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Hypnotic effects of melatonin depend on the environmental lighting conditions in the rat

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Abstract

Acute effects of exogenous melatonin have been widely reported to promote sleep or induce drowsiness in human. However, testing of the hypnotic effects of melatonin in nocturnal rodents has yielded contradictory results. The latter may be associated with differences in concentration, lighting conditions, time of administration of melatonin, and possibly the type of analysis. In this study, electroencephalogram (EEG) and electromyogram were recorded in pigmented male Brown Norway rats under both light-dark (LD) and constant dark (DD) conditions. Melatonin was administered intraperitoneally at a moderate dose of 3 mg/kg, at either 1 h after lights on under LD condition or 1 h after the activity offset under DD condition. The dosage is known to be able to entrain nocturnal rodents in DD conditions, but does not change sleep in rodents in LD. Only the rats under DD conditions showed a significant reduction in nonrapid eye movement (NREM) sleep latency, while the NREM sleep power spectrum remained unaffected. Under LD condition, melatonin did not alter NREM and rapid eye movement (REM) sleep latency, and had only minor effects on the NREM sleep EEG. Regardless of lighting conditions, melatonin administration resulted in less, but longer episodes for all vigilance states suggesting increased vigilance state consolidation. In the discussion, we compare our results with a summary of previously published data concerning the hypnotic effects of melatonin in polysomnographic/EEG-confirmed sleep in humans and nocturnal rodents. In conclusion, the hypnotic effect of exogenous melatonin in nocturnal rodents not only depends on the time of day, and concentration, but is also influenced by the lighting conditions. Regardless of inducing sleep or not, melatonin may consolidate sleep and through that enhance sleep quality.

KEYWORDS

dark, electroencephalogram, hypnotic effects, light, melatonin, rat, sleep

1 | INTRODUCTION

In mammals, melatonin (N-acetyl-5-methoxytryptamine) is secreted by the pineal gland during the dark period, and it is, therefore, also known as the "darkness hormone." This rhythmic secretion during the night by the pineal gland is controlled by the suprachiasmatic nucleus (SCN) and entrained by the environmental light-dark (LD) cycle.[1,2] Melatonin is involved in various developmental, physiological and behavioral processes, including pubertal development, thermoregulation, energy metabolism, antioxidant mechanisms, seasonal adaptation, analgesia, anticonvulsant mechanisms, entrainment of circadian rhythms, and acute hypnotic effects.[3–8] The administration of exogenous melatonin has beneficial effects on sleep in various conditions, including individuals with insomnia, jet lag, shift work, and circadian rhythm sleep disorders.[3,8–10]

It is generally thought that melatonin's working action is via melatonin receptors, MT_1 and MT_2, which are members of a family of G-protein coupled receptors.[11] Melatonin receptors are found in many brain areas, such as locus coeruleus, dorsal raphe nucleus, hippocampus, thalamus and SCN.[12–14] Exogenous melatonin is known to inhibit neuronal activity of SCN slices immediately,[15] which means that through the melatonin receptors in the SCN, melatonin can feedback on the circadian system. Quite surprising, knockout of both MT_1 and MT_2 receptors did not influence the amount of nonrapid eye movement (NREM) and rapid eye movement (REM) sleep in mice.[16] Similarly, in rats, pinealectomy failed to significantly alter the sleep architecture.[17] These findings suggest that absence of both receptors or melatonin has only a minor effect on sleep. Selectively knocking out or activating either MT_1 or MT_2, leads to significantly different and distinct outcomes, possibly due to the distinct locations of MT_1 and MT_2 receptors. For instance, MT_2 receptors are located in the reticular thalamus, an area associated with NREM sleep, while MT_1 receptors are found in the locus coeruleus and lateral hypothalamus, regions associated with REM sleep and sleep–wake maintenance.[12,18,19] Additionally, melatonin has a fivefold higher affinity for the MT_2 receptor than for the MT_1 receptor.[20] It has been proposed that MT_1 receptors are primarily involved in regulating REM sleep, while MT_2 receptor activation selectively increases NREM sleep.[21] This is supported by studies using gene-selective knockout mice and selective MT_1 or MT_2 agonists.[16,18] For instance, the selective MT_2 receptors agonist IIK7 can decrease the latency to and increase the duration of NREM sleep.[22] No effects on REM sleep duration have been observed in MT_2KO mice. These studies provide compelling evidence for the distinct and selective role of MT_2 receptors in NREM sleep. MT_1KO mice show reduced REM sleep and wakefulness,[16] furthermore, these mice also show a disrupted REM sleep circadian rhythm. These findings strongly suggest that the MT_1 receptor is not only involved in the regulation of REM sleep but also plays a crucial role in the circadian control of REM sleep. In addition, the possibility exists that the hypnotic action of melatonin directly concerns the neuronal level at nuclear binding sites, independent of membrane receptors.[23]

Unfortunately, in nocturnal rodents, application of exogenous melatonin has given conflicting results on sleep.[24–26] Unlike humans, these nocturnal rodents mainly sleep during the light phase, when melatonin is virtually absent, and sleep little in the dark phase, when the endogenous concentration of melatonin is increased. The action of exogenous melatonin in nocturnal rodents may be explained by the coincidence hypothesis, which holds that physiological responses to melatonin are linked to the existence of a diurnal rhythm in sensitivity to melatonin.[27] This diurnal variation in sensitivity to melatonin may depend on the light conditions. Light has a strong effect on sleep in nocturnal rodents, and may, therefore, modulate the effect of melatonin on sleep and wakefulness. In nocturnal C3H mice, it was found that the daily expression of MT_1 receptor mRNA and protein in the SCN differs between 12 h:12 h LD and constant dark (DD) conditions, with MT_1 melatonin receptor mRNA and protein expression being higher in the rest phase under DD conditions compared to the same phase under LD.[28] These differences in receptor expression level may make lower serum levels of melatonin still effective in mice under DD, as the lower level of melatonin is, in that case, sufficient to bind to the increased number of receptors to obtain similar effects as a higher level of melatonin under LD condition when receptor expression levels are lower. From this, we expect that the susceptibility to melatonin is higher under DD and that lower melatonin levels under DD, therefore, may have a similar effect as higher levels of melatonin under LD conditions. We want to test this hypothesis in the effect of melatonin on sleep.

To investigate this, we applied a relatively low concentration of melatonin (3 mg/kg) which in previous research did not induce sleep under LD conditions,[24] but was able to entrain circadian rhythms and induce phase shift under DD,[29,30] to investigate putative differences in sleep after treatment with melatonin between LD and DD conditions.

An additional source of conflicting results may be differences in the type of analysis performed. Most analyses applied only include changes in the amount of sleep and waking, whereas others, particularly in

humans include sleep latency in the analysis. Some analysis further includes changes in electroencephalogram (EEG) spectral power. We, therefore, calculated both sleep latency and the amount of sleep in different interval durations, and in addition, we performed an analysis of the vigilances state episodes and of the EEG power density spectrum.

In the discussion, we provide an overview of sleep analysis of the effects of melatonin, but limited to studies using EEG/electromyogram (EMG) confirmed sleep in nocturnal rodents and polysomnographically confirmed sleep in humans. The latter are the gold standard of vigilance state determination and ensure the most objective measure for sleep in these studies.

2 | MATERIALS AND METHODS

2.1 | Animals

Sixteen 12-weeks old male Brown Norway rats (Charles River) were used in this study. Since we wanted to test the effect of light conditions on sleep, we decided not to take an albino strain as is usually the case in rat sleep studies. The Brown Norway rat is a pigmented rat strain which has been used in sleep research before and displays normal sleep, but is known to have a slightly lower day–night amplitude in sleep–wake distribution compared to the more common albino strains,[31] which may also be reflected in the sleep–wake distribution presented here. Rats were group-housed under 12 h:12 h LD condition (100 lux at eye level), Lights on 8:00 lights off 20:00) with food and water ad libitum in a temperature-controlled room (21–22°C). All animal experiments were approved by the Central Committee on Animals Research (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2 | Surgical procedure and EEG recordings

At a body weight of approximately 300 g (16 weeks of age), the animals were deeply anesthetized with ketamine (Aescoket; 65 mg/kg) and Xylazine (Rompun, Bayer AG; 13.3 mg/kg) and surgery was performed as described earlier.[32,33] EEG electrodes (Plastics One) were screwed through the skull on the dura over the right parietal cortex (2.0 mm lateral to the midline, 3.5 mm posterior to bregma) and the cerebellum (at the midline, 1.5 mm posterior to lambda). Two wires with suture patches (Plastics One) were inserted between the skin and the neck muscle tissue for EMG recordings. The wire branches of all electrodes were set in a plastic pedestal (Plastics One) which was fixed to the skull with dental cement and three additional support screws. The rats were single-housed after surgery and were allowed to recover for at least 7 days in their home cage under 12 h:12 h LD condition. After full recovery, the animals were connected to the recording system by a flexible cable and a counterbalanced swivel system, and they remained in the recording chamber under either constant darkness or 12 h:12 h LD conditions for at least 1 week to familiarize with the recording conditions before the start of the recording. The recording chamber was equipped with a passive infrared sensor to record locomotor activity in the cage.[32,33]

2.3 | Experimental design

The animals were divided into two groups, one group was kept under constant darkness ($n = 8$) and another group was under 12 h:12 h LD condition ($n = 7$) for at least 1 week before treatment with melatonin. The animals' locomotor activity were recorded continuously to obtain an estimate of the circadian phase when the animals were in constant darkness. From this, onset and offset of rest and activity were determined and an F-periodogram analysis provided an estimate of the circadian period.[32,34] This enabled us to determine the time of melatonin or vehicle treatment for the next recording day.

Melatonin (M5250, Merck was dissolved in vehicle (15% ethanol) at a concentration of 3 mg/mL which was used previously in several studies.[24] At circadian time 1 (CT1, circadian time 12 is activity onset)/zeitgeber time 1 (ZT 1, zeitgeber time 0 is lights on), 1 h after lights on or the predicted offset of locomotor activity, the animals received either melatonin (3 mg/kg) or vehicle at a volume of 1 mL/kg body weight in an intraperitoneal injection under a randomized cross-over design. This timepoint was chosen to ensure that no endogenous melatonin was present at the time of injection in DD, and, therefore, all effects found are the result of the exogenous melatonin. In DD, the melatonin was given under dim red light conditions. At least 3 days were given between injections.

2.4 | EEG data acquisition and EEG power spectrum analysis

The EEG and EMG were simultaneously and continuously recorded for 24 h as previously described,[33]

amplified (amplification factor ~ 2000), band-pass filtered (EEG: 0.5–30.0 Hz, −40 dB/decade; EMG 15.0–40.0 Hz, −40 dB/decade), digitized (sampling rate 100 Hz) and automatically stored on hard disk (Spike2, Power1401, CED). A fast Fourier transformation routine with a 10-s window was performed offline (MATLAB, The MathWorks Inc.) to compute EEG power density spectra within the frequency range 0.1–25.0 Hz in 0.1 Hz resolution. The waking, NREM and REM sleep power spectrum were calculated relative to the vehicle NREM sleep power spectrum. To obtain relative power density spectra, it was necessary to obtain complete and clean recordings from all animals to enter the analysis. Due to problems with the quality of the recordings, one animal from the LD group did not contribute to the spectral analysis data.

2.5 | Data analysis

Three vigilance states (waking, NREM, and REM sleep) were scored offline in 10-s epochs. The manual scoring of vigilance states based on the EEG and EMG recordings was performed according to standardized criteria for rats.[32,33,35] Vigilance states could always be determined, but $3.27 \pm 0.54\%$ of total recording time was excluded for power spectral analysis because of artefacts. The average amount of the vigilance states (waking, NREM sleep, REM sleep, and REM sleep per total sleep time) were analyzed in 1-h intervals, 12-h intervals, and over 24 h. For more detailed analysis, 10-min values were calculated for the first 2 h after the injection.

To investigate the effect of melatonin on sleep homeostasis, we analyzed the EEG power density in the slow-wave range (SWA, 1.0–4.0 Hz) in NREM sleep as described previously.[32,33,35] The 10-min, 1-h, and 12-h values of SWA in NREM sleep were expressed relative to the average 24-h value of the vehicle day (=100%).

2.6 | Episode number, duration, and sleep onset

Analysis of vigilance state episodes gives detailed insight into sleep architecture and quality of sleep by determining the number as a measure for fragmentation and duration as a measure of state consolidation. Vigilance state episodes were determined with an algorithm described previously.[32] Episodes of each vigilance state were partitioned into 10 bins with an exponentially increased duration between 10 s and >2560 s. Average episode duration in 12-h values was calculated by dividing the total time of each vigilance state by the total episode number for the rest phase and active phase. NREM sleep onset was defined by the first NREM sleep episode that occurred after the injection with a minimum duration of 150 s. REM sleep onset was defined by the first REM sleep episode after the injection with a minimum duration of 10 s.

2.7 | Statistics

For data analysis, GraphPad Prism 8 (GraphPad software) was used. A two-way analysis of variance (ANOVA) was used to compare the effect of melatonin across time, EEG frequencies, episode duration bins, and environmental light conditions. In some cases, where missing values occurred in SWA in NREM sleep, a two-way ANOVA with main effects was used to compare the effect of melatonin. If the result was significant, a post hoc Bonferroni multiple comparisons corrected *t* test was performed, and the significant time points and EEG frequencies bins are reported in the results. The associated F-statistic is reported in all significant two-way ANOVA tests. One-way ANOVA was used to compared the difference between different injections and baseline condition, if the result was significant, a post hoc Dunnett's multiple comparisons test was performed. Anderson–Darling test and Shapiro–Wilk test were used for the normality test before the ANOVA. Paired student's *t* tests were used when distributions were normal; otherwise, the non-parametric Wilcoxon matched-pairs signed rank test was used to compare the difference between vehicle and melatonin administration conditions. In both LD and DD, the control and vehicle injection were performed on the same animals. This allowed to calculate a difference between vehicle and melatonin treatment for each individual animal. Subsequently, these differences were compared in an unpaired *t* test. The latter data are presented in the supplemental data set only when they yielded a significant result. We applied Grubbs' test to identify outliers inside the data set. If there was an outlier in the data set, the unpaired *t* test was applied on the cleaned data.

3 | RESULTS

3.1 | Long-term sleep–wake architecture and SWA in NREM sleep are not affected by melatonin

We first investigated the effects of 3 mg/kg of melatonin on sleep–wake amount under LD and DD

conditions. Melatonin administration did not significantly alter the 24-h pattern of waking, NREM sleep, REM sleep, and SWA in NREM sleep under either LD or DD conditions (Figure 1). Comparison with the undisturbed baseline conditions shows that handling and injection did indeed cause a change in sleep–wake distribution (Supporting Information S1: Figure S1).

We further analyzed the total amount and distribution of the vigilance states and SWA in NREM sleep in the rest phase and active phase under LD and DD conditions (rest phase: ZT 0–ZT12 for the LD condition, CT 0–CT12 for the DD condition; active phase: ZT 12–ZT 24 for the LD condition, CT 12–CT 24 for the DD condition). There was no significant difference in the total amount of waking, NREM sleep, REM sleep, and SWA in NREM sleep over the rest and active phase under LD and DD conditions between melatonin and saline treatment (Figure 2A–H). However, for the 12-h values there was a significant interaction of "melatonin" and "active/rest phase" in the ANOVA for REM sleep, indicating that the animals in DD showed less REM sleep than animals in LD, but no significant difference was identified between vehicle and melatonin administration (Figure 2C; interaction of "melatonin" and "active/rest phase," $F(1, 24) = 4.668$, $p = .0409$, two-way ANOVA). The first hour after melatonin administration under DD conditions, there appeared to be a difference from vehicle administration. During this period, there was a transient increase in NREM sleep and less time spent awake, but no significant interaction in the ANOVA was obtained and, therefore, there was no difference in the effect of melatonin between the light conditions (Figure 2I; factor "melatonin," $F(1, 26) = 5.139$, $p = .0319$, two-way ANOVA; vehicle: $85.6 \pm 5.8\%$, melatonin: $59.9 \pm 8.9\%$, $p = .0389$, Bonferroni multiple comparisons corrected t test; Figure 2J; factor "melatonin," $F(1, 26) = 5.522$, $p = .0267$, two-way ANOVA; vehicle: $13.1 \pm 4.8\%$, melatonin: $34.6 \pm 7.5\%$, $p = .0381$, Bonferroni multiple comparisons corrected t test). The amount of REM sleep and SWA in NREM sleep did not differ between melatonin or vehicle treatment under both LD and DD conditions in the first hour after treatment (Figure 2K,L), Moreover, the 24-h vigilance state values did not differ between control and melatonin treatment, irrespective of lighting condition, except for a significant effect of "lighting condition" in REM sleep, suggesting there was more REM sleep in the LD condition compared to the DD condition (Supporting Information S1: Figure S2).

3.2 | Melatonin administration has an acute sleep-inducing effect in constant darkness

We seemed to observed an acute sleep-inducing effect of melatonin during the first hour after the administration. Since the half-life of exogenous melatonin is 17–23 min,[36] we performed a detailed analysis of the vigilance states and SWA in NREM sleep during this time window for both lighting conditions.

We analyzed the data of the first hour after administration in 10 min intervals. Interestingly, we found that 3 mg/kg intraperitoneal injection of melatonin did not change the vigilance states and SWA in NREM sleep under LD condition (Figure 3A,C,E,G). In contrast, melatonin did seem to induce sleep under DD condition during this same period, and this effect lasted approximately 60–80 min after the injection (Figures 2I,J and 3B,D). As shown in Figure 2, waking and NREM sleep were significantly affected the first hour after melatonin administration in DD. The first hour following the melatonin injection, time spent awake was decreased compared with vehicle administration (Figure 3B; factor "melatonin," $F(1, 168) = 20.21$, $p < .0001$, two-way ANOVA; $p_{30\text{min}} = .0604$, $p_{40\ \text{min}} = .0367$, Bonferroni multiple comparisons corrected t test), and NREM sleep was significantly increased (Figure 3D; factor "melatonin," $F(1, 168) = 13.86$, $p = .0003$, two-way ANOVA; $p_{30\text{min}} = .0431$, $p_{50\ \text{min}} = .0954$, Bonferroni multiple comparisons corrected t test). Although we did not observe significant differences in specific 10-min time points for REM sleep and SWA in NREM sleep between vehicle and melatonin administration, we observed a main effect of melatonin from the results of a two-way ANOVA on the data obtained in DD (Figure 3F, factor "melatonin," $F(1, 168) = 9.117$, $p = .0029$, two-way ANOVA; Figure 3H, factor "melatonin," $F(1, 121) = 8.179$, $p = .0050$), however, no significant interaction between melatonin and the light condition was found in the ANOVA.

Most studies in humans have shown that melatonin does not significantly influence sleep–wake architecture, but it does shorten sleep onset latency. To be able to compare better with these results, we further investigated the effect of melatonin on the latency to enter NREM sleep and REM sleep. Interestingly, our data show that the administration of melatonin shortens NREM sleep latency by approximately 30 min compared to vehicle administration, under DD (Figure 3K and Supporting Information S1: Figure S3, melatonin: 25.52 ± 6.01 min, vehicle: 55.15 ± 9.53 min, $p = .0234$, Wilcoxon matched-pairs signed rank test), but not under LD condition. Nor was there any effect on REM sleep latency under either

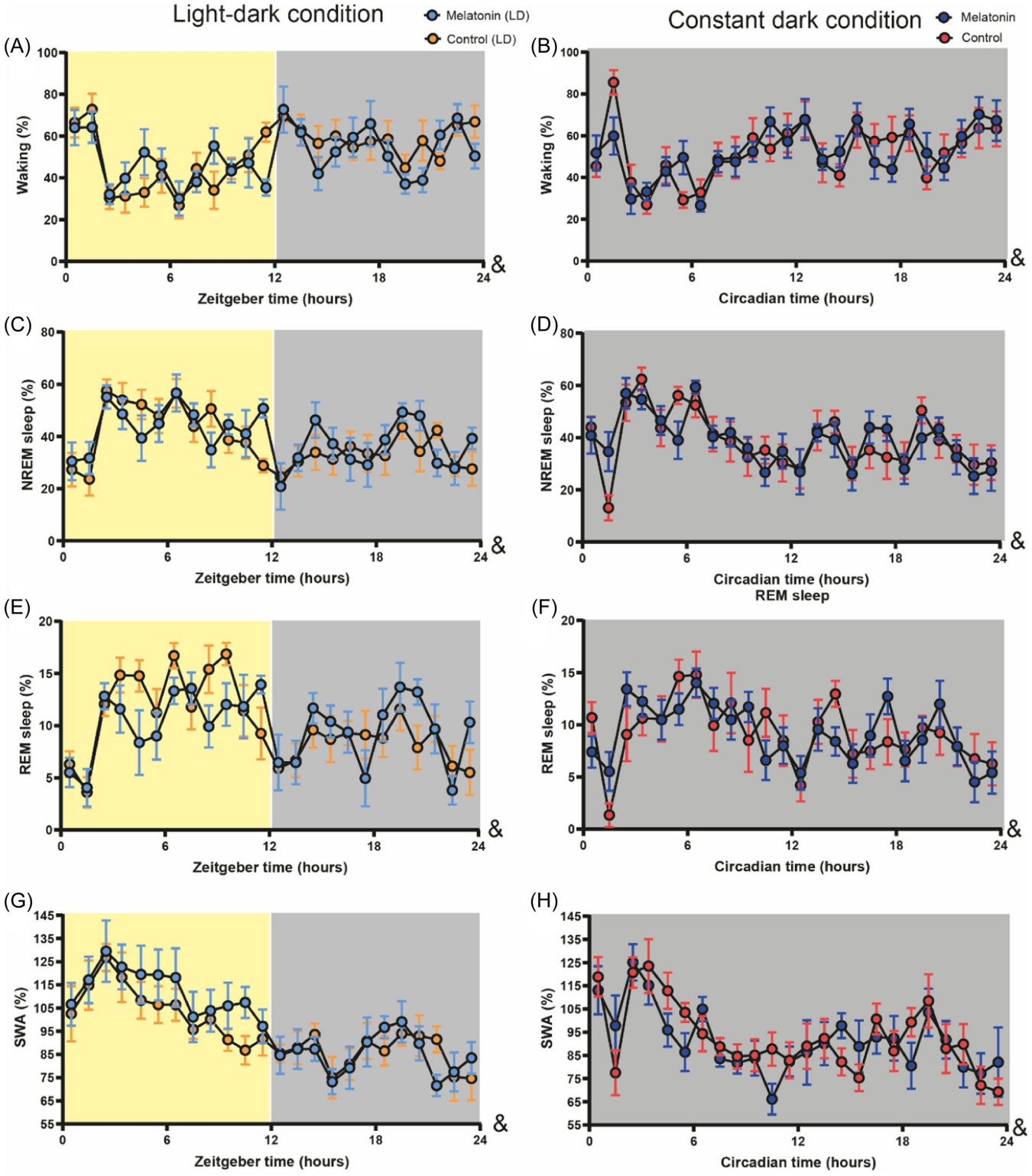

FIGURE 1 Effect of melatonin on vigilance states and slow wave activity in nonrapid eye movement (NREM) sleep under 12 h:12 h light dark (LD) and constant dark (DD) conditions over 24 h. (A–F) Time course of waking, NREM sleep, and rapid eye movement sleep in 1 h values for melatonin (light blue, $n = 7$; dark blue, $n = 8$) and vehicle (orange, $n = 7$; red, $n = 8$) administration under LD and DD conditions over 24 h. (G and H) Time course of slow wave activity (SWA) in NREM sleep in 1 h value for melatonin (light blue, $n = 7$; dark blue, $n = 8$) and vehicle (orange, $n = 7$; red, $n = 8$) administration under LD and DD conditions over 24 h. & indicates the significant main effect of "Zeitgeber/Circadian time" in a two-way analysis of variance. Yellow indicates light phase and gray indicates dark phase. Data are shown as mean ± SEM.

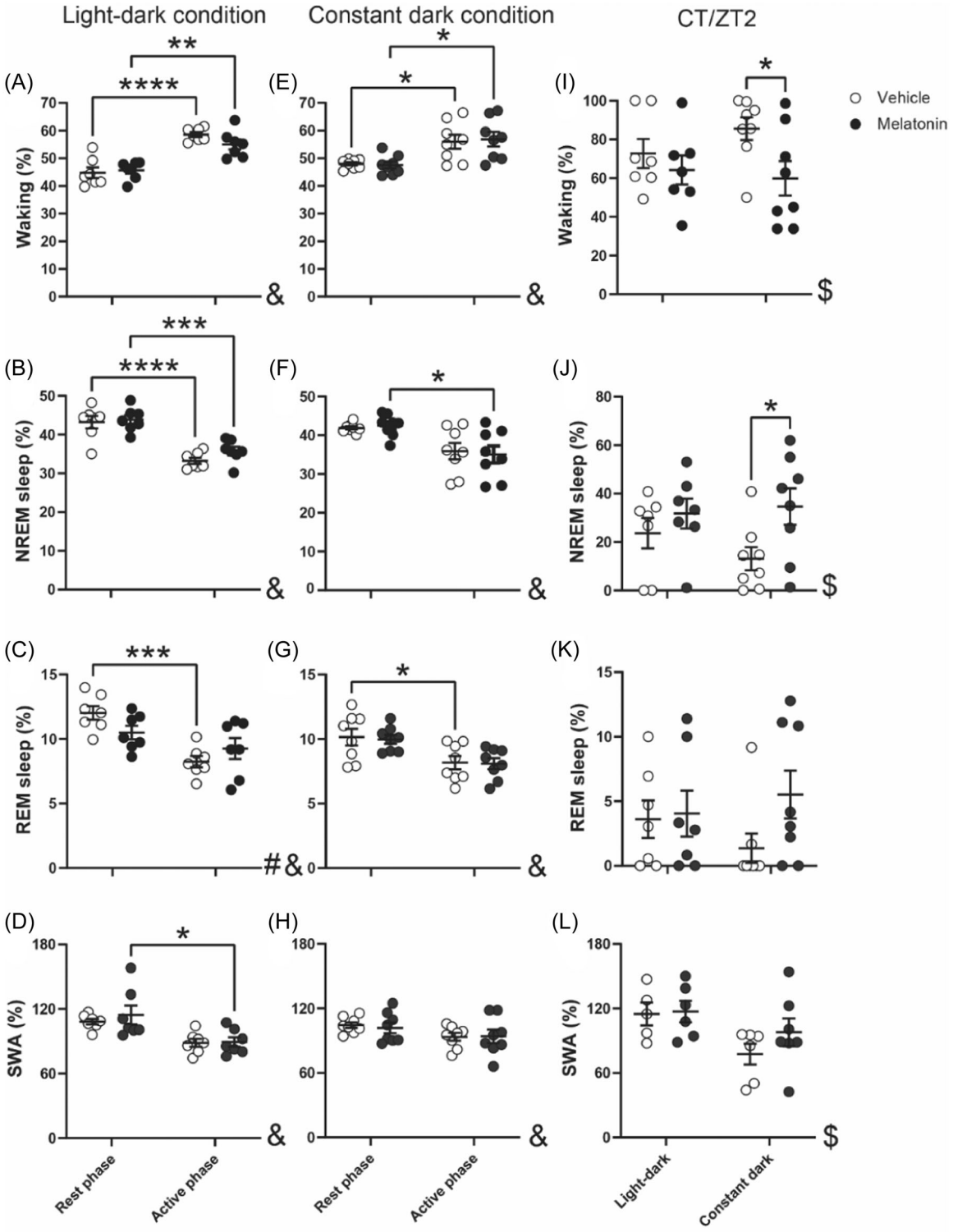

FIGURE 2 Vigilance states and slow wave activity in nonrapid eye movement (NREM) sleep of melatonin and vehicle treatment in the rest, and active phases and the first hour after injection. (A–H) Rest and active phase values of waking, NREM sleep, rapid eye movement (REM) sleep and slow wave activity in NREM sleep for melatonin (dark gray, $n_{LD} = 7$; $n_{DD} = 8$) and vehicle (white, $n_{LD} = 7$; $n_{DD} = 8$) administration under LD and DD conditions. (I–L) First-hour values after injection of waking, NREM sleep, REM sleep, and slow wave activity in NREM sleep for melatonin (dark gray, $n_{LD} = 7$; $n_{DD} = 8$) and vehicle (white, $n_{LD} = 7$; $n_{DD} = 8$) administration. & indicates significant main effects of "rest/active phase," $ indicates significant main effects of "melatonin," # indicates significant effects of the interaction of factors "melatonin" and "rest/active phase" in a two-way analysis of variance (ANOVA). Asterisks indicate significant differences between treatment or light conditions (*$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$; Bonferroni multiple comparisons t test after significant two-way ANOVA). Data are shown as mean ± SEM.

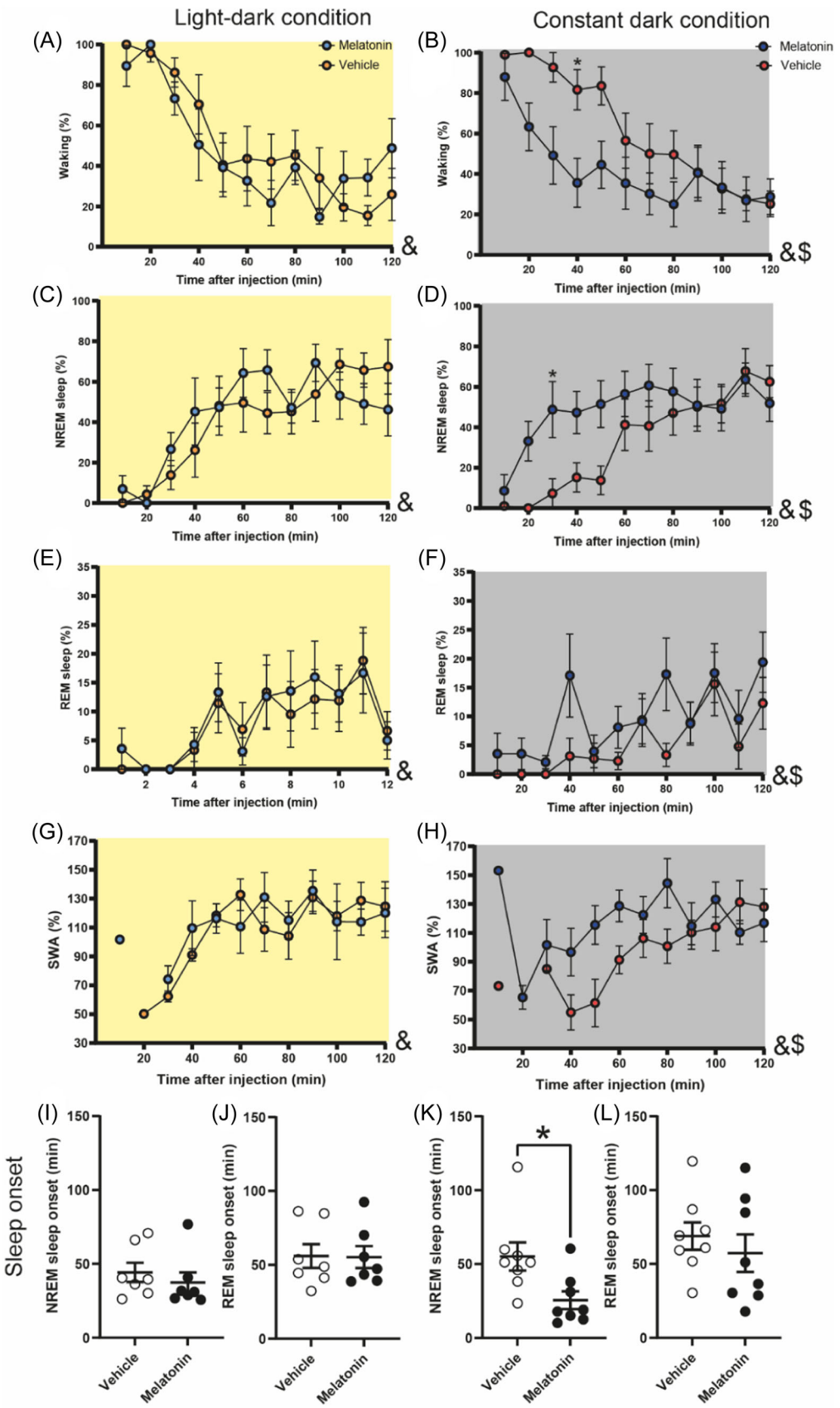

FIGURE 3 (See caption on next page).

LD or DD (Figure 3I,J,L and Supporting Information S1: Figure S3)

3.3 | Melatonin administration lengthens the episode duration of all vigilance states during the rest phase

Although melatonin did not significantly alter the amount of waking, NREM and REM sleep (Figure 1), it may, however, change sleep architecture without changing the total amount of the different vigilance states. We, therefore, in addition, analyzed the number and duration of the different vigilance state episodes. Surprisingly, melatonin administration significantly increased the episode duration of all states under both LD and DD conditions. This effect was limited to the rest phase (Figure 4). Episode duration was significantly increased for waking (Figure 4A, $p = .0004$, paired t test), NREM sleep (Figure 4C, $p = .0007$, paired t test), and REM sleep (Figure 4E, $p = .0038$, paired t test) under LD condition. Similarly, the episode duration was significantly increased for waking (Figure 4B, $p = .0078$, Wilcoxon matched-pairs signed rank test), NREM sleep (Figure 4D, $p = .0008$, paired t test), and REM sleep (Figure 4F, $p = .0078$, Wilcoxon matched-pairs signed rank test) during the rest phase under DD conditions. During the active phase, the mean duration of all vigilance states episodes was unchanged. The histogram of different vigilance state episode distributions is shown in Supporting Information S1: Figure S4.

3.4 | Melatonin induced minor changes in the EEG power density spectrum during the rest phase under LD and DD conditions

After observing a minor change in SWA under DD condition following melatonin administration, we aimed to analyze the effect of melatonin on the EEG power density spectrum. To achieve this, we analyzed the power density spectra (0–25 Hz) of waking and NREM sleep after both vehicle and melatonin administration during first hour and the first 11 h following NREM sleep onset, and expressed them relative to the average EEG power density of the NREM sleep EEG after the vehicle administration.

Given the acute effects of melatonin on shortening the sleep-onset latency, we hypothesized that the sleep homeostatic process may also be affected. Thus we analyze the EEG power density spectra in the first hour after sleep onset, providing us with more details about the quality of NREM sleep, waking, and REM sleep during this period. Melatonin had a minor effect on the waking EEG power density spectrum when administered under DD condition (Figure 5D, interaction between factor "melatonin" and factor "frequency," $F(249, 3500) = 1.993$, $p < .0001$, two-way ANOVA; $p_{0.1–0.7\,Hz} < .0261$, Bonferroni multiple comparisons corrected t test). There was a minor increase in the SWA of the NREM sleep EEG power density spectrum, around the 1.7 Hz frequency bin under LD (Figure 5B, factor "melatonin," $F(1, 3000) = 13.37$, $p = .0003$, two-way ANOVA; $p_{1.7\,Hz} < 0.0001$, Bonferroni multiple comparisons corrected t test). Melatonin also had a main effect on the REM sleep EEG power density spectrum when administered under DD condition (Figure 5F, factor "melatonin," $F(1, 3500) = 8.662$, $p = .0033$). These data indicate that although melatonin shortened sleep latency under the DD condition, it left the EEG power density spectrum basically unaffected and no differences were found between LD and DD in the effect of melatonin.

As we observed significant changes in the length of the vigilance state episode durations during the rest phase, we performed an analysis on the EEG power spectrum over the entire rest phase after injection (11 h). Here, we did not observe any significant effects of melatonin under either LD or DD conditions (Figure 5G–L). Nonetheless, there

FIGURE 3 Effect of melatonin on vigilance states and slow wave activity in nonrapid eye movement (NREM) sleep between ZT/CT1 and ZT/CT3 and NREM and rapid eye movement (REM) sleep onset latency. (A–H) Time course of waking, NREM sleep, and REM sleep in 10 min intervals for melatonin (light blue, $n = 7$; dark blue, $n = 8$) and vehicle (orange, $n = 7$; red, $n = 8$) administration during ZT 1–ZT 3 and CT 1–CT 3. & indicates a significant main effect of "time after injection," $ indicates significant main effects of "melatonin" in a two-way analysis of variance (ANOVA). Factors "melatonin" and "time after injection."* indicates significant differences between melatonin and vehicle administration ($p < .05$, Bonferroni multiple comparisons t test after significant two-way ANOVA). (I and J) NREM and REM sleep onset latency under light dark condition after melatonin (open cycle, $n = 7$) and vehicle (gray, $n = 7$) administration. (K and L) NREM and REM sleep onset latency under constant dark condition after melatonin (open cycle, $n = 8$) and vehicle (gray, $n = 8$) administration.
* indicates significant differences between melatonin and vehicle administration ($p = .0234$, Wilcoxon matched-pairs signed rank test). Yellow indicates light phase and gray indicates dark phase. Data are shown as mean ± SEM.

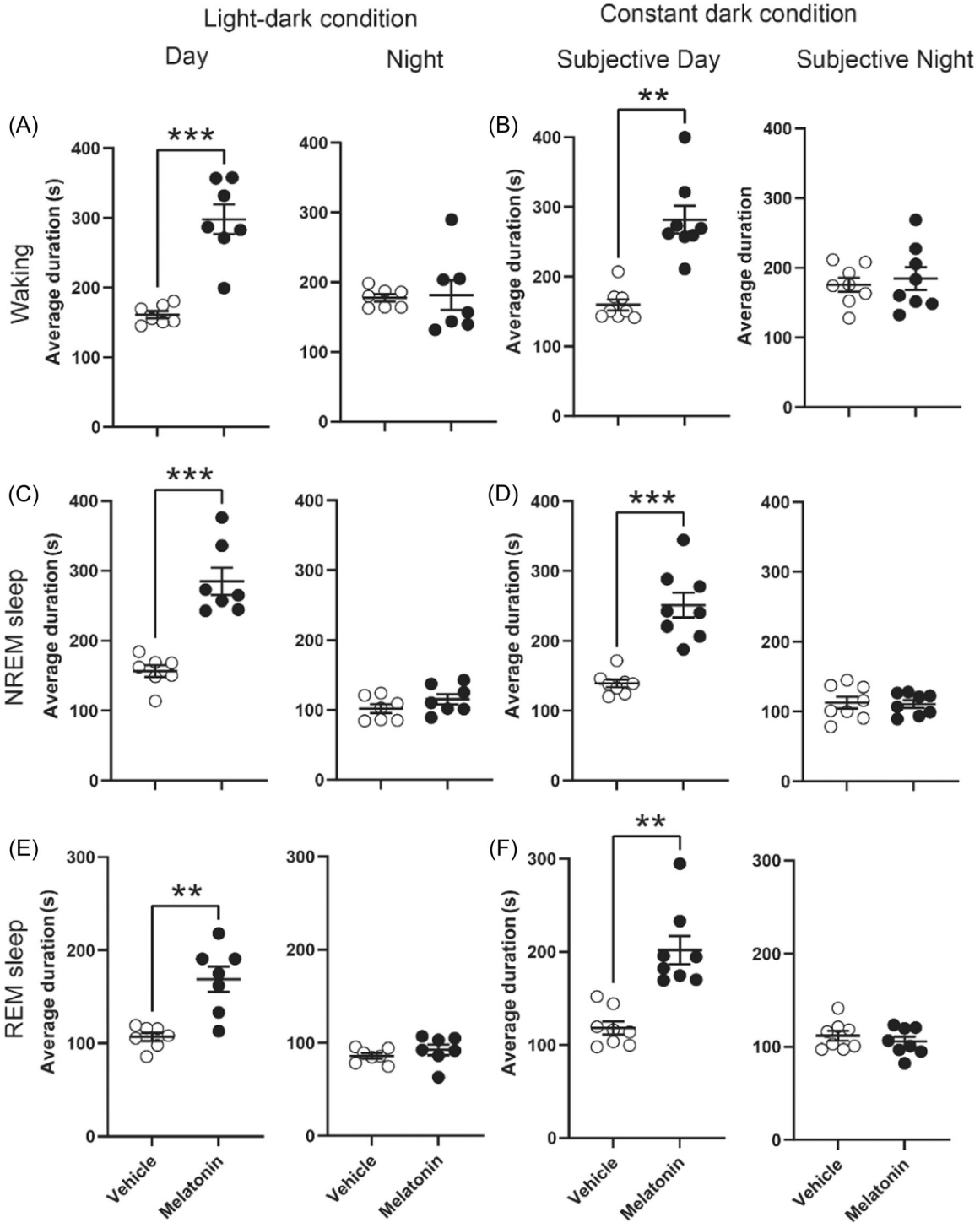

FIGURE 4 Effect of melatonin on episode duration of waking, nonrapid eye movement (NREM), and rapid eye movement (REM) sleep under light dark (LD) and constant dark (DD) conditions. (A–F) Episode duration (seconds) of waking, NREM sleep, and REM sleep during the day/subjective day and night/subjective night after melatonin ($n_{LD} = 7$; $n_{DD} = 8$) and vehicle ($n_{LD} = 7$; $n_{DD} = 8$) administration under LD and DD conditions. Asterisks indicate significant differences between melatonin and vehicle administration (**$p < .01$, ***$p < .001$; paired t test or Wilcoxon matched-pairs signed rank test depended on the normality of the data). Data are shown as mean ± SEM.

was a main effect of melatonin on the NREM sleep EEG power density spectrum under LD (Figure 5H, factor "melatonin," F (1, 3000) = 16.43, $p < .0001$, two-way ANOVA). This result implies that melatonin only showed a small acute effect on the EEG power density spectrum but did not change power density during the remaining rest phase under LD and DD.

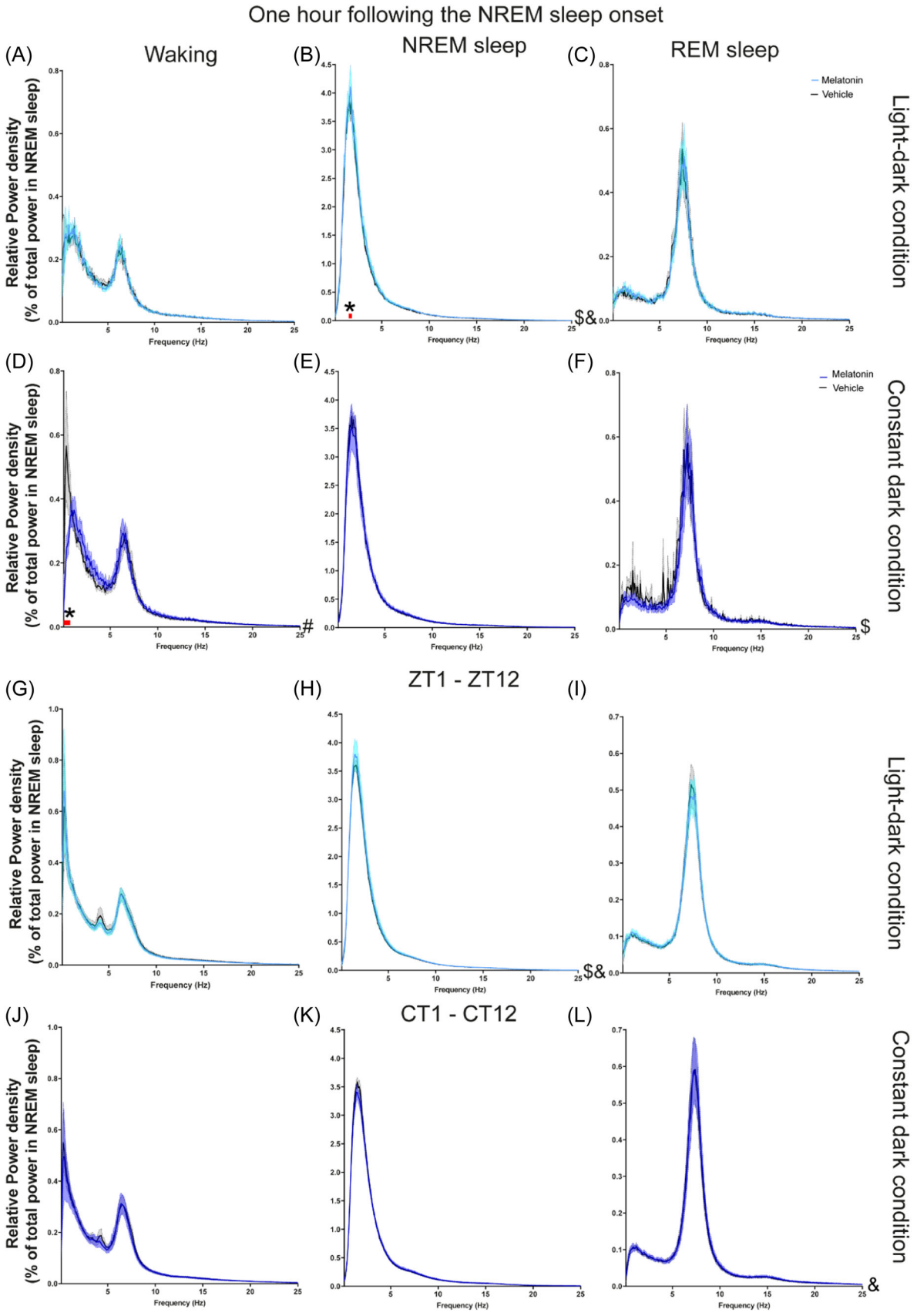

FIGURE 5 (See caption on next page).

4 | DISCUSSION

4.1 | Acute melatonin had different effects under different lighting conditions

We investigated the effects of a moderate dose of melatonin on sleep in LD and DD conditions in the Brown Norway rat. The dose we used is known to entrain animals in DD, but does not induce changes in sleep in LD conditions. The results show that the effect of melatonin on sleep can vary under different lighting conditions, and that larger effects with the same concentration of melatonin were observed under DD. These effects were mainly found in the latency to fall asleep, where application in DD significantly shortened sleep latency in the first 2 h after treatment. No effect on sleep latency or the amount of sleep was observed under LD conditions. In addition, in both lighting conditions, longer vigilance state episodes were found. There are several possible mechanisms that may explain this phenomenon related to changes in melatonin secretion or different sensitivity to melatonin under DD conditions.

4.2 | The effect of melatonin on episode duration

What did not differ between LD and DD conditions was the effect of melatonin on vigilance state episode duration. Melatonin treatment lengthened the duration of all the vigilance state episodes, suggesting a stabilization or consolidation of vigilance states by melatonin. In this context, exogenous melatonin seems to alleviate the sleep disruptions induced by handling and injecting, as episode durations after melatonin treatment were very similar to those found in undisturbed baseline conditions (Supporting Information S1: Figure S5). There is one study in rats with a similar analysis of vigilance state episode frequencies and durations, which showed that melatonin (5 mg/kg) did not change the mean episode duration of NREM sleep compared with vehicle under both L and D periods.[37] In addition, in humans it is more difficult to observe changes in vigilance state episode duration due to the small number of NREM-REM sleep cycles (four to five) per night.

Our finding may indicate that regardless of inducing sleep or not, melatonin may ameliorate sleep disturbances and enhance sleep quality by consolidating the vigilance states and reducing sleep fragmentation. The exact mechanism by which the brain regulates the duration of NREM and REM sleep remains unknown. Given that melatonin receptors are predominantly located in brain regions associated with NREM and REM sleep, it may be that melatonin mainly stimulates NREM and REM sleep stability with increased waking stability as a byproduct of this effect.

4.3 | The effect of melatonin on the EEG power density spectrum

In our hands, the influence of melatonin on the EEG power density spectrum was minimal. We observed a small increase in the SWA in NREM sleep 1 h after the sleep onset in LD, which is opposite to similar research in the literature, which reported reduced slow-wave activity in NREM sleep the first 2 h after treatment.[24]

When examining the power spectrum under the DD condition, it appears that melatonin did not influence the NREM sleep EEG power spectrum at all, in contrast to the small changes observed under LD. Effects were only evident in the waking and REM sleep power spectrum. Interestingly, over the whole subjective day, melatonin did not show any effect on the EEG. In this context, it is noteworthy that melatonin was able to shorten sleep onset latency without altering slow wave activity in NREM sleep. This suggests that the shortening of sleep latency is not related to increased sleep pressure, but may be related to changes in sleep timing mechanisms. Since it has been shown previously that it is possible to synchronize the circadian rhythm in constant darkness with the dose of melatonin that we applied,[29,30] it may be that the exogenous melatonin in DD influenced circadian timing of sleep. However, at this particular timepoint application of melatonin would likely have resulted in a

FIGURE 5 Effect of melatonin on the electroencephalogram power density spectrum of waking, nonrapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep. (A–F) Relative power spectrum in 1 h of waking, NREM sleep, and REM sleep for melatonin ($n_{LD} = 7$; $n_{DD} = 8$) and vehicle ($n_{LD} = 7$; $n_{DD} = 8$) treatment following the NREM sleep onset. & indicates a significant main effect of "frequency," $ indicates the significant main effect of "melatonin," # indicates a significant interaction between factors "frequency" and "melatonin" in the two-way analysis of variance. Red bar indicates the significant difference from Bonferroni multiple comparisons corrected *t* test. (G–L) Relative power spectrum of waking, NREM sleep, and REM sleep for melatonin ($n_{LD} = 7$; $n_{DD} = 8$) and vehicle ($n_{LD} = 7$; $n_{DD} = 8$) treatment during ZT 1 – ZT 12 and CT 1 – CT 12. Data are shown as mean ± SEM.

phase delay and, therefore, an increase in sleep latency, instead of a decrease. Therefore, the mechanism behind this decrease in sleep latency is probably not caused by a phase shift of the circadian clock.

4.4 | Differences between lighting conditions in sleep onset

The difference in the effect of melatonin between lighting conditions on sleep onset latency are probably caused by an interaction between light and melatonin. The finding that melatonin has a stronger effect on sleep onset latency in DD suggests that the sensitivity to melatonin is increased in DD. Changes in sensitivity are easiest accomplished by changes in receptor density.

The daily rhythm of melatonin binding site density in the SCN of rats was shown to have a higher density 1 h after lights on.[38] This daily variations in density would probably be involved in the responsiveness to melatonin treatment. Unfortunately not many studies exist that analyzed receptor density changes from LD to DD. There is one study in C3H mice which has analyzed the expression of MT_1 mRNA in the SCN in LD and DD.[28] The MT_1 receptor displayed different expression patterns, for example, with two peaks under LD conditions (near ZT2 and ZT14) and a single peak under DD near CT2. More remarkable is that the receptor density was increased by 20%–25% in DD. This may explain why the administration of the same dose of melatonin under LD or DD gives different results due to differences in receptor density. Together with our study, these studies suggest that the effect of melatonin on sleep is not only influenced by the dosage and the time of administration but also by the lighting environment, possibly through changes in melatonin receptor density. In our hands, melatonin is able to induce modest changes in sleep in rats in DD, but not in LD, as it shortens sleep latency in DD.

4.5 | Differences between diurnal humans and nocturnal rodents

The finding that melatonin can induce sleep in a nocturnal species is counterintuitive as melatonin is released during the night when nocturnal animals are mostly awake and active. This begs the question whether this finding is normal for nocturnal animals and whether they are different from diurnal animals, including humans. Data in the literature shows that distribution, elimination, and metabolism of melatonin are not constant processes but they display circadian fluctuations, with higher values during the activity phase for both diurnal humans and nocturnal rodents.[39–41] Several pharmacokinetic parameters in the rat after melatonin administration are found to depend on the time of the day. The metabolic clearance rate of melatonin is higher in the dark phase compared to the light phase in rats.[41,42] Earlier studies examining the effects of melatonin on sleep in rodents have produced conflicting outcomes, showing melatonin to be sleep-promoting, to have no effect or to be wake-promoting. These inconsistencies may be attributed to variations in EEG measurement and analysis criteria, diverse metrics used to assess hypnotic effects, varying melatonin doses, arousal status of the animals, and differences in routes and timing of administration.[43]

To address these challenges, we conducted a comprehensive analysis integrating data from 16 rodent studies and 10 human studies (see Figure 6 and Tables 1 and 2). To ensure that sleep is the variable that is measured, the studies were selected based on the criteria that they needed to contain EEG/EMG-confirmed sleep in nocturnal rodents, and polysomnographic recorded sleep in humans.

From this analysis, it becomes clear that in nocturnal rodents melatonin's primary effects are found to be sleep promoting or that it has no significant effect (Figure 6A). The sleep promotion in nocturnal rodents is most evident when melatonin was administrated in the first half of the light period. Among all of these 16 studies in rodents that applied a single dose of melatonin and analyzed EEG-confirmed sleep, only one study in rats demonstrated a wake-promoting effect, and this was with a relative low dosage. In contrast to the conflicting results observed in nocturnal rodents, the majority of studies involving humans show that melatonin induces sleep or drowsiness, with the exception of one study where administration of melatonin in the early morning was wake promoting (Figure 6B and Table 2). These findings highlight the consistency of melatonin's sleep-inducing properties in humans but also emphasize the importance of considering timing and dosage when studying its effects.

Adding to this complexity, several rodent studies have explored the direct infusion of melatonin into the brain to assess its hypnotic effects. One of these studies found that brain infusion of melatonin (500 pmol) promoted sleep in melatonin deficient C57/Bl mice.[19] Similarly, a study involving direct infusion of melatonin (1 μM, 300 nL) into the lateral hypothalamus in C57/Bl mice,[60] and a study that directly infused melatonin into medial preoptic area (0.2 uL per side) in Sprague–Dawley rats,[61] also showed a sleep-promoting effect. These findings suggest that targeting the melatonin receptor-rich hypothalamus induces sleep in mice and rats.

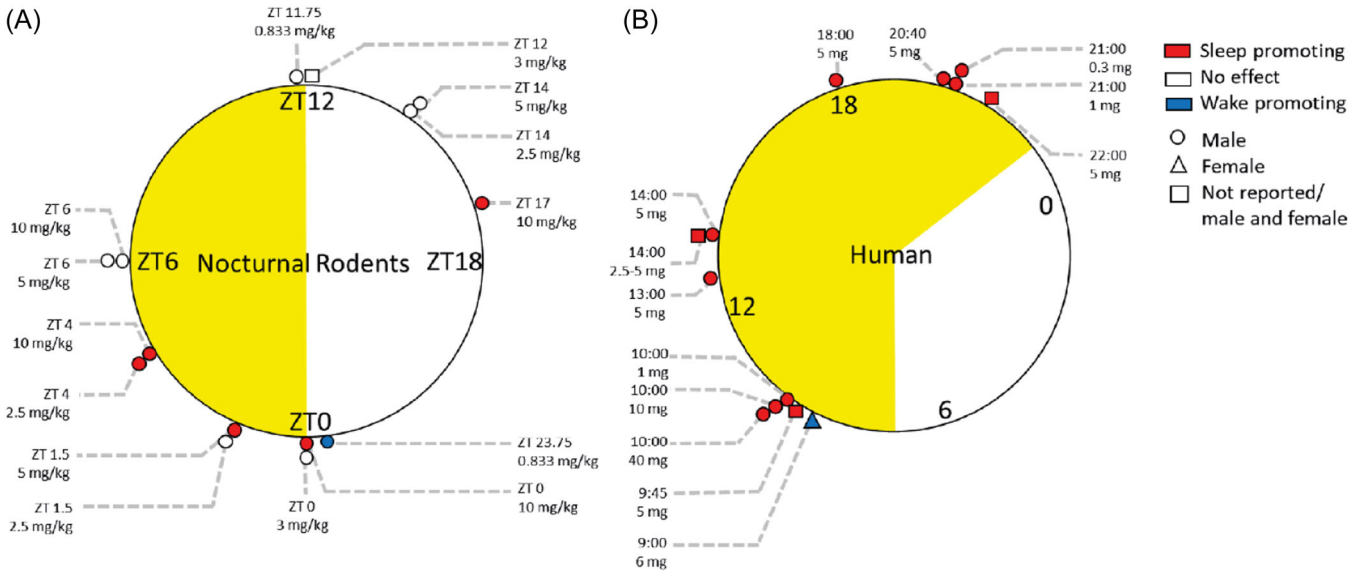

FIGURE 6 Exogenous melatonin in rodents and humans. Effect of a single dose of melatonin on electroencephalogram confirmed sleep in nocturnal rodents (A) and diurnal human (B). Yellow indicates the light period. Red indicates a sleep-promoting effect, white indicates no effect on sleep and wakefulness and blue indicates a wake-promoting effect. Circle indicates male, triangle indicates female, square indicates either not reported or includes both female and male. Details of the included studies can be found in Tables 1 and 2.

In this context, it is worth noticing that through the release of endogenous melatonin from the pineal gland, peak serum melatonin levels can reach around 30–70 pg/mL in various species, including humans, pigs, mice, rats, and reindeer.[62,63] Therefore, direct infusion of melatonin into the brain may correspond to a relatively high dose in local brain areas compared to systemic administration, or naturally occurring levels of melatonin. Furthermore, as depicted in Figure 6, most studies that utilized a dose of 10 mg/kg of melatonin, which is a relatively high dose, were able to induce sleep regardless of the time of day or species.

Another issue that is probably adding to the diversity in results and conclusions is that the definition of a sleep-promoting effect varies among different studies. In the present study, we considered melatonin to have a sleep-promoting effect based not only on an increase the overall sleep duration but also on shortening the sleep onset latency. This aligns better with findings from human studies where shortening of sleep onset latency is also considered indicative of a sleep-promoting effect. In this context, it is important to note that some studies in rodents have reported no hypnotic effect of melatonin, but also did not measure sleep onset latency,[24,37] emphasizing the importance of a clear definition of what is considered to be a sleep-promoting effect.

5 | CONCLUSION

We found in the present study that melatonin can be sleep inducing, and suggest that the effect of melatonin depends on both the dosage and the sensitivity of the brain at the time of administration. Sensitivity to melatonin may change under influence of light conditions as is suggested by the data in the present study in combination with previously published results. A review of the literature shows that sleep induction by melatonin in diurnal humans is found in a large majority of the available studies. In nocturnal rodents the effect of melatonin on sleep is less clear, but this also may depend on how sleep induction is defined. Measuring sleep onset latency in rodents, as is done in human studies may eventually give clearer and more comparable results in contrast to simply analyzing total amount of sleep, particularly because the effects of acute melatonin treatment on sleep seems to be short-lived. In this context, and in view of our findings that melatonin stabilizes vigilance state episodes, it is likely that acute treatment with melatonin is indeed capable of increasing sleep quality.

Data were generated by the authors and available on request from the corresponding author (permitted only for data types for which a community-recognized, structured repository does not exist).

TABLE 1 Effects of exogenous melatonin in rats' sleep and wakefulness.

Species	Sex M/F	Number	Light schedule	Light intensity	Concentrationi.p.	Injected time	Effect on sleep architecture, sleep latency, and EEG power spectrum	References
Wistar rats	Male	n.r.	12:12 LD constant light	150 lx	10 mg/kg 10 mg/kg	ZT0 CT 0	Shortened sleep onset increased slow-wave sleep, REM sleep, and total sleep.	Wang, Li, Wu, Yang, Zhang, et al.[44]
Wistar rats	Male	5	12:12 LD	n.r.	10 mg/kg	ZT 0	Sleep-promoting effect.	Wang, Li, Wu, Yang, Xu, et al.[45]
Sprague–Dawley SIVZ rats	Male	8	12:12 LD	300 lx	3 mg/kg	ZT 0	Not affect vigilance states. Reduced NREM power (1–8 Hz). No effect on cortical temperature.	Tobler et al.[24]
Wistar rats	Male	6	12:12 LD	130 lx	2.5 mg/kg 5 mg/kg	ZT 1.5	No sleep-promote effect similar results for L and D. Increased the number of sleep cycles. Increased total duration of REM sleep.	Mailliet et al.[37]
Sprague–Dawley rats	Male	10–12	12:12 LD	100–140 lx	10 mg/kg 2.5 mg/kg	ZT 4 ZT 4	Reduced time to sleep onset and time spent awake. Increased both slow wave and paradoxical sleep. Similar but smaller effect. Not altered normal EEG patterns.	Holmes et al.[46]
Wistar rats	Male	9	12:12 LD	50–120 lx	5 mg/kg 10 mg/kg	ZT 6	Not affect brain temperature nor sleep architecture.	Langebartels et al.[47]
Sprague–Dawley rats	Male	8	12:12 LD	n.r.	0.833 mg/kg	ZT 11.75	No effect on sleep.	Mendelson et al.[25]
Sprague––Dawley SIVZ).	n.r.	8	12:12 LD	100–300 lx	3 mg/kg	ZT 12	No significant changes in the EEG spectra.	Huber et al.[48]
Wistar rats	Male	6	12:12 LD	130 lx	2.5 mg/kg 5 mg/kg	ZT 14	No sleep-promote effect similar results for L and D. Increased the number of sleep cycles. Increased total duration of REM sleep.	Mailliet et al.[37]
Sprague–Dawley rats	Male	n.r.	12:12 LD	80 $\mu W/cm^2$	10 mg/kg	ZT 17	Reduced NREM sleep latency. Short-lasting increase in NREM sleep duration. No changes in REM sleep and latency.	Fisher et al.[26]
Sprague–Dawley rats	Male	8	12:12 LD	n.r.	0.833 mg/kg	ZT 23.75	Decreased NREM sleep and total sleep time.	Mendelson et al.[25]

Abbreviations: EEG, electroencephalogram; i.p., intraperitoneal injection; LD, light dark; n.r., not reported; NREM, nonrapid eye movement; REM, rapid eye movement.

TABLE 2 Effects of exogenous melatonin in humans' sleep and wakefulness.

Subjects	Sex	Number	Age	Dosep.o.	Administration time	Effect on sleep architecture, sleep latency, and EEG power spectrum	References
Reproductive and postmenopausal healthy volunteers	Female	26	n.r.	6 mg	9:00	No EEG changes in postmenopausal woman. Awaken effect in reproductive woman.	Staiko et al.[49]
Healthy volunteers	Male nine, female 14	23	21–53	6 mg	9:45	Increases in total sleep, decreases in sleep latency, increases in subjective drowsiness.	Paul et al.[50]
Healthy young volunteers	Male	8	18–30	1, 10, or 40 mg	10:00	Increased total sleep time. Decreased wake after sleep onset.	Hughes and Badia[51]
Healthy young volunteers	Male	n.r.	21–32	5 mg	13:00 18:00	Increased daytime sleepiness. Increase in theta/alpha frequencies of the waking EEG.	Cajochen et al.[52]
Healthy young volunteers	Male	8	Mean 20.3	5 mg	14:00	Decrease sleep latency onset.	Reid et al.[53]
Children	Male 42, female 26	68	Mean 8	2.5 mg <5 years 5 mg >5 years	14:00	Reduced sleep latency. No significant changes in sleep macro-structure.	Wassmer et al.[54]
Healthy young volunteers	Male	7	23–32	5 mg	18:00	Increased REM sleep (first REM sleep episode) EEG in NREM unaffected.	Cajochen et al.[55]
Healthy volunteers	Male	10	Mean 27	5 mg	20: 40	Reduced sleep latency. Reduced REM sleep latency.	Cajochen et al.[56]
Healthy young volunteers	Male	8	Mean 28.5	0.3 mg 1.0 mg	21:00	Sleep onset latency and latency to stage 2 sleep were significantly decreased. No change in sleep architecture.	Zhdanova et al.[57]
Alzheimer patients	n.r.	8	Mean 65	5 mg	22:00	Decreased REM sleep onset. Lower relative power and coherence of the β and γ EEG bands.	Cruz-Aguilar et al.[58] Cruz-Aguilar et al.[59]

Abbreviations: EEG, electroencephalogram; n.r., not reported; p.o., per os.

AUTHOR CONTRIBUTIONS

Yumeng Wang and Tom Deboer conceived and designed the study. Yumeng Wang performed the experiments and the analyses. Tom Deboer supervised the project. Yumeng Wang and Tom Deboer wrote, edited, and approved the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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