 0.09	326.7 ± 98.9 503.2 ± 144.7	1653 ± 315.7 2795 ± 484.4	119.2 ± 11.7 106.0 ± 9.18
Statistics	t = 2.4, **P = .02**	t = 1.81, P = .08	t = 1.49, P = .15	t = 1.46, P = .16	t = 1.04, P = .3	t = 2.06, P = .053	t = 0.8, P = .42
D. Nonresponsive Sham (n = 7) L5/L6 (n = 9)	28.71 ± 15.34 83.78 ± 21.57	1461 ± 916.8 2530 ± 802.3	47.77 ± 11.11 55.69 ± 13.64	0.12 ± 0.04 0.44 ± 0.10[b]	537.7 ± 260.2 744.1 ± 225.6	1894 ± 1043 3128 ± 751.5	157.8 ± 27.03 107.7 ± 17.1
Statistics	**t = 1.96, P = .06**	t = 0.87, P = .39	t = 0.43, P = .67	**t = 2.46, P = .02**	t = 0.6, P = .55	t = 0.98, P = .34	t = 1.63, P = .12

Group A. Basal burst-firing activity was evaluated in all recorded RTN neurons. Group B. Basal burst-firing activity in pinch-exciting RTN neurons. Group C. Basal burst-firing activity in pinch-inhibited RTN neurons. Group D. Basal burst activity in pinch-nonresponsive RTN neurons. An unpaired t-test was used to compare sham- vs L5/L6-operated rats for each burst-firing activity parameter. Data are expressed as mean ± SEM, with the number of neurons per group indicated in parentheses. Significant comparisons are highlighted in bold.

[a]P < .01 vs sham control.

[b]P < .05.

Abbreviation: RTN, reticular thalamic nucleus.

RTN Neuronal Subtypes Responded Differentially to the Mechanical Stimulation (pinch) in NP Vs Sham Animals

To further investigate the relationship between RT neurons and nociceptive signal modulation, we recorded spontaneous neuronal activity in the RT during contralateral mechanical painful stimulation (paw pinch). Interestingly, tonic firing rates responded in 3 different patterns to painful stimuli in both NP and sham animals: inhibition, excitation, or no response. We tested 30 RTN neurons to mechanical stimulation in sham-operated rats anesthetized with urethane. Their responses after the pinch were distributed as follows: 12/30 (40%) exhibited inhibition; 11/30 (36.66%) showed excitation, and 7/30 (23.33%) remained nonresponsive (Figure 4C). Conversely, in NP rats, out of the 27 RT neurons recorded, 10/27 (37.03%) displayed increased tonic firing activity, 8/27 (29.62%) showed inhibition, and 9/27 (33.33%) exhibited no changes following the pinch (Figure 4D).

We next compared the tonic firing activity of these 3 neuronal populations between sham and L5-L6 NP animals. Neurons excited by the mechanical stimulus displayed significantly higher mean firing activity rates in NP animals compared to sham controls ($t_{(19)}$ =3.06, P = .006; Sham mean = 3.24 Hz, n = 11, L5/-L6 mean = 9.67 Hz, n = 10; Figure 4E.1). Similarly, neurons inhibited by the pinch also exhibited elevated firing activity in NP animals relative to sham ($t_{(18)}$ =3.42, P = .003; Sham mean = 5.74 Hz, n = 12, NP mean = 14.79 Hz, n = 8; Figure 4F.1). Likewise, the firing activity of nonresponsive neurons was significantly higher in NP animals than in controls ($t_{(14)}$ =2.82, P = .012; Sham mean = 3.79 Hz, n = 7, NP mean = 16.85 Hz, n = 9; Figure 4G.1). Histograms representing these neuronal subtypes are shown below each graph (Figure 4E.2-G.2). We further analyzed the firing profiles of the 3 neuronal populations in sham control and NP animals before (baseline) and during the mechanical painful stimuli (pinch). For the neurons excited by the pinch, two-way RM ANOVA for the firing rate activity revealed no significant interaction between factors ($F_{(1,18)}$ = 0.21; P = .64), but an effect of condition (NP vs Sham; $F_{(1,18)}$ = 4.65; P = .04), and difference in pinch-evoke excitation ($F_{(1,18)}$ = 29.29; P = .0001) (Figure 4E.3). Likewise, the analysis of the neurons inhibited by the pinch indicated no significant interaction between factors ($F_{(1,18)}$ = 2.25; P = .15), but differences in condition (NP vs Sham; $F_{(1,18)}$ = 15.28; P = .001) and in pinch-evoke inhibition ($F_{(1,18)}$ = 27.67; P = .0001; two-way RM ANOVA) (Figure 4F.3). For the neurons nor-responsive to pinch, differences were found by condition (NP vs sham, $F_{(1,14)}$ = 6.19; P = .02) but no interaction ($F_{(1,14)}$ = 0.09; P = .75) and no effects of pinch-responsiveness ($F_{(1,14)}$ = 0.10; P = .75) were found (Figure 4G.3).

Finally, the burst-firing parameters (phasic activity) of RTN neurons across the three subpopulations (Table 1B-D) showed a significant increase in the number of burst between NP and sham animals in neurons excited and inhibited by pain stimulus (Excited: $t_{(19)}$ =2.32, P = .03; Inhibited: $t_{(18)}$ =2.40, P = .02), but not in the nonresponsive group ($t_{(14)}$ =1.96, P = .06). The bust rate (Hz) was higher in RTN neurons excited ($t_{(19)}$ =2.5, P = .02) and in nonresponsive neurons ($t_{(14)=}$2.46, P = .02) in the NP group compared to the sham group. Burst events were significantly increased in pinch-excited neurons ($t_{(19)}$ =2.58, P = .01) but not in the other two populations. No adaptive differences were observed in spikes per burst, burst duration, or coefficient of variation parameters across the 3 populations.

DISCUSSION

In this study, we demonstrated that NP in rats is associated with sleep disruption, characterized by decreased NREMS, increased sleep fragmentation (restlessness), and elevated delta and theta power during both NREMS and REMS. These EEG alterations in NP rats were accompanied by a higher tonic firing rate of GABAergic neurons in the RT, along with the increased number of bursts, but not the percentage of spikes per burst and burst duration. A lower proportion of neurons inhibited to pinch stimuli was also found in the NP group.

Previous studies have reported similar findings regarding the impact of chronic pain on sleep patterns in animal models. Most studies have demonstrated a significant reduction in NREMS and sleep fragmentation in various models of NP, including chronic constriction injury (CCI) and the polyarthritis model[41-47], and also found a disruption of NREMS in mice with NP, even if it was not paralleled by an increased delta power. However, some discrepancies exist. For instance, Kontinen et al.[48] did not observe such effects using the CCI model, while Monassi et al.[49] reported that only 30% of animals developed insomnia in a spared nerve injury (SNI) model. Similarly, Tokunaga and colleagues[50] found that only CCI rats placed on sandpaper as an aversive condition exhibited sleep disturbances, though the recording duration was limited to only 6 hours. Recently, Alexandre et al.[20] found that various NP models induce sleep fragmentation by increasing brief arousals from NREMS without altering total sleep time. In contrast, inflammatory or chemical pain models did not increase brief arousals. After SNI, the frequency of brief arousals rose during both the light (+ 24 ± 4%) and dark (+ 25 ± 9%) phases compared to baseline. Despite this increase, the EEG spectral profiles of brief arousals remained consistent pre- and post-SNI. The discrepancies between previous literature and our data may stem from differences in recording sensitivity or the use of an SNI model in mice rather than a spinal nerve ligation (SNL) model in rats, which may offer better signal quality. Our study confirms that the SNL model of NP is a robust and sensitive model for investigating sleep abnormalities in rats. Beyond sleep fragmentation, we observed reduced NREMS duration, increased wakefulness, more frequent NREMS and wakefulness episodes, and significant changes in EEG power spectra. The reduction in NREMS sleep quantity was also associated with a global increase in theta and delta power during both NREMS and REMS. Enhanced theta (4–8 Hz) oscillations in the primary somatosensory (SI) cortex have been previously observed in chronic pain patients using EEG[51,52] and in animal pain models.[53] Similarly, sleep recovery following sleep deprivation in chronic pain conditions is marked by increased slow-wave power (delta waves) and enhanced cortical synchrony,[54,55] reflecting sleep homeostasis or compensatory processes. In our study, elevated EEG delta power during NREMS might be linked to homeostatic mechanisms and abnormal activity of the RTN, while increased theta band power may reflect qualitative disruptions in sleep architecture.

The RTN is composed of GABAergic neurons that receive input directly from both the cortex and thalamus, and in turn, provide inhibitory output to thalamic nuclei, which subsequently project back to the cortex.[23,26,56,57] It is a thin, sheet-like structure topographically positioned between the cerebral cortex and dorsal thalamus, releasing GABA to regulate the activity of the dorsal thalamus.[58,59] The RTN is known to be involved in the generation of NREMS. According to Steriade,[21] during the NREMS, the RTN enters a phasic burst activity mode, with its GABAergic neurons projecting to the thalamic relay. The thalamic-cortical pathways then send signals to the cortex, which enters a silent mode interrupted by sporadic burst intervals.

Previous studies have reported that the $GABA_A$ receptor antagonist picrotoxin induces a “pain-like” behavior when

administered into the RTN of behaving rats.[60] Resting-state functional magnetic resonance imaging studies have shown that thalamocortical dysrhythmia and altered thalamic firing occur in individuals with NP.[52,61-63] Recent literature indicates that activating parvalbumin neurons in the rostro-dorsal sector of the RTN promotes pain sensitivity in rodents,[57,64] and that the RTN-lateral habenula circuit regulates depressive-like behaviors in chronic stress and chronic pain.[65] Although the mechanistic link remains speculative, our results reveal a neurophysiological correlation between RTN activity and pain condition, demonstrating a global increase in tonic firing activity in RTN neurons, which can suggest a compensatory mechanism to pain and sleep disruption, but with a partial adaptation of the phasic burst parameters. Tonic and burst firing are believed to serve distinct roles: tonic firing relays peripheral sensory signals to the cortex during wakefulness,[66,67] whereas burst phasic firing blocks sensory transmission from being relayed to the cortex during certain phases of sleep or deep anesthesia.[66,68,69] This is based on the observation that burst-firing events became more prevalent during sleep or deep anesthesia and are associated with deep sleep and lack of pain. Although tonic firing predominates during wakefulness, burst firing also plays critical roles, such as detecting new stimuli in the visual system[70] and facilitating the whisking behavior of mice.[71] A recent study[22] has found two neuronal profiles in the RTN following a transcriptomic analysis: *Spp1* (encoding secreted phosphoprotein 1) and *Ecel1* (encoding endothelin converting enzyme like 1) neurons. While the Spp1+ subpopulation of RTN neurons significantly reduced the power of delta rhythms during NREMS and the length of spindle oscillations, the Ecel1+ subpopulation increased the length of spindle oscillations without changing their numbers.[22] Burst firing of RTN neurons, known to be mediated by T-type Ca^{2+} channels, plays a crucial role in sleep spindle generation during NREMS.[72-74] Increased thalamic activity during pinch has been associated with antinociceptive effects,[75] suggesting that RTN burst activity may represent a compensatory antinociceptive response to chronic pain stimuli. Our in vivo recordings suggest that NP induces a general increase in RTN tonic firing, but the burst phasic activity is poorly compensated, since not all the burst parameters are significantly affected in NP animals (see Table 1). This finding may help explain why NP leads to disruption of the physiological NREMS. However, future studies utilizing optogenetic or DREADDs (designer receptors exclusively activated by designer drugs) techniques will be essential to confirm this hypothesis. In keeping, promoting thalamic bursting of RTN through optogenetic stimulation can reduce cortical theta oscillations and reverse nociceptive behavior.[76]

Moreover, the increase in fast waves, as opposed to slow delta waves at sleep onset induced by NP, may cause physiological arousal, thereby disrupting sleep initiation and maintenance.[42]

This work, for the first time, highlights the role of the RTN in pain and insomnia, emphasizing the need for further studies in humans. In humans, for example, lesions of the ventroposterolateral (VPL) thalamus, which receives inhibitory input from the RTN, cause Dejerine Roussy syndrome (or thalamic pain syndrome), a rare central NP syndrome, and patients with chronic pain have thalamocortical dysrhythmia, characterized by cortical disinhibition and nociceptive behaviors.[77] Thalamocortical dysrhythmia may result from increased bursting/firing in RT relay neurons, leading to excessive inhibition from hyperactive GABAergic projections[26,78] and hyperpolarization of VPL neurons. Disruption of the RTN is also one of the most severe thalamic alterations observed in fatal familial insomnia,[79] a neurodegenerative disorder caused by an abnormal variant in the prion-related protein (*PRNP*) gene.

The main limitation of this study is that it was conducted exclusively in male rats, despite evidence suggesting sex-specific differences in pain responses, with females often showing heightened pain sensitivity and more fragmented sleep patterns due to hormonal fluctuations.[11,80-82] Future studies will be designed to investigate sex-specific differences by incorporating female subjects at different phases of the estrous cycle. This approach could provide deeper insights into the neurophysiological mechanisms underlying pain-related sleep disruption and determine whether hormonal influences alter RTN neuronal responses to pain stimuli. Finally, while putative RTN neurons were identified by their electrophysiological characteristics, an in vivo immunohistochemical verification of neuronal subtypes was not feasible.

CONCLUSION

In conclusion, the chronic pain induced by the L5-L6 ligation NP model caused macro- and micro-level changes in the sleep patterns, reducing NREMS duration and increasing the sleep fragmentation, while enhancing the phasic and tonic activity of RTN neurons.

Future studies employing selective manipulation of RTN activity through optogenetics or DREADDs could determine whether suppressing RTN hyperactivity rescues sleep fragmentation and pain sensitivity. Such approach would provide deeper mechanistic insights into the observed relationship. Similar strategies have shown promising results in mice by modulating thalamocortical dynamics.[57] Establishing the mechanistic basis of RTN neuronal dysrhythmia in NP could lead to new therapies targeting thalamic circuits. For example, noninvasive neuromodulation techniques, such as transcranial magnetic stimulation or transcranial direct current stimulation, could be optimized to modulate RTN activity and restore sleep architecture. Interestingly, Czekus et al. highlighted the role of the RTN in regulating sleep architecture and homeostatic processes. Particularly, they demonstrated that oxidative stress-vulnerable Gclm knockout mice exhibited fragmented sleep architecture and impaired sleep homeostasis due to alterations in RTN-anterodorsal thalamocortical circuits. These impairments were reversed by administering N-acetylcysteine, an antioxidant, which normalized local neuronal dynamics.[83]
These findings suggest that targeting RTN dysfunction could be a promising therapeutic strategy for addressing sleep disturbances associated with NP in humans.

Author contributions

Martha López-Canul (Conceptualization [lead], Data curation [lead], Formal analysis [lead], Methodology [lead], Writing—original draft [lead]), Anahita Oveisi (Data curation [supporting], Investigation [supporting], Methodology [supporting], Writing—original draft [supporting]), Qianzi He (Investigation [supporting], Methodology [supporting]), Maria Luisa Viganó (Investigation [supporting]), Antonio Farina (Investigation [supporting], Methodology [supporting]), Stefano Comai (Data curation [supporting], Investigation [supporting], Writing—review & editing [equal]), and Gabriella Gobbi (Conceptualization [lead], Data curation [lead], Formal analysis [lead], Funding acquisition [lead], Investigation [lead], Methodology [lead], Project administration [lead], Supervision [lead], Writing—review & editing [lead])

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Conflicts of interest

G.G. is the inventor of patents on selective melatonin compounds and Founder of Cosmas Therapeutics. She is also the inventor of a patent on the use of LSD, which was licensed out to Diamond Therapeutics. Her lab received research grants from Diamond Therapeutics.

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Data availability

The data generated during this study are made available from the corresponding author upon reasonable request.

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