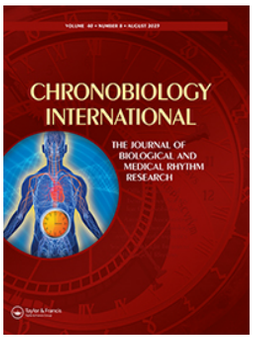

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Attenuation of homeostatic sleep response and rest-activity circadian rhythm in vitamin D deficient mice

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ABSTRACT

The link between vitamin D deficiency (VDD) and sleep disturbances has long been suggested. However, the direct causality between VDD, sleep disturbances, and circadian rhythm remains unclear. We aimed to characterize sleep-wake behavior and circadian rhythms in an animal model of VDD. VDD was induced by feeding vitamin D-deficient chow, and we analyzed sleep and circadian rhythm parameters. During light period, VDD mice exhibited reduced wake with more frequent wake bouts and increased NREM sleep time. However, during dark period, the wake EEG power spectrum peaked at theta band frequency, and slow-wave energy was suppressed in mice with VDD. Rest-activity analyses revealed increased circadian period, lower wheel counts, and more frequent and short activity bouts during VDD. Combining sleep and circadian data, we found significantly suppressed activities during the hours with a wake duration shorter than 30 minutes. Moreover, mice in VDD state exhibited a negative correlation between wake theta power and hourly wheel-running counts during dark period. Our data point to a direct link between VDD and disturbances in sleep and rest-activity circadian rhythm, featuring frequent wake bouts during the sleeping phase, reduced sleep pressure build-up in dark period, and reduced activity levels due to increased susceptibility to sleepiness.

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Vitamin D deficiency; sleep homeostasis; EEG; circadian rhythm; rest-activity

1. Introduction

Vitamin D synthesized by sun exposure to the skin is crucial in regulating calcium and phosphorus metabolism, which is necessary for maintaining bone health (Holick and Chen 2008; Lips 2001). Vitamin D receptors (VDRs) are expressed in many cells throughout the body (Wang et al. 2012), indicating that vitamin D may play an extensive role in the body. In modern society, lifestyle changes (Whiting et al. 2007) such as increased time spent indoors and using sunscreen (Matsuoka et al. 1987) have reduced sun exposure, resulting in the limited synthesis of vitamin D and a rising prevalence of vitamin D deficiency (VDD) globally (Gominak and Stumpf 2012; Holick 2008; Holick and Chen 2008). Studies indicate a correlation between VDD and several diseases. Specifically, these diseases include (Chatterjee et al. 2023), hypertension (Mirhosseini et al. 2017), cardiovascular disease (Wimalawansa 2018; Zhou et al. 2022), autoimmune diseases (Dankers et al. 2017), multiple types of cancer (Munoz and Grant 2022), and elevated mortality rates (Zittermann et al. 2012).

Many clinical studies have reported a correlation between VDD and sleep disturbance. Using polysomnography (PSG) and actigraphy, which measure sleep by assessing various parameters, it has been observed that VDD patients exhibit shorter sleep durations of less than 5 or 6 hours and a reduced proportion of rapid eye movement (REM) sleep (Bertisch et al. 2015; Massa et al. 2015; Piovezan et al. 2017). These clinical findings suggest a potential role for vitamin D in maintaining healthy sleep.

The two-process model of sleep regulation is a pivotal concept in sleep research, providing a substantial understanding of sleep regulation mechanisms (Borbely 1982). This model describes sleep regulation as a dynamic interplay between two fundamental processes: Process S and Process C. Process S represents the homeostatic sleep drive, which increases during wakefulness and dissipates during sleep, reflecting the need to balance sleep and wakefulness for optimal physiological functioning. On the other hand, Process C corresponds to the circadian rhythm, governs the regulation of the internal biological processes and alertness levels, coordinating the light-dark cycle of day and night and controlling various physiological patterns over a 24-hour period. This internal clock, driven by the suprachiasmatic nucleus (SCN) in the

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hypothalamus, oscillates in a roughly 24-hour cycle and aligns behavioral and physiological functions with the day-night cycle. The timing and structure of sleep result from the interaction between these two processes, where Process C modulates the expression and dissipation of Process S (Daan et al. 1984). Notably, these processes are not mutually exclusive but reciprocally influence each other to fine-tune sleep regulation, and any perturbation in either process could manifest as sleep disturbances.

Vitamin D plays a crucial role in the regulation of the circadian rhythm (Muscogiuri et al. 2019), significantly influencing the molecular clocks in the SCN (Lowrey and Takahashi 2004). Notably, adipose-derived stem cells (ADSCs) treated with active vitamin D3 exhibit increased rhythmicity and amplitude in the expression of BMAL1 and PER2, implying that vitamin D enhances daily rhythms through molecular clock regulation (Gutierrez-Monreal et al. 2014). Whole genome microarray analysis shows that VDD can significantly alter the expression of genes related to the circadian clock, including Npas2 and Per2 (Mengatto et al. 2011). Additionally, circadian disruption influences vitamin D levels. For instance, shift workers, who receive less sunlight, tend to have lower vitamin D levels compared to daytime workers (Mun et al. 2023). UVB light, vital for vitamin D synthesis, converts 7-dehydrocholesterol into previtamin D3, which then transforms into vitamin D3, or cholecalciferol (Holick 2007). Given that light both synchronizes circadian rhythms and triggers vitamin D synthesis, these two factors may closely and bidirectionally interact.

It has been suggested that there is a connection between VDD, sleep disturbances, and circadian rhythm, but determining VDD's precise role as a cause or contributing factor in any specific disease could be difficult. We hypothesized that VDD might directly impact sleep-wake behavior and circadian rhythm. To investigate this hypothesis, we characterize the sleep and circadian rhythm in the presence of VDD in an animal model.

2. Materials and Methods

2.1. Animals

Young adult male C57BL/6 mice used in this experiments (Assa et al. 2014; Dancer et al. 2015; Lagishetty et al. 2010; Parekh et al. 2017). The mice were individually housed and maintained under controlled conditions, including a standard 12-hour light-dark cycle (lights on from 7 AM to 7 PM), temperature (22 ± 2°C), and humidity (50 ± 10%) in specialized animal racks. Ambient light was provided by light-emitting diodes, yielding an intensity of approximately 200 lux at the level of the cage floor, characterized by a cool white chromaticity (Supplementary Figure S1). The mice had ad libitum access to food and water throughout the study. We provided regular chow (RodFeedTM, DBL, Eumseong, Republic of Korea) containing 8 IU of vitamin D3/g until the initiation of the dietary VDD induction. To induce VDD state, vitamin D-deficient chow (Cat. No. TD.89123, ENVIGO, Indianapolis, USA) was provided for six weeks based on previous literature (Dancer et al. 2015; Parekh et al. 2017). The regular diet was manufactured according to NIH-41 Open Formula and contained 62% carbohydrate, 20% crude protein, 4% fat, 8 IU/g of vitamin D, and others. VDD diet was composed of 64% carbohydrate, 18% crude protein, 10% fat, and others without vitamin D. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org). The Institutional Animal Care and Use Committee at the Gwangju Institute of Science and Technology (GIST) granted approval for all animal care and experimental procedures (GIST-2021-093). All methods were executed in accordance with institutional guidelines and regulations.

2.2. Stereotaxic Surgery

To obtain electroencephalogram (EEG) and electromyogram (EMG) recordings, 7–8 weeks-old mice were surgically implanted with electrodes for EEG and EMG under isoflurane anesthesia (2% isoflurane, delivered in oxygen). We implanted the electrodes on the skull in the frontal (anteroposterior = 1 mm, mediolateral = 1 mm) and the parietal areas (anteroposterior = −3.5 mm, mediolateral = 1 mm), referenced to an electrode over the cerebellum (5.3 mm posterior of bregma). Subsequently, EMG wire electrodes were inserted into the neck muscle. All wires connected to the electrodes were soldered to the head mount (Cat. No. 8402, Pinnacle Technology, Lawrence, KS, USA) and sealed with dental acrylic (Cat. No. 0921375, Bosworth Fastray; Keystone Ind, Gibbstown, NJ, USA). Pain management was ensured by administering a subcutaneous injection of ketoprofen (5 mg/kg dose) at the beginning and the day after surgery. Following their surgeries, mice were given a minimum recovery period of one week before conducting EEG recordings.

2.3. Electroencephalogram/Electroencephalogram Recordings

Sleep recording was conducted in a soundproof room, equipped with a ventilation and air-conditioning system to eliminate any potential sources of external stimuli. To

prepare for sleep recording, the head mount on the mice was connected to the preamplifier (Cat. No.8202, Pinnacle Technology) one day prior to recording, allowing the mice to habituate to the soundproof room environment. EEG and EMG signals were recorded using a signal acquisition system (Sirenia Acquisition 1.8.3, Sirenia Sleep Pro, Pinnacle Technology) with a 2000 Hz sampling rate. The sleep state of the recorded EEG signal was manually scored using analysis software (Sirenia Sleep Pro, Pinnacle Technology). Baseline EEG recordings were conducted on mice at 9 or 11 weeks of age, while a second EEG recording was taken at 22 or 24 weeks of age after induction of VDD.

2.4. Wheel-running activity recordings

After sleep recording, wheel-running activity was recorded in a light-controlled chamber (Circadian Cabinets, Actimetrics, Wilmette, IL, USA) inside the soundproof room. Mice were single-housed in cages with built-in wheels (Actimetrics, length × width × height: 29.5 × 11.5 × 12.0 cm; wheel diameter 11.0 cm) and free access to water and food during the recording period. In addition, the wheel-running activity could be viewed in real-time using a computer installed outside the soundproof room and analyzed with software (ClockLab Analysis Version 6, Actimetrics). Maintenance of mouse cages, such as cleaning and providing food and water, was done once a week a random timing during dark conditions with a dim red light. During the constant dark conditions, the cycle is divided into two phases: activity (α) and rest (ρ), based on the onset and offset times of wheel-running activities. The activity onset was determined by activity levels that exceed the threshold (i.e., the 20th percentile wheel-running activity level in ClockLab), while activity offset is determined by activity levels that drop below the threshold. The duration of alpha is calculated as the difference in time between activity onset and offset. An activity bout is defined as a period of activity during which the number of counts per minute remains above the threshold (20 counts/min) for longer than 21 minutes.

2.5. Experimental Schedule

Our study aimed to examine the impact of VDD on the sleep-wake behavior and circadian rhythm of mice. We recorded the spontaneous sleep-wake EEG and EMG signals and wheel-running activity both before and after inducing VDD in the mice (Figure 1a). The sleep recordings were divided into a 24-hour baseline, followed by a 6-hour period of sleep deprivation (SD) and a subsequent 6-hour period of recovery sleep (RS). This allowed us to investigate the characteristics of sleep and the homeostatic sleep response in the mice.

2.6. Statistic Analysis

All statistical analyses were performed using GraphPad Prism Version 9 (GraphPad Software, San Diego, USA). A two-way repeated measures ANOVA was used to assess the change of amount in sleep stages, SWE, and SWA and compare the peak frequency between the VDD states at different time points. In addition, we used student's t-test for rest-activity analysis, and pearson's correlation analysis to evaluate the simple linear correlation between values. Data are presented as mean ± standard error of the mean (SEM), and statistical significance was considered at $p < 0.05$.

3. Results

3.1. Decreased Wakefulness and Increased NREM Sleep duration in VDD mice

During 24-hour EEG recording, no significant difference was observed between the vitamin D conditions in the average time per hour spent in each state (Figure 1b–d). We then analyzed the amount of each stage in 6-hour intervals. The VDD status mice spend significantly less wakefulness at the end of light period (zeitgeber time, ZT 6–12) (23.9 ± 0.5%), compared to the baseline (30.1 ± 1.2%) ($F(3, 42) = 111.3$, $p < 0.001$; Figure 1e). Additionally, the VDD mice spend significantly more non-rapid eye movement (NREM) sleep (67.1 ± 1.5%) during the second half of light period compared to the baseline (60.5 ± 1.8%) ($F(3, 42) = 95.73$, $p < 0.001$; Figure 1f). And VDD mice showed a significant decrease in REM amount at the beginning of dark period (ZT 12–18) (1.0 ± 0.4%) compared to the baseline (2.5 ± 0.5%) ($F(3, 42) = 61.51$, $p < 0.001$; Figure 1g). These results suggest that VDD could alter the level of sleep and wake.

3.2. Increased the Number of Wakefulness Bouts in VDD Mice

To gain a more comprehensive understanding of the structure of each stage, we conducted a bout analysis. We divided EEG data into 10-second segments, each referred to as an epoch and defined one bout as the occurrence of three or more consecutive epochs of a particular stage. We observed a significant increase in the number of wake bouts in the VDD mice (ZT 0–6, 3.0 ± 0.3 vs. 1.9 ± 0.1; ZT 6–12, 3.4 ± 0.4 vs. 2.0 ± 0.4; ZT 18–24, 2.6 ± 0.3 vs. 1.7 ± 0.2) ($F(3, 42) = 1.597$, $p = 0.20$; Figure 2a). There was no statistically significant

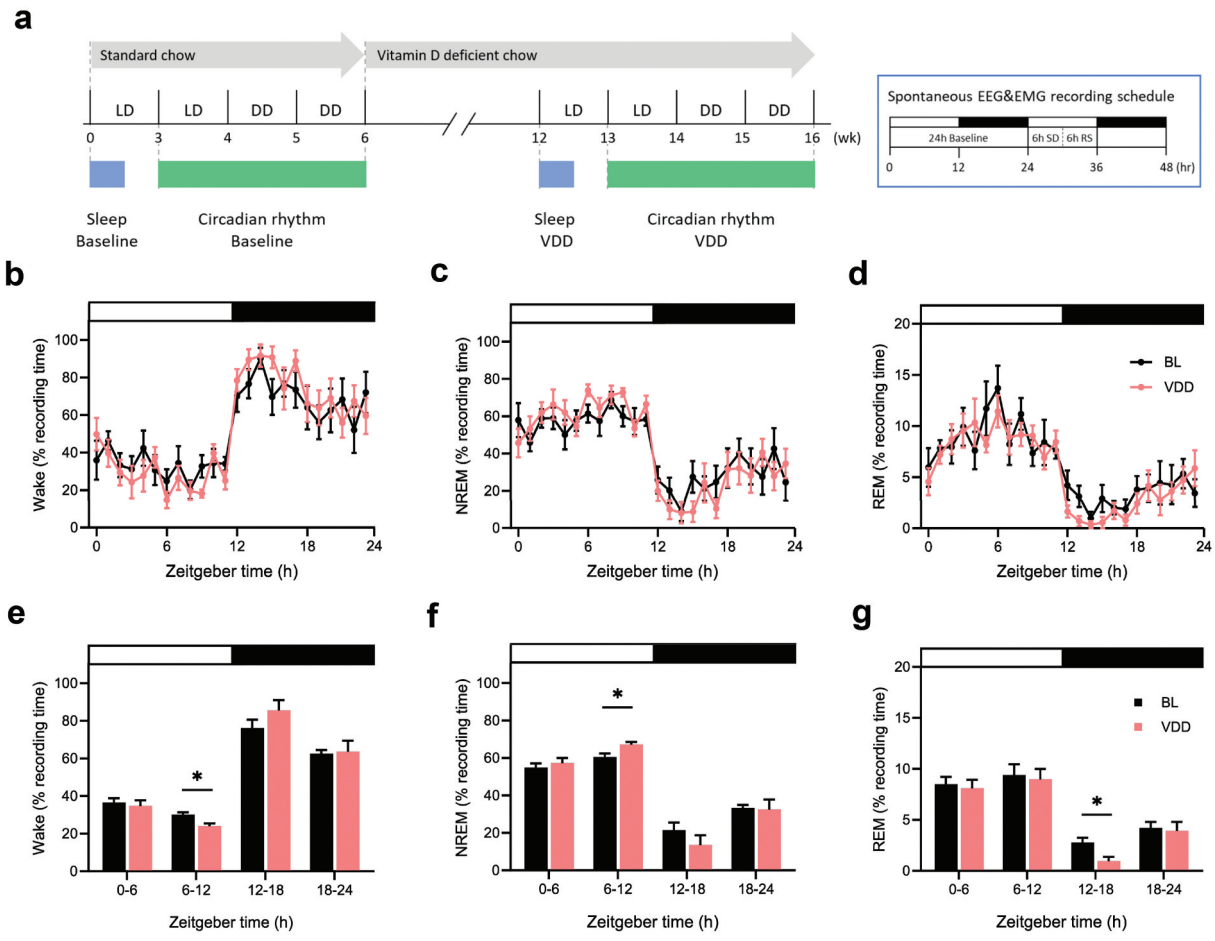

Figure 1. Increased NREM sleep and decreased REM sleep in VDD state. (a) schematic representation of the experimental design. Spontaneous EEG and EMG were recorded for sleep analysis (blue bar), with the recording schedule divided into a 24-hour baseline, followed by 6 hours of sleep deprivation (SD), and then a subsequent 6-hour recovery sleep (RS). Meanwhile, wheel running activity was recorded for rest-activity circadian rhythm analysis (green bar). Light-dark (12 h:12 h) and constant dark schedules are indicated as LD and DD, respectively. (b-d) proportions of wakefulness, NREM, and REM sleep across the 24-hour in the baseline and VDD. Data were expressed as the average time per hour spent in each state during consecutive 24-hour EEG recordings. (e-g) average percentage time spent in wakefulness, NREM, and REM sleep in 6-hour intervals. Open and closed horizontal bars represent light and dark periods, respectively. BL and VDD indicate baseline and deficiency states of vitamin D, respectively. Data are shown as means ± SEM ($n = 8$). *$P < 0.05$ by two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test and student's t-test.

difference between the baseline and VDD for the number of bouts of NREM and REM (Figure 2b,c). Moreover, we found no discernible difference in the duration of wake, NREM, and REM bouts between the baseline and VDD (Figure 2d,f). These findings suggest that VDD is linked to alterations in sleep architecture, characterized by a higher frequency of wake bouts during light period.

3.3. Wake EEG Peaked at Theta Band and Attenuated Slow Wave Energy During Dark Period in VDD Mice

EEG power spectrum analysis allows for the quantification of changes in the electrical activity of the brain during both sleep and wakefulness. EEG power was normalized to correct for power deviation between mice by dividing it by the sum of EEG power up to 50 Hz. During the 24-hour monitoring period, mice with VDD exhibited an increase in wake theta power within the 4–8 Hz range (Figure 3a), a slight reduction in NREM delta power within the 0.5–4 Hz range (Figure 3b), and a decrease in REM power (Figure 3c). Analysis of the EEG power spectrum revealed a significant peak in wake at the theta band of 7.28 Hz at the beginning of dark period (ZT 12–18) ($F(3, 39) = 4.891$, $p = 0.006$; Figure 3d). NREM and REM sleep showed no changes in peak levels (Figure 3e,f).

Slow wave activity (SWA) and slow wave energy (SWE) are essential measures to assess sleep depth and

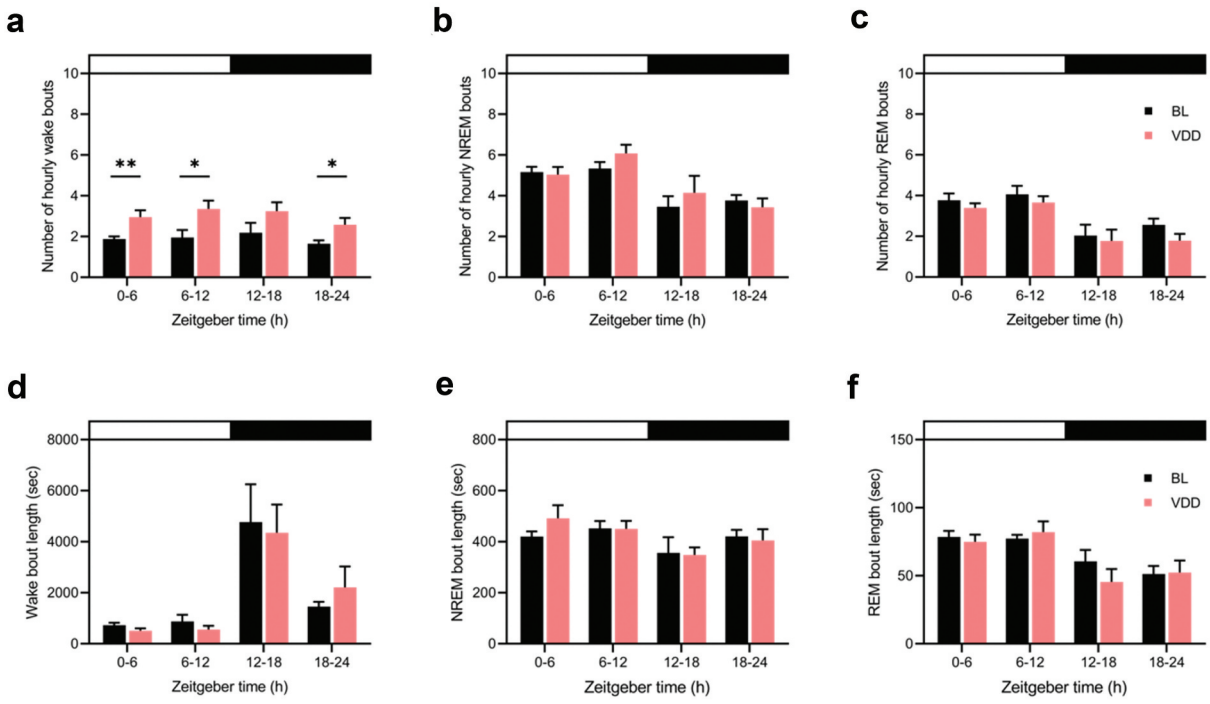

Figure 2. Increased wake bouts during light period in VDD. (a-c) average number of bouts per hour is shown in 6-hour intervals in each stage. (d-f) average bout length per hour is shown in 6-hour intervals in each stage. Open and closed horizontal bars represent light and dark periods, respectively. Baseline and VDD indicate baseline and deficiency states of vitamin D, respectively. Data are shown as means ± SEM ($n = 8$, each vitamin D status). *$P < 0.05$ and *$P < 0.01$ by student's t-test.

quality (Borbely and Achermann 1999; Huber et al. 2004; Vyazovskiy 2015). SWA represents the amount of EEG power in the slow wave frequency range (0.5–4 Hz) during NREM sleep. In addition, SWE is a factor that considers SWA and spent time in NERM. The attenuation of SWE was observed ($F(23, 299) = 9.906$, $p < 0.001$; Figure 3g), particularly at the beginning of dark period (ZT 12–18) in the VDD mice (1.9 ± 0.9 vs. 6.8 ± 1.4 a.u. *min) ($F(3, 39) = 47.05$, $p < 0.001$; Figure 3h). SWA was expressed as a percentage of the baseline SWA at the ZT point corresponding to the recovery sleep time (Figure 3i). These findings suggest that VDD could alter the EEG characteristics, resulting in a decrease in the degree of homeostatic sleep response.

3.4. Decreased the Circadian Period and Rest-activity of the DD Period in the VDD Mice

To further examine the impact of VDD on circadian rhythm, we monitored wheel-running activity in experimental mice and analyzed the features of rest-activity. Activity levels were assessed in both light-dark (LD) and constant darkness (DD) conditions to evaluate the impact of light exposure per se on the activity levels. The presence or absence of light did not significantly affect the activity levels, although significant differences were observed depending on vitamin D status in both LD and DD conditions (Supplementary Figure S2). The actograms visualize the change in activity patterns and amount over time, before and after inducing VDD through dietary manipulation (Figure 4a). Wheel-running activity was strongly entrained by the LD cycle, confirming that the activity started when the light was turned off. The Lomb-Scargle periodogram derived from data collected over a 14-day period in DD to identify the dominant period of the endogenous circadian rhythm (Figure 4b). The periodograms analysis showed a dominant period (τ), calculated individually for each animal and then averaged, resulting in 23.48 hours and 23.78 hours at baseline and VDD, respectively. A significant increase in the circadian period was observed in VDD (23.78 ± 0.09 vs. 23.48 ± 0.05 h) (Figure 4c). No significant differences in circadian amplitude were found between baseline and VDD conditions (Figure 4d). The phase angle, which is represented by the change in the acrophase value, was notably reduced in the VDD state (13.1 ± 5.5 vs. 31.5 ± 2.9 min/day) (Figure 4e). It was calculated by subtracting each mouse's period from the 24-hour cycle (13.1 ± 5.5 vs. 31.5 ± 2.9 min/day). The presence of VDD caused the alteration in the lengths of the active (alpha, α) and inactive (rho, ρ) periods. VDD state mice showed significantly shorter alpha (7.6 ± 0.7 vs. 9.9 ± 0.4 h) and longer rho (16.4 ± 0.7 h vs. 14.1 ± 0.4 h) (Figure 4f). The daily wheel

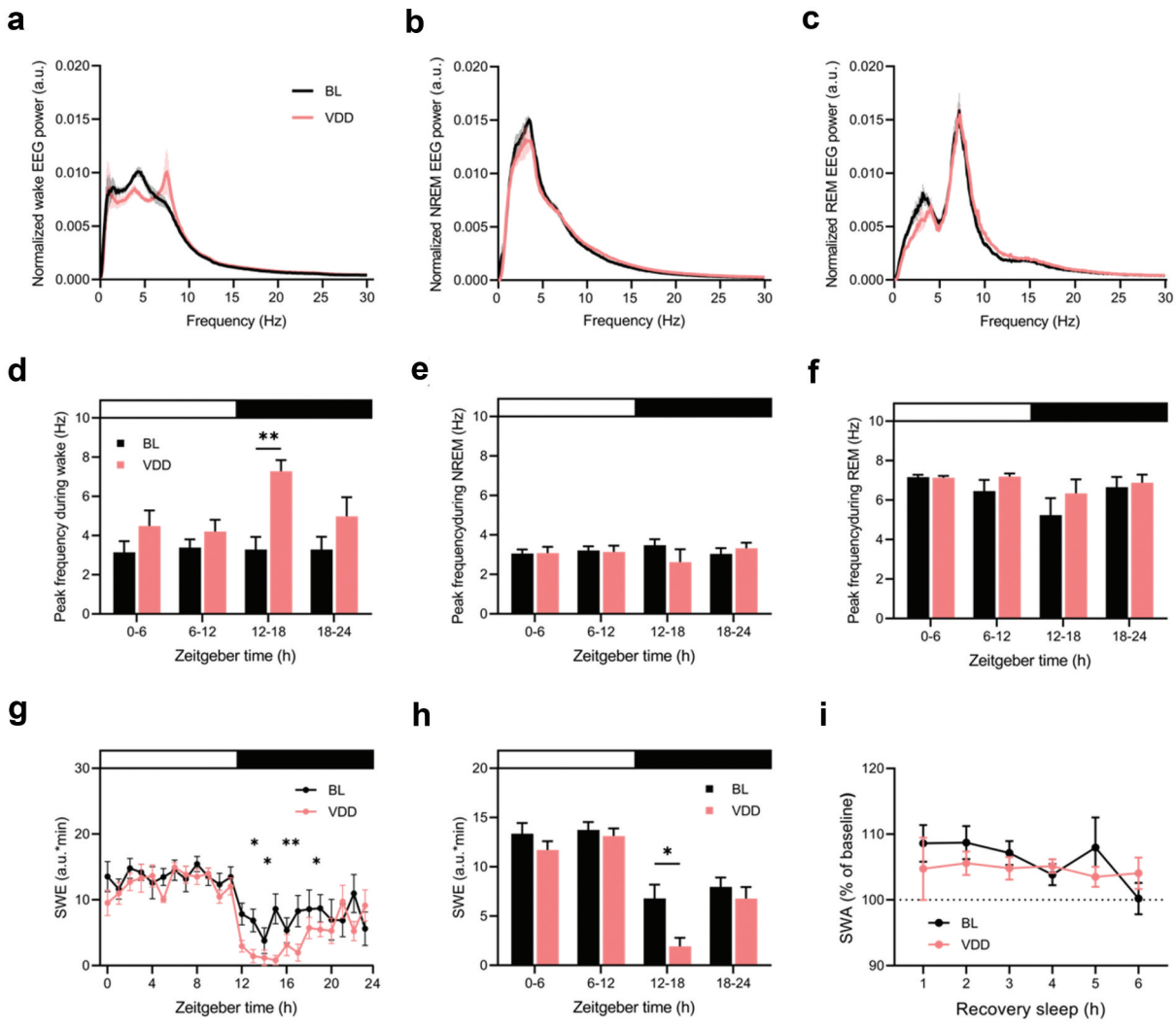

Figure 3. Wake EEG peaked at theta band and decreased slow wave energy during dark period in VDD state. (a-c) normalized EEG power spectrum of wakefulness, NREM, and REM sleep. The EEG power of each frequency band was normalized by total power up to 50 Hz. (d-f) peak frequency during each stage in 6-hour intervals. (g) time course of the changes in slow-wave energy (SWE) for 24 hours. (h) SWE in 6-hour intervals. Open and closed horizontal bars represent light and dark periods, respectively. (i) time course changes of slow-wave activity (SWA, 0.5–4 Hz) during 6-hour recovery sleep followed by 6-hour sleep deprivation. BL and VDD indicate baseline and deficiency states of vitamin D, respectively. Data are shown as means ± SEM ($n = 8$). *$P < 0.05$ and *$P < 0.01$ by two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test or student's t-test.

revolutions notable decreased in the VDD mice (Figure 4g). The VDD mice had less wheel activity (21.8 ± 1.6 vs. 31.5 ± 2.5 counts × 10^3) for 24 hours, which is a 36% reduction. In the alpha phase, VDD mice showed reduced wheel activity (17.1 ± 1.6 vs. 29.4 ± 2.4 counts × 10^3). In the rho phase, VDD mice had more wheel activity (4.7 ± 0.7 vs. 2.1 ± 0.4 counts × 10^3). In addition, the number of bouts of daily activity in VDD mice (4.9 ± 0.5 counts) was decreased compared to the baseline (3.8 ± 0.2 counts) (Figure 4h). The average length of circadian bout was shortened in the VDD mice (101.5 ± 16.9 vs. 208.0 ± 19.6 min) (Figure 4i). The number of wheel revolutions per bout was decreased in the VDD mice (2.9 ± 0.8 vs. 7.4 ± 1.2 counts × 10^3) (Figure 4j). These findings suggest that VDD may alter the activity and duration of the endogenous circadian cycle.

3.5. Suppressed Activity During Short Wake Periods with High Wake Theta Power in VDD mice

Combining sleep and circadian rhythm data, we analyzed the correlation between characteristics of sleep-wake behavior and circadian rhythms at baseline and during VDD. In dark period, mice at baseline displayed a relatively constant level of activity regardless of the duration of wakefulness. In contrast, VDD mice showed increased activity levels when wakefulness durations

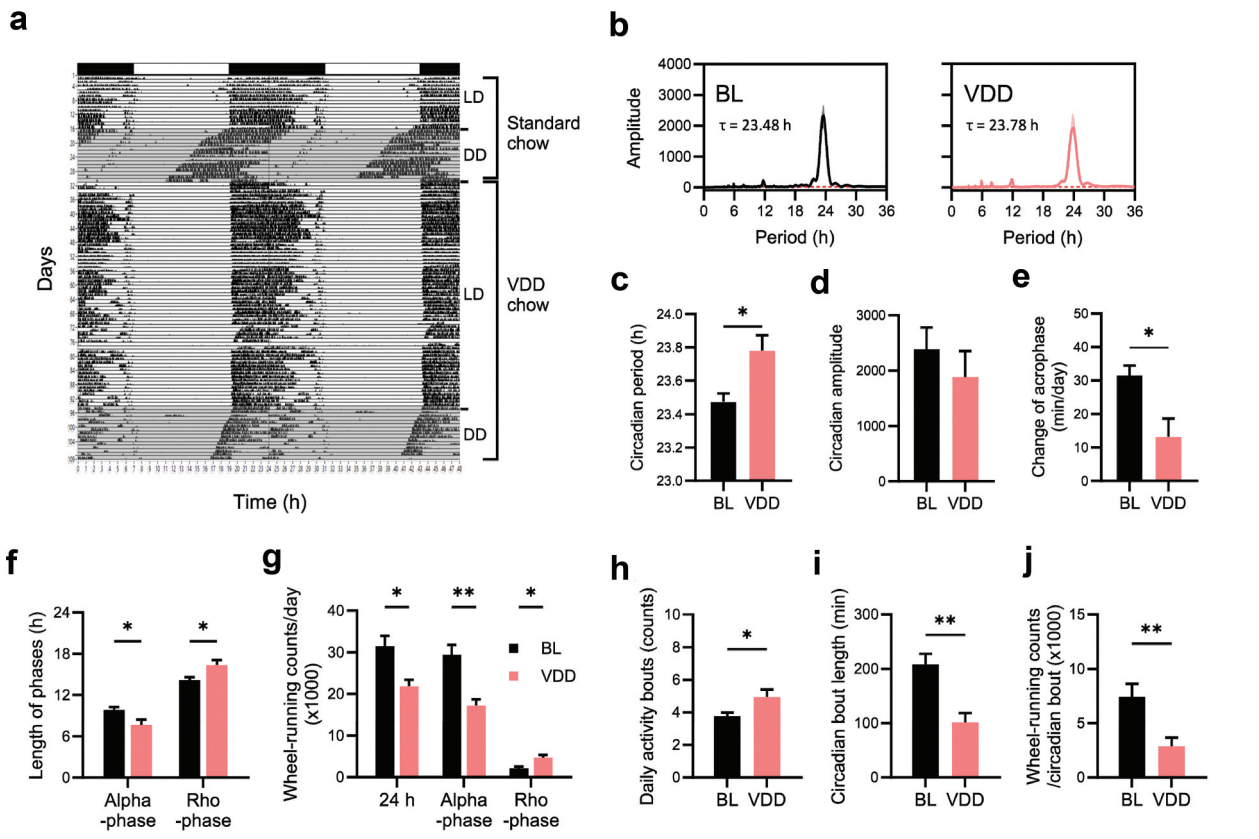

Figure 4. Decreased circadian period and activity in VDD. (a) representative double-plotted actogram according to changes in vitamin D-containing and deficient diets. Open and closed horizontal bars represent light and dark periods, respectively. Each horizontal lines corresponds to 48-hour period overlapping 24 hours. Vertical short bars on each row represent the wheel-running counts. Gray shaded areas indicate constant dark (DD) period. (b) periodograms in baseline (BL, left) and vitamin D deficiency state (VDD, right). The averaged period (τ) determined by lomb-scargle analysis is indicated in each periodogram. (c and d) comparison of circadian period and amplitude based on fourteen-day wheel-running counts data during DD condition. (e) change of acrophase values from a 24-hour period (min/day). (f) length of the alpha and rho phases in DD condition. (g) number of daily wheel-running counts during 24-hour period, alpha and rho phases between BL and VDD. (h to j) comparisons for counts, length of circadian bouts and wheel-running counts per circadian bout between BL and VDD states. Data are shown as means ± SEM ($n = 8$). *$P < 0.05$, *$P < 0.01$, **$P < 0.001$ by student's t-test.

were higher (Figure 5a). The VDD state mice exhibited a significant reduction in wheel activity (14.0 ± 3.2 counts) during spent wakefulness of less than 50% in dark period compared to the baseline (37.8 ± 3.9 counts) (Figure 5b). Moreover, when we analyzed the relationship between activity levels and wake theta power, a marker of sleep intensity, in the early dark period (ZT 12–18; Figure 5c), no correlation was shown at baseline between normalized wake theta power and hourly wheel-running counts ($r = -0.05$, $p = 0.7$), whereas mice in VDD exhibited a strong negative correlation ($r = -0.4$, $P = 0.005$).

4. Discussion

In this study, we found frequent wake bouts during light period, reduced sleep pressure build-up in dark period, a longer circadian period, and reduced activity levels due to increased susceptibility to sleepiness in VDD mice. Combining sleep and circadian data, we also found that VDD mice show suppressed activities during less awake hours. In addition, VDD showed a significant negative correlation between wake theta power and hourly wheel-running counts during dark period. Our data demonstrate that VDD resulted in significant alterations in sleep-wake behavior and daily rest-activity rhythm, indicative of changes in both homeostatic sleep regulation and circadian rhythm as described by the two-process model of sleep regulation.

VDR is a nuclear receptor expressed in cells throughout the body (Stumpf and Privette 1991; Wang et al. 2012), suggesting that vitamin D is involved in various physiological processes. An autoradiographic study conducted years ago revealed the distribution of VDR

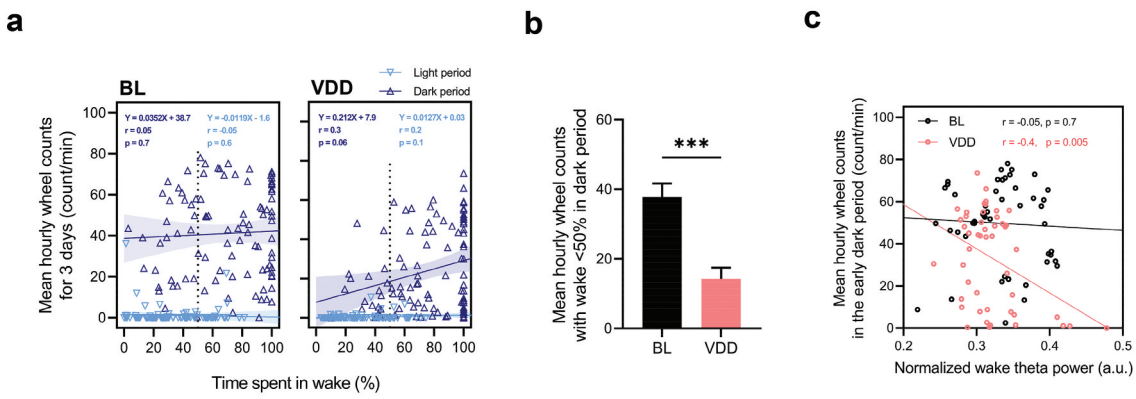

Figure 5. Suppressed activities during the less alert hours with higher wake theta power. (a) scatter plots for 3-day mean of hourly wheel counts and percent wake time. Linear regression lines and confidence intervals were depicted with solid lines and shaded areas in the corresponding color. Vertical dotted lines indicate 50% of wakefulness during each hour. (b) comparison of hourly wheel counts in the hours when mice were awake less than 50% of the hour during dark period. (c) correlation between normalized wake theta power (4–8 Hz) and mean hourly wheel-running counts in the early dark period (ZT 12–18). Data are shown as means ± SEM ($n = 8$). r, pearson's correlation coefficient; *$P < 0.001$ by student's t-test.

in several subcortical brain regions (Stumpf and O'Brien 1987), bed nucleus of the stria terminalis (BNST), thalamic reticular nucleus (TRN), dorsal raphe nucleus (DRN) and central nucleus of the amygdala (CeA). Intriguingly, these regions are known to be involved in regulating sleep-wake cycles (Kim et al. 2012; Kodani et al. 2017; Sanford et al. 2002; Smith et al. 2018). This connection implies that vitamin D might have a physiological role in sleep-wake behavior. Given the high expression of VDR in sleep-related brain regions, researchers have proposed that VDD could negatively impact sleep (Gominak and Stumpf 2012). While numerous clinical studies support this hypothesis, establishing causal relationships is challenging in observational studies due to confounding factors. Consequently, an experimental study using an animal model might be a more effective approach for investigating the causality in a controlled setting. Our experimental design, therefore, has the potential to unveil the direct impact of VDD on sleep-wake behavior and circadian rhythm. In addition, VDR knockout (VDR -/-) mice exhibit impairments in musculoskeletal development, along with physiological abnormalities such as hypocalcemia, hyperparathyroidism, and certain reproductive and immunological abnormalities (Burne et al. 2005; Mathieu et al. 2001). Given that constitutive knockouts influence developmental stages and could introduce confounding factors, inducing VDD through diet could be a reasonable methodological choice.

In clinical investigations, VDD was associated with poor nightly sleep and excessive daytime sleepiness (Lee et al. 2020; McCarty et al. 2012). Sleep propensity can be estimated by delta power during NREM sleep and theta power during wakefulness. However, it should be noted that wake theta power can represent two different meanings: first, increased sleep pressure, correlated with NREM delta power (Finelli et al. 2000), and second, increased walking or running behaviors (Coenen 1975; Kuo et al. 2010). Thus, our discovery of wake EEG power spectrum's peak frequency shifting from the delta to theta range during dark period should be interpreted cautiously. Because the wheel-running recording data showed lower activity levels during VDD, it is more plausible that the EEG peak shift was associated with high sleep pressure rather than increased activity. In other words, the VDD mice were awake or unable to sleep and tired during the first half of the dark period, potentially due to lower accumulated SWE during NREM sleep. Furthermore, VDD may increase AMP hydrolysis, leading to an increase in adenosine levels (Bagatini et al. 2019). Adenosine in the brain is associated with sleep pressure (Porkka-Heiskanen et al. 1997), suggesting that mice with VDD may exhibit heightened sleep propensity compared to baseline conditions. The slower accumulation of SWE observed during VDD implies that mice may be less efficient at dissipating sleep pressure, leading to an accumulation of unresolved sleep pressure and increased drowsiness during wakefulness. Our study noted some intriguing patterns in the VDD state: a diminished duration of wakefulness during the light period coupled with an increased number of wake bouts throughout the day. These results indicate more frequent transitions from wakefulness to sleep, hinting at a potential inability to sustain wakefulness due to hypersomnolence during wake periods. This phenomenon could be linked to the observed decrease in REM sleep during the active period. Additionally, the shift of peak

frequency in wake EEG to the theta range, particularly noted during the dark period, may be indicative of increased sleep propensity. Paradoxically, despite the high sleep needs, these mice exhibited reduced REM sleep. This pattern bears a resemblance to nighttime insomnia and excessive daytime sleepiness often reported in patients with VDD (Lee et al. 2020; McCarty et al. 2012).

The amount of SWA (0.5–4 Hz) during NREM sleep is characterized by high amplitude and low frequency on an EEG (Contreras and Steriade 1995), and the amount of delta activity observed during NREM sleep is considered to reflect the sleep pressure (Rodriguez et al. 2016). Our data shows a decrease in SWE, a value considering both the SWA and time spent in NREM sleep, suggesting a reduced build-up of sleep pressure during dark period. Several potential mechanisms could explain the decreased SWE in VDD mice. First, decreased calcium level caused by a reduced vitamin D level could be one factor for SWE decrease. Ca^{2+}-dependent hyperpolarization pathway has been proposed as a core mechanism of homeostatic sleep regulation (Ode et al. 2017; Shi and Ueda 2018; Tatsuki et al. 2016,2017), and it plays a vital role in generating slow wave sleep (SWS) firing patterns. Vitamin D is necessary for calcium absorption in the intestines, so its deficiency may lead to decreased calcium absorption and concentration (Bikle 2014; Holick 2007). Indeed, serum vitamin D and calcium levels were positively correlated in shift and non-shift workers, and the lower calcium levels were linked with the disruption of sleep and rest-activity circadian rhythm (Jeon et al. 2022). Therefore, it is plausible that the decrease in calcium caused by VDD could disturb the SWS firing pattern, reducing SWA in mice with VDD. Second, decreased synaptic strength caused by low vitamin D levels could reduce SWA. A level of synaptic potentiation during wakefulness is positively correlated with the increase in SWA during subsequent sleep (Tononi and Cirelli 2006). Additionally, extending the duration of wakefulness leads to an increased expression of synaptic potentiation markers, which subsequently enhances SWA during the following sleep episode (Cirelli and Tononi 2000). Since VDD disrupts the regulation of various brain proteins related to synaptic plasticity and neurotransmission (Almeras et al. 2007; Eyles et al. 2007), this decrease in synaptic strength may contribute to the decreased ability to generate SWA. These observations suggest that vitamin D might be integral to the proper functioning of the homeostatic sleep drive, and its deficiency could disturb this regulatory process, leading to imbalances in sleep and wakefulness.

Evaluating wheel-running activity is a well-established and reliable method for investigating rest-activity circadian rhythm in animal models. We measured wheel-running activity during a constant dark period, which reveals the animal's intrinsic endogenous rhythm. Our results confirmed that the VDD increased the circadian period and caused a slight decrease in circadian amplitude. In addition, shortened active alpha phase and decreased wheel-running activity were confirmed in VDD mice. The results suggest that VDD progression leads to decreased overall activity levels and lengthened circadian period, which may be affected by altered intracellular molecular clock regulation. It should be noted that wheel-running exercise can enhance the expression of neuroprotective proteins and improve motor and cognitive abilities (Zhou et al. 2017), potentially counteracting the negative impact of VDD. However, despite the neuroprotective benefits of wheel running, we observed significant alterations in the sleep and circadian rhythms in VDD mice. This fact reinforces our finding that VDD has a negative impact on these parameters. Recent research showed that impaired cytoplasmic homeostasis could cause circadian rhythm abnormality by interfering with the nuclear entry of PERIOD protein, a key component of the circadian molecular mechanism in SCN neurons (Beesley et al. 2020). Strong induction of autophagy results in a shortened circadian period, whereas inhibition of autophagy leads to a lengthened circadian period accompanied by weakened amplitude. Vitamin D activates autophagy by inhibiting mTOR through the activation of AMPK and inhibition of β-catenin /TCF4/GSK-3β signaling (Bhutia 2022; Kong et al. 2020; Wei et al. 2017). Research has also shown that VDD causes autophagy dysfunction (Lajtai et al. 2019; Zhao et al. 2017). Collectively, these evidence suggests that VDD can affect circadian rhythms through changes in autophagy levels. A longer period might be clinically associated with a delayed sleep phase (Chang et al. 2009; Micic et al. 2013), which increases the risk of several psychiatric disorders (Shirayama et al. 2003; Takaesu et al. 2022). It's worth noting that VDD could potentially disrupt the normal functioning of the circadian rhythm, leading to a misalignment in the sleep-wake cycle. Importantly, these two processes – the homeostatic sleep drive (Process S) and the circadian rhythm (Process C) – do not operate independently, but instead reciprocally influence each other to fine-tune sleep regulation. As such, the effects of VDD on these processes are likely to be intertwined. For instance, VDD could disturb the circadian rhythm, which may consequently exacerbate the imbalance in the sleep-wake cycle and contribute to the dysregulation of the homeostatic sleep

drive. In a similar vein, an imbalanced sleep-wake cycle could further misalign an already disrupted circadian rhythm.

We recognize several limitations in experiments that should be considered when interpreting our data. First, we were unable to present the concentration of serum vitamin D, as data could not be obtained due to insufficient establishment of the measuring method, despite collecting blood for concentration measurement. Nevertheless, we strictly followed the VDD protocol (Dancer et al. 2015; Parekh et al. 2017), which has been extensively validated in previous studies conducted under similar conditions, which may result in comparable results. In previous studies using the standard protocol, serum vitamin D levels of 8 and 9 nmol/L were observed, indicating a severe deficiency state, while 42 and 50.4 nmol/L were observed in each control. Although the criteria of vitamin D status are slightly different in vitamin D research, plasma 25-hydroxyvitamin D below 50 nmol/L but not less than 30 nmol/L was generally regarded as insufficiency, and below 30 nmol/L as deficiency (NIH Office of Dietary Supplements 2022). Given that vitamin D concentrations have been validated in previous studies employing the same protocol, we cautiously interpreted our data under the assumption that the mice used in our experiment were in a state of VDD. Furthermore, in the wild, fur-covered animals secrete a vitamin D precursor, 7-dehydrocholesterol, from sebaceous glands onto their fur (Joshi 2008; Loomis 1967). Exposure to UV light facilitates the transformation of this precursor into pre-vitamin D3, which is then ingested through grooming behaviors. In contrast, laboratory animals primarily obtain their vitamin D intake from their diet which contains cholecalciferol (vitamin D3), not a UV-dependent precursor. This ingested cholecalciferol is activated via the liver or kidney. Considering that the main source of vitamin D in a laboratory environment without UV exposure is feed, controlling vitamin D levels through diet is considered a reasonable approach to induce VDD. Secondly, EEG data for sleep analyses and wheel-running data were not collected simultaneously, which may hinder the interpretability of combining these data types to understand the interaction between sleep-wake behavior and circadian rhythm. By conducting the recording sessions in sound-proof rooms, we effectively minimized random noise, thereby enhancing the replicability of our recording system. However, it remained inevitable to subject the mice to different physical conditions between the two recording sessions, such as the use of tethering during EEG signal collection. For future research, it could be beneficial to employ a device that can simultaneously measure the core body temperature, EEG, and activity without the need for tethering. This approach could potentially enhance the reliability of the correlation between sleep and circadian rhythm, thereby providing more compelling evidence. Third, we conducted within-subject comparisons to observe differences in sleep and circadian rhythms before and after VDD induction. Age-matched control group could be more desirable for comparison, but we could not make the group in our experiment. Although aging significantly impacts sleep and circadian rhythm studies, all the subjects were within young adulthood. Prior studies have demonstrated no difference in sleep duration and slow-wave activity within the young adult range (McKillop et al. 2018; Wimmer et al. 2013). Furthermore, investigations of aging and its impact on sleep and circadian rhythms generally compare young adults aged around 5 months to old adults aged 15 months or more (Bruns et al. 2020; McKillop et al. 2018; Nakamura et al. 2015; Valentinuzzi et al. 1997; Wimmer et al. 2013). Nevertheless, because we could not exclude the possible influence of aging per se, we parsimoniously interpreted that the observed differences in sleep and circadian rhythm characteristics between BL and VDD were by VDD with potential confounding effect by aging. Fourth, vitamin D rescue experiments must be informative in the understanding of the roles of VDD in sleep and circadian rhythms. Sufficient condition test by supplementing vitamin D needs to be done in the future. Fifth, the nutritional composition of VDD diet utilized in our study was not identical to the regular diet. The optimal design for a VDD diet would maintain consistent nutritional compositions with exception of vitamin D content. In our study, the VDD diet contains a higher fat content, 10% as compared to the 4% in the regular diet. However, it should be noted that substantial effects associated with high-fat diets are generally evident when the fat percentage exceeds 35% (Duan et al. 2018). In addition, the caloric content of macronutrients has been calculated to be 3.64 kcal/g for the regular chow and 4.18 kcal/g for the VDD feed. Considering the daily caloric requirement of a mouse (161 kcal ME/BWkg$^{0.75}$/day), a mouse with a body weight of 29.8 ± 2.8 g would need between 10.30–12.33 kcal/day (Bernier et al. 1986). Mice typically consume 10 to 15 g of chow per 100 g of body weight per day (Huerkamp and

Dowdy 2008), which means a mouse weighing 29.8 ± 2.8 g would intake approximately 2.7 to 4.9 g daily. Factoring in an estimated daily consumption of around 3 g, the daily caloric intake at baseline would be 10.92 kcal, while for VDD diet it would be 12.54 kcal, both falling within the normal range of daily caloric intake. Thus, the potential of these dietary variations to serve as significant confounders in studies evaluating sleep and circadian rhythm appears to be limited. Finally, it is important to note that VDD can cause musculoskeletal weakness and pain (de Oliveira et al. 2017; Wintermeyer et al. 2016) which may also contribute to the observed reduction in circadian activity following VDD induction. The possibility of musculoskeletal weakness or pain as a confounding factor cannot be excluded. Further investigations are warranted.

In conclusion, our study reveals a significant relationship between VDD and sleep disturbances, along with diminished rest-activity circadian rhythmicity, thus highlighting the crucial role of vitamin D in maintaining healthy sleep and circadian function. We aimed to investigate the potential effects of VDD rather than conclusively determining its significance. Our study provides a unique opportunity to examine homeostatic sleep behavior and circadian rhythms independently. These two factors are usually intertwined and difficult to investigate separately due to the complex interaction of the two processes. Moreover, in human subjects, many confounding factors are difficult to be controlled strictly. We believe that utilizing an animal model in our experiments could unravel the separate influences of VDD on sleep and circadian rhythms. While further research is necessary to unearth the precise mechanisms underlying these associations, our findings lay a solid groundwork for future explorations in this field.

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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